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Original Article

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Mass spectrometry-based metabolomic profiling of prostate cancer - a pilot study

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Abstract

Aim: Prostate cancer (PCa) is the most commonly diagnosed non-skin cancer among men. Serum prostate-specific antigen level is used as a standard PCa biomarker for over 20 years. However, it has only 33% specificity and 86% sensitivity (for the cutoff value for prostate biopsy of > 4 ng/mL). This leads to overdiagnosis and overtreatment. In-depth insight into PCa metabolomics enables discovery of novel PCa biomarkers.

Methods: Metabolomic alternation in PCa serum, urine and interstitial fluid was examined using gold-nanoparticle-based laser mass spectrometry imaging. This study included 5 patients who underwent prostate biopsy with positive result, 5 patients with negative result and 10 healthy controls.

Results: Over two hundred differentiating metabolites (87 in urine, 54 in serum and 78 in interstitial fluid) were detected. Four, twenty two and ten metabolites from urine, serum and interstitial fluid respectively showed statistical significant differential abundance between cancer and control group.

Conclusion: Comprehensive metabolomic profile of PCa has been identified. Out of 36 metabolites, 20 were identified and should be further evaluated in clinical trials as a potential PCa biomarker. Urine concentration of triglyceride (12:0/20:1) showed over 10 times higher abundance in PCa samples in comparison to healthy controls and is considered the most promising potential biomarker.



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Keywords: Prostate cancer, biomarker, metabolomics, mass spectrometry

INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed non-skin cancer and third leading cause of mortality after lung and colorectal cancer among men in Western World. Overall, during lifetime, 1 in 6 men will develop PCa and 1 in 33 will die from this disease. Moreover, at presentation 16% of patients will have locally advanced or metastatic disease. Life expectancy depends mainly on the stage and varies between < 5 years for metastatic disease to minimal risk of death at 15 years for organ-confined disease^[1]. Historically PCa was diagnosed with digital rectal examination (DRE) followed by transperineal or digitally guided transrectal biopsy, often in advanced stage. Introduction of prostate-specific antigen (PSA) testing has revolutionized diagnosis of PCa and is shown to be the better predictor of cancer than DRE or transrectal ultrasound (TRUS). Traditional PSA cutoff value for prostate biopsy is > 4 ng/mL and has only 33% specificity and 86% sensitivity for detection of PCa. As a result patients with elevated PSA are overdiagnosed and overtreated. Decrease in mortality from PCa observed in most Western countries is considered partially to be the result of PCa screening with PSA. However, screening with PSA has become one of the most debated topics in urology and is dominated by the conflicting results of 2 randomized control trials: The European Randomized Study of Screening for Prostate Cancer (ERSPC)^[2], which found a survival benefit due to screening with PSA (with the greatest benefit within Goteborg arm of ERSPC trial^[3]) while the United States Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial^[4] did not. As ERSPC study shows, to save one patient from PCa specific death, 1,055 men need to be screened and 37 patients need to be treated. Recent studies concluded that screening is associated with increased diagnosis of PCa, no PCa-specific or overall survival benefit and increased risk of overdiagnosis and overtreatment^[5]. Therefore new diagnostic tools (multiparametric MRI) and biomarkers (PCA3, kallikreins and *TMPRSS2-Erg* fusion) are constantly being developed to increase sensitivity and specificity of PCa testing. Recently a number of novel PCa biomarkers have been developed. The most promising was developed by MDxHealth - SelectMDx. It is a genomic-based test that measures the expression of two PCa-related mRNAs in urine: *HOXC6* and *DLX1* in conjunction with clinical risk factors. It's main advantage is the ability to diagnose patients with clinically significant cancer (Gleason score ≥ 7) which would be selected for prostate biopsy and to avoid identification of indolent PCa. SelectMDx negative predictive value is 98%^[6]. According to Govers *et al.*^[7] application of SelectMDx in clinical practice contributes to increased quality-adjusted life years while saving healthcare costs.

Biomarker research is based on analyzing differences in abundances of varied substances between cancerous tissue or biofluid and their healthy counterpart. This comparative analysis approach identifies and classifies the individual molecules. However, recently, a novel approach have been proposed and is called network-based analysis. Instead of focusing on specific molecule, emphasis is placed on whole cellular pathway and key molecules which are altered in the disease. Search for potential biomarkers has been conducted at every molecular level: genome, transcriptome, proteome and metabolome. Genomic and transcriptomic microarray, real time PCR and next gen. sequencing, proteomic and metabolomic mass spectrometry and nuclear magnetic resonance spectroscopy in combination with advanced bioinformatics tools allowed detailed insight into cancerogenesis. Metabolome, which is collection of all metabolites produced within cell is an endpoint of the "omics cascade" (genomics, proteomics, metabolomics) pathway and in comparison to genes and proteins which are subjected to epigenetic processes and post-translational modifications, is the most accurate representation of phenotype^[8]. Recent advances in mass spectrometry enabled discovery of numerous metabolites specific for lung, colorectal, ovarian, prostate, renal, breast and liver cancer^[9-17]. Metabolomic studies of PCa have led to the discovery of sarcosine as a potential marker of PCa. It has been demonstrated that elevated level of serum sarcosine is associated with PCa^[18,19]. However the role of sarcosine as a potential PCa biomarker has been based on studies involving a small number of subjects and has been questioned following publication of the studies of larger cohort. de Vogel *et al.*^[20] examined serum of 3000

patients with PCa and 3000 controls and showed that elevated serum levels of sarcosine were associated with decreased risk of PCa.

The difficulty in developing PCa biomarker results from the heterogeneity of this type of cancer. It has been demonstrated that factors like obesity and hormonal profile may be associated with increased risk of high grade PCa. de Cobelli *et al.*^[21] demonstrated that high body mass index (BMI) is associated with upgrading and upstaging in patients with low-risk PCa. Moreover it has been shown that low testosterone level is a predictor of upstaging and upgrading in patients with low-risk PCa^[22]. This results support inclusion of BMI and testosterone level into selection criteria for active surveillance programs.

In this study we evaluate the role of mass spectrometry in the metabolomic study of PCa and the possibility to develop potential novel PCa biomarker.

METHODS

Protocol of this study was approved by the ethics committee at the University of Rzeszów, Poland (no. 14/06/2016). Specimens and clinical data related to this study were collected after signing informed consents. Data were prospectively collected from 10 patients with elevated PSA who underwent transrectal prostate biopsy and 10 healthy volunteers between December 2016 and February 2017 at Department of Urology, City Hospital in Rzeszów. Five out ten patients were diagnosed with PCa - 3 with Gleason 7, 1 with Gleason 8 and 1 with Gleason 9. Each biopsy was performed by the same urologist at our department with extensive experience in prostate biopsies (over 10,000 performed procedures) and was examined by dedicated genitourinary pathologist using 2005 International Society of Urological Pathology Gleason grading criteria. Exclusion criteria were: active acute urinary tract infection, bleeding disorder and bowel malignancies or inflammatory bowel diseases. Collected data included age, total PSA level, DRE examination result, TRUS calculated prostate volume and full pathological report. Standard 12-core TRUS guided biopsy was performed. Before procedure, single dose of i.v. ciprofloxacin was used as an antibiotic prophylaxis. Each core was placed into a separate container.

Decision to perform prostate biopsy was based on either elevated PSA level (higher or equal to 4.0 ng/mL) or positive DRE examination. For the purpose of metabolomic analysis, 10 mL of blood, 100 mL of urine and 50 µL of interstitial fluid (IF) extracted from each biopsy core were collected from each patient who has had prostate biopsy. Control group constituted of 10 young (< 40 years old, mean 28.7) healthy volunteers with average PSA of 0.7 ng/mL and average estimated PCa risk (using multivariable ERSPC cohort PCa risk calculator) of 2%. Each volunteer donated 10 mL of blood and 100 mL of urine, no biopsies were performed in this group. Whole blood was collected and centrifuged at 3000 rpm at room temperature for 10 min.

Preparation of samples for mass spectrometry imaging

Urine, serum and IF samples were immediately stored at -80 °C until use. Urine and blood plasma samples were diluted 600 times with water and placed on target plate (0.5 µL). Tissue samples were washed with 50 µL of water and directly placed on target plate (0.5 µL). All samples of the same type (for example all urine samples) were measured on the same target plate in one mass spectrometry imaging (MSI) experiment. Extracts were placed on target plate with the aid of 3D precision translation stage system with spot diameter ca. 1.5 mm.

Mass spectrometry measurements and data handling

Mass spectrometry (MS) analysis was performed using high-resolution laser desorption/ionization (LDI)-time-of-flight (ToF)-MS based on gold nanoparticle-enhanced target (AuNPET) plate^[23] and is presented in simplified form in flowchart in [Figure 1](#). AuNPET target plates of 2.5 × 3.5 cm size and ca. 0.8 mm thickness were used with Bruker NALDI adapter. AuNPET-based surface-assisted, LDI-ToF-MS experiments were

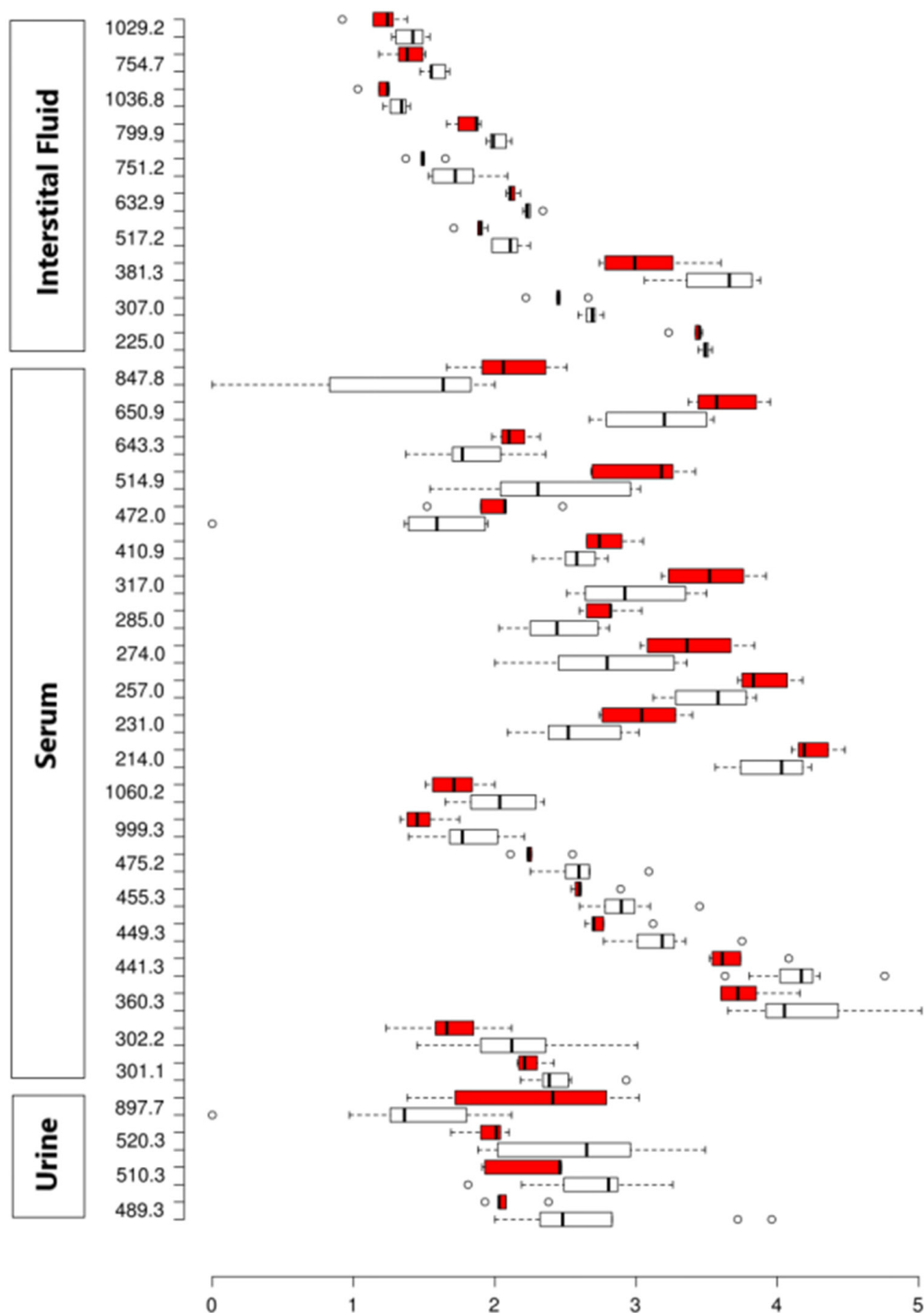


Figure 1. Box plot of all statistically significant ($P < 0.05$) metabolites. x-axis: log10 transformed mass spectrometry intensities (to reduce the impact of variability of high-intensity peaks, intensity axis has been transformed into a logarithmic one); y-axis: m/z values. Red boxes represent cancer, white boxes - control

performed using a Bruker Autoflex Speed ToF-MS in reflectron mode. Instrument was equipped with a SmartBeam II laser (352 nm). The laser impulse energy was approximately 60-120 μ J; the laser repetition rate

was 1000 Hz; and the deflection value was set to m/z 80 Da. The first accelerating voltage was held at 19 kV and the second ion-source voltage was held at 16.7 kV. The reflector voltages used were 21 kV (first) and 9.55 kV (second). Measurement range was m/z 80-2000.

Samples were placed on AuNPET plate semi-automatically with the aid of precise positioning of 3D translation stage which allowed precise orientation of commonly used pipette (Eppendorf Research plus). Application of described stage allowed much higher spot density and repeatability which would be rather impossible in manual manner. Semi-automated solution applied allowed measurements of over hundreds of samples on one custom-made target plates of 3.5×2.5 cm size.

For each sample spot, four measurement locations (pixels) were measured and resulting spectra averaged after preliminary re-calibration. Obtained quadruplicates were additionally recalibrated and normalized, the latter was based on three most abundant gold ion peaks.

Extracts were measured in MSI mode with 20,000 laser shots per individual pixel and four pixels per sample with 300 μ m-lateral resolution. Bruker's FlexImaging 4.0 software was used for setting of MSI experiments. Mass calibration was performed using internal standards (gold ions and clusters from Au^+ to Au_9^+). Imaging data were converted to Analyze 7.5 and then to mzXML format and processed in mMass software. Obtained spectra were processed by application of baseline correction and recalibration. Spectra from four pixels of each spot were averaged and then again recalibrated. Intensities of averaged spectra of spots were normalized based on arithmetical mean of intensities of Au^+ , Au_2^+ and Au_3^+ ions with the use of software developed by our group. Resulting ion lists were then assigned with the use of monoisotopic masses available for metabolites enclosed in Human Metabolome Database (HMDB) and Pathos database within 10 ppm mass accuracy windows.

Study design

Serum and urine were collected from all the patients and categorized as follows: (1) no PCa (healthy volunteers, $n = 10$ patients); (2) presumptive benign disease (negative biopsy, $n = 5$ patients); (3) PCa (positive biopsy, $n = 5$ patients). IF collected from each prostate biopsy core was categorized as follows: (1) presumptive benign disease (negative biopsy, $n = 5$ patients); (2) PCa (positive biopsy, $n = 5$ patients). Each sample was analyzed using standardized MS protocol. Serum and urine specimens collected from patients with biopsy confirmed PCa were compared with those from healthy volunteers (no biopsy in this group). Serum and urine from patients with negative biopsy (no PCa) were excluded from this study due to lack of certainty that there is no PCa in non-biopsied regions of prostate. IF from positive biopsy cores were compared with those from negative cores. Patient characteristics are shown in [Table 1](#).

RESULTS

All statistically significant metabolites ($P < 0.05$) are shown in [Table 2](#). Statistical analysis was performed using SPSS and R software package. Four, twenty-two and ten metabolites from urine, serum and IF respectively showed statistical significant ($P < 0.05$) differential abundance between cancer and control group via Mann-Whitney U test. In addition, metabolites with m/z values of 897.7 (urine) and 307.0 (IF) were positively correlated with prostate volume (Spearman's $\rho = 0.564$, $P = 0.028$ and 0.648 , $P = 0.043$ respectively). Differences between cancer/control intensities have been presented in the form of box- [\[Figure 1\]](#) and volcano-plot [\[Figure 2\]](#) graphs. Peaks obtained from MS analyses were identified and analyzed in the context of metabolic pathways in which they appear within Pathos and HMDB.

DISCUSSION

In this study, a new methodology for rapid metabolomic analysis of human physiological liquids is proposed. MS measurements in imaging mode based on LDI apparatus, with the use of AuNPET target plate, simplify

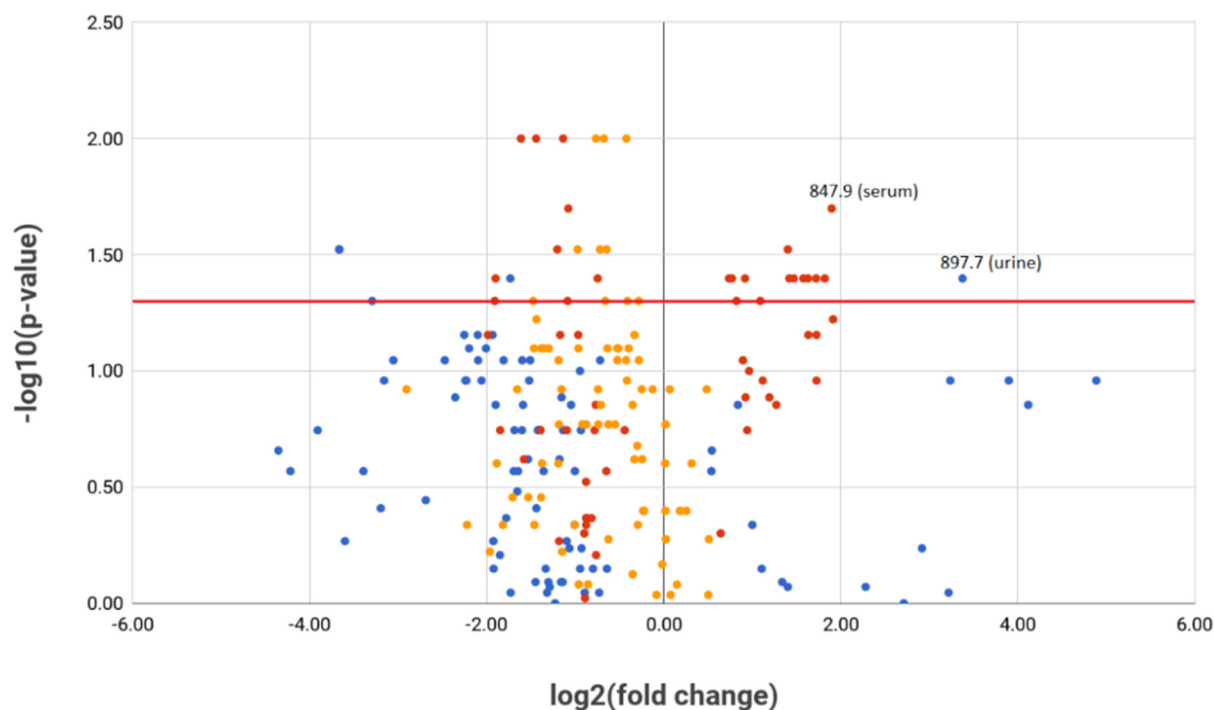


Figure 2. Volcano plot, all differentiating metabolites. Blue dots: urine metabolites; red dots: serum metabolites; orange dots: interstitial fluid metabolites. All substances above red horizontal line are statistically significant ($P < 0.05$). Two enumerated metabolites in upper right quadrant represent m/z values of most promising, potential urine and serum prostate cancer biomarkers

Table 1. Selected clinical characteristics of patients

Characteristics	Controls (no prostate biopsy) ($n = 10$)	Study group (prostate biopsy)	
		Positive ($n = 5$)	Negative ($n = 5$)
Age (years)			
Mean	28.7	71.4	66.8
PSA (ng/mL), n (%)			
PSA < 4	10 (100%)	0	0
PSA 4-10	0	2 (40%)	1 (20%)
PSA 10-20	0	0	3 (60%)
PSA 20-50	0	2 (40%)	1 (20%)
PSA > 50	0	1 (20%)	0
DRE			
Suspected of PCa	0	4	0
Normal	10	1	5
Prostate volume (mL), n (%)			
< 30 mL	10 (100%)	1 (20%)	1 (20%)
30-80 mL	0	3 (60%)	4 (80%)
> 80 mL	0	1 (20%)	0
PCa risk (%)			
Mean	2	77.2	42.6
Gleason grade, n (%)			
6	-	0	-
7	-	3 (60%)	-
8	-	1 (20%)	-
9	-	1 (20%)	-

PSA: prostate-specific antigen; DRE: digital rectal examination; PCa: prostate cancer

all of the necessary experimental procedures and thus greatly speed-up the experimental part of the analysis. For the imaging experiment presented in this work, the timeframe contained ~10 min of sample application

Table 2. Urine, serum and interstitial fluid metabolites ($P < 0.05$) sorted by m/z values, chemical formula, chemical class, metabolic pathway, cancer to control ratio and P -value

m/z	Chemical formula	Metabolite*	Metabolic pathway	Cancer to control ratio	P -value
Urine					
489.3	$[C_{27}H_{46}O_4S+H]^+$	Cholesterol sulfate	Cholesterol metabolism	0.08	0.03
510.3	$[C_{23}H_{45}N_5O_5+K]^+$	Ile-Ile-Lys-Val and other isomers	Peptide	0.30	0.04
520.3	$[C_{21}H_{33}N_11O_5+H]^+$	Ala-Arg-His-His and other isomers	Peptide	0.10	0.05
897.7	$[C_{55}H_{102}O_6+K]^+$	Triglyceride (12:0/20:1) and other isomers	Lipid metabolism	10.39	0.04
Serum					
302.2	$[C_{16}H_{31}NO_4+H]^+$	Nonanoylcarnitine	Carnitine-type compound	0.27	0.05
441.3	$[C_{22}H_{42}O_7+Na]^+$	Palmitoyl glucuronide	Fatty acid metabolism	0.33	0.01
449.3	$[C_{30}H_{50}+H]^+$	Squalene	Steroid biosynthesis	0.37	0.01
455.3	$[C_{27}H_{44}O+K]^+$	Calcitriol	Steroid biosynthesis	0.47	0.02
475.2	$[C_{18}H_{30}O_2+H]^+$	(9Z, 12Z, 15Z)-octadecatrienoic acid	alpha-Linolenic acid metabolism	0.45	0.01
999.3	-	unknown	-	0.44	0.03
214.0	$[C_6H_7O_6+K]^+$	Monodehydroascorbate	Ascorbate metabolism	1.77	0.05
231.0	$[C_6H_{12}N_2O_3S+Na]^+$	Ala-Cys and other isomers	Peptide	2.77	0.04
257.0	$[C_6H_8O_9S+H]^+$	Ascorbate 2-sulfate	Ascorbate metabolism	2.13	0.05
285.0	$[C_9H_{10}O_7S+Na]^+$	Homovanillicacidsulfate	-	1.89	0.04
410.9	$[C_4H_7O_8P+H]^+$	2-Oxo-3-hydroxy-4-phosphobutanoate	Vitamin B ₆ metabolism	1.67	0.04
515.0	$[C_{10}H_{15}N_4O_{13}P_3+H]^+$	dITP	Purine metabolism	2.99	0.04
643.3	$[C_{32}H_{44}N_8O_5+Na]^+$	Arg-Leu-Phe-Trp and other isomers	Peptide	1.71	0.04
650.9	-	unknown	-	2.65	0.03
711.0	-	unknown	-	3.53	0.04
847.9	-	unknown	-	3.73	0.02
Interstitial fluid					
225.0	$[C_7H_6O_6+H]^+$	Maleylpyruvate	Tyrosine metabolism	0.82	0.05
307.0	$[C_9H_{11}N_2O_8P+H]^+$	3,2',3'-Cyclic uridine monophosphate	Pyrimidine metabolism	0.61	0.03
517.2	$[C_{21}H_{36}O_2+H]^+$	Pregnanediol	Steroid hormone biosynthesis	0.59	0.01
632.9	-	unknown	-	0.75	0.01
751.2	$[C_{21}H_{34}N_{10}O_8+Au]^+$	Arg-Asp-Gln-His and other isomers	Peptide	0.51	0.03
754.7	-	unknown	-	0.64	0.03
799.9	-	unknown	-	0.63	0.01

*tentative identification

to the target and ~20 min of measurements. It should be noted that rapid analysis has additional advantages such as ability to preserve chemically labile low-molecular weight compounds. Additionally, it is of extreme importance that no chemicals were added to the analyzed samples. It is important to state that sample preparation was one of the simplest possible in terms of procedure as it required only dilution of unfrozen samples. For the first time, in this study, the AuNPET LDI-TOF-MS method was used to study metabolites originating from human urine, blood plasma and prostate IF. Methodology of measurements is shown on [Figure 3](#).

The decision not to examine prostate tissue but interstitial fluid was dictated by ethical and technical considerations. On one hand, fresh biopsy cores would have to be contained in high vacuum and thus they would be unfit for further pathological examination. Another possible analytical solution compatible with pathologist's examination is analysis of formalin-fixed/paraffin embedded cores. Unfortunately, only 40% of total metabolites is retained in tissue material for the mentioned type of analysis. In comparison to fresh/frozen specimens, it would introduce many contaminants like dimethyl sulfoxide, lauryl sulfate or melanin into MS analysis^[24]. IF which constitutes tumor microenvironment, often overlooked, has showed to be a highly valuable source of potential cancer biomarkers and a potential target for chemoprevention^[25,26].

Uncontrolled cancer cell proliferation requires efficiency in generation of energy. However, unfavorable tumor microenvironment (low level of oxygenation) impose activation of alternative metabolic pathways to

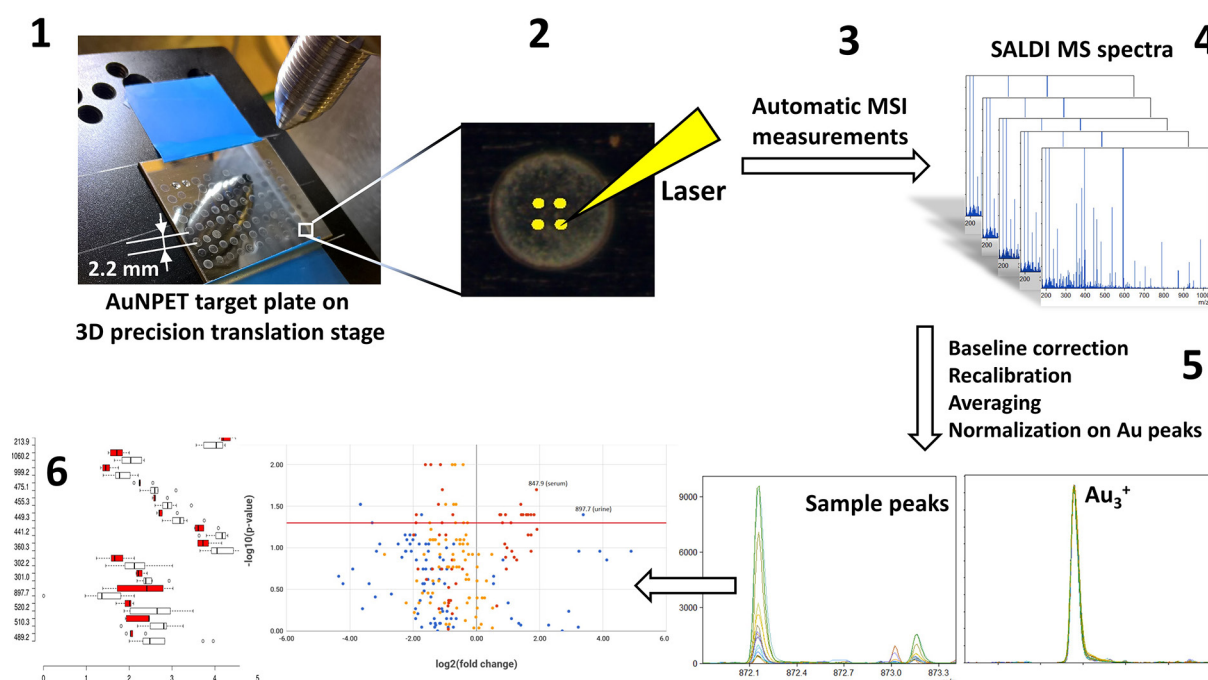


Figure 3. Flowchart for MSI analysis of interstitial fluid, urine and blood plasma samples on AuNPET target. Step 1: semi-manual application of diluted samples onto AuNPET target with the aid of 3D translation stage; 2: programming of four-pixel array per sample spot; 3, 4, 5: LDI MS measurements in imaging mode producing series of spectra; 6: data processing. MSI: mass spectrometry imaging; AuNPET: gold nanoparticle-enhanced target; SALDI: surface-assisted, laser desorption/ionization; LDI: laser desorption/ionization

ensure further development. First adaptive mechanism has been described by Otto Warburg in 1920 who discovered that malignant cells produce energy predominantly through anaerobic pathway (glycolysis), even in the presence of oxygen (Warburg effect). This increased utilization of glucose in cancer cells formed basis for positron emission tomography (PET), nuclear medicine imaging system, which uses radiolabeled analog of glucose to diagnose cancer. However, since then numerous studies have demonstrated association between alteration in glutamine, lipid and cholesterol metabolism and cancerogenesis. This is accompanied by increased uptake of exogenous lipids and activation of its intracellular synthesis. With respect to PCa, alterations of lipid and cholesterol metabolism has been broadly studied. Fatty acid beta-oxidation pathway is believed to be dominant energetic source for PCa, which is considered to be non-glycolytic type of tumor^[27,28]. This hypothesis is supported by low uptake of fluorodeoxyglucose on PET imaging^[29].

Dysregulation in cholesterol metabolism has been implicated in the pathogenesis of PCa. It is essential for cell proliferation by participation in membrane formation and steroid biosynthesis which is cornerstone of prostate cancerogenesis. It is also believed that it can play a major role in cancer progression to the castration resistant state by fueling intratumoral androgen biosynthesis in androgen deprived patient^[30,31]. This is possible due to activity of CYP17A1 enzyme, which is overexpressed in castrate resistant PCa^[32]. Cholesterol biosynthesis pathway including production of squalene by condensing two molecules of farnesyl pyrophosphate by squalene synthase (farnesyl diphosphate farnesyltransferase 1). Squalene is then transformed with the aid of squalene monooxygenase (SQLE) to lanosterol. In the final step, lanosterol is converted to cholesterol through a 19-step process.

Decreased cancer/control ratio of serum squalene was observed in this study. Increased utilization of squalene by PCa cell should be accompanied by increased activity of SQLE. This observation is consistent with the study by Stopsack *et al.*^[33] which showed that men with high SQLE expression are 8.3 times more likely to develop lethal PCa.

Elevated serum cholesterol and triglycerides (TG) has been linked with increased risk of developing aggressive PCa and recurrence after radical prostatectomy^[33-36]. However there is discrepancy between studies concerning association between serum lipids level and overall risk of developing PCa^[37-39].

Eberlin *et al.*^[40] showed that cholesterol sulfate (CS) which is the product of cholesterol sulfotransferase - SULT2B1 (within cells cholesterol occurs in form of esters, CS or hydroxycholesterol), accumulates in PCa and achieves higher concentration in comparison to benign prostate tissue. However, in our study, levels of CS in biofluids of cancer patients have not been elevated. Moreover, in comparison to control, a statistically significant decrease of intensity in urine has been observed ($P < 0.05$).

Another interesting aspect of affinity of PCa cells to adipose-rich environment is fact that most common metastatic sites are lymph nodes and bone marrow. This environment provides sufficient TG which are hydrolyzed to fatty acids to fuel mitochondrial beta-oxidation and ATP generation^[28,41]. This observation initiated research of new generation of drugs that target lipid metabolism^[28,42]. In this study increased intensity of TG in urine of PCa patients has been observed.

Fatty acids transport inside mitochondria is possible by its conjugation to carnitine. Giskeødegård *et al.*^[43] reported elevated serum level of four acyl carnitines in PCa patients in comparison to benign prostatic hyperplasia (BPH) control and Saylor *et al.*^[44] observed decrease in serum acyl carnitines concentration after 3 months of androgen deprivation therapy. In comparison, no acyl carnitines was elevated in our study, moreover, serum nonanoylcarnitine intensity of PCa patients was decreased in comparison to control ($P = 0.05$).

One of the most studied dietary-factor related with PCa risk is daily intake of omega-3 fatty acids, however results are still inconsistent. Only alpha-linolenic acid (ALA) out of all n-3 polyunsaturated fatty acids (ALA, eicosapentaenoic acid, docosapentaenoic acid, and docosahexaenoic acid) is essential (cannot be synthesized in the body and must be obtained from food). Recent meta-analysis showed only marginally significant negative association between PCa risk and intake of ALA and no significant association between ALA blood concentration and PCa risk^[45]. In this study serum ALA intensity was statistically significant lower in patients with PCa in comparison to control ($P < 0.05$).

Relationship between vitamin D (and its active metabolite - calcitriol) intake and its serum level and risk of PCa has been broadly studied. Vitamin D deficiency is associated with increased odds of PCa on biopsy and it is positively associated with higher PCa grade and stage^[46,47]. Moreover, calcitriol used alone or as a part of combination therapy is being studied in clinical trials to treat PCa^[48-50]. This is explained by the effect of vitamin D in promoting cellular apoptosis and differentiation and inhibiting cancer proliferation and angiogenesis. By decreasing expression of COX-2 gene it reduces proliferative stimulus of prostaglandin, a COX-2 product^[51,52]. Our study supports observation that serum vitamin D deficiency is relevant in pathogenesis of PCa.

Another interesting observation is higher abundance of vitamin C metabolites in the serum of patients with PCa. Relationship between daily intake of vitamin C and risk of developing PCa is highly debatable and the results are conflicting. Its antineoplastic properties are attributed to its antioxidative properties which protects DNA and RNA from oxidative damage. However at high doses it may act as a pro-oxidant rather than antioxidant^[53]. In our study elevated serum concentration of vitamin C metabolites in serum of patients with PCa has been observed.

The proliferative state of carcinoma requires both amino acids and nucleic acids to fuel cell division. We observed increased level of different amino acids in serum and simultaneously its decreased level in urine

and IF. Interestingly commercially available urine test for patients with negative DRE examination and modestly elevated PSA level has been developed which is based on a panel of four amino acids: sarcosine, alanine, glycine and glutamic acid (Prostarix™). Accumulation of modified nucleotides in a chemical process of deamination is characteristic for cellular processes involving DNA and RNA^[54]. Deaminated nucleotides (such as dITP) can be incorporated into either DNA and RNA which may result in nucleic acid instability, mistranslation and strand damage which may induce cancerogenesis^[55]. In this study we observed elevated level of dITP in serum of patients with PCa.

In conclusion, this study identifies comprehensive metabolomic profile of PCa. To the best of our knowledge, this is the first study which examined PCa IF. Moreover it combines simultaneous metabolomic analysis of serum, urine and IF, which gives most accurate insight into prostate cancerogenesis. Gold-nanoparticle-based laser MSI allowed identification of 20 metabolites which are up- and down-regulated. Identified metabolites mentioned in this study suggest their role in PCa pathogenesis and should be further evaluated in clinical trials as potential PCa biomarkers. The limitations of this study were the relatively small number of participants and the fact that small percentage of intensity values can be age-related for urine and plasma samples.

DECLARATIONS

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Authors' contributions

Sample collection: Ossoliński K, Ossolińska A, Ossoliński T

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Ethical approval and consent to participate

The authors state that they have obtained the appropriate consent of the local bioethics commission to carry out the research and in addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Consent for publication

Not applicable.

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Case Report

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Axillary recurrence after intramammary sentinel lymph nodes metastases with capsular extravasation

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Abstract

Axillary recurrence is a rare event in patients treated with sentinel lymph node biopsy alone with the majority occurring in the first 5 years after surgery. Intramammary lymph node (IMLN) can be the primary sites of metastasis and sentinel lymph nodes, but the clinical significance, including prognosis and therapeutic approach is yet unclear, even more with capsular extravasation. IMLN metastases are strongly correlated with axillary lymph nodes involvement and therefore a guide for further surgical management of the axillary nodes.

Keywords: Breast cancer, sentinel lymph node, lymph node dissection, metastasis, locoregional neoplasm recurrence

INTRODUCTION

Reported series of patients with intramammary lymph node (IMLN) diagnosed by final histological examination are small in number and clinical significance of metastasis is still unclear^[1]. We previously reported a case of conservative breast cancer surgery with 3 positive IMLNs demonstrating extracapsular extravasation (ECE). After multidisciplinary discussion, the patient was submitted to mastectomy and no further axillary dissection. Even though the 3 IMLNs were positive, pathology examination did not reveal any signs of malignancy in the mastectomy specimen. The patient underwent adjuvant chemotherapy, radiation and endocrine therapy^[2]. Here we report the new event after 2 years of follow up.



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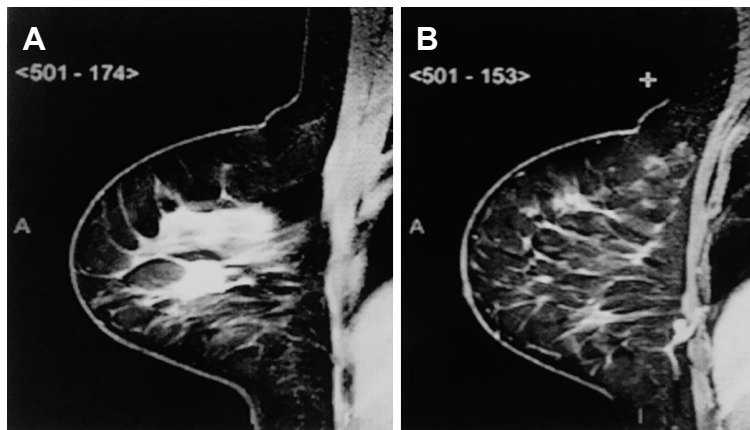


Figure 1. A: Tumor magnetic resonance imaging, axial; B: intramammary sentinel lymph node magnetic resonance imaging, axial view

CASE REPORT

A 44-year-old, post-menopausal female with a mammogram showing a partially defined lump of the right breast. Breast ultrasound (US) showed a circumscribed hypoechoic lesion, 1.4×1.1 cm, 3.8 cm away from the areolar complex, and also an adjacent hypoechoic microlobulated lesion of $1.4 \times 1.2 \times 1.4$ cm, both in the outer quadrants of the right breast. US-guided core-needle biopsy of the lesion revealed a high grade invasive ductal carcinoma (IDC), luminal B (estrogen receptor 30%, progesterone receptor and human epidermal growth factor receptor type 2 negative, and Ki-67 of 80%).

Magnetic resonance imaging (MRI) showed a mass with spiculated contours at early and heterogeneous post-contrast enhancement in the junction of the outer quadrants of the right breast, measuring $1.8 \times 1.6 \times 1.5$ cm [Figure 1A and B]. In addition, a circumscribed oval mass at early and homogeneous post-contrast enhancement, in lower inner quadrant, measuring $9 \times 5 \times 5$ mm and in close contact with the pectoralis muscle, which seemed to correspond to an IMLN. Second-look US was performed directly to this lymph node, which revealed a suspicious cortical thickening and a decreased hilum. A breast conserving surgery of the index lesion, plus radioguided occult lesion localization of the suspicious lesion in the lower inner quadrant and sentinel lymph node biopsy (SLNB), were performed. The pathology report of the lesions identified an IDC, no special type, histology and nuclear grade III and ductal carcinoma *in situ* associated. Three axillary sentinel lymph nodes (SLNs) were free of metastases, however the suspected lesion in the lower inner quadrant resulted in 3 IMLNs, all affected by cancer metastases with ECE [Figure 2A and B].

Published literature for IMLNs does not mention capsular leakage, thus, there is no consensus for the best treatment. Pathologists admitted margins in lymph nodes were not frozen, which did not make it feasible to know if the margins of the additional lesions were disease free. Multidisciplinary recommendation was mastectomy without axillary lymph node dissection (ALND) and immediate breast reconstruction with implants, which was performed two weeks after the patient's consent in regards to the unknown probability of further disease in the IMLNs. Pathology report revealed no evidence of malignancy. The patient underwent 4 cycles of anthracycline + cyclophosphamide followed by 12 cycles of taxanes and breast + axillary, internal mammary and supraclavicular drainage chain radiation therapy + tamoxifen. After 2 years of follow up, the patient suddenly developed axillary pain and fistulization accompanied by fever, and sought the emergency room immediately [Figure 3A]. US and breast MRI showed an axillary lymph node with high T2 signaling, measuring 4.0×3.6 cm in deep contact with pectoralis muscle and a circumscribed oval mass at early and homogeneous post-contrast enhancement, located in the level 2 of the axilla, measuring 1.0×0.9 cm.

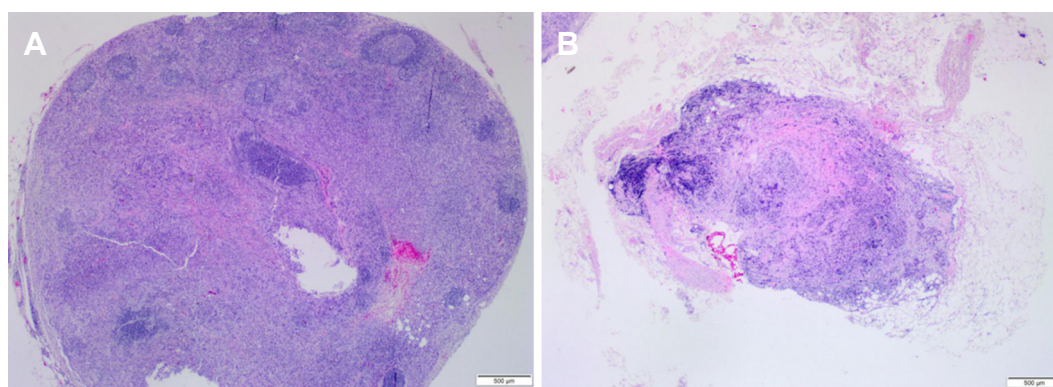


Figure 2. A: Intramammary lymph nodes affected by cancer metastases (3/3): capsular extravasation; B: capsular extravasation

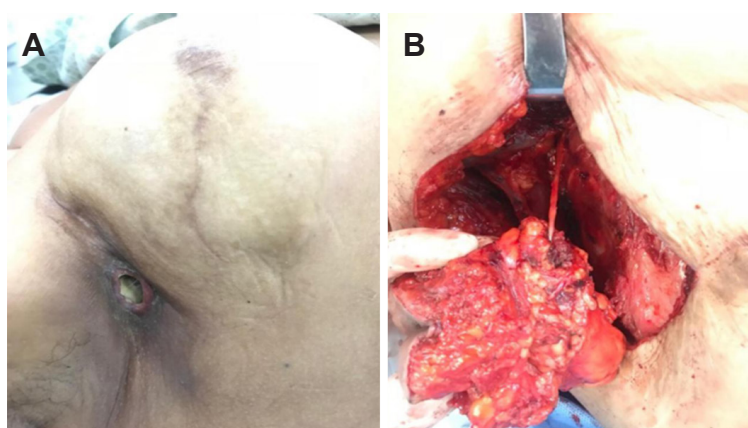


Figure 3. A: Clinical presentation of axillary recurrence with fistulization; B: axillary lymph node dissection

Removal of the implant and ALND with primary closure of the chest wall was performed. Pathology report showed fragment of fibroadipous and muscular tissue compromised by carcinoma, associated to intense chronic inflammatory process abscessed. Skin and 3 axillary lymph nodes level 2 and 3 free of metastasis [Figure 3B].

DISCUSSION

IMLNs are important because they can be the primary sites of metastasis and SLNs, and when the image is identified, the presence of metastasis is more frequent than in IMLNs detected incidentally^[1]. They are involved in a variety of clinical situations, including benign lesions, tumor metastases, breast lymphomas and breast cancer, where their importance is not fully established. Nevertheless, there is evidence that IMLNs are an independent factor for poor prognosis, and they may change therapeutic decisions^[3]. Patients with metastatic IMLNs had more aggressive cancers with lymphatic and vascular invasion as well as increased axillary lymph node metastases^[4]. In a multicenter study including 33,000 SLN-negative cases from 61 observational series analysis that did not go to ALND reported an axillary recurrence (AR) rate around 0.6%^[5]. The NSABP-32 trial showed that SLNB minimized side effects with equivalent survival and regional control than ALND in the SLNB-negative setting and then quickly became the standard of care in early stage breast cancer clinically node-negative^[6]. AMAROS trial included 1,425 patients with T1-2 breast cancer, no palpable nodes and positive SLNs. Patients were randomized to ALND or axillary radiotherapy and had a median follow up of 6.1 years. No differences in 5-year AR rate was observed between the ALND vs. radiotherapy group^[7].

A retrospective study from the National Cancer Data Base evaluated women with clinically node-negative breast cancer who had nodal macro or microscopic metastases in the SLN. There were no significant differences in AR with the addition or not of ALND in macrometastasis (1.2 vs. 1, $P = 0.4$) and micrometastasis (0.6 vs. 0.2, $P = 0.063$)^[8]. When IMLN metastases were identified in the breast specimens, it has been suggested that complete ALND may be based on the axillary SLN negative status and thus, ALND can be avoided in this setting^[9]. Even though our case has not demonstrated malignancy at the surgical site, ECE is considered a sign of worse prognosis, and might be the evidence that leads us to the AR of this patient. Moreover, there is not any standard definition of ECE in the literature. Some authors have used “focal” or “extended” definition with no description regarding adjacent sites of invasion eventually^[10]. Standard definition of ECE must be identified, because the ECE might be considered by future staging systems^[11].

However, it's impossible to conclude that IMLNs metastases featuring ECE might be an independent outcome factor and its clinical meaning is yet controversial and unknown.

DECLARATIONS

Authors' contributions

Sample collection: De Alcantara Filho PR, Bezerra SM

Analysis of medical aspects of research, data analysis: De Alcantara Filho PR

Availability of data and materials

All the data in this article is available to the readers.

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Ethical approval and consent to participate

Patient consented to participate in a research study.

Consent for publication

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Review

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Cancer stem cells, stemness markers and selected drug targeting: metastatic colorectal cancer and cyclooxygenase-2/prostaglandin E2 connection to WNT as a model system

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Abstract

Few studies have reported on the analyses of drugs targeting enriched populations of cancer stem cells (CSCs) as a means for identifying potent anti-CSC agents. This review evaluates recent information on the identification and functions of specific CSC surface markers, with particular emphasis on colorectal cancers and the screening of drugs to eliminate such cells. Many of these CSC markers are found commonly expressed on CSCs from different cancer types as well as embryonic stem cells. These markers are often related to hypoxic activation of the WNT/ β -catenin pathway, cyclooxygenase-2/prostaglandin E signalling and their relationship to LGR5. By effectively using drugs that inhibit these pathways to kill the CSC population, or otherwise forcing them out of dormancy into active cell division, cancers should become more susceptible to chemotherapy. Such combinational therapies targeting both CSCs and proliferating tumor cells should greatly improve upon the current basis for treatment.

Keywords: Cancer stem cells, colorectal cancer, markers, selective drug targeting

GENERAL BACKGROUND TO CANCER STEM CELLS

Our understanding of the roles played by cancer stem cells (CSCs), their importance during the progression of cancer and justification for why they should be specifically targeted to eliminate cancer as a disease remains limited. Evidence supporting the “cancer stem cell” hypothesis is mounting such that CSC existence



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has become generally accepted. Analysis of PubMed reveals the increasing importance of CSCs with over two thousand publications in 2008 increasing to nearly 7000 annually at the present time and 70,831 in total. The involvement of the CSC population and their contribution to the circulating tumor cells (CTCs) and metastatic cancers is also becoming established. After therapy, drug resistant and dormant CSCs reactivate as major contributors towards tumor recurrence, generating further tumor masses. From this latter perspective we propose that it will be essential to not only identify the CSCs, but also the means for selectively targeting these cells to eliminate them if we are to improve upon current cancer therapy. In recent years, several reviews have covered the identification of markers for colorectal CSC (CR-CSC)^[1], including that of the definitive colorectal cancer (CRC) stem cell marker, LGR5^[2-4].

In 2010, the question was posed: “How can we identify and analyze colon CSCs and what agents are being designed to kill this chemotherapy-refractory population?”^[5]. The CSC model accounts for tumor initiation, metastasis, drug resistance, and relapse and CSCs within the tumor bulk have the capacity to self-renew, differentiate, and give rise to new tumors (reviewed in^[6-8]). In the present systematic overview, the focus is the identification and drug targeting of CSC populations and developing strategies for improving the therapy of advanced stage colorectal cancer by eliminating CSCs. Controversy has grown around the proposal of targeting the CSC population and whether it would be beneficial to patient outcomes^[6]. In their treatise on the problems with the CSC model, these authors pointed out tumor heterogeneity and growth dynamics, which can be interpreted as cell plasticity in that cancer cells in a more differentiated state can re-enter the CSC pool. In addition, they argue that targeting a rare population of tumorigenic cells (such as CSCs) without consideration of the bulk of proliferating cells may not change patient outcomes^[6]. By necessity, the principle of clonal extinction applies in terms of the need to eliminate all of the cancer cells, including the CSCs and their differentiated progenitors if we are to overcome cancer^[7]. Simply put, one must kill the roots of the tree, or it will grow back [Figure 1] and was originally proposed as the “Dandelion hypothesis” for CSCs^[8]. This will require not only killing the rapidly dividing, more differentiated cancer cell population as is currently targeted by commonly used chemotherapies, but also the more dormant and drug resistant CSCs existing within the tumor population.

One of the key aspects of CSCs that should be borne in mind is their property of “stemness” and by this is meant not only their capability of self-renewal and differentiation, but also that despite being sourced from different tissue origins, the various types of CSCs with their less differentiated phenotypes, will share properties and markers in common with embryonic stem cells (ESCs), reflecting their similarity in terms of earlier stages of development^[9,10]. It is only when the CSCs differentiate that they will express greater sets of uniquely characteristic markers for the particular cell lineages that they encompass^[11]. It is in this light that the markers for the CR-CSCs are discussed below, and are discussed in reference to related findings of similar markers found expressed on the ESCs and CSCs alike across a range of different cancer types^[9,10,12].

CSC IDENTIFICATION AND GENERAL DEFINITION

The definition of CSCs is similar to that applied to the normal tissue stem cells: the ability of a small sub-population of cells existing within a tissue which when isolated and reintroduced into the host have the capacity to reform complete tissues containing the range of cellular phenotypes similar to the original tissue from whence the stem cells were derived^[12,13]. Thus, CSCs possess characteristics associated with normal stem cells, specifically multipotency in the ability to give rise to all cell types found upon pathological examination of an excised tumor^[11]. The most commonly used method for identifying and isolating CSCs is via their characteristic cell surface markers, many of which were originally discovered from studies of normal tissue development, including from hematopoiesis and embryonic stem cells and which are also found co-expressed on CSCs^[9].

The major surface markers that have been used to identify colorectal CSCs to enable their definitive isolation are the focus of this review, as well as improved means for testing anticancer cytotoxic drugs targeting

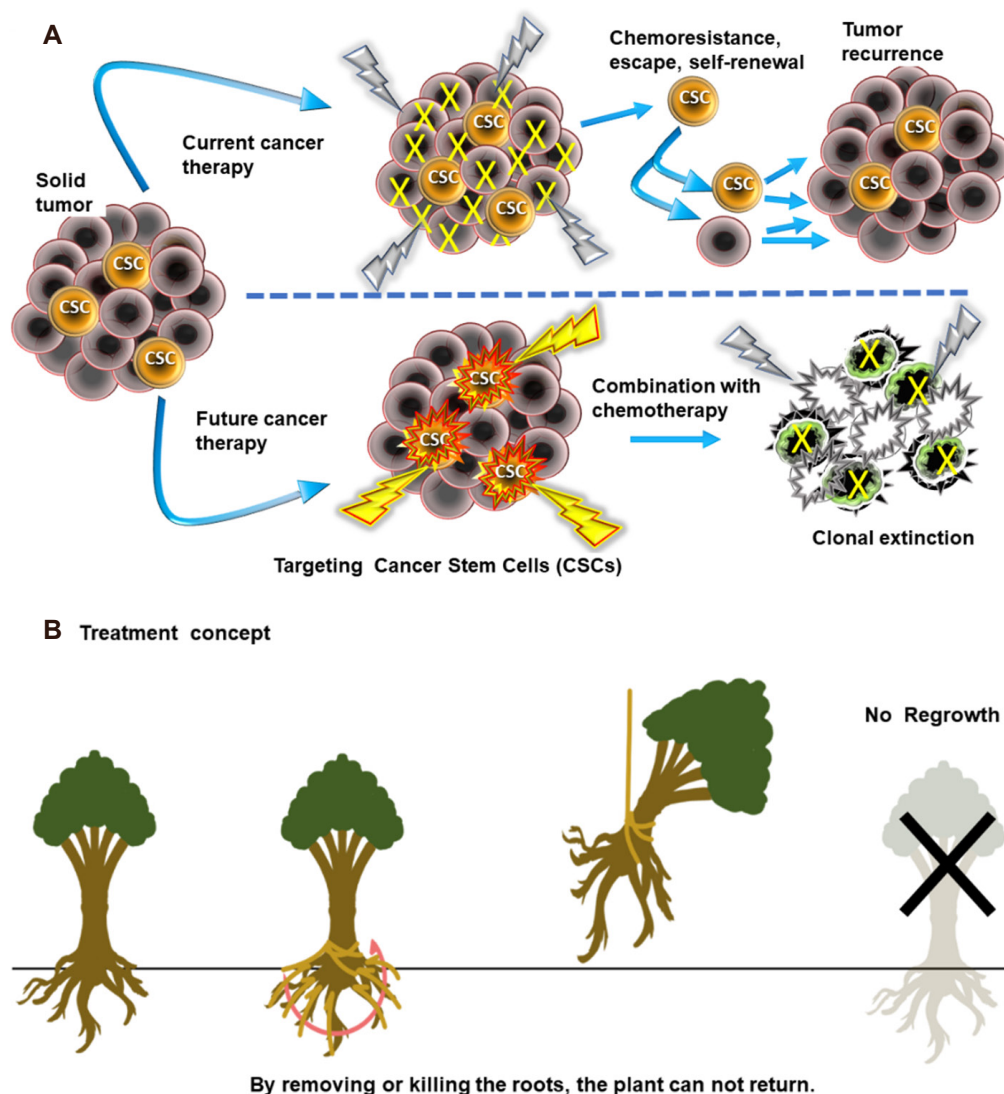


Figure 1. A: Targeting cancer stem cells: current treatments with conventional chemotherapy are not highly efficient against the cancer stem cells (CSCs) residing in tumors which resist chemotherapy or radiation and post-treatment will undergo self-renewal, differentiation and tumor regrowth or metastasize causing relapse and formation of additional tumors. However, drugs targeting the CSC population (such as celecoxib) can be used to eliminate the CSCs, either by direct cytotoxic effects or by sensitizing these tumor cells to other chemotherapy, resulting in the clonal extinction of the entire tumor cell population; B: The treatment concept of targeting CSCs (also known as the “Dandelion hypothesis”) by analogy proposes that by eliminating the roots (CSC population), the plant (tumor) can not become re-established whereas cutting the flowering stalks, stem or branches away will allow regrowth^[8]

their elimination. These findings should then be applicable to other CSC types. Historically, CSCs were extracted from solid tumors and partially purified as a poorly or negatively staining side population (SP) by flow cytometry, so-called because of their hallmark characteristic to exclude the nuclear DNA staining, fluorescent Hoechst series of dyes, such that CSCs could be separated from the bulk of other tumor cells that were highly positive for the nuclear DNA stain^[14]. These poorly stained “SP” CSCs often showed much greater stem-like self-renewal and increased tumor-initiating capability and were responsible for tumor recurrence because they were more resistant to many forms of chemotherapy^[14].

Other methods have also been used to enrich for CSCs, including growing tumor cells suspended in serum-free defined media or above agarose to form spheres or spheroids or by selective uptake of the Aldefluor™ stem cell stains because CSCs show greater expression of aldehyde dehydrogenase 1^[15]. The use of such methods for the identification and definition of CSCs has been complicated by observations that in the

case of leukemias, only a few cancer cells isolated from the blood of leukemic animals were sufficient for transferring the leukemia into naïve healthy animals receiving the transfused cells^[16]. Thus arose the basis for disputing the very existence of CSCs and whether all cancer cells are self-endowed with the capability of giving rise to whole tumors.

Since the discovery of CSCs, we have witnessed further complexities with the emergence of the epithelial to mesenchymal transition (EMT) hypothesis by which it was proposed that tumor cells transit back and forth between the two different states during the processes of invasion, migration and metastasis to form tumors at distant sites^[17]. However, this view has been made less certain given recent evidence showing that multiple phenotypes are present within both the tumors and CTCs, which exist rather as mixtures of cells including both epithelial and mesenchymal types^[18]. The emerging evidence from a range of studies would rather suggest a dynamic tumor heterogeneity with cancer cell plasticity existing as the consequence of genetic and metabolic changes, environmental differences and reversible adaptation of cellular properties, all proceeding within the context of a hierarchical system of tumorigenic CSCs differentiating into non-tumorigenic progeny (for reviews, see^[19-21]).

During the course of progression within the primary tumor microenvironment, with expanding growth and increasing cellular demands on the limited availability of nutrients and oxygen supply, there follow frequent rounds of intermittent hypoxia, hypoglycemia and acidosis occurring within the tumor microenvironment^[22]. Such conditions are the drivers for the production of greater numbers of the CSCs as a percentage of the total tumor population (reviewed in^[23]). In addition, such stressors within tumors give rise to cancer cells showing a higher capacity to invade into nearby tissues, extending beyond the tumor boundary, migrating from the site of the primary tumors into the circulatory system to produce metastases. In this manner, the tumor spreads into distant locations, some involving advanced release of exosomes, interactions with immune cells and extracellular matrix to lay down the framework for future tumor beds at these remote sites as premetastatic niches^[24].

Recent evidence has shown that the CTCs have a stem cell phenotype as evidenced by their expression of embryonic transcription factors (including OCT4, SOX2 and NANOG^[25]), and contain mixtures of epithelial and mesenchymal phenotypes (reviewed in^[26-28]). Furthermore, analyses of CTCs derived from the peripheral blood of colorectal cancer patients showed that they exhibit a more CSC-like phenotype^[28], including expression of CSC markers such as LGR5, establishing these as prognostic markers for disease progression and metastasis in CRC patients. From these observations, it would appear that the CTC population includes CSCs in their mix and that such cells are highly metastatic [Figure 2]. Despite these complexities, our ability to identify and target the CSC populations with specific cytotoxic drugs must improve if we are to eliminate the cause of cancers and their metastasis, given their significantly enhanced tumor-initiating potential.

CR-CSCS, WNT SIGNALLING AND SURFACE MARKERS IN COMMON WITH OTHER CANCER TYPES

The self-renewal of CSCs is regulated by various modulators, including WNT/ β -catenin, Notch and Hedgehog signalling, and at the transcriptional level by the pluripotency transcription factors such as OCT-3/4, KLF4, SOX2, and c-MYC, chromatin remodeling complexes, and non-coding RNAs (reviewed in^[29]). WNT signalling cascades cross-talk via the fibroblast growth factor (FGF), Notch, Hedgehog and transforming growth factor beta (TGF β)/bone morphogenetic protein signalling cascades to regulate expression of the functional CSC surface markers that have been identified, such as CD44, CD133 (PROM1), epithelial cell adhesion molecule (EPCAM) and LGR5 (GPR49) (reviewed in^[30]). The CSC regulatory signalling pathways such as WNT have become a target with implications for therapeutic interventions in cancers, albeit that its complexity poses significant challenges^[31]. The WNT/ β -catenin pathway is involved in the regulation of CSCs as with normal stem cells and their differentiation during embryogenesis and in the adult^[30,31].

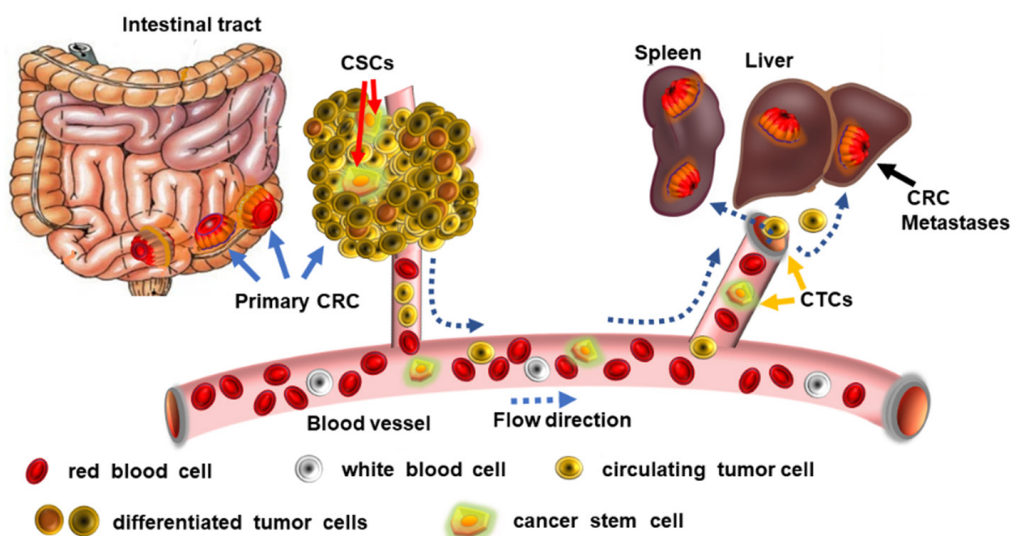


Figure 2. Circulating tumor cells (CTCs) include cancer stem cells (CSCs): tumor cells can migrate around the body via the blood vessels and this population includes CSCs that metastasize to invade into other organs, forming tumors by colonizing at distant sites such as in the spleen, and the liver. By applying drugs that are cytotoxic for CSCs (such as celecoxib) greatly reduces the incidence of tumor metastases and tumor recurrence. CRC: colorectal cancer

The great majority (> 90%) of colorectal cancers have mutations in one of two genes involved in the WNT signalling pathway: either in the adenomatous polyposis coli or β -catenin (CTNNB1) genes and this has meant that WNT signalling has become an important cancer therapeutic target^[32]. In 2016, studies examining the role of the Traf2- and Nck-interacting kinase (TNIK), an essential regulatory component of the WNT regulated T-cell factor-4 (TCF-4) and β -catenin transcriptional complex, showed that the small drug molecule NCB-0846 inhibited TNIK and abrogated colorectal cancer stemness^[33]. This drug was potent at inhibiting the growth of colon cancer patient derived xenograft tumor models and resulted in smaller tumors. Hence, based on the above studies it is clear that WNT signalling plays a key role in the regulation of CR-CSCs. We ascertain that the relationship of the CSC markers to colorectal cancer provides a unique opportunity for anticancer drug discovery and on this basis the CR-CSC markers that have been identified to date are discussed further in the next sections. Their relationship to the WNT/ β -catenin pathway for stem cell regulation is also highlighted where relevant.

The ATP binding cassette transporters/multiple drug resistance/P-glycoproteins

As described above, the ability to exclude the fluorescent nuclear DNA stains was an early hallmark of CSCs and has since been ascribed to their higher surface expression levels of the ATP binding cassette transporters (ABC) (a.k.a multidrug resistance or P-glycoproteins)^[34]. These proteins are expressed on the outer cell membrane and will pump compounds such as chemotherapeutic drugs or DNA stains out of the cancer cells. One member of the family, ABCG2 (a.k.a. breast cancer resistance protein) was shown to expel a wide variety of exogenous and endogenous compounds from liver CSCs^[35] and is prevalent on human colorectal cancer samples^[36]. Membranous ABCG2 levels of expression in colorectal cancer independently correlate with shortened patient survival times^[37].

The ABC transporters play significant roles in the distribution, absorption and elimination of substrate drugs and bestow multidrug resistance to cancer cells by maintaining the export or efflux of chemotherapeutic agents, preventing entry thereby avoiding attaining the toxic levels inside the cancer cells required to kill them. ABCG2 overexpression has also been observed in some human cancer cell lines and is widely expressed on liver cancer stem cells^[38]. Consequently, the ABC transporters are viewed and regarded as possible universal biomarkers for stem cells, both normal and cancer stem cells alike^[39]. Moreover, the

evidence supports ABCG2 as playing a vital role in enhancing stem cell proliferation and in the case of esophageal squamous carcinoma has been shown to be required for the maintenance of the stem cell phenotype^[39,40].

Therefore, ABCG2 has applications not only in the identification of the CSCs, but also for allowing the possibility of blocking its function as one way for improving selective drug targeting of tumors and their CSC population during the course of anticancer chemotherapy^[41]. Despite the supportive evidence that ABCG2 is a stem cell marker, there are limitations since the exact role of the ABCG2 signalling pathway and its regulation is not well understood^[42,43]. Although the molecular mechanisms regulating ABCG2 expression are not clearly understood, it is likely that the MYC oncogene plays an important role in promoting its expression in some cancers^[44].

In studies of “SP” cells isolated from human colorectal cancer cell lines, it was shown that expression of ABC transporter genes, such as ABCB1 and ABCG2 was significantly higher, linked with greater resistance to 5-FU and irinotecan, and higher activation of the WNT signalling pathway, than for the non-SP cells^[45]. In addition, silencing β -catenin expression to inhibit WNT decreased significantly more SP cells than non-SP cells, decreased transcription of the ABC transporter genes and the silenced cells became relatively sensitive to paclitaxel and irinotecan. Hence, these results indicate that ABC expression is regulated by WNT signalling. EMT induces a switch in WNT signalling from a β -catenin/E-cadherin/Sox15 transcription factor complex to the β -catenin/Twist1/TCF-4 complex, the latter of which then binds to promoters of the CSC-related genes^[46]. In this study, it was shown that Twist1 binding to β -catenin enhanced the transcriptional activity of the β -catenin/TCF-4 complex, including by binding to the proximal promoter region of the ABCG2 gene to promote ABCG2 expression as one CSC marker^[46].

Zhang *et al.*^[35] showed that the sensitivity of hepatocarcinoma cells to the chemotherapeutic drugs, doxorubicin or 5-fluorouracil inversely correlated with surface levels of the ABCG2 marker. The levels of ABCG2 expressed on cancer cells including colon cancer lines also closely correlated with tumorigenicity, drug resistance, proliferation and metastatic ability and therefore, the ABC transporters could be considered as critical and universal biomarkers for all CSCs^[34,35,47,48]. The results above of WNT signalling correlating with ABCG2 expression levels also support strategies aimed at inhibiting the WNT signalling pathway as a means for targeting chemotherapy-resistant colon cancer cells, including the CSCs.

CD133

CD133 (a.k.a. prominin-1) has been widely touted to be a CSC marker and was originally recognized as a stem cell marker found on rat neuroepithelial stem cells^[49]. CD133 and its role as a CSC biomarker has recently been reviewed^[50]. As a common biomarker, CD133 has been used for identifying many different types of CSCs, including those originating from gliomas^[51], colorectal^[10,52,53], lung^[54], liver^[38], and prostate cancer^[55]. However, CD133 has been shown to function in regulating glucose uptake for glucosamine production under conditions of increased glucose and glycolysis and is involved in autophagy^[56,57].

Despite the fact that CD133 can mark the tumor-initiating cell populations in several solid tumors, studies have shown that it does not play a crucial role in the process of CSC maintenance. Thus, unlike knocking down CD44 expression which inhibited tumorigenesis of CR-CRC cells, knocking down CD133 gene expression had no effect^[58]. CD133 expression is also not restricted to the stem cell population because both the CD133⁺ and CD133⁻ metastatic colon cancer cells were equally capable of initiating tumor production^[59]. Hence, CD133 expression is more a reflection of the level of glucose availability and is not necessarily a specific marker of CSCs.

CD44

CD44 is an extensively glycosylated surface protein as a major component of the extracellular matrix involved in cell-cell interactions, cell adhesion and migration and is one of the cellular receptors for

hyaluronic acid (HA), thereby linking binding to selectin, collagen, osteopontin, fibronectin and laminin in the extracellular matrix^[60]. HA binding to CD44 also facilitates complex activation of receptor protein tyrosine kinases of the epidermal growth factor receptor family in several types of cancer^[61]. Upon HA binding to CD44 on the cell surface, increased levels of cell proliferation and survival occur through downstream signal activation of the MAPK and PI3K/AKT pathways^[62]. Studies have also indicated that CD44 plays a significant role in the invasive and tumorigenic stemness capacity of several tumor cell types, including breast^[63], prostate^[64], pancreatic^[65] and mesothelioma^[66,67]. CD44 surface levels on tumor cells were shown to positively correlate with prostate cancer CTCs in the bloodstream of patients^[68].

Genetic knockdown of CD44 prevented the formation of tumors by colorectal CSCs^[58]. CD44 either alone or in combination with other cell surface markers can be used to enrich for CSCs from multiple tumor types including breast^[69,70], prostate^[71,72], colon^[73,74], pancreas^[65], and head and neck squamous cell carcinomas^[75]. However, given the relatively high levels of CD44 expressed on the cancer cell population, it would appear that CD44 is more a marker of the invasive, metastatic cell population and is not specific, per se for the CSCs.

CD13

The CD13 gene encodes the enzyme aminopeptidase N, a Zn^{2+} dependent membrane-bound ectopeptidase preferentially degrading proteins and peptides with an N-terminal neutral amino acid. CD13 is overexpressed in multiple cancer types as well as on the surface of vasculature endothelial cells in tumors undergoing angiogenesis, making it a promising target^[76]. CD13⁺ cells form clusters or foci within the tumors, are typically in the G₀ phase of the cell cycle and also found present in the fractionated “SP” of CSCs from hepatocarcinoma^[77,78]. Haraguchi *et al.*^[79] showed that CD13⁺ cancer cells surviving after chemotherapy or radiation treatment in tumors removed from hepatocarcinoma patients were found present within enriched fibrous capsular regions of the tumors and had decreased levels of DNA damage caused by reactive oxygen species (ROS). Thus, they proposed that the cancer cells appeared to be protected from treatment-induced apoptosis by expressing CD13. In their mouse xenograft models treated simultaneously with a CD13 inhibitor (either using a neutralizing antibody or the peptidase inhibitor, Ubenimex) and the genotoxic chemotherapeutic drug, 5-fluorouracil (5-FU), the combination diminished tumor levels significantly and was far more effective than 5-FU used alone^[79]. Their studies have since been confirmed in other studies where CD13 inhibitors were used as an adjuvant therapy together with ROS-inducing chemotherapy or radiation treatment as a potential treatment method that could improve survival rates of patients with hepatocarcinoma^[79-81]. The tripeptide NGR that targets CD13, conjugated with anticancer drugs has been similarly used to target human hepatocarcinoma growth by killing cancer stem cells and suppressing angiogenesis^[82]. Although CD13 expression has been associated with human colon cancer and poorer prognosis^[83], targeting its functional role as a marker on CR-CSCs has not yet been tested such that the usefulness of CD13 as a CR-CSC marker remains unclear.

LGR5

LGR5 is a member of the Leucine-rich-repeat-containing G-protein-coupled receptors (also known as GPR49) which belong to the seven transmembraneous G-protein-coupled receptor superfamily. The LGR1-5 family are regulatory receptors involved in WNT signalling^[84] and can bind to the furin-like repeat FU2 domains of the R-spondin 1-4 stem cell growth factors to potentiate WNT signalling^[85,86]. However, R-spondin 1 (RSPO1)/LGR5 can also directly activate TGF β signalling in a cooperative interaction with the TGF β type II receptor on colon cancer cells to enhance the TGF β -mediated growth inhibition and stress-induced apoptosis^[87]. Knockdown of *Lgr5* attenuated downstream TGF β signaling and increased cell proliferation, survival, and metastasis in an orthotopic model of colon cancer *in vivo*^[87]. Upon RSPO1 stimulation, LGR5 formed complexes with TGF β receptors^[87]. Hence, the net effects of LGR5 expression will also depend on its interplay with both the WNT and TGF β signalling systems as well.

LGR5 is found expressed in many organs including the brain, reproductive organs, mammary glands, the intestinal tract, stomach, hair follicles and the eyes^[88]. The *Lgr5* gene is itself a WNT signalling target and prostaglandin E2 (PGE2) treating colorectal cancer cell lines enhances LGR5 expression whereas *Lgr5* knockdown using siRNA inhibited the PGE2 survival response and induced cell death^[84]. These results suggest that the PGE2 promotion of colorectal cancer cell survival at least in part proceeds via increasing LGR5 expression. Given the relationship of LGR5 with WNT signalling and PGE2 production by cyclooxygenase-2 (COX-2), it is likely that NSAIDs cause regression in familial adenomatous polyposis patients by interfering with the LGR5/WNT mechanism for promoting cancer stemness and cell survival. X-gal staining using the transgenic *Lgr5* promoter driving β -galactosidase revealed that, following villus morphogenesis, *Lgr5* expression became restricted to dividing cells assembling in the intervillus region and within the distal small intestine^[89]. *Lgr5* deficiency is also known to cause premature Paneth cell differentiation within the small intestine, without affecting the differentiation of other cell lineages, nor the proliferation or migration of epithelial cells^[89].

LGR5 was shown to be a biological marker for intestinal stem cells in the murine small bowel of *Lgr5*-EGFP-IREScreERT2 mice, as well as being useful for cell lineage tracing studies where the LGR5⁺ stem cells developed into the different cell types comprising the intestinal crypt^[88]. LGR5 expression has been closely linked with tumorigenesis including that of liver, colorectal, as well as ovarian cancers (for review, see^[90]). In the normal intestine, LGR5 is solely expressed by the cycling crypt columnar cells and genetic lineage tracing studies identified these columnar cells in the base of the crypts as self-renewing, multipotent cells and are therefore considered to be genuine intestinal stem cells^[91,92]. LGR5 has also been shown to be expressed in the stem cell niche by actively cycling cells of the murine hair follicle where lineage tracing and transplantation studies revealed that the LGR5⁺ cells were retained for long periods and generated new hair follicles^[93]. LGR5 expression in multiple other organs has led to the proposal that it represents a global marker of adult stem cells (reviewed in^[94]).

Since LGR5 is involved in WNT signalling, it is not surprising that LGR5 and its ligands, the R-spondins are important for normal brain development^[95] and hypoxia stimulates neural stem cell proliferation by increasing hypoxia-inducible factor-1 α (HIF-1 α) expression and activating WNT/ β -catenin signaling^[96]. Hence, it is likely that WNT acts by increasing LGR5 expression to promote stemness and brain CSCs^[97]. LGR5 functions in the maintenance of brain CSCs and the transcribed levels of *Lgr5* is greater in these cells and in the case of glioblastoma has been associated with poorer prognosis^[97]. In addition, immunofluorescence staining revealed the localization of LGR5⁺ cells in sections of glioblastoma and *Lgr5* siRNA knockdown led to lower expression of the neuronal L1 cell-cell adhesion molecule^[97].

Most colorectal cancer cell lines and sporadic colonic adenomas exhibit significant LGR5 expression^[98], although this is linked with stemness and renewal but not tumor progression because overexpressing LGR5 caused greater cell-cell adhesion, reduced tumor growth, invasiveness, migration and metastasis^[98,99]. Recently, LGR5 was established as a definitive surface marker for colorectal CSCs (reviewed in^[90,100]), particularly when co-expressed with CD44 and EPCAM^[101]. In these studies, the triple positive cells exhibited more pronounced CSC-like traits with LGR5-positive subpopulations showing higher capacities for colony formation, self-renewal, differentiation, and tumorigenicity as well as higher expression of stemness genes than did any other subpopulation. Thus, LGR5 is a marker for early CSCs and relates to their WNT promoted self-renewing capacity. Enhanced LGR5 expression remains persistent during adenoma to carcinoma transition in human CRC samples, but markedly declines in the budding cancer cells at the invasive tumor fronts, and was not associated with either WNT- or EMT-signalling pathways^[98]. In the latter study, LGR5 overexpression attenuated proliferation, migration, and colony-forming capacities in colon cancer cells. Unfortunately, the levels of TGF β expressed by these cells were not analysed in these studies^[96,98,99] given the observations above that TGF β switches the role of LGR5 to inhibit growth. These

findings indicate that LGR5 can function as a tumor suppressor promoting maintenance of stemness during CRC progression and that LGR5 is a suitable marker of CSCs.

The most definitive studies for LGR5 as a CR-CSC marker were obtained by using a tamoxifen inducible suicide gene inserted into the *LGR5* gene of transgenic mice^[2,4] (reviewed in^[102]). The suicide gene could be triggered upon expression of LGR5 and activated by the injection of tamoxifen, resulting in death of the colon CSCs by tamoxifen. The more differentiated tumor cells do not transcribe LGR5 and hence, do not express the suicide gene, such that they survived. When the suicide mechanism was activated in growing tumors in mice, the LGR5-positive CSCs were eliminated. As long as the suicide mechanism was maintained then tumors regressed but when their suicide was no longer induced (by tamoxifen withdrawal), then differentiated tumor cells filled the void and reacquired CSC qualities with rapid tumor regrowth. Furthermore, it was shown that metastasis and growth of preestablished metastases was halted. These findings showed that unlike cancer at the primary tumor site, metastases were dependent on preexisting LGR5⁺ CSCs, so that once these CSCs were eliminated, reacquiring the CSC phenotype in the metastases was not possible^[4]. Hence, LGR5 is expressed in CR-CSC and these studies support the Dandelion hypothesis [Figure 1B], such that advanced metastatic CRC should be amenable to elimination by anticancer drugs that target the CSCs.

CD326 (EPCAM)

The surface marker, EPCAM (also referred to as 17-1A, TROP-1, CD326, GA733-2, KS1/4 or gp40) is a type 1 transmembrane glycoprotein and a calcium-independent homophilic cell adhesion receptor of 30-40 kDa, encoded by the *TACSTD1* gene^[103]. EPCAM is expressed by several human epithelial tissues, progenitor cells, cancer cells, stem and germ cells^[104]. This protein marker was discovered initially as an antigen expressed on colon cancers that was involved in cell-cell adhesion and the structure of EPCAM comprises an extracellular domain, thyroglobulin domains, epidermal growth factor domains, transmembrane domain and a short intracellular domain (ICD) consisting of 26 amino acids known as EpICD^[105,106]. *EpCAM* (*TACSTD1*) may be an oncogene as the encoded EpICD can be proteolytically released to signal into the cell nucleus by engagement with elements of the WNT signalling pathway including activation of the β -catenin/MYC pathway resulting in tumor cell proliferation (reviewed in^[106]). Expression of the EPCAM marker is elevated in malignant neoplasia and most cancers abundantly express EPCAM^[104].

In normal human cells, EPCAM is located in the intercellular spaces at the tight junctions formed between adjacent epithelial cells^[107]. Hence, it is inferred that EPCAM is sequestered in normal epithelial, whereas it becomes uniformly spread around the surfaces of cancer cells, where it is readily accessible to antibodies targeting it. The role of EPCAM in tumor progression and development is controversial, as is its relationship to another cell adhesion molecule, E-cadherin^[105]. EPCAM has been proposed to act as a molecule for homophilic cell to cell adhesion, which would prevent the occurrence of metastasis^[103]. It has a protective function in CRC since the deletion of *EpCAM* leads to an increased risk of developing CRC^[108] and when overexpressed on CRC cells, inhibits their metastasis^[109].

In contrast, EPCAM expression inhibits the extent of E-cadherin mediated cell to cell adhesion, enhancing metastasis^[110], although EPCAM has been proposed to bind to E-cadherin, both of which are important to the stability of the tight junctions (reviewed in^[107]). In addition, overexpressing EPCAM in tumor cells can enhance WNT signaling to promote the proliferation of cancer cells^[111]. In breast carcinoma, high levels of EPCAM expression are associated with poorly differentiated tumors^[112], development of larger cancers, nodal metastasis and poor survival of the patients^[113]. Marked EPCAM expression on breast carcinomas has been linked to poorer prognosis for both node positive and negative diseases^[114]. In 2009, the European Union approved the EPCAM antibody, Catumaxomab, a bivalent monoclonal antibody which cross-links between T cells (via CD3 receptor binding) and EpCAM on tumor cells in order to promote the elimination

of ascitic tumor cells. This therapy has contributed greatly to the management of breast cancer patients with malignant ascites and peritoneal carcinomatosis^[115]. EPCAM is also expressed by almost all human adenocarcinomas, retinoblastoma, hepatocellular and squamous cell carcinomas^[116].

EPCAM can be downregulated when cancer cells undergo EMT^[117], during the dissemination of cancer cells into other tissues. Hence, detecting cancer cells in blood as CTCs using EPCAM as a marker is likely to miss the invasiveness/migration events of tumor cells. EPCAM is also expressed by the normal breast epithelial cells, although its role in the homeostasis of normal breast tissue is unclear^[118]. In the human kidney 2 (HK-2) proximal cell line grown under hypoxic conditions, the activity of the *Epcam* promoter is increased two fold and in both HK-2 cells and primary kidney proximal cells, EPCAM protein expression is increased after hypoxia and reoxygenation^[119]. The widely distributed expression of EPCAM on most cancer cells makes EPCAM unsuitable as a specific marker of CSCs.

CD271 (p75NTR)

CD271 was identified as the low-affinity nerve growth factor receptor (LNGFR), also known as the p75 neurotrophin receptor (p75NTR) belonging to the tumor necrosis factor receptor and low-affinity neurotrophin receptor superfamily and is a marker for cells of neural crest embryonic origin^[120]. Previously, human CD271 was shown to be expressed on cells of the peripheral and central nervous system and was suggested to be involved in the survival, development as well as differentiation of neuronal cells^[121]. CD271 or LNGFR is also found expressed on a wide range of other cell types including follicular dendritic cells, mesenchymal stem cells (MSCs), astrocytes, Schwann cells, neural crest stem cells, oligodendrocytes, sensory and autonomic neurons and mesenchymal cells^[122].

Studies have shown that CD271 (LNGFR) is a predominant biomarker expressed by MSCs isolated from the bone marrow^[123]. The fibroblastic colony forming units (CFU-F) in MSC populations could be established only by the CD271⁺ cell fractionation, whereas no CFU-F's were obtained with the CD271⁻ cell population. CD271 is a highly specific marker expressed on the surface of freshly isolated bone marrow MSCs^[124]. CD271 may not be a specific marker for all tissues, because as a marker it failed to isolate MSCs from umbilical cord blood^[123].

CD271 was identified as a marker for the CSC population within human melanomas and its usefulness as a melanoma CSC marker has been well documented^[121] and was shown to be crucial for maintaining the tumorigenicity and stem-like properties of melanoma cells^[125]. CD271 has also been characterized as a stem cell marker for a small percent of cells in oesophageal squamous cell carcinoma^[126,127] and was associated with CSCs^[128] with high metastatic potential in CTCs from these cancer patients^[129]. CD271 confers an invasive and metastatic phenotype to head and neck squamous cell carcinomas through the upregulation of the EMT transcription factor, Slug^[130] which also promotes stemness. Thus, CD271 appears to be a marker of CSCs across a range of cancer types.

CD271 overexpression on melanoma cells suppressed *in vitro* activation of melanoma specific cytotoxic T lymphocytes^[131]. Moreover, the expression levels of PD-L1 and CD271 on the melanoma cells were both increased by IFN γ such that PD-L1 levels were related to those of CD271, and together with PD-L1 strongly and additively suppressed melanoma specific CTL activation^[131]. CD271 expression on melanoma cells has also been linked to p53 activity, the DNA damage response and chemotherapeutic drug resistance as well as migratory properties to form metastases^[132-134]. The higher expression levels of CD271 may determine specific properties of brain trophic metastatic melanoma cells^[135,136].

Hypoxia induced activation of the HIF-1 α and HIF-2 α as well as their target genes enhances tumorigenicity, self-renewal, resistance to chemotherapeutic drugs and metastatic potential and is associated with increased

surface levels of CD271 as a stem cell marker (reviewed in^[137,138]). However, CD271 expression has also been reported to inversely correlate with hypoxia (HIF-1 α expression) in skin samples from human melanomas analysed histologically at different stages of progression^[139] and likely reflects a delay in its expression after hypoxic induction. CD271 was noted to be highly expressed on the cells in the dermal nests of the biopsied melanomas from human skin samples^[139,140].

In this regard, hypoxia and HIF activation induce *Oct4* gene expression, which will in turn activate CD271^[141]. Thus, transfected gene expression of OCT4 caused melanoma cell dedifferentiation, acquiring features of CSCs such as multipotent differentiation capacity and expression of melanoma CSC markers, ABCB5 and CD271^[141]. Mechanistically, *Oct4*-induced dedifferentiation was associated with increased expression of endogenous stemness factors, OCT4, NANOG and KLF4, and changes in global gene expression enriched for general transcription factors. The reverse study, applying RNAi-mediated knockdown of *Oct4* in the dedifferentiated melanoma cells, led to diminished CSC phenotypes^[141]. Moreover, OCT4 expression in melanoma cells was induced by hypoxia and its expression was detected in a sub-population of melanoma cells from clinical samples^[141]. Overexpressing CD271 on human melanoma cell lines was shown to reduce their invasive and metastatic traits, whereas CD271⁺ melanoma cells showed less adhesive, more highly proliferative and invasive traits *in vitro* and *in vivo*^[142]. Mechanistically, similar to EPCAM, it was found that the CD271 ICD regulated proliferation^[143]. CD271, originally expressed in the epidermis of skin reconstructs, also disappeared when melanoma started to invade the dermis^[142]. Thus, CD271 has been identified as a suitable CSC marker, at least in the case of melanoma.

CD35 (Cripto-1)

Mouse and human Cripto-1 proteins range in sizes from 24 to 36 kDa and are found in complexes associated with other proteins of between 14 to 60 kDa. Cripto-1 is glycosylated at N- and O-linked asparagine and serine residues respectively^[144]. It is a member of the epidermal growth factor (EGF)-related family of growth factors, also known as the EGF-Cripto-1-FRL-1-cryptic (CFC) family. The EGF-CFC proteins are all associated with the cell surface and their structures including an amino terminal region, a modified EGF-like domain, a preserved CFC cysteine rich domain and a short hydrophobic COOH-terminus for attaching to the glycosylphosphatidylinositol (GPI) membrane anchor^[144]. The soluble, biologically active form of Cripto-1 generates from the detachment of the GPI by GPI-phospholipase D such that Cripto-1 can act either as a soluble ligand or a cell-membrane anchored protein^[144].

Cripto-1 was found highly expressed in a subpopulation (up to 60%) of human embryonal carcinoma cells which showed CSC-like properties, expressing OCT4, SOX2, and NANOG. Cripto-1 has a role in the maintenance of stem cells and their malignant progression^[145] as a multifunctional modulator during embryogenesis, as well as being important for oncogenesis^[144,146]. In the process of embryogenesis, Cripto-1 plays an important role in association with the TGF β ligand, Nodal^[147]. Cripto-1 is highly expressed in many different human tumors, findings consistent with its role as a marker of the undifferentiated CSCs^[148-150].

Cripto-1 is one of the master regulators of embryonic development along with the Notch/CSL, and WNT/ β -catenin systems^[151]. Hence, Cripto-1 is a surface marker for the identification of stem and quiescent cancer cells including the CSC phenotype in colorectal cancers and similar to the other CSC markers LGR5, EPCAM and CD271 described above, Cripto-1 expression is also up-regulated by hypoxia when HIF-1 α binds to the hypoxia response elements^[145,152] within the promoter of human or mouse Cripto-1 genes^[153]. Hypoxia can enhance production of beating cardiomyocytes and modulates the differentiation of mouse ESCs and Cripto-1 is required for the hypoxia-induced differentiation of mouse ESCs into cardiomyocytes, helping to regulate stem cell self-renewal and proliferation^[153]. It follows that Cripto-1 could be a potential marker for sub-populations of tumor cells with stem-like properties.

CD15 (stage-specific embryonic antigen 1)

Stage-specific embryonic antigen 1 (SSEA1; also called CD15 or non-sialylated Lewis X antigen) refers to a carbohydrate based antigenic epitope (3-fucosyl-*N*-acetyl-lactosamine) synthesized by fucosyltransferases (FUT4, FUT9) and is linked with cell adhesion, differentiation and migration. This marker is associated with the proteoglycan, phosphacan (a.k.a. protein tyrosine phosphatase receptor-Z1; RPTPZ1; PTPRZ1) which regulates the proliferation, self-renewal and differentiation of stem cells^[154]. RPTPZ1 is a receptor for the extracellular matrix protein, tenascin C recently linked with EMT, metastasis, recurrence and poor survival of CRC patients^[155].

CD15/SSEA1 is expressed by murine embryonal stem cells, murine embryonic carcinoma cells, as well as human and murine germ cells^[156]. SSEA1 has been widely used as a positive surface marker for undifferentiated normal stem cells from mice but not for human stem cells^[39]. Thus, upon differentiation of the murine embryonal stem cells or embryonal carcinoma cells, expression of SSEA1 is down-regulated, whereas during human embryonal stem or carcinoma cell differentiation, SSEA1 expression is increased. The SSEA-1 marker is commonly used for identifying stem cells because it can be readily detected using the monoclonal antibody, MC-480, as a cell surface antigen involved in cell differentiation. This monoclonal antibody has also been commonly used as a standard reagent for detecting embryonic stem cells^[157].

SSEA-1/CD15/FUT4 was found commonly overexpressed on metastatic CRC patients (43%) and was associated with lower levels of intratumoral CD3⁺ and CD8⁺ T cells, as well as poorer patient outcomes in terms of responses to treatment or progression-free survival (PFS) than those CRCs that were CD15/FUT4-low or negative^[158]. Studies of brain development have shown that expression of CD15/SSEA1 is predominantly associated with *O*-mannose-linked glycans of the phosphacan/RPTPZ1^[159] and high levels of RPTPZ1 expression have also been reported present in human CRC samples^[160]. Hence, although SSEA-1 may be a putative CSC marker in the case of murine CRC, it is likely to show a more widespread expression on human CRC cells.

COX-2 AND CELECOXIB AS AN APPROVED DRUG FOR COLORECTAL CANCER, ALSO TARGETS CSCS, INCLUDING CR-CSCS

Celecoxib was approved nearly 20 years ago by the USFDA for the treatment and prevention of some forms of colorectal cancers, including familial adenomatous polyposis and sporadic colorectal adenoma. The anticancer effects of celecoxib were claimed due to its potential for inhibiting the enzyme, COX-2 in the CRC cells^[23,161-163]. COX-2 (also known as prostaglandin-endoperoxide synthase 2) uses arachidonic acid to produce the prostaglandin H₂ required to synthesize the prostanoids including thromboxane, prostaglandins such as PGE₂ and prostacyclin. COX-2 is overexpressed in many cancer cell types, including CRC^[164] when compared to levels in the corresponding normal cells. Moreover, COX-2 was recently detected in the CTC populations from CRC patients, where COX-2 expression was associated with those tumor CTCs positive for vimentin and Twist as two markers detected co-expressed at a higher frequency in patients with metastases compared to those without (72.0% vs. 42.8%)^[165].

The role of COX-2/PGE in CRC and relationship to LGR5/WNT activation and survival of CSCs

The tumor cell inhibitory mechanism of celecoxib was proposed to involve counteracting the increased levels of COX-2 enzymatic function in colorectal cancer cells [Figure 3], thereby causing the suppression of cancer cell growth (^[166], reviewed in ^[167]). As outlined above, one of the mechanisms of action by inhibiting COX-2 likely involves interfering with the WNT/PGE₂ signaling pathways that would otherwise promote greater levels of LGR5 and cancer cell stemness. Among the tumor-sustaining prostaglandins produced by COX-2, PGE₂ is associated with enhancing cancer cell survival, growth, migration, invasion, angiogenesis, and immunosuppression^[168] and can act in an autocrine/paracrine manner to promote tumor growth and survival^[169] [Figure 3]. Inhibiting COX-2 with celecoxib would thereby block the growth stimulating effects

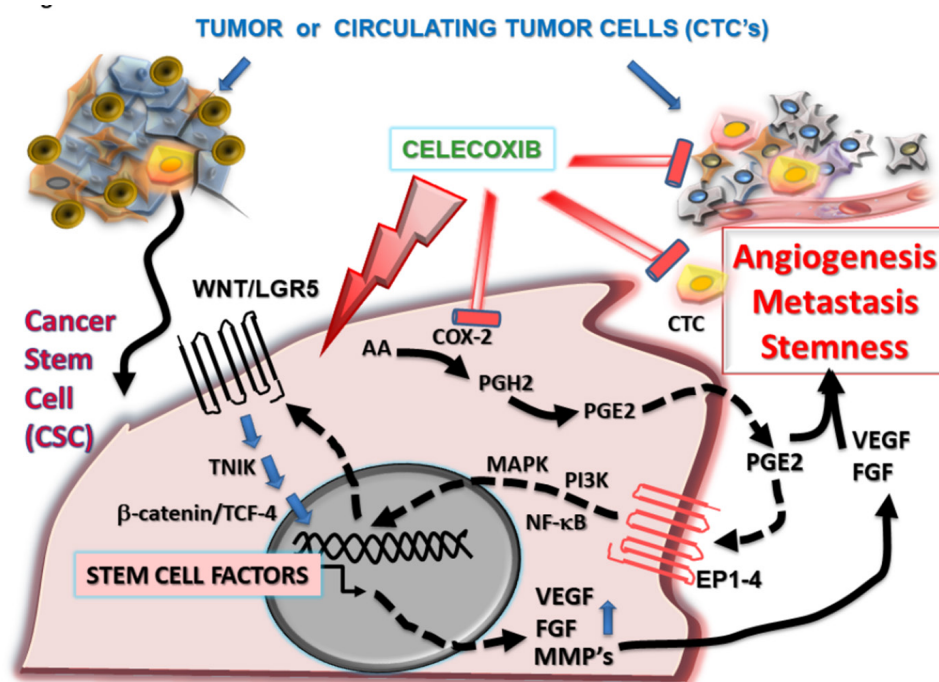


Figure 3. Celecoxib prevents CSC growth and tumor angiogenesis by inhibiting the COX-2/PGE2/VEGF and WNT/LGR5 stemness pathways. COX-2 is required for the production of prostanooids, including PGE2, which is released by tumor cells and stromal cells. PGE2 acts in an autocrine/paracrine manner binding to surface members of the prostanooid receptor family (EP1-4) to increase cancer cell stemness, angiogenesis (via production of VEGF and FGF), invasiveness (via matrix metalloproteinases MMP-2, MMP-9) and thereby promotes tumor metastasis. Celecoxib is an anticancer drug that is cytotoxic for CSCs, inhibiting COX-2/PGE2 production and stemness, thereby down-regulating the WNT/LGR5 signalling pathway, inhibiting angiogenesis and promoting the anticancer immune response. CSC: cancer stem cell; CTC: circulating tumor cell; VEGF: vascular endothelial growth factor; FGF: fibroblast growth factor; COX-2: Cyclooxygenase-2; PGE2: prostaglandin E2; PGH2: prostaglandin H2; TCF-4: T-cell factor-4; TNIK: Traf2- and Nck-interacting kinase; AA: arachidonic acid

derived from the COX-2 production of prostaglandins as cytokine-like factors stimulating growth, CSCs and metastases^[170,171]. Levels of PGE2 correlate with colonic CSC markers (CD133, CD44, LGR5, and SOX2 messenger RNAs) in human colorectal carcinoma samples suggesting that it is a marker for CR-CSC^[171]. Thus, PGE2 induced CSC expansion by activating nuclear factor-kappa B (NF-κB) via the PGE2 receptor 4 (EP4) and PI3K/MAPK signaling, promoting the formation of liver metastases by colorectal cancer cell lines injected into mice^[171].

Angiogenesis is one of the classical hallmarks of cancer, promoting new blood vessel formation from pre-existing vessels, thereby facilitating the supply of blood borne nutrients and oxygen to the tumor cells^[172]. As early as 1999, celecoxib was recognized as an important chemotherapeutic agent in colorectal cancer treatment^[173] because of its ability to potently inhibit angiogenesis by preventing vascular endothelial growth factor (VEGF) and FGF production, reducing growth of gastrointestinal cancer xenografts in nude mice^[174]. COX-2/PGE2 activation increases VEGF expression by colon cancer cells, thereby promoting tumor angiogenesis (reviewed in^[175,176]).

Evidence for COX-2/PGE playing a role in many other cancer types besides CRC and is a CSC drug target

These early findings have since been confirmed in many subsequent studies of other cancers as well including lowering microvessel density in gastric cancer models by inhibiting COX-2 mediated PGE2 production and VEGF expression^[177,178], and in head and neck^[179], pancreatic^[180], Lewis lung cancers and sarcomas^[181] (reviewed in^[182]). In more recent studies of dimethylhydrazine-induced colorectal cancer models, NSAIDs

like celecoxib decreased the rate of adenocarcinomas, markedly lowering angiogenesis parameters including VEGF, MMP-2, MMP-9 and MCP-1 levels^[174,183]. More importantly, inhibiting COX-2 with celecoxib was found to block the PGE₂-induced tumor repopulating capacity, which otherwise promoted chemoresistance in bladder cancer xenografts^[184] as well as CSC expansion and metastasis in colorectal cancer models^[171].

The role of COX-2 in CSC survival and repopulation after therapy has been reviewed elsewhere^[185], such that it has become clear that inhibiting COX-2 is an effective method for preventing treatment failure due to tumor repopulation. Moreover, celecoxib, as a selective COX-2 inhibitory drug, has also been reported to promote the apoptosis of colon cancer cells^[186]. The prostaglandin, PGE₂ produced by COX-2 activates pro-survival pathways preventing apoptosis of colon cancer cells^[187] (reviewed in^[168,188]). Celecoxib, by inhibiting COX-2 facilitates the activation of various apoptotic pathways in colorectal cancer cells, including p53, p38 and BAX pathways^[189].

Another aspect of the prostaglandins produced by COX-2 activity is that some, such as PGE₂, are pro-inflammatory cytokine-like factors that modulate the anticancer immune response^[190]. Celecoxib's anticancer inhibitory effects (resulting from lower prostaglandin levels) also include promoting an enhanced immune response. Thus, celecoxib inhibition of COX-2 prevents the switching from a Th1 to a Th2 immune response, where Th2 would be more favorable to cancer cell survival. The immune response switch to Th2 is induced by increased levels of COX-2 which also leads to decreased production of the NF- κ B activated cytokines that would otherwise induce Th1 proliferation, including interferon gamma (IFN- γ), tumour necrosis factor-alpha, and IL-2. Moreover, COX-2 activity promoted expression of cytokines including IL-6, IL-4, and IL-10 which preferentially activate Th2 type immune responses^[191], thereby enabling the cancer cells to evade the host immune system.

Tumor-associated COX activity in a mouse melanoma model driven by oncogenic mutation in *Braf* (similar to the situation in human melanoma) was shown to be a key suppressor of type II IFN (IFN- γ) and T cell-mediated tumor elimination, inducing an inflammatory signature more typically associated with cancer progression^[192]. COX-dependent immune evasion was also shown to be critical for tumor growth in a range of models including melanoma, colorectal, and breast cancers^[192]. Notably, tumor immune escape could be reversed by a combination of an immune checkpoint blockade inhibitor together with COX inhibitors. Hence, the COX-2/PGE pathway can promote tumor growth, survival and evasion of the immune response and highlights the importance of using NSAIDs such as celecoxib to inhibit the COX-2/PGE based activities in tumors.

We have shown that celecoxib has a fourth component to its anticancer function based on direct effects in killing cancer cells by targeting their mitochondria to increase the respiratory substrate driven production of reactive oxygen species such as superoxide, thereby activating the intrinsic apoptotic pathway^[23,193]. Although many of the NSAIDs show similar properties and ability to act as cytotoxic anticancer drugs (reviewed in^[194]), celecoxib was found to be by far the most outstanding^[23,193] and was highly effective at inhibiting growth of triple negative breast cancer spheroids^[195]. In this regard, in many animal models of cancer, celecoxib has been shown to chemosensitize cancer cells in a synergistic manner to enhance the cytotoxic effects of commonly used chemotherapies, such as the anthracycline drugs doxorubicin and epirubicin^[196-200] and platinum-based chemotherapies^[164,199,201-206]. In murine models of colorectal cancer, synergistic anticancer effects were obtained by combining celecoxib with the common CRC chemotherapeutic drug, 5-fluorouracil (5-FU)^[207] or with oxaliplatin^[208].

In human hepatomas, Chu *et al.*^[209] showed that celecoxib promoted death of the CSC population and suppressed stemness and progression by up-regulating the tumor suppressor function of the tyrosine phosphatase, PTEN. In related studies^[84,210], celecoxib was shown to target CSCs and suppress their self-renewal, sensitizing them against chemoresistance as well as inhibiting their EMT. Further, these studies

showed that celecoxib attenuated metastasis and tumorigenesis by inhibiting the synthesis of PGE₂, thereby down-regulating the WNT/LGR5 pathway activity, including for colorectal CSCs^[84]. Hence, celecoxib has shown a range of highly beneficial and intriguing properties as an effective anticancer drug in animal tumor models and provides an example of a drug affecting the WNT/LGR5 signaling pathway in CSCs to prevent CSC survival and metastasis.

Clinical studies of colorectal cancer patients receiving treatment with celecoxib over the long-term for several years have been successfully completed^[211-214]. In most of these studies, celecoxib was used in combination with chemotherapy and showed significant improvements with inclusion of celecoxib, resulting in decreased rates of metastases and incidence of recurrence. In a large trial from 2006, the results of the Adenoma Prevention with Celecoxib (APC study) showed that continued dosing (400 mg) of celecoxib daily in patients diagnosed with colorectal polyposis and treated over prolonged periods of three years significantly decreased the recurrence of adenoma and advanced adenoma, when compared to placebo^[215]. In follow-up studies, it was reported that although the effects of celecoxib diminished two years after treatment was halted, there was still a considerable treatment benefit at five years^[216,217]. Overall, the risk for advanced adenoma relative to placebo was lowered by 52% on low-dose 200 mg bi-daily (vs. 57% at 3 years) and 51% on high-dose 400 mg bi-daily (vs. 66% at 3 years)^[217]. Hence, long term use of celecoxib has consistently proven to significantly lower the formation of advanced colorectal cancers in such studies.

We have previously extensively reviewed the outcomes of human clinical trials with celecoxib to treat cancer and which have often shown that sustained, long-term use over several years provided significant benefits in terms of patient outcomes, particularly against metastatic recurrence of cancers^[23]. To summarize from the trial outcomes, unfortunately many such clinical trials underway during the last decade suffered from being prematurely terminated because of the growing recognition at the time of cardiotoxic side effects associated with the use of certain COX-2 inhibitors (coxibs), such as Vioxx (rofecoxib)^[218]. Although this resulted in a total ban for Vioxx, elevated cardiotoxicity was ruled out for celecoxib in 2015, when it was approved by the USFDA after it was found to be as safe as ibuprofen or naproxen^[219]. As an example of one prematurely terminated study, a Phase II trial was reported in 2018 concerning the effects of celecoxib (400 mg twice daily, every day) plus or minus chemotherapy with IFL (irinotecan, 5-fluorouracil (FU), and leucovorin; each cycle comprising IFL over 4 weeks then 2 weeks with no IFL) for previously untreated or unresectable metastatic CRC confirmed by biopsy^[155].

Unfortunately, because the Phase II trial was prematurely terminated, only short-term use of celecoxib in patients could be analyzed with follow up to 2 years, and which would not be expected to show significant effects based on the earlier findings outlined above. This Phase II study was further complicated by a protocol variation with the inclusion of 81mg aspirin added with celecoxib to mitigate against possible cardiotoxicity in the high-risk cardiovascular subjects. This trial was limited to a median of three treatment cycles (a total of ~ 20 weeks of daily celecoxib). Nevertheless, the results showed promise in that PFS and overall survival (OS) of patients was improved at 8.7 and 19.7 months, respectively when compared to the historical IFL alone treated controls of 7 and 15 months. After modifying the study protocol, the overall survival was ~91% at one year and 50% at 2 years (median OS = 24.2 months), and the authors could not exclude the possibility of added survival benefit with celecoxib slowing progression of disease. A larger, multicentred Phase III trial (Alliance/SWOG C80702) is underway with FOLFOX (5-FU + oxaliplatin + folinic acid = leucovorin) every 2 weeks for 24 weeks, plus celecoxib (400mg daily treatment extended to 3 years) with longer term follow-up to include 6-year survival and will complete in December 2019.

CONCLUSION

Based on the supportive findings reviewed here, the importance of cytotoxic drugs targeting the CSC population has become self-evident, with significant ramifications for the future of anticancer drug design

and treatment. It should be possible to identify and thereby improve the development of drugs used as biological modifiers to eliminate the CSC populations by relying on the selective biomarkers Cripto-1, ABCG2, LGR5 and CD271 to aid in the analysis of CSC drug sensitivity. From the range of evidence provided, it is apparent that such CSC markers are expressed in common across many cancer types and with ESCs. In this manner, the CSC biomarkers can be used to separate enriched CSC populations from the other non-tumorigenic cells and normal stem cells using cell-sorting technology to then identify those drugs with greater potential for specifically and selectively targeting the CSCs by activating their cell death programs. One such drug is celecoxib, with one of its properties being an inhibitor of the COX-2/PGE production by CSCs, otherwise required for promoting greater LGR5 expression and WNT signalling to enhance CSC proliferation.

Cancer treatments have repeatedly failed due to their inability to target and kill the CSCs because these cells are highly resistant to commonly used chemotherapies, having greater survival and metastatic properties. The CSCs can remain dormant, only to then become reactivated, with their capacity for self-renewal and extensive proliferation, metastasis and differentiation giving rise to recurrent tumors, even after extended periods post-treatment. Therefore, it will be essential to be able to identify the CSCs, isolate and study their characteristics in greater detail in order to develop better drug treatments, such as celecoxib, and where the evidence is compelling that such drugs can then either promote or induce the death of the CSCs, both in the circulation and in tumor niches.

DECLARATIONS

Authors' contributions

Both authors contributed equally to the writing, construction and editing of the manuscript as well as drawing of the figures.

Availability of data and materials

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Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

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Original Article

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A pilot study for distinguishing basal cell carcinoma from normal human skin tissues using visible resonance Raman spectroscopy

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Abstract

Aim: The aim of the study is to test visible resonance Raman (VRR) spectroscopy for rapid skin cancer diagnosis, and evaluate its effectiveness as a new optical biopsy method to distinguish basal cell carcinoma (BCC) from normal skin tissues.

Methods: The VRR spectroscopic technique was undertaken using 532 nm excitation. Normal and BCC human skin tissue samples were measured in seconds. The molecular fingerprints of various native biomolecules as biomarkers were analyzed. A principal component analysis - support vector machine (PCA-SVM) statistical analysis method based on the molecular fingerprints was developed for differentiating BCC from normal skin tissues.

Results: VRR provides a rapid method and enhanced Raman signals from biomolecules with resonant and near-resonant absorption bands as compared with using a near-infrared excitation light source. The VRR technique revealed chemical composition changes of native biomarkers such as tryptophan, carotenoids, lipids and proteins. The VRR spectra from BCC samples showed a strong enhancement in proteins including collagen type I combined



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with amide I and amino acids, and a decrease in carotenoids and lipids. The PCA-SVM statistical analysis based on the molecular fingerprints of the biomarkers yielded a 93.0% diagnostic sensitivity, 100% specificity, and 94.5% accuracy compared with histopathology reports.

Conclusion: VRR can enhance molecular vibrational modes of various native biomarkers to allow for very fast display of Raman modes in seconds. It may be used as a label-free molecular pathology method for diagnosis of skin cancer and other diseases and be used for combined treatment with Mohs surgery for BCC.

Keywords: Visible resonance Raman spectroscopy, human skin, basal cell carcinoma, principal component analysis, supports vector machine, molecular fingerprints, tryptophan, carotenoids

INTRODUCTION

Several studies have reported that skin cancer is in an increasing trend worldwide. Each year, new cases of skin cancer have been reported to have a greater incidence than breast, prostate, lung and colon cancers combined. Basal cell carcinoma (BCC) is the most common skin cancer, accounting for about 80% of the non-melanoma skin cancers (NMSC) which includes BCC and squamous cell carcinoma (SCC). Before the age of 65, 40%-50% Americans will have BCC or SCC skin disease occurring. There are about 4 million cases of BCC diagnosed in the USA annually^[1-4]. Although BCC lesions rarely metastasize and have a high curable rate, they frequently expand quickly and may not only become a cosmetic problem but infiltrate surrounding tissue causing functional problems. Therefore, early detection and rapid and accurate diagnosis become very important for treatment.

Currently, clinical routine diagnosis of skin cancer is performed using biopsy and histopathology. The gold standard method requires freezing or reagent preparation of the biopsy tissue prior to microscopic analysis. This requires skilled technicians and expert histopathologists to perform the diagnosis, as well as considerable time before the results are available. This leads to a diagnostic accuracy of about 80% depending on expertise and training^[5-9]. A new diagnostic technique that is more rapid with higher accuracy would be a great clinical advantage. A search is ongoing for a fast and accurate way for diagnosis while treating cancers.

An optical biopsy (OB) technique would offer many advantages including in situ evaluating lesions. There is a panoply of OB techniques, such as label-free native fluorescence, Raman spectroscopy (RS), optical coherent tomography, and other optical imaging techniques for *ex vivo* and *in vivo* cancer detection in human tissues and cells. These techniques have advanced significantly since the earliest reports in 1984, 1987, 1991 and 1992 by Alfano's group^[10-13]. In particular, OB techniques for *ex vivo* detection of a panoply of different cancer types such as NMSC have progressed in recently years. Some fluorescence spectroscopic techniques for diagnosis of BCC have been reported, such as fluorescence confocal microscopy that has been used to detect BCC skin cancer margin in Mohs excisions without the need of frozen and fixed section processing^[14-20]. RS technique has some unique features such as using intrinsic biomarkers and operating *in situ* and in a time period of many minutes or seconds. This has led to a rapid progress in seeking commercial instrumentation for researchers and clinical applications in BCC and other skin cancer diagnoses^[21-36]. Most of the reports demonstrated spectral differences between normal skin tissue and BCC lesions using near-infrared (NIR) laser excitation only. Some reported the use of high power and very long signal collection time (e.g., 300 mW of power and minutes of time) on cells and tissues^[24,25]. The NIR Raman method which uses excitation light sources at 671 nm, 785 nm, 830 nm or 1,060 nm has limitations for practical applications described as follows: (1) NIR Raman method only detects non-resonant biomolecule components; (2) NIR laser source requires long excitation time. Even a confocal micro Raman system has poor signal-to-noise (S/N) ratio since the spectral peaks are weak and not enhanced. Increasing excitation light intensity and

longer exposure time will limit the practical application^[24,26,30]; and (3) The NIR light penetrates relatively deep and excites a large volume in the skin compared to visible light. Thus, it is subjected to variations in the molecular content of the target tissue yielding a low selectivity distinguishing the abnormal from the normal target sites.

We have recently developed a novel visible resonance Raman (VRR) technique using 532 nm as the excitation source which can address the aforementioned limitations of NIR RS technique and extend the Raman technique so that it detects both non-resonant and resonant biomedical components. The vibrational resonance effect occurs in resonance Raman (RR) spectroscopy when the energy of the excitation approaches an optical transition energy level, which greatly enhances the scattering. The RR signal intensity may be enhanced 10^2 to 10^3 times, typically 10 to 100 folds, compared with non-resonance signal, since Raman scattering cross section is inversely proportional to the fourth power of the wavelength. In particular, RR using 488 nm and 532 nm excitation wavelengths gives almost seven and five times more intense Raman signal than using 785 nm excitation wavelength, respectively.

One will have many advantages of resonance Raman over conventional non-resonance Raman if 532 nm is used for excitation in biomedical diagnosis. Since cells and tissues contain many large biomolecular chromophores and other large conjugated molecules with multiple vibrational bonds, they experience stretching and bending vibrations that can be enhanced by the excitation laser and the RR spectra collected from them exhibit enhanced peaks. Specific biomolecules in the cell and organelles contain fluorophores, such as flavins, NADH, NAD, collagens, elastin, carotenoids, tryptophan, hemeproteins, mitochondria and cytochromes. The resonance enhanced signals from these biomolecules suggest that the 532 nm excitation wavelength matched (or closely matched) the molecular absorption bands for these biochemical compounds in the cells and tissues. For example, the metalloprotein such as hemoglobin, has one absorption band at 534 nm. Similarly, the mitochondrial electron transport protein, cytochrome c, has one absorption band at 552 nm (under the hypoxia conditions). The 532 nm is in the tail of the absorption band of flavins and lactate. Carotenoids and flavins have pre-resonance absorption bands about 480 nm and 500 nm which fall in the pre-resonance range of the excitation wavelength of 532 nm. The molecular vibrations of flavins were enhanced by more than 100 times. Therefore, the VRR spectral peaks are greatly enhanced, and much sharper than those from NIR non-resonance Raman. The first RR using 488 nm and 514 nm was noticed in cancer tissue by Alfano *et al.*^[11] in 1987. The VRR method has been used to produce enhanced S/N results in the biomedical fields. More recently, the VRR technique has been used on human brain, breast, vulnerable atherosclerotic plaque, gynecological tissues, gastrointestinal tissues, and atherosclerotic abdominal aortic tissues studies^[37-43]. The VRR spectra *ex vivo* exhibited native molecular signatures that could be used as optical molecular histopathological criteria to distinguish abnormal from normal tissues. The 532 nm wavelength is like a “magic” wavelength for tissue to generate extraordinarily high Raman signals for quasi real-time measurements. It is just inside the key fluorophores’ absorption and emission regions in tissue.

This study focuses on VRR spectral analysis of normal and BCC human skin tissues performed *ex vivo* using a confocal micro-Raman system. It investigates the depth dependence of BCC which reveals a process of status change, and demonstrates the discrimination and classification between normal and BCC skin tissues using molecular vibrational fingerprints and a statistical model. The statistical model is based on principal component analysis (PCA)^[44] and supports vector machine (SVM)^[43,45-48]. To the best of our knowledge, this study is the first to detail and discuss the depth dependence of BCC in correlation with statuses, and to distinguish BCC from normal skin tissue using the VRR technique. This is a pilot study on BCC skin cancer using VRR performed as a basis for longer studies using 532 nm and other visible laser wavelengths.

METHODS

RR spectra of sliced samples of human skin normal and BCC tissues were measured using a confocal micro-Raman spectrometer, with a 532 nm laser for excitation at low power (3.5 mW) for under 30 s. Fifty-five RR

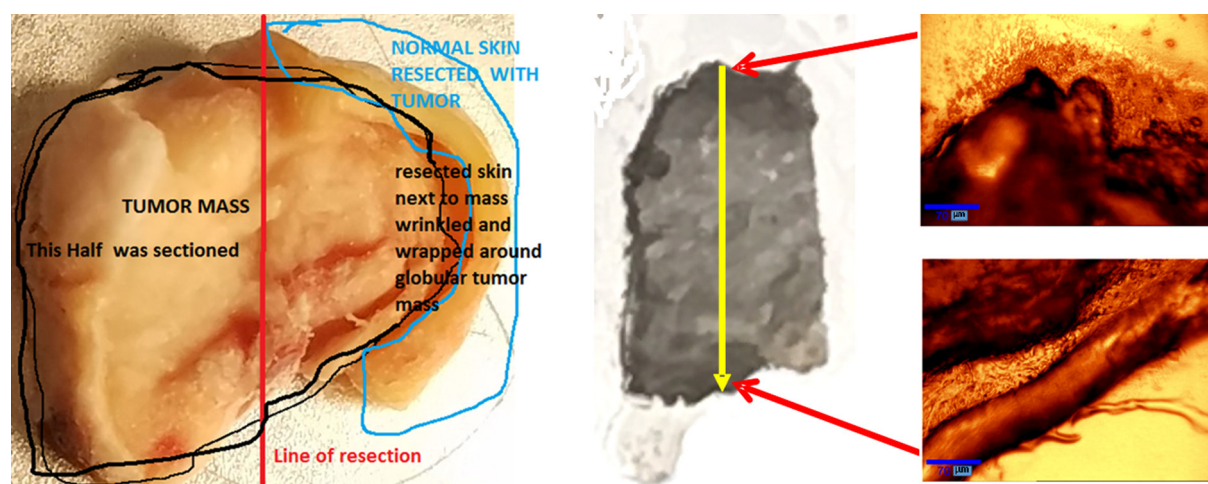


Figure 1. A photograph (side view) of the original human BCC skin specimen used in the experiments (left). The specimen was sliced in vertical direction indicated by the red line. Twenty-eight slices were made with 50 μm thickness for each slice. In the middle is the photograph of a piece of vertical slice of BCC with the size of 5.6 mm \times 8.3 mm \times 50 μm , where the yellow arrow indicates the direction of measurement steps in depth. The right images were taken from the top and bottom edges of the BCC slice. The red arrows show the sites at the top and bottom of the slice where the two images are taken. BCC: basal cell carcinoma

spectra were obtained and analyzed from thirty-six slices of skin tissue samples.

Tissue sample preparation

The human skin normal and BCC specimens were obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA) under the City College of CUNY Institutional Review Board (IRB). The BCC tissue was from the left ear of a 77-year-old white male. The normal skin sample was from the adjacent area of this BCC tissue. The RR spectra of sliced BCC and normal skin samples were measured, including 28 vertical section slices from one BCC sample and 8 horizontal section slices from one normal sample. Each slice is approximately 8.4 mm long and 5.6 mm wide with a thickness of 50 μm . One vertical section slice of BCC sample was selected for measurement at 6 steps of depths with 100 μm step size, and 9 steps of depths with 500 μm step size, starting from the top surface of the skin toward the bottom as shown in Figure 1.

The irregular-shaped skin specimens were snap-frozen with no chemical treatment. They were kept in a -80 $^{\circ}\text{C}$ freezer and shipped with dry ice for all tests. Tissue sections were cut with a thickness of 50 μm and mounted on uncoated glass microscope slides using Leica CM1080 Cryostats at -20 $^{\circ}\text{C}$. The specimens were thawed to ambient room temperature for the spectroscopic studies.

Measurement of RR spectra of BCC and normal skin sliced tissues

All of the RR spectra were collected directly from a region of interest on each sliced specimen. The raw RR spectrographs (without subtracting the baseline) were produced using the software of NGSlabSpec and ORIGIN 2015. A total of 55 spectra were collected from normal and BCC tissues at the following sites: 12 spectra from eight sections of normal specimen; 15 spectra from one vertical section of BCC sample that was selected for measurements at 6 depths with 100 μm separation, and 9 depths with 500 μm separation; and 28 spectra from the centers of 28 vertically sliced sections of BCC sample. Typical spectra are shown in Figure 2.

The instrument used in this study was WITec alpha300 Raman microscope system (WITec: Wissenschaftliche Instrumente und Technologie GmbH, Ulm, Germany), equipped with a Nikon 20X objective (Nikon Instruments, Melville, NY, USA). The excitation light source was a 532 nm solid-state diode laser (Verdi-2, Coherent Company, Santa Clara, CA, USA) with a maximum output power of 50 mW. Each piece of slices was placed on the stage of the system to collect data. The excitation beam was focused on the sample with

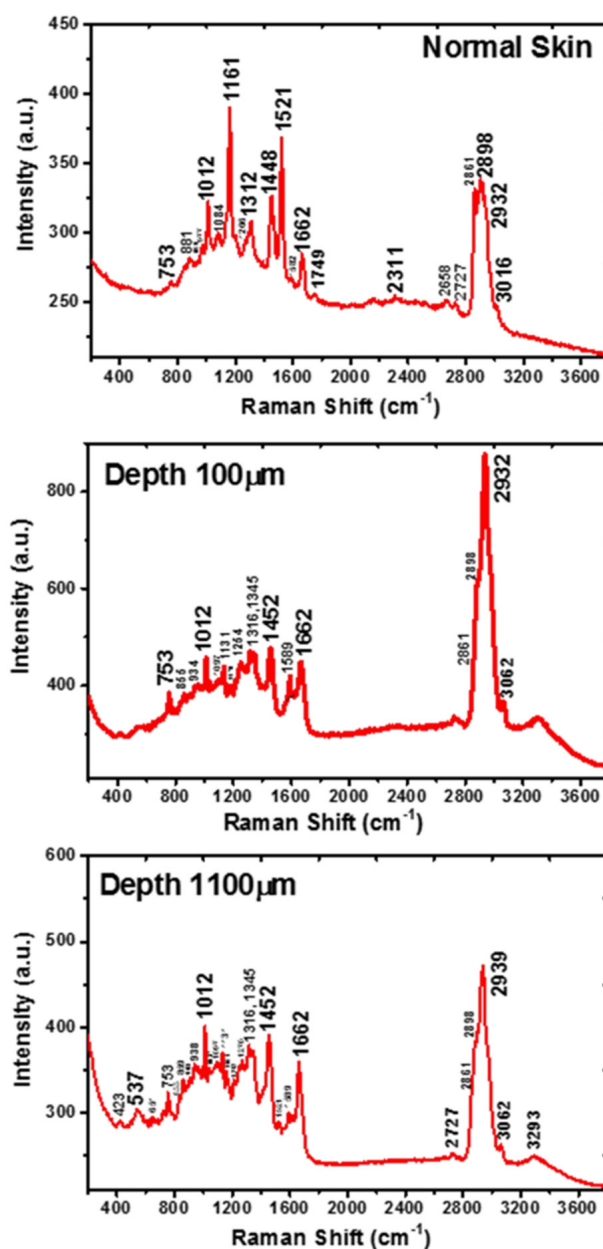


Figure 2. A set of typical resonance Raman raw spectra collected from a horizontal section of normal human skin sample, and a vertically sliced BCC skin sample measured at different depths. (Top), the spectrum was from dermis layer of normal skin showing nine feature peaks; (middle), the spectrum was from the vertically sliced BCC sample at a depth of 100 μm . There are eight characteristic peaks including increased peaks at 753 cm^{-1} and 1,589 cm^{-1} , but intense carotenoids peaks at 1,161 cm^{-1} and 1,521 cm^{-1} disappeared compared to the normal tissue (top); (bottom), the spectrum was from BCC sample at a depth of 1,100 μm , substantially similar to the depth of 100 μm , with six Raman peaks, but carotenoids peaks at 1,161 cm^{-1} and 1,521 cm^{-1} are present and obviously weaker than normal tissue sample (top). Those peaks of 753 cm^{-1} and 1,589 cm^{-1} greatly decreased in comparison with the depth of 100 μm . BCC: basal cell carcinoma

a spot diameter of 1 μm and the power of the beam at the sample position was kept at 3.5 mW. RR spectra were collected using a one-second integration time and 30 accumulations, with a system resolution down to the optical diffraction limit of ~ 200 nm. The spectra were collected over the spectral range of 400-4000 cm^{-1} . The spectral resolution was 2 cm^{-1} in the range of interest^[41,42]. All the spectra were collected at ambient room temperature. Student's *t*-test was used to determine if particular RR peak intensities between normal and BCC samples were significantly different.

RR spectral data analysis method by PCA-SVM

The baseline of each raw Raman spectrum was fitted to a polynomial using an asymmetric Huber function as the loss function^[49]. The difference between the raw spectra and the baselines were calculated. Each baseline-subtracted Raman spectrum was then normalized using its Euclidean norm, and used for subsequent analysis.

Raman peaks in the normalized baseline-subtracted Raman spectra were first investigated. Student's *t*-test was used to determine if particular RR peak intensities between normal and BCC samples were significantly different. Then unsupervised machine learning algorithms such as PCA were used to analyze the entire spectral data, reduce dimension and detect underlying spectral features.

PCA finds the uncorrelated components that explain the most variance in the signal. It has been widely used for various applications, such as spectroscopy^[50], face recognition^[51] and optical imaging^[52]. Mathematically, PCA solves an eigenvalue equation, and finds a set of orthonormal eigenvectors which are considered principal components (PCs) corresponding to the eigenvalues which are the variances of the PCs in the data. For Raman spectral data contained in a matrix $X_{M \times N} = \{x_1, \dots, x_N\}$, where M is the number of wavenumbers, and N is the number of spectra or samples (assuming $M > N$). PCA considers the spectral data x_i to be linear combinations of PC loadings $\{w_j\}$ with scores $\{h_{ji}\}$, i.e., $X_{M \times N} \approx W_{M \times N} H_{N \times N}$, where $W_{M \times N} = \{w_1, \dots, w_N\}$ and $H_{N \times N} = \{h_1, \dots, h_N\}$. To calculate the PCs, the data matrix is "mean centered" first, i.e., the mean of each row is calculated and subtracted off that row. Then an eigenvalue equation of the covariance matrix of the "mean centered" data matrix X' is solved to find the eigenvectors and the corresponding eigenvalues. The eigenvectors are the PC loadings, and eigenvalues are the variances explained by the corresponding PCs. In practice, this can be solved using singular value decomposition^[53] of the data matrix X' , i.e., $X' = U \Sigma V^T$, where U and V are left and right singular vectors, and $\sigma_i = \text{diag}\{\Sigma\}$ are the singular values. Columns of $U_{M \times N}$ are taken to be the PC loadings, i.e., $W = U$, eigenvalues $\lambda_i = \sigma_i^2$, $H = \text{pinv}(W)X$, where pinv denotes pseudo inverse, and $\text{pinv}(W) = (W^T W)^{-1} W^T$.

The PC scores contained in H are essentially the projection of the spectral data in matrix X onto the PCs or "eigenspectra". The PC scores h_i are a set of mixing coefficients of the PCs. These scores can be considered as the characteristic information of the spectra (samples) and used for classification. Alternatively, PC scores obtained from the mean-centered data matrix can also be used, and they are simply different from those obtained from the raw data with a shift in the origin.

Then the PC scores of different spectra (samples) were used for classification after standardization. The scores were standardized for each spectrum using the following formula: (score - "score mean")/"score standard deviation". An SVM with a linear kernel was used for classification. SVM attempts to find a hyperplane (a boundary line in two dimension) to separate two classes with the largest distance from the nearest class members (data points) which are called support vectors. Once the SVM classifier is trained, it is tested for classification using all the data points, which is called re-substitution validation. Various combinations of features were tested for classification. Since the contributions due to higher-order PCs significantly decrease according to the eigenvalues, limited number of PCs need to be evaluated and compared. More thorough search of optimal feature selection may be carried out^[47,48]. The classification performance of the SVM classifier was evaluated using statistical measures including sensitivity, specificity, and accuracy, along with the receiver operating characteristic (ROC) curve^[54,55]. To plot the ROC curve for the SVM classifier, the positive class (cancer) posterior probability (a data point classified into positive class) for each data point was calculated by using a sigmoid function to map the SVM scores which are the distances from the data points to the SVM separation line^[56]. Then the posterior probabilities were used to calculate the true positive rate (i.e., sensitivity) and false positive rate (i.e., 1 - specificity) by varying the threshold and generate the ROC curve for true positive rate vs. false positive rate. The area under ROC curve (AUROC)^[55,57] was calculated

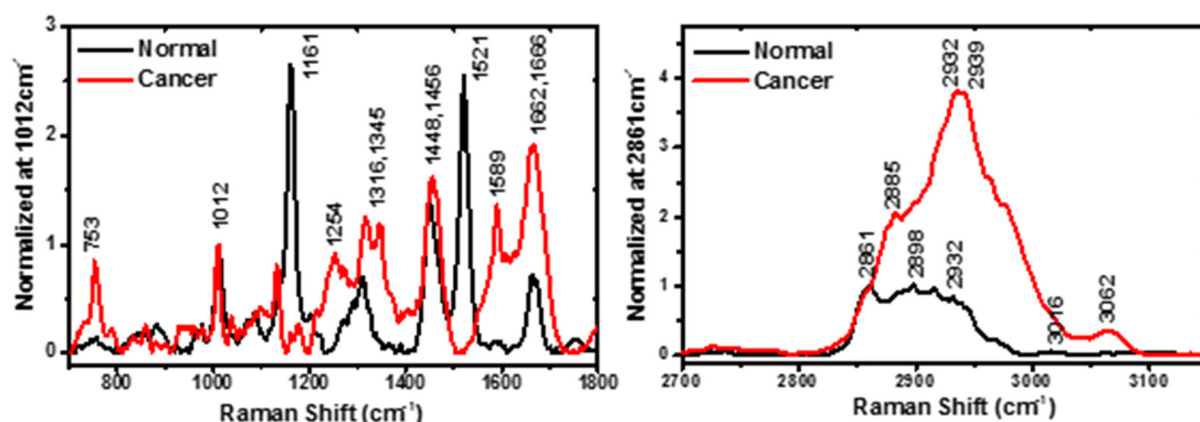


Figure 3. A set of typical resonance Raman spectra collected from a horizontally sectioned normal human skin sample, and a vertically sliced basal cell carcinoma skin sample measured at a depth of 100 μm . Both plots are displayed in the enlarged scale regions of low wave-number 700-1,800 cm^{-1} and high wave-number 2,700-3,150 cm^{-1}

to show the performance of the classifier. AUROC represents the probability that a classifier will rank a randomly chosen positive sample before a randomly chosen negative one. It is used as a global measure of classifier performance that is invariant to the classifier discrimination threshold and the class distribution. Perfect classification accuracy corresponds to an AUROC value of 1, while a random guess separation leads to an AUROC value of 0.5. To reduce bias in the classification with re-substitution, leave-one-out cross validation (LOOCV)^[58] was used to re-evaluate the classification performance. To perform LOOCV, each time one individual spectrum was removed from the dataset. The rest of the dataset was used to train an SVM classifier. The removed spectrum was then classified by the trained classifier for testing. This process was repeated for all spectra. In the end, sensitivity, specificity and accuracy were calculated based on the results of all testing as overall evaluation of the classification performance. All the computations for PCA-SVM were carried out in MATLAB.

RESULTS

RR raw spectra from horizontally sliced normal and BCC cancerous human skin samples in vertically section were measured. The distinctive Raman peaks that can be uniquely assigned to distinguish skin cancer lesions were obtained by the raw spectral profiles. The correlation between depth and the status of BCC cancer was found using the RR molecular fingerprints [Figures 2 and 3], by investigating the relative changes of biomarkers [Figure 4] and by calculating the ratios of peak intensities [Figure 5]. The classification of BCC cancer from normal skin tissues using PCA-SVM statistical method is shown in Figure 6.

Depth-dependent BCC assay: (1) VRR spectral fingerprints

RR spectral fingerprints of carotenoids: Figures 2 and 3 and Table 1 show the typical spectra from normal and cancerous BCC sliced skin samples. It revealed the process of evolution from normal to cancer and depth-dependence of cancer from different status with RR spectral fingerprints. The RR spectrum [Figure 2 (top)] was obtained from the center of the third piece of horizontally sliced normal sample with thickness 50 μm . The depth is around 150 μm in the normal skin tissue (corresponding to the lower epidermis layer and the dermis layer). The epidermis is usually ~ 0.1 mm thick, and ranges from 0.07 to 0.12 mm. The *dermis* is a layer of skin which is beneath the epidermis layer and is the thickest of the three layers (epidermis, dermis and hypodermis) of skin^[59-62]. The *dermis* is also called corium, whose thickness is 0.3-4.0 mm and it is composed of dense irregular connective tissue. So, we consider this normal sliced sample to be located at the dermis layer. In the RR spectrum of normal dermis skin, the resonance-enhanced intrinsic molecular fingerprints of β -carotenes (here we consider β -carotenes, because the β -carotenes and lycopene account

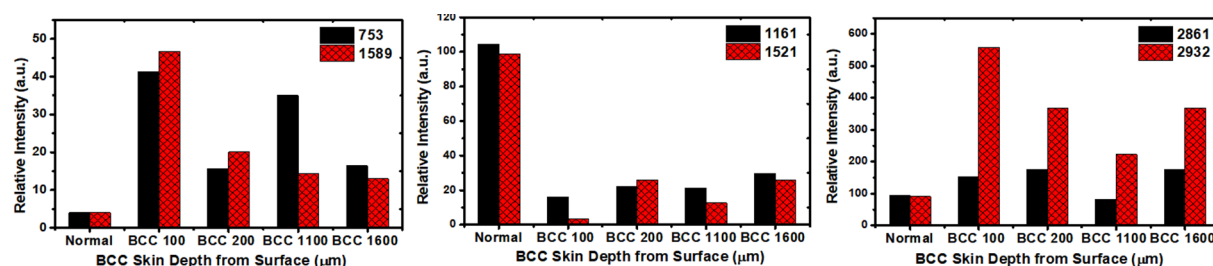


Figure 4. The relative concentration changes in biomarkers of tryptophan (left), carotenoids (middle) and lipids/lipoproteins (right) in the normal skin tissue and BCC skin tissues with depth-dependence. Dramatic changes on relative concentrations are shown for the lesion slices at the depth of 100 μm compared with other depths of 200 μm, 1,100 μm and 1,600 μm. Based on student's *t*-test, the difference between normal and BCC samples is statistically significant ($P < 0.05$) for biomarkers: 752, 1,161 and 1,521 cm^{-1} . The units of the numbers in the figure legends are cm^{-1} . BCC: basal cell carcinoma

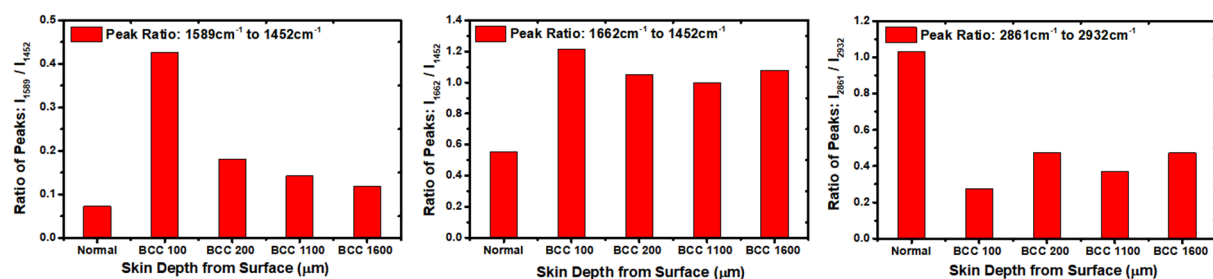


Figure 5. The changes in the resonance Raman fingerprints represented by the ratios of peak intensities of biomarkers. The 1,589 cm^{-1} mode is supposed to be arising from tryptophan; 1,452 cm^{-1} mode is assigned to fatty acids-lipid protein molecules; 1,662 cm^{-1} mode is amide I combined with type I collagen; the largest ratio (I1589 to I1452) changes occurred at a depth of 100 μm in the BCC tissue (left). Based on student's *t*-test, the difference between normal and BCC samples is shown to be statistically significant ($P < 0.05$) for all biomarkers. BCC: basal cell carcinoma

for about 60%-70% of the total of the five most concentrated carotenoids content in human organisms), at 1,161 cm^{-1} and 1,521 cm^{-1} are thought to play a significant role in the normal dermis skin anti-oxidant defense system, as shown in Figure 2 (top) and Figure 3 (left). These two resonance bands are active because carotenoids have a pre-resonance absorption band which falls in the pre-resonance range of the excitation wavelength of 532 nm^[37,63-67]. The RR peaks of 1,161 cm^{-1} and 1,521 cm^{-1} disappeared in the BCC sliced sample at a depth of 100 μm as shown in Figure 2 (middle) and Figure 3 (left). This shows a clear progression of the BCC tissue mutation process in comparison with normal skin tissue, which suggests it may be a lesion with very serious status. The spectrum shown in Figure 2 (bottom) is from vertically sliced BCC sample at a depth of 1,100 μm, substantially similar to the BCC sliced at a depth of 100 μm, with RR peaks present at 1,012 cm^{-1} , 1,452 cm^{-1} , 1,662 cm^{-1} , 2,861 cm^{-1} , 2,898 cm^{-1} and 2,932 cm^{-1} . But carotenoids peaks occurring at 1,161 cm^{-1} and 1,521 cm^{-1} are obviously weaker than normal tissue sample [Figure 2 (top)], which suggest this status may be a mildly lesion. Thus, we propose that the evolution of the RR spectra of biomarker carotenoids with the depth dependence revealed the correlation between depth and the process of status change. Carotenoids are the organic and natural fat-soluble pigments and exist in plants. Human beings can obtain carotenoids from diet, such as fruits and vegetables, and its concentration depends on their daily diet and stress factor^[67,68]. Carotenoids accumulate in the epidermis through (1) diffusion from the fat tissue, blood and lymph flows, or (2) secretion via sweat glands, and sebaceous glands onto the surface of the skin and subsequent penetration. The specific importance of carotenoids is to serve as a marker substance for the entire anti-oxidative network of human skin^[69,70]. Because antioxidants form protective chains in skin tissue, they act synergistically to protect each other against the destructive action of the free radicals, and mainly reactive oxygen species^[69-73].

RR spectral fingerprints of tryptophan: in contrast, the RR peaks of 1,161 cm^{-1} and 1,521 cm^{-1} diminished sharply for BCC tissues at a depth of 100 μm while the fingerprints of tryptophan peak at 753 cm^{-1} and 1,589 cm^{-1}

Table 1. Changes in intensities of peaks in the resonance Raman spectra of human normal skin tissues compared with basal cell carcinoma skin tissues in Figure 3

Peak position (cm ⁻¹)	Normal	BCC	Suggested molecular class
753		Enhanced	Trp. DNA
859			Polyadenylic acid, collagen type I, lactate
1,012			Trp.
1,161	Intense		Carotenoids
1,254		Enhanced	Amide III, Trp.
1,316, 1,345		Enhanced	Trp. collagen, A, G, T
1,452			Collagen type I, phospholipid
1,521	Intense		Carotenoids
1,588		Enhanced	Trp. heme class, Phe, A, G
1,662, 1,666		Enhanced	Amide I, collagen
2,861-2,892			Lipids, collagen
2,932, 2,939		Enhanced	Lipoprotein, collagen, lactate

BCC: Basal cell carcinoma; Trp: tryptophan; A: adenine; G: guanine; T: thymine; Phe: phenylalanine

greatly increased in BCC tissue at a depth of 100 μm in a more serious status [Figure 2 (middle), Figure 3 (left) and Table 1], but decreased in the BCC sliced sample at a depth of 1,100 μm in a mild status [Figure 2 (bottom)]. The RR spectra of key fingerprints of tryptophan with a main vibrational mode at 1,588 cm^{-1} (W8b) were observed^[66,74]. It was found that endogenous tryptophan and metabolites contributions were accumulated in the mode of 1,588 cm^{-1} in cancers and enhanced by resonance. Researchers have reported that heterocyclic amino acid tryptophan is a key factor during the metabolic process^[74,75]. This RR vibrational mode of 1,588 cm^{-1} indicates that tryptophan may be produced in human skin cancer in the kynurenine pathway of tryptophan metabolism involved in tumor progression according to our previous studies on human brain cancer^[75-77] and that the micro-environment of malignant tumor tissues may result from tryptophan radicals^[78-80].

RR spectral fingerprints of lipids and lipoprotein: the characteristic band of spectral peaks observed between 2,861 cm^{-1} /2,898 cm^{-1} and 2,932 cm^{-1} are attributed to the vibrations of methylene ($-\text{CH}_2$) from lipids and methyl ($-\text{CH}_3$) groups from lipoproteins [Figure 3 (right), Figure 4 (right) and Table 1]. The peak near 2,932 cm^{-1} , due to the asymmetric C-H stretching of methyl groups, showed a significant increase in BCC cancer tissue at a depth of 100 μm site, while the 2,861 cm^{-1} /2,898 cm^{-1} band relatively decreased in intensity in BCC cancer tissue at a depth of 100 μm in comparison to RR spectrum of the normal skin tissue.

Depth-dependent BCC assay: (2) relative content changes of molecular fingerprints

Figure 4 showed the relative content changes of biomarkers of carotenoids (left), tryptophan (middle) and lipids/lipoproteins (right) in normal skin tissue vs. BCC cancer skin tissues with depth-dependence. The dramatic changes in relative concentrations are shown in the slice at depth 100 μm which is a BCC lesion in comparison to other status BCC lesions at depths of 200 μm , 1,100 μm and 1,600 μm . These results are consistent with the analysis of RR spectral fingerprints in the previous section. The suggested reason for the spectral changes of carotenoids is that the concentration of carotenoids decreased in BCC cancer at the depth of 100 μm , which caused the RR peaks to become too weak to detect and induced a shift in chemical vibration bonds. This change in carotenoids may be due to the structural changes within the micro-environment of malignant lesion. All these observations point out the existence of fast activation and deactivation of Raman vibrational modes.

Depth-dependent BCC assay: (3) ratio of intensity of molecular fingerprints vibration modes

Figure 5 showed the RR fingerprints changes using the ratio of the intensities of RR peaks. The 1,589 cm^{-1} mode is attributed to tryptophan; while the 1,452 cm^{-1} mode is assigned to the fatty acid/lipid protein molecules and the 1,662 cm^{-1} mode is from the amide I combined with the collagen type I molecules. The largest ratio changes of (I_{1589} to I_{1452}), (I_{1662} to I_{1452}) and (I_{2861} to I_{2932}) occurred at a depth of 100 μm from the

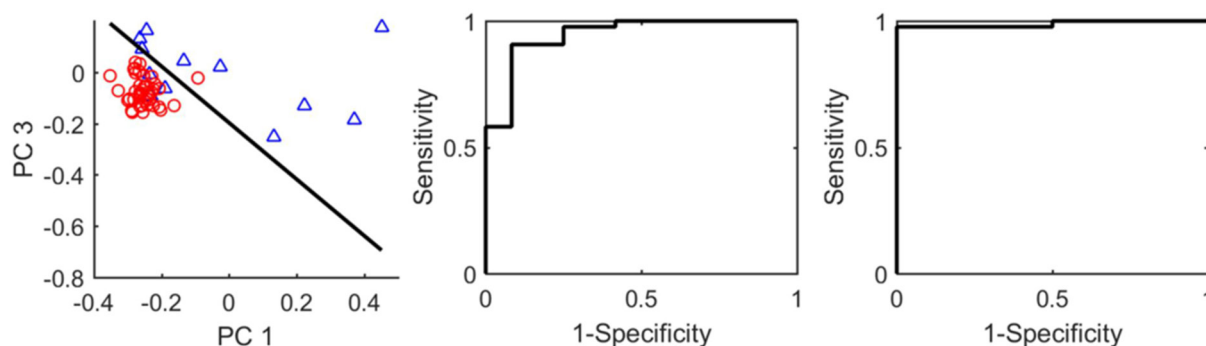


Figure 6. (left) is a scatter plot of scores PC1 and PC3 with the separation line (SVM classifier); (middle) ROC curve for the SVM classifier in the left panel; (right) ROC curve for the SVM classifier trained with PC1, PC3 and PC7. PC: principal component; ROC: receiver operating characteristic; SVM: supports vector machine

BCC tissue samples. These ratios of the intensities of the spectral peaks from the BCC cancer tissue at depth 100 μm are found to be much higher [Figure 5 (left and middle)] or lower [Figure 5 (right)] in comparison with those from normal skin tissue, therefore they may be used as a distinct marker to distinguish cancerous tissues from normal skin tissues. Such ratios provide an insight into the conformational changes occurring in biomolecules in these tissues. For instance, the Raman spectra of proteins (represented by the 1,662 cm^{-1} band) correspond to beta-sheets which provide insight into protein folding or the denaturation processes.

Classification based on RR spectral data of BCC and normal skin tissues by SVM

The spectral dataset including 12 spectra from sliced normal skin tissues, and 43 spectra from sliced BCC cancerous samples were analyzed using PCA. Fifty-five PCs were obtained with corresponding eigenvalues sorted in a descent order (the plots are not shown here). The first 10 PCs account for 97% of the total variance. If two PCs are selected for classification, first PC (PC1) and third PC (PC3) showed the best performance. The scatter plot of the PC scores along with a boundary line trained using SVM method is shown in Figure 6 (left). The sensitivity, specificity and accuracy of the classifier with re-substitution validation were calculated to be 97.7%, 75.0%, and 92.7%, respectively. The ROC curve was generated and shown in Figure 6 (middle). The AUROC was found to be 0.95. When PC1, PC3 and PC7 are used together for classification, it achieved optimal performance. Sensitivity, specificity and accuracy of the SVM classifier trained with all spectra were found to be 93.0%, 100%, and 94.5% with re-substitution validation. The ROC curve was generated and shown in Figure 6 (right). The AUROC was found to be 0.99. LOOCV achieved sensitivity 97.7%, specificity 66.7%, and accuracy 90.9%. The classification is shown to be effective for the diagnosis of human skin tissues using RR spectroscopy.

DISCUSSION

In conclusion, given our preliminary investigation, we have demonstrated how the molecular components and conformation change under different conditions of BCC skin cancer tissues, and shown that there is a correlation between the depth dependence of RR spectra and the status change of BCC tissue at a molecular level. At a depth of 100 μm , the VRR spectra from BCC tissue change significantly compared to the spectra from normal skin tissues due to the changes in the relative concentrations of tryptophan, carotenoids, lipids and proteins [Figures 2-5]. In addition, VRR technique with 532 nm excitation can effectively distinguish BCC from normal skin tissues. The PCA-SVM statistical analyses of the VRR data collected from human skin cancer and normal tissues were used to distinguish BCC lesions from normal skin tissues. It yielded a sensitivity, specificity and accuracy of 93.0%, 100%, and 94.5%, respectively, when compared with the histopathology analysis (as the “gold standard”) reports. This is the first evidence that the difference between human skin normal tissues and cancer lesions can be detected by VRR spectroscopy.

The VRR method has a high S/N ratio, and provides visually intuitive results for inspection. Even the raw RR spectra can be used for a direct comparison between cancerous and normal tissues. In contrast, it is much more difficult to inspect the raw Raman data acquired using NIR such as 785 nm excitation, because of the much lower S/N ratio. In fact, the raw Raman spectra are rarely provided in the literature^[81,82]. The VRR system also uses less power and shorter integration time to collect signals^[28,29,31,32], thus providing a safer and more suitable method for *in vivo* and real-time *in-situ* skin cancer diagnosis compared with other NIR or FT-Raman system. We believe we cannot obtain Raman spectra fast and at a local spot in a skin tissue to get cancer margins without using the 532 nm RR.

This pilot study shows that VRR technology may have broad clinical applications for real-time, and label-free detection of skin lesions and other human tumors such as breast and brain^[37-42,83,84]. A most recent reference to salient features of VRR in carotene in solution is given by Lu *et al.*^[85].

DECLARATIONS

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Authors' contributions

Read and revised the manuscript: All authors

Performed experiments, initiated the manuscript and performed data analysis: Liu CH

Performed the PCA-SVM analysis and wrote part of the manuscript: Wu B

Revised the manuscript: Sordillo LA

Performed experiments: Boydston-White S

Prepared the samples: Sriramoju V

Performed data analysis: Zhang C

Financially supported the study, participated in discussions and revised the manuscript: Beckman H

Participated in discussions and revised the manuscript: Zhang L, Pei Z, Shi LY

Is the principal investigator of the project, and supervised the entire study: Alfano RR

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

In this study, the human skin normal and BCC specimens were obtained from the NDRI (Philadelphia, PA). The experimental procedures were approved by the City College of the City University of New York, IRB office.

Consent for publication

Not applicable.

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Meeting Abstracts

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Meeting Abstracts of the 3rd International Workshop No-Cancer 2018 - understanding cancer cell biology to improve diagnosis and therapy

Novara, Italy; 28-30 Oct 2018; Published: 23 Jan 2019

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Editorial Note:

Cancer remains a leading cause of death, despite the undeniable progress made in the last decades in the diagnosis and therapeutic treatments. Further improvements are only possible if we understand more in depth how cancer develops and metastasizes, and how it produces its damaging effects on the whole organisms, including immune-suppression and cachexia, that eventually lead to patient death. The goal of the International Workshop No-Cancer 2018 “understanding cancer cell biology to improve diagnosis and therapy” held in 28-30 Oct, in Novara (Italy) is precisely to share the foremost updated knowledge on the biology of cancer and the emerging technologies for early detection of cancer cells and the new “chemo-free” therapeutic options.

This is the third international Conference on Cancer organized under the auspices of the Università del Piemonte Orientale and other Academic and Cultural institutions, and ideally follows the two previous ones “Basic to Translational Medicine 2016: focus on Cancer” and No-Cancer 2017 “From Cancerogenesis to Therapy: new paradigms, new opportunities”, held in Novara. As outlined in the title, the main objective of the present workshop No-Cancer 2018 is to discuss the current knowledge in Cancer Cell Biology with the aim of paving the design of more precise and non-invasive methods for early detection of cancer cells and of more effective and personalized therapeutic interventions.

Twelve internationally renowned speakers coming from Belgium, France, Italy, Germany, Spain, Portugal and United States will present their recent research in a two-day scientific marathon. In addition, eight short communications and thirteen flash communications have been selected from the submitted abstracts. The following topics are covered: Oncogenes, onco-suppressors and Non-Coding RNAs in Cancer development; Cancer Cell metabolism and Metabolic cross-talk between cancer cells and stromal cells; Role of diet and of microbiota in cancer progression and response to therapy; Novel non-invasive strategies for early detection of cancer, Genetic alterations predisposing to Cancer; Immune and Drug resistance; cachexia; Clinical trials with novel therapeutics. The invited lectures will focus particularly on ovary cancer, colon cancer, pancreatic cancer, breast cancer, prostate cancer, mesothelioma, and hemato-oncology.



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Besides the scientific sessions, participants also can enjoy the social events, which include the city tour, classical music concert and Gala dinner.

Scientific and Social event programs and the Photogallery of the Conference are available at www.isidorolab.com.

This Special Issue of *Journal of Cancer Metastasis and Treatment* is a collection of the articles and extended abstracts that refer to the lectures presented at the Conference. Commentaries and review articles related to the theme of the Conference are also included.

Ciro Isidoro and Gianluca Gaidano, Guest Editors

1. Role of canonical and non-canonical signaling in fibroblast to cancer - associated fibroblast transition

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Cancer is identified as the second leading cause of death worldwide with an estimated 9.6 million deaths in 2018. The mortality rate of cancer is primarily due to disease recurrence and therapy resistance. Recent studies have shown that the normal stroma in the cancer tissue is converted into an “activated” cancer-promoting niche by the cancer cells facilitating aggressive cancer growth. Normal fibroblasts in the vicinity of the cancer cells are primary recruits in this activation process. “Activated” fibroblasts, defined as cancer associated fibroblasts (CAFs), play a critical role in cancer progression, metastasis, and therapy resistance. Therefore, identifying the mechanisms underlying the conversion of normal cells to CAFs is of critical importance to define novel effective therapeutic targets. Using normal fibroblasts and patient-derived ovarian CAFs, we have identified a role for both lysophosphatidic acid-receptor (LPA) mediated canonical signaling pathways and lncRNA-hub mediated non-canonical signaling pathways in the induction and maintenance of CAF phenotype in normal fibroblasts. Our studies demonstrate the potential interplay between LPA-LPAR signaling-hub and lncRNA-signaling hub in the maintenance of the CAF-phenotype in ovarian cancer. Our results provide evidence that targeted inhibition of this signaling nexus in CAFs may represent an adjuvant therapy in ovarian cancer.

2. Obesity-associated alterations of adipose tissue microenvironment and colorectal cancer

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Introduction: Obesity, a low-grade inflammatory condition, is a major risk factor for the development of several pathologies including colorectal cancer (CRC). Adipose tissue (AT) is recognized as a key endocrine

organ regulating metabolic/immune processes with a central role in obesity-associated morbidities. AT is composed of different cell types, i.e., adipocytes and almost a full spectrum of immune cells whose function is changed in obesity. The AT inflammatory process affects adipocyte metabolism and secretory profile and promotes activation of AT resident immune cells. The obesity-associated changes of this tissue are consistent with an emerging concept that immune and metabolic systems are interconnected. Notably, dietary patterns have been associated with increased/decreased CRC risk highlighting the importance of nutrients in cancer prevention.

Experimental model: Visceral AT samples collected from lean and obese subjects affected or not by CRC were assessed for immune cell, inflammatory and fatty acid (FA) profile as well as secretory function.

Results: Alterations of AT microenvironment including FA profile, inflammatory status, immune cell and secretory pattern are found in obesity and CRC. Dietary polyunsaturated FA endowed with anti- or pro-inflammatory properties are able to attenuate or exacerbate, respectively, AT inflammation.

Conclusion: AT inflammation has a key role in carcinogenesis and hyper-activated inflammatory pathways in adipocytes can subvert immune surveillance. Dissecting the complexity of events associated with and/or driving cancer development in obesity will open new avenues for lifestyle-targeted CRC prevention strategies.

3. Monocarboxylate Transporters as targets for cancer therapy

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Preference for glucose metabolism is a common feature of cancer cells. They rely mainly on glycolysis for adenosine triphosphate (ATP) production, with increased glucose uptake and lactic acid production, leading to acidification of the microenvironment. This metabolic phenotype is associated with features of cancer aggressiveness, including invasion, metastasis, evasion from the immune system, angiogenesis and resistance to therapy. To cope with the high production of acids, cancer cells depend on the activity of proton exchangers and transporters, which export protons to the microenvironment. Among these, are monocarboxylate transporters (MCTs), which play a dual role in tumours, by removing lactate from the cancer cells and also helping in the regulation of intracellular pH. Thus, considering their role in cancer, MCTs represent attractive targets in cancer therapy.

We have studied the expression of MCTs in a variety of human cancer tissues including glioblastoma (GBM) by immunohistochemistry and also blocked MCT activity in different *in vitro* and *in vivo* models. MCT1 and MCT4 were found to be overexpressed in human GBM samples compared with diffuse astrocytomas and non-neoplastic samples. MCT1 targeting decreased cell glycolytic metabolism, migration, and invasion, induced cell death and sensitized GBM cells to temozolomide *in vitro*, and reduced tumour size and angiogenesis *in vivo*. Additionally, MCT1 is involved in the crosstalk between glioma cells and endothelial cells, and MCT1 targeting either in the tumour cells or endothelial cells decreased endothelial cell proliferation, migration and vessel assembly.

MCT1 is overexpressed in human gliomas and its inhibition decreased cancer cell aggressiveness and sensitized cancer cells to chemotherapy. Additionally, MCT1 mediates endothelial cell metabolic

reprogramming, and, thus, targeting MCT1 in both tumour cells and brain endothelial cells may be a promising therapeutic strategy for the treatment of GBM.

4. Metformin: toward drugging the metabolic control of epigenetics in cancer

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There is a growing appreciation that metabolic rewiring affects the epigenome in a manner that facilitates cancer formation, progression, and therapeutic resistance. An improved understanding of how the interplay between cell metabolism and the epigenome regulates major cell fate decisions such as cell differentiation, proliferation, and/or cell death, might radically amend the way we prevent and treat cancer. Metformin, a biguanide derivative that has long been a cornerstone in the treatment of type 2 diabetes (T2D), could help to accelerate the development of novel strategies capable of therapeutically tuning the metabolism-epigenome axis to battle cancer. This talk will summarize the most recent evidence collected in our laboratory unraveling the capacity of metformin to operate as a poly-therapeutic agent targeting the biologic machinery in charge of the metabolic recoding of cancer epigenetics. On the one hand, metformin can alter the abundance of mitochondrial metabolites that are substrates of chromatin-modifying enzymes (e.g., acetyl-CoA for histone acetyltransferases) by altering the energy status of the cell downstream of its primary inhibitory action on mitochondrial respiratory complex I. On the other hand, biocomputational approaches based on artificial intelligence coupled to experimental validation reveal that metformin is: (1) a direct SIRT1-activating compound that improves the catalytic efficiency of SIRT1-mediated deacetylation in cancer-prone conditions of low NAD⁺; and (2) a potent regulator of S-adenosyl methionine - mediated methylation reactions via direct and specific inhibition of a central reaction of the folate cycle, namely the conversion of serine to glycine by the mitochondrial serine hydroxymethyltransferase 2 enzyme. The biguanide metformin, which, sixty years after its introduction in Europe as a first-line therapeutic for T2D, may now be seen as an archetypal compound aiming at drugging the metabolism-epigenome axis in cancer.

5. PIK3C2G loss promotes pancreatic cancer development and metabolic rewiring

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Introduction: Pancreatic ductal adenocarcinoma (PDAC) is the most lethal cancer across the world, with incidence equaling mortality. A wealth of studies have identified the PI3K/mTOR axis as an important player in PDACs, impacting on tumor growth and metabolism. In particular, PI3K-C2γ, differently from other PI3Ks, is mainly expressed in the pancreatic tissue where it plays a critical role in controlling glucose metabolism.

Experimental model: Mouse model of PDAC (K-RASG12D/Trp53R172H/CrePdx1) was crossed with mouse strain lacking PI3K-C2γ expression. Mice were weekly followed for survival, tumor appearance and growth. Tumor lesions were evaluated by histopathological and immunofluorescence analysis. Functional *in vitro* and *in vivo* experiments were performed.

Results: PI3K-C2 γ loss was modeled by deleting *PIK3C2G* gene in a mouse model of pancreatic cancer (KPC). We found that its PI3K-C2 γ loss was sufficient to initiate and promote pancreatic tumor development, strongly reducing mice survival rate (18 weeks vs. 36 weeks) and driving rapid progression to PDAC. In pancreatic tumors reduced expression of PI3K-C2 γ expression was significantly associated with poor survival and resistance to chemotherapy. We reported that lysosomal localization of PI3K-C2 γ is responsible for mTORC1 inhibition under conditions of serum deprivation. In addition, we also observed that PI3K-C2 γ loss promotes the metabolic rewiring of PDAC, through the control of several metabolic factors, including glucose and monocarboxylate transporters and glycolytic enzymes (PKM2, HK2 and LDH). Furthermore, the use of rapalogs in KO KPC delay tumor progression.

Conclusion: PI3K-C2 γ is a novel tumor suppressor in pancreatic cancer and the metabolic phenotype of PI3K-C2 γ -deficient tumors can be exploited for specific therapeutic strategies.

6. Alleviating the Warburg effect: preliminary clinical results in advanced malignancies resistant to chemotherapy

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Chlorine dioxide and Methylene blue are known generators of free radicals. These free radicals are known to enhance the efficacy of the mitochondria.

The goal of this abstract is to describe the first fourteen patients with advanced incurable cancer treated, with a combination of metabolic treatment (lipoic acid, hydroxycitrate) and chlorine dioxide. All but one patient had failed conventional chemotherapy. Only three patients underwent concomitant conventional chemotherapy. There was no major side effect but nausea and diarrhea. All but one patient responded to treatment. Two more patients were treated with Methylene Blue and metabolic treatment for advanced tumors. One had low dose chemotherapy, the other one, with metastatic pancreatic cancer had no other treatment. Both responded. Rigorous clinical trials are warranted.

7. Ketogenic diets during radiotherapy against cancer

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The altered glucose metabolism of tumor cells is a long-known phenomenon that also contributes to resistance against radiotherapy by securing ATP production important for DNA repair, promoting tumor repopulation and increasing the pool of anti-oxidative molecules for protection against reactive oxygen and nitrogen species (ROS/RNS). In theory therefore, impairing the glucose metabolism of cancer cells is expected to lead to radiosensitization. Deduced from the hypothesis that cancer is a metabolic disease with mitochondrial dysfunction a hallmark of most tumor cells, ketogenic diets - i.e., diets systemically downregulating glycolysis and supporting ketone body and fat oxidation - have been proposed to selectively target tumor cells which in contrast to normal cells would be weakened when glucose and insulin levels are reduced. While many preclinical studies indeed found evidence for a reduction of tumor glycolytic

metabolism and growth through ketogenic diets, some others found no, or even pro-tumor effects. However, all studies published to date that combined a ketogenic diet with radiotherapy have found a radiosensitizing effect on tumor cells. Mechanisms include a selective increase in ROS and RNS production, ATP reduction and epigenetic impairment of DNA repair through histone deacetylase inhibition by the ketone body β -hydroxybutyrate, similar to the short chain fatty acid butyrate. The same mechanisms would not apply to normal tissue which is metabolically flexible and even able to initiate an anti-oxidative stress response when switching from glycolysis to mainly mitochondrial respiration fueled by fatty acids and ketone bodies. The hypothesis is therefore that combining a ketogenic diet with radiotherapy could benefit cancer patients in terms of larger tumor control probability for a given radiation dose without affecting or even decreasing the probability of normal tissue toxicity. While this hypothesis has been confirmed in preclinical studies, clinical data are limited thus far by small patient numbers, lack of control groups and lack of systematically collecting hard clinical endpoints such as local tumor control, overall survival and radiotherapy-induced side effects. However, there is some evidence that ketogenic diets could also benefit cancer patients in terms of better maintaining lean body mass which would be relevant especially for frail patient population such as those with head and neck or esophageal cancer. Given the proven safety of ketogenic diets when applied over several weeks in cancer patients, their combination with radiotherapy (as well as other established cancer treatments) deserves further study with a focus on important clinical endpoints.

8. Blocking the mitochondrial pyruvate carrier to inhibit lactate uptake by cancer cells and induce tumor radiosensitization

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Introduction: Lactate-based metabolic symbiosis between glycolytic and oxidative cancer cells is known to facilitate tumor growth. Compounds with the capacity to block this lactate exchange thus represent attractive therapeutic modalities to impact on tumor progression.

Experimental model: Several models, including *Xenopus* oocytes, 3D tumor spheroids and human tumor xenografts in nude mice, were combined with state-of-the-art metabolomics strategies (Seahorse respirometry, *in vitro* ¹³C tracing experiments, and *in vivo* hyperpolarized ¹³C-pyruvate monitoring) to characterize the mode of action of 7ACC2, an anticancer compound originally reported to block lactate influx but not efflux.

Results: We identified 7ACC2 as a potent inhibitor of the mitochondrial pyruvate carrier (MPC) activity which consecutively blocks extracellular lactate uptake by promoting intracellular pyruvate accumulation. Importantly, while both 7ACC2 and the MCT1 inhibitor AR-C155858 efficiently inhibited lactate influx, only the former could also block compensatory oxidative glucose metabolism. Moreover, while in 3D tumor spheroids MCT1 inhibition led to cystostatic effects, MPC activity blockade induced cytotoxic effects. This potent growth inhibitory action associated with exacerbated 7ACC2-mediated metabolic alterations (i.e., blockade of lactate- and glucose-fueled TCA cycle) led to a reduction in hypoxia as proven in spheroids via pimonidazole and carbonic anhydrase IX staining and *in vivo* through electron paramagnetic resonance

measurements. We showed that this induced tumor reoxygenation could benefit radiotherapy. Indeed, pre-challenge of tumor-bearing mice with 7ACC2 considerably improved the anticancer efficacy of either single high dose or fractionated low dose radiation therapy.

Conclusion: This study positions MPC as control point for lactate metabolism and expands on the anticancer potential of MPC inhibition.

9. The anti-neoplastic effect of triiodothyronine on hepatocellular carcinoma is preceded by reversion of the Warburg metabolism and inhibition of the pentose phosphate pathway

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Introduction: Liver carcinogenesis, from its very early steps, is characterized by reduced activation of thyroid hormone nuclear receptors (THR_s), and an altered glucose catabolism that leads to a Warburg metabolism and a strong induction of the pentose phosphate pathway (PPP). The administration of the THR_s agonist, triiodothyronine (T₃), reverts the preneoplastic hepatocytes to a fully differentiated phenotype and prevents the development of hepatocellular carcinomas (HCC), but its effects on glucose metabolism have not yet been fully explored. Given the strong link between THR_s activation and metabolism, the aim of this study is to verify whether T₃ is able to restore the physiological PPP activity and energetic metabolism in neoplastic liver.

Experimental model: Rats were subjected to the Resistant-Hepatocyte model of liver carcinogenesis and treated with T₃ at 10 weeks, when preneoplastic nodules reached the maximal expansion, or at 10 months after initiation, when HCCs appeared. Rats were killed after 4 or 7 days of T₃ treatment.

Results: The treatment with T₃ for 7 days resulted in a reduction in the number of preneoplastic lesions. This effect was preceded by a change in the expression of key genes regulators of the glucose metabolism. The immunohistochemical and enzymatic study of liver sections confirmed that the reversion of the Warburg phenotype and inhibition of the PPP occurs before the disappearance of the preneoplastic lesions. The same effect was evident also in fully developed HCCs.

Conclusion: Our results indicate that, at least in part, the antineoplastic effect of T₃ depends on the ability of the activated THR_s to revert the Warburg phenotype together with an inhibition of the PPP.

10. The interaction between the transcription factor STAT3 and the Ca²⁺ channel IP3R3 regulates Ca²⁺ release from the endoplasmic reticulum and apoptosis

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In many tumors the signal transducer and activator of transcription (STAT) 3 has been found to be constitutively active exerting two different functions, both required for cell transformation: (1) activating transcription of its canonical target genes; and (2) regulating energy metabolism and mitochondrial functions. These different activities are mediated by distinct phosphorylation events. In particular, phosphorylation on residue Y705 is responsible for the nuclear activities, while phosphorylated S727 is involved in regulating OXPHOS activities via mitochondrial localization.

We recently reported the localization of STAT3, in both its serine- and tyrosine-phosphorylated forms, to the endoplasmic reticulum (ER) in breast cancer cell lines displaying constitutive activation of this factor. In these cells, S727-phosphorylated STAT3 is able to reduce ER Ca^{2+} storage and release, thus contributing to resistance to Ca^{2+} mediated apoptosis upon oxidative stress stimuli. Indeed, Ca^{2+} release from the ER represents a crucial event in the regulation of life/death decision of cells, and its regulation is mainly exerted by inositol 1,4,5-trisphosphate receptors, and in particular by type 3 (IP3R3), a calcium channel that in response to IP3 stimulation allows Ca^{2+} transfer from the ER to the mitochondrial associated membranes. We observed that STAT3 can physically interact with IP3R3. Moreover, STAT3 silencing increases IP3R3 expression by decreasing IP3R3 proteasoma-mediated degradation, suggesting that constitutively active STAT3 can inhibit apoptosis by regulating IP3R3 abundance and activity. Accordingly, we found an inverse correlation between IP3R3 and STAT3 protein levels in basal-like breast cancers, a cancer subtype that often shows constitutive STAT3 activation. All together, these data indicate that S727-phosphorylated STAT3 can promote IP3R3 degradation via direct interaction, thus reducing Ca^{2+} exit from the ER and conferring resistance to apoptosis.

Thus, in addition to the previously characterized nuclear and mitochondrial functions, we described a novel contribution of STAT3 constitutive activation in conferring aggressiveness to breast cancer cells, and in particular to basal-like breast cancers. This is exerted via localization of STAT3 to the ER, interaction with IP3R3 and regulation of its degradation, that leads to decreased calcium release and thus resistance to apoptosis.

11. Targeting acidic, nutritional and oxidative stresses in cancer

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In metazoans, sensing the availability of oxygen and key nutrients (glucose, amino acids, fatty acids) is integrated with growth factor and hormone signaling. This multiple nutrient and energy checkpoint converges on the activation of the master protein kinase TORC1, critical for engaging cells in the cell cycle and promoting growth. Cells have evolved sophisticated regulatory systems to rapidly respond to several lethal stressors including metabolic acidosis, nutritional depletion and reactive oxygen species. Cancer cells respond in multiple ways to escape and thrive these microenvironment stresses thus offering several strategies to combat cancer resilience before and after therapeutic treatment.

In this lecture we will discuss how we can exploit cancer vulnerabilities (metabolic tumor acidosis, amino acid depletion and oxidative stress) to propose novel anticancer targets capable to either arrest tumor growth or to kill cancer cells.

12. The role of diet related short chain fatty acid acetate in colorectal cancer: therapeutic implications

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Colorectal cancer (CRC) is an important public health concern worldwide, particularly among populations that adopt Western-style diets. The use of dietary propionibacteria found in dairy products, which produce short-chain fatty acids (SCFA), has been suggested as a possible strategy in the prevention and therapy of CRC. The SCFA acetate has been proved by us and others to induce apoptosis in CRC cells. Our group has been focusing on unravelling the mechanisms underlying acetate-induced apoptosis and on understanding the precise role of acetate in CRC cells.

We showed that acetate induces partial lysosome membrane permeabilization with specific cathepsin D (CatD) release to the cytosol in CRC cells. We verified that CatD has an anti-apoptotic role by the degradation of damaged mitochondria when autophagy is impaired, protecting CRC cells from acetate-induced apoptosis. Moreover, we demonstrated that acetate enters CRC cells by a sodium dependent monocarboxylate transporter 1 and passive diffusion by aquaporins. We also found that MCT-1 and/or MCT-2 seems to mediate acetate transport in CRC cells exposed to acetate. Additionally, we observed that acetate upregulates MCTs expression and promotes plasma membrane re-localization of MCT-1 and triggers changes in glucose metabolism. Further, we explored the combined treatment of acetate with the glycolysis inhibitor 3BP and we demonstrated that 3BP potentiates acetate-induced apoptosis in CRC cells.

Our results established a protective role of CatD in acetate-induced apoptosis which could negatively impact the efficacy of acetate. Thus, the use of CatD inhibitors in combination with strategies to increase acetate concentrations in the colon, like nutraceuticals, should be explored. Our findings also support a novel approach for CRC therapy based on the association of acetate with 3BP or other anti-cancer agents whose transport is mediated by MCTs.

13. Implementing new diet formulations in order to shape microbiota and reverse chemoresistance in the frame of pancreatic cancer

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Introduction: Despite recent advances in treatment options, pancreatic cancer (PC) remain a highly malignant disease and due to its poor prognosis it is ranked as the fourth leading cause of cancer-related deaths worldwide. We have previously demonstrated that manipulating the dietary intake of total amount of calories (using a short-term fasting cycles approach) or replacing carbohydrate with resistant starch have the potential to improve the efficacy of chemotherapy against PC. The aim of this talk is to clarify the effect of the engineered resistant-starch (ERS) mimicking diet on the growth of cancer cell lines *in vitro*, on the composition of fecal microbiota, and on tumor growth in an *in vivo* pancreatic cancer mouse xenograft model. Moreover we will shed light on the interaction between the microbiota and chemotherapeutic drugs.

Experimental model: Pancreatic cancer xenograft mice were subjected to different dietary approaches such as intermittent fasting cycles or ERS diet to assess tumor growth as compared to mice fed with a standard diet. The composition and activity of fecal microbiota and metabolomics profiles were further analyzed by next generation sequencing (NGS) technology and LC-MS analysis of serum respectively.

Results: Pancreatic cancer cells cultured in fasting mimicking medium and ERS diet-mimicking medium impacted the proliferation pathway and the nutrients sensitive pathway decreasing the levels of phospho-ERK1/2 and phospho-mTOR. As compared to those cultured in standard medium. Consistently, xenograft pancreatic cancer mice subjected to modifies dietary approaches displayed significant retardation in tumor growth and a significant shift in microbiota profiles towards butyrate producer's microorganisms.

Conclusion: It is important to modulate dietary habits and apply new dietary intervention in order to modulate the composition and metabolism of mouse fecal microbiota which is one of the key organs involved in the chemotherapy response. All these results indicate that engineered dietary interventions may potentially reveal useful in fighting human cancers and could be supportive as a synergistic approach to improve the efficacy of existing drugs in pancreatic cancer therapy.

14. Effects of selective targeting of mitochondria in experimental cancer and chemotherapy-induced cachexia

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Cachexia is a syndrome frequently occurring in cancer patients. The main features are loss of muscle mass and function associated with negative energy balance. In this regard, cachectic tumor-bearing animals show altered mitochondria and reduced oxidative capacity in the muscle. Mitochondrial homeostasis could be further impaired by chemotherapy administration, contributing to muscle wasting.

The present study aimed at evaluating the effects of a mitochondrial-targeted compound (SS-31) on muscle wasting in mice bearing the C26 tumor in the presence or in the absence of chemotherapy [oxaliplatin + fluoruracil (OXFU)].

Reduced body weight, food intake, muscle mass and function were observed in the untreated tumor hosts. Lifespan in OXFU-treated C26 hosts was almost doubled with respect to untreated animals, however muscle wasting was exacerbated. SS-31 administration was able to counteract body weight loss, anorexia and the reduction of myofiber dimensions, while the protection against muscle mass depletion was only slight. As for markers of mitochondria, both treated and untreated C26 hosts showed that the expression of PGC-1 α , cytochrome c, SDH and cardiolipin levels were reduced compared to control animals, and not modified by SS-31. In OXFU-treated tumor-bearing mice, also the SDH total activity and ATP content were reduced. SS-31 increased SDH activity and ATP content in untreated C26 hosts. The modulations of the mitochondrial compartment in the C26-bearing mice were also associated with markedly decreased protein synthesis, that improved after administration of SS-31.

In conclusion, these results suggest that targeting mitochondria could be an effective strategy to counteract cancer cachexia, partially preventing the loss of body weight and food intake, improving muscle atrophy, muscle oxidative capacity and energy wasting in mice hosting the C26 tumor in the absence of

chemotherapy. Further investigation and treatment optimization is required in order to demonstrate a potential effectiveness of SS-31 in the chemotherapy-treated tumor-bearing mice.

15. L1CAM gene overexpression is associated with platinum-resistance in high-risk endometrial carcinoma

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Introduction: L1 cell adhesion molecule (L1CAM) expression has been reported associated with high-grade disease and non-endometrioid histology, as well as poor prognosis, in endometrial carcinoma (EC). These high-risk EC types have frequently already spread outside the uterus when diagnosed and, after an extensive surgery, are often treated with chemo and radiation therapy. We hypothesized that L1CAM gene expression could discriminate, among poor outcome EC patients, those who do and who do not respond to adjuvant platinum-based chemotherapy.

Experimental model: Using an efficient multiplex qRT-PCR, we test L1CAM mRNA expression on 117 EC and 16 normal endometrial (NE) flash-frozen tissues, with HPRT1 and PPIA as reference genes.

Results: L1CAM mRNA was significantly overexpressed in EC compared to NE tissues ($P = 0.02$), significantly upregulated in G3 vs. G1-2 ECs ($P < 0.001$) and in non-endometrioid vs. endometrioid ECs ($P < 0.001$). Our analysis showed no difference in L1CAM expression of stage I-II vs. stage III-IV ECs ($P = 0.5$). Of the initial 117 EC patients, 47 received chemotherapy on adjuvant setting and were classified as platinum-sensitive and platinum-resistant patients, based on PFI > 12 months and < 6 months, respectively. L1CAM gene was significantly overexpressed in resistant vs sensitive EC ($P = 0.001$). Moreover, by means of a multivariate logistic regression model, we found L1CAM gene overexpression as an independent indicator of the probability to harbor a platinum-resistant EC ($P = 0.047$, OR = 3.5). In addition, univariate and multivariate survival analysis showed L1CAM gene upregulation associated with poor outcome, in terms of progression-free survival and disease-specific survival.

Conclusion: Our results suggest L1CAM gene expression as a potential prognostic marker and a predictive biomarker of platinum-response in high-risk EC patients.

16. Resveratrol counteracts ovarian cancer cell migration stimulated by interleukin-6 by limiting glucose uptake

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Introduction: IL-6, a pro-inflammatory cytokine produced by cancer-associated fibroblasts, increases the proliferative and invasive properties of ovarian cancer cells. Glucose metabolism is altered in ovarian cancer cells and permits fast proliferation and survival. Resveratrol (RV) is a naturally occurring polyphenol with the potential to inhibit cancer cell migration.

Results: Here, we found that IL-6 enhances ovarian cancer cell migration, while RV and deprivation of glucose reduce cell motility. In particular, IL-6 stimulates glucose uptake along with cell migration, while RV abrogates this effect through the reduction of GLUT1 plasma membrane translocation and glucose internalization. Further, the cells exposed to IL-6 at the migration front show an increased expression of N-cadherin over E-cadherin, and this effect is reverted by RV exposure. Accordingly, the expression of TWIST1, a regulator of epithelial-to-mesenchymal transition (EMT), is reduced by RV and deprivation of glucose. Transcriptomic and microRNomic analyses revealed that RV up-regulates a subset of miRNAs that have several glucose metabolism regulators as targets. In particular, we found that RV abrogates the transcription of ZEB1, Hexokinase 2 and FOXM1.

Conclusion: Our data indicate that RV counteracts glucose metabolism negatively impinging on ovarian cancer cell migration induced by IL-6.

17. FXYD5 is a predictor of short-term survival in high-grade serous ovarian carcinoma

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Introduction: High-grade serous ovarian carcinoma (HGSOC) is generally associated with a very dismal prognosis. Nevertheless, patients with similar clinicopathological characteristics can have markedly different clinical outcomes. We aim to identify the molecular determinants influencing survival by comparing the gene expression patterns of two patient cohorts characterized by extreme overall survival.

Experimental model: We determined the gene expression profiles of 12 HGSOC long-term and 27 short-term survivors (training set) by microarray chips. By a generalized linear model with cross-validation, we generated a prognostic gene signature that was further evaluated on the entire "The Cancer Genome Atlas" (TCGA) ovarian cancer dataset. The resulting genes were then verified on an independent cohort of 29 HGSOC flash frozen samples (validation set) by RT-qPCR, and in a panel of 38 formalin fixed paraffin embedded HGSOC tissues by immunohistochemistry (IHC).

Results: We identified a ten-gene prognostic signature able to correctly assign 98% of patients of the training set within their survival class. By "in silico" validation on TCGA microarray dataset, we confirmed the overexpression in short term survivors of FXYD domain containing ion transport regulator 5 (FXYD5),

one of the 10 top score genes of the signature. The prognostic power of FXYD5 was also successfully validated, both at mRNA and protein level by RT-qPCR and IHC, respectively on the training and validation sets. Moreover, FXYD5 overexpression was significantly associated with platinum resistance and cancer progression.

Conclusion: We demonstrated the consistent overexpression of FXYD5 in HGSOC short-term survivors compared to long-term ones. FXYD5 may become a useful predictive marker for a more accurate selection of HGSOC patients for adjuvant treatments, and a possible target for antibody-drug conjugated anticancer agents, as recently demonstrated for thyroid cancer cell lines.

18. Claudin-7 downregulation is predictive of distant metastases in high-grade serous ovarian carcinoma patients

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Introduction: High-grade serous carcinoma (HGSOC) is the most frequent and lethal ovarian carcinoma histotype. Although the abdominal dissemination is considered the most common, distant metastases occur in about 30% of patients with newly diagnosed or recurrent HGSOC. No tumor marker can currently predict the risk of distant metastasis in HGSOC. Tight-junction protein claudin-3, -4 and -7 are frequently dysregulated in HGSOC and functionally related to cancer progression to a metastatic disease. Here we analyze claudin-3, -4 and -7 expression as markers of distant metastasis.

Experimental model: Claudin expression was evaluated in 105 primary HGSOC tissues, 14 normal ovarian and 26 normal fallopian tube epithelia by quantitative RT-PCR and immunohistochemistry, and correlated with clinicopathological features. Gene set enrichment analysis was performed on microarray-generated gene expression data to investigate key pathways in patients with distant metastasis.

Results: Claudin-3, -4 and -7 expression levels are decreased in HGSOC compared to normal tubal epithelium, currently considered alternative source of such tumors. Decreased expression of claudin-7 is seen in tumors from women who develop distant recurrence ($P = 0.016$), mainly by hematogenous route ($P = 0.025$). The estimated reduction in the probability of distant disease is of 39% per unit increase in the level

of claudin-7 (AUC 0.659, $P = 0.03$). Genes involved in hypoxia and angiogenesis processes result strongly associated to hematogenous recurrence ($P = 0.012$).

Conclusion: Claudin-7 decreased expression in primary tumor tissues is a significant predictor of distant metastasis in HGSOC patients.

19. Glutamine synthetase-negative multiple myeloma cells secrete glutamate and shape the bone marrow niche

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Altered metabolism, a hallmark of cancer, also impacts on non-cancer cells of tumor microenvironment, driving them towards a pro-tumor behavior. In multiple myeloma (MM) patients, bone marrow glutamine (Gln) is lowered, while glutamate (Glu) and ammonium increase. In most MM patients, Glutamine Synthetase (GS), the enzyme that catalyzes Gln synthesis from Glu and ammonium, is lowered in neoplastic plasma cells. GS is also down-regulated during normal osteoblast differentiation from mesenchymal stromal cells (MSC). Since bone lesions of MM patients are characterized by impaired osteogenesis, we hypothesize that MM cells negatively affect osteoblasts through the peculiar low-Gln, high-Glu bone marrow microenvironment and the manipulation of GS expression in niche cells.

Human MM cell lines (HMCLs) and immortalized MSC were grown in Glu-free α MEM and used in mono- or co-cultures. Gene expression was assessed at either mRNA and protein level. Amino acid levels were determined with a colorimetric test (extracellular) and LC-MS/MS (intracellular).

Analysis of a dataset of 323-sample plasma cell dyscrasias indicated that GS expression is downregulated during MM progression, with less than 20% MM patients with GS-positive CD138⁺ cells. Also most HMCLs, but not MM1.S and U266, have a negligible GS expression. While both low- and high-GS HMCLs exhibited a high consumption of Gln and a comparable glutaminolysis, low-GS HMCLs exploited roughly 50% of Gln to secrete Glu. Consistently, the activity of the Glu exchanger xCT was higher in low-GS MM cells. GS is strongly induced in MSC when co-cultured with MM cells.

These preliminary results are consistent with the hypothesis that low GS expression, Gln addiction and Glu secretion of MM cells are functional to osteoblastic differentiation impairment in the BM niche.

20. Evaluation of the interaction among HPV16 E6 and E7 oncoproteins and the DNA damage sensor 53BP1 in 2D and 3D epithelial cultures by the proximity ligation assay

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Introduction: Human papillomaviruses (HPV) group several viruses are able to infect squamous stratified epithelia and cause benign papillomas, warts and anogenital lesions. They also correlate to oropharyngeal

and anogenital malignancies, mainly promoted by the high risk (HR) α -HPV16 E6E7 oncoproteins. Despite scientific progresses and the development of vaccines, these tumors are still common and represent one of the major causes of women's death. Host's cell replication fidelity depends by the DNA damage response (DDR). Unlike from other DNA viruses, HR-HPVs encourage cells proliferation without inactivating the DDR: the mechanisms at the basis haven't been clarified yet. During HPV16 infection, E6 binds and degrades p53 through the E6AP LXXLL domain. Similarly, E7 competes with E2F1-pRb interaction, thus inactivating pRb, and promoting the linking the pRb-like proteins CBP/p300 and p107, that also harbor a LXXLL sequence. Unfortunately, E6 E7 role in the DDR activation is not elucidated yet.

Experimental model: To gain new information, we reproduced an *in vitro* 3D HPV16-E6E7 infected epithelium, already characterized for HPVs studies, and checked for cellular and viral markers, among them HPV16E6E7 oncoproteins and the double strand breaks sensor 53BP1; we then made a Co-IF for E6 and E7 with 53BP1. Since E6 and E7 both interact with LXXLL containing proteins, we analyzed 53BP1 BRCT2 domain and we explored the binding hypothesis via the *in situ* PLA technique in 2D in CaSki and E6E7HPV16 keratinocytes and in the 3D model.

Results: The *in vitro* infected epithelium resembles the *in vivo* tissue. E6E7HPV16, both in basal and differentiated strata, induce a 53BP1 increase in nuclear foci. After highlighting E6 and E7 co-expression with 53BP1 and a LKVLL sequence within the 53BP1 BRCT2 domain, we demonstrated the binding in all the employed cellular models.

Conclusion: Our results add new information on HPV16 oncoproteins capability in overcoming cellular defense strategies.

21. Iron metabolism regulates cancer related skeletal muscle wasting

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Introduction: Cancer associated cachexia is a syndrome characterized by a significant weight loss, due to metabolic changes affecting skeletal muscle and adipose tissue²⁰¹⁸. Given the importance of iron in controlling energy metabolism, we speculated that decreased iron availability occurring in cancer might contribute to skeletal muscle atrophy.

Experimental model: *In vitro* experiments are performed with C2C12 myotubes, while *in vivo* experiments are conducted using Colon-26 carcinoma bearing BALB-C mice.

Results: Interestingly, using Colon-26 carcinoma bearing mice, we found strong alterations in protein and gene expression of iron homeostasis key players in skeletal muscle, notably a downregulation of transferrin receptor 1 (TFR1, the main importer of iron) and an increase of the iron exporter Ferroportin. Coherently, we observed a decreased iron content in both skeletal muscle and spleen while serum iron is increased, suggesting a global mobilization of iron. To further confirm our hypothesis, we created iron-deprived models *in vitro* using several iron chelators (Desferoxamine, BPS) or by TFR1 knockdown with siRNA and, in line with our *in vivo* observations, iron depletion in C2C12 myotubes directly promoted atrophy. Finally, we were able to prevent myotube atrophy by restoring iron transport.

Conclusion: Taken together, these results highlight a previously unknown role for altered iron homeostasis in cancer-induced muscle wasting and provide a potential new therapeutic target.

22. Liquid biopsy applications in lymphomas

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Liquid biopsy is an emerging tool across many types of cancers. This technique consists in the analysis of biomarkers released by tumor cells in the peripheral blood. In lymphomas, the most studied biomarker is circulating tumor DNA (ctDNA), shed into the bloodstream (e.g., plasma) by tumor cells undergoing apoptosis.

Analysis of plasma ctDNA analysis allows serial monitoring of the disease genotype over time and the assessment of the entire tumor heterogeneity at different anatomic sites. Consistently, ultra-deep targeted next generation sequencing of ctDNA from diffuse large B-cell lymphoma (DLBCL) patients correctly identified DLBCL-associated mutations. Moreover, plasma ctDNA genotyping also allows for the recovery of mutations that are undetectable in the tissue biopsy, conceivably because, due to spatial tumor heterogeneity, they are restricted to clones that are anatomically distant from the biopsy site. In Hodgkin lymphoma (HL), the rarity of neoplastic cells in the biopsy has so far limited genomic studies. By using a highly sensitive and robust deep next-generation sequencing approach for ctDNA, the current knowledge of HL has been refined. In addition, similarly to DLBCL, also in HL a fraction of mutations has been detected only in ctDNA but not in the tissue biopsy.

Regarding disease monitoring during the course of treatment, CT/PET scans do not capture all patients designated to relapse. This gap may be filled by ctDNA analysis providing higher sensitivity and ease of sample collection in a radiation free manner. In DLBCL, the 2.5-log drop of ctDNA concentration after two cycles of treatment is an independent predictor of response. Similarly, in HL the 2-log drop of ctDNA after two courses of chemotherapy associated with complete response and complemented the results obtained by the CT/PET scan. Incorporation of both CT/PET and ctDNA monitoring into clinical trials may guide future personalized risk-directed approaches of treatment.

23. *BIRC3* mutations stratify a poor prognostic subgroup in Fludarabine-Cyclophosphamide-Rituximab treated chronic lymphocytic leukemia

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Introduction: The current shift of therapy of chronic lymphocytic leukemia (CLL) towards novel targeted agents mandates the identification of new molecular predictors. The aim of this study is to identify new molecular predictors in FCR treated patients and to assess the biological features underlying chemo-refractoriness to FCR.

Experimental model: A retrospective multicenter cohort of 287 untreated CLL receiving first-line therapy with FCR was subjected to a targeted NGS approach in 24 most recurrently mutated genes in CLL. The entire non-canonical NF- κ B pathway was assessed by Western blot and by real-time PCR.

Results: *SF3B1* and *NOTCH1* were the most frequently mutated genes identified in 13.9% and in 13.6% of patients respectively, followed by *TP53* in 9.4% and *ATM* in 6.9%. *BIRC3* was mutated in 3.1% of patients. By univariate analysis adjusted for multiple comparisons, only *BIRC3* mutations (median PFS of 2.2 years; $P < 0.001$) and *TP53* mutations (median PFS of 2.6 years; $P < 0.0001$) identified patients who failed early FCR. By multivariate analysis, *BIRC3* mutations maintained an independent risk of progression, with a HR of 2.8 (95%CI 1.4-5.6, $P = 0.004$). In addition, *in vitro* studies showed that fludarabine-induced apoptosis in *BIRC3* mutated cells was comparable to samples harboring *TP53* mutations. Western blotting analysis of the non-canonical NF- κ B pathway showed that *BIRC3* mutated cells was significantly enriched of non-canonical NF- κ B target gene and addicted of MAP3K14 overexpression.

Conclusion: *BIRC3* mutations identify a very poor prognostic subgroup of patients that fails FCR similar to patients harboring *TP53* abnormalities. If validated, *BIRC3* might be used as a new molecular predictor to select high-risk patients for novel frontline therapeutic approaches. From the biological point of view, *BIRC3* mutations enhance the non-canonical NF- κ B signaling pathway promoting survival, proliferation and chemo-refractoriness.

24. Peptide nucleic acid-based targeting of microRNAs: possible therapeutic and diagnostic applications for glioblastoma

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Introduction: MicroRNAs (miRNAs) are small noncoding RNAs regulating gene expression by sequence-selective targeting of mRNAs, leading to translational repression or mRNA degradation. Low miRNA expression is associated with accumulation of target mRNAs; high miRNA content causes low expression of target mRNAs. In cancer, microRNAs are associated with tumor onset and progression.

Experimental model: Targeting oncomiRNAs and metastamiRNAs with biomolecules interfering with their biological activity is of interest and peptide-nucleic acids (PNAs) might be useful. PNAs are DNA analogues in which the sugar-phosphate backbone has been replaced by *N*-(2-aminoethyl) glycine units, hybridize to RNA with high efficiency, are resistant to proteinases and nucleases, and have been proposed as excellent tools for alteration of gene expression. We have developed novel delivery strategies for PNAs targeting miRNAs, based on the use of PNAs linked to a poly-arginine R8 peptide tail for efficient cellular delivery. As far as cancer-related model systems, we focused on PNAs targeting miR-221 and miR-222 in glioblastoma cells.

Results: In a first study, a combined treatment of U251, U373 and T98G glioma cell lines was performed with different anti-miRNA PNAs (against miR-221, miR-222 and miR-155). Increased pro-apoptotic effects were obtained with the co-administration of both anti-miR-221 and anti-miR-222 PNAs, or anti-miR-221 and anti-miR-155 PNAs. In a second study, we demonstrated synergistic effects of co-administration of corilagin and a PNA targeting miR-221. In a third approach we performed a combined treatment of glioma U251 cells with the pro-apoptotic pre-miR-124 and the PNA targeting miR-221, showing induction of apoptosis at very high levels.

Conclusion: PNAs might be a relevant therapeutic tool for anti-cancer miRNA-therapy based on inhibition of oncomiRNA and metastamiRNAs, as well as mimicking tumor-suppressor miRNAs (funded by AIRC IG13575 and Horizon 2020 Project ULTRAPLACAD).

25. Liquid biopsy-based colorectal cancer diagnosis: analysis of a limited panel of miRNA in mice bearing colorectal carcinoma tumor xenografts and in Human plasma samples

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Introduction: Due to their high stability in body fluids, circulating tumor microRNAs are proposed as promising biomarkers useful for early tumor diagnosis, prognosis, monitoring, and to predict therapeutic response, in non-invasive liquid biopsy. We investigated the release of miR-141-3p, miR-221-3p and miR-222-3p previously associated to colorectal cancer (CRC).

Experimental model: We employed droplet digital PCR to quantify the amount of miRNAs released in: (1) supernatants of three human CRC cell lines (HT-29, LoVo and Ls174T); (2) in plasma of nude mice inoculated with the same three cell lines, in order to obtain tumor xenograft models; (3) in plasma isolated from a heterogeneous group of CRC patients.

Results: MicroRNAs miR-221-3p and miR-222-3p (but not miR-141) in cellular supernatants were proportional to the cellular levels. Interestingly, all three miRNAs are released in plasma of xenografted mice. Using plasma samples from CRC patients, we found that only in 57% of the cases it was possible to identify a differential expression of at least one of the miRNAs with respect to control subjects.

Conclusion: Our data demonstrate that, despite the three selected miRNAs are not only present in CRC cells and tissues but are also released in extracellular environments, they are not informative for a high proportion of CRC patients. NGS allowed to expand the number of miRNAs differentially expressed in CRC samples. A novel set, constituted of 12 miRNA was demonstrated to be of diagnosis relevance in 94% of CRC patients samples (funded by AIRC IG13575 and Horizon 2020 Project ULTRAPLACAD).

26. Sensitivity to asbestos is increased in patients with mesothelioma and pathogenic germline variants in BAP1 or other DNA repair genes

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Introduction: Malignant Pleural Mesothelioma (MPM) is a rare, aggressive cancer caused by asbestos exposure. A genetic predisposition has been suggested to explain the occurrence of multiple cases in the same family and the observation that not all individuals highly exposed to asbestos develop the tumor. Germline variants in *BAP1* are responsible for a rare cancer-prone syndrome (*BAP1*-TPDS) that includes mesothelioma in its cancer constellation. *CDKN2A* and several DNA repair genes have been reported as further predisposing genes.

Results: We searched for *BAP1* germline variants in 25 new probands with suspected *BAP1*-TPDS and we found a new pathogenic germline variant that affects splicing: c.783+2T>C. We calculated that the prevalence of the truncating variants in patients with familial MPM and MPM and other tumors is 7.7% (3/39). Cumulative asbestos exposure was assessed quantitatively in our cohort of patients to compare patients carrying pathogenic variants in *BAP1*, *CDKN2A* and DNA repair genes ($n = 14$) to patients without variants in 94 cancer predisposing genes ($n = 67$). We showed that patients with variants in *BAP1*, *CDKN2A* and DNA repair genes had a lower asbestos exposure than non-mutated patients ($P = 0.00002$).

Conclusion: Our results suggest that other genes could be involved in the genetic predisposition to mesothelioma. These data support the hypothesis of an increased asbestos sensitivity in patients with germline variants in *CDKN2A*, *BAP1* or DNA repair genes. According to the concept of BRCAness, patients with germline mutations in genes involved in homologous recombination repair may respond to drugs (e.g., PARP inhibitors) that induce synthetic lethality, similarly to patients with familial ovarian cancer and *BRCA1/BRCA2* inherited mutations.

27. Resveratrol reverts the EMT phenotype induced by lysophosphatidic acid in ovarian cancer cells through restoration of autophagy

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Introduction: Ovarian cancer emerges as a highly aggressive metastatic disease characterized by a high grade of lethality due to its asymptomatic nature and the late diagnosis, when cancer cells already spread in distant organs. Cancer progression is facilitated by pro-invasive factors, released by ovarian cancer cells and CAFs, that promote the Epithelial-to-Mesenchymal transition (EMT). Lysophosphatidic acid (LPA), a bioactive phospholipid abundantly present in ascitic fluid and plasma of ovarian cancer patients, stimulates the invasiveness of cancer cells. Resveratrol (RV), a natural-occurring polyphenol, is attracting the interest of many researchers due to its several anti-cancer properties. Of note, RV is a strong autophagy inducer.

Results: We found that LPA elicits cell migration in a panel of ovarian cancer cell models characterized by different grades of malignancy. In parallel, LPA induces EMT through the inhibition of autophagy in the cancer cells at the migration front. Interestingly, RV restores autophagy and halts ovarian cancer cell motility even in the presence of LPA.

Conclusion: Our findings indicate that restoration of autophagy by RV prevents LPA-induced tumor invasion and suggest that there is a functional cross-talk between autophagy and EMT phenotype.

28. Spiperone, an antipsychotic, induces colorectal carcinoma cell death by a calcium-mediated apoptosis

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Colorectal cancer (CRC), one the most common malignancies, with increasing incidence and mortality rate worldwide, requires novel therapeutic strategies for its treatment. As developing new drugs is a time- and cost-intensive process with unexpected side effects, drug repositioning provides an effective strategy for accelerating drug development. Some antipsychotics currently used in human therapy reported potential anti-tumoral activity, thus in this study we intended to investigate their activity against CRC and CRC-stem cells (CRC-SCs).

We screened a panel of commercially available psychotropic drugs for their antitumoral activity on HCT116 cell line. Regardless for their receptor specificity, the viability data showed that this cell line was sensitive to just some drugs, especially spiperone, with an IC_{50} of 4 $\mu\text{mol/L}$. Spiperone was also highly toxic for CRC-SCs, inducing cell death by both apoptosis and necrosis with an $IC_{50} < 6 \mu\text{mol/L}$. To verify its mechanism of action, we investigated the role of spiperone on Ca^{2+} homeostasis in HCT116 by using the Fura-2 probe. We observed that it increases cytoplasmic Ca^{2+} in CRC cells in a dose dependent manner. Experiments performed in the absence of extracellular Ca^{2+} demonstrated that spiperone increased cytoplasmic Ca^{2+} originating from endoplasmic reticulum (ER); this was further confirmed by the induction of ER depletion with 2,5-tBHQ (specific SERCA inhibitor) followed by spiperone treatment resulted in an accentuated store operated Ca^{2+} entry response. Restoration of extracellular Ca^{2+} in the presence of spiperone further increased cytoplasmic Ca^{2+} , suggesting that this drug is also active on plasma membrane Ca^{2+} channels.

Concluding, our data suggest that the anti-tumoral activity of spiperone might be due to Ca^{2+} movements through ER and plasma membrane channels. Further experiments are needed to better clarify the mechanism of action of spiperone in Ca^{2+} signalling and its possible repurposing as an antineoplastic drug.

29. Circulating tumor cells in patients with non small cell lung cancer: pilot study, initial results

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Introduction: Lung cancer is the main cause of cancer mortality worldwide and the number of new cases is still rising. Early detection might be paramount to diagnose curable stages. There is growing interest about isolation of circulating tumor cells (CTC). This pilot study aimed at isolating epithelial putative CTCs in peripheral blood of early stage lung cancer patients using Smart BioSurface CTC assay, a nanoparticle-

coated slide that quickly immobilizes living nucleated cells, thus avoiding pre-selection and any change in cell structure and biology.

Experimental model: Twenty-two patients undergoing surgical lung resection have been included in the preliminary study. Blood sample was collected from patients and the white blood cells fraction was let adhere on SBS-CTC slide. The cells were stained with anti-CD45 and anti-pan-CK antibodies. Positive cells (putative CTCs) were detected with an automated fluorescence microscope, isolated by laser capture microdissection and characterized for gene mutations.

Results: All patients were positive for at least 1 epithelial cell (putative CTC)/mL of blood, though no correlation between the number of epithelial cells and the stage of the disease was observed. NGS analysis revealed gene variants associated with tumors in all patients, of whom 13 patients had mutations in genes that are specifically associated with lung cancer. One patient carries BRAF V600E mutation, that has been identified also in the tissue biopsy.

Conclusion: Epithelial cells are present in the blood of lung cancer patients. Further analyses of the genotype and phenotype are in the process to determine whether these cells are indeed CTCs.

30. Benefit sharing and globalisation of industry sponsored clinical trials for breast cancer research

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Introduction: The burden of cancer is the greatest and rising most rapidly in low-income and middle-income countries (LMICs). The survival of breast cancer is especially poor in LMICs due to late stage presentation and inadequate access to therapy. Whereas low-income countries (LICs) suffer from a generalized lack of access to cancer care, in middle-income countries (MICs) such services and facilities may exist. However, highly-priced innovative medicines are often only affordable for certain subsets of the population, and good outcomes remain biased toward those who can pay. We carried out comprehensive analysis of the geographic distribution of breast cancer clinical trials involving at least one LMICs clinical site and trials evaluating costly innovative medicines.

Methodology: Data were extracted from clinicaltrials.gov registry (as of 30 Jun 2018). Advanced search filters used: (1) condition/disease: breast cancer; (2) study type: Interventional studies; (3) phases: I to IV; (4) funder type: industry. Countries were classified by income according to the World Bank (Fiscal year 2019).

Results: We analysed 1,746 trials. The fraction of phase III trials involving MICs sites was 55.36% (155/280) in which Lower-MICs (L-MICs) were 27.14% (76/280) and Upper-MICs (UMICs) were 54.64% (153/280). Smaller proportions of Phase I and II trials were conducted in MICs i.e. 5.92% (26/439) and 16.23% (161/992). Phase IV trials that involve MICs were 31.43% (11/35). No trials were conducted in LICs. L-MICs countries with the highest number of trials were India ($n = 63$) and Ukraine ($n = 56$) followed by Philippines ($n = 23$), Egypt ($n = 17$), and Pakistan ($n = 10$). For U-MICs, Russian Federation ($n = 141$), Brazil ($n = 113$), China ($n = 112$), followed by Mexico ($n = 88$) and Turkey ($n = 66$).

Conclusion: Our analysis of the case of industry sponsored clinical trials with new, innovative medicines for breast cancer confirms previous observations with rarer blood cancers, that such trials are increasingly

globalized, i.e., delocalized to countries that do not have the means to ensure (universal) access to high-cost medicines. Although substantial numbers of anti-cancer medicines are nowadays included in national lists of LMICs, their affordability and accessibility at country-level are often far from ensured. The relevance of benefit sharing in international research is still poorly understood. A legal framework formulating benefit-sharing requirements in international research is necessary.

31. *TP53* analysis in hematological malignancies

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Introduction: Chronic lymphocytic leukemia (CLL) is the most common type of adult leukemia in the Western World. *TP53* disruption (e.g., 17p del and/or *TP53* mutations) is associated with chemo-refractoriness and is now used in the clinical practice as a predictive biomarker. Moreover, also in myelodysplastic syndrome (MDS) *TP53* seems to be associated with an inferior outcome. Based on these observations, the objective of the project is to characterize *TP53* mutations in a prospective cohort of CLL and MDS patients to better define the frequency and the clinical implications of *TP53* mutations.

Experimental model: The study is based on the analysis of 53 CLL and 26 MDS patients. The mutational analysis of the *TP53* gene was performed using genomic DNA extracted from tumor cells. Peripheral blood was collected for CLL patients, while bone marrow for MDS patients. The *TP53* gene was analyzed by Sanger sequencing from exons 2 to 11.

Results: In the CLL group 5/53 (9.4%) cases showed *TP53* mutation. All mutations were missense and were reported in the IARC database. All *TP53* mutations predicted functional consequences and were all reported as pathogenic mutations. At diagnosis, *TP53* mutations associated with advanced age ($P = 0.031$), advanced Binet stage ($P = 0.021$) and higher $\beta 2$ -microglobulin levels ($P = 0.018$). In MDS 2/26 (7.7%) patients harbored *TP53* mutation. These two mutations were missense and were validated by the IARC database. Both of *TP53* mutated patients were treated with azacitidine but progressed early after treatment start.

Conclusion: In this prospective CLL cohort, *TP53* mutations reflect the mutation profile already reported, with the novel finding of the correlation between *TP53* and higher $\beta 2$ -microglobulin levels. In MDS, *TP53* seems to be associated with an advance disease and with early progression after therapy. Longer follow up is needed for univariate and multivariate survival analysis stratified according to *TP53* mutations.

32. Epigenetic changes in ovarian cancer cells subjected to starvation or to the caloric restriction mimetic Resveratrol

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Introduction: Biological processes are regulated through epigenetics, including chromatin remodelling (methylation, acetylation, etc.) and non coding RNAs. The correct balance of these mechanisms is crucial for the maintenance of cellular homeostasis and its dysregulation is correlated to many disorders such as cancer. Nutrient availability has a great impact on the metabolic pathways involved in cancer cell proliferation. Incubation in Earle’s Balanced Salt Solution (EBSS), a culture medium containing 1% glucose in absence of serum and amino acids, mimics the condition of starvation. Resveratrol (RV), a dietary polyphenol acting as a protein (caloric) restriction mimetic makes the cells unable to uptake nutrients for their metabolism.

Results: Here, we report on the changes of the miRNAs in ovarian cancer cells subjected to amino acid starvation or to RV. In this fasting condition, cancer cells promote autophagy as a pro-survival mechanism and eventually exit the cell cycle to undergo a dormant state. RV has a major impact on miRNome involved in autophagy and apoptosis compared to the one observed in EBSS-treated cells.

Conclusion: Our data support the view that RV treatment can be more effective than nutrient starvation, thus it can substitute it in order to induce a dormant-like state in cancer cells.

33. *KMT2D* mutations and *TP53* disruptions are poor prognostic biomarkers in MCL receiving high-dose therapy: a fil study

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Introduction: In recent years, the outcome of mantle cell lymphoma (MCL) has improved, especially in younger patients, receiving cytarabine-containing chemoimmunotherapy and autologous stem cell transplantation. Nevertheless, a proportion of MCL patients still experience early failure. The aims of the study are to identify biomarkers anticipating failure of intensive chemotherapy in MCL.

Experimental model: We performed target resequencing and DNA profiling of purified tumor samples collected from patients enrolled in the prospective FIL-MCL0208 phase III trial (NCT02354313, high-dose chemoimmunotherapy followed by autologous transplantation and randomized lenalidomide maintenance).

Results: Mutations of *KMT2D* and disruption of *TP53* by deletion or mutation associated with an increased risk of progression and death, both in univariate and multivariate analysis. By adding *KMT2D* mutations and *TP53* disruption to the MIPI-c backbone, we derived a new prognostic index, the “MIPI-genetic”. The “MIPI-g” improved the model discrimination ability compared to the MIPI-c alone, defining three risk groups: (1) low-risk patients (4-year PFS and OS of 72.0% and 94.5%); (2) intermediate-risk patients (4-year PFS and OS of 42.2% and 65.8%); and (3) high-risk patients (4-year PFS and OS of 11.5% and 44.9%).

Conclusion: Our results: (1) confirm that *TP53* disruption identifies a high-risk population characterized by poor sensitivity to conventional or intensified chemotherapy; (2) provide the pivotal evidence that patients harboring *KMT2D* mutations share the same poor outcome as patients harboring *TP53* disruption; and (3) allow to develop a tool for the identification of high-risk MCL patients for whom novel therapeutic strategies need to be investigated.

34. *In vitro* and *in vivo* activity of biomimetic magnetic nanoparticles for drug delivery in presence of gradient magnetic field

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Nanotechnology and nanoparticles (NPs) have become very attractive for their applications in different fields, comprising biology, medicine and oncology. In this context, magnetite nanoparticles are even more interesting as they can be manipulated by an external magnetic field, besides being multifunctional platforms.

Herein, we describe a drug delivery system based on biomimetic magnetic nanoparticles (BMNPs) synthesized in presence of MamC protein from *Magnetococcus marinus* MC-1. MamC controls the

morphology and size of the crystals (~40 nm), that are paramagnetic at room and body temperature. Because of this protein, BMNPs have a negative surface charge at physiological pH values that allow an efficient coupling with different molecules.

These BMNPs were functionalized with the chemotherapy drug doxorubicin (DOXO) and their ability to respond to an externally applied gradient magnetic field (GMF) was studied both *in vitro* and *in vivo*. Naïve BMNPs were cytocompatible on 4T1 cells, while the DOXO-BMNPs were toxic starting from short exposure times when a GMF is applied. This allowed a faster interaction between BMNPs and cells (Perls Blue Staining) as well as a faster delivery of the DOXO to cell nuclei (confocal analysis). When DOXO-functionalized or not functionalized BMNPs were i.v. injected in mice bearing 4T1 cells-induced tumors, the application of the magnet on the tumors for 1 h reduced their size. Moreover, the presence of DOXO on BMNPs enhanced this effect.

All together, these data suggest that tumor attack by combined strategies (chemotherapeutic drug and magnetic field) could represent a promising approach for cancer therapy. Future steps will be the possibility to apply an alternating magnetic field to BMNPs to induce hyperthermia, to which tumor cells are more sensitive than healthy cells, and to enhance a more efficient localized release of the drug at the tumor site.

35. Novel diacylglycerol kinase alpha inhibitors for X-linked lymphoproliferative disease 1 therapy

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Background: X-linked lymphoproliferative disease 1 (XLP1) is a primary immunodeficiency due to mutations in the SH2D1a gene, encoding SAP. SAP deficiency perturbs TCR signalling and results in a constitutive diacylglycerol kinase alpha (DGK α) activity that impairs CD8⁺ T cell restimulation induced cell death (RICD). Indeed, pharmacological inhibition of DGK α in XLP1 animal models limits CD8⁺ T cell expansion and interferon- γ production, suggesting the development of DGK α inhibitors for XLP1 therapy.

Experimental model: To find new DGK α inhibitors, we used a 2D/3D *in silico* approach based on chemical homology with the two commercially available DGK α inhibitors (R59922 and R59949). The library was screened for inhibitory activity at 100 μ mol/L. Active compounds were tested at concentration from 0.1 to 100 μ mol/L to estimate the IC₅₀. Furthermore, we tested the most active compounds in RICD assay using SAP silenced lymphocytes as XLP1 model.

Results: Out of the resulting 127 compounds, ritanserin (serotonin antagonist) and compound01 (uncharacterized molecule) were highly specific for DGK α and showed superior potency compared to R59022 and R59949. In cellular models of XLP-1, both ritanserin and compound01 restored RICD of SAP-deficient CD8 $^{+}$ without significant toxicity. Moreover, compound01 doesn't perturb serotonin signalling. Thus, we executed compound optimization of compound01 that yielded compound02-07 (synthesized compounds). All those compound01 derivatives are highly specific to DGK α without perturbing serotonin signalling.

Conclusion: Concluding our work allows us to propose a pharmacological model for the rational design of DGK α inhibitors and may contribute to the development of innovative therapies for diseases characterized by RICD resistance such as XLP-1.

Original Article

Open Access



A non-toxic approach for treatment of breast cancer and its metastases: capecitabine enhanced photodynamic therapy in a murine breast tumor model

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Abstract

Aim: Breast cancer (BCA) in women is a leading cause of mortality and morbidity; distant metastases occur in ~40% of cases. Here, as an alternative to ionizing radiation therapy and chemotherapy and their associated side effects, we explored a new combination approach using capecitabine (CPBN) and aminolevulinic acid (ALA)-based photodynamic therapy (PDT). We had previously developed a combination PDT approach in which 5-fluorouracil (5FU), a differentiation-promoting agent, increases the levels of protoporphyrin IX (PpIX) in cancer cells when given as a neoadjuvant prior to aminolevulinic acid (ALA). However, 5FU can be toxic when administered systemically at high levels. We reasoned that CPBN, a known chemotherapeutic for BCA and less toxic than 5FU (because CPBN is metabolized to 5FU specifically within tumor tissues), might work equally well as a PDT neoadjuvant.

Methods: Murine 4T1 BCA cells harboring a luciferase transgene were injected into breast fat pads of female nude mice. CPBN (600 mg/kg/day) was administered by oral gavage for 3 days followed by intraperitoneal ALA administration and PDT with red light (633 nm) on day 4. Tumor growth and regression were monitored *in vivo* using bioluminescence imaging. Histological changes in primary tumors and metastases were assessed by immunohistochemistry after necropsy.



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Results: CPBN pretreatment of 4T1 tumors increased cellular differentiation, reduced proliferation, raised PpIX levels, enhanced tumor cell death, and reduced metastatic spread of 4T1 cells post-PDT, relative to vehicle-only controls.

Conclusion: The use of CPBN as a non-toxic PDT neoadjuvant for treatment of BCA represents a novel approach with significant potential for translation into the clinic.

Keywords: Photodynamic therapy, protoporphyrin IX, aminolevulinic acid, breast cancer, metastasis, cell death, 4T1, *in vivo* imaging system

INTRODUCTION

Breast cancer (BCA) is the most common type of malignancy in women, with 1.7 million new cases diagnosed worldwide each year^[1] and it is also a leading cause of mortality among women, accounting for over half a million deaths annually^[1-3]. The main cause of death is not the primary tumor, but distant metastases that create significant morbidity. Although 80% of BCA patients receive adjuvant chemotherapy, ~40% of those patients relapse and ultimately die due to metastatic disease^[4]. While 5-year survival rates of BCA patients are increasing overall due to advanced therapeutic techniques, relatively low survival rates and poor prognoses are still observed in patients with metastatic BCA^[4]. BCA cells metastasize mainly to the lung, liver, brain and bone; metastases to the skin (cutaneous metastasis) occur in ~20% of cases^[4]. Local relapse and chest wall metastases occur at a rate of 5%^[4]. The chest wall tumors are notable because they can be very painful, and are hard to treat due to their resistance to chemotherapy^[5,6]. Ionizing radiation therapy (RT) has been successfully used for the treatment of cutaneous chest wall metastases; however, multiple rounds of therapy are required and are typically associated with side effects such as radiation dermatitis, blistering and chronic ulcers. An alternative to RT, constituting a safer treatment that might be given either alone or together with chemotherapy, is urgently needed.

Photodynamic therapy (PDT) is a non-mutagenic, non-scarring treatment modality for cancer. PDT employs a photosensitizer (PS) and visible light in the presence of oxygen to kill tumor cells^[7-9]. During PDT, two steps provide dual selectivity. First, the cancer cells accumulate and retain PS to a greater extent than normal cells. Second, focusing the light source onto the tumor further enhances tumor specificity. The PS is activated, triggering cell death by releasing free radicals^[8]. In the early history of PDT, the PS was given systemically and carried a high risk of increased skin phototoxicity (sunburn). To reduce toxicity and improve tumor selectivity, a newer mechanism of PDT that uses a prodrug, aminolevulinic acid (ALA) instead of the pre-formed PS, was developed^[10,11]. ALA, given systemically, orally or topically, is taken up and enzymatically converted into protoporphyrin IX (PpIX) within mitochondria. PpIX is then activated by strong visible light to generate reactive oxygen species that kill the cancer cells^[9,12,13]. Skin cancers are easily illuminated from the surface using red light that penetrates up to 1 cm into tissue. PDT is now successfully used in Europe to treat superficial basal cell carcinoma and squamous cell carcinoma (SCC)^[14]. PDT has several advantages over other modalities: (1) unlike surgical excision, PDT is non-scarring (and may actually inhibit fibrosis^[15,16]); and (2) unlike RT, PDT can be repeated multiple times and because PDT targets mitochondria rather than DNA, there is minimal risk of genetic mutations.

Although PDT is currently employed mostly for skin cancers, it carries tremendous potential to shrink or eliminate BCA and its associated metastases; this research area has only been explored in a preliminary fashion^[17,18]. Up until now, the need for specialized equipment and better-standardized protocols has delayed the acceptance of PDT as a main line of treatment for BCA and its metastases. In a recent clinical trial in China, ALA-PDT used in combination with RT to treat cutaneous BCA metastases, showed a better complete response (50% vs. 20%) and a reduced time to clearance (110 days vs. 175 days) vs. RT alone^[19]. It was also shown that lowering the photosensitizing drug dose and the light fluence rate can improve the clearance

of BCA chest wall lesions^[19]. Here, we seek to apply a different combination therapy PDT approach to BCA. Using pre-clinical murine models and subsequent clinical trials in patients with skin cancer, we have established that certain neoadjuvant agents can significantly improve PpIX levels and distribution within tumors when combined with ALA-PDT^[9,20]. In this study, we explore an approach that combines two FDA-approved drugs [capecitabine (CPBN) and ALA] and a standard light source used in dermatology, to deliver a safer, more efficacious, and more convenient treatment for localized BCA tumors and metastases in mice.

The rationale for our approach is based upon several relatively new scientific principles. In the past, several PDT regimens have been explored with different PS molecules, routes and doses of PS, and various wavelengths, illumination protocols and oxygen concentrations (hypoxia *vs.* normoxia), in attempts to improve PDT responses^[7-9]. However, since cell physiology (particularly the state of tumor differentiation) plays an important role in PDT outcome, we considered biomodulation with neoadjuvants given prior to PDT as another avenue for optimization^[9]. Towards this goal, we pioneered a concept called “differentiation-enhanced” or “combination PDT”. Thus, cancer cells of different tissue origins, when pre-treated with differentiation promoting agents, show elevated accumulation of PpIX and enhanced cell death after ALA-PDT^[9]. Three such agents, methotrexate^[21,22], vitamin D (calcitriol)^[23] and 5-fluorouracil (5-FU)^[24] were shown to improve tumor responses in skin, prostate and BCA models when used as neoadjuvants for PDT. Pretreatment with any of these agents resulted in 3- to 5-fold upregulation of mitochondrial PpIX production^[9]. The effects on PpIX levels were selective for tumor cells and did not occur in adjacent skin^[24,25]. We recently worked out the molecular mechanisms for this effect. Coproporphyrinogen oxidase, a heme enzyme located upstream of PpIX, is upregulated; ferrochelatase located downstream of PpIX, is decreased; and the net effect favors PpIX buildup^[23,26]. An important feature of these neoadjuvants is that they drive cancer cells toward a more highly differentiated state, as determined by the elevated expression of E-cadherin in tumors. Using a BCA model (MDA-MB-231 cells implanted in breast fat pads of nude mice), we showed that pretreatment with calcitriol (the active form of Vit D) prior to ALA-PDT resulted in an enhanced therapeutic response relative to ALA-PDT alone^[25]. Using topical 5-FU, we recently completed a clinical trial in patients with actinic keratosis (AK; pre-SCC), and showed that combination PDT with topical 5-FU significantly enhanced the PDT efficacy as determined by increased clearance of the AK lesions^[20].

A major reason for PDT treatment failure is the heterogeneity of PpIX distribution within the target tissue. Some microanatomical pockets, located deep inside the tumor, produce minimal PpIX due to poor PS penetration and suboptimal physiological response, thereby escaping PDT photodamage^[26]. When 5-FU is given as a neoadjuvant prior to ALA, PpIX levels are raised in the vast majority of tumor cells, resulting in enhanced cell death following PDT in both skin tumors and in murine breast tumors^[24]. However, the systemic dose of 5-FU required to achieve this effect is quite high and might be toxic in humans. In the current study, in order to circumvent toxicity, we used an alternative drug that is FDA-approved for treatment of metastatic BCA^[27-29]. CPBN is a precursor form of 5-FU that is selectively converted into the active end product, 5-FU, primarily in cancer cells. CPBN is converted to 5-FU in three enzymatic steps requiring carboxylesterase, cytidine deaminase (CDA), and thymidine phosphorylase (TYMP)^[28,30]. Cancer cells have relatively high levels of CDA and TYMP, explaining the increased sensitivity of cancer cells to CPBN, and the low adverse effects of this drug on non-tumoral tissues^[28]. To test and develop a combination approach with CPBN and PDT, we used a mouse BCA model (the 4T1 cell line; a triple-negative BCA equivalent to human stage IV) that has already been previously utilized in many studies of BCA^[31,32]. The long-term goal of our study was to develop a better mechanistic understanding of the CPBN-PDT approach, for possible translation of this concept to the clinic for treating localized BCAs.

METHODS

Cell culture

4T1, a murine breast carcinoma line (a triple-negative breast cancer and stage IV human BCA equivalent) and Bioware® Brite Cell Line 4T1-Red-FLuc, were purchased from animal type culture collection and Perkin

Elmer Inc., respectively, and cultured as per the instructions provided by the source/vendor.

4T1 murine breast tumor model

Murine 4T1 cells (0.5×10^6) and 4T1-Red-Fluc cells (0.005×10^6) were resuspended in the growth medium and injected into breast fat pads of female nude mice. Visible/palpable tumors developed in 3-5 days post injection. All experimental procedures involving mice were preapproved by the Institutional Animal Care and Use Committee of the Cleveland Clinic.

Pretreatment with CPBN

CPBN (Sigma Aldrich, 600 mg/kg/day) was dissolved in 5% gum arabic-sodium citrate solution (vehicle) and was given by oral route (gavage) once daily for 3 days. Control mice received vehicle solution only. On day 4, ALA was administered through intraperitoneal route (IP; 200 mg/kg in phosphate buffered saline) for 4 h and the mice were either exposed to red light (633 nm) for PDT, or euthanized for tumor harvest and histological and immunohistochemical analyses^[23,24]. The dose of oral CPBN chosen for our study (600 mg/kg/day for three days) was based upon 80% of the maximum tolerated dose determined in nude mice by Kolinsky *et al.*^[33], maximum tolerated dose 700 mg/kg/day when given for 7 days. Note that dose translation between CPBN (Xeloda®) doses used in humans^[34,35] and biologically equivalent doses in mice is not straightforward, and typically involves body surface normalization methods^[36,37].

Imaging of tumor growth/regression and metastases using *in vivo* imaging system (IVIS® spectrum)

4T1 tumor bearing mice were injected with *D*-luciferin (IP; 150 mg/kg; Perkin Elmer Inc.) and imaged under continuous isoflurane anesthesia using the *in vivo* imaging system (IVIS spectrum; Perkin Elmer Inc.) following the manufacturer's instructions. Bioluminescence imaging signals were recorded as bioluminescence units (BLUs, photons/s/cm²/sr), and used to analyze tumor growth/regression and metastases to distant sites using Live Imaging Software^[25]. Digital images were captured 5 min after *D*-luciferin injection, along with a radiance calibration standard scale, at each weekly time point during tumor growth/regression experiments. Tumor-associated signals (emitted light units) from each region of interest (ROI) were normalized per this radiance standard scale.

Treatment with PDT

Mice were anesthetized using intraperitoneal ketamine and xylazine. Anesthetized mice with 4T1 tumors (\pm CPBN pretreatment and after 4 h of ALA), were exposed to 100 J/cm² of 633 nm light using a LumaCare® xenon source (LumaCare). The light source was calibrated using a FieldMate® laser power meter (Coherent). Mice were either imaged at different time points to analyze the therapeutic response and metastases to distant sites or sacrificed and tumors harvested at 24 h post PDT for histological and immunohistochemical analyses^[23,24].

Imaging of PpIX in 4T1 tumors by confocal microscopy

Tumor cryosections (10 μ m) were placed on glass slides, briefly air dried, mounted under coverslips with Vectashield (Vector Laboratories), and viewed on a confocal microscope (Leica Microsystems; 40 \times magnification; excitation at 635 nm and image collection at 650-780 nm). Quantitation of relative PpIX levels from digital photomicrographs was performed using IP Lab software^[23,24].

Histology, immunohistochemistry and cell death analyses

The 4T1 tumors and distant metastatic sites were harvested at different time points, fixed in formalin, paraffin-embedded and sectioned (5 μ m) following a standard protocol. Hematoxylin and eosin staining, immunofluorescence staining, and cell death analysis [terminal-deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)] were performed as described^[23,24].

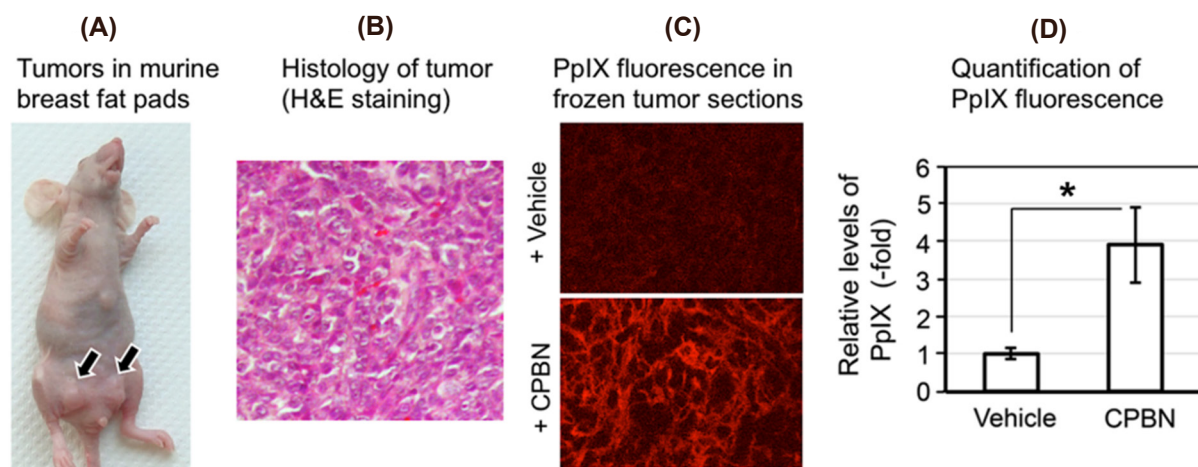


Figure 1. Analyses of protoporphyrin IX (PpIX) in murine breast tumor model. (A) Photograph of a nude female mouse with 4T1 subcutaneous tumors in breast fat pad; (B) hematoxylin and eosin (H&E) stained 4T1 tumor section showing histological details of the murine breast carcinoma shown in (A); (C) confocal micrographs of frozen sections of 4T1 tumors after oral treatment with vehicle (top) or capecitabine (CPBN) (bottom) followed by aminolevulinic acid; red signal is mitochondrial PpIX; (D) quantitation of PpIX-specific fluorescence from confocal micrographs shown in (C); integrated fluorescence intensity was measured using IPLab software. Mean SEM (18 images from 6 tumors) from three independent experiments is shown. $*P = 0.0001$

Statistical analyses

Statistical analyses were performed using a two-sided *t*-test to compare differences between PpIX levels, expression of markers by immunohistochemistry, cell death and tumor bioluminescence. $P \leq 0.05$ was considered statistically significant.

RESULTS

Experiments were designed to test our hypothesis that oral CPBN, given as a differentiation-promoting agent (instead of administering its final product 5-FU [Anand, 2017 #100; Maytin, 2018 #114]), may enhance the PS levels, subsequent cell death response and will impact distant metastases following ALA-PDT in a murine 4T1 breast tumor model.

Combination of oral CPBN before ALA enhances PpIX levels in 4T1 tumors

To study if oral CPBN can successfully replace its relatively toxic final product 5-FU for the purpose of promoting differentiation prior to ALA-PDT, a murine breast tumor cell line (4T1) equivalent to stage IV human triple negative breast carcinoma was injected in the breast fat pad of female nude mice to generate a murine breast tumor model [Figure 1A and B]. Following the injection of 4T1 cells, mice with visible and palpable 4T1 breast tumors [Figure 1A] were given either CPBN or vehicle (orally by gavage, once a day for 3 days), followed by intraperitoneal ALA administration on the 4th day. Tumors were harvested and frozen sections were analyzed by confocal microscopy using excitation and emission settings that allow visualization of PpIX-specific fluorescence. In the 4T1 tumors from mice treated with oral CPBN, PpIX was more abundant and also present throughout the tumor at much higher levels, as compared with vehicle treated (control) tumors [Figure 1C; bottom *vs.* Top]. Digital quantification of the PpIX fluorescence signal from confocal images confirmed this observation by showing a statistically significant increase of ~4-fold in PpIX levels in CPBN treated tumors [Figure 1D].

CPBN pretreatment enhances differentiation, inhibits proliferation and enhances cell death following PDT

5FU, a chemotherapeutic drug and the final product of CPBN metabolism specifically converted in the tumors due to high levels of CDA, and TYMP^[28,30], is known to downregulate DNA synthesis through

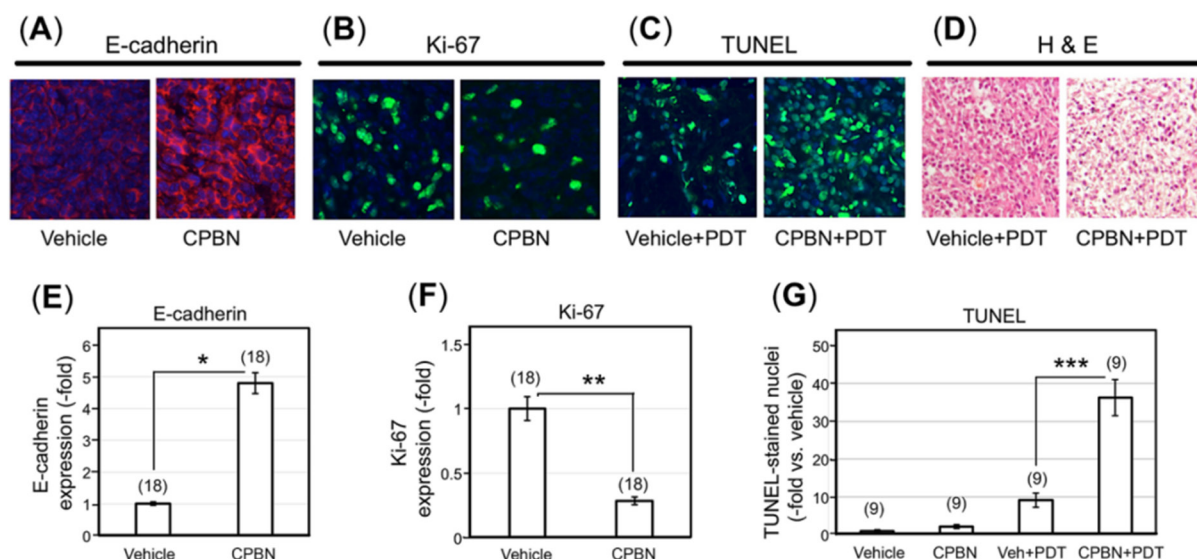


Figure 2. Physiological and cell death responses to capecitabine pretreatment and photodynamic therapy in murine breast tumor model. Photomicrographs showing 4T1 tumor sections immunohistochemically stained for (A) E-cadherin (E-cad), a marker of differentiation; (B) Ki67, a marker of proliferation, following vehicle or capecitabine (CPBN) treatment; (C) Cell death analysis by terminal-deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) labeling of apoptotic nuclei at 24 h post photodynamic therapy (PDT), following vehicle or CPBN pre-treatment. Nuclei in images shown in (A-C) were stained with 4',6-diamidino-2-phenylindole (DAPI); (D) hematoxylin and eosin (H&E) staining of tumor sections showing enhanced cell death at 24 h post PDT in tumors pretreated with CPBN or vehicle alone. Graphs show quantitation of changes in (E) E-cadherin signal; (F) Ki67 signal; and (G) TUNEL positive nuclei per high power field. All graphs show Mean \pm SEM from multiple images (number of images shown in parentheses) from three independent experiments. P values from an unpaired two-sided t -test are shown: * $P = 1.57 \times 10^{-11}$; ** $P = 1.20 \times 10^{-8}$; *** $P = 6.02 \times 10^{-9}$.

inhibition of thymidylate synthase (TS) and *de novo* synthesis of thymidylate acid^[38]. In addition to its anti-proliferative effect, 5-FU induces epithelial cell differentiation^[9]. The physiological effects of CPBN pretreatment on the 4T1 tumors were analyzed by immunostaining of tumor sections for expression of E-cadherin and Ki67, markers of differentiation and proliferation, respectively [Figure 2]. As anticipated, relative to vehicle treated tumors, CPBN pretreatment led to a 5-fold increase in differentiation [E-cadherin expression; Figure 2A and E]. In the same set of tumors, CPBN pretreatment resulted in a 70% reduction in cell proliferation [Ki67 expression; Figure 2B and F].

To assess the effects of combination therapy upon 4T1 intratumoral cell death, CPBN + PDT treated tumors along with vehicle- and CPBN-treatment controls were harvested 24 h after PDT and histological sections were analyzed by TUNEL assay and by hematoxylin and eosin staining. TUNEL is a technique in which cleaved inter-nucleosomal DNA fragments are enzymatically labeled in dying cells. CPBN pretreated tumors showed an approximately 4-fold increase in TUNEL positive nuclei, relative to their vehicle treated PDT controls by 24 h post PDT [Figure 2C and G]. Histomorphological details of CPBN-PDT induced cell death in 4T1 tumors were analyzed by comparing hematoxylin and eosin stained sections from tumors treated with CPBN + PDT or with PDT alone [Figure 2D]. At 24 h following PDT, all tumors displayed numerous apoptotic cells with shrunken, pyknotic nuclei, intracellular vacuoles, and large areas of cell loss (empty spaces), but these features were more pronounced in the CPBN pretreated tumors, similar to quantitation of cell death features by hematoxylin and eosin reported previously^[25,39].

Non-invasive visualization and monitoring of tumor growth and regression after PDT using IVIS

Using the IVIS Spectrum® device, growth and/or regression of orthotopic 4T1 breast tumors in nude mice from different treatment groups (vehicle, CPBN, vehicle + PDT and CPBN + PDT) was monitored non-invasively. A genetically modified version of 4T1 cells (4T1-red-fluc Bioware® Brite cell line), which had been stably transduced with the red-shifted firefly luciferase gene from *Luciola Italica* (Red-FLuc), was

used to monitor the effect of vehicle or combination PDT on tumor growth and regression. These cells were implanted in mammary breast fat pads of nude mice and tumor growth was followed non-invasively by IVIS. The bioluminescent images were captured on: (1) a day after implantation; (2) prior to PDT (pre-PDT); (3) one-week and (iv) two-week post PDT. Growth/regression after PDT was quantitated using BLUs from individual ROI from images captured at different time points during an experiment. [Figure 3A](#) shows representative examples of nude mice carrying 4T1 breast tumors imaged just before, one week, and two weeks after PDT, along with vehicle- or CPBN-only treatment groups that did not receive PDT. The bioluminescence signal (pseudo-colored rainbow scale) represents the radiance (photons/s/cm²/sr) emitted from 4T1 tumors due to the reaction between luciferin substrate (delivered via i.p. injection) and luciferase enzyme (expressed in the 4T1 cells).

In the vehicle-only group of mice, the luciferase signal increased continually to 4.6-fold by 2 weeks, reflecting unrestricted growth of 4T1 cells [[Figure 3A](#); top left set of images, and [Figure 3B](#)]. In a CPBN-treated group of mice that received only CPBN (600 mg/kg/day) once daily for 3 days, the luciferase signal by week 2 was increased by 13.6-fold as compared to the pre-PDT signal [[Figure 3A](#), top right set of images, and [Figure 3B](#)]. The ~4-fold induction in growth of 4T1 tumors following CPBN treatment, relative to the vehicle only group, was an unanticipated observation. CPBN, a chemotherapeutic drug approved for the treatment of BCA in humans with anti-proliferative physiological effects, should have reduced the tumor growth, but we note that the incongruent finding might possibly be due to a smaller number of mice ($n = 4$) in this particular treatment group. In the vehicle + PDT group, the rise in bioluminescent signal (2.5 and 9-fold over baseline at weeks 1 and 2, respectively) reflected continued growth and inadequate response of 4T1 tumors to PDT alone [[Figure 3A](#), left bottom set of images, and [Figure 3B](#)]. In contrast, the last group of mice that received CPBN + PDT was the only treatment group that showed no tumor growth [[Figure 3A](#), right bottom set of images, and [Figure 3B](#), fourth set of bars]. Thus, the combination of CPBN with PDT showed a much better therapeutic response than PDT alone. Our observation of tumor bioluminescence was only possible up to two weeks post-treatment. At weeks 3 and 4, the signal was often blocked or reduced due to a combination of crust on the dying tumor, increased tumor size, and probably uneven uptake of luciferin due to tumor hypoxia and cell death. We are currently studying the mechanistic details of these effects, with special interest in regulation of apoptotic cell death following CPBN-PDT.

Distant 4T1 metastases: bioluminescent, histological and immunohistochemical analyses after PDT treatments

The 4T1 BCA line used in this study has been shown to metastasize widely to distant organs (lymph node, lung, liver, bone, spleen and skin) at later stages of tumor development^[40-42]. Here we investigated the effect of CPBN-PDT on distant metastases in nude mice, using luciferase-expressing 4T1 cells implanted at a single site on the animal. Metastatic spread to lung, lymph nodes, spleen and skin was observed at three weeks after implantation of the primary tumor site into the ventral breast fat pads [[Figure 4](#)]. In addition to the obvious signal at the site of injection, 4T1 cells were also observed to spread in the abdominal cavity and at other distant sites, reflecting likely metastases of tumor cells to lungs, lymph nodes and spleen [[Figure 4](#)]. Signals from some specific anatomical locations corresponded to cervical, axillary, sciatic and inguinal lymph node sites. Cutaneous metastases were also seen both visually on the skin (data not shown), and confirmed by IVIS imaging that revealed luminescent signals originating from cutaneous metastatic lesions [[Figure 4C](#), rightmost mouse].

To analyze the effect of combination therapy upon BCA metastases, mice from different treatment groups were examined for the incidence of distant metastases. The mice from three treatment groups, i.e., vehicle, CPBN and vehicle + PDT showed a similar incidence of metastases ranging from 60%-65% [[Figures 4A-C and 5A](#)]. By contrast, mice from the CPBN + PDT treatment group showed significantly less metastatic spread [[Figure 4D](#)], corresponding to only a 17% incidence of metastases [[Figure 5A](#)]. The metastatic tumor

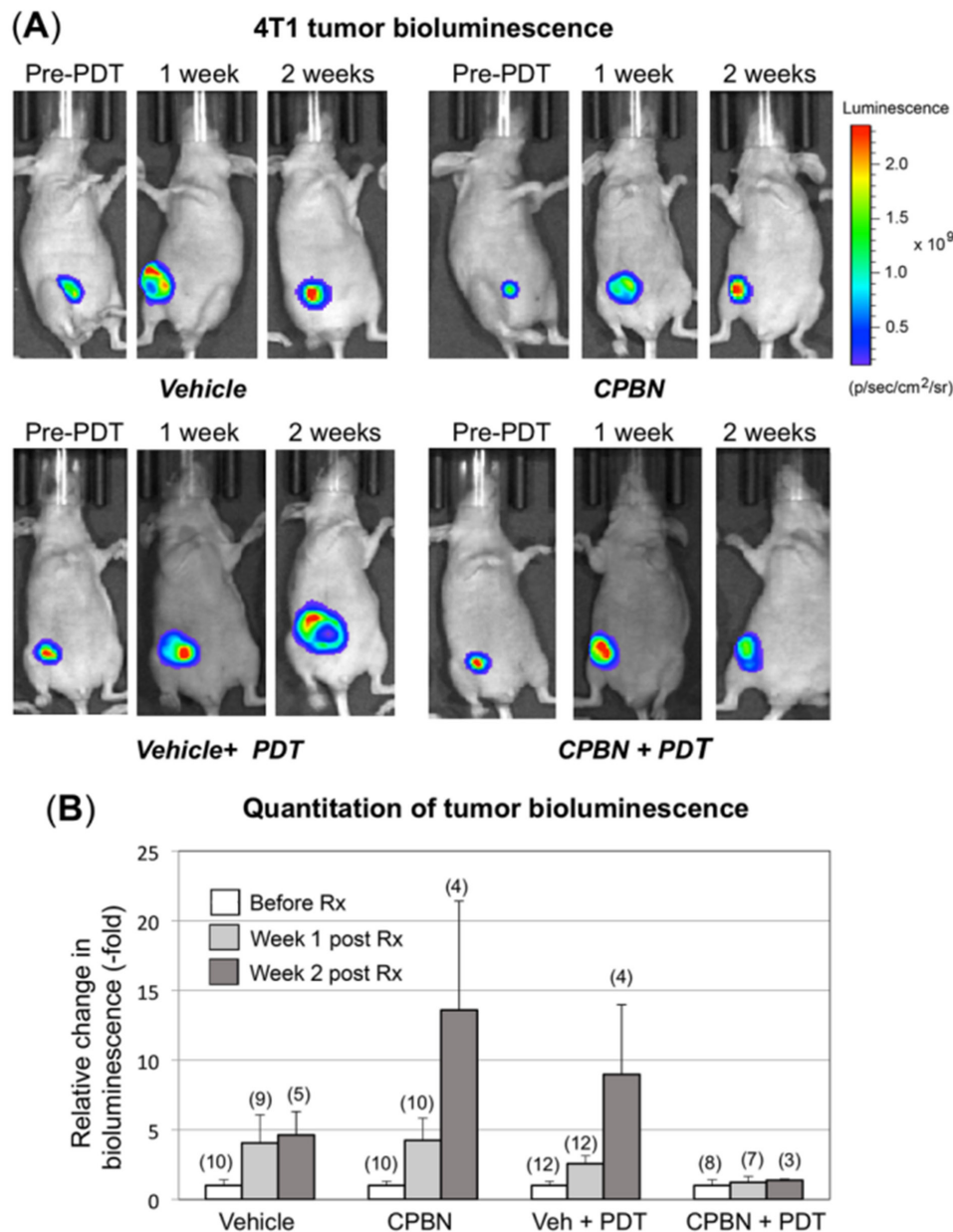


Figure 3. Monitoring of therapeutic responses to capecitabine and/or photodynamic therapy (PDT) in murine breast tumor model using an in vivo imaging system (IVIS). 4T1 murine breast cancer cells (stably transfected with luciferase) were implanted in the breast fat pad of nude mice and monitored for bioluminescence signals originating from tumors, using an IVIS instrument. (A) Tumor growth or shrinkage after 1 or 2 weeks of vehicle- or capecitabine (CPBN)-only, ALA + PDT or CPBN + PDT, was monitored by injection of luciferin, and measurement and quantitation of luminescence output using Live imaging software as described^[25]. A typical radiance scale, common to most of the bioluminescent images, is shown in (A); (B) quantitation of bioluminescent light units, relative to pre-PDT tumor luminescence from each treatment group, is shown as fold increase. Bars representing mean (\pm SEM), from the number of mice shown in parentheses on top of the bars (pooled from four independent experiments) are shown. The differences observed in the relative bioluminescence between vehicle + PDT and CPBN + PDT at one ($P = 0.14$) and two ($P = 0.20$) weeks post PDT, were not statistically significant

load among mice from different treatment groups was compared by analyzing cumulative bioluminescence originating from different ROIs on each mouse. Mice from vehicle-only, CPBN-only, PDT-only treatment groups, showed a similar metastatic load [Figure 5B]. However, the CPBN + PDT treatment group showed a significantly reduced metastatic load of only 14% [Figure 5B].

To further confirm that the bioluminescence observed in Figure 4 did indeed represent tumor metastases, various organs were necropsied and stained with hematoxylin and eosin. Our histological and

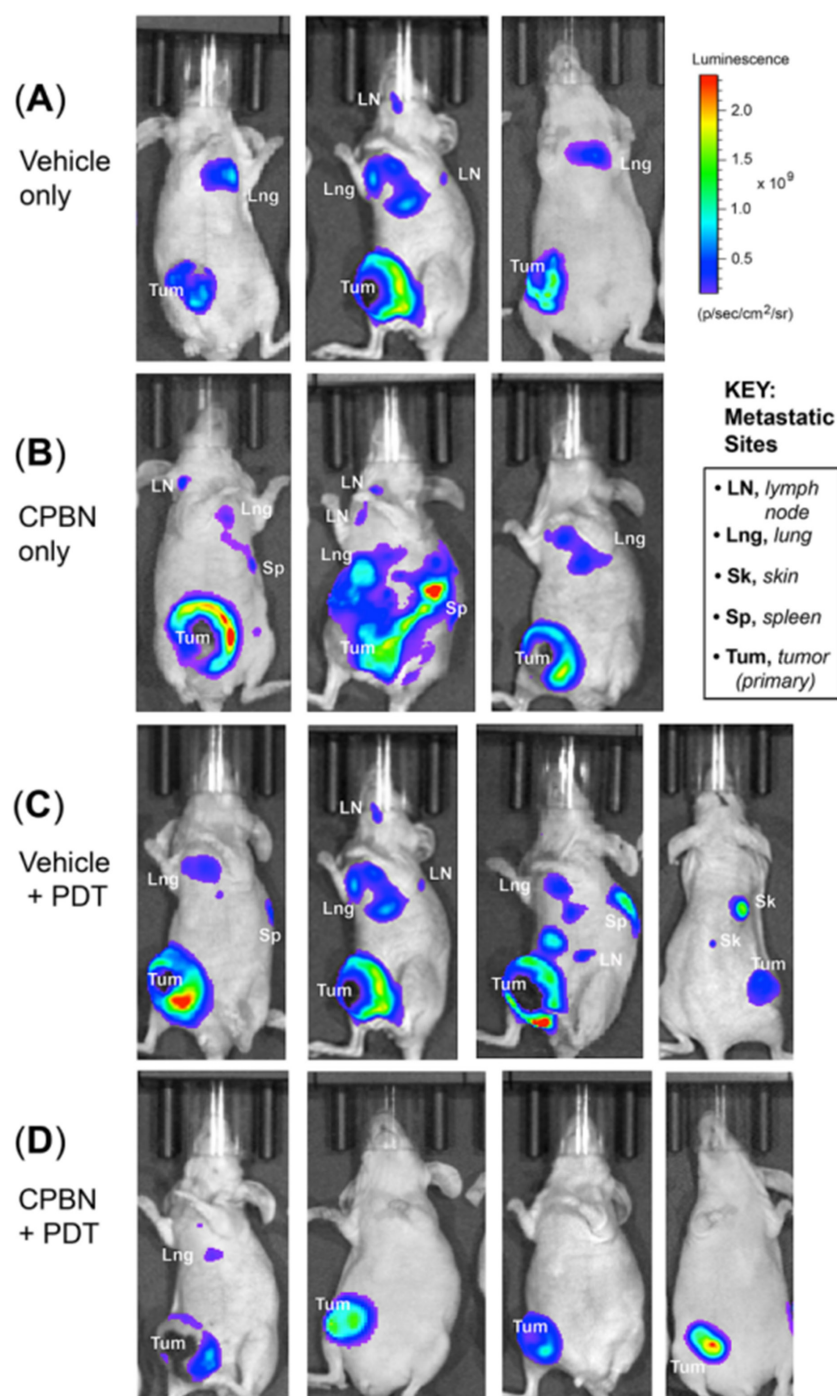


Figure 4. Monitoring of distant metastases of breast tumor cells in nude mice treated with conventional or combination photodynamic therapy (PDT). 4T1 murine breast carcinoma cells were implanted in the breast fat pad of nude mice, and distant metastases were monitored by bioluminescence signals originating from metastatic sites using an *in vivo* imaging system instrument. Metastatic spread of 4T1 tumor cells (3 weeks post PDT) in mice representing (A) vehicle only; (B) capecitabine (CPBN) only; (C) vehicle + PDT; and (D) CPBN + PDT are shown. Observed organ sites of metastases, including lymph nodes (LN), lung (Lng), spleen (Sp) and skin (Sk) are indicated. Note the significant reduction in the incidence of metastases in CPBN + PDT treated mice

immunohistochemical analyses were only able to reveal 4T1 metastases in the lungs and skin, possibly due to the difference in sensitivity of detection between IVIS and hematoxylin and eosin methods. IVIS is an *in vivo* optical imaging system capable of detecting signal from as few as 3 cells implanted in a mouse^[43], as compared to a few hundred cells required to form a tumor nest that can be detected histologically by

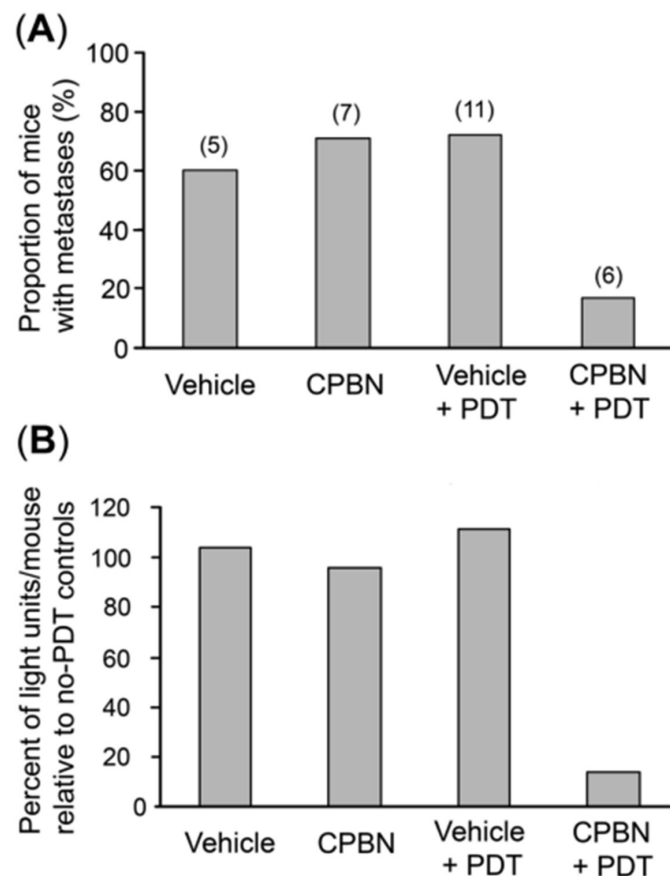


Figure 5. Analyses of incidence of metastases, and total metastatic load, in nude mice with 4T1 breast tumors. Mice from four different treatment groups [vehicle only, capecitabine (CPBN) only, vehicle + photodynamic therapy (PDT) and CPBN + PDT] were quantitatively analyzed as follows. (A) Incidence of metastases by week 3. Note that the first three treatment groups showed 60%-70% of mice with metastases, whereas the CPBN + PDT treatment group showed only 17% of mice with metastases; (B) metastatic tumor load per mouse, calculated by comparing the cumulative bioluminescence units (BLUs) from each mouse with the average cumulative BLUs from no-PDT controls. The metastatic load of 14% in CPBN + PDT group is much lower than the other three groups (96%-111%)

hematoxylin and eosin. [Figure 6A](#) clearly shows the presence of metastatic tumor nests (marked with dotted lines) in the lung of a mouse that was positive by IVIS imaging. Keratin 14 (K14, a marker for epithelial breast tumors at ectopic sites; [Figure 6A](#)), was expressed only within tumor nests in the lungs of tumor-bearing mice and not in the lungs from control mice [[Figure 6A](#); bottom]. Comparing this to the primary tumor (which also stained strongly for K14; [Figure 6B](#)), the pattern of K14 expression was interesting, in that the most intense expression was observed in the tumor periphery, with far fewer positive cells in the central/stromal (hypoxic) tumor regions ([Figure 6B](#); top vs. bottom), possibly due to hypoxia and necrosis in the center and the proliferative nature of the tumor periphery, respectively. This observation further supports the assertion that the primary breast tumor was the origin of the K14-positive, metastatic tumor nests in the lungs. Histology of cutaneous metastatic lesions also clearly confirmed the presence of metastatic carcinoma in skin ([Figure 6C](#); righthand image marked with dotted lines). Further exploration of how CPBN-PDT exerts its inhibitory effect on 4T1 metastatic spread is underway.

DISCUSSION

In this study we have shown, in a murine model of BCA, that pretreatment with CPBN prior to ALA-mediated PDT causes selective enhancement of PpIX levels within 4T1 tumors, and improves the treatment outcome of PDT by enhancing tumor cell death. We also showed, using histological and

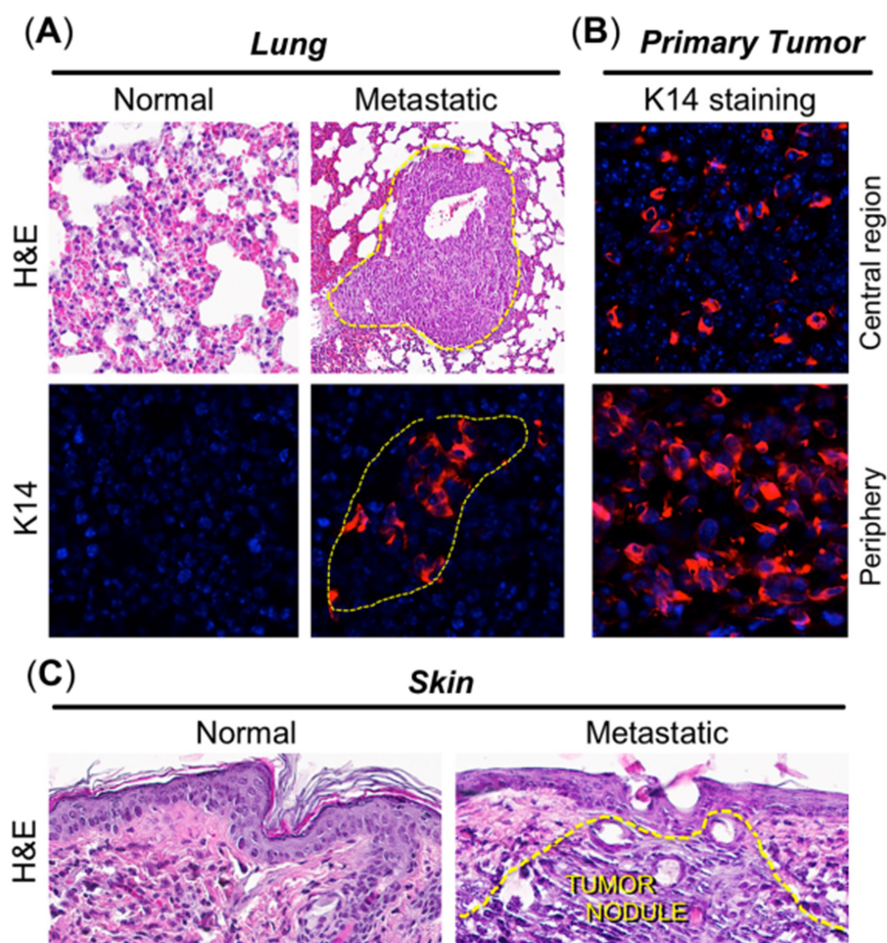


Figure 6. Histological and immunohistochemical analyses of distant metastases in the 4T1 breast tumor model. Tissue biopsies from organs positive for bioluminescence, along with primary tumors, were harvested three weeks after photodynamic therapy. By histological analyses, metastases were detectable in lungs and skin only. (A) Metastatic nodules were visible in lungs via hematoxylin and eosin (H&E) staining, and by immunostaining with antisera to keratin 14 (K14; red color), as delineated by dotted yellow lines; (B) positive K14 staining in primary breast tumor, to confirm that lung tumor nests originated from the primary breast tumor. K14 staining was weaker in the tumor center (top panel) than in the tumor periphery (bottom panel), possibly due to hypoxia and necrosis in the center *vs.* proliferation at the tumor periphery; (C) a cutaneous metastasis, detected visually and by *in vivo* imaging system, revealed obvious infiltrating tumor cells when stained with H&E (right panel, dotted yellow lines)

immunohistochemical analyses, that the CPBN-PDT combination enhances the differentiation state of 4T1 carcinoma cells within the tumors. Finally, using *in vivo* bioluminescent monitoring, we showed that the combination approach appears more effective than ALA-PDT alone, retarding the growth of the primary tumor, and also reducing metastatic spread.

The observed beneficial effect of neoadjuvant CPBN on the therapeutic outcome of PDT may involve several underlying mechanisms that we previously identified in studies with other differentiation-promoting drugs such as methotrexate, vitamin D and 5-FU^[9]. The first apparent mechanism, an inhibition of tumor cell proliferation [Figure 2B and F], is an anticipated effect of 5-FU since the latter inhibits TS and induces growth arrest by mis-incorporation of FU into DNA^[38]. The second mechanism, enhanced differentiation of tumor cells, is a less understood effect whose basis is unclear since the induction of growth arrest is often associated with induction of differentiation. The third mechanism, enhancement of PpIX accumulation, is actually now fairly well understood from our previous work^[24]. PpIX is produced by enzymatic conversion of ALA through the heme biosynthetic pathway. 5-FU, the final product of CPBN metabolism, has been

shown to exert its effects on PpIX levels in murine and human SCC tumors by upregulating the levels of two rate-limiting enzymes of the heme pathway, coproporphyrinogen oxidase (upregulation) and ferrochelatase (down regulation), in a way that favors accumulation of higher PpIX levels inside the tumor cells' mitochondria^[24]. The ultimate consequence of elevated PpIX levels following CPBN pretreatment, is a significant increase in tumor cell death after PDT. This is due not only to higher PpIX concentrations within individual cells, but also to a more homogeneous distribution of high PpIX throughout the tumor [Figure 1C]. Following PDT, the loss of tumor cells through apoptosis was demonstrated via TUNEL and hematoxylin and eosin staining; thus, apoptosis clearly contributes to better outcomes such as retardation of tumor growth in the CPBN-PDT treatment group. Our data regarding inhibitory effects of the CPBN-PDT regimen upon metastases are particularly exciting. Although our sample sizes were relatively small and the data should be considered somewhat preliminary, the CPBN-PDT combination appears to be more effective at preventing metastatic spread than either CPBN or PDT alone [Figures 4 and 5].

The clinical implication of this study is the possibility that a new combination approach might bring PDT closer to the forefront of options available for management of BCA. Mainline treatment options available for BCA today include RT, surgery, chemotherapy, biological therapy (targeted agents), and hormonal therapy. As approaches to BCA disease management have evolved, the focus has shifted toward breast preservation, placing less emphasis on surgical and ablative approaches. In this setting, PDT, represents a promising treatment and management strategy for the following reasons: (1) Unlike RT, PDT does not cause fibrosis and scarring; (2) PDT can be repeated many times since it targets mitochondria rather than DNA, and thereby poses minimal risk for inducing mutations and secondary tumors; (3) PDT has already been successfully tried as a means to control recurrent BCA, especially for recurrences on the chest wall (which are extremely resistant to chemotherapy for unknown reasons). Thus, a recent clinical trial from China used ALA-mediated PDT in combination with RT for BCA metastases of the chest wall, and showed that the alternating use of PDT and RT improved the complete response rate, and reduced the time to clearance, when compared to RT alone^[19]. Increased use of PDT as a tissue-sparing adjunct for RT, would reduce the amount of radiation-induced fibrosis and vascular damage (scarring, ulceration, dermatitis) suffered by BCA patients, thereby representing a meaningful advance in clinical oncology; (4) it might be possible to boost the efficacy of PDT, by using neoadjuvant CPBN, to the extent that CPBN-PDT becomes useful as a monotherapy. At the very least, CPBN-enhanced PDT would be more effective as an RT-sparing approach when used together with RT; (5) future clinical translation of a CPBN-PDT approach is likely to be relatively easy, since CPBN (trade name Xeloda) is already a well-established, FDA-approved agent for the treatment of metastatic BCA.

In conclusion, based on our previous work showing that a combination of 5-FU given prior to PDT, leads to enhanced ALA-mediated PpIX levels and greater tumor cell death following PDT in several different epithelial cancers, we hypothesized here that CPBN might be a useful alternative to 5-FU as a neoadjuvant with PDT for BCA. Because 5-FU might be quite toxic at the high systemic levels required to modulate PpIX levels in BCA tumor, we reasoned that CPBN, a precursor to 5-FU, might serve as a safer yet equally effective alternative. In this study, we compared the responses of 4T1 tumors when treated with either conventional ALA-PDT, or with ALA-PDT preceded by oral CPBN. Our results revealed the following major findings: (1) CPBN-enhanced PDT can be used to significantly increase the accumulation of PpIX in murine breast tumors, and therefore represents a non-toxic alternative to 5-FU; (2) the pretreatment of 4T1 tumors with CPBN causes increased differentiation and decreased proliferation; (3) CPBN-enhanced PDT improves photodynamic killing of the breast tumor cells; (4) a combination regimen using CPBN and PDT significantly reduces distant metastases in the 4T1 tumor model. In summary, this treatment combination has the potential to be an effective therapy for BCA metastases, and potentially for other types of cancer. A more extensive version of this preclinical study would be immensely exciting and provide an additional rationale for designing a clinical trial based upon this concept.

DECLARATIONS

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Authors' contributions

Conception and design, gather experimental data, analysis, interpretation and writing: Anand S

Gather experimental data: Yasinchak A, Bullock T, Govande M

Conception and design, analysis, interpretation, funding and writing: Maytin EV

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Wrong place at the wrong time: how retrograde trafficking drives cancer metastasis through receptor mislocalization

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Abstract

Retrograde trafficking is a well-regulated, multi-component pathway that can result in endosomal trafficking to the trans-Golgi network, the perinuclear space, or the nucleus. Either clathrin or the retromer complex can travel with proteins endocytosed from the plasma membrane, guided by Rabs (including 5, 6, 7, 9, 22A), interacting with a host of sorting nexin proteins, and fusing with Golgi-specific anchors to allow transport of activated receptor tyrosine kinases to a potential end within the nucleus. Amplification in these constituents is common in cancer, leading to increased retrotranslocation and a reduction in degradation of receptor tyrosine kinases, an event highly associated with cancer metastasis. Here, we review the role of retrograde trafficking in altering transmembrane receptor localization and activity and the relationship to metastasis, focusing on all four members of the ErbB family, with comparison to other receptor tyrosine kinases including the insulin receptor and fibroblast growth factor receptor, as well as other transmembrane proteins dysregulated in metastasis. By examining how these receptors are being alternatively trafficked and the cancer-associated events resulting from this process, we hope to identify novel therapeutic targets.

Keywords: Retrograde trafficking, receptor tyrosine kinase, metastasis, nuclear receptor



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INTRODUCTION

Retrograde transport is a process that involves secretory trafficking pathways from endosomes to the trans-Golgi network (TGN), the Golgi to the endoplasmic reticulum (ER), and within the perinuclear space, designed to maintain a steady-state localization of proteins^[1-4]. Upon internalization of a receptor, toxin, or other constituent subject to intracellular trafficking, cargo is transported to the early endosomes. There, cargo can be recycled to the plasma membrane (with or without passing through the recycling endosome), transferred to a late endosome en route to the lysosome for degradation, or subject to retrotranslocation to the trans-Golgi^[5]. Within the Golgi proteins can undergo post-translational modifications, be distributed to their appropriate cellular localization (e.g., polarized membrane), or sent to the ER for re-folding or ER-assisted degradation (ERAD). In this review, we focus on retrograde trafficking, not for the purposes of protein homeostasis, but rather examining how activated receptor tyrosine kinases are trafficked towards the nucleus upon internalization, where a host of signal transduction, DNA binding, and transcription result in metastatic events. In multiple kinase receptor families, including the ErbB family, this alternative trafficking results in endosomally-captured active receptors in the perinuclear space and nucleus. This trend is not specific to solely receptor tyrosine kinases, as we also discuss the retrotranslocation of other transmembrane proteins, such as the interleukin-2 receptor (ILR2) and the transforming growth factor β (TGF- β) receptor. In addition to perinuclear localization, retrotranslocation is frequently associated with the upregulation and constitutive activation of the phosphatidylinositol-3-OH kinase (PI3K)/protein kinase B (AKT) pathways, known drivers of cancer metastasis. Taken together, we present the concept that retrotranslocation of activated proteins, particularly receptor tyrosine kinases, augments AKT signaling and increases nuclear transcription, resulting in cancer metastasis.

MEDIATORS OF RETROTRANSLOCATION

Internalization

Internalization of cargo, the first conserved step of retrograde trafficking (receptor tyrosine kinases require activation, toxins do not) occurs in two primary formats - clathrin-mediated endocytosis (CME) or retromer-assisted. Toxins (such as shiga, ricin, or cholera) and many receptor tyrosine kinases are subject to retrotranslocation from the early endosome in clathrin-coated endosomes, while transmembrane receptors such as wntless, β -secretase (BACE1), and TGN38 interact with the retromer complex from the early endosome^[6-11]. Trafficking constituents such as mannose 6 phosphate receptor and Vsp10 are directed from late endosomes via the retromer complex^[12-14].

CME occurs when clathrin triskelions form a bilayered coat of polyhedral lattices surrounding an inner coat of clathrin adaptors. Adaptors are then responsible for interacting with the cytoplasmic domains of membrane-bound receptors, and in some instances, even appear to promote the binding of clathrin directly to the receptor^[15]. Clathrin can interact not only with the cytoplasmic domain of receptors, but also with post-translational modifications, and at the interface between endosomes and the TGN^[3,16]. It has been demonstrated that clathrin is frequently required for retrograde trafficking, both for endogenous cargo and plasma-membrane-localized proteins^[3,17].

The retromer

In contrast to clathrin-mediated retrotranslocation, proteins can also be trafficked via the retromer complex. The retromer is a three-protein complex consisting of Vsp26, 29, and 35, capable of binding the cytoplasmic domain of transmembrane proteins located in endosomes without interacting with clathrin. Once bound, the retromer interacts with a dimer of sorting nexin (SNX) family members (either SNX1/2 and SNX5/6) to induce membrane curvature and direct retrograde trafficking away from lysosomal degradation^[1,18]. This trimer interacts with GTPases; Rab5 when interacting with the early endosome or Rab7 with the late endosome^[2]. SNX proteins directly interact with the membrane, with a Bis/amphiphysin/Rvs domain to sense

and stabilize membrane curvature and a PX domain capable of interacting with phosphoinositides (PtdIns) to determine the localization of early endosomes^[18-20]. Alteration of SNX proteins can directly affect receptor tyrosine kinase activity, as loss of SNX1 activity is correlated to a reduction in lysosomal degradation of epidermal growth factor receptor (EGFR)^[21-23].

Rab GTPases and retrotranslocation

Whether cargo enters the system via CME or SNXs, it will be found associated with Rab GTPases. The Rab family of proteins - highly specialized in location and function, are frequently amplified but not mutated in metastatic cancers, leading to increased availability of retrograde trafficking vesicle fusion components, thereby promoting unabrogated receptor signal transduction and uninhibited nuclear colocalization^[24-27].

Rab proteins are GTPases involved in vesicular transport, providing membrane identity and tethering vesicles prior to fusion^[26,28]. They are capable of interacting with other coat components, motor proteins including kinesins and myosins, and soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs)^[26]. Despite strong homology among the family members, each Rab confers a unique function to the vesicles they mediate in response to specific activators^[29]. Two Rabs in particular are known mediators of retrotranslocation: Rab22A which acts in early endosome-TGN trafficking, and Rab6A which is involved in Golgi-ER transport.

Rab22A is involved in clathrin-independent endocytosis, associating with both early and late endosomes, but not lysosomes. When bound to GTP and active, Rab22A is capable of binding to early endosome antigen 1 (EEA1; a marker unique to the endosomal sorting complex where most transmembrane receptors pass through during endocytosis), directing the activator for Rab5 to promote early endosome fusion, a necessary step in retrotranslocation^[30]. Rab22A expression is increased in lung, liver, ovarian, renal cell carcinoma, and melanoma^[30,31]. Rab22A is also commonly overexpressed in breast cancer and associated with decreased patient survival. A HIF1 α -dependent Rab, Rab22A is responsible for generating hypoxia-induced vesicles, that when present promote breast cancer cell invasion through cytoskeletal alterations^[32]. Using miRNAs to suppress the expression of Rab22A, studies have demonstrated that miR-373 can inhibit ovarian cancer, miR-203 inhibits osteosarcoma, and miR-204 inhibits renal cell carcinomas, all in a Rab22A-dependent manner^[31,33,34].

Rab6A is a GTPase associated with highly dynamic vesicles subject to retrograde transport from the Golgi to the ER^[35]. Part of the recycling endocytic pathway, Rab6A is known to function within the cis- and trans-Golgi, involved in both intra-Golgi and coat protein complex-1 (COP-1)-mediated Golgi-ER transport^[36-38]. Frequently dysregulated and associated with poor prognosis in cancer, it also presents with increased expression in human epidermal growth factor 2 (HER2+; ErbB2) breast cancer patients^[35,39].

One of the primary functions of the Golgi is to aid in post-translational modifications (such as glycosylation) of proteins, particularly for activation of receptor tyrosine kinases, which allows for ligand binding, receptor trafficking, and internalization^[40]. As previously discussed, a key element of retrotranslocation is the ability of vesicles to fuse with the TGN, instead of trafficking for lysosomal degradation. GOLPH3, a resident Golgi protein, localizes to the TGN and can directly interact with the retromer complex through Vsp35. Responsible for driving cell proliferation, tumor development, and anchorage independent growth both *in vitro* and *in vivo*, GOLPH3 is an oncogene amplified in more than 30 percent of lung, ovarian, and breast cancer cases (as high as 56 percent of lung cancers). Previously demonstrated in yeast that the retromer complex is involved in mammalian target of rapamycin (mTOR) signaling, high expression levels of mTOR have been similarly observed in GOLPH3-amplified human tumor tissues, resulting in increased pAKT activity^[40]. Not only is increased pAKT associated with high metastatic potential in breast cancers, but mutations in an anchor protein such as GOLPH3 could result in endosomal retention of receptors by inhibiting their transition out of endosomes, leading to prolonged signal transduction and metastasis^[41,42].

An event frequently observed in cancers such as breast, pancreatic, and lung is the localization of activated receptor tyrosine kinases in the perinuclear space^[43-45]. While not all of these receptors have been shown to pass through the Golgi, they do appear to be subject to retrotranslocation as many of them are found in the nucleus, including fibroblast growth factor receptor (FGFR), members of the ErbB family, and TGF- β receptor, all of which will be further discussed below^[41,46,47].

Here, we explore a range of transmembrane receptors associated with cancer and metastasis, examining their endogenous trafficking pathways, conditions in which this trafficking may be altered, and the relationship between retrotranslocation, nuclear localization, and metastasis.

INSULIN RECEPTOR

The insulin receptor (InsR) is a receptor tyrosine kinase known to traffic to the perinuclear space (rather than the TGN) and colocalize with SNARE proteins through a targeting motif in the carboxy terminus^[48]. This perinuclear colocalization occurs in a subset of storage vesicles near the Golgi, driven by the high rates of intravesicular cycling associated with InsR, as only 2-5 percent of the receptor is maintained at the cell surface^[49,50]. Intracellular InsR promotes the constitutive activation of AKT, resulting in glucose transporter 4 translocation, enhanced anchorage-independent growth, and altered acinar structure formation^[50-52].

FGFR

FGFR is a receptor tyrosine kinase highly involved in epithelial to mesenchymal transition (EMT) and tumorigenesis, promoting signal transduction through mitogen-activated protein kinase (MAPK) and β -catenin activity^[53]. Stimulation with ligand FGF1 results in receptor retention in Rab5-positive endosomes in the perinuclear space (undetermined if in the Golgi apparatus) and promotes trafficking to the nucleus, an event modulated by importin- β ^[46,54]. When bound to the ligand FGF2, FGFR is capable of inducing c-Jun and cyclin D1 mRNA expression by acting as a transcription factor at the FGF2 promoter, an event similar to that seen in EGFR^[54-56]. Nuclear FGFR is highly associated with AKT-driven breast cancer metastasis to the lung and pancreatic cancer cell invasion^[57,58]. Importantly, neither the FGF ligands nor FGFR contain a canonical nuclear localization sequence (NLS)^[55], indicating that the nuclear localization is a result of retrotranslocation driving activated receptors from endosomes in the perinuclear space toward the nucleus.

ERBB RECEPTORS

EGFR

ErbB1, also known as HER1 or EGFR is a single pass transmembrane receptor tyrosine kinase subject to significant post-translational modifications, which affect both its activation and trafficking. Upon binding a ligand, EGFR is subject to homo/heterodimerization, transphosphorylation, and is primarily trafficked to the lysosome^[59,60]. It is also capable of retrotranslocation, recycling, and other trafficking pathways, all of which are highly dependent on the localization of EGFR within the cell and additional signal transduction pathways activated in the cell^[61-63].

EGFR can bind a range of ligands, including epidermal growth factor (EGF), TGF- α , heparin-bound-EGF (HB-EGF), β -cellulin (BTC), epiregulin (EPR), and amphiregulin (AR). Upon binding a ligand, ErbB receptors dimerize and are subject to internalization and trafficking. Ligand binding triggers membrane invagination within at least 30 s, a transient process that resets within approximately 15 min after exposure. All ligands promote the transport of EGFR from the plasma membrane to EEA1-positive endosomes, though AR is slightly less efficient at this^[62,64]. EGF, HB-EGF, and BTC promote EGFR trafficking to the lysosome for degradation, while TGF- α and EPR binding primarily promote EGFR recycling^[62]. EGFR-EGF binding promotes activation of multiple signaling pathways including MAPK, PI3K, phospholipase C (PLC γ), and AKT, leading to a host of potentially oncogenic activities when left unregulated. EGFR has been implicated

in organ morphogenesis, maintenance and repair pathways, tumor progression, and metastasis through upregulated EGFR signaling^[65]. EGFR signaling is potentiated by phosphorylation of tyrosines found in the cytoplasmic domain, whose activation is determined by ligand and dimerization partner. Phosphorylated tyrosines act as docking sites for many proteins and interactors, such as Src homology 2 or phosphotyrosine binding domain-containing proteins, including growth factor receptor-bound protein 2, Src-homology and collagen, PLC γ , PI3K subunit p85, GTPase activating protein, Cbl, and adaptor protein-2 (AP-2). EGFR phosphorylation remains active in endosomal vesicles, where EGFR is hyperphosphorylated, allowing for continued signal transduction^[66-70]. Signaling through PLC has been shown to be preferentially activated when at the cell surface, while MAPK signaling continues to occur while in intracellular vesicles^[71].

PI3K signaling is also preferentially activated in endosomes, as Vieira *et al.*^[66] demonstrated PI3K activation is unnecessary for internalization and instead plays a role in endosomal trafficking of EGFR. Similarly, Garay *et al.*^[67] indicated preferential AKT signaling when EGFR is internalized in clathrin-coated vesicles rather than at the plasma membrane through siRNA against clathrin.

Vesicular EGFR alternatively trafficked away from the lysosome (demonstrated with a SNX1-directed siRNA) results in augmented AKT signaling in non-small-cell lung cancer^[72], and we have previously demonstrated that endosomally retained and mislocalized EGFR is capable of upregulating AKT activity, resulting in increased metastatic potential, as well increased nuclear localization of transcription factor TAZ (transcription co-activator with a PDZ-binding domain), an event associated with cancer stem cells^[42,73]. AKT is involved in driving EMT through disassembly of cell-cell junctions and upregulation of SNAIL, confirmed by loss of EMT phenotype in prostate cancer cells when treated with AKT inhibitors^[74]. AKT1 kinase activity and AKT2 overexpression are associated with ovarian, breast, and thyroid cancers - three cancer types commonly hallmarked by high EGFR expression^[75-81]. While AKT activation is strongly associated with the inhibition of apoptosis, at least one study has found endocytosed EGFR that does not traffic to the lysosome correlates with an induction of apoptosis^[82]. More work to determine the mechanisms of these events will be required to fully understand these processes.

Though EGFR is primarily targeted to the lysosome to prevent constitutively activated signal transduction, when stimulated by EGF, it has been shown that a fraction of EGFR will be transported to the Golgi and the ER, carried via COP-I vesicles en route to trafficking to the nucleus^[83,84]. Studies have shown that after 20 min of EGF stimulation, 10 percent of EGFR colocalizes to the Golgi and remains phosphorylated, even in the presence of protein synthesis inhibitors, indicating that the retrograde trafficking observed is not due to post-translational modifications^[85].

Upon translocation to the Golgi, the amino-terminal domain of EGFR sits within the lumen of the Golgi, leaving the carboxy-terminal domain exposed in the cytoplasm to interact with importin β -1 via the NLS^[84]. All ErbB family members can enter the nucleus, with full-length, phosphorylated EGFR entering via the Sec61 translocon and interacting with importin α - β 1 complexes^[43,86,87]. EGFR is capable of transactivational activity, but lacks a DNA-binding domain, providing a need for transcription co-factors^[88]. Upon entry into the nucleus, EGFR can associate with transcription co-factors such as signal transducer and activator of transcription 3 (STAT3) to co-regulate inducible nitric oxide synthase expression and E2F1 to promote cell cycle progression, as well as the proliferation marker Ki-67^[88-90]. Nuclear EGFR interactions with proliferating cell nuclear antigen (PCNA) also promote cell cycle progression, while increasing PCNA phosphorylation and the DNA damage response^[91,92]. Nuclear EGFR has been found to be associated with more than 40 percent of breast cancer tumors and 35 percent of esophageal squamous cell cancers, correlated to shorter overall survival in patients and increased metastatic potential, respectively^[93,94]. Nuclear EGFR also contributes to cetuximab and gefitinib resistance, likely due to the restricted access of the therapeutics to the target protein^[41,85]. Finally, the role of retrotranslocated EGFR as a transcriptional co-factor further exemplifies its kinase-independent function, highlighting the need for therapeutically targeting this process.

We have previously demonstrated the localization of EGFR in the nucleus when interacting with the oncogenic adaptor glycoprotein mucin1 (MUC1). Notably, when in the presence of MUC1, EGFR is able to bind to chromatin and act as a co-transcriptional activator by colocalizing to the transcriptional start sites with phosphorylated RNA polymerase II^[56]. EGFR has also been shown to interact with cyclin D1 in the nucleus and cyclin D1 expression is increased in EGFR-dependent mouse models and breast cancers^[56,95-97]. Also seen in MUC1-expressing, EGFR-driven breast cancer mouse models were high rates of lung metastases (96 percent showing distinct pulmonary foci and the remaining 4 percent unspecified adenocarcinomas). Alternatively, in the absence of MUC1 and nuclear EGFR, no lung metastases were observed. Taken together, nuclear EGFR, particularly in the presence of MUC1, promotes EGFR transcriptional activity, increases cyclin D1 expression, and drives metastasis.

To elucidate the mechanism by which MUC1 promotes nuclear localization of EGFR, we have also demonstrated MUC1 and EGFR colocalize in EEA1-positive endosomes that are retained in the perinuclear space and actively are trafficked away from the lysosome. This allows for signal transduction to remain unattenuated, resulting in a MUC1-dependent increase in breast cancer cell migration rates, an effect completely eliminated by the introduction of a retrograde trafficking inhibitor, Retro-2. Inhibiting retrotranslocation of EGFR, even in the presence of MUC1, results in a reduced migratory phenotype through cytoskeletal rearrangement and reduction of focal adhesion kinase (FAK)-positive structures^[41].

EGFR is a pleiotropic signal transducer with a highly conserved activation and endocytosis pathway. However, during cancer, a high correlation is observed between retrograde trafficking and nuclear localization, resulting in increased metastatic events, both *in vitro* and *in vivo*.

ErbB2 receptor

ErbB2 (HER2, Neu2) is another member of the ErbB family, well-studied for its role in driving cancer through increased cell proliferation, resistance to apoptosis, and migration. A transmembrane protein found on the basolateral surface of cells, ErbB2 lacks the ability to bind ligands, therefore relying on other receptors for dimerization. While EGFR is primarily driven to the lysosome as part of its endogenous trafficking, ErbB2 is resistant to downregulation of itself or its heterodimerization partner, instead subject to rapid recycling through CME^[98].

ErbB2 activation is dependent upon heterodimerization with another ErbB receptor, as it lacks a ligand-binding domain to allow for self-activation. It can also become active through extensive overexpression, which promotes the formation of ErbB2 heterodimers responsible for enhancing the signal transduction associated with their dimer partner, an event seen in colon, gastric, prostate, and breast cancers^[59,61,99]. ErbB2 overexpression in breast cancer is so well-characterized that it is responsible for an entire classification: HER2+; studies have shown it is amplified in more than 20 percent of breast cancer tumors, making it a strong predictor of survival and time to relapse^[100,101].

Upregulation of ErbB2 is known to promote lymph node metastasis, as well as significantly increase metastasis rates to the brain, liver, and lung^[102]. Like all members of the ErbB family, ErbB2 can enter the nucleus, where it can interact with promoters, including matrix metalloproteinase 16, p53-related protein kinase, and cyclooxygenase-2 (COX-2). The COX-2 gene is known to drive metastasis in cancer cells and co-expression of these two proteins has been reported in colon, cholangiocarcinoma, and breast cancers^[103]. Exploring further, Edwards *et al.*^[104] reported that prostate tumors with increased HER2 copy number also had increased levels of COX-2 expression while Thorat *et al.*^[105] and Glynn *et al.*^[106] demonstrated that COX-2 levels are highest (if not statistically significant) in HER2+ breast cancer (vs. luminal A/B and triple negative breast cancers) and that positive COX-2 expression in HER2+ patients is directly correlated to increased phosphorylation of AKT and poor survival outcomes^[104-106].

ErbB2 overexpression is seen in a variety of cancers and is strongly associated with metastatic events and inhibited degradation of activated receptors. Lacking intrinsic ability to self-phosphorylate, ErbB2 receptors bind with other members of the ErbB family to induce activation and potentiate signal transduction, frequently resulting in increased COX-2 and AKT activity due to nuclear localization, events commonly seen in metastasis.

ErbB3 receptor

ErbB3 is a catalytically inhibited member of the ErbB family, bearing a mutated tyrosine kinase domain and relying on heterodimerization partners for phosphorylation. As such, ErbB3 frequently presents with decreased levels of ubiquitination in comparison to EGFR, emphasizing the inefficiency of ErbB3 to be targeted to the lysosome^[107]. Heterodimers between ErbB2 and ErbB3 lead to strong activation of the MAPK and PI3K/AKT pathways, promoting survival and proliferation by ErbB2 and ErbB3, respectively^[108-110]. Through these signaling pathways ErbB3 activity is associated with loss of cellular differentiation and increased expression of MUC1 in carcinomas, as well as driving proliferation in ErbB2-positive breast cancer cells lines^[108,111]. ErbB3 is subject to significantly slower rates of endocytosis than EGF-bound EGFR due to the anti-internalization regions within the C-terminus, allowing for longer periods of receptor activation (when in a heterodimer)^[71,112]. This delayed trafficking results in perinuclear accumulation, thereby allowing nuclear localization via retrotranslocation^[113]. Once in the nucleus, ErbB3 presents in an activated, uncleaved format, capable of associating with the Cyclin D1 promoter to drive cell proliferation, similar to the mechanism seen in EGFR^[54,56,114]. Clinical data has shown in prostate cancer, nuclear localization of ErbB3 present in 100 percent of hormone-refractory samples and 40 percent of hormone-sensitive samples, in contrast to the negligible amount seen in normal prostate tissue, directly associated with disease progression risks^[115]. Nuclear ErbB3 also presents at higher frequency in prostate cancer metastases (particularly bone) than in primary tumor sites^[116], indicating that retrotranslocated ErbB3 is likely involved in the metastasis of prostate cancers.

ErbB4 receptor

The fourth and final member of the ErbB family, ErbB4 maintains homology to ErbB1- capable of binding ligands (EPR, HB-EGF, BTC, and neuregulins 1-4) and a tyrosine kinase domain capable of catalytic activation^[112,117]. Once bound to a ligand, ErbB4 promotes signal transduction through a host of pathways, including PLC γ , PI3K, and STAT^[118].

Post-activation, ErbB4 is subject to dual-protease cleavage in both the extracellular domain and the transmembrane domain, resulting in an active, cleaved cytoplasmic domain capable of nuclear translocation (upon binding to neuregulin). ErbB4 activity is highly associated with poor prognosis and increased metastasis in Ewing sarcoma, activating PI3K-AKT and altering FAKs to drive invasion^[119]. Expression of ErbB4 also increases in colorectal cancer as disease stage progresses, indicating a potential role in driving cancer progression, though its expression is associated with improved prognosis in estrogen-receptor positive breast cancers^[120,121]. Highly enriched in neuronal plaques of patients with Alzheimer disease, nuclear localization can lead to transcriptional activation of genes that regulate neurodegeneration^[118]. To achieve nuclear localization, it is possible that active, cleaved ErbB4 reaches the Sec61 translocon in the ER and is processed while avoiding ERAD-ubiquitin-associated proteolysis, in a mechanism similar to EGFR nuclear translocation^[86,118].

In addition to the tyrosine kinase receptors described above, at least two non-tyrosine kinase receptors, ILR2 and TGF- β receptor, also undergo retrotranslocation in cancer.

ILR2

Found in fibroblasts, epithelial, and neuronal cells, ILR2, upon ligand binding is internalized and targeted for degradation through acidification, similar to EGFR and the transferrin receptor^[59,122,123]. ILR2 has also

demonstrated an ability to undergo endocytosis in the absence of clathrin or AP-2 at relatively uninhibited rates, likely due to the nature of the receptor to promote T-cell proliferation - a system that would benefit from redundancies^[122].

When undergoing non-CME, ILR2 is internalized through detergent-resistant membrane domains, similar to the mechanism employed by cholera toxin^[124,125]. Upon entry into the cell, vesicles containing ILR2 are found in the glycolipid rich areas of the cell, particularly in the perinuclear spaces and near the organelle responsible for glycolipid modification, the Golgi^[124]. Retention of the receptor in the perinuclear space is common in cancer, as ILR2 expression can be a predictor of patient survival and is significantly reduced at cell surface levels in advanced ovarian cancers^[126,127].

TGF- β RECEPTOR

TGF- β is a secreted ligand involved in modulating cellular growth and arrest, differentiation, and immune responses, capable of binding to type I or type II single-pass transmembrane receptors^[47,128-130]. Unlike other receptors presented in this review, TGF- β receptor can be internalized with or without ligand binding, resulting in either CME-assisted recycling or non-CME degradation^[47].

When subject to CME, TGF- β receptor is sorted into EEA1-positive vesicles where it acts to activate SMAD2 and potentiate TGF- β signal transduction. Activated receptors localize to the perinuclear space and remain in endosomes, a phenotype also seen with the transferrin receptor and which we have previously shown to occur with EGFR in breast cancer^[41,47]. Alternatively, TGF- β receptor can also be sorted via lipid rafts to interact with SMAD7. In either scenario, SMAD signaling nuclear translocation require internalization of TGF- β receptor^[131,132].

Taken together, a range of transmembrane proteins are subject to retrotranslocation. While some localize to the perinuclear space and can be internalized into the nucleus, such as InsR, FGFR, TGF- β receptor, and ILR2, others can enter the Golgi, such as ErbB family members, and remain active and promote metastasis. ErbB receptors also localize to the nucleus, resulting in transcriptional activity of cancer genes and pathways.

THERAPEUTIC TARGETING OF RETROTRANSLOCATION

Given the metastatic and oncogenic activity associated with nuclear localization of activated receptor tyrosine kinases driven by retrograde trafficking, it is important to explore potential mechanisms by which retrotranslocation could be inhibited as a therapeutic target.

It has been demonstrated that introduction of retrograde inhibitors can drastically reduce the cytotoxic effects of toxins like ricin, cholera, or shiga, which rely on retrograde trafficking to be distributed throughout cells. A compound designated Retro-2 works to inhibit toxin trafficking from the early endosome to the trans-Golgi interface, actively protecting cells from ricin toxicity 2-3 fold *in vitro* and almost 50 percent of mice from airborne exposure to the ricin toxin (in comparison to the 11 percent that survived in the absence of retrograde inhibition)^[133]. Retro-2 does not inhibit plasma membrane budding or endosome formation; rather it inhibits transport of endosomes to the TGN without affecting Golgi morphology (or that of EEA1 or Rab11). Endocytic degradation and recycling pathways also remain unaltered. However, SNARE proteins syntaxin 5 and 6 were subject to alterations in localization, indicating Retro-2 may work to inhibit retromer trafficking of endosomes or inhibit interactions of endosomes with the Golgi^[133].

We have previously demonstrated the importance of retrograde trafficking in promoting a migratory phenotype in association with the presence of MUC1^[41]. Treatment of breast cancer cells *in vitro* with the retrograde trafficking inhibitor Retro-2 led to the inhibition of MUC1-driven migration and re-introduction

of EGFR trafficking to the lysosome for degradation. Given this, a potential therapeutic option may be to inhibit retrograde trafficking of EGFR, possibly through the use of Retro-2, though no studies have yet examined the effects in humans.

Alternative to inhibiting retrograde trafficking, current research has demonstrated an effort to capture the retrotranslocation mechanisms of toxins and repurpose them for drug delivery. Using the non-toxic subunit B of Shiga-like toxin, various attempts at conjugating therapeutics, incorporating nanoparticles, or developing fusion proteins have been attempted (reviewed in Luginbuehl *et al.*^[134]).

Should inhibition of retrograde trafficking prove untenable in patients, a secondary approach to inhibition of the oncogenic activity driven by nuclear receptor tyrosine kinases would target receptors within the nucleus. Nuclear EGFR has been shown to associate with promoter regions of a variety of proteins, including cyclin D1, activated STAT3, E2F1, DNA-dependent protein kinase, and other nuclear targets, while nuclear ErbB2 is capable of interacting with COX-2, all resulting in increases in tumorigenesis, proliferation, metastasis, chemoresistance, and radioresistance^[106,135]. ErbB2-EEA1 complexes are capable of nuclear transport, and given the high affinity of EGFR-MUC1 complexes in EEA1 positive vesicles in the perinuclear space of cells after extended exposure to EGF ligand, it is feasible that EGFR endosomal machinery is promoting nuclear localization of EGFR in a similar mechanism^[41,87,136].

Radiation treatment of cells is known to drive EGFR to the nucleus as part of the DNA-repair pathway mechanism, and studies have shown that treatment of irradiated cells with cetuximab will inhibit EGFR trafficking to the nucleus in both lung carcinoma and breast cancer cell lines^[137]. Radiation treatment, along with neuregulin stimulation or trastuzumab will also promote retrograde trafficking of ErbB2-ErbB3 dimers to the nucleus, visible by super-resolution confocal microscopy as demonstrated by Pilarczyk *et al.*^[138]. Nuclear import might also be inhibited by targeting the importin- β 1 molecule through treatment with small molecule inhibitors such as Karyostatin1A to disrupt importin interactions with the GTPase Ran^[139].

Treatment of cells with 1,25-dihydroxyvitamin D was found to prevent EGFR from entering the nucleus by promoting intracellular localization of inactive, unphosphorylated EGFR, even if bound to a ligand, to early endosomes. By doing so, 1,25(OH)₂D₃ downregulated the oncogenic activity associated with nuclear EGFR without targeting vesicles to the lysosome^[140]. This treatment could feasibly be used to target two processes associated with cancer progression - inhibit nuclear targeting of EGFR and convert endosomally localized EGFR to an inert state, essentially nullifying the need for lysosomal degradation.

CONCLUSION

Retrograde trafficking of transmembrane proteins, with an emphasis on receptor tyrosine kinases, results in alternatively trafficked activated receptors accumulating in the perinuclear space of cells. The primary effect of these undegraded, endosomally-retained, actively signaling receptors is the unabrogated transduction of the PI3K/AKT pathway - resulting in upregulation of cancer metastasis. Not only that, but for those receptors that transit through the Golgi or come in sufficient proximity to a nuclear pore, active receptors can now act as transcriptional activators to a host of oncogenic activities. Seen in cancers ranging from Ewing sarcoma to breast to prostate and more, it is clear this ubiquitous mechanism of translocation is responsible for trafficking receptors to the wrong place at the wrong time, driving cancer metastasis. Future therapeutics may choose to inhibit retrograde trafficking to prevent mislocalization or focus instead on targeting receptors after their localization has already been altered. Given current treatment plans for many cancers now involve adjuvant therapies, the most efficient method for overcoming metastasis-promoting retrograde trafficking may involve some combination of both.

DECLARATIONS

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Authors' contributions

Written and edited manuscript: Maisel SA, Schroeder J

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Both authors declared that there are no conflicts of interest.

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Not applicable.

Consent for publication

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Case Report

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Microinvasive breast cancer with supraclavicular lymph node and ovarian metastasis as clinical findings: a rare case report

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Abstract

Breast cancer is a common malignancy among women. Due to the improvement of social awareness and advances in imaging technologies, significant achievements are obtained at its diagnosis and treatment each passing day. A 54-year-old, multiparous and postmenopausal woman, who presented with a palpable lymph node in the right supraclavicular region and a right adnexal mass but had no findings from the breast examination, is reported in this article. Following advanced assessment, metastatic carcinoma was identified in the lymph node biopsy and the adnexal mass. During the exploration for the primary origin, microinvasive breast cancer was diagnosed following mammographic imaging and an excisional biopsy from the right breast. Microinvasive breast cancer, which did present itself with clinical findings with metastases despite the lack of local findings, was discussed with the review of the literature.

Keywords: Microinvasive breast cancer, imaging, ovarian metastasis

INTRODUCTION

In the United States, one in every eight women has the risk to experience breast cancer in her lifetime. Breast cancer is one of the most common cancers and it is often stated as the second cause of cancer related



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deaths among women^[1]. Microinvasive breast cancer makes 0.7%-2.4% of all cases, it is a rare subgroup of breast cancer. The term of “microinvasive breast cancer” was used by Logios in 1982. Later, the World Health Organization defined it as an invasive carcinoma of the breast with no invasive focus measuring more than 1 mm^[2]. The prognosis of microinvasive breast cancer is open for discussion, since it is rare and difficult to diagnose. Generally, it is known that their clinical behavior is between invasive carcinoma and carcinoma *in situ*. Lymph node metastasis, which is one of the most important prognostic factors of breast cancer, is rare among microinvasive breast cancers (0%-25%) and there is an expectation of better clinical course^[3]. On the other hand, although there are very few in the literature, there are reported cases of aggressively progressive microinvasive breast cancers that presented clinical findings with lymph node involvement and distant organ metastasis^[4].

In this article, we present a case of microinvasive breast cancer that presented clinical findings with supraclavicular lymph node involvement and ovarian metastasis without any findings from breast examination. We found it to be interesting, due to the rarity of such cases.

CASE REPORT

A 54-year-old patient (Gravida 4, parity 3, 3 years since menopause, gave birth to her last child 20 years ago) was visited due to the complaint of a palpable lymph node at the right supraclavicular region. Due to the finding of a metastatic carcinoma at the lymph node after thin needle aspiration biopsy, she was investigated with imaging and systemic examination in order to find the primary focus. Systemic examination only demonstrated a palpable lymph node, which was 2 cm × 3 cm in size, at the right supraclavicular region. No other abnormal clinical finding was detected. A solid, mobile mass was found at the right adnexal region during gynecologic examination. The mass was 6 cm × 7 cm in size. Pelvic ultrasonography of the left adnexa and the uterus was normal regarding the patient's age. The lesion, which seemed to be originating from the right ovary was measured to be 65 mm × 72 mm in size. No free abdominal fluid was present. Ultrasonography of the upper abdomen and the physical examination of the breast was normal. Cervical smear, endometrial sampling, endoscopy of the gastrointestinal system, routine biochemical tests and complete blood count resulted in the normal range.

Apart from elevated CA15-3 concentration (157 IU/mL) and erythrocyte sedimentation rate (72 mm per 30 min); no abnormal blood parameters were present. Bilateral mammography was reviewed as Breast Imaging Reporting and Data System - 4B for the right breast (possible malignant findings), and therefore excisional biopsy (surgical margin clear of disease) was performed. Following radiological examinations showed no residual lesions. Pathological examination resulted as: microinvasive breast carcinoma, nuclear grade II. Immunohistochemistry results were as follows: p63 (+) at myoepithelial cells, estrogen receptor (ER) (+) for about 5%-10% of the cells at the ductal carcinoma *in situ* (DCIS) focus, progesterone receptor (PR) (-) for the cells at the DCIS focus.

Positron emission tomography-computerized tomography (PET-CT) showed increased F-18 fluorodeoxyglucose (FDG) uptake at both supraclavicular lymph nodes, especially more prominent on the right cervical level. In addition, increased uptake of FDG was also present at the lymph nodes in thorax and abdomen. The mass lesion, whose margins were not distinguishable from the uterus, was located in the right adnexal region and showed increased F-18 FDG uptake. These radiological findings in the right adnexal region supported the diagnosis of primary gynecological malignancy.

The case was reviewed in the gynecological oncology council of our institution. The decision was to perform diagnostic laparotomy in order to confirm the primary focus. The patient underwent surgery. A firm, solid mass with lobulated margins and 6 cm × 7 cm in size was observed during laparotomy ([Figure 1](#): macroscopic appearance of the tumor located at the right adnexal region). The contralateral ovary

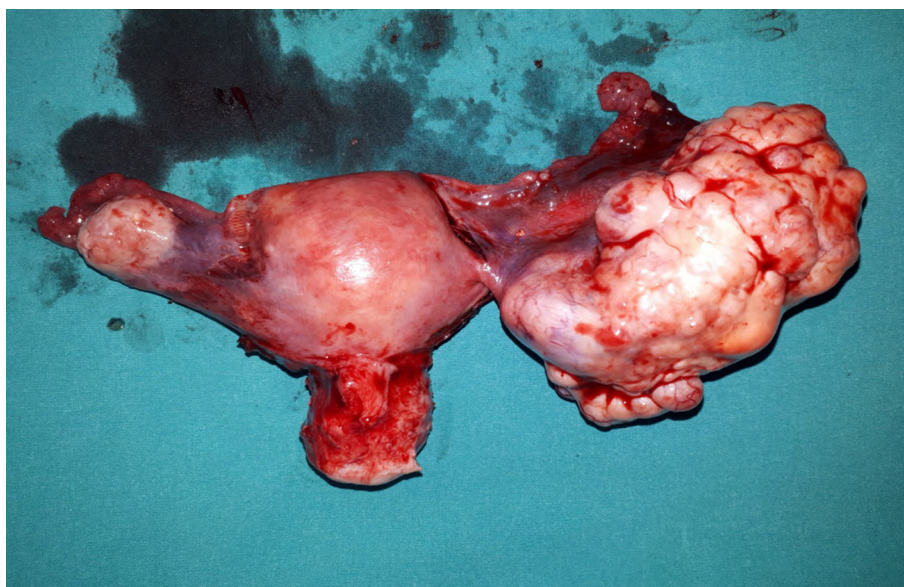


Figure 1. Macroscopic image of the tumor located in the right ovary

and genital structures, pelvic structures, organs of the upper abdomen were in normal appearance. Frozen examination of the mass from the right adnexal region was reported as malignant metastatic tumor. Due to this result staging surgery was performed.

The paraffin sections of the removed mass were examined by our pathology department and the diagnosis of metastatic carcinoma at the right ovary was confirmed (Figure 2: the microscopic image of the metastatic tumor located at the right ovary). The morphology of the metastatic tumor was reported as lobular carcinoma and it was concurrently examined with the histopathology of the previously removed excisional biopsy material from the right breast. After the examination, the ovarian mass was diagnosed as a metastasis of the primary breast cancer. The immunohistochemical findings at the ovarian tumor are as follows: ER positive, PR negative, Pax8 negative, Wilms' tumor 1 negative, gross cystic disease fluid protein-15 focal positive, GATA3 positive, epithelial membrane antigen positive, Ber-Ep4 positive; these findings supported the diagnosis for a malignant epithelial tumor but excluded a primary ovarian carcinoma. The lesion at the right breast, which appeared as a single focal lesion in imaging studies, was removed with clear margins. Therefore, no secondary surgery for local control was considered. After the abdominal surgery, the patient received the following chemotherapy treatments: 8 cycles of herceptin and docetaxel and subsequently 8 cycles of trastuzumab and pertuzumab. She undergoes examination every three months and has been disease - free for 12 months as this report is written.

DISCUSSION

At the present day, more patients are diagnosed with microinvasive breast cancer due to the broad usage of mammography. Most of the microinvasive breast cancer cases (80%) do not have palpable masses in the breast and are diagnosed with mammography^[2]. Nevertheless, microinvasive breast cancers constitute only a small part of invasive breast cancers (0.7%-2.4%) and confusion is still present about their prognosis^[1]. According to many studies, microinvasive breast cancers are reviewed in a spectrum between the early invasive breast cancer and *in situ* carcinoma of the breast, and excellent survival expectations are reported^[1-4]. However, in some cases, local and distant recurrences are reported during follow-up despite this high prognostic expectation^[5,6]. Involvement of axillary lymph nodes, which is a very important prognostic factor during the treatment and follow-up of breast cancers, is reported to be between 0 and 25 percent of microinvasive breast cancer cases^[3]. Involvement of axillary lymph nodes is crucial regarding local and

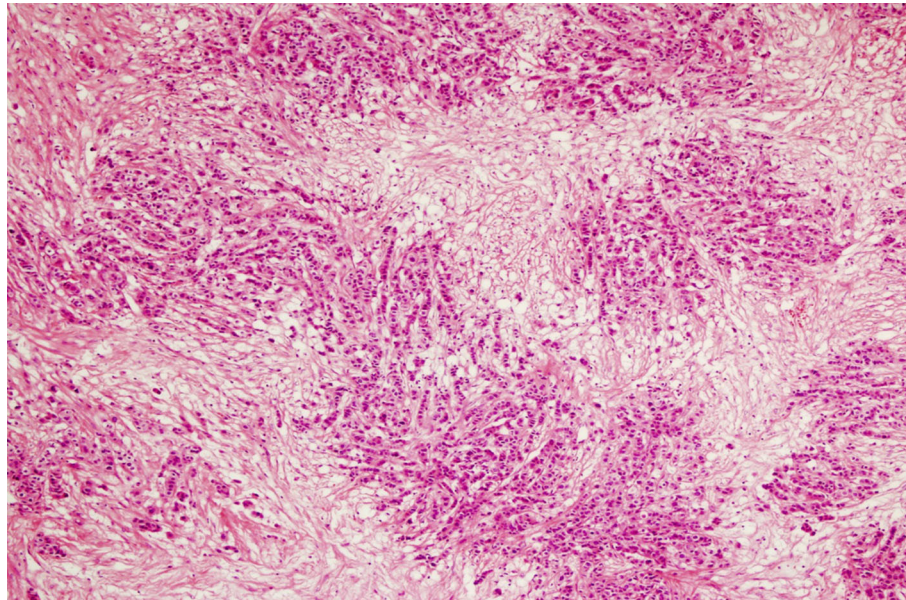


Figure 2. Microscopic image of the tumor H&E × 100

distant recurrences, as it is with invasive breast cancers. Expectation of five-year survival in microinvasive breast cancer is 95.2%-95.9% when there is axillary lymph node involvement; whereas the five-year survival rate is 100% for axillary lymph node-negative cases^[3-7]. Axillary lymph nodes were negative for tumors in our case.

In spite of the advancements in its diagnosis and treatment, breast cancer continues to be fatal. Because of the fact that it can spread both locally and distantly simultaneously, breast cancer is considered to be a systemic disease from the beginning.

Three point five to ten percent of the breast cancer cases present findings with distant metastasis at the moment of diagnosis^[8,9]. Microinvasive breast cancer presented clinical findings with supraclavicular lymphadenopathy and adnexal mass. Positive ER (which was focally 5%-10% positive in this case), negative PR and increased expression of human epidermal growth factor receptor 2 (HER2) may be the molecular features that are responsible for such aggressive spreading of the tumor^[10]. One to seventeen percent of all malignant ovarian tumors are metastatic. Breast, gastric and colon cancers are the most common cancers that metastasize to the ovary^[11]. Contrary to our case, 70% of the metastatic ovarian tumors are bilateral. Distinction of primary and metastatic tumor is essential for determining the treatment approach and prognosis. Tumors metastasizing to the ovary from non-genital organs are associated with poor prognosis. Five-year survival rate for primary ovarian cancer is 40%, whereas in case of metastasis from non-genital organs the rate becomes 12%-24%, and the mean survival is 15 months^[11,12]. Survival depends on the prognosis of the primary tumor and when the tumor presents findings with metastases before itself, the prognosis is poorer^[12].

Debulking surgery has a role in patients with metastatic ovarian tumors that originate from non-genital organs, however it is not established that which patients are going to benefit from this technique of surgery^[12,13]. Yet we consider it worthwhile, because it reduces the required amount of time for final diagnosis and the tumor burden prior to chemotherapy. The patient undergoes regular clinical controls and has been disease free for 12 months.

Concerning the diagnosis and management of primary ovarian tumors, serum CA 125 levels are among

important markers. Still, it is difficult to tell the same for metastatic tumors^[13,14]. Except elevated CA 15-3 levels, other tumor markers (CA 125, CA 19-9, carcino-embryonic antigen, alpha-fetoprotein) were in normal range in our case.

As patient history, laboratory findings and physical examination, radiological examination is also an important means while detecting the primary focus of the tumor. In addition to ultrasonography, mammography, magnetic resonance imaging and CT; PET-CT is also very helpful. Especially during the postmenopausal period, when the ovaries are non-functional, the diagnostic power of PET-CT is higher^[11]. Mammography manifested the findings for breast cancer rather clearly. The expectation for broad involvement of lymph nodes and presence of distant metastases was low at the stage of microinvasive breast cancer, therefore the possibility of the right ovarian mass being a second primary malignancy was considered. Although the imaging methods were very revealing regarding the extensiveness of the lesion, they were inadequate for the distinction between primary and metastatic tumor. Consequently, final diagnosis was established with laparotomy^[15]. Presence of an adnexal mass, while there is a diagnosis or possibility of breast cancer, should be evaluated carefully regarding increased risk of malignancy.

Distant organ metastasis is beyond expectation at the moment of diagnosis for microinvasive breast cancers. Nevertheless, ovarian metastasis should be kept in mind provided there is involvement of regional lymph nodes and the tumor is histologically lobular type^[8,9,16]. Despite the fact that it was not tested at our case, if the immunohistochemistry of the breast tumor is negative for hormone receptors and shows findings like increased expression of Ki-67 and HER2, it should be alarming for increased metastatic potential^[4,10,16].

Microinvasive breast cancers constitute a rare subgroup of invasive breast cancers. Since their histological features are better acknowledged and imaging technologies are more enhanced, more patients are diagnosed with the disease in recent years. While it presents a fair survival expectation, systemic spread and metastases may occur in very early phases of microinvasive breast cancers, when it is accompanied with regional lymph node involvement and poor prognostic immunohistochemical factors. Hence, testing for immunohistochemical and molecular markers may provide more information regarding the biology and behavior of this tumor^[10]. Metastases from non-genital organs to the ovary are associated with poor survival. Due to the expediting effect on the process of diagnosis and treatment, laparotomy and laparoscopy should be performed promptly as definitive methods. Multidisciplinary approach is the most important principle for the diagnosis and treatment of a malignancy that presents itself with metastases, also for the investigation of the primary focus.

DECLARATIONS

Authors' contributions

Wrote the manuscript: Kurt S, Timur HT

Provided data regarding the patient's treatment in the department of Obstetrics and Gynecology: Kurt S, Timur HT, Saatli HB

Provided information about the patient's treatment in the department of General Surgery: Sevinc AI

Provided the pathological findings and other information from the department of Pathology: Ulukus EÇ

Availability of data and materials

Not applicable.

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None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

The consent form was signed by the patient herself. We foresee no ethical issues regarding the publication of this case report.

Consent for publication

The consent form was signed by the patient herself.

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Review

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Studies of postpartum mammary gland involution reveal novel pro-metastatic mechanisms

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Abstract

Postpartum involution is the process by which the lactating mammary gland returns to the pre-pregnant state after weaning. Expression of tumor-promotional collagen, upregulation of matrix metalloproteinases, infiltration of M2 macrophages, and remodeling of blood and lymphatic vasculature are all characteristics shared by the involuting mammary gland and breast tumor microenvironment. The tumor promotional nature of the involuting mammary gland is perhaps best evidenced by cases of postpartum breast cancer (PPBC), or those cases diagnosed within 10 years of most recent childbirth. Women with PPBC experience more aggressive disease and higher risk of metastasis than nulliparous patients and those diagnosed outside the postpartum window. Semaphorin 7a (SEMA7A), cyclooxygenase-2 (COX-2), and collagen are all expressed in the involuting mammary gland and, together, predict for decreased metastasis free survival in breast cancer. Studies investigating the role of these proteins in involution have been important for understanding their contributions to PPBC. Postpartum involution thus represents a valuable model for the identification of novel molecular drivers of PPBC and classical cancer hallmarks. In this review, we will highlight the similarities between involution and cancer in the mammary gland, and further define the contribution of SEMA7A/COX-2/collagen interplay to postpartum involution and breast tumor progression and metastasis.



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Keywords: Postpartum involution, postpartum breast cancer, Semaphorin 7a, cyclooxygenase-2, collagen, metastasis

BREAST CANCER METASTASIS AND POSTPARTUM BREAST CANCER

In the United States, breast cancer remains the second leading cause of cancer related death in women, with the majority of these deaths resulting from metastatic disease. Breast cancer is a heterogeneous disease with five identified molecular sub-types: luminal-A, luminal-B, HER2-enriched, basal-like, and normal-like^[1]. Treatments for hormone receptor positive and HER2 amplified cases include targeted therapies that, while initially successful, often result in the development of resistance, thereby increasing the likelihood of metastasis^[2]. Breast cancers lacking estrogen receptor, progesterone receptor, and HER2 amplification are defined as triple negative breast cancers (TNBC) and account for 15%-20% of breast cancer diagnoses. Patients with TNBC not only have lower survival rates due to increased early metastasis, but also lack a targeted therapeutic option^[3,4]. This evidence underscores the necessity of novel models for the identification of molecular drivers of breast cancer progression, therapy resistance, and metastasis.

Multiple epidemiological studies highlight the effect of pregnancy on breast cancer risk. While first pregnancy at an early age confers a lifelong protective effect against breast cancer, all women who have given birth undergo a transient period of increased risk^[5-9]. Previously, women diagnosed within five years of most recent childbirth were found to be at higher risk for developing metastatic breast cancer than nulliparous women^[5-12], a risk that persists even after adjustments have been made for variability in hormonal receptor status, HER2 status, age, histological grade, tumor size, node status, and year of diagnosis^[10,13]. More recent results have revealed that the probability of metastasis is increased for women diagnosed with breast cancer between 0-5 and 5-10 years postpartum. Thus, we now define breast cancers diagnosed within 10 years of most recent childbirth as postpartum breast cancer (PPBC)^[10,13]. By this definition, PPBC accounts for over half of all breast cancers diagnosed in women under age 40 in two independent cohorts^[10,13]. Since patients with PPBC have significantly worse outcomes, regardless of numerous clinical parameters, it has been hypothesized that pro-tumorigenic changes in the breast tissue following pregnancy may persist for an extended period and accelerate PPBC progression. Consistent with this hypothesis, Asztalos *et al.*^[14] identified a breast cancer associated genetic signature in the normal breast tissue of parous women that persists for up to 10 years after childbirth. Further evidence of a postpartum tumorigenic signature is supported by multiple pre-clinical models, where implantation of tumor cells into rodent mammary glands after weaning facilitates tumor cell growth, invasion, and metastasis. Additional studies of the mammary gland after lactation have revealed that these phenotypes are driven, in part, by mammary and tumor specific increases in pro-inflammatory cyclooxygenase-2 (COX-2), fibrillar collagen, semaphorin 7a (SEMA7A), bone marrow derived stromal and macrophage populations, lymphangiogenesis, and circulating estrogens^[15-22]. In this review, we will explore some of the mechanisms by which post-lactational changes in the mammary gland facilitate breast cancer progression and metastasis, with a focus on the roles of collagen, COX-2, and SEMA7A in cell death, extracellular matrix (ECM) and vascular remodeling, and macrophage infiltration.

PRO-TUMORIGENIC ROLES OF INVOLUTION ASSOCIATED PROGRAMS

For a detailed review of the events that occur during embryonic and adult mammary gland development, see Macias and Hinck^[23]. Briefly, mammary gland development begins during embryogenesis where ectodermal placodes invade into the mammary mesenchyme to form a rudimentary ductal tree^[24]. This primitive structure persists until puberty where upregulation of growth hormone and estrogen coordinate ductal morphogenesis to form the extensive epithelial ductal network that fills the mammary fat pad. Mammary epithelial structures are bi-layered, meaning they consist of both luminal cells and basally restricted myoepithelial cells that contact the basement membrane^[25]. Full differentiation of the mammary

gland is not achieved until pregnancy, where progesterone and prolactin coordinate differentiation of the alveolar structures that are responsible for milk storage and secretion; in addition, continued branching morphogenesis occurs to prepare the gland for lactation^[23,26]. During lactation, oxytocin stimulates the contraction of myoepithelial cells in response to suckling, resulting in milk delivery via the nipple^[27]. When lactation ends, the gland must cease milk production and return to the pre-pregnant architecture through postpartum involution.

Postpartum involution involves the upregulation of tumor-promotional factors in the mammary epithelium and surrounding stroma. As primary components of the mammary ECM, collagen proteins, and the fibroblasts that produce and remodel them, have been extensively studied for their role in breast tumor progression and metastasis^[28,29]. The 28 types of collagen proteins share a triple α -helix as part of their structure, and they can be broadly classified as fibrillar or non-fibrillar based on their assembly^[30]. For additional information on collagen and its assembly, see Mouw *et al.*^[30]. In this review, we will focus on fibrillar collagen, as this is the most abundant form in the mammary gland^[31]. Collagen I expression is both spatially and temporally regulated, and its tight control is essential for the proper mechanosignaling required for normal mammary gland development and function^[31]. In fact, dysregulation of the primary collagen I receptor, $\alpha_2\beta_1$ integrin, can alter mammary ductal branching and promote tumor formation^[32,33]. The contribution of collagen to tumor progression is emphasized by the prognostic value of collagen I mRNA in clinical outcomes of breast cancer^[34] and evidence that increased collagen density promotes local breast cancer invasion and distant metastasis^[35]. One way that collagen deposition is regulated during involution is by the pro-inflammatory enzyme COX-2.

COX enzymes were first identified as the targets of inhibition by aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs)^[36]. COX enzymes exist in two isoforms, both of which bind to cell membranes where they catalyze the metabolism of free arachidonic acid (AA) to prostaglandins (PGs) via phospholipase A2^[37,38]. First, COX enzymes rapidly catalyze the formation of an unstable intermediate, prostaglandin G2 (PGG2), from AA. PGG2 is then rapidly converted to prostaglandin H2 (PGH2) via COX-mediated peroxidase activity. Finally, specialized prostaglandin synthases result in the conversion of PGH2 to specific, biologically active PGs^[37-39]. Though initially believed to function identically, later investigations revealed important differences between COX-1 and COX-2^[40]. COX-1 is constitutively expressed as a regulator of tissue homeostasis^[41], whereas COX-2 is not normally expressed in adult tissues, with the exception of the central nervous system^[42], kidneys^[43], and male reproductive organs^[40,44]. Unlike COX-1, COX-2 expression is regulated by mitogens, hormones, and cytokines, and it is also correlated with cancer progression^[45,46]. However, it is the product of COX-2 activity - PGE2 - that serves as the active effector of pro-tumorigenic signaling. Once synthesized, PGE2 can bind to specific E-prostanoid receptors on the cell surface to activate pathways associated with survival and inflammation^[47]. COX-2/PGE2 signaling has been described in multiple models of cancer, including oral, breast, prostate, and colorectal, with documented roles in tumor initiation, invasion, immune evasion, cell survival, metastasis, vascular remodeling, cancer stem cells, and drug resistance; for further review, see Hashemi Goradel *et al.*^[46] and Stasinopoulos *et al.*^[48]. In addition to the feedback between COX-2 and collagen deposition in involution and in breast cancer^[18], we have also published a connection between COX-2 and tumor cell invasion through expression of the neuronal guidance protein, SEMA7A^[49].

SEMA7A, or CD108w, was first recognized for its expression on lymphocytes^[50]; however, a role for SEMA7A in cancer was later identified through its association with Plexin-C1, the receptor for the viral homolog of SEMA7A. Engagement of Plexin C1 by viral semaphorin inhibits dendritic cell adhesion and motility via alterations to the actin cytoskeleton^[51]. Similarly, in melanoma, SEMA7A/Plexin-C1 engagement inhibits cell migration via inactivation of the cofilin pathway^[52]. Cofilin activation, which normally generates free actin filaments required for cell migration^[53] is considered a major component of the metastatic cascade^[54]. SEMA7A/Plexin-C1 mediated cofilin inactivation led to the identification of Plexin-C1 as a novel tumor

suppressor^[53]. Consistent with this finding, Plexin-C1 expression is frequently lost in melanoma^[53]. Conversely, during development, SEMA7A signals through its other known receptor, β 1-integrin, to promote axon outgrowth via activation of the mitogen activated protein kinase (MAP-K) pathway^[55]. Unlike Plexin-C1, SEMA7A-mediated activation of β 1-integrin has been shown to promote cancer progression. Our lab and others have shown that SEMA7A- β 1-integrin binding promotes cell migration, invasion, metastasis, and neo-vasculogenesis of the blood and lymphatic vessels^[49,56-58]. SEMA7A has also been implicated in multiple models of fibrosis (reviewed below), supporting additional roles in inflammation and fibrillar collagen deposition. The expression of SEMA7A, COX-2, and collagen during postpartum involution and their known roles in facilitating tumor progression suggest these molecules and their interplay may be important drivers of PPBC.

PRO-TUMORIGENIC MECHANISMS OF CELL DEATH DURING INVOLUTION

Programmed cell death is essential for preventing the aberrant cellular phenotypes that arise when cells acquire DNA damage and mutations. Resistance to cell death may, consequently, result in the propagation of potentially harmful mutations that allow cells to bypass checkpoints meant to prevent unregulated growth and division. Resistance to cell death is thus defined by Hanahan and Weinberg^[59] as one of the original hallmarks of cancer. For a detailed review of cell death and cancer, see Ichim and Tait^[60]. In tumor cells, resistance to cell death plays an important role beyond tumor initiation, affecting drug-resistance, recurrence, and metastasis. Despite continuous advances in targeted chemotherapy, treatment efficacy tends to decline over time as a result of the ability of tumor cells to suppress apoptotic pathways, upregulate DNA repair, and genetically adapt to escape death. Tumor cells with repopulating capacity may also acquire the ability to resist death in response to chemotherapy, thereby constituting a major mechanism of cancer recurrence. Mechanisms by which tumor cells resist chemotherapy are further summarized by Al-Dimassi *et al.*^[61]. Active survival signals from the mammary ECM are required to override the default programming of normal adherent cells to die. When these cells become detached, they die in the absence of ECM signaling by a specialized form of programmed cell death called anoikis^[62]. Anoikis-resistance is a critical requirement for the successful metastasis of circulating tumor cells, a feature that may be shared by some of the cells that survive involution^[63]. Postpartum involution is characterized by two major waves of apoptosis, culminating in the death of over half of the mammary epithelium^[64]. As a minority of mammary epithelial cells (MECs) survive this process, postpartum involution represents an excellent model for studying the mechanisms by which cells acquire resistance to cell death in apoptotic environments. In this section, we will review the major mechanisms regulating cell death during postpartum involution and potential contributors to cell death resistance during tumorigenesis.

Postpartum involution occurs in two primary phases. In the first, or reversible phase, accumulation of milk protein, a process known as milk stasis, results in the detachment and shedding of secretory alveolar epithelial cells to the lumen. Milk stasis induces the first wave of cell death, in part, through the local expression of leukemia inhibitory factor (LIF). LIF is a primary activator of the master apoptotic regulator of involution - signal transducer and activator of transcription 3 (STAT3)^[65]. Other important activators of STAT3 at the onset of postpartum involution include transforming growth factor- β (TGF β), Janus kinase-1 (JAK-1), and Snail2 (SLUG)^[66-68]. Activation of STAT3 promotes postpartum mammary gland involution by shifting the balance of pro- and anti-apoptotic signals in favor of programmed cell death. STAT3 coordinates the first wave of epithelial apoptosis through activation of pro-apoptotic Bcl-2 family members, upregulation of PI3K inhibitory subunits, and downregulation of MAP-K survival signaling^[66]. STAT3 further promotes cell death during involution by mediating the formation of triglyceride-containing vacuoles. Triglycerides within these lysosomal-like vacuoles become metabolized into free fatty acids which interact with and distort lysosomal membranes. This results in the leakage of cathepsin proteases into the cytosol, and ultimately, lysosomal-mediated programmed cell death^[69]. The role of STAT3 in regulating apoptosis during involution has recently been comprehensively reviewed by Hughes and Watson^[66]. STAT3 has a well-defined role in

cancer progression, with over 40% of breast cancers presenting with constitutive STAT3 activation. Because STAT3 activates multiple signaling pathways, aberrant activation can promote multiple changes associated with cancer, including altered cell cycle dynamics, EMT, angiogenesis, and interestingly, resistance to cell death^[70,71]. Therapies targeting STAT3 may, thus, be of important therapeutic value for PPBC patients. For more about the role of STAT3 in breast cancer, see Segatto *et al.*^[70].

Removal of the overwhelming number of dead cells from the gland requires the Rac-1 mediated switch of MECs from a secretory to a phagocytic phenotype - a process which is essential for proper remodeling in the second phase of involution^[72]. Despite the massive wave of cell death that occurs during the first phase, if suckling resumes within this window (48-72 h, in mice), involution can be reversed, and lactation can proceed. This reversibility is due, in large part, to the expression of tissue inhibitors of metalloproteinases (TIMPs)^[73]. TIMPs preserve the reversibility of gland regression by delaying irreversible tissue remodeling events through the inhibition of matrix metalloproteinases (MMPs)^[74]. Interestingly, TIMP expression may also serve as an additional mechanism to regulate epithelial apoptosis, as TIMP3 has been implicated in the regulation of cell death in a tumor necrosis factor (TNF)-dependent manner^[75]. The number of compensatory mechanisms that exist to activate apoptosis underscore the importance of postpartum involution to future rounds of successful lactation. At approximately day three of involution in mice, downregulation of TIMPs results in activation of the MMPs that degrade the mammary ECM, causing MECs to lose contact with their underlying basement membrane^[73]. In the absence of pro-survival signaling from the ECM, detached MECs die by anoikis, thus comprising the second wave of cell death^[62,73]. Similar to the first phase, clearance of dead cells during the second phase of involution is largely mediated by phagocytic MECs, with additional limited support from professional phagocytes. Milk fat globule epidermal growth factor 8, which works by recognizing phosphatidylserine on the outer leaflet of the plasma membrane of dying cells, is also critical for apoptotic cell clearance during the second phase^[76].

What allows some cells to die and others to live during involution remains largely unanswered. To date, more than 50 mammary specific knockouts have been generated that exhibit alterations in postpartum involution - either delayed or premature - that are consistent with the proposed role for each molecule in the process. For example, activation of pro-survival pathways, such as Akt1, and/or deletion of death inducing genes, such as Bax, results in delayed involution, while deletion of Akt1 and anti-apoptotic Bcl-x results in premature involution^[77-80]. See Radisky *et al.*^[80] for an extensive review of these models. As pro-survival signals progressively decline and pro-apoptotic signals increase during the first phase of involution, the mammary basement membrane and ECM become the primary mediators of cell survival^[81]. Yet, during the second phase, the basement membrane and ECM are degraded by proteases. Previously, SEMA7A mRNA expression was shown to increase in whole mammary extracts during the early phase of involution, and its expression was attributed to its immunomodulatory role^[82]. Recently, we published that SEMA7A is expressed on Epcam+ MECs during the remodeling phase of involution^[57]; however, the downstream mechanisms activated by epithelial SEMA7A are not well understood. Interestingly, SEMA7A is a ligand for β 1-integrin, which is the receptor for ECM molecules that normally facilitate epithelial cell attachment. It is therefore possible that SEMA7A may activate β 1-integrin signaling and provide a pro-survival mechanism to overcome anoikis. Furthermore, β 1-integrin mediates the activation of known survival pathways including MAP-K and AKT, which affects survival via stabilization of NF- κ B and expression of COX-2. Thus, SEMA7A may promote anoikis-resistance during involution via activation of β 1-integrin in a manner that is independent of ECM; however, additional investigation is required.

The role of COX-2 during involution has also largely been attributed to its role in the modulation of the immune milieu^[83]. Yet, COX-2 also contributes to cell survival mechanisms and collagen remodeling via PGE₂, which promotes cell growth and proliferation, modulates collagen expression levels, and upregulates proteinase expression. Studies investigating the effects of postpartum involution in TNBC progression

have revealed a feed-forward mechanism by which fibrillar collagen deposition during involution requires COX-2 expression. COX-2 further promotes increased fibrillar collagen^[18], which is directly associated with tumor progression and poor prognosis in breast cancer patients^[31,84]. Cooperation between fibrillar collagen and COX-2 may therefore contribute to the pro-tumorigenic nature of the involuting mammary gland. Similar to postpartum involution, wound healing is a biological process where cell death is accompanied by collagen remodeling. In the “phoenix rising” model of cell death, discovered in a model of wound healing and further characterized in cancer, dying cells send signals to stem and progenitor cells to increase their proliferation, thereby coordinating cell growth with death^[85,86]. In dying cells, activation of caspases 3 and 7 results in the release of calcium-independent phospholipase-2, which increases the production and release of AA from cell membranes. Ultimately, COX-2 and PGE2 synthase convert free AA to PGE2, which increases stem and progenitor cell proliferation to promote tissue regeneration^[85]. Though the phoenix rising model, to our knowledge, remains uninvestigated during mammary involution, there are numerous similarities between the wound healing program and postpartum involution, including apoptosis, clearance of damaged and dead cells, and activation of similar inflammatory programs. This suggests that this mechanism of coordinated cell death and tissue regeneration may be conserved in mammary development. Additionally, these mechanisms seem to be particularly important for the growth and survival of stem and progenitor cells in response to apoptotic stimuli, thus representing a mechanism of tumor recurrence in response to chemotherapy. The preceding data may help to further explain the role of collagen, COX-2, and SEMA7A in facilitating tumor progression following lactation.

EXTRACELLULAR MATRIX AND REMODELING DURING POSTPARTUM INVOLUTION AND CANCER

In their updated review of the hallmarks of cancer, Hanahan and Weinberg described the tumor microenvironment (TME) as a critical mediator of cancer progression due to its ability to supply cancer cells with signals that promote inflammation, induce angiogenesis, and confer resistance to cell death^[87]. The TME consists of all the cells, ECM molecules, vasculature, and proteins that surround a tumor. The cross-talk between these components and the tumor can affect tumor growth, survival, metabolism, metastasis, response to treatment, and recurrence. The contributions of the TME to cancer progression are comprehensively reviewed in a recent special issue of *Nature Reviews Cancer*^[88]. In this section, we will briefly discuss some of the similarities between the tissue microenvironment of the involuting mammary gland and the TME.

ECM fragments, which can be used as diagnostic markers of disease, are known contributors to cancer for their ability to participate in cell signaling events and modulate gene expression^[31,89]. An investigation of the tumor-promotional aspects of involution revealed that tumor cells co-cultured with ECM isolated from involuting rat mammary gland promoted tumor cell invasion, whereas tumor cells co-cultured with ECM isolated from nulliparous rat mammary glands did not^[90], suggesting that involution-derived ECM promotes tumor cell invasion and metastasis. In further support, orthotopic injection of breast cancer cells mixed with ECM isolated from the mammary glands of rats undergoing postpartum involution also confirmed that involution derived ECM promotes metastasis^[90]. Additional investigations have confirmed that the involution microenvironment mirrors the typical breast TME based on their shared availability of ECM fragments, dramatic increases in fibrillar collagen, and increased MMP activity^[90-92]. Specifically, studies of stromelysin-1 (MMP3), the primary active MMP during the tissue remodeling phase of involution, have characterized it as a potent mediator of EMT and other early oncogenic events in the mammary gland^[93]. Furthermore, elevation of fibrillar collagen results in increased collagen crosslinking and ECM stiffening - characteristics known to accompany breast tumor progression^[89,94-96]. Increased collagen deposition and cross-linking further promote tumor cell invasion and metastasis by providing a structural network for tumor cell migration^[97].

Transforming growth factor β (TGF β), well-known for its paradoxical role in breast tumor progression, is a primary regulator of collagen deposition in the mammary gland^[98]. At approximately 8 h post weaning, TGF β becomes activated and exerts tumor suppressive effects via activation of apoptotic programming^[73,99]. Conversely, when normal cells begin to take on tumorigenic phenotypes, TGF β promotes cancer progression by enhancing tumor cell survival and contributing to the maintenance of cancer stem cell populations^[100]. TGF β is also essential for wound healing, where it stimulates ECM deposition via fibroblast activation^[101]. Interestingly, TGF β dependent fibroblast activation during involution may be COX-2 dependent, as NSAID treatment decreases fibroblast activation *in vivo*. Furthermore, NSAIDs inhibit fibroblast mediated fibrillar collagen deposition during involution and in a model of PPBC^[15]. Additionally, TGF β is known to positively regulate SEMA7A in pulmonary fibrosis, where SEMA7A is critical for ECM deposition via activation of the PI3K/AKT pathway^[102]. Given its role in ECM deposition, SEMA7A expression during the second phase of involution suggests it may also play a role in mammary gland tissue remodeling^[57]. SEMA7A can affect ECM remodeling through its ability to recruit fibroblasts and immune cells to fibrotic sites, and it has been further implicated in fibrosis models in the liver, kidney, and in glial scar formation^[103-108]. Contradictory to this role, when endogenously expressed on fibroblasts, SEMA7A can maintain fibroblast homeostasis and reduce pro-fibrotic markers^[109], indicating context dependent roles for SEMA7A-mediated signaling. In cancer, fibrillar collagen coordinates upregulation of COX-2 on tumor cells, further promoting tumor cell invasion and metastasis^[7], and we have published that COX-2 drives SEMA7A expression^[49]. Thus, fibrillar collagen, COX-2, and SEMA7A may be a part of a feed-forward loop that ultimately results in cancer cell invasion and metastasis. Additional studies, however, are needed to better understand the hierarchy and cross-talk between these molecules in the context of postpartum involution and breast cancer progression.

Altering fibrillar collagen deposition provides a route for tumor cell migration and invasion, but also changes the signals received by cells from the surrounding environment. Signals from the ECM are communicated to cells by integrins. Integrins are heterodimeric ($\alpha\beta$) transmembrane receptors with 18 known α and 8 β integrin subunits, resulting in 24 possible heterodimeric integrin receptors^[110]. Proper expression and signaling of integrins is essential for cell survival and adhesion, and integrin dysregulation can promote cancer via activation of pathways that affect survival, EMT, and migration^[28,111-115]. Specifically, improper integrin signaling and expression can result in the loss of normal epithelial cell polarity and attachment, in addition to the over-activation of focal adhesion kinase and subsequent downstream signaling pathways that promote cell survival, migration, invasion, and ultimately metastasis^[116]. While some integrin pairs are highly specific in their substrate recognition, others can recognize a number of substrates from the ECM, as well as foreign molecules such as snake venom, viral particles, and pathogens^[117,118]. Alternative ligand-binding partners and/or differential integrin expression can elicit different signaling pathways; thus, when either ligand or integrin profiles are altered, cellular signaling pathways can become aberrantly activated or inhibited. Abnormal SEMA7A expression during involution and/or cancer may promote tumorigenesis via activation of β 1-integrin and downstream pathways. SEMA7A binds to β 1-integrin via the RGD binding site located on the SEMA domain; however, this site is buried in the crystal structure when SEMA7A is bound to Plexin C1. The ability to differentially regulate tumor progression could, therefore, be explained by binding of SEMA7A to its different receptors^[119,120]. Further, while SEMA7A binds to α 1 β 1-integrin on inflammatory macrophages^[121], the α -binding partner needed for SEMA7A-mediated breast cancer progression is unknown. As reviewed above, one consequence of SEMA7A- β 1-integrin signaling is fibrillar collagen deposition. Collagen can also activate integrins and modulate their associated signaling pathways, primarily through α 2 β 1 integrin, which is often upregulated on cancer cells of epithelial origin^[122]. Collagen binding to α 2 β 1 integrin on tumor cells promotes cellular invasion, which helps cells navigate through the collagen I rich mammary TME and distant metastatic sites, such as the bone^[122]. Interestingly, α 2 β 1 integrin has been shown to increase COX-2 expression in intestinal epithelial cells^[123], leading to activation of downstream signaling events associated with tumor promotion. While these results further support a link between COX-2 and collagen, α 2 β 1 integrin has also been recognized as a metastasis suppressor in breast cancer. The

complex interplay between integrins, collagen, COX-2, and SEMA7A is, thus, likely to be context-dependent, and additional studies are necessary to understand the role of this signaling axis in mediating tumor cell invasion and metastasis in breast cancer.

MACROPHAGES IN POSTPARTUM INVOLUTION AND PPBC

Macrophages are the phagocytic immune cells that mediate the removal of foreign pathogens, dead cells, and debris. Classically-activated macrophages, also known as M1 macrophages, are activated in response to pathogen-associated cytokines, most often IFN- γ and lipopolysaccharide. M1 macrophages are largely considered to be “anti-tumor” based on their expression of the pro-inflammatory cytokines, interleukin-1 (IL-1), IL-12, TNF- α , and inducible nitric oxide synthase - all of which have been shown to oppose tumor progression^[124,125]. In contrast, Th2 family cytokines induce the maturation of alternatively-activated, or M2 macrophages, and cause their release of anti-inflammatory mediators that support tumor cell survival^[21,126]. M2 macrophages promote tumor cell growth, invasion, and metastasis, via their secretion of IL-10, TGF β , and MMPs. Though the M1/M2 system is useful for broadly classifying macrophages, this taxonomy fails to capture the plasticity and diversity characteristic of this cell type. For the purpose of this review, however, we will use M1 and M2 to broadly classify anti- and pro-tumor macrophages, respectively. Multiple studies support the role of macrophages as critical mediators of metastasis^[21,127-129]. In models of gastric and breast cancer, M2 macrophages are recruited by tumor cells, where they activate MAP-K signaling to promote the motility of disseminating tumor cells^[127]. Further, macrophages appear to be critical for the migration of the majority of ductal carcinoma *in situ* cells, as only 10% are motile when macrophages are absent^[130]. The critical contribution of macrophages to tumor cell metastasis is further evidenced by studies in the MMTV-PyMT mouse model of breast cancer, where knockout of colony stimulating factor-1 (CSF-1), a secreted glycoprotein that induces the differentiation of hematopoietic stem cells to macrophages, correlates with a near complete elimination of tumor cell metastasis^[21,131]. Macrophages, therefore, represent a diverse population of cells that can promote or inhibit tumor progression based on the context of their environment.

Macrophages are the primary immune cells present during mammary gland postpartum involution, and because of their role in facilitating tumor metastasis, represent a potential contribution to the highly metastatic nature of PPBC. Though known primarily for their phagocytic capacity, macrophages only play a minimal role in the clearance of apoptotic cells during involution^[21,132,133]. Despite their limited role in phagocytosis, M2 macrophages are essential for the epithelial apoptosis and tissue remodeling characteristic of postpartum involution^[134]. At the peak of apoptotic cell clearance, macrophages exist at relatively low levels, as MECs represent the primary phagocytes. At day 6 of involution, however, the peak of mammary tissue remodeling, M2 macrophages exist at 6 times the level of those in the nulliparous mammary gland, while classically-activated M1 macrophages remain at consistent levels throughout pregnancy, lactation, and gland regression^[21]. F4/80, a general marker of mature mouse macrophages, marks more than the sum of M1 and M2 macrophages during involution, suggesting there are additional macrophage populations present in the involuting mammary gland^[20]. Our lab has recently identified a population of macrophages that also express the lymphatic endothelial marker, podoplanin (PDPN)^[57]. In culture, SEMA7A drives the expression of PDPN on macrophages and promotes their migration and adherence to lymphatic vessels^[57]. Because macrophages have proven to be a critical part of the metastatic cascade by facilitating intravasation into tumor associated blood vessels^[135], SEMA7A-mediated macrophage lymphatic mimicry may also facilitate intravasation into lymphatic vessels, providing another explanation for the high rates of metastasis associated with PPBC. This is further supported by the prognostic value of a combined genetic signature of CD68, PDPN, and SEMA7A in predicting decreased distant metastasis free survival in a cohort of 600 human breast cancer cases^[57]. SEMA7A further regulates macrophages by serving as a strong activation factor for monocytes, promoting both chemotaxis and secretion of inflammatory cytokines, in addition to upregulation of granulocyte-macrophage CSF (GM-CSF), supporting an additional role for SEMA7A in macrophage differentiation^[136].

Another important macrophage regulator during postpartum involution and breast cancer is COX-2. Previous studies in breast cancer models have shown that COX-2 expression increases with cancer stage, and its expression levels can indicate breast cancer progression, recurrence, and metastasis^[137]. Recently, COX-2 expressing tumor associated macrophages (TAMs) have been shown to promote the metastatic potential of breast cancer cells via secretion of IL-6 and subsequent activation of AKT signaling in cancer cells^[138]. Further, expression of COX-2 in stromal TAMs results in upregulation of COX-2 in breast cancer cells, thereby shifting polarization of local macrophages toward the M2 phenotype. In addition to its association with tumor promotional CD163+ TAMs, COX-2 expression in the stroma is further associated with increased collagen alignment in invasive breast cancer^[139]. TAMs are known to associate with dense regions of collagen in breast cancer in the same way M2 macrophages associate with fibrillar collagen during involution. In the MMTV-PyMT model, macrophages associated with fibrillar collagen have been shown by intravital imaging to migrate across collagen fibers, suggesting that one mechanism by which macrophages promote metastasis is by supporting the migration of tumor cells across collagen networks^[21,140]. SEMA7A, COX-2, and collagen all represent important effectors of macrophage-mediated tumor cell growth, survival, and metastasis. As macrophages are considered essential for successful metastasis, targeting the molecules responsible for alternative macrophage activation, survival, and chemotaxis may be critical for the successful treatment of metastatic disease.

ENDOTHELIAL VESSEL FORMATION

Blood and lymphatic vessels form two similar, yet distinct, organ systems that assemble into extensive networks throughout the body to support development and survival. Blood vessels provide tissues with oxygen and nutrients, while the primary functions of lymphatic vessels are immune cell trafficking and removal of excess interstitial fluid from tissues. Blood and lymphatic vessels are lined with blood endothelial cells and lymphatic endothelial cells, respectively, and are both surrounded by a thin layer of smooth muscle. Some lymphatic vessels have unique “button-like” junctions that differ from the more continuous “zipper-like” junctions of the blood vasculature and established lymphatic vessels. These specialized junctions are covered with a flap that opens and closes to allow fluid and cells to pass without affecting vascular integrity. Vascular networks are highly dynamic, expanding and retracting as tissues change in response to normal developmental processes or pathologies. Indeed, a widely accepted hallmark of cancer is the ability of tumors to induce angiogenesis, or the development of new blood vessels from existing vasculature^[59]. Tumors must acquire pro-angiogenic abilities in order to grow beyond 1-2 mm³^[141]; otherwise, the tumor will die by necrosis or apoptosis^[142,143]. In rat mammary tissues, we observe an overall net increase in lymphatic vessel density (LVD) during involution when compared to a lactation timepoint^[16]. In contrast, blood vessel density (BVD) drops dramatically after lactation, suggesting an initial period of regression before increasing in a manner similar to LVD^[144]. During involution, the highest vasculature densities peak at day 10, followed by a slight decrease in the fully regressed gland [Figure 1]. This is consistent with published studies from our group and others describing increased pro-angiogenic and pro-lymphatic signaling during postpartum involution, and in postpartum tumors compared to non-postpartum controls^[14,16,22,145]. Interestingly, fibrillar collagen, COX-2, and SEMA7A all have established roles in endothelial vessel formation. Studies using artificial collagen matrices have shown that collagen increases angiogenic responses from endothelial cells by providing the support needed for sustained endothelial cell growth and the formation of endothelial networks^[146]. Angiogenesis can also be regulated by mechanical stiffness within the small microvessel environment^[147], and breast tumors are often stiffer than neighboring normal tissues by up to 6-fold^[84,148]. For a more comprehensive review on collagen and angiogenesis, see Fang *et al.*^[149]. These studies demonstrate how the ECM in the TME can modulate vessel formation and alter the tumor’s blood supply.

If a tumor outgrows its blood supply and loses its access to oxygen, it can become hypoxic. Under normal oxygen content conditions, termed “normoxia,” the transcriptional regulator hypoxia-inducible factor-1

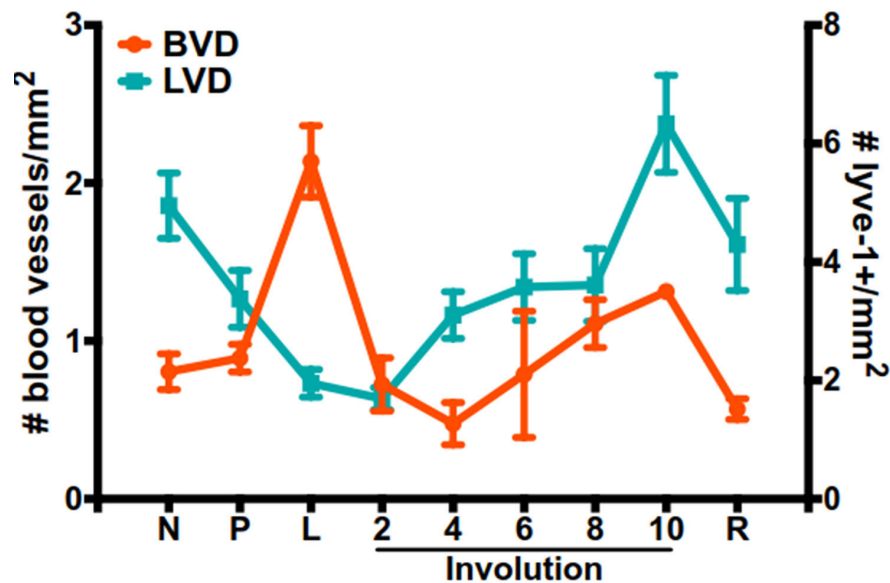


Figure 1. BVD and LVD during mammary gland development. BVD measured as number of blood vessels per millimeter and LVD measured as number of lyve-1 positive vessels per millimeter in nulliparous (N), pregnant (P), lactating (L), involuting (days 2-10) and regressed (R), mammary glands (data adapted from Lyons *et al.*^[16] and Ramirez *et al.*^[144]). BVD: Blood vessel density; LVD: lymphatic vessel density

(HIF-1) is unable to affect its targets; for further review of this topic, see Masson and Ratcliffe^[150]. HIF-1 is comprised of two subunits - ARNT/HIF-1 β , which is constitutively expressed, and HIF-1 α , which is an oxygen-sensitive subunit. During normoxia, HIF-1 α is hydroxylated by prolyl hydroxylase domain containing proteins, ubiquitinated by von-Hippel-Lindau protein, and rapidly degraded. During hypoxia, however, HIF-1 α is not hydroxylated and degraded, but instead, translocates to the nucleus, heterodimerizes with ARNT, and induces transcription of its target genes. HIF-1 affects a variety of targets, including pro-angiogenic genes like vascular endothelial growth factor-A (VEGF-A)^[151]. In breast cancer cells, COX-2 can induce inflammation-associated HIF-1 activity, resulting in the expression of pro-angiogenic genes^[48]. Further, HIF-1 and COX-2 maintain a positive feedback loop, as HIF-1 can also induce expression of COX-2^[152]. Utilizing NSAIDs to target COX-2 activity can inhibit angiogenesis, demonstrating the potential for therapeutic intervention^[152-154]. Corroborating our findings that SEMA7A may be involved in COX-2 associated pathways, HIF-1 can directly upregulate SEMA7A in endothelial cells^[155]. SEMA7A can elicit the release of pro-inflammatory cytokines and cause increased endothelial barrier permeability^[156,157]. SEMA7A can also promote angiogenesis in a hypoxia-independent manner in murine mammary carcinoma and in the cornea by stimulating macrophages to produce pro-angiogenic molecules, such as CXCL2/MIP-2^[158] and VEGF-A^[159].

In addition to their roles in angiogenesis of the blood vasculature, collagen, COX-2, and SEMA7A also have known roles in lymphangiogenesis. In breast cancer, increased LVD, lymph node involvement, and lymph vessel invasion are predictive of higher risk of metastasis; further, increased LVD and lymph node metastasis are commonly observed in PPBC^[16]. While the role of collagen in lymphangiogenesis has not been extensively characterized, one study has shown that collagen I increases lymphangiogenesis and angiogenesis in mouse embryoid bodies under hypoxic conditions^[160]. Therefore, it is plausible that other fibrillar collagens may contribute to lymphangiogenesis during mammary tumorigenesis. COX-2/PGE2 signaling also promotes production of pro-angiogenic VEGF-A and pro-lymphangiogenic VEGF-C and VEGF-D^[161]; further, lymphangiogenesis during wound healing is dependent on COX-2 activity^[162]. Moreover, COX-2 has been implicated in lymphangiogenesis in other cancer types, including cervical and gastric^[163,164]. We published that inhibition of COX-2 with celecoxib and NSAIDs results in decreased LVD, tumor cell

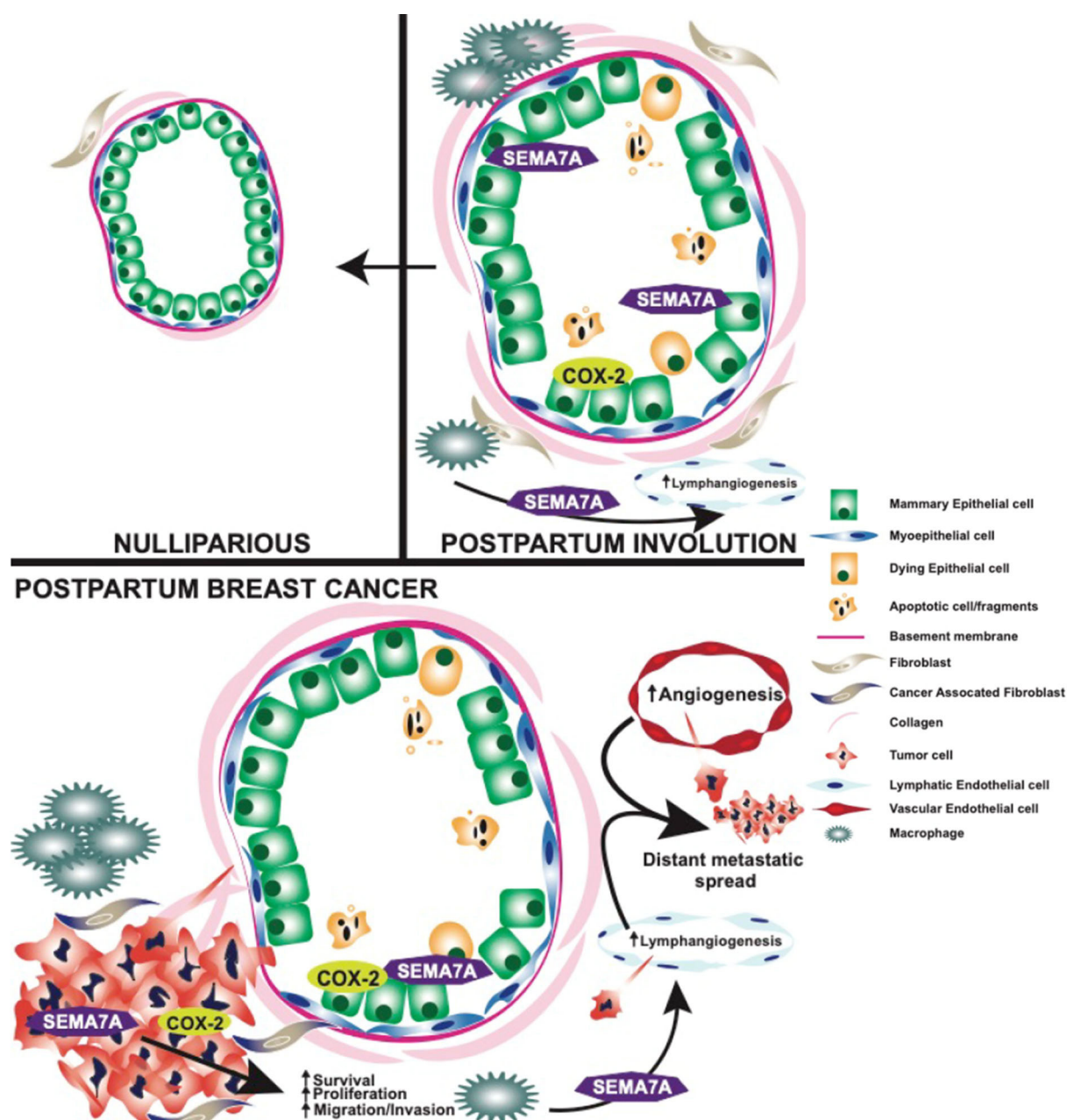


Figure 2. Graphical summary of pro-tumorigenic and metastatic roles of COX-2 and SEMA7A during postpartum involution and postpartum breast cancer. SEMA7A: Semaphorin 7a; COX-2: cyclooxygenase-2

invasion into lymphatics, and metastasis during PPBC^[16]. For more detailed information on COX-2 and lymphangiogenesis in breast cancer, see Lala *et al.*^[165]. Finally, SEMA7A has a functional role in lymphatic vessel modulation, as we recently published that SEMA7A promotes tumor-associated lymphangiogenesis via macrophage-mediated lymphatic vessel remodeling during postpartum involution and breast cancer^[57]. These findings suggest an additional mechanism by which SEMA7A, COX-2, and collagen promote tumor progression and metastasis.

CLINICAL RELEVANCE/CONCLUSION

Women with postpartum breast cancer face a disease that carries three times the rate of metastasis and death relative to women who have never been pregnant and those diagnosed outside of the postpartum

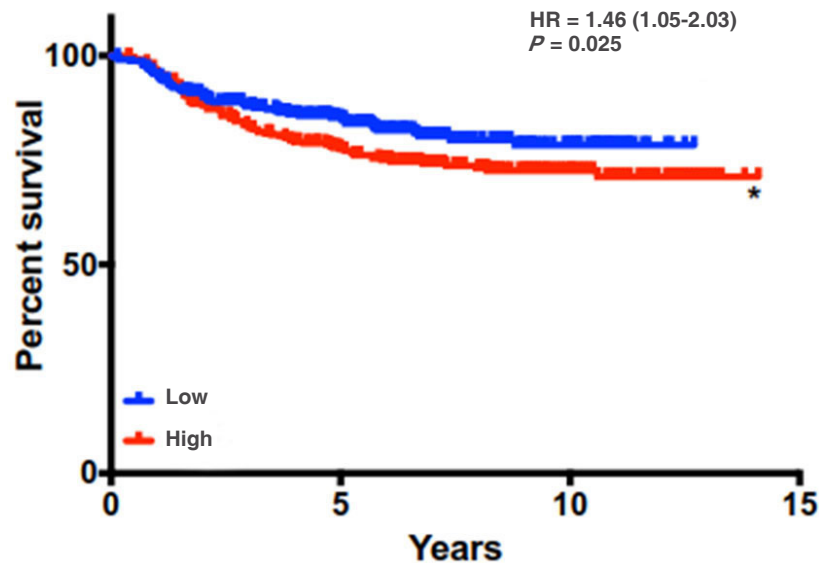


Figure 3. Distant metastasis free survival is decreased with high levels of expression of SEMA7A, COX-2, and COL1A1 signature. DMFS analysis using KmPlot ($n = 664$) * $P < 0.05$

window. One physiological event these women have in common is postpartum involution - a process that results in the upregulation and activation of tumor-promotional factors in MECs and the mammary stroma. Identification of the genetic engines that drive PPBC is critical to the development of targeted therapies for postpartum patients. In this review, we have highlighted potential roles for collagen, COX-2, and SEMA7A in driving some of the pro-metastatic aspects of involution [Figure 2]. Previously published results indicate overall survival is generally decreased for breast cancer patients with high collagen, COX-2, and SEMA7A expression, suggesting that these mechanisms are important mediators of breast cancer metastasis^[18,49]. Interestingly, while individual expression of each molecule does not predict for metastasis using KM Plotter analysis^[166], the combination of high SEMA7A, COX-2, and COL1A1 mRNA expression results in significantly decreased distant metastasis free survival for breast cancer patients in this dataset [Figure 3]. Thus, studies rooted in understanding the contributions of postpartum involution associated programs to breast cancer metastasis are likely to also be applicable to general breast cancer metastasis, and perhaps to other cancer types.

Based on the cooperation between SEMA7A, COX-2, and collagen, a multi-targeted therapy to affect the individual molecules and their interplay would likely be more effective than targeting one, alone. The potential of COX-2 as a therapeutic treatment has been investigated in multiple models of cancer. In fact, the COX-2 inhibitor, celecoxib, has been successful in the treatment of a specific type of colorectal cancer - familial adenomatous polyposis - in both adults and children^[167,168]. Targeting COX-2 in breast cancer, by celecoxib or other NSAIDs, may inhibit tumor cell dissemination by reducing the expression of tumor-promotional collagen. Targeting SEMA7A in conjunction with already established therapies, such as NSAIDs, may also increase the efficacy of these treatments in women with breast cancer. Ideally, the characterization of tumor-promotional factors in the postpartum mammary gland may also lead to preventative therapies aimed at reducing the risk for PPBC. NSAIDs may further represent a safe candidate for preventative therapy during involution via inhibition of COX-2 mediated collagen upregulation and alternative macrophage activation. The topics covered herein highlight both the potential contribution of the SEMA7A/COX-2/Collagen relationship to PPBC, and the importance of PPBC models to the discovery of new molecules and pathways that can be exploited as novel therapeutics.

DECLARATIONS

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Authors' contributions

Conception and design: Wallace TR, Tarullo SE, Lyons TR

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Manuscript writing/editing: Wallace TR, Tarullo SE, Crump LS, Lyons TR

Read and approved the final manuscript: Wallace TR, Tarullo SE, Crump LS, Lyons TR

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Radiotherapy of brain metastases from non-small cell lung cancer

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Abstract

Brain metastases risk at the time of diagnosis or during the course of disease is high in non-small cell lung cancer (NSCLC). Even the incidence of brain metastases has increased in recent years, due to detection of smaller asymptomatic lesions with MRI screening as well as improved survival as a consequence of developments in systemic therapies. In the last decade, there have been many trials in the management of NSCLC patients with brain metastases, questioning the role of adjuvant whole brain radiotherapy (WBRT) after surgery or stereotactic radiosurgery (SRS), WBRT, compared to best supportive care in patients not amenable to surgery, aggressive local therapies in solitary brain metastases, postsurgical cavity SRS, SRS in non-oligometastatic patients, cranial radiotherapy in patients with driver mutations, tyrosine kinase inhibitors, immune check point inhibitors and the impact of therapies on neurocognitive functions and quality of life. The main objective of this review is to provide an update on current trends in radiotherapy in the management of newly diagnosed brain metastases from NSCLC.

Keywords: Radiotherapy, whole-brain radiotherapy, stereotactic radiotherapy, stereotactic radiosurgery, brain metastases, lung cancer



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INTRODUCTION

Brain metastases are the most frequent malign brain tumors. Lung cancer is the leading primary tumor composing 40%-50% of cases^[1,2]. In non-small cell lung cancer (NSCLC) brain metastases risk at the time of diagnosis is 15%-20%, even the risk increases to 50%-60% during their course of disease in patients with epidermal growth factor receptor (EGFR) mutation or anaplastic lymphoma kinase (ALK) rearrangement^[3,4].

Incidence of brain metastases has increased in recent years as a result of detection of smaller asymptomatic lesions with MRI screening as well as improved survival as a consequence of developments in systemic therapies. In addition to these, improvements in imaging technology, surgery, radiotherapy, targeted therapies, even immunotherapy and deep understanding of treatment related toxicities have resulted in evolvement of current trends in management of brain metastases^[5,6].

Although brain metastases is an indicator of poor outcome^[7,8], it's shown that a subgroup might have longer survival, e.g., EGFR mutated and ALK rearranged NSCLC^[4,9]. In this context, to assist the selection of personalized therapy several prognostic indices have been developed; e.g., recursive partitioning analysis, diagnosis-specific graded prognostic assessment (DS-GPA) and more recently graded prognostic assessment for lung cancer using molecular markers (Lung-mol GPA)^[10-12]. Optimal treatment planning should consider both patient (age, performance status, expected life span) and tumor related factors (number and volume of brain metastases, extracranial disease control, molecular subtype).

Historically, whole brain radiotherapy (WBRT) or best supportive care were the only treatment modalities for patients with brain metastases. The aim of WBRT was to treat both microscopic and macroscopic metastases. But treatment to whole brain parenchyme reduces distant in-brain recurrences with the cost of cognitive toxicity^[6]. In the last decade, there have been many trials in management of NSCLC patients with brain metastases, questioning the role of adjuvant WBRT after surgery or stereotactic radiosurgery (SRS), WBRT compared to best supportive care in patients not amenable to surgery, aggressive local therapies in solitary brain metastasis, postsurgical cavity SRS, SRS in non-oligometastatic patients, cranial radiotherapy in patients with driver mutations, TKI, immune check point inhibitors and the impact of therapies on neurocognitive functions (NCF) and quality of life (QOL).

The main objective of this review is to provide an update on current trends in radiotherapy in the management of newly diagnosed brain metastases from NSCLC.

Solitary metastasis: WBRT vs. WBRT plus surgery

Until Patchell *et al.*^[13] randomized trial of surgical resection followed by WBRT vs. WBRT only in patients with solitary brain metastasis, WBRT was the standard treatment. The findings of increased median survival (10 months vs. 4-6 months), decreased brain recurrences (20% vs. 52%), increased functional independence and lower risk of neurological deaths with surgery in conjunction to WBRT, lead to increased role of aggressive local therapies in selected patients with solitary and oligo brain metastases^[14-17].

Solitary metastasis: adjuvant WBRT plus surgery vs. surgery only

The success of aggressive local treatment and fear of long-term neurologic toxicity led the authors to question the role of adjuvant WBRT in oligometastatic patients. Patchell *et al.*^[18] randomized the patients with solitary brain metastasis after surgery to WBRT or observation. Postoperative WBRT reduced both local and distant brain failure rates significantly compared to observation arm (10% vs. 46%, $P < 0.001$), (14% vs. 37%, $P < 0.01$), respectively. Neurologic death rate was 3 times higher in the observation arm. But overall survival (OS) and survival with functional independence were the same^[18].

Solitary metastases: surgery vs. SRS

In the absence of head to head randomized data, most of the retrospective studies comparing surgery vs. SRS reported similar outcomes in brain control or survival in solitary resectable brain metastases^[19,20]. It's hard to make a decision when there is no indication for surgical decompression or need for histologic diagnosis. One small randomized study with 64 patients with solitary metastases, compared SRS to microsurgery plus WBRT. OS and 1 year local control were similar^[21]. Recently, in secondary analysis of EORTC 22952 trial in abstract form which randomized patients to SRS or surgery with or without adjuvant WBRT, it was reported that early control was better with SRS but late control favored surgery^[22].

Solitary metastasis: only SRS after surgery

Postoperative SRS vs. WBRT after resection of brain metastases

The absence of survival advantage and recognized neurotoxicity of adjuvant WBRT after surgery have led to postoperative SRS to surgical cavity^[6]. But there has been some challenges in the use of SRS without WBRT after surgical resection. First, the use has increased in trend although the data have been mainly derived from retrospective series^[23,24] and one prospective phase II trial^[25]. The results of these studies report around 80% local control in 1 year suggest similar control rates with adjuvant WBRT. The only randomised data on this subject has been available from Kepka's multicenter trial, which did not demonstrate the non-inferiority of postsurgical SRS compared to postsurgical WBRT in patients with solitary brain metastasis. The difference in neurocognitive failure at 6 months (primary end-point) was in favour of WBRT. Besides, although it was not the primary end-point; the patients in postsurgical SRS arm compared to postsurgical WBRT were found to have increased neurological deaths (66% vs. 31%, $P = 0.015$, HR: 2.51) and decreased 2 years overall survival (10% vs. 37%, $P = 0.046$, HR: 1.8), respectively^[26]. The second challenge is that there have been some reports on dural-based leptomeningeal recurrences along the surgical tract after postoperative SRS^[27,28]. Atalar *et al.*^[27] reported 13% leptomeningeal recurrence rate at a median of 5 months after SRS directed to postsurgical resection cavity. It's hard to define target volume for postoperative SRS. Tumor cell dissemination along the surgical tract is another challenge. Recently, Soliman *et al.*^[29] reported a consensus guideline on delineation of CTV for postsurgical cavity SRS.

Preoperative SRS

In the context of leptomeningeal recurrences after postoperative SRS, preoperative SRS followed by surgical resection in 48 h is evolving as a new paradigm. The theoretical benefits are decreased radionecrosis and decreased leptomeningeal recurrences as a result of better definition of target volume by delineation of intact metastasis, a smaller margin volume and sterilization of surgical bed^[6,30]. The results of preoperative SRS in 47 oligometastatic (1-3 brain metastases) patients before surgical resection have been reported by Asher *et al.*^[5] NSCLC patients were 37.2% of the cohort. The median dose was 14 Gy. The expansion margin of gross target volume to planned target volume was not used. There wasn't any difficulty during surgery or increased postoperative complication. In spite of the inclusion of relatively large lesions (median dimension 3.04 cm and median volume 8.49 cm³), local control rates were 97.8%, 85.6% and 71.8% at 6, 12 and 24 months, respectively. Six of the 8 local relapsing patients were the ones who had either significant dural attachment or adherence to dural veins which might make an implication on the need for higher doses in such cases instead of dose reduction. There wasn't any leptomeningeal failure which confirms the hypothesis of the study. The authors concluded that preoperative SRS might be a better treatment at least for "high risk" patients^[5]. A phase II trial is going on this subject^[31].

Oligometastases: WBRT alone vs. WBRT plus SRS boost

In line with the intensification of local therapy Radiation Therapy Oncology Group (RTOG) conducted a phase 3 trial (RTOG 9508) which randomized 331 patients with 1-3 brain metastases to WBRT vs. WBRT plus SRS. Lung cancer patients were 64% of the patient cohort. SRS boost in conjunction to WBRT showed survival benefit in patients with 1 metastatic lesion (6.4 months vs. 4.9 months, $P = 0.0393$). The addition of

SRS resulted in increased local control in all patients (82% vs. 71%, $P = 0.01$). There were improvement in survival, local control, performance status and decreased steroid dependence in NSCLC patients with 1-3 in post hoc analysis^[17]. In the secondary analysis, the patients were re-stratified according to GPA scoring system. There were 211 lung cancer patients out of 252 (84%) re-analysed patients. It was found that SRS boost after WBRT resulted in increased survival in patients with GPA 3.5-4.0 score (good prognosis); the median survival was 21 months in WBRT + SRS vs. 10.3 months in WBRT only. The benefit was irrespective of the number of metastases^[32].

Oligometastases: omitting adjuvant WBRT after surgery or SRS

The indication of adjuvant WBRT after surgery or SRS has been evolving. The rationale of omitting immediate WBRT after surgery or SRS with close follow-up lies on the availability to treat in-brain recurrences with salvage focal therapies or WBRT at relapse with the aim of avoiding neurocognitive dysfunction. To meet this challenge four phase 4 randomised trials and a metaanalysis was carried out^[33-37].

In this concept, the first randomized trial came from Japan (JRSOG-99 trial). The primary end point was the survival difference between SRS and WBRT plus SRS, but at interim analysis it was realized that sample size would be unachievable and accrual was terminated. Aoyama *et al.*^[33] reported same survival rates (8 months vs. 7.5 months, $P = 0.42$) and same neurologic death rates (19% vs. 23%) in spite of higher local recurrences (27% vs. 11%) and distant brain recurrences (64% vs. 41%) in 132 patients with 1 to 4 brain metastases treated with SRS alone vs. WBRT plus SRS. The authors claim that SRS alone might be a viable approach if the patient is compatible with close brain monitoring for the early detection of recurrence to have the ability for early salvage^[33]. In a secondary analysis, 88 NSCLC patients were poststratified by DS-GPA and survival was the primary end-point. Forty seven patients with favorable DS-GPA score (2.5-4.0) had better OS with the addition of WBRT (16.7 months vs. 10.6 months, $P = 0.04$)^[38].

From MD Anderson, Chang *et al.*^[34] reported the second randomized trial: 58 patients with 1-3 brain metastases were randomized to SRS alone vs. SRS followed by WBRT. The primary end-point of the study was decline in neurocognitive function measured by Hopkins Verbal Learning Test-Revised at 4 months compared to baseline but was terminated early due to high probability (96%) of inferior outcome in learning and memory at 4 months in SRS plus WBRT arm. Local and distant in-brain controls were higher in SRS plus WBRT arm compared to SRS only (100% vs. 67% and 73% vs. 45%, respectively). An addition of WBRT resulted in less need for salvage therapies (11% vs. 90%) but higher risk for neurological deaths in SRS plus WBRT group (HR: 2.1, 95% CI 0.8-6.0, $P = 0.15$) and median survival was higher in SRS only group (15.2 months vs. 5.7 months). Higher survival in SRS only group was explained by higher rate of local surgical salvage and ability of the patients to have systemic chemotherapy 1 month earlier and median 2 more cycles^[34].

EORTC 22952 phase III randomized trial was the third with the largest patient number, unique in enrolling both SRS and surgically resected patients. Besides, in contrast to other two studies, it included only patients with systemic disease under control. The design was more complex; randomizing adjuvant WBRT vs. observation after surgery or SRS in systemically controlled 359 patients with 1-3 brain metastases. The primary end-point was survival time with functional independence. The local (surgery: 27% vs. 59%, $P < 0.001$; SRS: 19% vs. 31%, $P = 0.04$) and distant brain recurrences (surgery: 23% vs. 42%, $P < 0.008$; SRS: 33% vs. 48%, $P = 0.023$), need for salvage therapy (16% vs. 51%) and neurological death rates because intracranial progression (28% vs. 44%) were decreased with addition of WBRT after surgery or SRS compared to observation but without difference in median duration of functional independence (9.5 months vs. 10 months, $P = 0.71$) and survival (10.9 months vs. 10.7 months, $P = 0.89$)^[35]. In secondary analysis of EORTC 175 NSCLC patients were re-analyzed to evaluate the benefit of WBRT on survival of patients with favorable prognosis (high GPA); there wasn't any significant survival difference between patients with high or low GPA scores^[39].

As seen from these three randomized controlled trials, intracranial control (both local and distant sites) was almost equal in both SRS and SRS plus WBRT groups but survival outcomes were conflicting though none of these trials were intended to address survival as the primary endpoint; both JRSOG and EORTC trials reported same survival rates with both treatment approaches, whereas MD Anderson trial reported higher survival in SRS only group. An individual patient data metaanalysis considering JRSOG, MD Anderson trials and only 199 patients treated with SRS plus or minus WBRT in EORTC was performed by Sahgal *et al.*^[40] It was reported that, in patients with single brain metastasis, overall survival was higher and distant in-brain recurrence was lower compared to the patients with 2-4 metastases. SRS alone resulted in survival advantage in patients ≤ 50 years of age compared to their age-matched control treated with SRS plus WBRT without increase in distant brain recurrences. But there was no significantly higher neurological death in patients ≤ 50 years treated with SRS only (39% vs. 22%). In patients > 50 years of age distant in-brain recurrence risk was significantly higher in SRS only group but this increased recurrence did not translate to any survival disadvantage. When the data was re-evaluated considering only NSCLC patients the results were similar. But the authors emphasize that firm conclusion concerning histology could not be done because of limited sample size and reflection of subset analysis. As a conclusion, the authors suggest that SRS might be the treatment of choice in patients ≤ 50 years with 1-4 metastases^[40].

Recently, Brown *et al.*^[37] have reported the effects of adjuvant WBRT on cognitive function in a randomized phase III (N0574-Alliance) trial in patients with 1-3 metastases. The primary end-point was the amount of neurocognitive decline at 3 months compared to baseline. The local control was increased at 1 year with addition of WBRT to SRS (85% vs. 51%). There wasn't any difference in terms of survival (10.1 months vs. 7.5 months)^[37]. Recently secondary analysis of N0574-Alliance has been reported in 126 NSCLC patients to determine if WBRT might be associated with prolonged survival in NSCLC patients with good performance status. The patients were scored according to DS-GPA scores. There was no significant differences in OS between SRS only or WBRT plus SRS groups in NSCLC patients with favorable DS-GPA scores. This study further supports the approach of SRS alone in the majority of patients with limited brain metastases^[41].

Neurocognitive impairment following WBRT after surgery or SRS vs. observation after surgery or SRS

The impact of adjuvant WBRT on NCF have been utilized in these previously mentioned 4 studies by different methods. JRSOG trial evaluated the Mini Mental State Examination (MMSE) results in SRS only and SRS plus adjuvant WBRT groups in long term. They revealed that the control of metastases increased the MMSE scores in 2-3 months after radiotherapy in both groups and but in late-term (36 months) deterioration of NCF in WBRT group was prominent. In the post hoc analysis, average time to neurocognitive decline was shorter in SRS only group compared to SRS plus WBRT group 7.6 months vs. 16.5 months, respectively. The authors claim that distant in-brain recurrence might have a bigger negative effect on neurocognition and control of metastases is the most important factor in stabilizing cognitive function^[42]. But it should be mentioned that MMSE has been accepted as having lower sensitivity in detection of NCF changes^[43]. Besides, the paucity of the data on neurocognition in this study prevents us from making a conclusion on the effect of WBRT on NCF^[34].

MD Anderson trial reported significant decrease in recall memory and learning at 4 months after WBRT plus SRS compared to SRS alone patients (52% vs. 24%) despite the decreased local and in-brain recurrences as described previously. The authors claimed that adverse effects of WBRT on neurogenesis in hippocampus might have a greater effect on neurocognition than in-brain recurrences and related salvage therapies seen more often in SRS only group^[34].

QOL analysis of EORTC 22952 trial declared a significant neurocognitive decline at 12 months and lower QOL scores in WBRT arm after surgery or SRS^[44].

Table 1. Summary of randomised trials comparing stereotactic radiosurgery to stereotactic radiosurgery plus whole brain radiotherapy

Study	Patient number	Lung cancer patients (%)	# of brain metastases	Type of test	Local relaps (%)		Distant relaps (%)		Median OS (month)		Neurologic death (%)		Decline in NCF/QOL	
					SRS	SRS + WBRT	SRS	SRS + WBRT	SRS	SRS + WBRT	SRS	SRS + WBRT	SRS	SRS + WBRT
JRSOG-99, Aoyama <i>et al.</i> [33] 2006	132	66	1-4	MMSE	27	11	64	41	8	7.5	19	23	Deterioration is earlier in SRS only group at 7.6 month at 16.5 month	
MD Anderson, Chang <i>et al.</i> [34] 2009	58	67	1-3	HVLT-R	33	0	55	27	15.2	5.7	SRS + WBRT risk for death HR: 2.1, 95% CI 0.8-6.0		24%	52%
EORTC22952, Kocher <i>et al.</i> [35] 2011	359	53	1-3	EORTC QLQ C-30 QLQ BN-20	31	19	48	33	10.7	10.9	44	28	Global health at 9 month. 63 52 Physical function at 8 weeks 52 42 Role fxn at 8 weeks 64 58 Cognitive function at 8 weeks 81 74 Cognitive function at 12 months 80 69	
Alliance N0574, Brown <i>et al.</i> [37] 2016	213	68	1-3	FACT-Brain ADL Index HVLT-R GPT, COWAT TMT-A and B	27	10	30	8	10.4	7.4	NA		NCF at 3 months 64% 92% $P < 0.001$ QOL change from baseline -0.1 -20 $P = 0.001$	

SRS: stereotactic radiosurgery; WBRT: whole brain radiotherapy; OS: overall survival; NCF: neurocognitive functions; QOL: quality of life; MMSE: Mini Mental State Examination; HVLT-R: Hopkins Verbal Learning Test-Revised; FACT: functional assessment of cancer therapy; ADL: activities of daily living; GPT: Grooved Pegboard Test; COWAT: Controlled Oral Word Association Test; TMT: Trail Making Test Part A and B

Alliance trial evaluated the amount of decline in neurocognitive function at 3 months compared to baseline with 6 different cognitive tests. The WBRT and SRS group showed significant decline compared to SRS alone group (92% vs. 64%, $P < 0.001$) at 3 months. Among long term survivors, cognitive decline rates in SRS only and SRS plus WBRT groups were 45% vs. 94% ($P = 0.007$) in 3 months and 60% vs. 94% ($P = 0.04$) in 12 months, respectively, despite the increased local control in 1 year with the addition of WBRT (85% vs. 51%). In each cognitive test there was higher deterioration in SRS plus WBRT group, reaching statistical significance for immediate memory (30% vs. 8%, $P = 0.004$), delayed memory (51% vs. 20%, $P < 0.001$) and verbal memory (17% vs. 2%, $P = 0.01$), respectively [37].

The summary of these four trials can be seen in Table 1. As a whole, adjuvant WBRT reduces distant brain recurrence approximately by 50% and increases local control approximately by 15%-30%, without any survival benefit [6]. Of note, as mentioned before, none of these trials were powered to evaluate survival difference. One explanation for the lack of survival benefit for adjuvant WBRT is the uncontrolled extracranial disease which is the proximate cause of death. But even the largest EORTC trial that enrolled only patients with systemic disease under control to eliminate the competing risk of death from extracranial disease and to be able to detect the survival advantage of WBRT if any, did not find any difference. Secondary analyses of JRSOG 99 showed survival benefits of WBRT among NSCLC patients with high DS-GPA scores (good prognosis). But, secondary analyses of EORTC and Alliance trials failed to confirm this result. On the other hand, efficacy of local salvage therapies in SRS only arm might also be attributed to the lack of survival benefit [6]. Taken together, in the absence of survival advantage and recognized neurotoxicity of WBRT the results of these 4 trials suggest that in single or 24 brain metastases, SRS alone might be the

preferred strategy in amenable patients willing to have frequent brain surveillance with MRI.

Similarly, the American Society for Radiation Oncology (ASTRO) does not recommend routine adjuvant WBRT in oligometastatic brain metastases in its choosing wisely initiative organisation^[45].

On the other hand there have been several opposers to this approach, because they claim that there should be a trade between the risk of neurocognitive dysfunction and the risk of in-brain recurrences which could adversely worsen cognitive function even more than WBRT that might not be reversed by salvage therapies^[46,47].

Solitary or oligometastatic or non-oligometastatic: WBRT only

Brain metastases from the primary tumors occur mainly by hematogenous route making the whole brain under the risk for micrometastatic disease. This concept has been the rationale of WBRT even in case of small solitary metastasis but recently has started to be questioned in the context of true oligometastatic brain disease^[33]. In patients with life expectancy less than 3 months and/or poor performance status, short course WBRT or best supportive care with corticosteroids are reasonable options^[48].

A phase III non-inferiority Medical Research Council trial (QUARTZ), comparing best supportive care alone and best supportive care plus WBRT in NSCLC patients with brain metastases not amenable to surgery or SRS, reported similar survival and QOL. Solitary metastasis rate was 30% but median number of metastatic lesions and distribution of patients among two treatment arms according to number of metastatic lesions were not available in the data^[49]. But there has been some debates on this article; 38% of the cohort had poor performance, the median survival was only 8 weeks which was much inferior compared to previous data^[7]. Besides, due to heterogeneity of prognosis in patients with brain metastases with different primaries the results can not be generalized^[6].

Hippocampus sparing WBRT

WBRT related neurocognitive complications have led to the development of new radiotherapy techniques; hippocampus sparing WBRT (HS-WBRT) is one of them. The rationale lies on that; new memory is composed by neural stem cells located in the subgranular zone of the hippocampus and there is a dose-response-related risk of decline in NCF due to radiation dose received by the HP^[50]. Until recently, there have been only 2 prospective data on this subject. The first one, single-armed phase II RTOG-0933 trial, reported no increased risk of recurrence with HS-WBRT and better preservation of cognitive function with this technique compared to historical controls^[51]. The second data is from Oehlke *et al.*^[52] the progression in the hippocampus avoiding area was 2 (10%) of 20 in 40 weeks. But recently, the preliminary results of a randomized trial (NRG-CC001) composed of 518 randomized patients to memantine and HS-WBRT arm vs. memantine and WBRT arm have been presented in the last ASTRO meeting. HS-WBRT has prolonged time to neurocognitive failure, decrease in neurocognitive function at 6 months was 59.5% vs. 68.2% (HR: 0.76, $P = 0.03$) favoring HS-WBRT without any difference in intracranial brain recurrences or overall survival ($P = 0.208$ and $P = 0.307$, respectively)^[53].

Memantine and WBRT

Memantine has been tested in prevention of cognitive dysfunction as a neuroprotective agent in a randomized placebo controlled trial (RTOG 0614) and shown to be effective in decreasing the rate and delaying the time to cognitive decline^[54]. There is an ongoing randomized clinical trial comparing memantine and WBRT with or without HS-WBRT^[55].

Non-oligometastatic: SRS without WBRT

There have been growing data on SRS without WBRT in patients with more than 3 brain metastases although the maximum number of metastases appropriate for SRS has not been established, yet^[49]. The

Japanese Leksell Gamma Knife Society reported the results of a prospective observational multicenter phase 2 (JLGK0901) study in which survival outcomes and treatment related toxicity of SRS without WBRT in 1,194 patients with single, 2-4 and 5-10 brain metastases were evaluated. Patients with solitary brain metastasis had the longest survival (median 13.9 months, 95% CI 12.0-15.6). In this non-inferiority study, overall survival, local failure, distant in-brain recurrences, neurological death and toxicity were found to be similar both in patients presenting with 2-4 and 5-10 metastases. There was not any survival difference in lung cancer patients with 2-4 or 5-10 metastases either^[56]. An update of this study which was planned to see the radiation related complications and changes in NCF in long-term reported that toxicity and MMSE scores were similar among the three groups^[57]. There have been two clinical trials recruiting patients with estimated study completion date of 2022. One of them led by National Cancer Information Center, is randomizing patients with 5-15 brain metastases to SRS or WBRT plus memantine. Overall and neurocognitive deterioration free survival are the primary endpoints^[58]. The other one is from Dana-Farber Cancer Institute and randomizing patients with 5-20 brain metastases to SRS or WBRT plus hippocampal sparing in available cases, the QOL is the primary end-point^[59].

SRS vs. fractionated SRS

In the absence of randomized trial comparing SRS vs. fractionated SRS (FSRS); FSRS might be the option with lower risk of radionecrosis in large lesions^[60].

TKI and radiotherapy

NSCLC patients having driver mutations (EGFR mutation and ALK rearrangement) in NSCLC have increased risks of brain metastases both at the time of diagnosis and during the course of the disease^[3,4]. TKI targeting EGFR and ALK pathways have resulted in encouraging intracranial control rates compared to chemotherapy^[61].

Withholding radiotherapy in asymptomatic patients with driver mutations

Recently reported three phase II trials advocate withholding radiotherapy (SRS or WBRT) until progression if small asymptomatic brain metastases are present in an EGFR mutation positive NSCLC patient^[62-64]. There are several explanations; first, targeted therapies have greater intracranial activity compared to historical chemotherapeutic agents. Secondly, prolongation of survival with these agents allows time for manifestation of neurocognitive side effects if radiotherapy is the treatment of choice^[65]. But patients should have the willingness to close surveillance with MRI, though the optimal interval is unknown. Contrary to these trials, recently, a retrospective multiinstitutional analysis reported inferior survival with up-front EGFR-TKI in EGFR mutant NSCLC patients with brain metastases, both up-front SRS and up-front WBRT were found to be associated with higher survival compared to up-front EGFR-TKI in multivariate analysis, (HR: 0.39, 95% CI 0.26-0.58 and HR: 0.70, 95% CI 0.50-0.98), respectively^[66]. The debate will be going on until a randomized trial's results, comparing up-front radiotherapy followed by EGFR-TKI to up-front EGFR-TKI followed by radiotherapy at progression in EGFR mutated NSCLC patients.

A retrospective analysis of NSCLC patients with brain metastases and ALK rearrangement, showed prolonged time to intracranial progression with up-front cranial radiotherapy adjunct to first generation ALK inhibitor (crizotinib) compared to patients without cranial radiotherapy, (13.2 months vs. 7 months, respectively)^[67]. Second and third generation ALK inhibitors have better blood brain barrier penetration kinetics which might change the indications for cranial radiotherapy in the near future^[61]. Updated ALEX trial comparing crizotinib vs. alectinib in stage IIIB/IV (asymptomatic brain metastases were allowed) reported median PFS 27.7 months with alectinib vs. 7.4 months with crizotinib (HR: 0.35, 95% CI 0.22-0.56), cranial response rates were 59% vs. 26% and complete cranial response rates were 45% vs. 9% in patients presented with brain metastases at the time of diagnosis, respectively^[68]. As a result of these striking results, postponing the cranial radiotherapy might be reasonable in ALK rearrangement positive patients if Alectinib was the treatment of choice in first line.

Sequencing of radiotherapy and TKI

It's recommended to withhold TKI during WBRT to decrease unexpected toxicity which was observed with concurrent use of erlotinib and WBRT^[69]. If SRS were the treatment of choice, similarly withholding TKI and resuming one day after SRS is usually recommended in the absence of data.

Immune checkpoint inhibitors and radiotherapy

Although the prospective data is lacking, the data on immune checkpoint inhibitors targeting the programmed cell death 1 pathway (PD1) in NSCLC patients with brain metastases has been growing. In a phase II trial it was reported that pembrolizumab showed 33% intracranial response rate^[70]. But nivolumab led to discontinuation of treatment in 58% of patients, because of progression of neurologic symptoms which might be the reflection of pseudoprogression or hyperprogression^[71]. A recent retrospective study reported higher OS in multivariate analysis that concurrent use of anti-PD1 and SRS resulted higher OS compared to sequential use ($P = 0.006$) or SRS alone ($P = 0.002$) or anti cytotoxic T-lymphocyte-associated protein 4 ($P = 0.045$). Concurrent use also reduced distant intracranial relapses^[72].

Sequencing of radiotherapy and immune checkpoint inhibitors

Several retrospective data have reported no increased toxicity with the concurrent use of immunotherapy and cranial radiotherapy in NSCLC, though prospective studies are needed to confirm this finding^[73]. There have been several clinical trials recruiting patients testing the efficacy of immunotherapy in combination with radiotherapy in NSCLC patients with brain metastases (NCT02978404, NCT02858869, NCT02696993)

CONCLUSION

Taken together, these trials suggest survival advantage of surgery or SRS in selected patients with solitary metastasis but no survival advantage of certain treatment option in oligometastatic patients; they differ only in terms of local or distant in-brain control. Besides, QOL assessments have inconsistent results and has yet to be defined. Optimal treatment planning should consider both patient (age, performance status, expected life span) and tumor related factors (number and volume of brain metastases, extracranial disease control, molecular subtype) by using new prognostic models which is in line with personalised medicine and tailored therapy approach rather than “one size fits all”. In light of today's knowledge, it is quite likely that the trade would be going on between doctors and patients, considering QOL as an outcome of neurocognitive function that might be deteriorated either by symptomatic brain recurrence(s) or treatment related morbidity.

Surgical cavity directed SRS seems to be effective in local control and preservation of NCF compared to WBRT but the optimal sequencing (postoperative or preoperative) should be defined.

In non-oligometastatic patients the role of SRS has been evolving, the results of randomized studies might help in decision making.

Targeted therapy and immune checkpoint inhibitors have resulted in increased intracranial activity compared to chemotherapy, but the evidence is not strong enough to defer local therapy and the use as up-front therapy. Also, there are ongoing trials exploring the technique and the methods to spare the NCF.

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Authors' contributions

All authors contributed equally to the article.

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All authors declared that there are no conflicts of interest.

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Review

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Is active targeting of brain metastases of breast cancer superior to passive targeting?

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Abstract

Brain metastasis is a major cause of death in patients with solid cancers. Breast cancer cells have high tendency to migrate towards brain. Cancer cells within brain are characterized by severe aggressiveness and inaccessibility. Currently, breast cancer and its metastasis are the second leading cause of death among women. Tumor microenvironment and blood brain barrier (BBB) represent great obstacles in targeting breast cancer and its metastasis. Chemotherapy is a safer treatment modality for brain metastasis compared with risky surgical resection and brain radiotherapy. Unfortunately, conventional chemotherapy lack penetration of BBB and suffer from multiple resistance mechanisms. Current treatment technologies for brain metastases of breast cancer have limited long-term success and numerous side effects, illustrating the urgent need for novel smart strategies. Various novel drug entities and nanosystems have been employed to improve diagnosis and targeted treatment of breast cancer and its metastasis. Immunotherapy agents and small tyrosine kinase inhibitors have been shown to reduce tumor size and increase survival in patients with breast cancer, but still poorly penetrate BBB. Tailored sized nanoparticles to some extent crossed brain tumor barrier and enhanced drug accumulation in tumors by taking advantage of enhanced permeability and retention. Furthermore, various active targeting strategies have been adopted to improve accessibility to brain malignancies. Therefore, to achieve enhanced antitumor therapy against



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breast cancer and its brain metastasis, multi-talented delivery systems are urgently needed for optimal treatment. This review focuses on the various active and passive targeting technologies for the treatment of breast cancer brain metastases in the past decade. A comprehensive summary and examples along with pros and cons of each system will be discussed. Different treatment modalities and nanotechnology facilities will be demonstrated to aid in designing the optimal smart, safe, targeted and effective systems to combat brain metastases of breast cancer.

Keywords: Active targeting, passive targeting, brain metastasis, breast cancer

INTRODUCTION

Despite the recent pharmaceutical and medical advances in treatment and diagnosis of different carcinogenic tissues and their metastases, brain and central nervous system (CNS) primary and metastatic tumors remain a tough hurdle to overcome due to physiological and anatomical barriers represented by blood brain barrier (BBB) and the aggressiveness and continuous adaptive evaluation of this tumor tissue.

As systemic therapy of metastatic breast cancer advances, survival rates of patients are enhanced. On the other side, the incidence of brain and CNS complications increases^[1]. In historical series, the risk of developing brain metastasis has been estimated among women with stage IV breast cancer to range from 10% to 16% among living making it the second most common cause of metastatic brain tumor after lung cancer (10%-25%)^[2-4]. The development of breast cancer brain metastases (BCBM) tends to vary by the subtype. Forty-six percent of patients with advanced triple negative breast cancer, and up to thirty-seven percent of patients with HER2-positive breast cancer relapse due to intracranial metastases, despite control of the peripheral tumors^[5-8].

Treatment options are limited and usually involve multimodality approaches that include surgery, radiotherapy, radiosurgery, and rarely systemic therapy, depending on the number of CNS lesions^[9,10]. The unpredictable extent of therapeutics that can reach tumor vasculature of BCBM has been the challenge in developing effective treatments for these patients^[11,12]. BBB has always been a challenge to drug delivery to brain tissues^[13-16]. However, during brain metastases, the structure and integrity are altered forming a “Blood Brain Tumor Barrier” (BBTB)^[17]. The leaky structure of BBTB is an appealing strategy to target brain tumors^[18]. Approaches to deliver drugs into the brain are being extensively studied [Figure 1].

Beside the common technologies including viral vectors and nanoparticles, novel non-invasive techniques such as ultrasound alteration and magnetic stimulation have been studied to temporally open the BBB to enhance brain drug uptake. In this review, we discuss passive and active targeting BCBMs.

BBB AND DRUG DELIVERY

The BBB, as a morphologic and physiologic barrier, protects the brain tissue from the peripheral vasculature, protecting normal brain function by impeding most compounds from transiting from the blood to the brain^[13,16,18]. There is a common misconception about the nature of BBB as a protective barrier or sac protecting the brain underneath it. Perhaps, the term BBB was the main reason of such misconception. The BBB is mainly a fortified special vascular structure in the brain different from the common vascular structure in the rest of the body. Mainly, the brain capillary endothelial cells (BCECs) form the BBB, besides other cell types such as pericytes, astrocytes, and neuronal cells; playing an important role in the function of the BBB^[19]. BCEC have tight junctions that prevent paracellular transport of small and large (water soluble) compounds from the circulation to the brain except for some very small or gaseous molecules, such as water and carbon dioxide^[17,19,20]. The components of the BBB continuously adapt in response to various physiological changes in the brain^[21] [Figure 1].

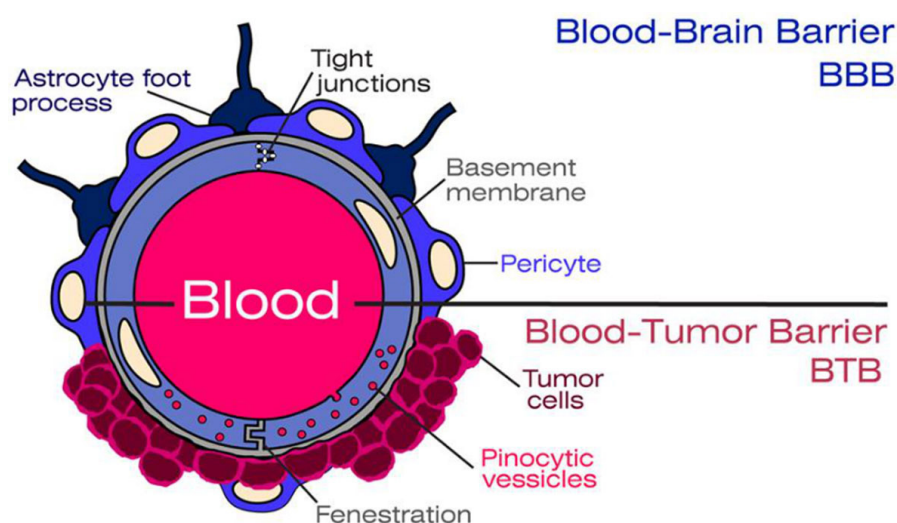


Figure 1. The vascular and structural components of the blood brain barrier (BBB) and the blood brain tumor barrier

Similarly, BCECs restrict drug distribution into the brain by limiting drug diffusion from blood to brain. In addition to these physical barriers, cells, like pericytes, and astrocyte foot processes, express several drug metabolizing enzymes that inactivate drugs reducing the distribution of active drugs to the brain. Thus, complicating barrier mechanisms & the presence of both drug-uptake and drug efflux mechanisms such as multidrug transporters including P-glycoprotein (ABCB1/MDR1) and ABCG2. Some compounds pass the BBB by passive non-saturable diffusion, some by active transport; others are actively excluded, and still, others enter through endocytosis^[12].

BRAIN METASTASES OF BREAST CANCER

The occurrence of brain metastases depends on how breast cancer cells adapt to and survive in foreign environments^[22]. Specific homing molecules mediate organ-specific metastasis formation on the heterogeneous tumor cell surface^[23]. Investigating throughout the metastatic cascade is one area of research that can lead to the development of effective treatment.

Research has shown that at the time of initial diagnosis of primary cancer, circulating tumor cells can be found in the bloodstream of patients^[24]. Breast cancer stem cells are characterized by the ability to self-renew along with their high level of resistance to radiation and chemotherapeutic agents^[25]. After detachment of tumor cells from the primary lesion, invasion into the CNS and bypassing the BBB occur, followed by sustained proliferation towards the formation of brain metastases^[26,27]. Studies have suggested that brain metastases originate from cells as a primary mass or lymph nodes or from other visceral metastases^[28].

Disruption of the BBB was shown to involve mediators of extravasation through non-fenestrated capillaries. A number of mediators have been identified to assist breast cancer cells crossing the BBB, including cyclooxygenase-2, heparin-binding epidermal growth factor-like growth factor, and the $\alpha 2$, 6-sialyltransferase ST6GALNAC5^[29-34].

The recently identified extracellular (circulating or exosomal) miRNAs were shown to mediate the ability of metastatic breast cancer cells to target the distant brain endothelium and vasculature^[35]. The metastatic BC cells in brain express high levels of anti-plasminogen activator serpins as a shield to escape from the reaction of brain stroma, conferring the adherence of infiltrating breast cancer cells to the surface of capillaries and the growth on the vasculature^[36].

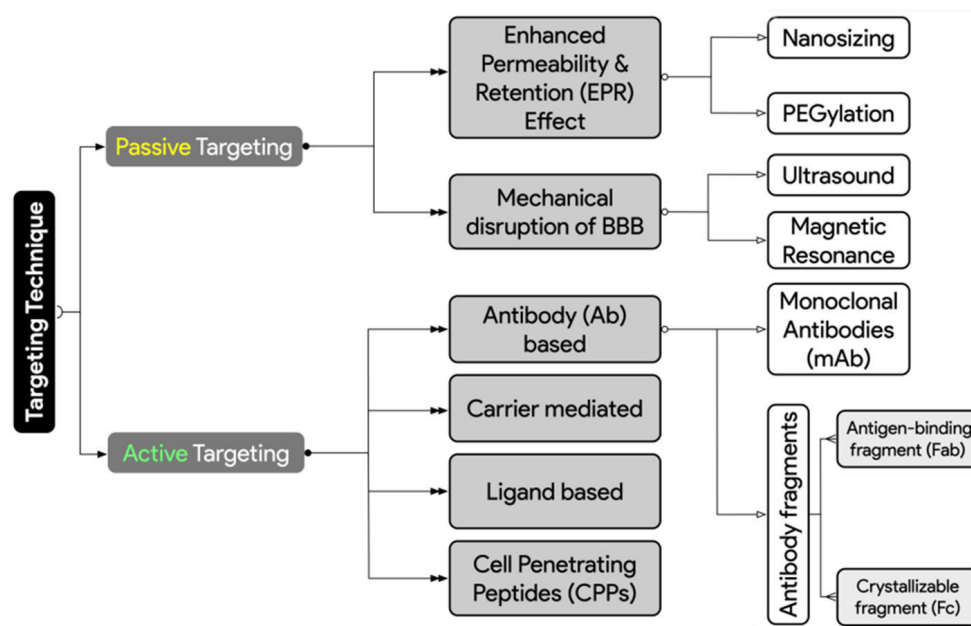


Figure 2. Different adopted strategies for actively and passively targeting brain metastases of breast cancer to overcome the blood brain barrier (BBB) hurdles

Brain metastases of breast cancer are also subject to the limited permeability characteristics of the BBB in spite of the formation of the BBTB with altered integrity. MRI data have shown that not all brain metastases display elevated BBTB permeability^[37]. The changes in BBTB vascular permeability are usually not homogenous throughout the lesion^[38,39]. It was noticed that brain metastases from HER2+ breast cancers infiltrate brain tissue crossing the endothelial cells without disrupting the BBB, unlike the brain metastases from triple negative or basal-type breast cancers that often disrupt the BBB^[8,40] [Figure 1].

TARGETING BRAIN METASTASES

Although, there are no FDA-approved systemic treatments for BCBM to date^[41], patents and studies in the past years have shown promising progress and well-established techniques to overcome the BBB/BBTB^[42]. There are two general strategies adopted to facilitate crossing the BBB; invasive and non-invasive techniques^[43]. The invasive techniques rely primarily on disrupting the BBB integrity by direct intracranial drug delivery through intracerebroventricular, intracerebral or intrathecal administration, osmotic pumps or biochemical means^[43]. But all these approaches are severely limited by poor distribution into brain parenchyma^[44]. On the other hand, non-invasive methods include drug moieties modifications through the transformation of the drug into lipophilic analogs, prodrugs, chemical drug delivery, carrier-mediated drug delivery, receptor/vector-mediated drug delivery and intranasal drug delivery^[43,45].

Diffusion of substances into the brain can be divided into paracellular and transcellular. Generally, BBB targeting strategies can be categorized into passive (transcellular lipophilic pathway) and active targeting (mainly; transcytosis)^[46]. Figure 2 summarizes the targeting techniques covered in this article.

Passive targeting

Enhanced permeability and retention phenomenon

Passive targeting depends mainly on the preferential accumulation of drug molecules into tumor cells^[47]. Enhanced permeability and retention (EPR) phenomenon based on the nanometer size range of the nanoparticles and two fundamental characteristics of the neoplastic tissues, namely, the leaky vasculature and impaired lymphatic drainage. EPR was first described by Maeda and Matsumura^[48]. The selective high

local concentration of nanosized anticancer drugs in tumor tissues enhances the therapeutic effect with minimal side effects in both preclinical and clinical settings^[49].

Various important factors such as circulation time, targeting and the capability to overcome BBB are heavily reliant on the shape, size and the surface area of these particles. In passive diffusion, lipid solubility, molecular weight, and the presence of tight junctions greatly affect the degree of permeation^[50,51].

Conventionally, a particle must be at least 10 nm in diameter to avoid clearance by first-pass renal filtration and the optimal size range of 100-180 nm will ensure longer circulation time, increased accumulation within the tumor mass and lower renal clearance^[52,53]. Moreover, surface characteristics also play a very important role in determining the extent of internalization of these nanoparticles into cells. Relatively, the surface can be modified by the polymer composition, thus governing an extra amount of hydrophobicity or hydrophilicity to these particles^[51]. [Supplementary Table 1](#) illustrates some of the papers using passive targeting technology in targeting BCBM.

Nanotechnology as a tool for targeting EPR

Nanotechnology paved the road towards safer and more likely effective cancer treatment strategies that can overcome some of the hurdles for drug delivery. Nano-medicines primarily aim to improve the circulation time of the conjugated or entrapped (chemo-) therapeutic drugs^[54]. Examples of nanoparticles commonly used include: polymeric particles, polymeric micelles, dendrimers, and liposomes. Nanoparticles represent versatile tools to encapsulate various types of drugs, either hydrophilic or hydrophobic moieties altering their physicochemical parameters and pharmacokinetics profile^[55].

Beside increasing drug-site contact time, some polymers used in nanoparticles formulations such as polylactic-co-glycolic acid (PLGA) have high cell adhesion property, increasing the drug concentration gradient at the adhesion site by longer drug carrier contact time with the targeted cells^[56].

However, nanoparticles can show some serious adverse effects^[57]. Adverse effects of nanoparticles depend on individual factors such as genetics, existing disease conditions, exposure, nanoparticle chemistry, size, shape, agglomeration state, and electromagnetic properties^[57]. The key to understanding the toxicity of nanoparticles is their size; nanoparticles are smaller than cells and cellular organelles, which allow them to penetrate these biological structures, disrupting their normal function^[57]. Examples of toxic effects include tissue inflammation, and altered cellular redox balance toward oxidation, causing abnormal function or cell death^[57]. Furthermore, a major drawback is the difficulty in scaling up the formulation and its transformation to clinical use, due to high cost and instability during storage. The NP materials must be biocompatible and safe when administered^[58].

Liposomes

Liposomal (Liposome: lipid vesicle in Latin) nanocarrier systems are vesicular lipid bilayer colloidal spheres formed by self-assembly^[59]. Without the use of surface modification by a hydrophilic polymer such as polyethylene glycol (PEG), the biological half-lives of liposomes are very short due to several factors including the tendency of the liposome to exchange lipid materials with cell membranes and their uptake by phagocytes. Mohammad *et al.*^[60] noticed increased survival rates in animals treated with liposomal irinotecan (nal-IRI) that sustained the accumulation of irinotecan and its active metabolite in the brain metastases lesions, unlike non-liposomal irinotecan whose distribution in the CNS was hampered by BBTB and various efflux proteins. The size of nal-IRI was between 100-110 nm, which enhanced its preferential accumulation within tumor due to EPR through the leaky vasculature of the tumor^[60]. Drawbacks of liposomes involved their rapid uptake by the Reticulo-Endothelial System (RES) and consequent removal from circulating blood.

PEGylation

The main pharmacokinetic outcomes of PEGylation include: changes occurring in overall circulation life-span, tissue distribution pattern, and elimination pathway of the parent drug/particle^[61]. PEG mainly protects these particles from being phagocytosed by natural particle eliminating mechanisms, mainly organs of the RES^[62].

Pegylated nanoparticles are characterized by hydrophilic surfaces that circumvent opsonization and decrease their clearance by macrophage, which results in prolonged circulation^[51]. PEGylation has been shown to alter the pharmacokinetics of doxorubicin considerably; the total clearance was significantly reduced^[63]. In the early 1990's, PEGylated polymeric vesicles were introduced by Yokoyama *et al.*^[64], which represented an important milestone in the synthesis of long-circulating liposomal formulations (STEALTH® liposomes). Liposomal formulations are the first novel controllable carrier systems to be sold in the market for cancer (Doxil®, PEGylated liposomal formulation encapsulating doxorubicin(PLD)^[65-67]. PLD showed reduced cardiotoxicity and prolonged activity.

Anders *et al.*^[68] encapsulated doxorubicin in pegylated liposomes (PLD) that achieved 20 folds higher concentration of doxorubicin within intracranial tumors compared to non-liposomal doxorubicin, whose distribution was compromised by BBTB. Moreover, co-administration of PLD and BBB permeable ABT-888, an inhibitor of a poly (ADP-ribose) polymerase, showed better survival as compared to non-liposomal doxorubicin and ABT-888.

Nektar™ Therapeutics has several patents on PEGylation bioconjugation aiming to modify the pharmacokinetic profile^[69-75]. Adkins *et al.*^[76] formulated NKTR-102, an Irinotecan-PEG conjugate linked with a hydrolysable ester bond, whose polymer moiety resulted in prolonged circulation and subsequently increased tumor localization. The innovative nature of this PEGylated system is the adoption of the three-dimensional (3D) branching technology in the PEGylation to provide superior pharmacokinetics and pharmacokinetic parameters. The 3D propriety Nektar PEG technology offers weeks of half-lives compared to days in conventional PEG^[76]. The preferential accumulation of NKTR-102 within brain metastases via EPR, its reduced clearance and ability to escape P-glycoprotein mediated efflux resulted in continuous release of the active metabolite SN38 and better therapeutic efficacy.

Nektar™ Therapeutics is not the only pharmaceutical company relying on innovative PEGylation technologies for passively targeting brain metastases of breast cancer. Other companies, such as 2-BBB, is investing heavily on the use of PEGylation bioconjugated with liposomal vesicular carrier systems to passively target brain metastases of breast cancer^[77]. BBB adopts a propriety G-Technology® for targeting the brain. 2-BBB's G-Technology® empowers sustained delivery of systemically administered therapeutics to the brain with high safety and efficacy profiles. The G-Technology® uses PEG and glutathione bioconjugated to active pharmaceutical ingredients loaded liposomal vesicular structures. Polyethylene glycol (PEG) is attached to the liposomes to provide a prolonged circulation time in the blood stream with sustained half-life pharmacokinetic profile. Glutathione is bioconjugated to the PEG molecules to offer targeted safe and effective delivery of the therapeutics across the BBB^[78].

2-BBB has adopted its G-Technology® in developing two lead clinical programs targeting multiple indications of brain cancers and neuroinflammatory diseases. 2-BBB's lead product 2B3-101 (glutathione PEGylated liposomal doxorubicin) combines the G-Technology® with the existing chemotherapeutic agent doxorubicin for the possible treatment of brain metastases and glioma. It has completed a Phase I/IIa trial treating patients with various forms of brain cancer. 2-BBB's second promising lead product is 2B3-102. 2B3-102 is a glutathione PEGylated liposomal methylprednisolone. It applies the anti-inflammatory glucocorticoid methylprednisolone to combat acute and chronic neuro-inflammation associated with several CNS

indications including acute relapses of multiple sclerosis, optic neuritis, neuromyelitis optica and uveitis. 2B3-201 has completed a Phase I clinical trial in healthy volunteers^[77].

2-BBB is a clear example of industry-driven research aiming at developing innovative passive targeting technologies to brain and brain metastases. They use triple passive targeting techniques to offer optimal safety and efficacy profiles, Glutathione passively targeting the cancerous intracellular reductive environment, PEGylation and liposomal vesicular carrier system^[77].

Nucleic acid

RNA interference (RNAi) is an endogenous pathway for post-transcriptional silencing of gene expression that is triggered by double-stranded RNA, including endogenous microRNA (miRNA) and synthetic short interfering RNA^[79]. MicroRNA-122 mediated RNAi brings new prospects^[80]. Zhang *et al.*^[81] miRNA-1258 in BMBC cells inhibited heparanase which regulates many molecules involved in angiogenesis and metastasis of the tumor.

Advanced physically manipulated systems to disrupt BBB

Another modality of addressing BBB passively is advanced physically manipulated systems that can be tightly mediated by stimuli to treat diseases specifically and with a controlled dosage of drugs. Physical manipulation can be achieved based on ultrasound, electricity, magnetism and photonic emission technologies^[82]. Davalos *et al.*^[83] applied pulsed electric fields into brain tissue of an animal, to cause temporary disruption of the BBB in a volume of brain tissue near the source of the pulsed electric fields over a specified time interval.

The use of focused ultrasound (FUS) combined with circulating microbubbles is a non-invasive method that increases the permeability of BBB and improves outcomes of trastuzumab; this technique was used by Park *et al.*^[84] to improve outcomes with trastuzumab in a breast cancer brain metastasis model. Similarly, FUS in combination with microbubbles was able to temporarily disrupt the BBB enhancing the anti-tumor efficacy of the two anti-HER2 agents combination therapy^[85]. Moreover, reversible disruption of the BBB by bursts of low frequency MRI-guided ultrasound enhances the brain delivery of monoclonal antibody - Herceptin (trastuzumab) in mice^[86,87].

Active targeting

Active targeting was proposed for improved targeting efficacy^[47]. These strategies consist in incorporating affinity molecules or taking advantage of influx transport systems expressed within the BBB/BBTB depending on specific interactions of ligand-receptor and antibody-antigen^[47,88,89]. Also, several ligands have been studied and utilized to shuttle nanoparticles, antibodies, and drugs across the BBB and into the brain cells^[90] [Figure 1]. Supplementary Table 2 summarizes recent studies of actively targeted treatments for BCM.

Receptor-mediated transcytosis/ligand-based

An overexpression of receptors or antigens in cancer acts as a potential target to achieve efficient drug uptake via receptor-mediated endocytosis^[47]. Moreover, receptor-mediated transcytosis (RMT) allows for BBB transport of various macromolecules after initial binding of a targeting ligand to a receptor expressed on the brain endothelial cells^[91]. Tumor-targeting ligands such as peptides and antibodies may effectively aid certain cytotoxic agents (either biological or synthetic) to deliver to the tumor cells, thereby improving therapeutic efficacy while limiting the exposure of normal tissues to the cytotoxic agents^[92]. The transferrin receptor, the low-density lipoprotein receptor-related protein 1 (LRP-1), the insulin receptor and the nicotinic acetylcholine receptors are examples of receptor expression on the BBB.

AngioChemTM Inc. developed a series of aprotinin polypeptides (Angiopeps)^[93]. Angiopep-2, a 19-amino-acid peptide, is one of the promising vectors designed to target the LRP-1 receptor, to mediate transcytosis across the BBB. It is derived from the human Kunitz domain^[94]. Angiopep-2 can facilitate brain-targeted drug delivery through LRP-1-mediated transcytosis. Regina *et al.*^[95] demonstrated that a conjugate between angiopep-2 and an anti-HER2 mAb results in a new chemical entity, ANG4043. ANG4043 retains *in-vitro* binding affinity for the HER2 receptor and antiproliferative potency against BCBM rat model. This study showed increased uptake in brain endothelial cells and enhanced BBB permeability compared to poor brain penetration of anti-HER2 mAb alone. Similarly, Thomas *et al.*^[96] showed that ANG1005 shows significantly improved delivery to brain and brain metastases of breast cancer compared to free paclitaxel in mice bearing BCBM.

On the other hand, Orthmann *et al.*^[97] formulated rigid and fluid liposomes entrapping Mitoxantrone and equipped with a 19-mer angiopeptide as a ligand. Angiopeptide bearing fluid liposomes showed *in vitro* the highest cellular uptake and transcytosis. They were significantly better than the corresponding ligand-free fluid liposomes and ligand-bearing rigid vesicles however; the improvement was mainly depending on liposomal fluidity while the targeting contributed only to a minor degree. In 2016, Orthmann *et al.*^[98] encapsulated oxaliplatin (OxP) in liposomes then bound angiopep-2 to the vesicular surface. They determined that the newly developed OxP liposomes significantly improved the treatment of subcutaneously and intracerebrally growing breast cancer, but the targeted angiopep-equipped liposomes showed no superior effect *in vivo*.

Melanotransferrin (hMTf) is another target to the LRP-1 receptor, which was shown to deliver doxorubicin across BBB^[99,100]. In order to overcome the trastuzumab inability to cross the BBB and treat brain metastases of HER2+ breast cancer, BT2111, a novel bioconjugate of trastuzumab was developed by BiOasis Inc., on the hMTf (p97) TranscendTM vector platform^[101]. This platform is an example of an actively targeted immunotherapy, aiding in enhancing the immunotherapy targetability, ability to cross the BB and clinical efficacy via the use of the targeting moiety hMTf.

Another complex process involves binding to a primary, tumor-specific receptor activating endocytosis; using “Tumor-penetrating peptide”^[102]. The prototypic peptide of this class, iRGD (CRGDKGPDC), contains the integrin-binding RGD motif^[103]. The integrin-binding RGD sequence motif binds to $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins, which are specifically expressed in tumor endothelial cells^[102]. Hamilton *et al.*^[104] demonstrated that a single dose of iRGD had a significant effect on metastatic tumor progression and nonproliferative cancer cell retention when applied early in course of tumor development. Proteolytically processed iRGD also exerts anti-metastatic activity by binding to neuropilin-1 and activating an endocytic bulk transport pathway through tumor tissue. The iRGB platform offers an innovative dual targeting tool to target the tumor portion via the integrins and the RGB motif^[103].

Carrier-mediated transcytosis

Naturally, carrier-mediated transcytosis (CMT) enables spontaneous internalization of small biomolecules^[105,106]. CMT takes advantage of the immunological surveillance system of the brain, using circulating phagocytic cells such as monocytes or macrophages as Trojan horse to deliver drug molecules into the brain^[107]. Such cells have a tendency to endocytose colloidal materials, for example, nano or microparticles, liposomes, and subsequent exocytosis to release drug and/or colloidal materials to external media^[108]. Fidler and colleagues provided evidence that macrophages of blood monocyte origin can infiltrate experimental brain metastases while the BBB is intact.

In 2012, Choi *et al.*^[109] reported the first successful demonstration of the active delivery, using macrophages, of nanoparticles to brain metastases. Activated macrophages not only cross the BBB but they envelop the

metastatic cells delivering the loaded nanoparticle to less than a cell width away from the nearest metastatic cell^[109].

Cell penetrating peptides

Cell-penetrating peptides (CPP), that show great capacity in BBB transport, have the ability to transport protein or peptides into cells in a nonspecific, receptor-independent manner and non-immunogenic when compared with antibodies^[110]. Considering their smaller size (up to 30 amino acids in length), cationic and/or amphipathic CPPs have a greater potential to penetrate the BBB than other transport systems. Short peptides, as targeted drug delivery vehicles, appear to have some advantages owing to their small size, efficient tissue penetrability, and minimal toxicity and immunogenicity.

The first CPP, trans-activator of transcription (TAT), derived from human immunodeficiency virus-1, can be efficiently taken up from the surrounding media^[111]. Morshed *et al.*^[112] used PEGylated gold nanoparticle conjugated to TAT peptide as well as doxorubicin. This formulation offered extensive accumulation of particles throughout diffuse intracranial metastatic microsatellites. Moreover, it was shown to destabilize a brain capillary monolayer increasing its permeability. Fu *et al.*^[113] combined brain metastatic breast carcinoma cell (231-BR)-binding peptide BRBP1, a cell penetrating peptide TAT, and a proapoptotic peptide KLA. The composite selectively homed to the tumors in vivo where it induced cellular apoptosis without significant toxicity on non-tumor tissues. Angiopep-mediated targeting also can be considered as one of the most promising ways to reach the CNS for the treatment of brain cancer or brain metastases^[114].

Conclusion and future directions

There is a global need for effective and safe pharmaceutical chemotherapeutic agents that have the potential to target tumors like BCBM.

Conventional chemotherapy, radiation therapy and immunotherapy offers promising options for treating brain metastases, which is traditionally treated with surgery.

In 2005, the chemotherapy temozolomide (Temodar®) was approved to treat glioblastoma (GBM) patients. However, over 50% of GBM tumors generate a DNA repair protein (methylguanine methyltransferase) that effectively combats and neutralizes temozolomide chemotherapy. There are two FDA approved immunotherapies for brain and nervous system cancers and metastases. Bevacizumab (Avastin®) and Dinutuximab (Unituxin®). Several other immunotherapies are being used to treat different types of brain cancers in clinical trials. However, there are no FDA-approved systemic treatments specific to BCBM to date^[41]. Improved insights into the microenvironment and metastatic cascade processes have resulted in the development of several novel chemotherapeutic and immunotherapeutic drugs and strategies^[35,36,38,39,54]. Such novel chemotherapeutic and immunotherapeutic strategies adopt active and passive targeting strategies to enhance the clinical treatment effects.

Surveying the published research papers and patents also revealed a major drawback in the methodology design, which is the lack of standardization of efficacy and safety profiles. Various sophisticated nano-systems and conjugates have been studied to either actively or passively target BCBM. However, most of these complex delivery systems fail to reach the market due to high cost, instability and difficulty of scaling up. In addition, majority of the reagents used in the formulation of such novel therapeutic systems to improve the stability; are not included in the FDA approved inactive ingredient database. The main question of this review is to query the preferential advantage of either active or passive targeting in combating brain metastases of breast cancer. Such question is a major ongoing debate as revealed by the recent patents and papers portfolio.

Passive targeting could be a cheaper, long-term effective and safe technique to target tumor cells without triggering resistance for active targeting moieties. Numerous significant downsides in active tumor-targeted drug delivery were identified^[115-120]. Interestingly, passive targeting can be tailored and designed via simple ideologies and techniques such as simple PEGylation, size modifications or even shape customization. It was shown that slender shape and particles modifications such as drifting from the conventional spherical particulate structures, can yield high effectual passive targeting^[121].

Active targeting shows significant results in combating cancer initially with long-term degraded efficacy profile, most potentially attributed to multidrug resistance^[119]. Additionally, the marginally augmented efficacy of active targeting strategies does not substantiate the extremely higher cost, difficulty in clinical application and the high complexity level of the carrier system^[54,78,115-120,122,123]. Interestingly, passive targeting can be tailored and designed via simple ideologies and techniques such as simple PEGylation, size modifications or even shape customization.

Multiple researchers suggested the preferential long-term advantage of passive targeting over active targeting such as Lammers *et al.*^[122] and Rosenblum *et al.*^[123]. Such researches highlighted the need for future realistic and scalable efforts to address some of the conceptual drawbacks of drug targeting to tumors such as resistance, and that strategies should be developed to overcome these deficiencies. Such research presented evidence that passive targeting yield very comparable results to active targeting on the short term and superior results on the long term^[54,78,115-120,122,123].

Some of the main drawbacks of engineered nanotechnologies, either active or passive, are the poor robust and cost-effective toolbox to characterize nanomaterials and to quantify exposure in test systems (dosimetry), lack of standard techniques and methods for test, hazard, and risk assessment strategies and lack of specific safety assessment strategies in evaluating nanotoxicities. However, passive targeting offers less problematic strategy compared to active targeting engineered nanotechnologies in terms of nano-safety and its quantitative characterization^[124].

Passive targeting could provide an applied direction for the advance of novel management tackles and therapeutics for brain metastases of breast cancer for researchers worldwide, paving the road to affordable, scalable, stable, efficient and safe management strategies. Such observations were evident from the multiple passive targeting products under Phase II and Phase III clinical trials from multiple pharmaceutical firms such as Nektar Therapeutics, BiOasis Inc. and 2-BBB. Such companies have invested heavily into the development of new formulations that combat brain barriers, targeting the brain and accumulates in tumor tissue rather than normal cells, to enhance the nano-safety profiles to chemotherapeutics. This may not be only attributed to their need to expand their portfolio to increase their commercial value for possible products buyouts and licensing by big pharmaceutical firms, but the safety and efficacy potential of such passively targeted nanotherapeutics in the treatment strategies of brain metastases of breast cancer compared to current conventional modalities and research-driven active-targeting initiatives.

Such conclusions have driven the industry to invest heavily on passive targeting compared to active targeting. A major focus by industry is the successful transformation of such novel technologies from bench to bedside with reasonable cost, scale-up abilities and formulation robustness and reproducibility, which are achievable via passive targeting technologies compared to active targeting. Active targeting ligand post-insertion and labeling techniques need to be extensively researched for ease of application for active targeting to gain industrial momentum. Conclusively, patents and products under development should focus on simple passively targeted bioconjugate structures, which are easily synthesized with high yield, reduced cost and high stability profile of the final formulation.

DECLARATIONS

Authors' contributions

Manuscript writing: Kamal NH, El-Amrawy F, Ali HA, Nounou MI

Manuscript proofing and revisions: Edafiogho I, Nounou MI

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Review

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Metabolic rewiring of stem cells and differentiated cells in cancer: the hypothetical consequences of a GABA deficiency in endocrine pancreas

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Abstract

The carcinogenic mechanism proposed considers that stem cells committed to repair tissues and differentiated cells, acquire different metabolic properties, if there is an associated GABA deficiency suppressing a control system of the endocrine pancreas. This control system mediated by GABA, released with insulin, normally turns off glucagon and somatostatin release when insulin is released. A consequence of the GABA deficiency in pancreas and adrenals is a hybrid insulin-glucagon-somatostatin message, received by new mitotic stem cells displaying then a hybrid metabolic rewiring. This gives them a selective metabolic advantage over differentiated cells that become insulin resistant and only receive the glucagon- somatostatin part of the hormonal message. Indeed, their insulin receptors are desensitized by the persistent leakage of insulin resulting from the GABA deficiency that fails to close the insulin release mechanism. Thus differentiated cells are simply rewired to be plundered by stem cells. The metabolic advantage gained by stem cells blocks their own differentiation and maintains their mitotic capacity. Inevitable mutations of mitotic cells follow, the immune system is unable to eliminate a geometrically increasing number of altered stem cells, a selection of the most aggressive but metabolically successful population takes place when cancer is declared.



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Keywords: Cancer metabolism, endocrine pancreas, adrenals, GABA, stem cells, differentiated cells

INTRODUCTION

Since the early observations of Warburg on cancer metabolism showing the lactic acid increase even in the presence of oxygen (Warburg effect)^[1,2], until the seminal works of Mazurek and Eigenbrodt^[3] and Eigenbrodt *et al.*^[4] on pyruvate kinase (PK) M2 blockade in tumors, much progress has been made on metabolic rewiring processes in cancer^[5,6]. An increasing number of works are interestingly gathered in special issues on cancer metabolism^[7]. However, they deserve to be included in a more general frame proposing a mechanistic explanation for metabolic rewiring processes in cancer. In parallel, there is great interest for diets, natural products and life styles that would decrease cancer incidence. Presumably, the idea is that a diet might influence metabolism and the epigenetic control of genes that have been implicated in cancer incidence. It is for example shown that a simple dietetic mixture: curcuma and poly unsaturated fatty acid, gives protection against a carcinogen (Dimethyl Benz Anthracene) in animal models^[8]. There are several observations linking nutrition and cancer, reminding that nutrition has evidently metabolic consequences that may change the incidence of diseases, including cancer.

Paradoxically, even viral or cellular oncogenes that initially pointed toward a viral or genetic cause for cancer, reinforced the metabolic cancer model, because the genes that were up-regulated by the viral or cellular oncogenes encoded for proteins supporting the different steps of signaling pathways similar to the one activated by insulin, via tyrosine kinase receptors. With insulin signaling, glycolytic metabolism evidently came back on the stage. However, a general frame that would explain the metabolic rewiring process in cancer, its effects on stem cells and differentiated cells remained to be proposed; this was started in previous works^[9-13] and is continued in this presentation.

A surprisingly low number of metabolic “switch compounds” control the direction of major metabolic pathways in a network of enzymes that support the different metabolic finalities reached by a cell in response to its receptors. The cascades of signals that follow coordinate the enzymes and the pathways direction towards a specific metabolic finality responding to a physiological situation. Schematically, the finalities to reach are: (1) the catabolic production of nutrients (glucose and ketone bodies) when food gets scarce; (2) the production of energy, with a possible selection of the energetic source (glucose, fatty acids, eventually ketone bodies); (3) the anabolic synthesis of new constituents for new mitotic cells, which requires energy and the substrates for making these constituents. In addition, anabolic hormones increase glycogen and lipid tissue stores when food is available. These metabolic finalities are controlled by the ON or OFF status of major enzymes, activated or inhibited by phosphorylation via their respective protein kinases or phosphatases, themselves controlled by “switchboard” kinases and phosphatases activated by specific receptors. In previous works we have analyzed the phosphorylation status of key enzymes in given physiological situations covering the finalities considered. A coherent set of phosphorylation of these enzymes was associated to the action of catabolic hormones (glucagon, epinephrine and cortisol). The phosphorylation status was opposite for anabolic hormones such as insulin or IGF. The conclusion was that in cancer, enzymes such as PK and pyruvate dehydrogenase (PDH) remained phosphorylated and blocked in their catabolic configuration, even if the cell had switched from catabolism to anabolism; as if the action of a switch board phosphatase had failed. In such condition, the cell had to rewire its metabolic pathways in order to by-pass the PK and PDH “bottle necks”, the resulting metabolism was favorable to its development at the detriment of other differentiated cells that become their nutritional reservoir; this perverted finality seems to be a starter for cancer. We shall first consider the status of major control switches, for the three major metabolic finalities and then analyze the unique hybrid configuration of these switches in cancer, showing how pathways are reconnected in mitotic stem cells, or in differentiated cells. It will become apparent that a non-coherent

mixed hormonal signal is received by new mitotic cells with new receptors, while differentiated cells with desensitized insulin receptors, resistant to insulin, will respond preferentially to catabolic hormones and to growth hormone (GH). We have in earlier works considered that a failure of the endocrine pancreas, would explain the aberrant hybrid hormonal signaling that supports cancer metabolism. Indeed, insulin secreting beta cells in the pancreas possess a major GABA control system that normally turns off glucagon releasing alpha cells and somatostatin releasing delta cells, when beta cells release insulin and GABA. The released GABA also closes via auto receptors of beta cells, the release of insulin. Thus a GABA deficiency would not only fail to turn off glucagon release when insulin is released, sending a dual hybrid glucagon-insulin message, but also maintain a steady leakage of insulin that desensitizes with time, insulin receptors. New mitotic stem cells with new insulin receptors, not yet desensitized for insulin will then respond to both insulin and glucagon, while other differentiated cells constantly submitted to an insulin leakage become resistant to insulin. The stem cells will have to rewire their metabolic pathways in response to the hybrid message; they gain a special metabolic advantage over differentiated cells that are simply plundered, since they only respond to the catabolic component of the hybrid message.

The carcinogenic mechanism proposed considers that stem cells committed to repair tissues after a variety of injuries and differentiated cells develop different metabolic features, if there is an associated GABA failure in the endocrine pancreas^[9,10]. The pancreatic GABA deficiency hypothesis that fully explained the observed metabolic rewiring in cancer was initially published in reference 9; the hypothesis was confirmed and strengthened by a set of epidemiological observations that were gathered in reference 10. These observations also included the more controversial role of some pesticides that may affect GABA, a point that was not particularly appreciated. The metabolic advantage given to stem cells by the pancreatic deficiency blocks their differentiation and maintains their mitotic capacity; while differentiated cells are plundered. Inevitable mutations follow, while the immune system becomes unable to eliminate a geometrically increasing number of altered stem cells, a selection of the most aggressive and metabolically successful population starts cancer. Presumably, this metabolic trigger for carcinogenesis starts years before cancer appears, correcting the GABA pancreatic failure, or neutralizing eventual auto-antibodies against glutamate decarboxylase (GAD), or suppressing its inhibition, might delay or avoid cancer. When cancer is declared present therapies might be backed-up by a metabolic treatment aiming to reverse the rewiring process that gave to tumor cells their metabolic advantage.

METABOLIC SWITCHES ORIENT PATHWAYS TOWARDS DIFFERENT METABOLIC FINALITIES

We represented in [Figure 1](#), the endocrine pancreas, with its beta, alpha and delta cells that secrete respectively insulin, glucagon and somatostatin (STH). The GABA release from beta cells inhibits alpha and delta cells (via GABA A receptors) when insulin is released; and puts an end to insulin release (via GABA B auto receptors). GABA also inhibits epinephrine release from adrenals. We describe with different colors the pathways that operate for the different metabolic finalities that a cell is susceptible to reach, and give in the corresponding colored boxes the status (increase +, decrease -, or 0 not relevant) of the selected switches. First finality: The production of nutrients; triggered by catabolic hormones, glucagon, epinephrine, and cortisol (yellow pathways and yellow box [Figure 1](#)). When food gets scarce, the pancreas senses the drop of blood glucose, hyperpolarized beta cells retain GABA, no longer spilled over alpha cells and their relative depolarization triggers the release of glucagon. The action of glucagon on liver glucagon Gs coupled receptors, or that of epinephrine on beta adrenergic receptors in muscles, stimulates adenylate cyclase and the synthesis of cAMP. The latter, activates PKA serine kinase then Src tyrosine kinase, eliciting in fine, via specific protein kinases, the phosphorylation of a set of enzymes that are ON or OFF after phosphorylation. Glycogen phosphorylase a, is ON, supporting glycogenolysis, while PK and PDH are OFF closing the entry of the citric acid cycle, this spares oxaloacetate (OAA) at the start of the neoglucogenic pathway; pyruvate carboxylase (Pcarb) and phosphoenolpyruvate carboxykinase (PEPCK) are both activated. The blockade of PK by phosphorylation, avoids a reconversion of phosphoenolpyruvate (PEP) into pyruvate (PYR). Muscle

proteolysis provides amino acids, the transamination of alanine feeds with PYR the neoglucogenic route. Since cAMP inhibits Fructose 2-6 bis phosphate (Fru2-6 bisP), it cancels the inhibition of neoglucogenesis and blocks glycolysis; glucose is now produced. We have to add that hypoglycemia decreases glycolysis in erythrocytes, which form less 2-3 DPG. Thus, oxygen is retained by hemoglobin and less is delivered and reduced by other cells, their NADH/NAD ratio increases, closing their citrate condensation that starts the Krebs cycle, more OAA goes to neoglucogenesis. Glycogenolysis is activated as well (phosphorylated phosphorylase a, is ON), while phosphorylated glycogen synthase is OFF. The other nutrient are ketone bodies; we know that PKA and Src activate a protein LKB1 by phosphorylation, it will stimulate AMP kinase, which inhibits acetyl CoA carboxylase (ACC) at the start of the fatty acid synthesis pathway. The inhibition of ACC decreases the product malonyl CoA, which is an inhibitor of the mitochondria carnityl transporter of fatty acids, allowing then fatty acid to enter in mitochondria. There, beta oxidation forms acetyl CoA, then ketone bodies, acetoacetate, particularly in liver. The malonyl CoA inhibition of the fatty acid transporter blocks the degradation of fatty acid when their synthesis is activated and vice versa. The ketone bodies are delivered to other cells, where they give back acetyl CoA by the reverse pathway (thiolase operating in both directions).

Second finality: the production of energy, and selection of glycolytic or fatty acid sources of acetyl CoA. Even when glucagon, epinephrine and cortisol are released for making glucose and nutrients, muscles can reverse the situation and keep their glycolytic pathway operational, with little or no neoglucogenesis; on the other hand, glycogenolysis is still possible (Figure 1 red pathways and red box). This muscle exception is presumably linked to the fact that muscles have to be ready for escaping predators. How are the effects of cAMP on neoglucogenesis cancelled? Muscle activity is associated to the movement of charges along T tubules until reticulum sacs with ryanodine receptors in the depth of the muscle, the depolarization releases calcium in the cytosol initiating muscle contraction. Calcium will also stimulate a phosphodiesterase (PDE) converting cAMP into AMP, which restores the synthesis of Fru2-6 bisP, the allosteric activator of glycolysis, and inhibitor of neoglucogenesis. In parallel, calcium stimulates calcineurin phosphatase, which dephosphorylates an inhibitor (I1) of a major phosphatase PP1 that gets activated, leading in fine to the dephosphorylation and activation of PK and PDH, which opens the glycolytic supply of acetyl CoA to the Krebs cycle. The citrate condensation starts, (the NADH/NAD ratio decreases via oxygen reduction). Then, since ATP is low and AMP elevated, the inhibition isocitrate dehydrogenase by ATP is cancelled and the citric acid cycle "turns", which decreases the citrate efflux from mitochondria and closes the fatty acid synthesis route (via ATP citrate lyase and ACC). The elevated AMP activates AMP kinase, which inhibits ACC; a low level of malonyl CoA may now open the beta oxidation of fatty acid and the fatty acid source of acetyl CoA, when glucose is missing. Well, exactly the same glycolytic energetic finality (red pathway Figure 1) will eventually operate in anabolism when insulin is released; because insulin elicits the activation of a phospholipase that splits phosphatidyl inositol (4-5) Bis P (PIP2) into diacyl glycerol (DAG) and inositol 1,4,5 Phosphate (IP3). The latter, will act on IP3 receptors of the reticulum, releasing calcium in the cytosol. Calcium stimulates as described above, a PDE neutralizing the inhibitory effect of cAMP on Fru2-6 bisP synthesis, and activates calcineurin, then PP1, PK and PDH, which stimulates glycolysis and opens the glycolytic entry of the Krebs cycle. But here is a new regulator to consider: DAG. If the level of DAG is low, presumably when DAG is converted into monoacyl glycerol, the glycolytic route forms acetyl CoA as above (Figure 1 second red box). However, if DAG increases, it will activate protein kinase C (PKC) and the latter elicits the synthesis of a new inhibitor of PP1 known as CPI 17, which keeps PK and PDH phosphorylated and inhibited, and closes the glycolytic supply of acetyl CoA. The fatty acid source of acetyl CoA will have to be opened. Since AMP is elevated, it stimulates AMP kinase, which inhibits ACC and the fatty acid synthesis pathway. With malonyl CoA being low, fatty acid beta oxidation is now permitted, opening the fatty acid supply of acetyl CoA, (Figure 1 black pathway and black box). The third finality: the anabolic synthesis of new constituents depends essentially on Insulin and IGF. It is represented by the green pathways, plus the glycolytic red pathway, and green box for switches. The effect of insulin on its tyrosine kinase receptor elicits anabolism: The synthesis of glycogen, of fatty acids and triglycerides (TAG), of

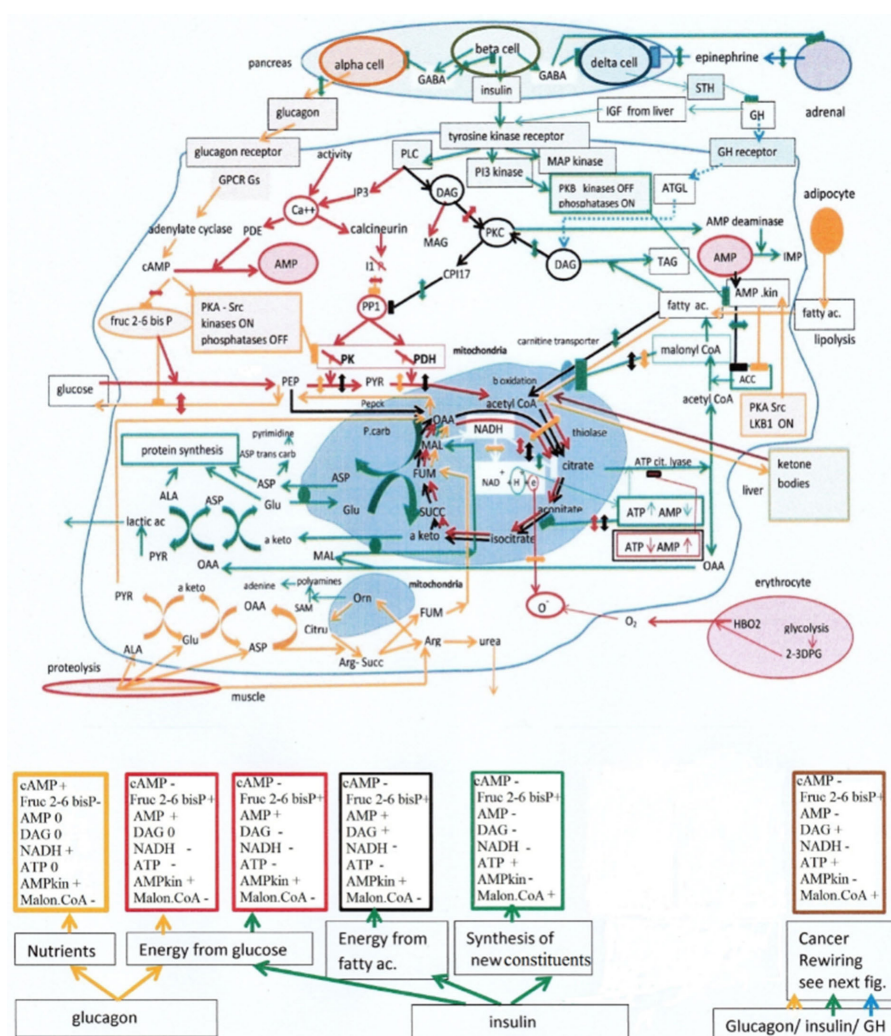


Figure 1. Top: endocrine pancreas, the alpha cell releases glucagon; it is turned off by GABA co-released with insulin from beta cells. In this way when anabolic insulin is ON catabolic hormones are OFF, GABA also inhibits epinephrine release from adrenals. Moreover, GABA turns off somatostatin release from delta cells, which stimulates growth hormone and IGF. The cell below the pancreas displays different metabolic finalities that can be reached; these are controlled by a few “switch compounds” themselves controlled by the endocrine pancreas. These compounds are listed in the boxes below the cell. The yellow pathways represent the nutrition finality forming glucose and ketone bodies; it is activated by catabolic hormones that control the switch compounds in the yellow box (+ is increase, - decrease, 0 no change or not relevant). The second and third metabolic finalities represented by the red pathways is the production of energy from glucose, in the case of glucagon or insulin, note in the corresponding red boxes the difference for diacylglycerol DAG. The next finality is the production of energy from fatty acids it is represented by the black pathways and the black box for switch compounds. The last finality represented by the green pathways and green box for the switches, is the anabolic synthesis of new constituents, it is essential for mitotic cells. The last box in brown shows for comparison the switches for mitotic tumor cells, specially studied in Figure 2. DAG: diacyl glycerol; PK: pyruvate kinase; PDH: pyruvate dehydrogenase; GH: growth hormone

proteins. Insulin also inhibits the opposite effects: glycogenolysis, neoglucogenesis, lipolysis and proteolysis mediated by catabolic hormones. Insulin stimulates glucose uptake, facilitating the incorporation of the glucose transporter. The influx of glucose in cells is also regulated by different affinities of liver glucokinase lower than the equivalent brain enzyme hexokinase; which serves the brain first with glucose. In parallel, insulin activates the MAP kinase mitotic and PI3kinase routes, supporting mitosis and cell survival (recall that each step of these pathways was up-regulated by viral or cellular oncogenes). We have mentioned above, the downstream stimulation by insulin, of a phospholipase C that generates IP3 and DAG. We recall that IP3 elicits the release of internal calcium, activating a PDE hydrolyzing cAMP, which cancels the inhibition of Fruc 2-6 bisP synthesis, leading to an increased glycolysis. Moreover, calcium helps the incorporation of glucose transporters in cells that have no constitutive transporter in their membrane. But the stimulation

of PKC by DAG will then stimulate AMP deaminase that converts AMP into IMP; which decreases AMP and the stimulation of AMP kinase, cancelling its inhibitory effect on ACC and fatty acid synthesis that gets activated. Moreover, insulin-mediated PKB effects are opposite to those of PKA that stimulated AMP kinase. One may then expect that the inhibition of AMP kinase (low AMP and inhibition by PKB) will open the fatty acid synthesis pathway; the malonyl CoA intermediate increases, closing the fatty acid carnityl transporter and the beta oxidation source of acetyl CoA. It is thus necessary to open back the glycolytic source of acetyl CoA that had been closed via DAG PKC stimulation forming the CPI17 inhibitor of PP1, which blocked PK and PDH. Now that the synthesis of fatty acids and triglycerides is activated by insulin, DAG will be converted into triglycerides and decrease, suppressing the stimulation of PKC by DAG and the CPI17 inhibition of PP1, which opens back PK and PDH and the glycolytic source of acetyl CoA (red pathway [Figure 1](#)), while the full anabolic effects of insulin take place. Note that the efflux of citrate from mitochondria is facilitated by the blockade of isocitrate dehydrogenase by ATP (the ATP/AMP ratio is elevated). When energy is missing, this ratio decreases, the citrate efflux from mitochondria declines, closing the citric supply to the fatty acid synthesis route, while the citric Krebs cycle starts turning again; the acetyl CoA may come again from fatty acid beta oxidation or from glucose, depending on the level DAG, as indicated in the corresponding black or red boxes.

Well, one of the keys for understanding the rewiring process taking place in cancer is the fact that tumor cells do maintain the synthesis of fatty acids and triglycerides, which automatically closes the fatty acid source of acetyl CoA, but tumor cells are unable to open back the glycolytic source of acetyl CoA; as if they were unable to decrease its DAG blocker, in spite of its conversion into triglycerides. A new supply of DAG kept PKC and CPI 17 stimulated, inhibiting PP1, then PK and PDH, which closes the glycolytic source of acetyl CoA. With these two sources of acetyl CoA blocked, the only possible way for tumor cells to get acetyl CoA and survive, comes from ketone bodies provided by liver cells. It is then essential to find out what keeps DAG elevated, in spite of its consummation by the active lipid synthesis pathway? Remember that carcinogenic phorbol-esters act similarly to DAG on PKC; this is discussed in the next section.

A CARCINOGENIC METABOLIC REWIRING PROCESS DESCRIBED IN STEM CELLS AND DIFFERENTIATED CELLS

We have again represented in [Figure 2](#), the endocrine pancreas but here is a blockade of GABA release, which might be the consequence of auto-antibodies against GAD that synthesizes GABA; remember that diabetes Type 1 is provoked by such antibodies that kill beta cells^[14,15]. The GABA deficiency may have other causes, vitamin B6 deficiency (it is the co-factor of GAD) or a GAD mutation as in Stiff person syndrome, or result from pesticides and compounds that affect GABA uptake (betel nut addictions) and metabolism^[10]. The most compelling fact in support of the hypothesis is that smoking (a major risk factor for numerous cancers, including pancreatic) impairs the GABA system. In brain, the increase of glutamate and decrease of GABA prolongs the activation of the dopaminergic rewarding sensation elicited by nicotine. In the periphery, the effects of smoking increase the systemic levels of epinephrine and norepinephrine. Whatever is the cause of the pancreatic GABA deficiency, it leads to an abnormal release of glucagon even when insulin is released, sending to cells a hybrid catabolic - anabolic message. There is also an effect on beta cells that become unable to terminate insulin release, a constant leakage of insulin will occur and desensitize insulin receptors, recalling much aspects of type 2 diabetes or metabolic syndrome. The GABA deficiency should increase somatostatin release from delta cell, but this is counteracted by an increased epinephrine release from adrenals due to the GABA deficit; thus, epinephrine strongly inhibits the delta cell and STH release, increasing the action of GH. It is not the same for alpha cells that are stimulated by epinephrine.

Below the GABA deficient pancreas, three cells are represented: the top one is a stem cell rewiring its metabolic pathways into a carcinogenic mode after receiving the hybrid hormonal message from the deficient pancreas, the cell below is a liver differentiated cell, rewiring its metabolism while receiving the hybrid hormonal message after becoming insulin resistant. The bottom cell is of a muscle fiber also receiving

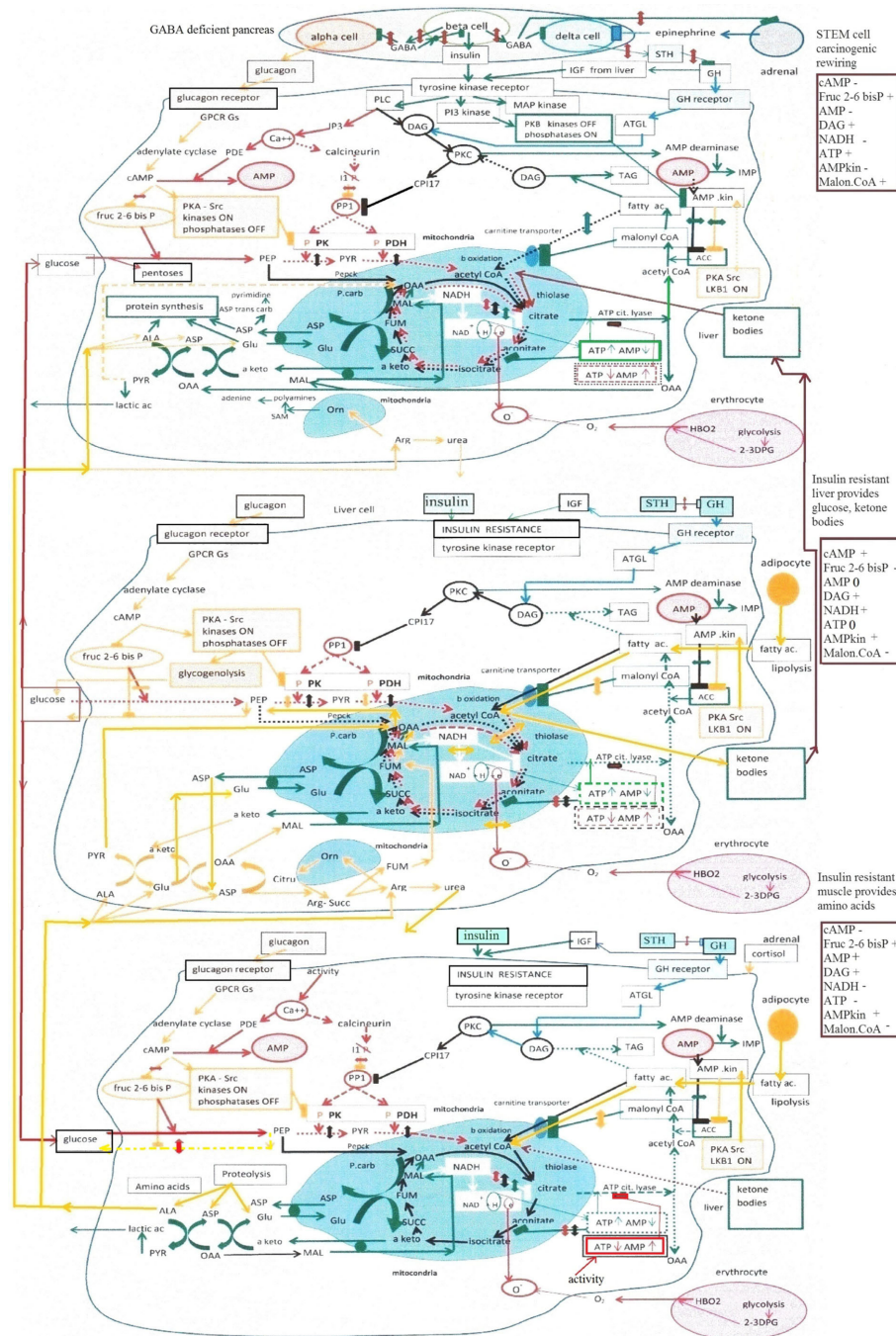


Figure 2. Top: endocrine pancreas with its GABAergic controls OFF. This leads to a leakage of insulin rendering differentiated cells resistant to insulin; in addition, GABA fails to block glucagon release when insulin is released, sending a hybrid catabolic-anabolic hormonal message. The GABA deficiency increases epinephrine release from adrenals, which inhibits delta cells and somatostatin, activating growth hormone. The first cell is a new mitotic stem cell with new insulin receptors it receives the dual catabolic - anabolic message. PKA, Src are activated closing pyruvate kinase and pyruvate dehydrogenase and the glycolytic supply of acetyl CoA; but the increase of IP₃ and calcium mediated by insulin, boosts glycolysis until the pyruvate kinase bottle neck, by increasing the fructose 2-6 bis Phosphate activator. Indeed, calcium stimulates a phosphodiesterase that decreases cAMP, cancelling its inhibitory effect on fructose 2-6 bis Phosphate synthesis. Note that growth hormone activates the production of DAG via ATGL, which stimulates PKC and CPI17, inhibiting PP1 phosphatase, which maintains the glycolytic bottle neck, in spite of the conversion of DAG into triglycerides. Insulin elicits fatty acid synthesis by inhibiting via PKB, AMP kinase, which suppresses the inhibition of acetyl CoA carboxylase; the automatic increase of malonyl CoA closes the fatty acid transporter and the fatty acid source of acetyl CoA. Since the glycolytic source of acetyl CoA was off as well, the stem cell becomes dependent of ketone bodies reconverted into acetyl CoA to feed their citrate condensation, and support the synthesis of fatty acid and lipids for new mitotic cells. Note the supply of amino acids the synthesis of proteins, the transamination, and the release of lactic acid. The next cell is a liver cell resistant to insulin; it receives the catabolic glucagon part of the pancreatic message and is programmed for making glucose and ketone bodies. The fatty acid synthesis is OFF (ACC inhibition by AMP kinase) while fatty acid supplied by adipocytes, form acetyl CoA by beta oxidation, the decreased citrate condensation inhibited by NADH, leaves more acetyl CoA for the ketogenic pathway. The increased NADH comes from a lower oxygen supply and reduction. PKC-CPI17 is stimulated by DAG coming from ATGL stimulated by growth hormone. Muscle, provides amino acids supporting neoglucogenesis, note the transamination the urea cycle. The bottom cell is a muscle cell, also insulin resistant, while receiving the catabolic message (through epinephrine). Muscle activity releases calcium in the cytosol stimulating a phosphodiesterase hydrolyzing cAMP, which cancels the inhibition of fructose 2-6 bis Phosphate formation, activating glycolysis. The glycolytic bottle neck, is maintained because the inhibitor CPI17 of PP1 is again formed via PKC stimulation by DAG, produced by ATGL activated by growth hormone. The citrate condensation is not inhibited by NADH (the latter decreases since lactic acid and myoglobin pull more oxygen). The citric acid cycle turns, activity lowers the ATP/AMP ratio. Cortisol activates proteolysis providing amino acids to the liver. Dotted lines are interrupted pathways. Blocks adjacent to each cell indicate the switch compound levels. DAG: diacyl glycerol; PK: pyruvate kinase; PDH: pyruvate dehydrogenase; GH: growth hormone; ATGL: adipose triglyceride lipase

the hybrid hormonal message with insulin resistance. The full lines are operational pathways; the dotted lines are pathways that have been closed. Each block adjacent to the cell gives the expected switch status in cancer: [Figure 2](#).

In the top stem cell, glucagon activates via GPCR Gs receptors, adenylate cyclase and PKA-Src signaling as for the nutritional finality, but insulin elicits the formation of IP₃ and DAG. The release of calcium mediated by IP₃ activates the PDE hydrolyzing cAMP into AMP, the associated increase of Fruc 2-6bisP stimulates glycolysis, while calcium triggers the incorporation of glucose transporters in the membrane, rendering the cell avid for glucose. But the increased DAG stimulates PKC and the synthesis of the CPI17 inhibitor of PP1^[16], which maintains the phosphorylation of PK and PDH and closes the glycolytic source of acetyl CoA. Accumulated above this bottle neck, PEP will be converted into OAA. Then, PKC will stimulate AMP deaminase and 5' nucleotidase^[17,18], which decreases AMP and the stimulation of AMP kinase; but here, AMP kinase is already directly blocked by PKB that is activated by the insulin signal. This blockade of AMP kinase cancels its stimulation by PKA-Src via LKB1. The inhibition of AMP kinase activates ACC, eliciting the synthesis of fatty acid, while the malonyl CoA intermediate increases and turns off the beta oxidation of fatty acids, which closes the fatty acid source of acetyl CoA. With the two sources of acetyl CoA closed, the only possible supply of acetyl CoA are ketone bodies provided by liver cells. Acetoacetate is taken up and reconverted back into acetyl CoA (via beta hydroxybutyrate dehydrogenase, ketoacyl CoA transferase and thiolase that are reversible). With OAA coming from PEP above the bottle neck via PEPCK, and acetyl CoA provided by ketone bodies, the citric Krebs cycle starts. The ketone bodies supply was the only way for these cells to survive. The citric condensation reaction is activated; since oxygen reduction pulls electrons from NADH converted to NAD, which removes the NADH brake over the citrate condensation reaction. Note that the release of lactic acid generates an acid gradient around the cell, pulling oxygen from erythrocytes via the Bohr Effect. The ATP/AMP ratio being elevated isocitrate dehydrogenase is inhibited favoring the citrate efflux from mitochondria, which feeds via ATP citrate lyase, and ACC, fatty acid synthase; and the synthesis of fatty acids, then triglycerides, to make new membranes for mitotic cells. The conversion of DAG into TAG should decrease DAG and put an end to the stimulation of PKC and CPI17 and to the inhibition of PP1 that closed the glycolytic source of acetyl CoA. But this does not occur in this case, because growth hormone activated by the decrease of STH mediated by epinephrine, stimulates an enzyme: adipose triglyceride lipase (ATGL) that forms more DAG, maintaining in this way the stimulation of PKC and CPI17, which closes the glycolytic acetyl CoA supply, via PP1 inhibition that fails to activate PK and PDH; (recall once more that phorbol ester carcinogens stimulate PKC as DAG). The release of epinephrine that blocked STH release from delta cells and increased GH is a consequence of the GABA deficiency that increases epinephrine release from adrenals.

Another point to mention is that the inhibition of AMP kinase discussed above, suppresses as well the inhibition of mTOR, which liberates a factor (eIF4E) from its binding protein phosphorylated by mTOR; this factor eIF4E will then initiate the synthesis of proteins. Amino acids are provided by muscle cells, and processed via the transaminases and the mitochondria shuttle, feeding also the synthesis of adenine and pyrimidine bases. The transamination chain forms pyruvate and in fine lactic acid. In addition, the avid uptake of glucose supports the pentose phosphate pathway, forming necessary nuclear components (ribose 5P) for mitotic cells. The box adjacent to the top cell gives the status of switches for this stem cell that rewires its metabolism into a carcinogenic way.

The next cell in [Figure 2](#) is a differentiated liver cell rendered resistant to insulin; this is due to the leakage of insulin resulting from the pancreatic GABA deficiency. However, this cell responds to the glucagon part of the hybrid pancreatic message. The Gs coupled glucagon receptor is activated, adenylate cyclase forms cAMP, and PKA-Src are operational; cAMP inhibits the formation of Fruc 2-6bis P, closing glycolysis while opening neoglucogenesis. In parallel, PKA-Src will phosphorylate and inhibit PK and PDH closing the entry of the citric acid cycle. The cell receives amino acids from muscle proteolysis; the amino acids follow the

transamination chain as indicated, forming pyruvate that feeds, via Pcarb and PEPCCK the neoglucogenic pathway. There is also an activation of the urea cycle. In parallel to neoglucogenesis, glucose is also produced by glycogenolysis (phosphorylase a, is ON). The transaminations are interacting as indicated in Figure 2, with the mitochondria shuttle. Note that PKA-Src activates LKB1, which stimulates AMP kinase, this closes the fatty acid synthesis pathway (AMP kinase inhibits ACC). Now fatty acids provided by adipocyte lipolysis, get transported in the liver cell mitochondria since malonyl CoA is low, their beta oxidation forms acetyl CoA. With the NADH being elevated, the citrate condensation is inhibited. Thus, acetyl CoA in excess will follow the ketogenic route giving acetoacetate. Note that growth hormone stimulates ATGL and DAG formation, which stimulates PKC and the CPI17 inhibitor of phosphatase PP1, maintaining the inhibition of PK and PDH, which closes the glycolytic entry of the citric acid cycle. The stimulation of PKC should activate AMP deaminase, decreasing AMP and the stimulation of AMP kinase, but AMP kinase is already efficiently activated by LKB1, which maintains the inhibition of the fatty acid synthesis pathway. Note also that the ATP/AMP ratio that controls the citrate efflux via the inhibition of isocitrate dehydrogenase by ATP is not operational here, since the above reaction, the citrate condensation, is already inhibited by an elevated NADH/NAD ratio. Presumably a low supply of glucose decreases 2-3DPG in erythrocytes, less oxygen is delivered and reduced by the liver cell and fewer electrons are pulled away from NADH, which inhibits the citrate condensation reaction. Probably, less lactic acid is released by liver cells, since pyruvate feeds the neoglucogenic route, rather than lactate dehydrogenase, which locally decreases the Bohr Effect and oxygen supply to the liver. This liver cell is programmed to feed the above stem cell with nutrients, glucose and particularly ketone bodies, while receiving amino acids from muscle, and fatty acids from adipose tissue.

The bottom cell in Figure 2 is a muscle cell; again, it is resistant to insulin like other differentiated cells, and receives the catabolic part of the hybrid pancreatic message. For muscles, epinephrine rather than glucagon, activates via Gs coupled beta adrenergic receptors, adenylate cyclase and PKA-Src. But here, there is a muscle specificity that has to maintain glycolysis as discussed earlier. Muscle activity releases calcium from internal stores, stimulating a PDE that hydrolyses cAMP, this cancels the inhibition by cAMP on Fruc2-6 bis P formation; the increase of this allosteric activator of glycolysis at the phospho fructokinase 1 step, boosts glycolysis, while calcium stimulates the membrane incorporation of the glucose transporter. In addition, the alpha epinephrine receptors of Go or Gi type are predominant in muscle limiting the increase cAMP, via Gs coupled beta adrenergic receptors. Calcium should also activate calcineurin phosphatase and neutralize the inhibition of PP1 by I1 in order to open back PK and PDH by de-phosphorylation, but here the activation of PKC by DAG forms the CPI 17 inhibitor of PP1 closing the glycolytic acetyl CoA supply. This DAG is formed by ATGL stimulated by GH; we explained above that the GABA deficit elicits the release of epinephrine that blocks efficiently delta cells, decreasing STH, which stimulates GH and ATGL. As occurred for the liver cell, the LKB1 stimulation of AMP kinase inhibits ACC and closes the fatty acid synthesis route, which automatically opens the beta oxidation of fatty acids into acetyl CoA. But in contrast to the liver cell, the citrate condensation is operational in muscle. It is activated by the decrease of NADH resulting from the increased reduction of oxygen. Indeed, the lactic acid released supports the Bohr Effect, which liberates more oxygen from oxy- hemoglobin in acid conditions. Moreover, muscle myoglobin pulls more oxygen into the muscle. The condensation of acetyl CoA coming from fatty acids, and OAA provided by PEP, above the PK bottle neck, forms citrate. The efflux of citrate will not feed the fatty acid synthesis, we have seen that the pathway was closed (via the activation of AMP kinase by LKB1) thus citrate will be processed by the Krebs cycle. Muscle activity consumes ATP and forms AMP; since ATP is lowered, it does not inhibit isocitrate dehydrogenase, the citric acid cycle “turns”. Another essential point is that cortisol stimulates proteolysis in muscle; indeed, glucagon increases ACTH release and the release of cortisol from adrenals. Note that the amino acids will feed the liver cell forming glucose, and the stem cell synthesizing its proteins; follow the interaction of the amino acids with the transaminases and mitochondrial shuttle, and the lactic acid release for both stem cells and muscle supporting locally a Bohr Effect. In liver cells, pyruvate will preferentially follow the neoglucogenic route, and less goes to lactate dehydrogenase.

CONCLUSIONS AND PERSPECTIVES

An overview of the carcinogenic mechanism proposed, considers that stem cells receive an anabolic message: “divide and make the constituents for new cells” in order to replace altered cells by whatever cause. These stem cells will then have to synthesize fatty acids and lipids for making new membranes. Fatty acid synthesis automatically closes via malonyl CoA, their beta oxidation, and shuts the fatty acid source of acetyl CoA. Since there is an associated pancreatic GABA failure, there is a catabolic message sent in parallel to insulin, and glucagon cuts the glycolytic supply of acetyl CoA as well, by eliciting the phosphorylating of PK and PDH. Normally this occurs in neoglucogenesis and should be reversed by the anabolic insulin message; but here, this does not occur. Insulin did stimulate via IP₃ and calcium the PDE that hydrolyses cAMP, which boosted glycolysis by increasing Fruc2-6 bisP, and calcium did stimulate glucose uptake by incorporating transporters in the membrane, but the end of glycolysis remains OFF, closing the glycolytic supply of acetyl CoA. The persistent blockade of the de-phosphorylation of PK and PDH and their inhibition, comes from an elevated DAG that maintains the stimulation of PKC and CPI 17 inhibitor of PP1, which fails to activate PK and PDH. What kept DAG elevated is again a consequence of the GABA failure. Indeed, the GABA deficiency increases epinephrine release as well, which turns off STH and thus activates GH; the latter will stimulate ATGL, which increases DAG that stimulates PKC, closing the glycolytic source of acetyl CoA. Since the two sources of acetyl CoA, from fatty acids, and from glycolysis are blocked, the mitotic stem cell will have to get acetyl CoA from ketone bodies, reconverted into acetyl CoA by the reversible ketogenic pathway. This is possible if liver cells are maintained in catabolism to form ketone bodies. Well, this is again a consequence of the GABA failure, because a persistent leakage of insulin desensitizes their insulin receptors, rendering differentiated cells resistant to insulin, while responding to glucagon. Adipocytes provide fatty acids that will form in the liver these ketone bodies. As for the muscles, which are also insulin resistant, they receive the catabolic message through epinephrine and cortisol, the beta oxidation of fatty acids operates in muscles, but since the citric acid cycle Krebs cycle “turns” in muscles, acetyl CoA condenses with OAA coming via PEPCK and forms citrate, rather than ketone bodies. In muscles an intense proteolysis takes place, they provide amino acids to stem cells for making their proteins, and to liver for neoglucogenesis. The adipocytes respond preferentially to the catabolic component of the hybrid pancreatic message (they become insulin resistant) and provide fatty acids to liver and muscle. The amino acids processed by the transamination chain form pyruvate, and lactic acid is released by stem cells and muscles. In liver, a large part of this pyruvate feeds the neoglucogenic route.

Thus, the new mitotic stem cells that have not been chronically desensitized for insulin receive the dual hybrid message and the metabolic advantage they gain over the differentiated cells that are plundered, opens the road for these stem cells to cancer. Their differentiation is blocked, their number increases geometrically, inevitable mutations select the most aggressive population, the immune system fails to eliminate such cells.

A comment of some perspectives might be useful; the finding that diabetes type 1 result from the auto-immune destruction of beta cells by anti-GAD antibodies is certainly a major discovery, particularly if we consider that GABA is an essential regulator in the endocrine pancreas. These anti-GAD antibodies were detected in most cases, and were even implicated in type 2 diabetes. An interesting attempt to prevent diabetes using a “GAD vaccination” for preserving beta cells, did not yet give the expected results. Presumably, the network of anti-GAD antibodies and anti-idiotypic antibodies is an equilibrium that is difficult to displace. There is still some condition to be found and hopefully a preventive vaccination will someday become efficient. This question is particularly relevant here, since we suspect that the GABA deficiency of beta cells in the pancreas is the starting point of a metabolic rewiring process of stem cells and differentiated cells that leads to the carcinogenic transformation of stem cells. Hence, preserving the pancreatic GABAergic system^[19] may not only prevent diabetes but presumably cancer as well. It would be interesting to look for eventual anti-GAD antibodies in cancer as it was done for diabetes. One may also

hope that neutralizing these antibodies would give protection. The GABA deficiency may have other causes, a vitamin B6 deficiency for example, since it is the co-factor for GAD. Indeed, conditions that inhibit GAD or neutralize vitamin B6 (pesticides, amines and others) have been found to be carcinogenic^[10]; this is also the case for diseases that genetically affects GAD. In Stiff person syndrome for example, the neurological symptoms prevail, but the cancer incidence is more elevated. It would be an interesting perspective to look for anti-GAD antibodies long before cancer develops and eventually find out procedures for neutralizing them, and develop “an anticancer vaccination”. It is certainly a difficult project, particularly if we consider the difficulty encountered for the diabetes type 1 vaccination attempt. Checking GABA metabolism and preserving this control system of the pancreas that depends of vitamins (B6) and nutrition, might decrease cancer incidence, particularly if its alteration by pesticides and other compounds that affect GAD is avoided. Moreover, it might be an interesting perspective to explore the pancreatic GABA system in relation to cancer, using noninvasive imaging methods for GABA as those developed for brain.

The other more practical perspective is to try to normalize the rewiring process that was presented in detail, even though many parts were deduced, and require more experimental work. A few compounds such as isocitrate, lipoic acid and others, gave encouraging results in animal cancer models (the list of compounds is given in previous publications)^[5,11,13,20]. However, only a small number of potentially useful compounds were tested, in spite of the fact that many more should be given for normalizing the complex rewiring processes associated to cancer. One would gain to suppress the metabolic advantage given to stem cells by cutting their supply of ketone bodies; or to block the effect of GH on ATGL, which increases DAG, inhibitors of ATGL have been developed^[21]; or to control the cAMP and AMP ratio, and AMP kinase^[22]; or the mitochondria citrate efflux^[23]; or to decrease the DAG activation of PKC, there are several PKC inhibitors discussed in reference^[13]; or to inhibit the fatty acid synthesis pathway. A final remark concerns the direct effects of GABA on pancreatic ductal adenocarcinoma and presumably on other cancers. Apparently, GABA B receptor activation mediates a decrease of adenylate cyclase that attenuates the progression of pancreatic cancer^[24]. This does not contradict the fact that a GABA deficiency of Beta cell fails to terminate insulin release, which desensitizes insulin receptors, while glucagon is released, leading to the described metabolic rewiring process that initiates cancer.

The best mixtures that will reverse the rewiring process still remain to be compared, in animal cancer models, then in properly built clinical trials. It is also essential to find an adequate diet and control the amino acid supplied, in order to attenuate the effects of the metabolic rewiring process in cancer; and then develop a metabolic treatment with non-toxic mixtures that would normalize cancer metabolism. In our opinion, this would be useful for backing up, if necessary, present cancer therapies.

DECLARATIONS

Authors' contributions

Israël M solely contributed to this study.

Availability of data and materials

Not applicable.

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Conflicts of interest

The author declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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A review of the effects of healthcare disparities on the experience and survival of ovarian cancer patients of different racial and ethnic backgrounds

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Abstract

Ovarian cancer (OC) is a serious condition that often presents at advanced stages and has high mortality rates, with the current mode of early-stage screening lacking sensitivity and specificity. OC often presents asymptotically, which renders early diagnosis difficult. Furthermore, many patients lack significant risk factors or family history of the disease. Five-year survival rates differ between patients with OC among racial, ethnic, and social groups as a result of different social barriers. This review article aims to present the currently existing data regarding health care disparities among OC patients of different ethnic, demographic, and socioeconomic backgrounds, and what next steps should be taken to better understand and eventually eliminate these potentially devastating health care disparities. Increasing data support the notion that a combination of genomic, socioeconomic status, social factors, and cultural differences lead to differential treatments and therefore health care disparities. While genomic and biological factors are important, language barriers, geographic and travel barriers, differences in comorbidity likelihood between populations, and different treatment plans seem to have a greater impact on 5-year survival rates of patients from diverse backgrounds. Language barriers limit a shared-decision model of care. Transportation limitations and geographic differences can lead to limited follow-up and insufficient care in resource and equipment restrictive settings. Patients with these barriers also tend to have a higher incidence of comorbidities that raise the mortality rate of OC. Further research needs to explore effective



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solutions to bridge health care disparities and understand why they occur.

Keywords: Ovarian cancer, healthcare disparities, public health, racial disparities, epidemiology, literature review

INTRODUCTION

Ovarian cancer (OC) is the most lethal gynecologic malignancy and tends to develop as a result of atypical cells in the epithelium of the distal fallopian tubes^[1]. Worldwide each year there are an estimated 200,000 women diagnosed with OC and 125,000 deaths from the disease^[2]. In the United States, the American Cancer Society estimated that in 2017, there would be 22,440 new cases of OC and 14,080 deaths associated with the disease^[3]. One in 78 American women will develop OC in their lifetime^[2]. Five-year survival rates vastly differ among early stage and late-stage disease. For instance, with advanced stage III and IV disease, in which disease has spread beyond the pelvis to lymph nodes or the abdominal cavity or lungs, the mortality rate is around 70% whereas in early stages when the disease is confined to the ovaries or pelvis mortality rates are much lower at around 10%-40%^[1,4,5]. Early diagnosis of OC when the disease is limited to the ovaries is difficult because, in early-stages, patients tend to be asymptomatic. This is further complicated by the lack of screening or diagnostic tests, which aid in early diagnosis^[1,4-6]. Early diagnosis is prudent since the mortality rate after treatment or interventional therapy of early-stage OC is much lower^[1,5,6]. By the time patients develop symptoms, such as abdominal pain or swelling, the vagueness of these symptoms often complicates and delays diagnosis. These symptoms tend to be attributed to aging, menopause, dietary changes, stress, depression or gastrointestinal issues. Patients with more apparent symptoms such as a pelvic mass, abdominal pain, bloating, abdominal swelling, early satiety or urinary symptoms often have a more advanced disease, with symptoms usually due to the development of large mass and ascites^[7]. This is further complicated by the fact that screening tests for OC, such as tumor markers or imaging, often lack both sensitivity and specificity. As a result, a physician's ability to detect OC in its early-stages is limited by the low-performance measures of these currently available screening tests^[5], leaving most patients undiagnosed. Patients with lack of access to tertiary medical care are more susceptible to late-stage diagnosis, associated with far higher mortality rates^[1,5,8]. The current standard is to test for high levels of cancer antigen 125 (CA-125), an ovarian cell glycoprotein^[5]. However, high levels of CA-125 tend to be correlated with late-stage OC^[5]. Eighty percent of patients with late-stage OC have elevated values of CA-125, whereas only 10% of patients with early-stage OC have elevated values of CA-125^[5]. Furthermore, certain histologic types of OC, such as mucinous or endometrioid type tumors may not have elevations in CA-125. Another factor limiting the utility of CA-125 screening is that high levels of CA-125 can be seen in a variety of other benign gynecologic and non-gynecologic malignant conditions. Non-malevolent conditions such as endometriosis, fibroids, and pregnancy can result in elevations in CA-125^[4]. Other malignant conditions, which can result in elevations in CA-125, are breast, pancreatic, lung, gastric and colorectal cancers^[4]. Additional diagnostic techniques such as ultrasound evaluation for masses, may aid in diagnostic precision but also lack sensitivity and specificity. Consideration of other risk factors for OC such as genetic susceptibility, strong family history, and nulliparity may influence the detection of OC in patients^[1,9]. At present, the standard tests and techniques to screen for this devastating disease are ineffective, and studies show that routine screenings are not recommended for the general population^[4,8-10].

Standard treatment for OC is a combination of surgery and chemotherapy^[4]. Surgical exploration is performed if there is sufficient suspicion for OC based on an initial evaluation. The goal of surgery is to confirm if malignancy is present and if so to proceed with surgical staging and cytoreduction. Per National Comprehensive Cancer Network (NCCN) guidelines, adjuvant therapy, which consists of platinum-taxane based combination chemotherapy, is necessary in most cases and depends on the stage of the disease^[5]. Women with clinical suspicion of OC should be referred to a gynecologic oncologist for counseling and surgical treatment. Evidence shows that prognosis is improved when a gynecologic oncologist performs surgical staging and cytoreduction^[1,2,6]. Unfortunately, despite initial therapy, the majority of women with

advanced-stage OC will relapse and require additional treatment. The likelihood for recurrence depends on many factors, including distribution of disease at initial presentation, the success of initial surgical cytoreduction, rapidity of CA-125 resolution, and treatment response after primary therapy^[5]. However, a predictive marker for recurrence has not been prospectively verified. Patients should be followed closely by a gynecologic oncologist to detect these signs of recurrence. The management of patients with relapsed disease varies based on the platinum-free interval or months since last platinum treatment. Platinum free interval has been shown to correlate with progression-free survival and overall survival, as well as response to subsequent treatment. The complexity of managing patients with relapsed disease underscores the importance of treatment plans to include a gynecologic oncologist. Mortality rates for advanced stage disease are around 70%, and these rates differ between patients with OC among racial, ethnic, and social groups could be a result of different social barriers^[1,4,5].

EPIDEMIOLOGY OF OC

OC is the sixth most common cancer in women and the seventh most common cause of cancer death^[11]. There is substantial geographic variation in OC incidence and mortality. Higher rates are seen in the United Kingdom, Northern Europe, Australia, and the USA, with lower rates observed in Asia, China, and Africa^[11]. A recent study examining the international assessment of OC incidence and mortality confirmed these findings, demonstrating the lowest incidence of OC in China, and the highest in Russia and the United Kingdom^[12]. Similarly, other studies have confirmed that countries with a predominantly Caucasian population such as Europe, the US, Canada, and Australia, have a higher incidence of OC and that the incidence is lower in countries with other ethnic groups such as Asia, Brazil, and Mexico^[12]. These findings are consistent with the fact that within the USA, rates of OC are higher among white women than black women^[11]. Incidence rates of OC in the US are also lower in Asian/Pacific Islanders, American Indians/Alaskan Natives that are also consistent with previous international studies. In the US the incidence rate per 100,000 women of OC is 13.5 in whites compared to 9.9 in Asian/Pacific Islanders, 10.6 in American Indians/Alaska Natives, and 10.0 in blacks and 11.6 in Hispanics^[12]. In other terms, compared to black and Hispanic women, the risk of OC is 40% greater in white women^[2]. The exact reason for this racial disparity in incidence is unknown however racial disparities in certain risk factors for OC may account for this variation. For instance, this variation in geographic incidence rates of OC may be attributed to genetic risk factors, such as the higher incidence of OC among women with Ashkenazi Jewish ancestry^[13]. Among women with OC with Ashkenazi Jewish ancestry, 40% have a mutation in *BRCA1* or *2* when diagnosed with OC^[2]. Other risk factors for OC such as obesity, endogenous or exogenous hormonal exposure, parity, and dietary factors such as smoking, alcohol use, caffeine consumption are highly related to cultural habits and lifestyle practices that differ across different ethnic groups and may account for this geographical variation^[2,12]. Racial discrepancies of rates on gynecologic surgery such as tubal ligation and hysterectomy, which are known protective factors against OC, may also account for some of these variations in OC incidence^[2]. Similarly, rates of breastfeeding and combined oral contraceptive pill use, also known protective factors against OC, are highly related to cultural practices and may differ across ethnic groups. Overall the incidence of OC has gradually declined in most developed countries such as North America and Europe since the 1990's^[14]. Conversely, less developed countries with recent economic growth and lifestyle changes have seen increases in incidence rates^[14].

DISPARITIES IN THE SURVIVAL AND EXPERIENCE OF OC PATIENTS

The overall difficulty of early detection, early diagnosis, and the subsequent optimum treatment for patients with OC is exacerbated by the social disparities that exist in underserved communities. These disparities, in turn, lead to differences in survival rates and treatment. The existing literature supports the hypothesis that the risk of all-cause mortality in African American OC patients is roughly 1.3 times higher when compared to Caucasian women with OC, even when the access to care is equal^[15]. The mortality rates have increased

specifically for African American populations over time^[15,16]. A study conducted by Srivastava *et al.*^[16] demonstrated that from 1992 to 2008 the 5-year survival rates for Caucasian women rose from 40.7% to 45%, yet 5-year survival rates for African American women declined over that same time period from 47.9% to 40.3%. A similar study found that the 5-year survival rate for African American women had fallen to 36% from 2006 to 2012, while all races combined had a 45% 5-year survival rate, with Caucasian women having the highest 5-year survival rate at 46%^[16]. This variation in survival rates appears to be due to a decreased likelihood of receiving guideline-recommended care^[17]. Other studies have suggested that after controlling for factors like socioeconomic status (SES), patients with the same stage of OC have similar survival rates^[3,18]. Studies suggest that patients of a lower SES are receiving care less in line with NCCN guidelines and have decreased access to preventative medicine and genetic testing, which could contribute to delayed presentation. However, most studies are limited by their inability to detect if a patient's SES and a delay of presentation to the clinic are correlated, which could, in turn, affect 5-year survival rates^[3,18]. The current literature on the different survival rates of OC between diverse populations and the frightening statistics facing our underserved communities introduces a series of compelling questions. Why are patients with low SES presenting to the clinics with a more advanced disease? What preventative factors (i.e., the difference in the fund of knowledge, access, communication and financial resources) impact these differences in survival? Are there certain factors that are creating more disadvantages for patients who are underserved than other factors? What can be done to effectively mitigate these disparities? Studies have tried to control for such factors, with little success in large part because a multitude of critical contributing confounding variables is at work, not just one sole factor that is key in precluding underserved communities from attaining equal outcomes^[18,19]. Currently there is a need for increased randomized control studies and interventional studies that could shed more light on the impact of these social determinants of health, however the challenge is to isolate certain variables in these studies and have a sample size large enough to draw impactful conclusions.

GENOMIC DISPARITIES AMONG OC PATIENTS

The lifetime risk of developing OC is low, at 1.5%, and the vast majority of patients who develop OC comprise sporadic cases with no significant family history of the disease^[1]. In contrast, the remaining group of patients with deleterious genomic germline mutations (i.e., *BRCA1* and *BRCA2* genes) carry a much greater lifetime risk for breast cancer and OC (i.e., 50%-85% and 20%-40%, respectively)^[1,20-23]. Patients with a known genetic susceptibility to OC are typically followed closely with a combination of pelvic examination, ultrasound imaging, and CA-125 testing in order to detect abnormalities at early curable stages. These patients are also typically offered risk-reducing surgery, which consists of removal of the fallopian tubes and ovaries at the completion of childbearing in order to prevent the onset of disease. Patients in underserved communities may have lack of access to this type of preventive medical care. Among these OC patients with deleterious germline mutations, a significantly decreased overall survival and the worse prognosis is reported in OC patients from underserved populations^[24-26]. Bandera *et al.*^[27] reported an increased OC risk, associated with a worse survival rate, in underserved communities after controlling for confounding variable. Factors, such as medical comorbidity risk, and prevalence of *BRCA1* and *BRCA2* mutation, pointing instead to other contributing etiologic factors, such as quality of care and delay in care, to explain the significantly poorer outcome. Although a higher incidence of unmanaged medical comorbidities (i.e., hypertension, coronary artery disease, and diabetes) may be associated with an increased OC risk, these medical comorbidities alone cannot explain the poor outcomes in underserved populations^[16,27-29].

PUBLIC HEALTH, SOCIAL DISPARITIES, AND LANGUAGE BARRIERS AFFECT OUTCOMES IN OC PATIENTS

High adiposity and inflammatory diets consisting of high sugar intake are associated with an increased risk of developing OC^[16,27-30]. A systematic review of 28 studies reported a statistically significant, association between obesity [body mass index (BMI) 30 kg/m² or more] and OC^[31]. Another large prospective study

found that the risk of death from OC was higher in women with the greatest BMI (35-40 kg/m²) compared with those of normal weight (BMI 18.5-24.9 kg/m²)^[32]. African American and Hispanic women have a disproportionately higher rate of obesity and have higher BMI (> 40) which may correlate with a higher incidence mortality of OC^[16,27,28,33]. A correlation also exists between obesity and socioeconomic position, suggesting that more overweight and obese people are from underserved populations with poorer access to healthcare^[34]. In addition to limited access to quality healthcare, obese patients may be more difficult to diagnose with OC due to greater difficulty in assessing vague symptoms and limited utility of current diagnostic approaches in these patients. Studies indicate that women who were considered overweight and obese had symptoms such as abdominal swelling and discomfort months before the diagnosis of OC^[35]. Healthcare providers can impact meaningful change through education to patients of the positive impact of a low inflammation diet. Similarly, patient education regarding weight loss through healthy diet and exercise can meaningfully impact patient's health and reduce risk factors for OC.

Patients with a limited English proficiency have lower health literacy rates, less access to healthcare systems, and poorer outcomes^[36]. Many of these patients use ad-hoc medical interpreters, such as staff, friends or family members, who are not officially licensed to serve as an interpreter for a clinical interaction. The use of an ad-hoc interpreter leads to miscommunication and is ineffective and result in poor patient outcomes. One study confirmed that the use of an ad-hoc interpreter leads to a higher rate of error than using no interpreter at all^[37]. Schwei *et al.*^[36] reported that patients rarely, if ever, request interpreter services. Even the use of trained medical interpreters may result in impaired physician-patient communication. Kamara *et al.*^[38] analyzed 24 audio recordings of cancer genetic counseling consultations from 13 different patients and six different counselors conducted in Spanish with the assistance of 18 telephone interpreters at two large public hospitals^[38,39]. The primary causes of impaired communication through the use of interpreters were erroneous hypothetical explanations, and misinterpretation by the interpreter, resulting in a significant departure from the shared-decision model of healthcare^[37,38,40]. The interpretations did not assist patients in their decision-making process. Furthermore, physicians found that even with the use of interpreters it was difficult to understand the decision of a patient. There were instances in which interpreters did not interpret a patient's response or question, a marked departure from a high-quality mutual discussion, and a shared-decision model of healthcare^[36-38,40]. Therefore, patients often consented to the provider's recommendations regardless of their comprehension of the treatment plan and limiting patient autonomy. Low English proficiency patients are more likely to have a limited health literacy and increased dissatisfaction with the health system overall^[36-38,40]. These data suggest that public health, social disparities, and language barriers negatively impact outcomes in OC patients.

GEOGRAPHIC AND TRAVEL FACTORS THAT IMPACT OC PATIENTS

Disparities also exist in patients' geographic environments, impacting patient outcomes. Patients from lower socioeconomic communities have access to fewer clinics, with fewer resources and compromised diagnostic capability^[19,29]. Sakhuja *et al.*^[19] reported that African American OC patients lived in geographic areas that had fewer oncology centers and fewer centers with ultrasound, and fewer subspecialists^[1,5,19,29]. Statistics have consistently shown that patients with OC are best treated by a gynecologic oncologist and that tertiary care medical centers have the resources and capabilities to better serve these patients. Lack of access to subspecialty care negatively impacts survival from OC. Hence, the likelihood of a patient presenting to a physician with insufficient clinic resources is statistically much greater in a lower socioeconomic setting. Moreover, OC patients who are presented to general hospital clinics have greater odds of late-stage diagnoses and increased mortality rates than those who present to subspecialty clinics^[18,19].

Srivastava *et al.*^[16] reported that only 22% of Caucasian and 14% of African American OC patients would be willing to drive more than 20 miles for treatment, which also correlates with data suggesting that the larger the distance between a patient and their gynecologist, the greater the mortality rates^[16,41,42]. Guidry *et al.*^[43] surveyed 593 cancer patients in Texas, of which 38% of the Caucasians surveyed, 55% of

the African Americans, and 60% of the Hispanic respondents reported that they had poor access to a vehicle^[16,41,43]. The net result to the underserved communities was geographic and transportation barriers, greater reschedules, no-shows, delayed care or missed or delayed medication use^[16,41]. Hence, these geographic and transportation factors lead to delivery of substandard care and negatively, impact survival rates^[19,29,41]. Across 25 different studies, patients reported that transportation was a major barrier to their healthcare^[41]. These data indicate that geographic and travel factors have a negative impact on OC patient outcome.

DIFFERENT OC PATIENTS RECEIVE DIFFERENT TREATMENT

Patients from different socioeconomic backgrounds and cultures experience a difference in quality treatment. African American women from underserved communities overall have lower 5-year survival rates from OC than their Caucasian counterparts, attributed to treatment delays^[16]. Bristow *et al.*^[44] reported that despite equitable access to healthcare, patients who hailed from lower SES status were less likely to receive treatment adhering to the NCCN guidelines^[29]. Furthermore, a clear difference exists between populations regarding access to quality of healthcare, impacting the outcome of an OC patient. To understand the source of healthcare disparities is the key in order to pursue strategies to correct these disparities and eventually create initiatives to eliminate them.

An increasing body of literature suggests that patients from a lower SES receive substandard care, in large part from a general lack of proper facilities and equipment to carry out standard procedures^[1,16,29,44]. Unfortunately, patients from underserved communities, have poorer access than affluent communities to high-quality healthcare^[19,29]. African American and Hispanic patients were less likely to receive accurate staging for OC than Caucasian patients^[29]. African American patients with OC also tend to receive less proactive treatment plans than Caucasian patients^[16,19]. A study conducted at a large, high volume medical center showed that African American OC patients were less likely to undergo surgery than Caucasian OC patients overall, 61% and 77% respectively, and less likely to undergo chemotherapy^[16,19,29]. Furthermore, underserved patients are much less likely to enroll in clinical trials and experimental treatments. In a study that analyzed over 400 clinical trials from The Gynecologic Oncology Group (GOG) from 1985 to 2013, 83% of participants were Caucasian, and only 8% African American, and 2.2% Hispanic^[29,42,45]. As compared with the expected Centers for Disease Control projections for minority participation in clinical trials, actual African American participation was 15 times lower than expected for ovarian GOG trials^[45]. The GOG also reported that the rate of participation for African American patients has been decreasing over time, demonstrating that participation from African American communities has decreased 2.8 times from 1994-2002 to 2009-2013^[45]. There are many possible explanations for poor participation by minority groups in clinical trials include lack of access to centers, which offer these trials, lack of understanding regarding the purpose and potential benefits of these trials, and lack of means such as transportation to effectively participate in these trials. Due to decreased levels of education, patients may have diminished understanding of how the treatment works and therefore cannot understand how these treatments are different from previous ones and might have decreased efficacy. Additionally, cultural and spiritual differences in these communities could also contribute to the disparity. Members of these communities have been spurned by healthcare institutions and other institutions in the past, which could result in an overall distrust in providers or healthcare institutions. All of these aspects stack the odds against minority communities from receiving proper treatment.

Armstrong *et al.*^[46] reported that only 37% of patients received genetic counseling prior to testing for the *BRCA1/2* gene, and of those that did receive counseling, most were Caucasian women (69%) who were married (76%), with high income (55%) and with a college degree (81.4%)^[1,46]. African American women have a much lower rate of testing particularly in limited-resource settings, which is important because they have a similar or higher incidence of *BRCA* mutation than their Caucasian counterparts^[1,20-23]. It is estimated that roughly 12.4% of African American patients with breast cancer under 50 carry the

mutation^[1]. Limited resource settings are less likely to have a genetic counselor, and therefore patients are less likely to receive counseling support. All patients with OC should receive genetic counseling according to the NCCN guidelines. By undergoing genetic testing and genetic counseling, care providers can have a better understanding of the type of tumor that a patient is presenting with^[21,38]. If the patient has a known *BRCA1* or 2 mutation, then a provider has increased indications of what targeted therapies may work^[1,20-23,26]. A *BRCA1* or 2 mutation may result in errors with homologous recombination mechanisms in the DNA repair system, consequently there are downstream genes that are more likely to be mutated as well. These downstream genes have been studied extensively and as a result, target-therapies have been developed targeting these mutations. Additionally, patients who receive genetic counseling and genetic testing have increased understanding of their cancer, which can help make more informed decisions when it comes to their care^[21,38]. They have increased understanding about the progression of disease, their risks of developing cancer and their risks of passing this mutation onto their offspring. By providing this information, the decision-making model is shifting from one of paternalism to a shared-decision model, and allows for patients to be much more cognizant of early signs of developing cancer to begin treatment earlier, engage in more preventative treatment measures, and make care decisions in a timely manner that are in line with their values^[1,38]. However, in this case patients with decreased SES and therefore decreased access to genetic testing and counseling providing care teams have less information on base treatment, which essentially limits patient involvement in their own care^[1,19]. Proper genetic counseling prior to testing is a critical component of delivering this test and understanding the results.

Peres *et al.*^[47] reported that women taking an aspirin regimen for cardiovascular diseases or a daily nonsteroidal anti-inflammatory drug for arthritis had a decreased risk of developing epithelial OC, which stood at 44%, and 26%, respectively. However, a significant disparity still exists between the compliance rates for Caucasian and African American populations (44% vs. 29%, respectively) for these regimens^[48]. Decreased compliance with these medications reduces the protective factor of these agents against OC. African American and Hispanic women are much less likely to use oral contraceptives, a known reducer of OC risk^[1,49]. These data indicate that patients from underserved communities receive less preventive care than other patients.

CONCLUSION

Eliminating healthcare disparities is critical in order to ensure optimal outcomes in all patients with OC. Identifying what the healthcare disparities are is critical to their elimination. A paradigm shift, which leads to redistribution in the allocation of healthcare resources to create more equality across populations, will eliminate healthcare disparities.

Future research must focus upon the underlying genetic components that contribute to healthcare outcomes. Research that will elucidate tumor and population-specific molecular modifications to genes and proteins may positively impact the outcomes of patients with OC. The contribution of changes in dietary considerations (i.e., low sugar), language barriers, and geographic differences to the elimination of healthcare disparities requires additional research.

Physicians can impact the elimination of healthcare disparities through patient education (i.e., dietary practices), effective use of interpreters, and outreach to resource-poor communities with less access to high-quality healthcare. Population data demonstrate that the allocation of important equipment and resources (i.e., ultrasound machines, special genetic counselors) to support community primary care physicians, and the number of obstetric and gynecological specialists in the community will favor a lower incidence of late-stage diagnosis of OC^[19]. Patients hailing from lower SES and underserved communities may be at an additional disadvantage when they are excluded from promising investigational clinical trials. Hence,

the concept of the mobile clinic may bring high-level care directly to communities obviating the need for patients to travel long distances to find high-quality healthcare^[19].

In the short-term and long-term, a robust and sustainable solution is the creation of an inclusive culture that supports the elimination of healthcare disparities and discrimination among different racial, ethnic and social groups^[50]. The net result would be the replacement of patient isolation in underserved communities with access to quality healthcare, including access to promising clinical trials to treat cancer.

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Authors' contributions

The authors listed equally contributed to the concept, design, data collection, drafting, revision, and approval processes of this literature review piece.

Availability of data and materials

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Original Article

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Sequential treatment with doxorubicin and zoledronic acid has no additive effects in an aggressive model of established bone metastases

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Abstract

Aim: Bisphosphonates are used as an adjuvant treatment in breast cancer bone metastasis patients, often simultaneously with chemotherapeutic agents. Interestingly, their sequential combination has been reported to have synergistic anti-tumor effects on bone metastases in preclinical models. We studied the effects of doxorubicin (DOX) and zoledronic acid (ZOL) and their combination on established bone metastases in the MDA-MB-231(SA) GFP bone metastasis model.

Methods: Tumor burden and osteolytic bone lesions were quantitated by fluorescence imaging and radiography, respectively. The mice were randomized in four groups receiving vehicle, DOX, ZOL or both DOX and ZOL in a sequential combination on day 14. Serum marker of osteoclast number was followed weekly, and blood ionized calcium was measured at sacrifice. Bone and tumor area, apoptosis and proliferation of tumor cells were analyzed from histological sections.

Results: ZOL prevented hypercalcemia and osteolytic lesion progression, whereas DOX induced apoptosis in the MDA-MB-231(SA)GFP cells. However, neither of the treatments alone nor in sequential combination were able to reduce tumor burden in bone. Furthermore, no additive effects on tumor cell apoptosis were observed in the



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combination group.

Conclusion: No additive effects in combination of DOX and ZOL were observed in this aggressive model of breast cancer bone metastasis.

Keywords: Combination treatment, breast cancer, bone metastasis, MDA-MB-231(SA), doxorubicin, zoledronic acid

INTRODUCTION

Despite major advances in the treatment of primary breast cancer within the past decades, treatment of advanced breast cancer with bone metastases is still palliative in nature, as no curative treatments are available. Inhibitors of bone resorption, such as zoledronic acid (ZOL) or denosumab are used as adjuvant treatment in breast cancer patients with bone metastases, often simultaneously with chemotherapeutic agents such as doxorubicin^[1,2]. In these patients bisphosphonates decrease bone pain and skeletal-related events, but do not increase the overall survival^[3]. However, before the formation of bone metastases, bisphosphonate treatment in combination with standard chemotherapy may prevent the occurrence of bone metastases in postmenopausal, early stage breast cancer patients^[4]. Many preclinical studies in established experimental breast cancer bone metastasis models have reported direct anti-tumor effects of bisphosphonates alone, such as increase of apoptosis in intraosseous tumors and improved survival^[5-7], but we have reported no effects on survival in a model of established bone metastases^[8].

Bisphosphonates seek and incorporate into the hydroxyapatite in bone matrix. Therefore, it has remained an unanswered question how tumor cells in soft tissues can be exposed to effective concentrations of bisphosphonates. It is also possible that what was believed to be a direct anti-tumor effect is indeed, indirect. It has been shown that macrophages take up bisphosphonates in cytotoxic quantities, and tumor growth in soft tissues can be inhibited due to the lack of tumor-associated macrophages^[9,10].

Some exploratory preclinical studies investigating the effects of combinations of aminobisphosphonates and chemotherapeutic agents have suggested additional benefits. Sequential treatment with doxorubicin (DOX) followed by a single dose of ZOL 24 h later was reported to decrease tumor volume and proliferation and to increase apoptosis as well as mouse survival with established bone metastases with MDA-MB231/BO2 breast cancer cells^[11]. In addition, a single cycle of sequential administration of DOX and ZOL in a very early phase, 2 days after cancer cell inoculation, was reported to decrease intraosseous tumor area in an intracardiac mouse model using MDA-MB231 cells with green fluorescent protein (GFP)^[12]. Three additional studies by the same group with MDA-MB436 breast cancer cells showed synergistic effects on tumor volume reduction with the weekly administration of DOX and ZOL in combination, both on bone metastasis and primary tumor^[13-15]. In these studies, the combination treatment reduced the intraosseous tumor area and increased survival compared to DOX or ZOL monotherapy. The mode-of-action of the combination is not understood, but increased uptake of ZOL by tumor cells was observed with the sequential combination^[14]. Because of the varying results of anti-tumor effects of bisphosphonates, we felt a need to investigate these effects in a fairly aggressive and widely used model of breast cancer bone metastases, utilizing the human MDA-MB231(SA) GFP cells in nude mice. The treatment schedule was otherwise the same as used by Ottewell *et al.*^[13], but the treatment was started 3 days earlier to accommodate the more aggressive model.

METHODS

Compounds and cell line

DOX (Sigma-Aldrich, Saint Louis, MO, USA) was dissolved in saline (0.9% NaCl, Baxter, Deerfield, IL, USA) to concentration of 0.5 mg/mL. ZOL (Zometa®, Novartis Pharma GmbH, Basel, Switzerland) infusion

concentrate (0.8 mg/mL) was diluted in saline to obtain the concentration of 0.02 mg/mL. Saline was used as a vehicle control. MDA-MB231(SA)GFP^[8] were authenticated using short tandem repeat analysis (GenePrint10 system, Promega, Madison, WI, USA) at Institute for Molecular Medicine Finland (FIMM, Helsinki, Finland).

***In vivo* study**

The animal experiment was approved by the Animal Experiment Board of Finland and performed according to European Union directive 2010/63/EU. Five-week-old female athymic nude mice (Hsd: Athymic nude-nu, Harlan Laboratories B.V./Envigo, Horst, The Netherlands) were used. The mice were housed in groups of five in Scantainer™ (Scanbur, Karlslunde, Denmark) and fed an autofluorescence-reducing chlorophyll-free purified diet (AIN-76A, Special Diets Services, Witham, UK). The animals were weighed three times per week and daily during the last five days. On day 0, 10⁵ MDA-MB231(SA)GFP breast cancer cells were inoculated into the left cardiac ventricle of the animals, as described previously^[16,17]. The mice were anesthetized with 4 mg/kg xylazine (Rompun Vet 20 mg/mL, Bayer Animal Health GmbH, Leverkusen, Germany) and 75 mg/kg ketamine (Ketalar 10 mg/mL, Pfizer, NY, USA) intramuscularly (im) for inoculations and imaging procedures. Buprenorphine (Temgesic, Invidior UK Ltd, Slough, UK) was used as analgesic at 0.1 mg/kg subcutaneously (s.c.) and was administered once before the intracardiac inoculations and twice a day during the last five days of the study. The mice were randomized into four treatment groups (*n* = 8) on day 14 based on tumor growth and osteolytic lesion size as well as body weight. Tumor growth and osteolytic lesion size were measured by fluorescence imaging and X-ray analysis, respectively, on study day 14 and at sacrifice. The mice were sacrificed on day 25, except the 2-3 mice per group, which were sacrificed earlier due to weight loss over 20% or paraplegia. All the mice were included in the endpoint analyses. The mean day of sacrifice was very similar in all groups ranging from 24.13 in the DOX group to 24.63 in the ZOL group.

The control group received vehicle 5 mL/kg s.c. on day 15 and intraperitoneally (i.p.) on days 15 and 22. The DOX group received half of the maximal tolerated dose, DOX 2.5 mg/kg i.p. on days 15 and 22. The ZOL group received a single dose of ZOL 0.1 mg/kg s.c. on day 15. The DOX + ZOL group received DOX (2.5 mg/kg i.p.) on days 15 and 22 and a single dose of ZOL (0.1 mg/kg s.c.) 24 h after the first dose of DOX, i.e., on day 16 (schedule of the combination group dosing is shown in [Figure 1](#)).

Blood samples were collected from saphenous vein to 100 µL Microvette serum tubes (Sarstedt AG&Co, Nümbrecht, Germany), on days 1, 9, 17 and 24. Terminal blood samples were obtained by cardiac puncture. Five intact animals of the same strain and age were sacrificed on day 25 in order to obtain the baseline level for blood Ca²⁺ (ionized calcium). Femur and tibia were collected for histomorphometric analyses.

Fluorescence imaging

Metastatic growth of disseminated breast cancer cells was followed by fluorescence imaging using the LT 9 GFP imaging system LT-MACIMSYSPUSC (Lighttools Research, Encinitas, CA, USA) on days 10, 14 (randomization), and after sacrifice. Autofluorescence was reduced using the chlorophyll-free diet. The animals were anesthetized and imaged in both the ventral and dorsal position. The imaging settings (exposure 2.34 s, gain 6.7 and offset 238) were the same for all animals. The area of fluorescence was determined from the images using MetaMorph™ software (Molecular Devices, Sunnyvale, CA, USA).

Radiography

Development of bone lesions was monitored by radiography on days 10, 14 (randomization), and prior to sacrifice. The animals were anesthetized and radiographed (31 kV, 10 s, 2× magnification) in a prone position using the faxitron specimen radiographic system MX-20 DC-2 and Specimen Radiography Beta 2.0.0 software (both from Faxitron Bioptics, LLC, Tucson, AZ, USA). Lesion number and area were determined in tibias and femurs and the resulting data were analyzed using MetaMorph™ software.

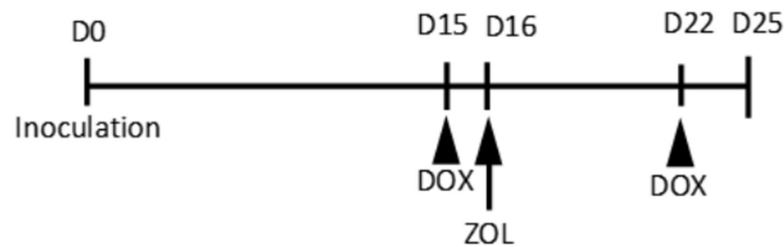


Figure 1. Dosing schedule of the doxorubicin (DOX) + zoledronic acid (ZOL) group

Histomorphometric analyses

After fixation in 10% neutral buffered formalin and decalcification in 10% ethylenediaminetetraacetic acid for two weeks, the bone samples were processed to paraffin blocks. Four μm thick midsagittal sections were obtained from the left hind limb (tibia and femur) and stained with hematoxylin, eosin, Orange G and phloxine B (HE + Orange G)^[16], tartrate-resistant acid phosphatase (TRAP)^[6], Ki-67 (primary antibody clone SP6, Nordic BioSite AB, Täby, Sweden) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) methods (*in situ* cell death detection kit, APTM, Roche Diagnostics GmbH, Basel, Switzerland). Tumor area as well as trabecular and cortical bone areas were analyzed from the HE + Orange G stained slides as follows: first, micrographs were taken with a Leica DM4000 B Research Microscope (Leica Microsystems, Wetzlar, Germany), then brightness and contrast were optimized using the same settings for all images. Next, the tumor area was quantitated by drawing and bone area was quantitated by color thresholding using MetaMorphTM software as described^[18]. Areas expanding 5 mm from the articular surfaces of femur and tibia were analyzed. From a TRAP stained section, the number of osteoclasts at the tumor-bone interface in distal femur and proximal tibia was counted as described^[18], the whole section was analyzed using the MetaMorphTM software. Apoptotic cells in tumor were manually counted as TUNEL stained cells with apoptotic morphology as described^[6]. The whole tumor area was analyzed using a 20 \times objective Leica DM4000 B Research Microscope.

Biomarker analyses

Ca^{2+} concentration (corrected to pH 7.4 by the internal algorithm of the instrument) in whole blood was determined using a blood gas analyzer (ABL835 Flex blood gas analyzer, Radiometer Medical ApS, Bronshøj, Denmark) immediately after terminal bleeding. A comparison to the normal level of ionized calcium was performed using the values obtained from five intact animals of the same strain, sex and age. Serum tartrate-resistant acid phosphatase 5b (TRACP 5b) activity as a marker of osteoclast number was measured in serum samples obtained on days 1, 9, 17 and 24 using the MouseTRAP kit (IDS, Boldon, UK).

Statistical analyses

Statistical analysis was performed with SPSS (version 14.0). All statistical analyses were performed as two-sided tests and $P < 0.05$ was considered statistically significant. The normality of residuals and homogeneity of variances were examined using Kolmogorov-Smirnov and Levene's tests, respectively. If these assumptions were fulfilled as such or after appropriate transformation, one-way ANOVA followed by Dunnett t -test was used. If the assumptions were not fulfilled even after transformation, the non-parametric Kruskal-Wallis test followed by Mann-Whitney U -test was used. Fischer Exact test was used for comparison of proportions.

RESULTS

Effects of DOX and ZOL on body weight and tumor characteristics

Body weight of the mice decreased during the last five days of the study due to the disease progression. Although the weight loss was more pronounced in the DOX treated groups, statistically significant differences compared to vehicle group in the body weight change from day 0 were not observed [Figure 2B].

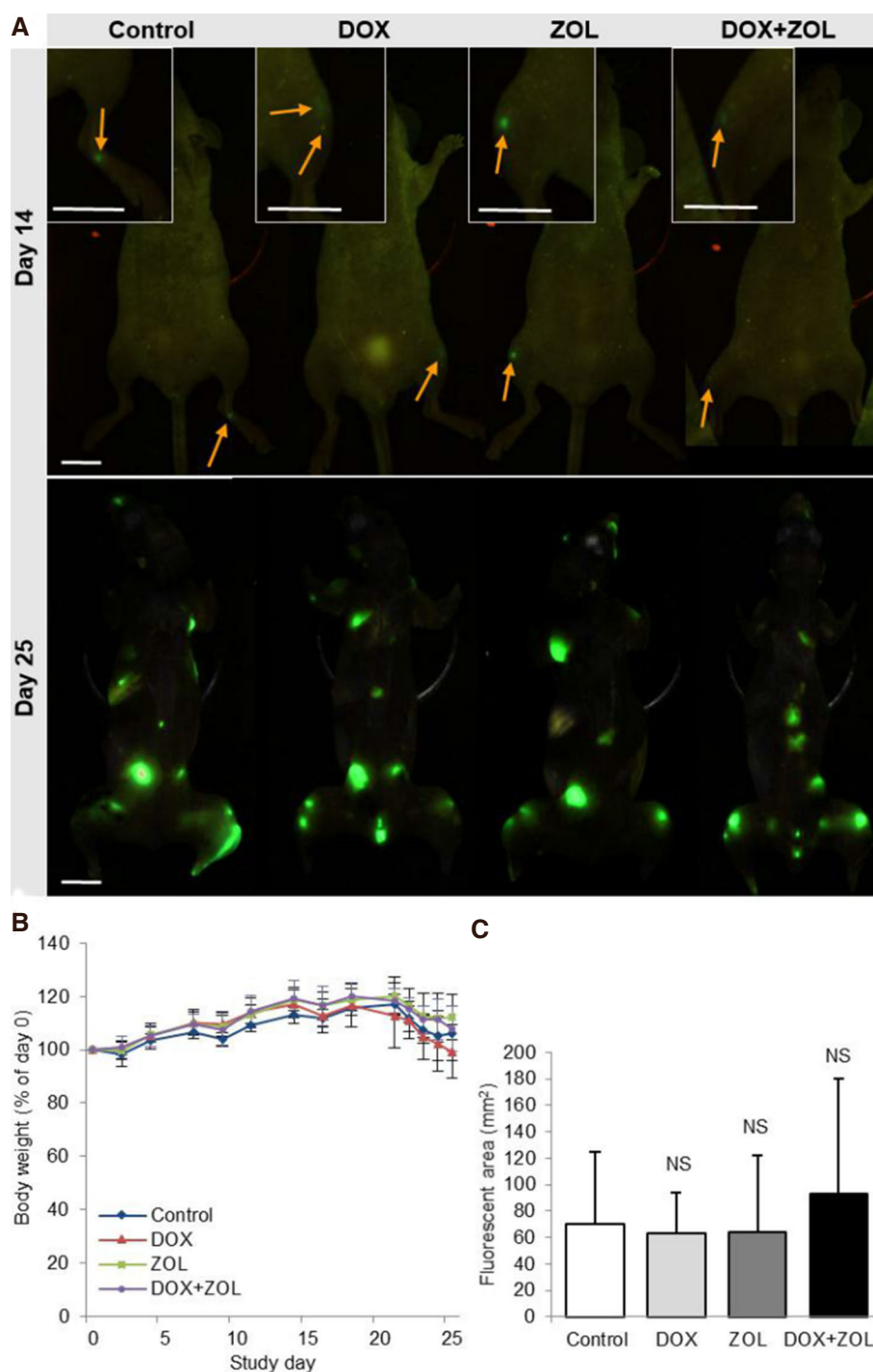


Figure 2. Body weight and MDA-MB231(SA) tumor burden. Whole body tumor burden was analyzed by fluorescence imaging. A: Representative images showing established metastatic foci before treatment (day 14) and at sacrifice (day 25); B: body weight during the study relative to body weight at the day of tumor cell inoculation. Significant differences in body weight at sacrifice relative to baseline were not observed; C: whole body tumor burden did not differ between the groups as measured by fluorescent imaging. Data are expressed as mean \pm SD, $n = 8$ animals. Scale bars 10 mm, arrows tumor foci *in vivo*. NS: non-significant; DOX: doxorubicin; ZOL: zoledronic acid

Anti-tumor effects of DOX, ZOL, and their sequential combination were studied by (1) measuring whole body tumor burden by fluorescence imaging; and (2) tumor area in bone by histomorphometry [Figures 2C and 3D, respectively]. According to fluorescence imaging at sacrifice, none of the treatments affected the

Table 1. The number of bone and soft tissue metastases were counted from green fluorescent protein images taken from dorsal and ventral sides of each animal

Groups		Vehicle	DOX	ZOL	DOX + ZOL
Bone	Mean	6.63	6.75	5.87	7.0
	SD	2.33	2.38	3.91	4.0
	ANOVA	$P = 0.913$			
Soft tissue	Mean	0.50	0.75	0.50	0.43
	SD	0.76	1.03	0.76	0.79
	ANOVA	$P = 0.884$			

DOX: doxorubicin; ZOL: zoledronic acid

whole body tumor burden [Figure 2C]. Furthermore, there was no difference in the numbers of metastases to bone or soft tissue sites between the treatment groups, based on fluorescence imaging [Table 1].

In accordance to these results, none of the treatments decreased the tumor area in bone analyzed from histological sections [Figure 3D]. All sections except sections from one animal in the DOX + ZOL group, contained tumor tissue. In many sections the tumor filled the entire intraosseous area. A thin layer of tumor on the periosteal surface resulting from disruption of the cortical envelope was sometimes observed. The anti-tumor effects were further examined by counting the number of apoptotic tumor cells and the proliferation index in histological samples [Figure 3E and F]. Even though the intraosseous tumor area was not affected, the number of apoptotic cancer cells analyzed by TUNEL staining was increased in the DOX and DOX + ZOL groups [Figure 3E]. However, the sequential combination of DOX and ZOL did not increase the amount of apoptotic cells compared to DOX alone.

Effects of DOX and ZOL on bone

The effects of DOX and ZOL on bone were studied by measuring osteolytic lesion areas observed in X-ray images and trabecular bone area in histological sections. All mice in the control and DOX groups, 12.5% of the mice in the ZOL group, and 42.9% of the mice in the DOX + ZOL group had osteolytic lesions at sacrifice [Figure 4A]. The proportion of mice with osteolytic lesions was significantly lower in both groups treated with ZOL compared to the control group ($P < 0.001$ for ZOL and $P = 0.026$ for DOX + ZOL). Osteolytic lesion area measured from X-ray images was radically lower in the ZOL and DOX + ZOL groups compared to the control group [Figure 4B]. ZOL also increased trabecular bone area by a factor of 2 analyzed by histomorphometry and DOX + ZOL by a factor of 1.5 compared to control. However, in the DOX + ZOL group statistically significant difference to the control was not reached [Figure 4C].

Effects of DOX and ZOL on TRACP 5b and Ca^{2+} concentration in the blood

Because breast cancer patients with bone metastases often have hypercalcemia and increased serum TRACP 5b has also consistently been reported in these patients, we measured Ca^{2+} in blood and serum TRACP 5b from the mice. In addition, osteoclasts were counted at tumor-bone interface of TRAP-stained sections. We found that ZOL alone rapidly decreased serum TRACP 5b values as early as two days after dosing, on day 17. Both the ZOL and DOX + ZOL groups showed decreased TRACP 5b on day 24 compared to the control group [Figure 5B]. The number of osteoclasts was 25%-50% lower at the tumor-bone interface in the ZOL and DOX + ZOL treated groups compared to the vehicle group [Figure 5C], but the differences were not statistically significant ($P = 0.595$ and 0.142 , respectively). Both groups also had lower blood Ca^{2+} compared to the control group (5d). The blood Ca^{2+} in ZOL and DOX + ZOL groups was similar to the baseline Ca^{2+} measured from five healthy animals of same age, sex and strain (1.268 mmol/L, SD 0.0205), whereas the control group had higher blood Ca^{2+} compared to the healthy animals ($P = 0.038$). In the DOX group 4/8 animals were above the range of healthy mice (> 1.29 mmol/L), but the group average did not differ from the control group.

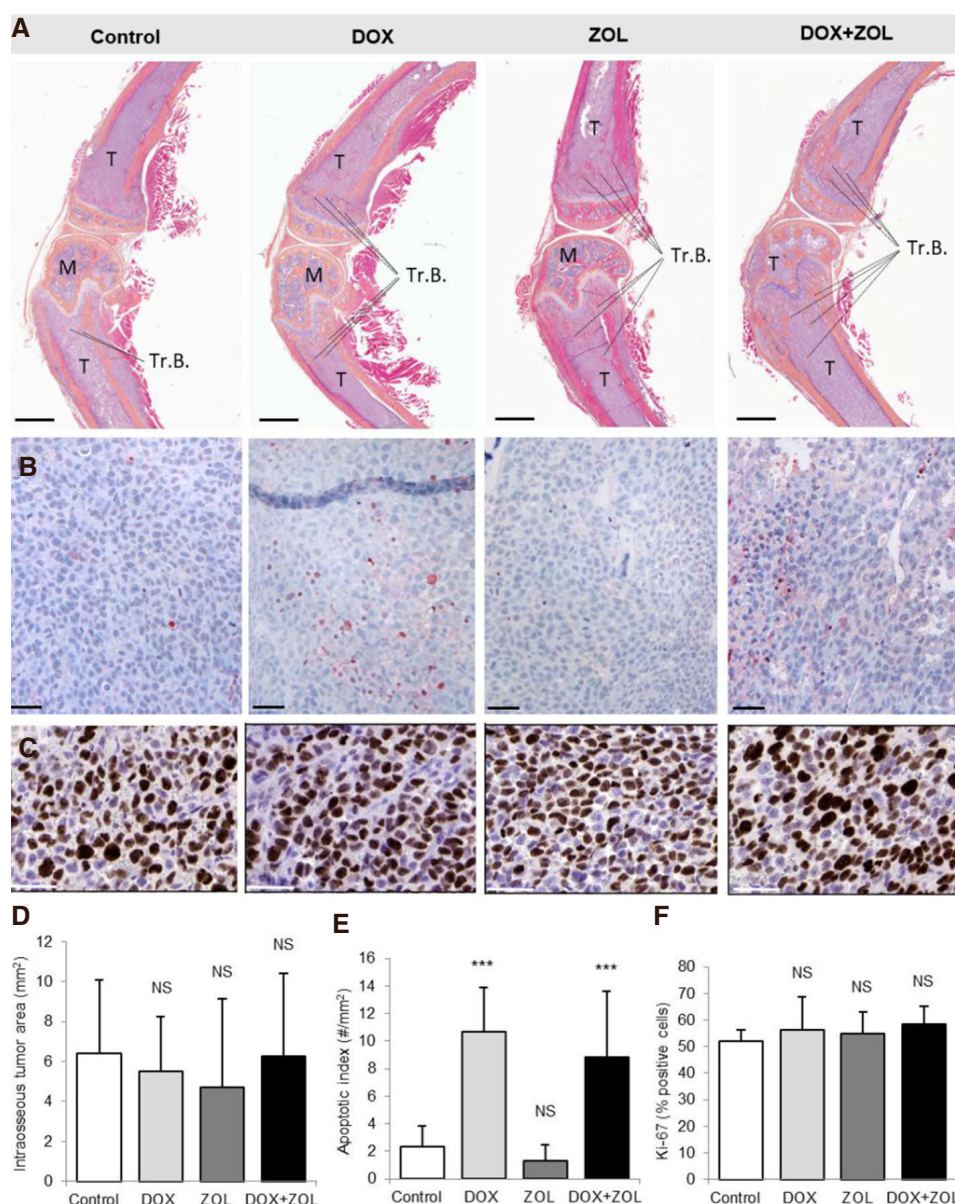


Figure 3. Intraosseous tumor area, apoptosis and proliferation. Tumor burden in bone was analyzed in histological sections of the left hind leg. Apoptotic tumor cells were stained using the TUNEL method and stained cells with apoptotic morphology were counted. Proliferation index was analyzed from Ki-67 stained sections. A: Representative images of HE + Orange G stained sections showing tumors in bone at sacrifice (day 25); B: representative images of the TUNEL stained sections; C: representative images of the Ki-67 stained sections; D: intraosseous tumor area did not differ between the groups; E: apoptotic tumor cells relative to tumor area were increased in both groups receiving DOX, but not in the ZOL group; F: proliferation index did not differ between the groups. Data are expressed as mean \pm SD, $n = 8$ animals. Scale bars 1 mm (A); 50 μ m (B and C). ***Significantly different from control group ($P < 0.001$); NS: non-significant; T: tumor; M: marrow; Tr.B.: trabecular bone; HE: hematoxylin-eosin; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling; DOX: doxorubicin; ZOL: zoledronic acid

DISCUSSION

Direct anti-tumor effects of bisphosphonates on established tumors in bone have been reported in many preclinical studies^[5-7]. Decrease in tumor growth has been observed also in a model with defective osteoclasts^[19]. However, findings have not been positive in all models, and clear clinical proof has been lacking. Additive or synergistic effects when combined with chemotherapeutic agents observed in some preclinical models of bone metastases as well as primary tumors have further fueled the interest in the anti-tumor actions of bisphosphonates. Several clinical studies with combination of neoadjuvant

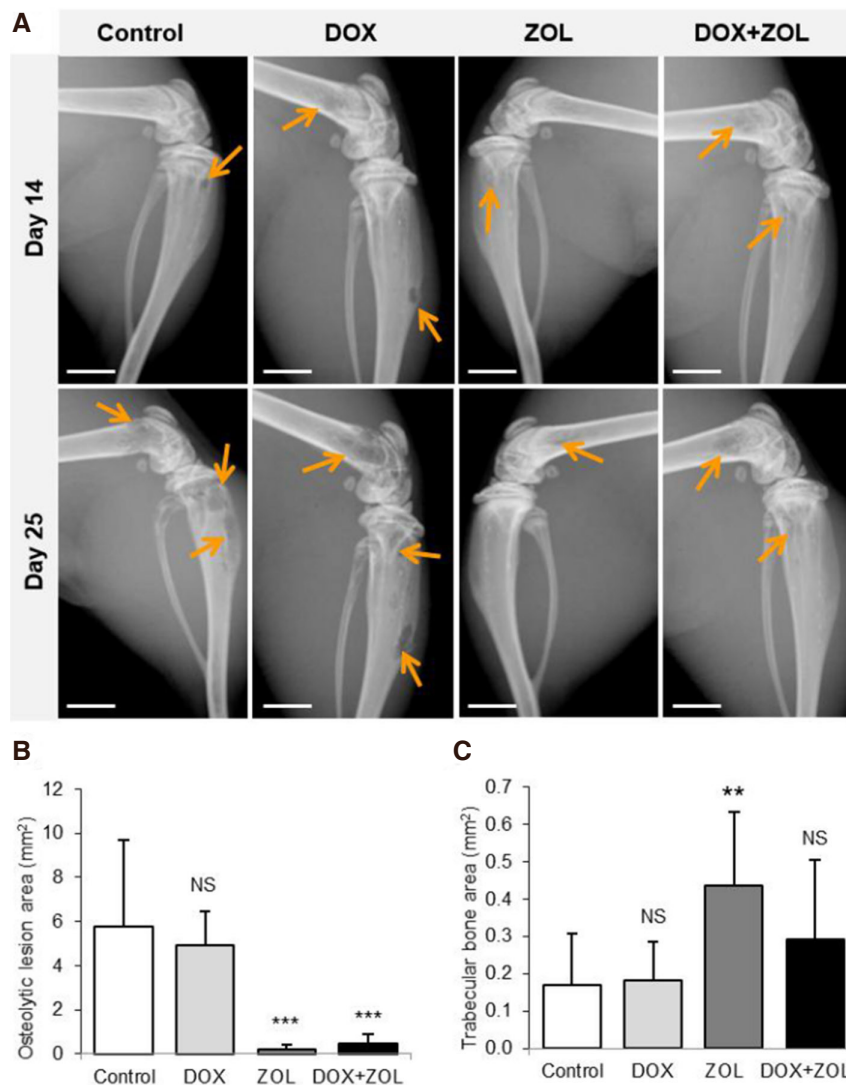


Figure 4. Osteolytic lesions, trabecular bone volume and the number of osteoclasts at the bone-tumor interface. Osteolytic lesion area was analyzed from X-ray images of both hind limbs, trabecular bone area and the number of osteoclasts in the bone-tumor interface in midsagittal histological sections of the left hind limb. A: Representative images showing established osteolytic lesions before treatment and at sacrifice; B: osteolytic lesion area was decreased in the ZOL and DOX + ZOL groups as measured by radiography; C: trabecular bone area was increased in the group receiving only ZOL, but the effect was not statistically significant in the DOX + ZOL group. Data are expressed as mean \pm SD, $n = 8$ animals. Scale bars 2 mm, arrows osteolytic lesions. **Significantly different from control group ($P < 0.01$); ***significantly different from control group ($P < 0.001$); NS: non-significant; DOX: doxorubicin; ZOL: zoledronic acid

chemotherapeutic agent and ZOL have been reported. A retrospective analysis of sequential treatment showed benefit in a patient subgroup of postmenopausal women with estrogen receptor negative breast cancer^[20]. Subsequent randomized controlled trials have suggested benefit, but failed to show statistically significant results^[21-23]. This study aimed to examine whether a sequential combination of DOX and single dose ZOL has synergistic effects in the widely used MDA-MB-231(SA) bone metastasis model.

Aminobisphosphonates, such as ZOL, inhibit the mevalonate pathway and thus protein prenylation, resulting in inhibition of many subsequent cellular processes and induction of apoptosis in *in vitro* studies, but the results of *in vivo* studies are less conclusive^[24]. ZOL alone did not affect tumor burden, proliferation or apoptosis, which is in line with previous findings, including models using MDA-MB231 cells or its sublines^[5,8,11,12]. It is also in line with clinical data of ZOL not increasing overall survival in patients with

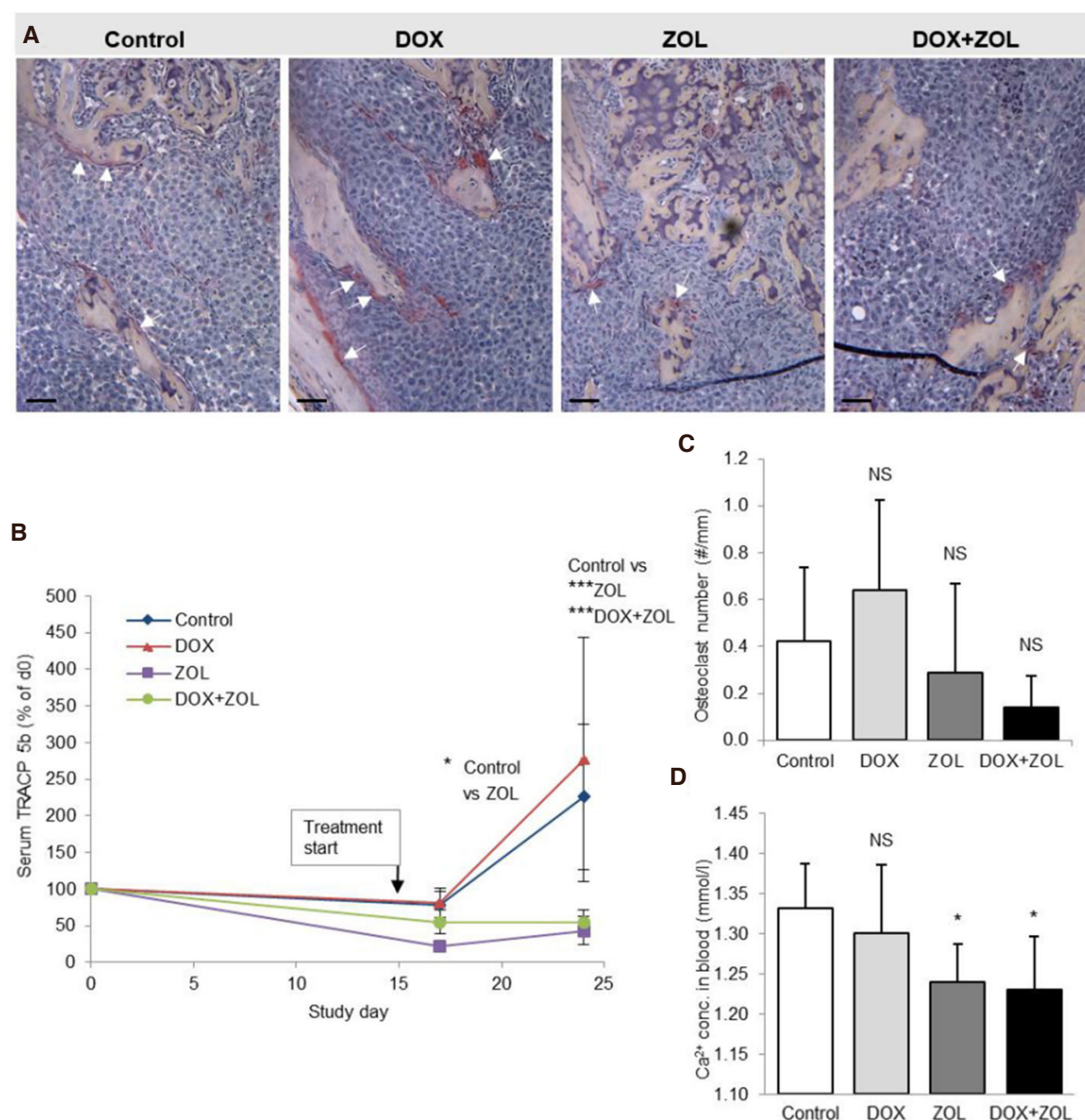


Figure 5. Osteoclasts and blood Ca²⁺. Osteoclasts were counted from TRAP stained sections and serum TRACP 5b activity was measured at three time points (before inoculation of cells, days 17 and 24) as a systemic marker of osteoclast number. Blood Ca²⁺ was measured from terminal blood samples for detection of hypercalcemia. A: Representative images of the TRAP staining; B: statistically significant changes were not observed in the number of osteoclasts at tumor-bone interface; C: serum TRACP 5b was decreased in the ZOL group already on day 17 and in the ZOL and DOX + ZOL groups on day 24 compared to the control group; D: hypercalcemia was prevented in the ZOL and DOX + ZOL groups. Data are expressed as mean \pm SD, $n = 7-8$ animals. Scale bars 50 μ m, arrows osteoclasts. *Significantly different from control group ($P < 0.05$); ***significantly different from control group ($P < 0.001$); NS: non-significant; TRAP: tartrate resistant acid phosphatase; TRACP 5b: tartrate resistant acid phosphatase 5b; DOX: doxorubicin; ZOL: zoledronic acid

established bone metastases^[3]. DOX on the other hand did increase the number of apoptotic cells, as expected. We did not observe any other significant effects with DOX on tumor growth or proliferation index, which may have been due to a suboptimal dose of DOX. Higher doses of DOX are able to decrease tumor burden in the same model^[25]. However, the dose in this study was set at a low level to enable observation of possible synergistic effects. Likewise, Ottewell *et al.*^[11] used a suboptimal dose of DOX and observed synergistic effects on tumor volume and number of apoptotic cells in bone. They did not observe any effects of DOX alone on apoptosis by caspase-3 staining or on tumor burden in the intravenous MDA-

MB231BO2 or intracardiac MDA-MB231 models. In contrast, we observed clear induction of apoptosis with DOX alone, but we did not observe any potentiation of apoptosis induction with the sequential combination. Furthermore, the sequential DOX + ZOL treatment did not decrease tumor area in our study, despite of increased apoptosis of cancer cells induced by DOX. The absence of any observed decrease in the GFP positive area representing total tumor burden supports the histological data.

The metastatic behavior between cell sublines can be strikingly different. Sublines of the MDA-MB231 cells derived through *in vivo* selection for bone metastases behaved differently, some formed metastases in adrenal glands and others in bone, some were fast with a high frequency and others were slow with a low frequency of metastases^[26]. However, the bone or adrenal metastatic sublines of the MDA-MB231 had similar growth rate when inoculated subcutaneously demonstrating that the cells were not generally more aggressive. Importantly, the bone metastatic subline cells exclusively induced osteoclast mobilization and recruitment^[26]. The MDA-MB231(SA) model is more aggressive and progresses faster than the MDA-MB231BO2 model, as the median survival time is 25 days in the former^[8] and 60 days in the latter^[11], with the same number of inoculated cells. However, the different inoculation route is likely to play a role in the disease progression. It is possible that more cells are seeded to the bone marrow through the intracardiac route, than through the intravenous route, where the cells have to pass through the lung capillaries first. The MDA-MB231(SA) cells have been found to express, e.g., more serine and interleukin-11 compared to the parental MDA-MB231 cells^[27,28], whereas the MDA-MB231BO2 cells were found to have decreased expression of the miRNA30 family regulating several osteomimicry genes, compared to the parental cells^[29]. Although the history of the two sublines is very different, the BO2 subline being derived through *in vivo* selection and the cells from San Antonio having occurred spontaneously, a common finding for both MDA-MB231(SA) and -BO2 sublines compared to the parental line is the strong downregulation of miRNAs 200a and 429^[28,29]. These miRNAs are involved in the epithelial-to-mesenchymal transition^[30]. Positive effects on tumor growth and survival have also been observed in a model of MDA-MB436 intratibial tumors using intensive, weekly sequential administration of DOX + ZOL^[14]. The MDA-MB436 cells are triple negative like MDA-MB231 cells, and both represent the claudin-low subtype.

As expected, ZOL efficiently prevented the progression of osteolytic lesions as both monotherapy and in combination with DOX. However, trabecular bone area was not statistically significantly higher in the DOX + ZOL group compared to the control group. The lesions visible in X-ray images present mainly lesions in cortical bone, whereas the reduction of trabecular bone is typically not so clearly observed in the X-ray images. This could partly explain the difference in radiography and histological data. Furthermore, a lesion has lost 50% of the bone volume by the time it is visible with planar X-ray imaging. MicroCT analysis would provide volumetric information of both cortical and trabecular compartments. Unfortunately, this imaging modality was not available for this study. Furthermore, there were no statistically significant differences in the numbers of osteoclasts at the tumor-bone interface between the groups. The variation of osteoclast number measurement was high, affected by the variable amount of measurable tumor-bone interface in the samples. The reason for not observing significant reduction in numbers of osteoclasts at the tumor-bone interface might be that the ZOL-induced reduction in the number of osteoclasts has been reported to be transient, observed three days after ZOL dosing to mice, but non-observable already five days after ZOL administration due to the formation of new osteoclasts^[31]. Consistently, serum TRACP 5b was decreased three days post-dosing compared to the control group. Serum TRACP 5b remained low at later time points in the groups treated with ZOL, suggesting that the osteoclasts may have been smaller^[32], or that the number of osteoclasts along normal bone surface at tumor-free areas was lower. Indeed, Brown *et al.*^[12] have reported decreased number of osteoclasts in tumor-free bone but not in tumor-bearing bone 13 days after administration of ZOL. Very importantly, however, the function of the osteoclasts at the tumor-bone interface was inhibited, as shown by the decreased osteolytic lesion area in the ZOL and DOX + ZOL groups, the increased trabecular bone area in the ZOL group, and the prevention of hypercalcemia in the ZOL and DOX + ZOL groups.

High variation was a problem in this study and may have masked treatment effects on osteoclast number. Larger groups and more sensitive methods, such as μ CT and luciferase labelled cells could have helped in reducing the variation. Intraosseous tumor area can only be analyzed by histology, which was restricted to analyzing the midsagittal sections in order to standardize the analysis. However, analyzing several levels could have reduced the variation. In order to further clarify the reasons underlying the insensitivity of the MDA-MB231(SA) tumors in bone to the combination treatment, as well as the unresponsive portion of patient population, the ZOL uptake of the MDA-MB231(SA) cells after DOX administration requires investigation. Longer treatment schedules with repeated cycles of sequential combinations would better present the clinical situation, but such studies are unfortunately not possible in this model due to the time frame of disease progression. Furthermore, the strong bone forming ability of the young, fast growing mice used in this model presents a major difference to the elderly breast cancer patients. New bone formed after the ZOL administration is not protected by ZOL, and is thus vulnerable to cancer induced degradation. This could have mitigated the effects of ZOL in this study, but because the treatment period is so short, the amount of new bone formed in that time period is not likely to influence the results.

In summary, we examined the anti-tumor effects of a single dose of ZOL alone and in sequential combination with DOX on established bone metastases in the intracardiac MDA-MB231(SA) model. DOX induced apoptosis in the tumor cells and ZOL prevented tumor-induced bone destruction. However, anti-tumor effects of ZOL were not observed, nor additive or synergistic effects of the sequential combination of DOX and ZOL.

DECLARATIONS

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Authors' contributions

Conception and design of the study, data analysis and interpretation: Suominen MI

Data acquisition, provided administrative, technical, and material support: Rissanen JP, Käkönen R, Halleen JM

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Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon request.

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Suominen MI, Rissanen JP, Halleen JM and Härkönen P are voting stock holders of Pharmatest Services Ltd. Käkönen SM and Käkönen R are voting stock holders of Aurexel Life Sciences. Halleen JM is a consultant of and receipt of royalties from IDS Plc.

Ethical approval and consent to participate

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Consent for publication

Not applicable.

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Review

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Targeting histone lysine-specific demethylase KDM1A/LSD1 to control epithelial-mesenchymal transition program in breast cancers

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Abstract

Epithelial-mesenchymal transition (EMT) is a plastic and reversible process, essential for development and tissue homeostasis. Under pathological conditions, EMT causes induction of tumor growth, angiogenesis and metastasis. According to its reversible nature, the EMT program is associated with vast epigenetic changes. Targeting the epigenetic network that controls the EMT pathway in disease progression is a novel promising strategy to fight cancer metastasis. The impact of alterations in histone methylation in cancer has led to the identification of histone methyltransferases and demethylases as promising novel targets for therapy. Specifically, the lysine specific demethylase 1 (LSD1, also known as KDM1A) plays a pivotal role in the regulation of EMT. Here we present an overview of the causative role of LSD1 in the EMT process, summarizing recent findings on its emerging functions in cell migration and invasion in breast cancer.

Keywords: Lysine specific demethylase 1, KDM1A, epithelial-mesenchymal transition, breast cancers, metastasis, invasion, LSD1-complex

INTRODUCTION

Breast cancer is the most frequent cancer in women worldwide. It has a predictable incidence of 246,660 new cases (29% of all sites cancers) and 40,450 estimated deaths (14% of all sites) in 2016 in the United States^[1].



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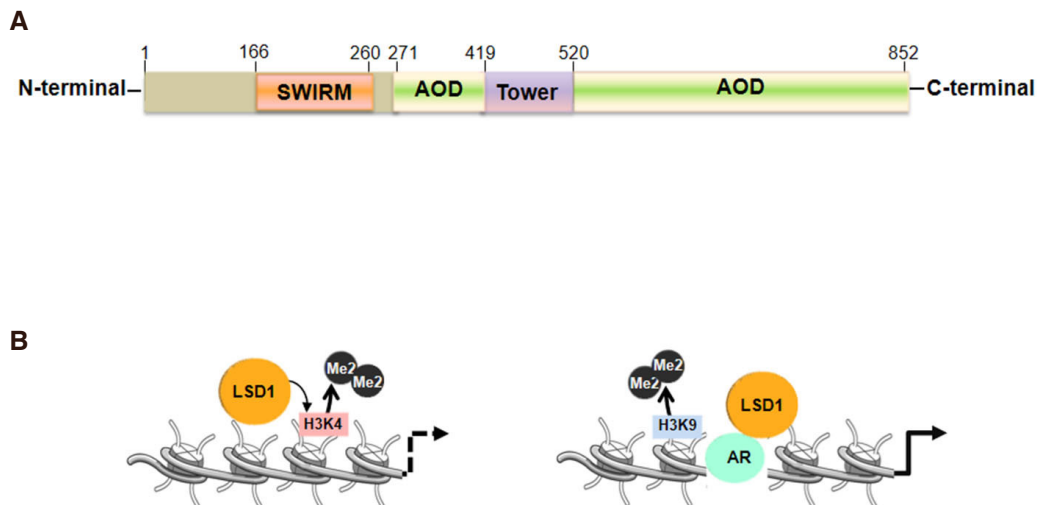


Figure 1. Lysine specific demethylase 1 (LSD1) protein domains and structure. A: In orange the SWIRM domain, in green the AOD domains and in yellow the Tower Domain; B: LSD1 selective substrate specificities: LSD1 represses promoter and enhancer activities through the demethylation of histones lysines, H3K4 or in cooperation with the androgen receptor (AR), activates transcription through Histones H3K9 demethylation

The major causes of death in breast cancer patients are the result of metastasis in distant organs^[2]. Metastasis is a complex biological process, which occurs through a series of steps: (1) rising of a locally invasive and migratory behavior, (2) arriving to the blood vessel and into the circulation via the blood flow, (3) halting in the distant organ, (4) surviving the initial stress, and (5) reinitiating outgrowth in distant stroma^[2,3]. Metastasis involves a multitude of molecular signals^[3]. Single cells leaving the primary tumor undergo epithelial to mesenchymal transition (EMT), consisting in the loss of epithelial polarity and the achievement of a mesenchymal morphology^[4]. The opposite process, the mesenchymal to epithelial transition, takes place when metastatic cells switch back to an epithelial state in order to colonize secondary sites^[5].

The mechanism of EMT is currently a major focus in metastasis research. The knowledge of its mechanisms is still fragmentary and more in-depth studies are needed in order to improve therapeutic approaches and influence, in the long-term, control of breast cancer outcome^[6,7].

According to the reversible nature of EMT-associated processes, epigenetic mechanisms, such as DNA methylation and histone modifications, exert a great influence on the EMT program^[8-11]. Particularly, wide ranges of studies demonstrate that the lysine specific demethylase 1 LSD1, also known as KDM1A, plays a pivotal role in the EMT regulation^[12-15].

High levels of LSD1 expression in various tumors, including breast cancer, are correlated with poor prognosis^[16-19]; several works reported that the ablation of LSD1 in breast cancer cells inhibits the invasion capabilities and suppresses their metastatic potential^[20-22].

LSD1 is classified as an amine oxidase that uses FAD as the co-factor for its enzymatic action^[23]; LSD1 is composed by three major domains: an N-terminal SWIRM (small alpha-helical domain), for protein stability; a central protruding Tower domain and a C-terminal amine oxidase like domain [Figure 1A]; LSD1 is able to demethylate mono and di-methylated lysine 4 and 9 of the histone H3 (H3K4, H3K9)^[23-27]. Exerting its activity LSD1 can function as co-repressor, removing H3K4 methylation on gene promoters and enhancers, or co-activator, by removing H3K9 methylation, indicating that substrate specificity and the binding to different interactors define its biological outcome [Figure 1B]^[12]. Furthermore, LSD1 regulates methylation dynamics of non-histone proteins^[16,28-33].

This review summarizes recent advantages in understanding the mechanisms through which LSD1 controls EMT in breast cancer, and discusses how these findings could be used to establish a new approach for therapeutic intervention in breast cancer.

LSD1 MODULATE THE EMT PROCESS BY ITS PARTICIPATION IN DIFFERENT COMPLEXES

In human cancers, EMT contributes to tumor progression and invasion of surrounding tissues and confers chemo-resistance. Morphological and phenotypic changes are orchestrated by transcriptional reprogramming in which several transcription factors, known as EMT-TF^[34], have a role in silencing of epithelial genes (e.g., E-cadherin, Occludin) and in the activation of mesenchymal genes (e.g., N-cadherin, Vimentin, Fibronectin)^[35]. EMT is characterized by reprogramming of epigenetic marks: following the induction of the EMT by the transforming growth factor beta (TGF- β), a global increase of trimethylation in H3K4 and H3K36 histones and a decrease in the dimethylation of H3K9 are observed^[34]. McDonald *et al.*^[35] demonstrated that these epigenetic modifications depend largely on LSD1, and that loss of LSD1 functions affects EMT-driven cell migration and chemo-resistance.

Taking in consideration different studies, it is clear that LSD1 may function as enhancer or inhibitor of EMT functioning in a cell type-specific fashion, probably due to the cells genetic background and depending on its interacting partners^[36]. A number of studies have described LSD1 as a critical player in epigenetic reprogramming during EMT. Two independent reports demonstrated that LSD1 physically associates with SNAIL1 (snail family transcriptional repressor 1) in breast cancer cells^[15,34,37]. The members of the SNAIL family of zinc finger transcription factors (Snail, Slug and Smuc) control the invasive phenotype and metastasis in several types of cancers, included breast cancer. In the EMT pathway, SNAIL family proteins repress the expression of epithelial genes, such as E-cadherin (CDH1), through an LSD1-dependent molecular mechanism. It has been shown that LSD1, interacting with Snail, leads to CDH1 repression^[15,34] [Figure 2A]. In particular, it has been defined that the N-terminal SNAG domain of Snail is essential and responsible for the interaction and recruitment of LSD1 to gene promoters. Notably, SNAG domain of Snail1 resembles a histone H3-like structure, thus, it acts as a pseudo-substrate “hook” for LSD1 to recruit it together with CoREST to the Snail1 target gene promoters [Figure 2A]. The inhibition of the Snail/Slug-LSD1 interaction, using pharmacological LSD1 inhibitors or a cell-permeable peptide corresponding to the SNAG domain of Slug, suppresses motility and invasiveness of breast cancer cells^[34].

Notably, high expression of LSD1 or ERR α associates with breast cancer poor prognosis, decreased survival, cancer progression and metastasis, and LSD1 has been found to protect the ERR α protein from proteasome degradation^[38]. The ERR α -LSD1 complex regulates a subset of genes involved in migration and invasiveness in breast cancer. The LSD1-ERR α complex binds the TSSs of common target genes and this interaction induces LSD1 to demethylate H3K9 leading to transcriptional activation^[39] [Figure 2B].

Among co-regulated genes LSD1-ERR α enhances the expression of the matrix metalloproteinase 1 (MMP1), a secreted protein involved in extracellular matrix degradation and cell invasion, and this can account for the capacities of LSD1-ERR α to induce tumor progression^[39].

Although the majority of work indicates that LSD1 is a key positive regulator of the EMT program, in cooperation with other proteins such as the NuRD complex, it could promote opposite effects. It has been reported that the LSD1/NuRD complex inhibits TGF- β signaling pathway, reducing breast cancer metastatic potential^[20] [Figure 2C].

LSD1 has been found to have a role also as epigenetic regulator of EMT-TFs transcription factors expression [SNAIL, zinc finger E-box-binding homeobox 1 (ZEB1) and ZEB2]^[34]. It has been reported that in breast cancer stem cells LSD1 interacts with the Ubiquitously transcribed tetratricopeptide repeat, X chromosome

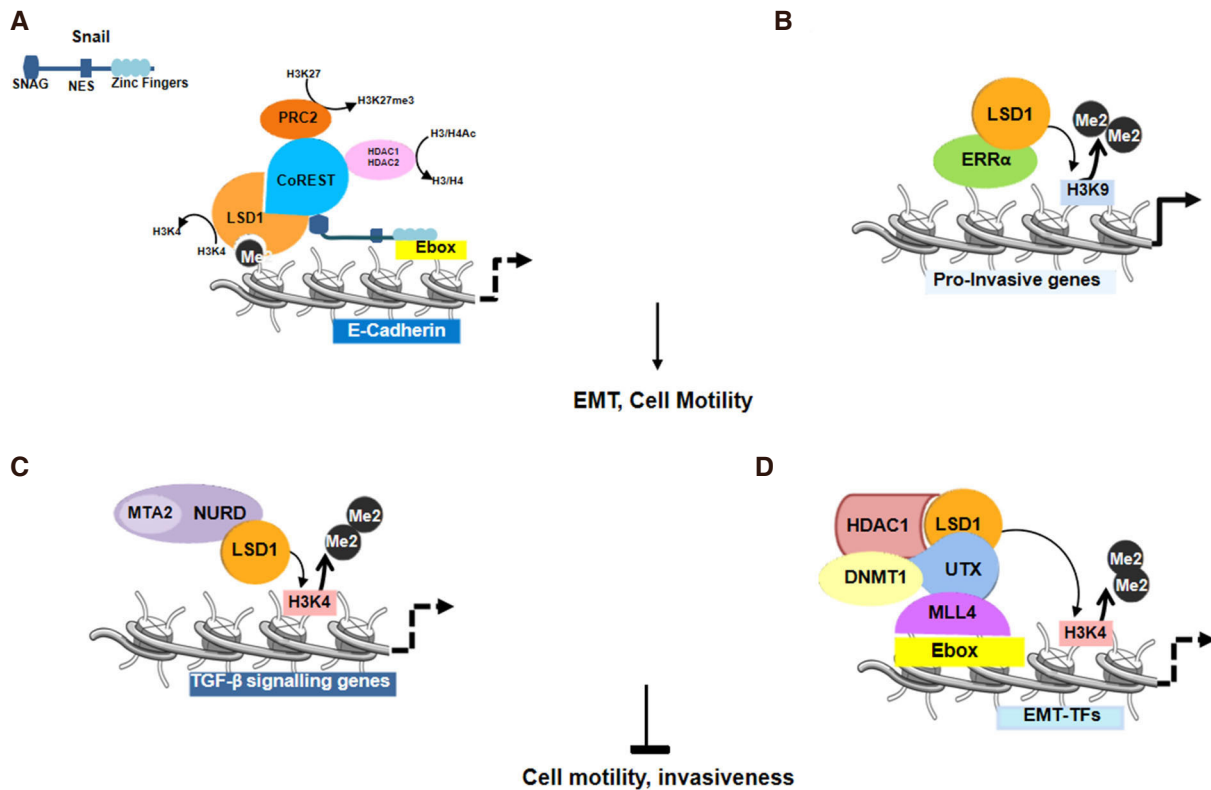


Figure 2. Lysine specific demethylase 1 (LSD1) regulates cell motility and epithelial to mesenchymal transition (EMT). A: Snail is stabilized through the formation of a ternary Snail-LSD1-CoREST complex. SNAG domain of Snail binds LSD1 and recruits it together with CoREST to E-boxes of Snail target gene promoters. LSD1 binds the histones H3 tail and removes the activation marks on H3K4. HDAC1 and 2 deacetylate histones 3 and 4 (H3/H4), subsequently PRC2 directs the trimethylation of H3K27; B: H3K9 demethylation activity by LSD1 in complex with ERRα leads to transcriptional activation of pro-invasive genes; C: LSD1 inhibits EMT process through binding to NuRD impairing TGF-β signaling genes expression. D LSD1 with UTX functions as epigenetic silencer of EMT TFs

(UTX)^[14]. UTX functions as lysine-specific demethylase on H3K27me2/H3K27me3. Choi *et al.*^[14] demonstrated that the role of UTX in epigenetic silencing of EMT-TF is not due to its H3K27 demethylation activity but to the ability to disrupt c-Myc and MLL4 recruitment on the E-boxes of their promoters. LSD1 recruited by UTX on the E-boxes form a transcriptional repressive complex with histone deacetylase 1 (HDAC1) and DNA methyltransferase 1 (DNMT1) [Figure 2D]. LSD1 together with UTX in this case of human breast cancers functions directly as epigenetic silencer of EMT TFs and consequently acts as tumor suppressor^[14].

LSD1 POST-TRANSLATIONAL TARGETING IN EMT MODULATION

Several studies established that LSD1 activity and stability is altered by post-translational modifications^[40,41]. LSD1 is acetylated in epithelial, but not in mesenchymal cells. In particular, the acetyltransferase MOF (or KAT8, lysine acetyltransferase 8) is critically involved in LSD1-induced EMT, acting on LSD1 acetylation^[21]. MOF-mediated acetylation of LSD1 on lysines 432, 433 and 436 alters the association between LSD1 and target chromatin loci, increasing H3K4me2 levels and expression of epithelial markers. Moreover, while LSD1 is ubiquitously expressed, MOF is enriched in epithelial cells, but down-regulated in mesenchymal cells^[21]. Thus, MOF-mediated acetylation of LSD1 may represent a crucial regulatory switch that, modulating LSD1 control of the EMT process. Accordingly, in epithelial breast cancer cells, MOF blocks LSD1 association with epithelial gene promoters, increases H3K4me2 levels at these loci and activates E-cadherin expression^[21] [Figure 3A].

In addition, recent evidences highlight that phosphorylation of LSD1 on Serine-111 (analogous to murine serine-112) is crucial for its activity in the EMT programs. It has been reported that PKCα (Protein kinase

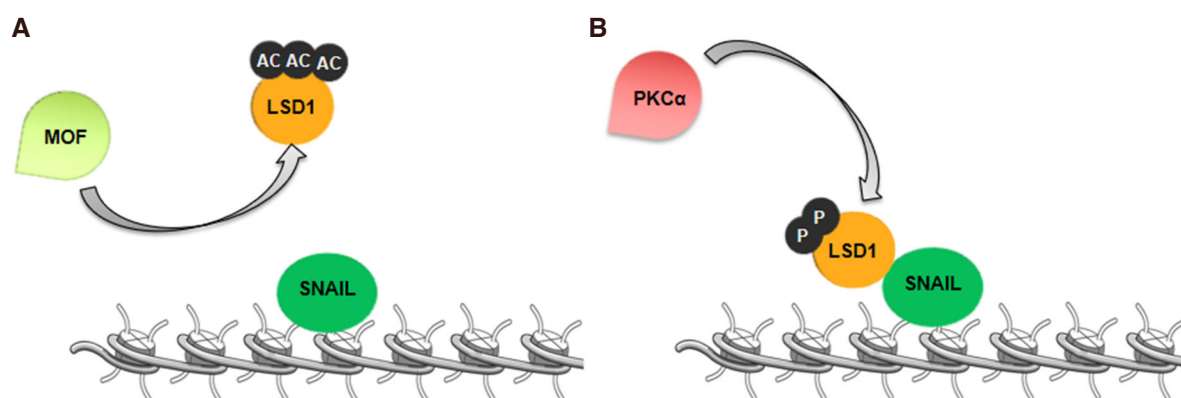


Figure 3. Lysine specific demethylase 1 (LSD1) is differently modified at post-translation level in epithelial and mesenchymal cells. A: In epithelial cells, MOF acetylates LSD1, and this modification induces the dissociation of LSD1/SNA1 complex. Then, LSD1 cannot block CDH1 transcription; B: in mesenchymal cells, PKC α phosphorylates LSD1. LSD1 phosphorylated interacts with SNA1 in order to silence CDH1 gene

C alpha), that phosphorylate LSD1 at Serine-111, is required for LSD1 association with the E-cadherin promoter^[42]. Specifically, LSD1-mediated demethylation at E-cadherin promoter is PKC α dependent and knockdown of PKC α impairs EMT induced by LSD1 overexpression in breast cancer cells [Figure 3B]. Another study reports that, upon induction of EMT, LSD1 is transiently induced and regulates epigenetic changes that result in transcriptional activation of several EMT-related genes in breast cancer cells. Phosphorylation of LSD1 at Serine-111 by a different PKC isoform, the PKC- θ (Protein kinase C theta), regulates its pro-EMT function^[22]. Indeed, PKC- θ co-localizes with LSD1 in mesenchymal-like, but not in epithelial breast cancer cells and co-binding with LSD1 occurs predominantly at promoter regions of genes activated during EMT. Moreover, LSD1 phosphorylated at Ser-111 is enriched in chemo-resistant breast cancer cells with mesenchymal phenotype after therapy. *In vivo* studies also demonstrate that targeting LSD1 in combination with chemotherapy significantly reduces tumor growth by inhibiting the mesenchymal properties generated by chemotherapy alone^[22].

Thus, targeting specific LSD1 post-translational modifications may be instrumental to modulate LSD1 functions and poses the basis to develop selective therapeutic tools.

LSD1/HIF-1 α AXIS IN BREAST CANCERS

Several studies demonstrate that LSD1 acts also on several non-histone substrates, such as lysine 370 on p53, which allows the binding of 53BP1^[28,43]. Furthermore, LSD1 regulates the protein stability of the DNMT1^[29], E2F1 (E2F transcription factor 1)^[30], MYPT1 (myosin phosphatase)^[31], STAT3 (signal transducer and activator of transcription 3)^[32] and HIF-1 α (hypoxia inducible factor 1 subunit alpha)^[33,44] through its non-histone demethylase activity. LSD1 demethylation of non-histone substrates confirms its versatility to regulate different processes. One of the most important LSD1 non-histone substrate is HIF-1 α , the master transcriptional regulator of developmental response to hypoxia^[45]. High levels of HIF-1 α have been strongly implicated in cancer biology, as well as a number of other pathophysiologicals, vascularization and angiogenesis, metabolism, cell survival, and tumor invasion. HIF-1 α protein accumulates under a hypoxic microenvironment, and it appears increased in various cancers. HIF-1 α plays a crucial role in adaptive responses of the hypoxic tumor cells and it functions as a transcriptional activator of genes that regulate biological processes required for tumor survival and progression. Furthermore, high levels of HIF-1 α induce up-regulation of different EMT-associated transcription factors, which in turn activate or repress EMT-associated signaling pathways and modulate EMT-associated inflammatory cytokines^[46]. In breast cancer cells, EMT is prompted by ZEB1-MYB-E-cadherin signaling under hypoxic stress, which induces higher expression of ZEB1 and lower expression of MYB^[47]. In particular, HIF-1 α promotes EMT through the regulation of E-cadherin, SNAIL, ZEB1, TWIST (twist family bHLH transcription factor 1), and

transcription factor 3 (TCF3, also known as E47)^[47-49]. In this contest, LSD1 has a pivotal role in HIF-1 α post-translation regulation. LSD1 demethylates lysine 32 (K32) of HIF-1 α , while the same site can be methylated by SET9 inducing HIF-1 α protein degradation^[50] [Figure 4A].

Moreover, in breast cancer, it has been reported that LSD1 regulates indirectly HIF-1 α at post-translation level influencing its stability and avoiding its degradation. In particular, LSD1 upregulates HIF-1 α demethylating the RACK1 (receptor for activated C kinase 1) protein (a component of the HIF-1 α ubiquitination machinery) on lysine 271 (K271). After that, the degradation of HIF-1 α is suppressed. These results indicate that in breast cancer the levels of FAD in the cells and the FAD-dependent LSD1 activity on RACK1-K271me2 could be the main determinants of HIF-1 α stability during prolonged hypoxia^[51] [Figure 4B]. Accordingly, it has been shown that LSD1 inhibitors also impair the accumulation of HIF-1 α , which is implicated in the activation of EMT in breast cancer. Therefore, even if at the moment there is a lack of evidences for an *in vivo* link between LSD1/HIF-1 α /EMT, this pathway needs to be further explored since FAD modulation could represent a potential therapeutic strategy to regulate LSD1 and HIF-1 α pathway.

CONCLUSION

Oncogenic roles of LSD1 in the pathogenesis of different epithelial cancers, such prostate, bladder, liver, non-small cell lung cancer and neuroblastomas, have been widely reported.

In breast cancer, LSD1 expression increases with cancer progression and its overexpression is positively correlated with the estrogen receptor negative status; high levels of LSD1 are a considerate molecular marker for predictive aggressive biology in ER-negative^[17,24] and basal-like^[17] breast cancer. Moreover, the depletion of LSD1 reduces proliferation and invasiveness of breast cancer cells *in vitro*.

In breast cancer, the oncogenic role of LSD1 depends on its versatility to interact with different partners. Current clinical trials utilizing epigenetic drugs for combination therapy have been shown to be promising in treating metastatic cancers. Vasilatos *et al.*^[52] suggest that the combination therapy of LSD1 and HDAC inhibitors leads to expression activation of genes such as E-cadherin in Triple-negative breast cancer (TNBC)^[52-54]. More recently, Yang *et al.*^[55] proposed that LSD1 interacts with SIN3A/HDAC complex, which plays a role in EMT-induced cancer stemness inhibiting a series of genes, such as TERT (Telomerase reverse transcriptase), CUL4A (Cullin4A), TGFB2 (Transforming growth factor beta 2), MDM2 (Mouse double minute 2 homolog), RHOA (Ras homolog family member A) and HIF-1 α ^[55].

Progresses have been made in drug targeting for breast cancer over the years. Current clinical treatments typically involve surgery if disease is promptly diagnosed. Moreover, depending on molecular characteristics of injury, breast cancer surgery may be followed by radiation, chemotherapy, hormone therapy and targeted therapy. Nevertheless, the major limitation of targeted anticancer therapies is the intrinsic or acquired resistance^[56].

Epigenetic therapies are prime candidates for adjuvant treatments to improve cancer therapy efficacy. Thus, establishing how exactly LSD1 regulates cell proliferation and invasive capacity will potentially facilitate the development of epigenetic therapies to attenuate tumor progression and metastasis in human breast cancer.

Recent studies identify LSD1 as a potent inhibitor of anti-tumor immunity and responsiveness to immunotherapy. LSD1 inhibition leads to double-stranded RNA stress and activation of type 1 interferon, which stimulates anti-tumor T cell response and represses tumor growth^[57].

Qin *et al.*^[58] have recently shown that the inhibition of LSD1 reactivates key immune checkpoint regulator and cytotoxic T cell-attracting chemokines in TNBC to immune checkpoint blocking antibodies. *In vivo*,

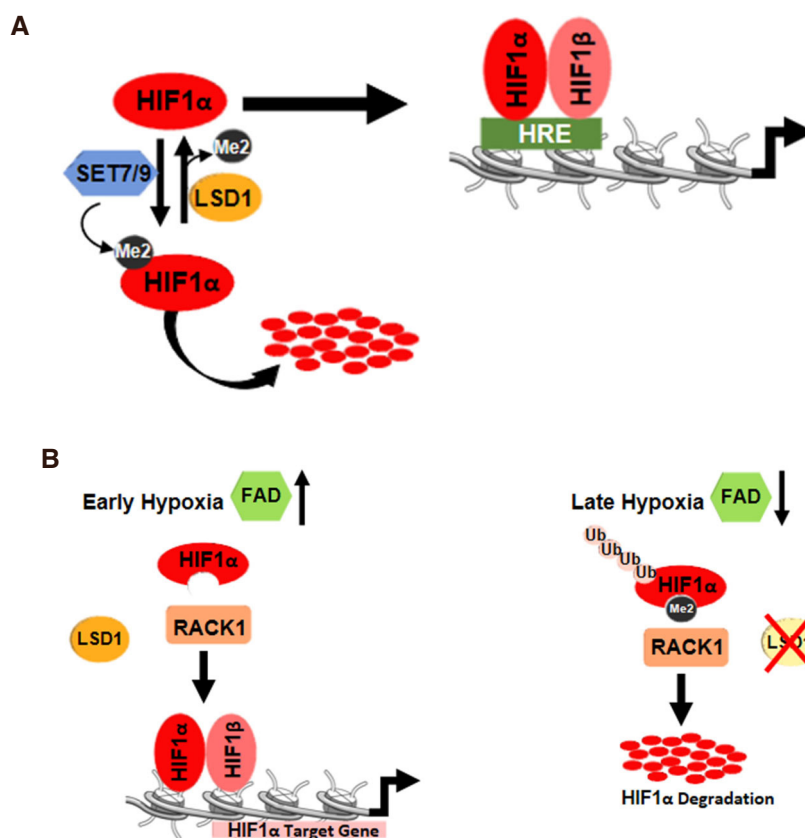


Figure 4. Non-histone lysine specific demethylase 1 (LSD1) substrate: hypoxia inducible factor-1 alpha (HIF-1α) protein stability is modulated by LSD1. A: Methylation-demethylation by SET7/9 and LSD1 respectively regulates HIF-1α degradation; B: LSD1-mediated regulation of HIF-1α protein stability during hypoxia: In early stage of hypoxia, the LSD1 (FAD-dependent) demethylase activity protects HIF-1α from RACK1-mediated degradation and HIF-1α activates transcription of its target genes. In prolonged hypoxia, the absence of FAD impairs FAD-dependent LSD1 activity on HIF-1α with consequent RACK1-dependent HIF-1α protein degradation

the inhibition of chemokine receptors, by combined treatment with PD-1 antibody and LSD1 inhibitors, suppresses tumor growth and metastasis^[58].

In conclusion, LSD1 inhibitors represent a promising epigenetic approach to treat breast Cancer. Furthermore, for a possible therapeutic goal, developing new drugs that target LSD1 and its partners or immune modulators would carry high innovation and translational potential.

DECLARATIONS

Authors' contributions

All authors participated in drafting the article and to its critical revision and approved the final version to be published.

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

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Review

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hnRNP E1 at the crossroads of translational regulation of epithelial-mesenchymal transition

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Abstract

The epithelial-mesenchymal transition (EMT), in which cells undergo a switch from a polarized, epithelial phenotype to a highly motile fibroblastic or mesenchymal phenotype is fundamental during embryonic development and can be reactivated in a variety of diseases including cancer. Spatio-temporally-regulated mechanisms are constantly orchestrated to allow cells to adapt to their constantly changing environments when disseminating to distant organs. Although numerous transcriptional regulatory factors are currently well-characterized, the post-transcriptional control of EMT requires continued investigation. The hnRNP E1 protein displays a major role in the control of tumor cell plasticity by regulating the translational control through multiple non-redundant mechanisms, and this role is exemplified when E1 is absent. hnRNP E1 binding to RNA molecules leads to direct or indirect translational regulation of specific sets of proteins: (1) hnRNP E1 binding to specific targets has a direct role in translation by preventing elongation of translation; (2) hnRNP E1-dependent alternative splicing can prevent the generation of a competing long non-coding RNA that acts as a decoy for microRNAs (miRNAs) involved in translational inhibition of EMT master regulators; (3) hnRNP E1 binding to the 3' untranslated region of transcripts can also positively regulate the stability of certain mRNAs to improve their translation. Globally, hnRNP E1 appears to control proteome reprogramming during cell plasticity, either by direct or indirect regulation of protein translation.

Keywords: Breast cancer, tumor progression, epithelial-mesenchymal transition, cancer stem cells, transforming growth factor- β , translation, hnRNP E1, *PCBP1*



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INTRODUCTION

Epithelial-mesenchymal transition in tumor progression and metastasis

Metastasis represents a critical step in tumor progression and accounts for more than 90% of cancer-induced mortality^[1]. Despite the tremendous efforts made by the scientific community over recent decades, the cellular and molecular events that control tumor cell plasticity remain incompletely elucidated.

It has been shown that the epithelial-mesenchymal transition (EMT) is essential in embryonic development and in tumor metastasis and is among the mechanisms deemed critical in tumor cell plasticity. EMT consists of a fine-tuned phenotypic switch, characterized by the loss of apical-basal polarity and cellular adhesion in epithelial cells^[2,3]. Cells undergoing transition gradually express mesenchymal features, such as enhanced cytoskeletal rearrangement and extracellular matrix (ECM) degradation, both essential for cell motility [Figure 1].

EMT is not a unidirectional mechanism. The transition is a fine-tuned, reversible mechanism that allows cells to switch between epithelial and mesenchymal phenotypes while manifesting all intermediate phenotypic shades^[2,4]. The reverse mechanism, known as mesenchymal-epithelial transition (MET), allows reversion to the differentiated phenotype. Reversion is important for the potential formation of metastases that can occur as tumor cells attempt to relocate to distant organs to develop secondary tumors. Due to its transience, its presence in multiple-states, and its reversible nature, EMT is technically challenging to observe throughout tumor progression *in vivo*^[4]. Nevertheless, it is clearly demonstrated that transitioned cells harbor higher invasive capacities^[5-7]. In the early steps of metastasis, epithelial cancer cells must acquire the ability to separate from the primary tumor^[4,8]. Such departure may occur as single cells or as clusters of cells, and always requires the loss or the alteration of cell-to-cell and cell-to-matrix interactions^[9].

In the current model, EMT-positive tumor cells displaying newly-acquired mesenchymal features invade their surrounding environment and intravasate into the circulatory system. Cancer dissemination in this model results from ECM degradation and increased motility^[10,11]. It was also proposed that the survival of circulating tumor cells (CTCs) in the blood stream was enhanced by the phenotypic plasticity observed during EMT^[12-15]. Following dissemination into the circulation, CTCs extravasate and colonize distant organs to ultimately form secondary tumors. The ultimate metastatic colonization occurs through the re-epithelialization of cells by MET and is followed either by a proliferative cycle with subsequent drug resistant secondary tumor growth, or by a dormant cycle with latency of tumor relapse. Although there are countless reports demonstrating that genetic mutations are recurrently arising in many types of primary tumors, the attempts to identify genes that are recurrently mutated in the genomes of metastasized cells have consistently failed until now. Such observations are advocating for the prime role of cell plasticity in tumor cells dissemination. However, the experimental evidences of the casual function of EMT in metastasis formation remain to be clearly demonstrated. The explicit role of EMT in tumor progression remains actively debated but its implication in the increased resistance seen in both conventional and targeted antitumor therapies is currently well-accepted by the scientific community^[12,14,16-21]. It seems now clear that the controversy around the role of EMT in tumor dissemination might be attributed to its non-linear and multi-modal nature and that these many intermediate stages of EMT may occupy different regions on such a multi-dimensional landscape^[22]. Many groups are still investigating the role of EMT in tumor cells dissemination, and at this point, multiple hypotheses are still emerging to underline the mechanisms involved in the successful dissemination of tumor cells beyond the current EMT/MET view^[23].

Heterogeneous nuclear ribonucleoprotein E1

Heterogeneous nuclear ribonucleoproteins (hnRNPs) encompass a large family of RNA-binding proteins (RBPs) that contribute to multiple aspects of nucleic acid metabolism. These aspects include alternative splicing, mRNA stabilization, transcriptional control, and translation regulation. The coding sequences

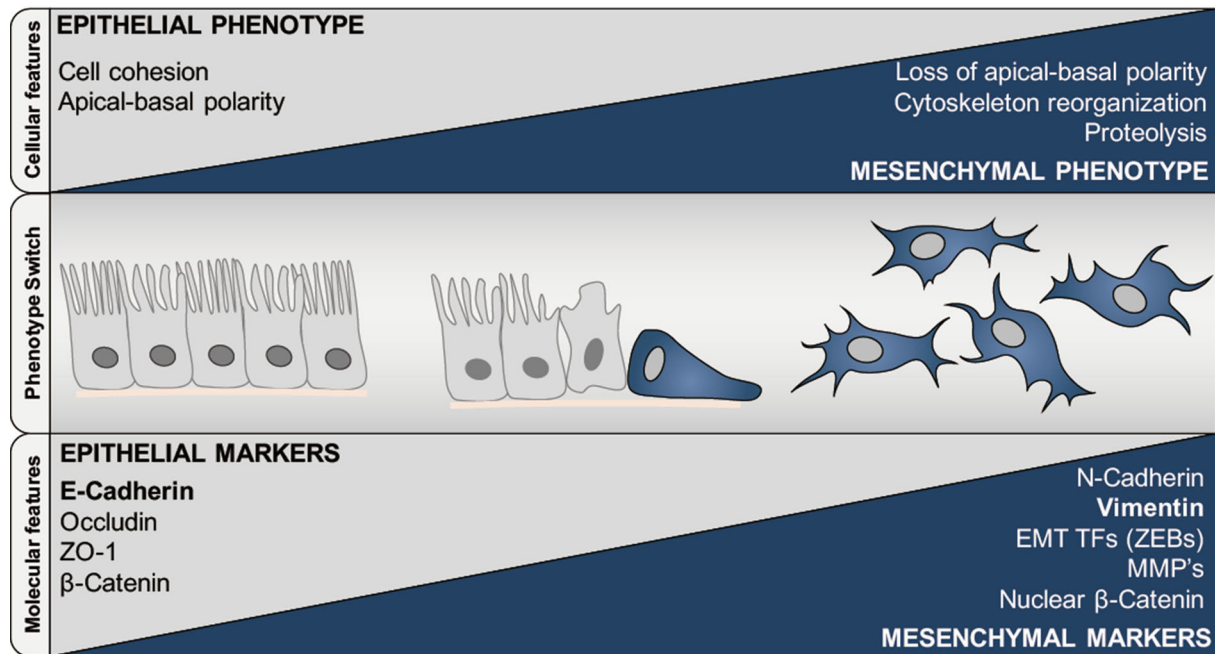


Figure 1. Epithelial-mesenchymal transition relies upon a gradually orchestrated switch from a polarized, epithelial phenotype to a highly motile fibroblastic or mesenchymal phenotype. Epithelial cells are polarized with strong cell-cell cohesions and are organized by multiple cell junction proteins such as E-Cadherin, Occludin, Zonula Occludens, β-catenin and other epithelial markers. During EMT, tumor cells lose their epithelial features and acquire a mesenchymal phenotype, which promotes their motility and invasive capacity. The switch is acquired through a deep reprogramming of the transcriptional landscape and involves activation of EMT transcription factors such as ZEBs, reorganization of cytoskeletal components by regulation of proteins such as Vimentin, and modulation of expression/secretion of invasion-mediating proteases such as matrix metalloproteinases

of hnRNPs reveal a modular structure consisting of one or more RNA-binding motifs, and at least one auxiliary domain that regulates protein-protein interactions and subcellular localization^[24]. Indeed, all hnRNPs contain RNA binding domains that may include RNA recognition motifs (RRM), the quasi-RRM, glycine-rich domains and KH domains. Contrary to RNA-binding domains, auxiliary domains are divergent in protein sequence and are unstructured but are highly involved in regulating subcellular localization and other biological features. Most of the hnRNP proteins contain nuclear localization signals and are therefore mainly located in the nucleus during steady state. However, they can translocate into the cytosol via signaling pathway activation or by recruitment by other proteins. Importantly, for most of the hnRNPs, cellular functions are tightly regulated through post-transcriptional modifications including but not limited to methylation, phosphorylation, ubiquitination and sumoylation.

Poly(rC)-binding protein 1 (PCBP1 also called hnRNP E1) belongs to a hnRNP family that is composed of hnRNP K/J and the alpha-complex proteins (PCBP1-4α or CP1-4). Proteins in this family contain three hnRNP K homology (KH) domains for RNA-binding. The human PCBP1 gene encodes the hnRNP E1 protein, and was initially defined as clone sub2.3, with poly(C)-binding activity and observed similarity to hnRNP E2^[25]. hnRNP E1 harbors three highly conserved KH domains, KH1 to KH3. KH1 and KH3 domains were initially predicted to bind to RNA and *in vitro* studies later showed that the KH2 domain of hnRNP E1 also binds to RNA to regulate protein translation^[26-28]. The hnRNP E1 and hnRNP E2 proteins share 82% amino acid identity, with an even higher level of conservation (93%) for their KH domains^[24].

The subcellular localization of hnRNP E1 is predominantly nuclear, and demonstrates accumulation within nuclear speckles, with precise sites of accumulation observed with splicing factors prior to nascent transcript loading^[29]. The nuclear localization of hnRNP E1 is abolished by Actinomycin D^[30]. Since splicing factors undergo continuous and rapid nucleo-cytoplasmic shuttling^[31], and because splicing is coupled to

transcription, RNA polymerase inhibition results in the cytoplasmic accumulation of many splicing factors. Such observations support the primordial nuclear role of hnRNP E1 in pre-RNA splicing^[32]. The subcellular localization of hnRNP E1 is also governed by cell signaling stimuli. It was demonstrated that SMAD3 and hnRNP E1 were induced to govern alternative splicing, mediated by their colocalization in SC35 (also known as SRSF2)-positive nuclear speckles, downstream of EGF and TGF β , respectively^[33]. Even if it is to a lesser extent, hnRNP E1 is also clearly observed in the cytoplasm, and its presence potentially correlates to its function as a translational regulator^[29,34,35].

REGULATION OF TRANSCRIPTION

HnRNP E1 functions as a regulator of gene expression at the transcriptional level, although this does not appear to be its primary role. Recombinant cloning of the four members (PCBP1 to PCBP4) of the poly(C) binding protein family (PCBP) demonstrated that transcriptional activity of the mouse μ -opioid receptor (MOR) gene increased due to interaction between PCBPs and the 26-bp polypyrimidine stretch in the MOR proximal promoter^[36]. PCBPs can bind either to single-stranded or to double-stranded DNA^[27]. hnRNP E1 also exhibits a mild but consistent activation of the promoter of the BRCA1 gene^[37]. Finally, hnRNP E1 was found to regulate eIF4E transcription^[38]. Recruitment of hnRNP E1 to the eIF4E basal element (4EBE) in the eIF4E promoter region occurs downstream of serum or EGF-mediated cellular stimulation, and this mechanism requires Pak1-dependent phosphorylation of hnRNP E1 protein^[38]. These findings suggest that both hnRNP E1 and its phosphorylation downstream of growth factor-induced signaling play a regulatory role in eIF4E transcription in mitogen-stimulated cells.

TRANSLATION REGULATION

The hnRNP E1 protein regulates translation through either direct or indirect mechanisms. Examples of the most common mechanisms include regulation of mRNA stability, direct control of the ribosomal machinery, or the generation of RNA species that prevent miRNA-mediated translational repression of specific mRNAs.

Control of mRNA stability

The role of hnRNP E1 in RNA stability is exemplified by a broad spectrum of mRNA interactions. Mainly, hnRNP E1 regulates gene expression via binding to specific AU-rich elements (AREs) or U-rich elements located in the 3' untranslated regions (UTR) of target mRNAs. For instance, the binding of hnRNP E1 to p27^{kip1} 3' UTR via its KH1 domain stabilizes p27^{kip1} mRNA, fueling p27^{kip1} protein expression by enhancing its translation prior to degradation. The upregulated p27^{kip1} protein consequently inhibits cell proliferation, cell cycle progression, and tumorigenesis, and can concurrently promote cell apoptosis under paclitaxel treatment^[39]. Interestingly, other cyclin-dependent kinase inhibitors such as p21^{Waf1} are also regulated through hnRNP E1 mediated mRNA stability. Co-immunoprecipitation of p21^{Waf1} mRNA from MDA-MB-468 breast cancer cells using hnRNP E1 antibody suggests that hnRNP E1 protein binds to one or more motifs distributed throughout the p21^{Waf1} 3' UTR to stabilize the mRNA^[30].

The eNOS mRNA 3' UTR contains multiple evolutionarily conserved pyrimidine (C and CU)-rich sequence elements that are both necessary and sufficient for mRNA stabilization. The hnRNP E1 protein binds to these 3' UTR elements. Hence, hnRNP E1 is recruited to a stabilizing RNP complex that protects eNOS mRNA from the inhibitory effects of its antisense transcript sONE, and from 3' UTR-targeting small interfering RNAs (siRNAs) and miRNAs^[40]. HnRNP E1 regulates the stability of p63 mRNA via binding to a CU-rich element (CUE) within the p63 3' UTR^[41]. The p63 protein contribution to EMT could vary according to the biological context. It has been shown that p63 directly modulates the tumor cell plasticity by either attenuating EMT in human prostate cancer cells or promoting tumor cell invasion during human and mouse breast tumor cells dissemination^[42,43]. Phosphorylation of hnRNP E1 also contributes to stabilization

of mu opioid receptor (MOR) mRNA via interaction with ARE RNA-binding protein 1 (AUF1) and poly A binding protein (PABP)^[44].

Alternative polyadenylation

The addition of the poly-(A) to the messenger RNA 3' UTR is a co-transcriptional process occurring in the nucleus. As 3' UTRs contain cis elements that are involved in various aspects of mRNA metabolism, 3' UTR alternative polyadenylation (APA) can affect post-transcriptional gene regulation considerably in various ways, including modulation of stability, translation, nuclear export and cellular localization of mRNA. 3' UTR-APA can also affect the localization of the encoded protein^[45]. Polyadenylation of mRNA is a two-step process consisting of endonucleolytic cleavage and addition of an untemplated poly(A) tail.

The role for hnRNP E1 in the regulation of alternative polyadenylation has been established in an *in vitro*-transcribed and polyadenylated alpha-globin 3' UTR assay^[46]. Furthermore, a screening study for alternative polyadenylation utilizing RNA interference (RNAi) identified hnRNP E1 as the second highest factor in the control of polyadenylation signal usage. The mechanism by which hnRNP E1 modulates polyadenylation has yet to be characterized^[47].

Direct control of translation machinery through BAT elements

As we and others have previously demonstrated, the regulation of gene expression at the post-transcriptional level plays an indispensable role in TGF β -induced EMT and metastasis^[30,34,48-52]. We identified a transcript-selective translational regulatory pathway in which a ribonucleoprotein (mRNP) complex binds to a 33-nucleotide TGF β -activated translation (BAT) element in the mRNA 3' UTR and silences the translation of a cohort of mesenchymal protein-encoding mRNAs. hnRNP E1 is a critical component of the BAT-binding mRNP complex^[53]. Silenced mRNAs include Disabled2 (Dab2) and Interleukin-like EMT-inducer (ILEI), which are involved in mediating EMT^[34,51,54,55]. Furthermore, TGF β activates a kinase cascade terminating in the phosphorylation of hnRNP E1 protein at Serine 43. This phosphorylation occurs by isoform-2 specific activation of Akt and induces the release of the mRNP complex from the BAT element. This results in the reversal of translational silencing of mesenchymal protein-encoding transcripts that are required for EMT. By using a genome-wide combinatorial approach involving expression, polysome profiling and RIP-Chip analysis, we have identified the members of the cohort of translationally regulated mRNAs that are induced during TGF β -mediated EMT^[53].

At the molecular level, the eukaryotic elongation factor-1 A1 (eEF1A1) is an important additional functional component of the mRNP complex. We have previously demonstrated that the BAT element, hnRNP E1 and eEF1A1 form a ternary complex that mediates translational silencing at the translational elongation step^[35]. In non-stimulated cells exhibiting epithelial characteristics, hnRNP E1 binds to eEF1A1 and blocks progression of the 80S ribosome by preventing the release of eEF1A1 from the ribosomal A-site following GTP hydrolysis. EMT induced by either TGF β or hnRNP E1 knockdown disrupts the mRNP complex, allowing eEF1A1-mediated translational elongation of mesenchymal transcripts to proceed^[34,35] [Figure 2].

This mode of translational regulation represents an unusual case of dependency upon either agonists or stimuli to upregulate translation through 3'-UTR elements. Thus, the elucidation of this post-transcriptional regulatory pathway identified an "EMT gene signature" and provided mechanistic information as to how cell plasticity could be tightly regulated. Taken together, this work underscores the contribution of the non-phosphorylated hnRNP E1 protein to maintenance of epithelial cell integrity under normal conditions. During tumor-related epithelial plasticity, hnRNP E1 also acts as the trigger for the reversal of translational silencing, resulting in a fine-tuned, spatio-temporally controlled increase in mesenchymal protein expression.

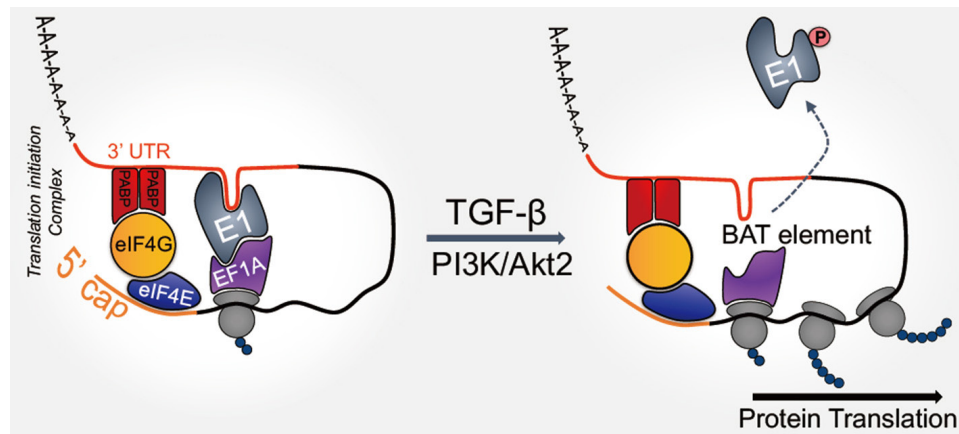


Figure 2. Molecular mechanism of hnRNP E1-mediated translational silencing. The eukaryotic elongation factor-1A1 (eEF1A1) forms a complex with hnRNP E1 and the BAT element, and silences specific protein expression by stalling the elongation of their translation by ribosomes. Given the necessity for cognate-codon interaction with the ribosomal A site, it is likely that the formation of the BAT mRNP complex occurs post-delivery of the aminoacyl-tRNA to the ribosome. The ability of the BAT mRNP complex to inhibit eEF1A1-dependent elongation suggests that the 3'-UTR is interacting with the 5'-UTR in a circularized model to facilitate its proximity to the 80S ribosome^[35]. It has been suggested that translatable mRNAs are likely to be found in circular forms due to interaction between PABP, eIF4G, and the cap-binding protein eIF4E^[56]

Indirect control of translation through alternative splicing

Alternative splicing regulates over 90% of multi-exon protein-coding genes in humans^[57]. Abnormal regulation of alternative splicing often produces disease-specific protein isoforms^[58,59]. Additionally, genome-wide analysis has identified tens of thousands of “splice variant” mRNAs that are enriched in a wide range of human diseases^[60,61]. The hnRNP E1 protein is well documented for its repressive role in alternative splicing mechanisms as they apply to human health and disease. For instance, hnRNP E1 represses tumor cell invasion by inhibiting the alternative splicing of CD44^[62]. It was therefore demonstrated that enforced hnRNP E1 expression inhibited CD44 variants expression in HepG2 human liver cancer cells while knockdown of endogenous hnRNP E1 induced these variants splicing^[62]. Interestingly, another study based on *in vitro* and *in vivo* models of breast cancer progression demonstrated the switch of CD44 expression occurring from variant isoforms to the standard isoform during EMT. This isoform switch to CD44s was essential for cells to undergo EMT and was required for the formation of breast tumors that display EMT characteristics in mice^[63]. hnRNP E1 also binds to the growth hormone receptor pseud exon and prevents its usage to allow the expression of a functional protein^[64]. Disruption of hnRNP E1 binding and subsequent activation of an alternative splicing event responsible for Laron syndrome was demonstrated either by hnRNP E1 knockdown or by alterations to the genomic pseudosite. We also recently reported the binding of hnRNP E1 to a pre-RNA pseudosite in the serine/threonine-protein phosphatase 1 regulatory subunit 10 (*PNUTS*) transcript^[30]. The hnRNP E1 protein binds to a conserved BAT element that is similar in structure to those observed in the 3'-UTRs of the mesenchymal encoding mRNAs discussed above. The loss of hnRNP E1 binding to the alternative splicing site of *PNUTS* following hnRNP E1 knockdown, phosphorylation, and/or cytoplasmic translocation activates usage of the pseudosite and generates an alternatively spliced isoform of *PNUTS*. This alternative *PNUTS* isoform does not encode a functional protein, but rather a non-coding isoform of the gene. Functionally, the lncRNA-*PNUTS* acts as a decoy for miRNA-205 and binds to the miRNA causing a decrease in miRNA-205 bioavailability. This abolishes translational inhibition of mesenchymal factors such as ZEB mRNAs that would otherwise be suppressed in epithelial cells^[30]. Since alternative splicing and generation of lncRNA-*PNUTS* is an early event in TGFβ-mediated EMT, the lncRNA-*PNUTS* likely operates as a transient inhibitor of the miRNA-205 to allow for the temporal upregulation of ZEBs and subsequent regulation of downstream EMT events. Indeed ZEBs proteins are reciprocally linked in a feedback loop with the miR-200 family, each strictly controlling the expression of the other^[18,65]. In this way, a transient, but nevertheless, strong decrease in miR-205 bioavailability, sufficient to activate the ZEB proteins, would allow for transcriptional repression of the miR-200 family or other miRNAs

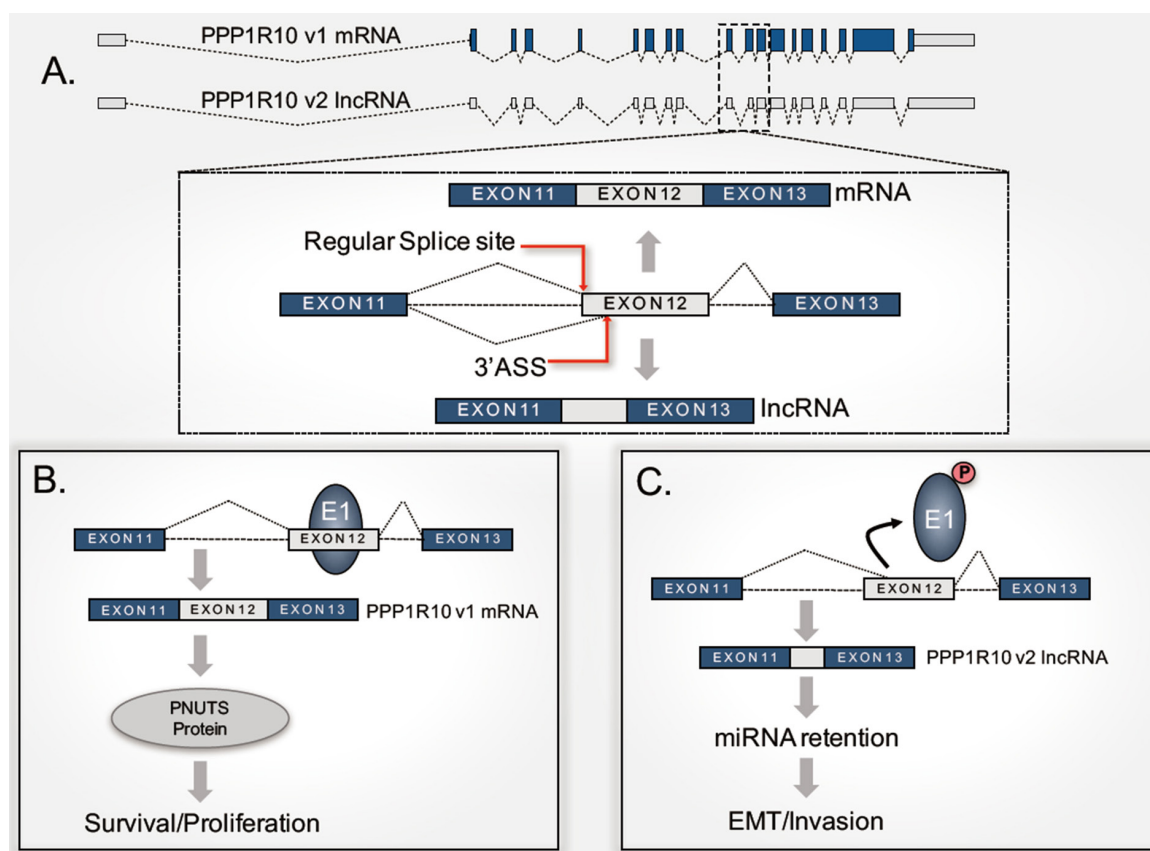


Figure 3. Molecular mechanism of hnRNP E1-mediated alternative splicing of *PNUTS*. A: The PPP1R10 (*PNUTS*) gene locus can encode either a protein coding mRNA or a non-coding RNA isoform. The *PNUTS* gene locus is highly conserved between human and mouse and expresses both coding and non-coding transcripts. The lncRNA-PNUTS is generated by the usage of the 3' alternative splice site (3'ASS) located at the 5' end of exon 12. This usage leads to the change of the open reading frame and the generation of a premature stop codon; B: the binding of hnRNP E1 to a BAT consensus element located in the alternative splicing site results on its masking and prevents its usage to generate the *PPP1R10* mRNA translated into the PNUTS protein; C: loss of hnRNP E1 binding to the alternative splice site uncovers it and allows its usage by the spliceosome machinery. The lncRNA-*PNUTS* acts as a decoy for miRNA-205 and thus allows the de-repression of ZEB protein translation. Reactivated expression of ZEB proteins induces the shutdown of epithelial markers such as E-Cadherin, allowing EMT to proceed

such as miR-183 or miR-203 thereby further stabilizing ZEB proteins and reinforcing the EMT process^[30]. Moreover, it has been suggested that the feedback loop of miR-200/ZEB also generates hybrid phenotypes of the cells during EMT-mediated tumor cell plasticity^[66] [Figure 3].

CONCLUSION

For many years, characterization of the role of RBPs in tumor biology and cell plasticity resulted in substantial progress, and the investigation of hnRNP E1 provided understanding of many facets of its molecular function in cells. The function of the PCBP1 gene encoding the hnRNP E1 protein was first demonstrated through characterization of its role as a negative regulator of alternative splicing. Since then, many additional roles have also been discovered, and most of them appear to have critical participation in the maintenance of cell phenotype integrity^[34,39,67]. At the molecular level, the ability of hnRNP E1 to specifically bind to mRNA species often leads to a direct or indirect regulation of their translation. This occurs either by controlling processivity of ribosomal machinery, stabilizing mRNA, or locking/unlocking dormant translational controls. Since it is well established that hnRNP E1 controls cell plasticity in health and disease through multiple fine-tuned regulatory mechanisms, it will be essential to develop investigations involving novel therapeutic strategies. Moreover, targeting the KH domain of hnRNP E1 may be relevant.

However, because hnRNP-E1 is pleiotropic and ubiquitous, confining therapeutic strategies to tumor cells may be challenging.

The phenotypic changes observed during cell plasticity and tumor progression demand radical proteomic reprogramming of cells concomitant with a modulation of the codon usage by the translational machinery. Accumulation of evidence advocates for acknowledgement of a primary role for hnRNP E1 in cell plasticity, and this is reinforced by identification of mechanisms involving hnRNP E1 that directly or indirectly converge upon the translational control of plasticity-associated proteins. We therefore propose that hnRNP E1, together with its associated proteins, acts as a hub that orchestrates the demands of proteome reprogramming during health and disease-associated cell plasticity.

DECLARATIONS

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Authors' contributions

Wrote the manuscript: Grelet S

Validated the manuscript: Howe PH

Availability of data and materials

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Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

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Original Article

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AF1q inhibited T cell attachment to breast cancer cell by attenuating Intracellular Adhesion Molecule-1 expression

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Abstract

Aim: To investigate whether AF1q, overexpressed in metastatic cells compared with the primary tumor cells, plays a pivotal role in breast cancer metastasis.

Methods: To investigate whether AF1q has a responsibility in the acquisition of a metastatic phenotype, we performed RNA-sequencing (RNA-Seq) to identify the gene signature and applied the Metacore direct interactions network building algorithm with the top 40 amplicons of RNA-Seq.

Results: Most genes were directly linked with intercellular adhesion molecule-1 (ICAM-1). Likewise, we identified that ICAM-1 expression is attenuated in metastatic cells compared to primary tumor cells. Moreover, overexpression of AF1q attenuated ICAM-1 expression, whereas suppression of AF1q elicited the opposite effect. AF1q had an effect on ICAM-1 promoter region and regulated its transcription. Decreased ICAM-1 expression



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affected the attachment of T cells to a breast cancer cell monolayer. We confirmed the finding by performing the analysis on Burkitt's lymphoma.

Conclusion: Attenuation of ICAM-1 by AF1q on tumor cells disadvantages host anti-tumor defenses through the trafficking of lymphocytes, which affects tumor progression and metastasis.

Keywords: MLLT11, AF1q, intercellular adhesion molecule-1, breast cancer, metastasis, RNA-sequencing

INTRODUCTION

While primary lesion breast tumors are not fatal to the patient, the metastatic breast tumors are. For this reason, breast patients are often given other treatments after surgery to try to eliminate metastatic tumor cells that might have spread to other organs. Although the prognosis for breast cancer has improved during the last decade, we still lack specific treatments that target metastases. The discovery of such treatment will be of great benefit to patients.

The *AF1q* gene, located in chromosome 1, band 21, is an MLL fusion partner that was identified in acute myeloid leukemia patients with t (1; 11) (q21; q23) chromosomal abnormality^[1]. The function of AF1q is not yet fully known; however, elevated AF1q expression is associated with poor clinical outcomes in various malignancies. We have demonstrated that the enhanced AF1q expression promotes cell proliferation, migration, sphere formation, and chemo-resistance *in vitro* and *in vivo* breast cancer models^[2]. During the course of our study, we made an exciting discovery that AF1q physically interacts with TCF7, a key factor in the Wnt signaling pathway that enhances its activation^[2]. The Wnt signaling pathway plays a leading role in various processes that are important for cancer progression, including cancer initiation, growth, cell death, differentiation, and metastasis^[3]. Strikingly, we observed that AF1q-positive cancer cells are significantly more prevalent at metastatic sites than in primary breast tumors, when comparing paired samples from breast cancer patients^[2]. Moreover, the Wnt signaling pathway was activated by AF1q crosstalk to the STAT3 pathway via the PDGF/PDGFR cascade^[4]. The PDGF-B/PDGFR signaling cascade was activated upon enforced AF1q expression and this caused an increase in STAT3 DNA binding activity through *Src* kinase action in cancer cells^[4]. Moreover, AF1q is one of the genes that are differentially expressed between highly metastatic breast cancer cells and their parent cells^[2]. Importantly, when breast tumor cells were treated with doxorubicin or etoposide, endogenous AF1q expression was further activated, implying that current chemotherapeutics may increase the risk of metastasis of breast cancer. Taken together, characterization of AF1q-induced breast cancer and new treatment strategies for hyperactive AF1q expression patients are urgently needed.

Intercellular adhesion molecule-1 (ICAM-1) plays a key role in inflammatory conditions, nervous system development, and immune response through antigen recognition and lymphocyte surveillances^[5,6]. There is still controversy regarding the contribution of ICAM-1 expression to tumor progression. Expression of ICAM-1 on tumor cells negatively correlates with tumor progression and development, including tumor size and lymph node metastasis^[7]. A better prognosis was also reported for patients with ICAM-1 positive tumors compared with ICAM-1 negative tumors^[8,9]. However, *in vitro* studies demonstrated that ICAM-1 overexpression can positively affect tumorigenicity^[10]. Despite the controversy, it is clear that ICAM-1 plays a pivotal role in immune response. ICAM-1 levels on tumor cells stimulate T-cell receptor-mediated cellular immune response^[11]. Recent studies revealed that ICAM-1 expression plays an important role in interactions between lymphokine-activated killer cells and cancer cells^[12,13]. These results suggest that a decrease of ICAM-1 may be one of the mechanisms by which tumor cells escape cell-mediated cytotoxicity and lysis by the host cellular immune system.

Metastatic cancer cell, MDA-MB-231LN, is subtype of MDA-MB231 breast cancer cells and it has shown enhanced tumor growth and widespread metastasis than parents cell line in xenograft models^[14]. We observed AF1q, a metastasis enhancer, is highly expressed in metastatic cancer cells (MDA-MB-231LN) than in the primary tumor cells (MDA-MB-231). In this study, we investigated whether AF1q is responsible in the acquisition of metastatic phenotype using RNA-sequencing (RNA-Seq) and applied the Metacore direct interactions network building algorithm. Intriguingly, most genes were directly linked with ICAM-1. Likewise, we identified that ICAM-1 expression is attenuated in metastatic cancer cells compared to primary cancer cells.

In addition to AF1q oncogenic functions, we demonstrated that intensity of AF1q expression in metastatic sites is higher than in primary sites^[2]. Thus, similarly to that reported for certain oncogenes (i.e., Myc and Ras), AF1q has been shown to be endowed with a dual function in malignancy, being a protein apparently involved in both initiation and promotion of cancer progression through regulation of ICAM-1 expression. Our results suggest that targeting AF1q is valuable in developing treatments for breast cancer metastasis.

METHODS

Cell lines and cell culture conditions

MDA-MB-231 was purchased from American Type Culture Collection (ATCC). MDA-MB-231-luc-D3H2LN (MDA-MB-231LN) was purchased from Caliper Life Science. The cells were maintained as a monolayer culture in DMEM, supplemented with Glutamax and penicillin-streptomycin (Invitrogen). Fetal bovine serum (FBS) (Thermo Fisher Scientific) was added to the media. Burkitt's lymphoma cell lines, Ramos, Akata, Mutu, Raji, Jiyoye, BL-5, BL-7, and BL-8, were maintained in RPMI-1640, supplemented with Glutamax, penicillin-streptomycin, and 10% FBS (Thermo Fisher Scientific).

Whole blood was collected from healthy volunteers at James Graham Brown Cancer Center, University of Louisville with donors' written consent. The CD4-positive and CD8-positive human T cells were purified from buffy coats via positive selection using a 1:1 mixture of CD4- and CD8- MicroBeads (Miltenyi Biotec) according to the manufacturer's protocol. Isolated T cells were maintained in RPMI-1640, supplemented with 300 IU/mL IL-2 (R&D) and 10% FBS (Thermo Fisher Scientific). All cells were maintained at 37 °C under a humidified atmosphere of 5% CO₂.

RNA-Seq

Total RNA were isolated from MDA-MB-231 and MDA-MB-231LN cells with the use of the Purelink RNA min Kit (Thermo Fisher Scientific) in triplicates. Of total RNA, 1 µg was depleted of cytoplasmic and mitochondrial ribosomal RNA using the Illumina Ribo-Zero Gold rRNA Removal Kit (Human/Mouse/Rat) (Illumina). The rRNA-depleted RNA was ligated with Illumina adapters and further processed for sequencing following the Illumina TruSeq Stranded Total RNA library preparation kit (Illumina). All samples were pooled and a 75-cycle single-end sequence run was performed using the Illumina High Output Kit v2 on the Illumina NextSeq 500 platform.

RNA-Seq data analysis

RNA-Seq data were mapped using UCSC human genome, hg38, with STAR (version 2.5.2b). The read count and reads per kilobase of transcript per million mapped reads (RPKM) were calculated on the basis of the human GRCh38 Ensembl Release 91 gene annotation. To search differentially expressed genes, Cuffdiff (version 2.2.1) was used. For further analysis, 1485 significantly differentially expressed genes were selected as following criteria, when the value of averaged RPKM from three replicates is greater than or equal to 1 in at least one of the two samples, when the absolute value of log₂ (fold-change) is greater than or equal to 1, and when false discovery rate is less than or equal to 0.01.

Tumor data selection

We selected data from The Cancer Genome Atlas (TCGA) project to represent the breast invasive carcinoma (BRCA). We downloaded publicly available 1,222 RNA-Seq data of 1,092 breast cancer cases from TCGA database. Using FPKM values from all 1,222 samples, the expression level of AF1q was compared to that of ICAM-1.

RT and real-time PCR analysis

Total RNA was subjected to reverse transcription (RT) with a High capacity RNA-to-cDNA kit (Thermo Fisher Scientific), and the resulting cDNA was subjected to real-time polymerase chain reaction (RT-qPCR) analysis with BrightGreen qPCR master mix (ABM) and a StepOne real time PCR system (Thermo Fisher Scientific). The amplification protocol comprised 40 cycles of incubations at 95 °C for 30 s and at 60 °C for 60 s. PCR primer sequences (forward and reverse) were as follows: 5'-TGAGTACAGCACCTTCAACTTC-3' and 5'-GGGAAAGGAGTGGAAGGAAG-3' for AF1q; 5'-CAATGTGCTATTCAAAGTCCCC-3' and 5'-CAGCGTAGGGTAAGGTTCTTG-3' for ICAM-1; 5'-CAGAGGGCTACAATGTGATGGC-3' and 5'-GCTGAGGATTTGGAAGGGTG-3' for HPRT1.

Plasmid construction

Full-length AF1q cDNA was inserted into pLUTdNB, which is a pTRIPZ base modified doxycycline-inducible vector by cloning the HindIII and XhoI fragments as previously described. Short hairpin RNA (shRNA) of AF1q was purchased from Open Biosystems. Empty pLUTdNB, and pTRIPZ-scramble were used as controls for pLUTdNB-AF1q, and pTRIPZ-AF1q shRNA, respectively.

Viral production and infection

Lentivirus was produced by co-transfection of HEK 293T cells (ATCC) with the lentiviral constructs pVSV-G and psPAX2 (Addgene). Transfections were carried out using Lipofectamine 2000 (Invitrogen). For enforced AF1q expression or shRNA targeting, cells underwent lentiviral transduction and were selected using 1 µg/mL puromycin (Thermo Fisher Scientific) as previously described^[15]. After antibiotic selection, cells were cultured in complete medium including 1 µg/mL doxycycline (Thermo Fisher Scientific) for AF1q or shRNA induction.

Western blot

For blots of whole-cell lysates, cells were lysed directly in GLB buffer (2% SDS, 10% glycerol and 50 mmol/L Tris, pH 6.8), boiled, and separated by electrophoresis on a 4%-12% SDS-PAGE gradient gel. Proteins were transferred to PVDF membrane (Millipore) and blocked in 5% skim milk (Bio-Rad) in 0.05% PBST. Rabbit monoclonal anti-AF1q, ICAM-1, and β-actin antibodies were purchased from Abcam. After the appropriate antibody incubations, an enhanced chemiluminescence (Denville) system was used for developing blots.

Reporter gene construct, transfection, and Luciferase assay

Dual-Luciferase reporter assays were performed using an ICAM-1 reporter plasmid. To construct reporter plasmids, 850 bases of an ICAM-1 promoter fragment were cloned to between the XhoI and HindIII sites of the pGL4-Luciferase reporter plasmid (Promega). Briefly, 1×10^4 cells were plated in each well of a 96-well plate. The next day, the reporter plasmid (100 ng) and *Renilla* plasmid (10 ng, Promega) were co-transfected into the cells with lipofectamine 2000 (Thermo Fisher Scientific). After 48 h, cells were washed with PBS and assayed with the Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions. Luciferase activity was determined using Synergy H1 Multi-Mode reader (Biotek). Quantitation of luminescent signal from reporter plasmid was normalized by quantitation of the luminescent signal from *Renilla*.

Surface and intracellular staining

Cells were harvested using non-enzymatic dissociation solution (Corning) and washed once with PBS. For staining of ICAM-1, cells were incubated in Human Fc Block (BD) for 10 min. Thereafter, cells were stained

with FITC-conjugated antibody to ICAM-1 in the presence of Fc Block for 30 min at 4 °C. Cells were then washed 3× with PBS and analyzed by FACS Caliber (BD). Data analysis was performed using Flowjo V10 software (Ashland).

Monocyte adhesion assay

Isolated T cells were incubated with Cell Tracker (Thermo Fisher Scientific) for 30 min at 37 °C and then 10⁵ cells per well were incubated on MDA-MB-231LN monolayers in HBSS supplemented with 2 mmol/L calcium and magnesium for 1 h. After removing nonadherent cells, the number of Cell Tracker-labeled cells bound to the MDA-MB-231LN cells was quantified under fluorescent microscopy.

Immunohistochemistry

Tissue samples were fixed in 4% paraformaldehyde, embedded in paraffin, and then 4-μm sections were prepared. Sections were de-waxed and a steamer pre-treatment in Tris/EDTA buffer (DAKO) was performed. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide in PBS. For blocking steps, avidin (Sigma-Aldrich), biotin (Sigma-Aldrich) in PBS, and a super block (IDlabs Biotechnology) were used. Rabbit monoclonal AF1q antibody and mouse monoclonal ICAM-1 antibody in a 1:200 dilution were incubated at 4 °C overnight. IHC detection was performed with the IDetect Super Stain System HRP (IDlabs Biotechnology). Specific signals were amplified using 3-amino-9-ethylcarbazole (IDlabs Biotechnology) under visual control, followed by counterstaining with hematoxylin.

Statistical analysis

Quantitative data are presented as means ± SD and were subjected to analysis of variance followed by a *t* test with the use of Prism V.7 software (GraphPad Software). A *P* value of < 0.05 was considered statistically significant.

RESULTS

Next-generation sequencing and transcriptome data analysis

We observed AF1q, a metastasis enhancer, was highly expressed in metastatic cells, MDA-MB-231LN (invasive subline from MDA-MB-231), than in the primary tumor cells (MDA-MB-231) [Figure 1A]. To validate the results of RNA-seq, we performed RT-qPCR analysis using randomly selected genes [Figure 1B]. Both MDA-MB-231 and MDA-MB-231LN cell lines were subjected to RNA-Seq analysis with three biological replicates for each cell line. The 75 bp-long single-end sequencing reads were mapped to the human genomic reference using STAR aligner. More than 27.7 million sequencing reads were mapped to genome on each replicate (averaging 35.3 million mapped reads) [Supplementary Table 1]. Out of the 58,289 annotated genes, 18,042 (31.0%) by Ensemble were expressed with more than 10 read counts on at least one of our replicates [Supplementary Table 2]. Only uniquely mapped reads were used further for searching differentially expressed genes using Cuffdiff. Total 1,485 genes were selected as significantly differentially expressed with the criteria explained above. Among those, 762 genes showed increased value of RPKM in MDA-MB-231LN cell line compared to MDA-MB-231 and 723 genes showed the opposite. Row-scaled RPKM value of each replicate represents that the differential expression of genes is consistent throughout replicates of each cell line [Figure 1C].

With those differentially expressed genes, pathway analysis was performed using Metacore. By selecting top 40 network objects resulted from the pathway analysis [Table 1], direct interaction network building algorithm was applied. Interestingly, pathway process showed that most genes were directly linked with ICAM-1 [Figure 2A]. In addition, we found out that ICAM-1 transcript level was significantly decreased in MDA-MB-231LN cell line compared to MDA-MB-231 confirming that ICAM-1 expression is attenuated in metastatic cell line [Figure 2B].

To explore the relationship of AF1q with ICAM-1 in breast cancer, we used 1,222 RNA-seq data of 1,092 breast cancer cases from TCGA database. Using FPKM values from all 1,222 samples, the expression level of

Table 1. Top 40 network objects ranked by occurrence in top 50 pathway maps

Ranking	Network objects	Number of maps containing network object	Ranking	Network objects	Number of maps containing network object
1	IL-6	24	10	TGF- β receptor type II	8
2	ICAM-1	20	11	NFKBIA	7
3	IL-1 beta	18	11	MHC class II	7
4	p38 MARK	16	12	WNT5A	6
5	VCAM-1	15	12	GRO-2	6
6	VEGF-A	12	12	Tcf (Lef)	6
6	c-Myc	12	12	Bcl-XL	6
7	GM-CSF	11	12	WNT	6
7	CCL2	11	13	GRO-3	5
8	I-kB	10	13	PLAUR	5
8	PTCH1	10	13	IL-1 α	5
8	SHH	10	13	SNAIL1	5
9	MMP-1	9	13	PKA-cat	5
10	Fibronectin	8	13	MMP-13	5
10	TCF7L2	8	13	PDGF-R- β	5
10	Frizzled	8	13	CSF1	5
10	TLR2	8	13	PKA-reg	5
10	GLI-2	8	14	Jagged1	4
10	Lef-1	8	14	TRF2	4

Network object ranking reflects the popularity of each network object in pathway map. Smaller rank value means that network object occurs in greater number of maps. ICAM-1: intercellular adhesion molecule-1

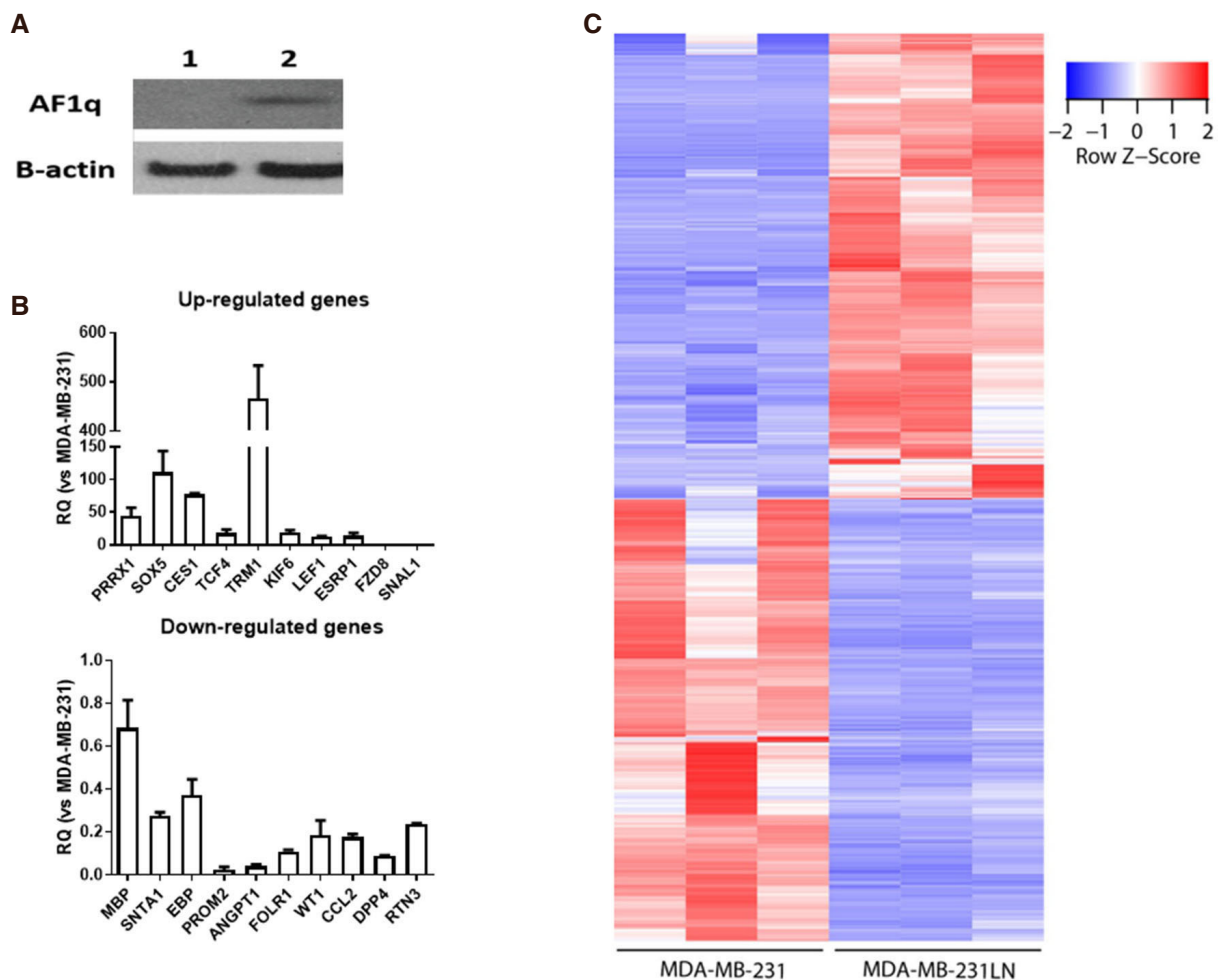


Figure 1. A: AF1q expression status in breast cancer cell lines (1: MDA-MB-231, 2: MDA-MB-231-luc2-LN); B: validation of relative expression of gene obtained from RNA-seq by qPCR. qPCR analyses were performed as described in the method section using randomly selected 20 genes (upregulated genes 10 and downregulated genes 10). Relative expression values are presented as an average \pm SD of three biological replicates; C: significantly expressed genes. 1,485 genes are significantly expressed in both cell lines with three biological replicates, respectively. Among those, transcripts of 762 genes increased in MDA-MB-231LN cell line and those of 723 genes decreased

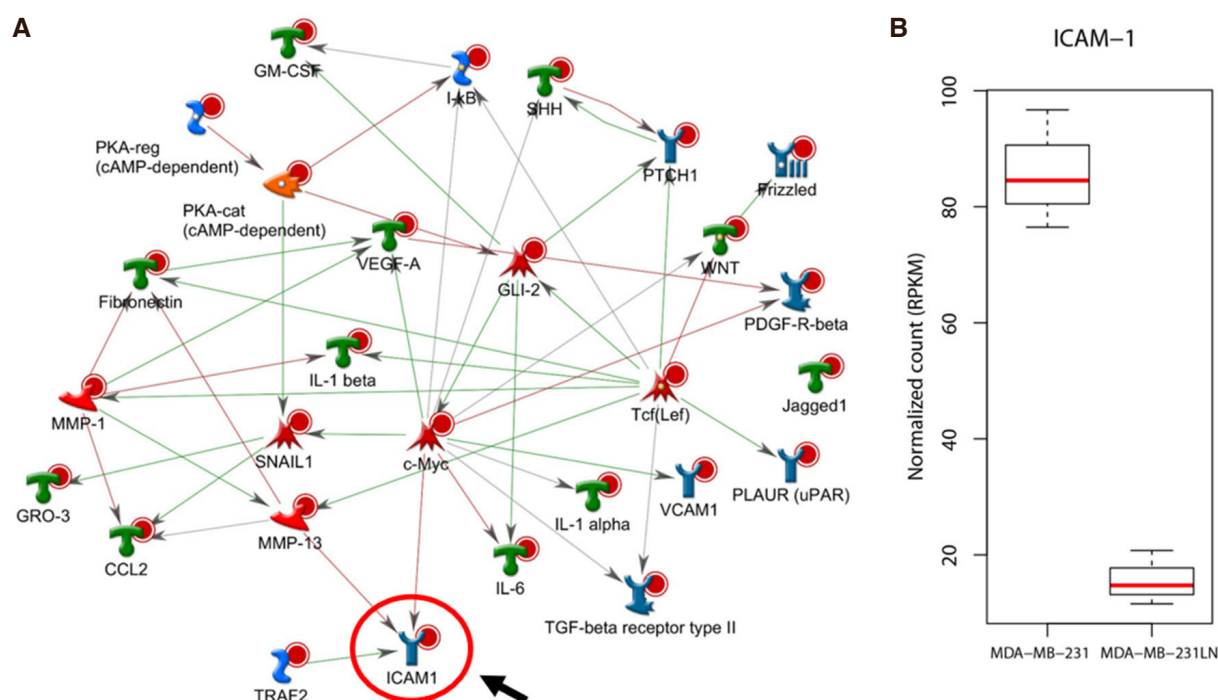


Figure 2. Functional interaction network analysis. A: 1,485 significantly selected genes were further analyzed for pathway process using Metacore. Top 40 network objects were, then, used for building direct interactions. ICAM-1 is directly linked with most genes and positioned at the end of the pathways. Red line represents suppression, blue line represents activation; B: RNA-seq shows that expression level of ICAM-1 is drastically decreased in MDA-MB-231LN cell line compared to MDA-MB-231

AF1q was compared to that of ICAM-1. Vast majority of the samples did not show clear correlation between the expression levels of AF1q and ICAM-1 [Supplementary Figure 1]. Interestingly, however, all those with high FPKM values of AF1q showed low expression level of ICAM-1 supporting our observation that overexpression of AF1q attenuated ICAM-1 expression. Although majority of samples did not show clear correlation, this finding is consistent with our previous report that the role of AF1q is a co-factor rather than a transcription factor^[2].

ICAM-1 is transcriptionally regulated by AF1q

To demonstrate that the AF1q expression was involved in ICAM-1 gene expression in breast cancer, we first experimentally overexpressed or suppressed AF1q expression in MDA-MB-231LN. We used a lentiviral transduction system to overexpress and suppress AF1q with endogenous AF1q expression. As shown in Figure 3A and B, overexpressed AF1q (AF1q) remarkably decreased ICAM-1 mRNA and protein expression, compared to that of control (Ctrl). The ICAM-1 expression, however, was increased by the suppression of AF1q with shRNA (shAF1q) than control (shCtrl). These results indicate that AF1q regulates ICAM-1 expression in transcription. FACS analysis comparing ICAM-1 surface expression on cells show that the attenuation of ICAM-1 on the surface of MDA-MB-231LN in response to AF1q was also confirmed [Figure 3C].

We assessed whether AF1q influences ICAM-1 promoter activity by performing a Luciferase reporter assay. We first experimentally overexpressed or suppressed AF1q expression in HEK293, then, HEK293 cells with a construct containing 850 bases of an ICAM-1 promoter fragment. Overexpressed AF1q displayed 0.7 fold lower luminescence. However, the luminescence was 1.4 fold higher when AF1q expression was suppressed by shAF1q [Figure 3D].

ICAM-1 plays an important role in T cell adhesion and cytotoxicity

Because ICAM-1 is associated with the recognition of T cells, we wanted to determine whether ICAM-1 dysregulation by AF1q is essential for the T cell attachment to breast cancer cells. To demonstrate that

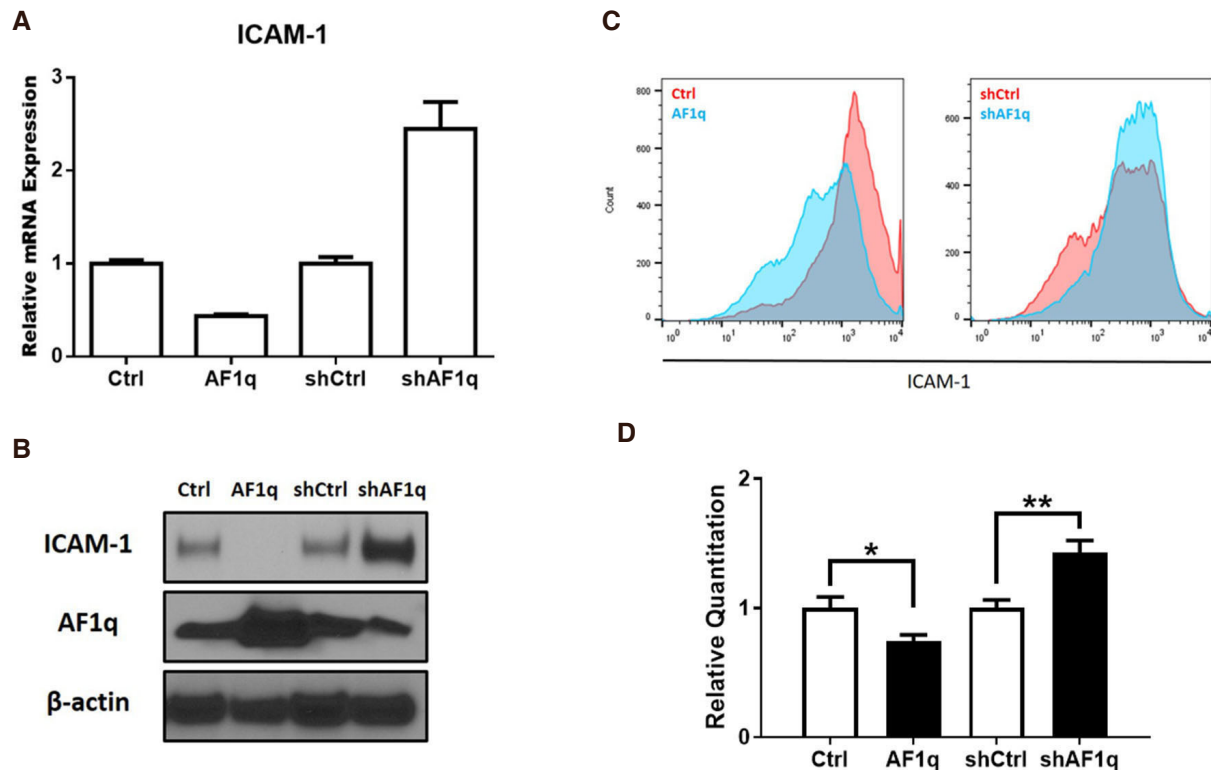


Figure 3. Transcriptional regulation of ICAM-1 expression by AF1q in breast cancer. A: ICAM-1 mRNA expression was quantified using qPCR in MDA-MB-231LN cells engineered to overexpress or suppress AF1q; B: the blot shows ICAM-1 protein expression; C: MDA-MB-231LN cells were stained with ICAM-1 mAb and flow cytometry analysis were performed using FACS Calibur. On left panel, Red is profile of the MDA-MB-231LN/Ctrl and Blue is MDA-MB-231LN/AF1q. On the right panel, Red is MDA-MB-231LN/shCtrl and Blue is shAF1q cells. Representative data of three similar experiments are shown; D: Luciferase activity in HEK293T cells transfected with reporter constructs of the proximal promoter of ICAM-1. Renilla Luciferase activity was used to normalize the promoter activity

ICAM-1 plays a critical role in the adhesion of T cells, we performed an *in vitro* adhesion assay. The attachment between breast cancer cell monolayer and T cells was significantly reduced by AF1q-induced ICAM-1 attenuation, while enhanced ICAM-1 expression by AF1q suppression increased the attachment [Figure 4A]. The attachment between T cells and breast cancer cell monolayer was significantly reduced by pretreatment with a blocking antibody to ICAM-1 [Figure 4B].

Cell cytotoxicity assays are shown in Figure 4C where the corresponding MDA-MB-231LN sublines were used as the target cell. Assays were carried out using *ex vivo* expanded T cells derived from healthy donors. These data indicate that ICAM-1 can function as an important determinant of a tumor cell's sensitivity to T cell-mediated killing.

AF1q reciprocally regulate expression of ICAM-1

We extended our finding to Burkitt's lymphoma. It is well known that downregulation of ICAM-1 in Burkitt's lymphoma enhances the probability of escape of tumor cells from T cell surveillance^[16]. RT-qPCR and Western blot analysis showed that the AF1q expression at both the mRNA and protein levels reciprocally regulated the expression of ICAM-1 in Burkitt's lymphoma cell lines [Figure 5A and B]. We validated our finding in Burkitt's lymphoma patient samples. To investigate the expression levels of AF1q and ICAM-1 in Burkitt's lymphoma patient samples, immunohistochemical staining of patient tissues was performed. We have observed identical results from the IHC study [Figure 5C]. These findings are consistent with observation in breast cancer cells.

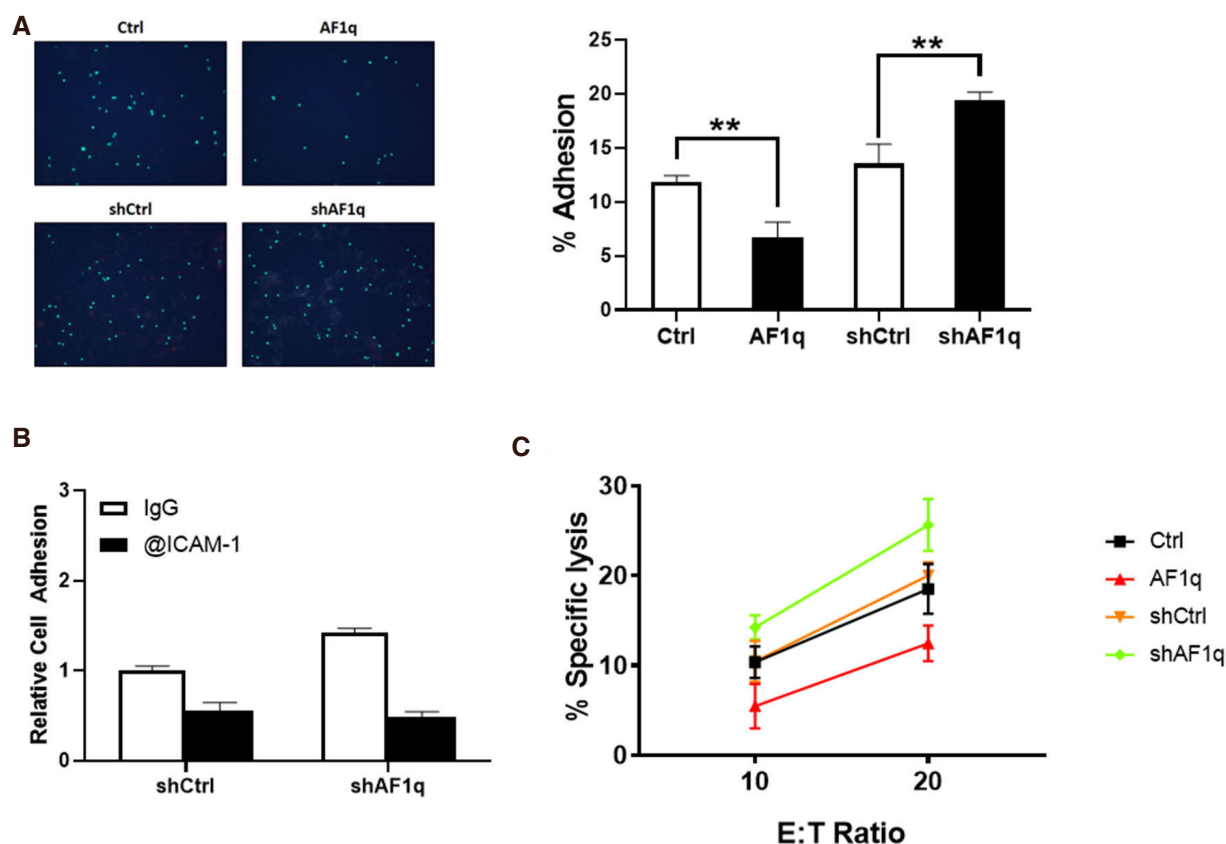


Figure 4. ICAM-1 is important for attachment and cytotoxicity of T cells to breast cancer. A: Labelled T cells attached to MDA-MB-231LN cells monolayer. AF1q significantly reduced the number of attached T cells to the monolayer. When AF1q expression was suppressed, the number of attached T cells was increased. The Bar graph represents the % adhesion cells of total cells; B: ICAM-1 blocking antibody significantly reduced the number of attached T cells to the monolayer; C: a 72 h cytotoxicity assay was performed in 24 well plates where co-cultured at 10:1 or 20:1 E:T ratio with MDA-MB-231LN cells

DISCUSSION

We have here identified ICAM-1 which reciprocally regulated by AF1q was associated with metastasis of cancer cells. Although elevated AF1q expression is associated with poor clinical outcomes in various malignancies^[4-6], the function of AF1q is not yet fully understood. Our findings further explain why high AF1q expression is associated with poor clinical outcomes. However, greater mechanistic understanding of how AF1q is involved in promoting cancer metastasis is necessary before these laboratory investigations can be translated into clinical interventions. Thus, it is very important to continue to investigate the underlying biological functions of AF1q and its association with breast cancer metastasis.

We observed that nuclear factor-kappa B (NF- κ B) activity was attenuated in response to AF1q expression in breast cancer cells (data not shown). NF- κ B translocated to the nucleus binds to the proximal NF- κ B consensus sequence of the ICAM-1 promoter and binding of NF- κ B to the proximal binding site of the ICAM-1 promoter induces transcriptional activity^[17]. Published reports show that Wnt/ β -catenin negatively regulates NF- κ B activity through a β -catenin-NF- κ B interaction in colon and breast cancer cells^[18,19]. β -catenin can physically complex with NF- κ B, resulting in a reduction of NF- κ B DNA binding, transactivation activity, and target gene expression^[20]. Activated β -catenin is associated with repressed NF- κ B activity in human cancer cells^[20]. Interestingly, the interaction between them is only indirect, as these two proteins do not bind to each other without a helper protein. Structurally, AF1q has highly acidic peptide regions highly conserved between species that fulfill the criteria for an acidic blob, a typical feature for cofactors. We also noted an internal peptide repeat within the sequence of 90 amino acids of human AF1q, which is located at both ends of the peptide, indicative of similar binding interfaces^[2]. Previously,

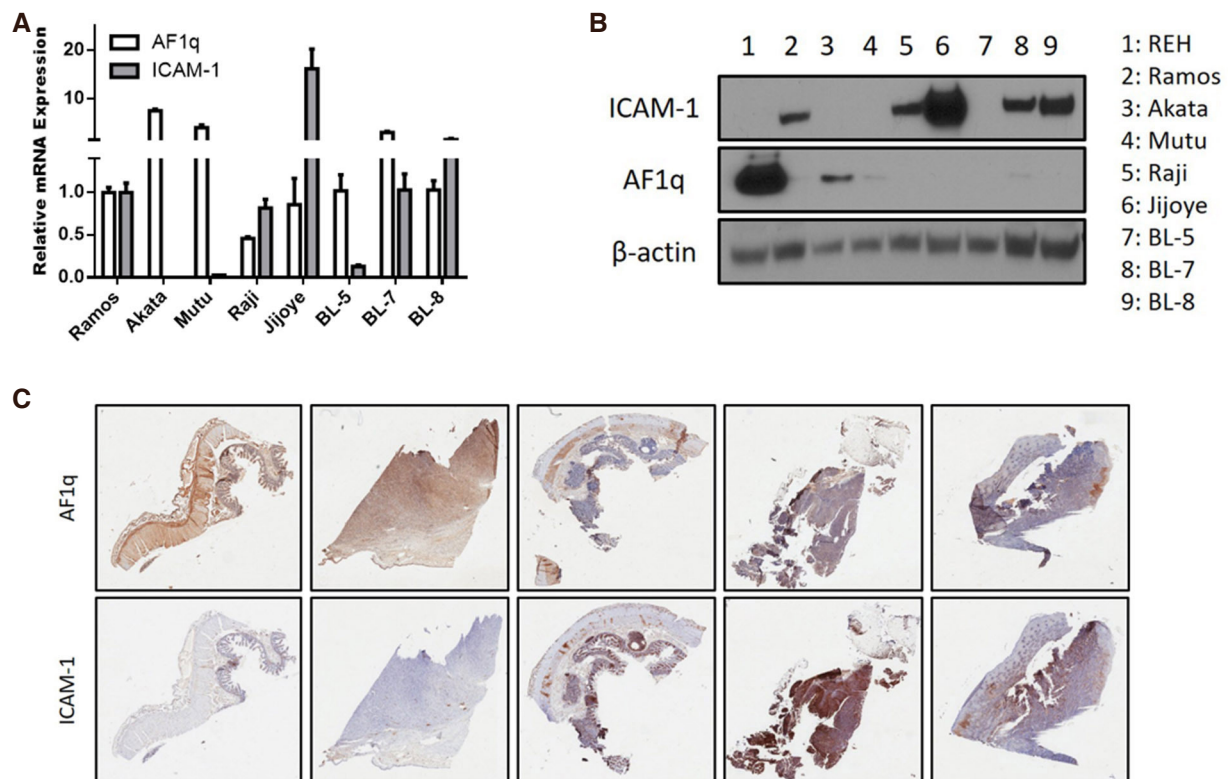


Figure 5. AF1q reciprocally regulates the expression of ICAM-1 in Burkitt's lymphoma. A: ICAM-1 mRNA expression was quantified using qPCR in MDA-MB-231LN cells engineered to overexpress or suppress AF1q; B: Western blot analysis of ICAM-1 and AF1q in Burkitt's lymphoma cell lines; C: representative images of AF1q and ICAM-1 staining in human Burkitt's lymphoma tumor metastasis tissues. High IHC staining of AF1q in tissue samples show low ICAM-1 expression

we reported that AF1q enhances the TCF7/LEF/β-catenin complex binding affinity as a cofactor. When we performed immunoprecipitation with NF-κB antibody in cancer cells overexpressing AF1q, we observed that β-catenin and AF1q were pulled down together (data not shown). However, it is not clear yet whether AF1q promotes protein interaction between β-catenin and NF-κB. These results suggest that activated β-catenin by AF1q would archive higher affinity to bind with NF-κB.

Cancer cells utilize multiple mechanisms to prevent host immune cells from exercising their antitumor activities. Many of these mechanisms are now known on a cellular and molecular level. These mechanisms, which enable the tumor to escape from the host immune system and to progress, are being intensively investigated in hope of finding therapeutically safe and effective inhibitors able to counteract tumor-induced immunosuppression. Tumor escape has been a major problem in cancer immunotherapy, and it has been held responsible for the failure of many immune interventions in cancer. For this reason, it is important to study and understand the various suppressive pathways human tumors utilize.

Tumors use blood vessels to supply themselves with oxygen and nutrients as well as for waste removal^[21]. Lymphocytes also use blood vessels as the gateway where integrin interactions with endothelial cell adhesion molecules are required to infiltrate into the tumor^[22,23]. The downregulation of ICAM-1 by several inhibitory mechanisms limiting T cell transendothelial migration have been described^[24-26]. Also, other componets of the tumor stroma and cancer-associated fibroblasts, can suppress T cell infiltration, which can influence cancer progression and metastasis^[27].

The interaction between tumor and lymphocytes through ICAM-1 plays an important role in leukocyte adhesion, transduction, and cytotoxicity^[28-30]. For example, tumor clones from melanoma metastasis split

into 2 groups with high and low susceptibility to killing by IL-2 activated lymphocytes. The subset with low lysability expressed ICAM-1 at levels 10 fold lower than those of tumor clones with high lysability^[31]. These results suggest that a constitutively high expression of ICAM-1 on tumors would be the parameter contributing to the high lysability of these tumor cells by any effector.

This study investigated the role of AF1q-attenuated ICAM-1 in progression and metastasis of breast cancer. Based on published reports, ICAM-1 strongly stimulates metastasis but also regulates lymphocyte infiltration via interactions between immune cells and malignant cells. This suggests that ICAM-1 needs an on-off switch for cancer progression and metastasis. It needs “off” to escape from host immune surveillances system in the initial phase of cancer, but “on” to invade and grow afterwards. Our results suggest that AF1q is a switch for ICAM-1 expression. Therefore, AF1q is a promising target for developing treatment for breast cancer metastasis.

DECLARATIONS

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Authors' contributions

Conception and design: Park J, Tse W

Collectively performed experiments: Park J, Hwang JY, Thore A, Kim S

Statistical analysis: Park J, Hwang JY

Writing, review, and/or revision of the manuscript: Park J, Hwang JY, Togano T, Hagiwara S, Park JW, Tse W

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Window of opportunity trials in head and neck cancer

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Abstract

Head and neck squamous cell carcinoma (HNSCC) has a large global burden of disease and poor survival outcomes. Recent targeted therapies and immunotherapies have been explored in HNSCC, but there has been limited translation to clinical practice outside of recurrent or metastatic cases. Window of opportunity settings, where novel agents are administered between cancer diagnosis and planned definitive therapy, have begun to be employed in HNSCC. Tumor tissue biopsies are obtained at diagnosis and after the investigation treatment, along with other biospecimens and radiographic exams. Thus, this study design can characterize the safety profiles, pharmacodynamics, and initial tumor responses to novel therapies in a treatment-naïve subject. Early window studies have also identified potential biomarkers to predict sensitivity or resistance to treatments. However, these early investigations have revealed multiple challenges associated with this trial design. In this review, we discuss recent window of opportunity trials in HNSCC and how they inform design considerations for future studies.

Keywords: Window of opportunity trial, head and neck cancer, biomarker, translational research, preoperative, oncology, trials



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INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) imparts significant morbidity and mortality as the sixth most common cancer in the world^[1]. In the United States alone, over 10,000 deaths and 51,000 new cases were estimated to occur in 2018^[1]. Nonspecific symptoms often lead to advanced stages at clinical presentation and thus poor survival outcomes, with an average 5-year survival in the United States of 65%^[2]. Surgical extirpation is a widely employed curative approach for advanced HNSCC, but there is often a time lapse of several weeks for preoperative workup and planning. Window of opportunity or “window trials” [Figure 1] leverage this time, where normally no treatment is rendered, in order to trial novel agents without delaying standard of care therapy^[3] in the context of a tumor microenvironment and human pathophysiology that cannot be replicated in preclinical models. Tissue is widely available for study, given the necessity of a biopsy for initial pathologic confirmation of the diagnosis and the subsequent curative therapy. Recent genomic studies have highlighted a number of potential molecular alterations in HNSCC, which can provide valuable targets which can be studied through window trials. Additionally, immunotherapies that have shown promise in recurrent or metastatic cases can be studied in treatment-naïve subjects through this approach. Of note, neoadjuvant trials typically do not qualify as window trials, as neoadjuvant therapies are given preoperatively typically with the goal of a measurable pathologic or clinical response. Some recent neoadjuvant trials in HNSCC, however, have followed window trial methodology, in that a tumor response to therapy did not preclude or delay surgery. These trials have shed light on the safety, possible efficacy, and potential patient selection biomarkers for the therapies employed. Thus, in this article, we review select neoadjuvant and window trials in HNSCC and discuss potential future directions.

RECENT WINDOW TRIALS OF TARGETED THERAPIES

Genomic methodologies have characterized numerous molecular alterations in HNSCC, many critical in tumor cell survival and proliferation pathways, that could form the basis for targeted therapies^[4-6]. However, translation of these findings into clinical practice has been slow.

ANTI-EPIDERMAL GROWTH FACTOR RECEPTOR BASED WINDOW TRIALS

Currently, the only molecularly targeted drug approved for HNSCC is cetuximab, a monoclonal antibody that targets the EGFR, an antiapoptotic transmembrane protein which is amplified or overexpressed in the vast majority of HNSCC and is correlated with reduced survival^[7]. In a phase III trial, cetuximab has been shown to improve overall survival in HNSCC when combined with radiotherapy, although only a fraction of patients receive benefit^[8]. Thus, identifying biomarkers that predict response to cetuximab is an ongoing area of study that potentially can be explored in a window of opportunity setting.

Schmitz *et al.*^[9] administered cetuximab to 33 subjects planned for curative surgery and compared radiologic tumor response via 18-fluorodeoxyglucose positron emission tomography (¹⁸FDG-PET) and tumor cellularity to 5 control subjects who did not receive any drug preoperatively [Table 1]. No treatment-limiting adverse events were noted, and there was a high rate of tumor response by ¹⁸FDG-PET (90% in cetuximab group vs. 0% in the control group). Tumor cellularity was correlated with ¹⁸FDG-PET standardized uptake values. As expected, cetuximab administration decreased pEGFR and phosphorylated extracellular signal regulated kinase expression, but neither of the biomarkers correlated with ¹⁸FDG-PET avidity. Cetuximab was also studied in a window of opportunity setting by Ferris *et al.*^[10], who noted an objective response in tumor size by computed tomography (CT) in a third of evaluable patients. Larger numbers of circulating EGFR-specific T cells^[10] as well as HLA class I upregulation^[11] were correlated with response to cetuximab.

Erlotinib is another EGFR inhibitor that has been approved in other cancers such as non-small cell lung cancer and pancreatic cancer. An uncontrolled neoadjuvant trial conducted by Thomas *et al* administered erlotinib in 35 subjects with advanced nonmetastatic HNSCC who were awaiting surgery^[12]. Four subjects

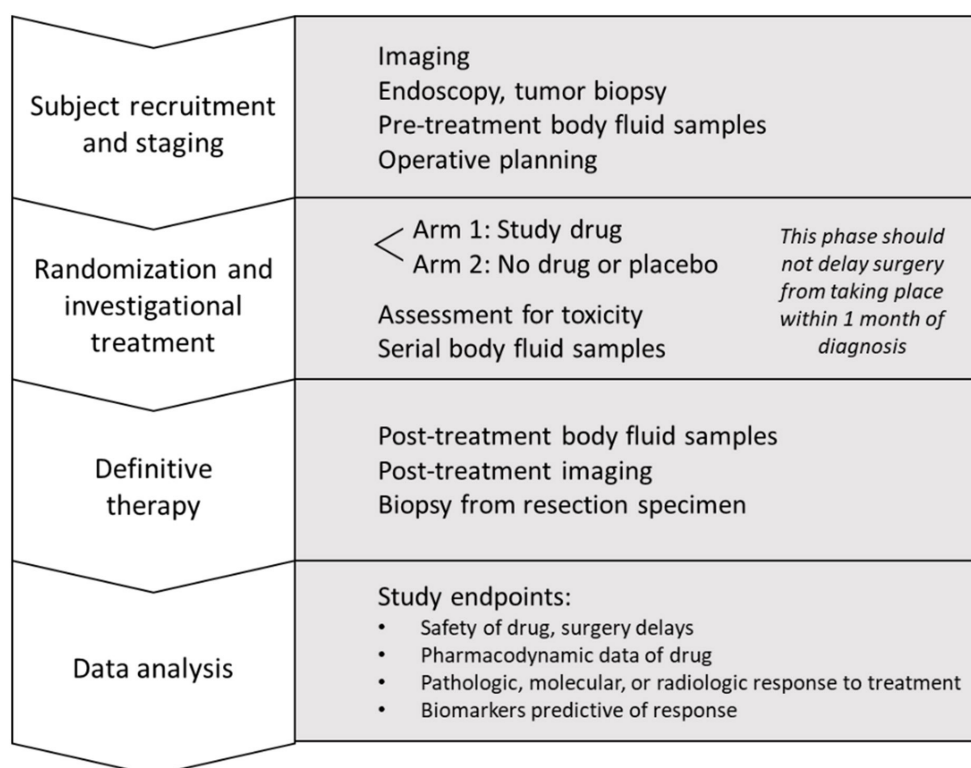


Figure 1. The typical format of a window of opportunity study

withdrew consent, and three subjects stopped treatment entirely due to grade 2-3 toxicities. Notably, length of treatment varied between enrolled subjects, with three subjects restarting treatment at a lower dose after grade 2-3 toxicities from the starting dose of erlotinib. Of 31 evaluable patients, decreased tumor size was seen in 9 subjects. Of multiple biomarkers studied, only the pre-erlotinib immune response score for p21^{waf}, or cyclin-dependent kinase inhibitor 1, was significantly correlated with response to treatment.

Cyclooxygenase-2 (COX2) pathways are also upregulated in HNSCC, and concurrent targeting of EGFR and COX pathways has shown synergistic effects in preclinical models^[13]. Thus, in a randomized double-blind window trial by Gross *et al.*^[13], 47 subjects received either erlotinib, erlotinib plus sulindac (a non-selective COX inhibitor), or placebo. One subject discontinued treatment for grade 2 anxiety, and another had their erlotinib dosage decreased for grade 2 mucositis. The primary endpoint of the Ki67 proliferation index, a cellular marker of proliferation, was only evaluable in 27 subjects. There was an ordered significant reduction of Ki67 between the erlotinib-sulindac combination *vs.* erlotinib alone, with no change in Ki67 attributable to the placebo group. No biomarkers tested appeared to mediate the decrease in Ki67, although higher pSrc expression was correlated with smaller decreases in Ki67. No clinical outcomes were evaluated.

Building from these results, Bauman *et al.*^[14] randomized subjects to a placebo arm or erlotinib with or without dasatinib, a small molecule inhibitor of Src family kinases. No significant treatment-altering toxicities were seen in any arm of the study. Erlotinib with or without dasatinib was correlated with a significant reduction in tumor size by response evaluation criteria in solid tumors (RECIST) measurement techniques using baseline and preoperative CT scans. Dasatinib did not appear to provide synergistic effects. No significant changes in expression levels of potential biomarker proteins was observed. Pretreatment mitogen-activated protein kinases (MAPK) and signal transducer and activator of transcription 3 expression were correlated with erlotinib sensitivity and dasatinib resistance respectively. Interestingly, the Ki67 proliferation index did not correlate with change in tumor size.

Table 1. Window trials of targeted therapies in head and neck squamous cell carcinoma

Ref.	Agent(s)	n*	Inclusion criteria	Duration	Outcome(s)	Biomarkers	Toxicity/surgery delays
Day <i>et al.</i> ^[17]	Rapamycin	16 (21 [‡])	Stage II-IV OC/OP	21 days	Tumor size (clinically, CT/RECIST)	NS	
Machiels <i>et al.</i> ^[15]	1. Afatinib 2. No drug	25 5 (30)	Stage II-IV OC/OP	14 days	¹⁸ FDG-PET response (EORTC, RECIST); DCE-MRI; DW-MRI	TP53 genotype, hypoxia screen	G3 diarrhea, renal failure (<i>n</i> = 1), surgery delay (<i>n</i> = 3)
Bauman <i>et al.</i> ^[14]	1. Erlotinib 2. Desatinib 3. Combination 4. Placebo	14 15 14 15 (56)	Stage I-IV OC/OP/L	2-21 days	Tumor size (CT)	pMAPK, pSTAT3	None
Uppaluri <i>et al.</i> ^[16]	Trametinib	20 (20)	Stage II-IV OC	7-16 days	Tumor size, SUV (PET/CT; WHO); tumor stage	NR	G4 duodenal perforation (<i>n</i> = 1), G2 nausea (<i>n</i> = 1); 3 patients stopped treatment
Ferris <i>et al.</i> ^[10]	Cetuximab	40 (40)	Stage III-IV OC/OP/L/HP	21-28 days	Tumor size (CT); progression free survival	EGFR-specific T cell counts	NR
Gross <i>et al.</i> ^[13]	1. Erlotinib 2. Erlotinib + Sulindac 3. Placebo	19 16 12 (39 [†])	Stage II-IVA OC/OP/L/HP	7-14 days	Ki67 proliferation index	pSrc	G2 anxiety (<i>n</i> = 1 stopped treatment), G2 mucositis (<i>n</i> = 1 decreased dosage)
Schmitz <i>et al.</i> ^[9]	1. Cetuximab 2. No drug	33 5	T1-T4 OC/OP/L/HP	21 days	¹⁸ FDG-PET response (SUV); tumor size (CT/MRI); tumor cellularity	NS	None
Thomas <i>et al.</i> ^[12]	Erlotinib	35	T2-T4 OC/OP/L/HP	18-29 days	Tumor size (CT)	p21 ^{waf}	G3 pruritis and G2 rash (<i>n</i> = 6; <i>n</i> = 3 stopped treatment)

Studies listed by date published. ‡*n* = 21 and *n* = 37 listed as accrual number and actual enrollment on ClinicalTrials.gov, with *n* = 16 included in the published manuscript; †accrual number modified based on discontinuation of parent study; *sample sizes listed include actual number of subjects, with the amount necessary for full accrual in parentheses if published. Biomarkers listed in the table include biologic characteristics statistically associated with sensitivity or resistance to the tested therapy. Toxicities only include those attributed to or possibly attributed to the drug being studied that are grade (G) 3 or higher or caused treatment dosage reduction or discontinuation. Ref.: reference; HNSCC: head and neck squamous cell carcinoma; OC: oral cavity; OP: oropharynx; P: pharynx; HP: hypopharynx; L: larynx; CT: computed tomography; ¹⁸FDG-PET: 18-fluorodeoxyglucose-positron emission tomography; SUV: standardized uptake value; DCE-MRI: dynamic contrast enhanced magnetic resonance imaging; DW-MRI: diffusion-weighted MRI; RECIST: response evaluation criteria in solid tumors; EORTC: European Organization for Research and Treatment of Cancer; WHO: World Health Organization; NS: not significant; NR: not reported

In another recent multicenter window study, Machiels *et al.*^[15] randomized 30 subjects to afatinib (an irreversible second generation inhibitor of the EGFR-family of receptor tyrosine kinases) or no drug prior to surgery. There were several afatinib-related adverse events, leading to discontinuation of the drug in one patient and a delay of surgery by 24 days in one subject, as well as delayed surgery with continuation of afatinib in two additional subjects. Radiologic response was seen in 16 of 23 evaluable subjects in the afatinib arm by ¹⁸FDG-PET and in 5 of 23 subjects by RECIST criteria. Tumor protein p53 (*TP53*) wild type allele and a hypoxia expression screen were associated with ¹⁸FDG-PET results but not responses by RECIST criteria.

OTHER TARGETED WINDOW TRIALS

Uppaluri *et al.*^[16] hypothesized that MAPK/extracellular-signal-regulated kinase (ERK) pathway could be targeted in oral cavity HNSCC. They performed a window trial^[16] of trametinib, an inhibitor of MAPK/ERK kinase, that resulted in decreased tumor size by FDG avidity by PET/CT and tumor downstaging in approximately half of the 17 evaluable subjects. There were, however, 3 subjects who discontinued the study, including one who suffered a grade 4 duodenal perforation. While there was biochemical evidence of a suppressed MAPK/ERK pathway in a third of evaluable patients, no clear correlation between biochemical results and responsiveness to trametinib was drawn.

Recently, Day *et al.*^[17] undertook a single-armed window trial of rapamycin, an inhibitor of the mammalian target of rapamycin pathway that is dysregulated in the majority of HNSCC. Their inclusion criteria differed

Table 2. Window trials of immunotherapies in head and neck squamous cell carcinoma

Ref.	Agent(s)	n*	Inclusion criteria	Duration	Outcome(s)	Biomarkers	Toxicity/ surgery delays
Bell <i>et al.</i> ^[27]	Anti-OX40 (MEDI6469)	17 (55 [‡])	Stage III-IV	5-6 days	TIL counts and expression profiles	MHC I genes	None
Colevas <i>et al.</i> ^[26]	Anti-PD-1 Ab	NR [‡]	NR	NR	TIL counts and expression profiles	Novel PET imaging	NR
Shayan <i>et al.</i> ^[28]	Motolimod + cetuximab	14	Stage III-IV OC/ OP/L/HP	15-22 days	TIL counts, circulating leukocytes, immune effector cell biomarkers		Unspecified cetuximab toxicity (n = 1, withdrew from study)
Ferris <i>et al.</i> ^[25]	Nivolumab	29 [‡]	T1+N1+ OC/P/L	15 days	Tumor size (CT) Pathologic response Tumor PD-L1 expression Immune correlates	NR	Grade 3-4 adverse events (n = 4)
Uppaluri <i>et al.</i> ^[24]	Pembrolizumab	21 [‡]	Stage III-IV HPV negative	NR	High-risk pathologic features Pathologic treatment response Tumor staging	Baseline PD-L1 expression	None
Berinstein <i>et al.</i> ^[23] , Wolf <i>et al.</i> ^[22]	IRX-2	27	Stage II-IV OC/ OP/L/HP	21 days	Tumor size (CT/MRI; RECIST), TIL counts		Postoperative wound infection (n = 1)
Timar <i>et al.</i> ^[21]	1. IL-2 2. Historical pathologic controls	19 20	T2-3 OC	21 days	Pathologic analysis, Tumor dimensions (MRI)	CD4:CD8 ratio	None

Studies listed by date published. ‡Active study on ClinicalTrials.gov; *sample sizes listed include actual number of subjects, with the amount necessary for full accrual in parentheses if published. Biomarkers listed in the table include biologic characteristics statistically associated with sensitivity or resistance to the tested therapy. Toxicities only include those attributed to or possibly attributed to the drug being studied that are grade (G) 3 or higher or caused treatment dosage reduction or discontinuation. Ref.: reference; HNSCC: head and neck squamous cell carcinoma; OC: oral cavity; OP: oropharynx; P: pharynx; HP: hypopharynx; L: larynx; TIL: tumor infiltrating leukocyte; CT: computed tomography; PET: positron emission tomography; MRI: magnetic resonance imaging; RECIST: response evaluation criteria in solid tumors; NR: not reported

from the previously discussed window trials in that subjects were either planned for curative surgery ($n = 15$) or chemoradiation ($n = 1$). There was one grade 3 hypokalemia reported but no resultant delays in surgery. Decreased tumor size was seen in 14 of 16 subjects clinically and 4 of 16 patients by RECIST criteria. Ki67 was significantly decreased in all patients.

Ongoing targeted therapy window trials in HNSCC without published results include use of olaparib, a poly-ADP ribose polymerase inhibitor, and AZD6738, a serine/threonine-specific protein kinase inhibitor (NCT03022409).

RECENT WINDOW TRIALS OF IMMUNOTHERAPIES

Studies have shown impairment of the innate and adaptive immune systems in HNSCC patients^[18]. Immunotherapies are designed to sensitize the body's immune system to the tumor and to counteract various strategies that tumors use to evade immunologic detection. With the recent FDA approval of nivolumab^[19] and pembrolizumab^[20] for patients with recurrent/metastatic HNSCC, there has been expansion of phase II window of opportunity trials utilizing immunomodulating drugs [Table 2]. In 2005, Timar *et al.*^[21] administered an interleukin-2 (IL-2) treatment to subjects with oral cavity cancer prior to surgery. Treatment consisted of peritumoral and perilymphatic injections 5 times per week over 3 weeks, along with a preceding intravenous cyclophosphamide administration and oral indomethacin and zinc sulfate medications. Matched historical pathologic specimens were used as controls. No treatment related adverse events were reported. Partial or complete response as judged by histopathologic examination or tumor dimensions on magnetic resonance imaging (MRI) were observed in 8 of 19 subjects treated with IL-2. Additionally, increased CD4+:CD8+ ratios were observed in treated subjects, although a statistically significant ratio increase between responders and non-responders was observed only in the tumor stroma.

In a later study, Wolf *et al.*^[22] utilized subcutaneous injections of IRX-2, a biologic composed of a mixture of purified cytokines, along with cyclophosphamide, indomethacin, and zinc in a cohort of 27 patients with HNSCC. There were no significant adverse events related to treatment noted. Of 23 evaluable subjects, 4 had an objective decrease in tumor size, although this did not constitute a true partial response by RECIST criteria. Increased lymphocytic infiltration into tumors was associated with increased response and overall survival^[22,23].

More recently, Uppaluri *et al.*^[24] presented preliminary results from an ongoing single-armed trial of advanced HPV negative HNSCC subjects who received neoadjuvant pembrolizumab (an anti-PD-1 antibody) that was continued post-operatively. No serious drug-related adverse events were reported. Significantly decreased high-risk pathologic features, pathologic treatment response, and clinical-to-pathologic downstaging was observed among the 21 subjects. Baseline high tumor expression of the programmed cell death protein ligand (PD-L1) was correlated with pathologic treatment effect. Ferris *et al.*^[25] presented interim results of a window trial of nivolumab (another anti-PD-1 antibody) for HNSCC. In half of the 23 evaluable subjects, tumor dimensions were reduced after treatment. As part of another ongoing neoadjuvant trial, Colevas *et al.*^[26] are administering anti-PD-1 antibody prior to planned curative surgery or radiation in HNSCC. They presented results from a single subject where their novel nuclear medicine imaging test correlated with tissue markers of immunologic activity.

A novel antibody MEDI6469, an OX40 (CD134) agonist, was also studied in a window of opportunity setting by Bell *et al.*^[27]. No significant adverse events were reported, and immunologic response was detected in 4 of 17 subjects. There was a significant difference between responders and non-responders in genes associated with major histocompatibility complex (MHC I)-mediated antigen processing.

Shayan *et al.*^[28] combined motolimod, a small molecule agonist of the toll-like receptor 8, along with cetuximab in 14 patients planned for curative surgery. One subject withdrew from the study due to an unspecified cetuximab toxicity. Study results showed that the expected increase in suppressive co-signaling molecule expression induced by cetuximab monotherapy was counteracted by the addition of motolimod, resulting in increased circulating EGFR-specific T cells and greater tumor infiltration of leukocytes.

OTHER WINDOW TRIALS

While not a classic targeted therapy or immunotherapy, metformin, an anti-hyperglycemic, has been shown to be associated with improved outcomes in HNSCC^[29]. It is postulated that metformin's metabolic effects through inhibition of mitochondrial oxidative phosphorylation may be proapoptotic in HNSCC. Curry *et al.*^[30] executed a single-armed window trial of metformin among 39 subjects with HNSCC of the oral cavity or larynx, each who took between 9-24 days of the drug without significant side effects. Markers of increased apoptosis and altered stromal metabolism were identified in the 33 evaluable subjects.

The results of several additional targeted therapy and immunotherapy trials have yet to be published. Those listed in ClinicalTrials.gov are briefly reviewed in [Table 3](#).

CONSIDERATIONS IN DESIGNING FUTURE WINDOW TRIALS

Window trials offer the opportunity to study the safety, mechanism, and efficacy of novel agents in treatment-naïve HNSCC. The studies outlined here have demonstrated the overall safety of each agent studied, with limited numbers of treatment-related adverse events and no clear post-operative complications attributable to the investigational drug. They have also confirmed the intended knockdown of upregulated pathways in HNSCC with targeted therapies and have shed light on the immunomodulatory mechanisms behind newer immunotherapies. Promising preliminary data reveal clinical, radiologic, and pathologic responses in some treated subjects along with possible biomarkers predictive of sensitivity or resistance to

Table 3. Ongoing window trials in head and neck squamous cell carcinoma

Type	Principal investigator/institution	Agent(s)	Inclusion criteria*	NCT
Targeted therapy	Duvvuri/University of Pittsburgh	AZD6738, olaparib	Newly diagnosed, treatment naive Planned surgery/biopsy + adjuvant RT and/or chemo	03022409
Immunotherapy	Wolf/University of Michigan	IRX-2	Stage II-IVA OCSCC Treatment naive KPS \geq 70% Adequate hematologic, hepatic, and renal function	02609386
Immunotherapy	Worden/University of Michigan	Pembrolizumab	Any T stage with \geq N2 disease T4 disease, any N stage T3 OCSCC, any N stage Clinical evidence of ECE ECOG 0-1	02641093
Immunotherapy	Neskey/Medical University of South Carolina	Nivolumab	Newly diagnosed, treatment naive, T2-T4, M0 OCSCC; or Recurrent/persistent locoregional T2-T4 OCSCC initially treated with surgery alone, ECOG 0-1	03021993
Immunotherapy	Schoenfeld/Dana-Farber Cancer Institute	Nivolumab \pm Ipilimumab	\geq T2 \pm \geq N1 surgically resectable OCSCC ECOG 0-1 Adequate hematologic, hepatic, and renal function	02919683
Immunotherapy	Porosnicu/Wake Forest	Durvalumab	Surgically resectable OCSCC/OPSCC No prior immunotherapy or RT ECOG 0-1 Adequate hematologic, hepatic, and renal function	02827838
Immunotherapy	Curry/Thomas Jefferson	Durvalumab \pm Metformin	Surgically resectable HNSCC ECOG 0-1 Body weight > 30 kg Adequate hematologic, hepatic, and renal function	03618654
Targeted therapy/immunotherapy	Ferris/University of Pittsburgh	Motolimod and Cetuximab \pm Nivolumab	Treatment naive Stage II-IVA HNSCC Planned surgical resection ECOG 0-1 Adequate hematologic, hepatic, and renal function	02124850

*Inclusion criteria abbreviated. See ClinicalTrials.gov for full inclusion and exclusion criteria, as well as primary endpoints for each trial. HNSCC: head and neck squamous cell carcinoma; NCT: ClinicalTrials.gov identifier; OCSCC: oral cavity SCC; OPSCC: oropharyngeal SCC; P: pharynx; HP: hypopharynx; L: larynx; KPS: Karnofsky performance status; ECOG: Eastern Cooperative Oncology Group Performance Scale; RT: radiotherapy; ECE: extracapsular extension

the studied agents, although work remains to duplicate and understand these results.

By definition, window trials occur in a short timeframe, which requires careful coordination to obtain the desired imaging studies, tumor tissue, and serial biologic samples. As the authors of a recent study discuss^[15,31], this can be difficult in a patient population that often has socioeconomic and adherence challenges with an already complicated diagnosis and treatment strategy to discuss. For this reason and because patients may be hesitant to take an investigational drug that should not be marketed to improve clinical outcomes in the research setting, accrual can take longer than expected. Accrual goals were not uniformly available for the studies included in this review, and many unpublished planned window trials may have failed due to poor accrual. Narrowing subject selection to specific tumor sites (i.e., oral cavity, oropharynx, larynx, or hypopharynx) or immunogenomic profiles may further elongate recruitment timelines.

Pre- and post-treatment tissue is readily available by nature of the window of opportunity design, but the timing, selection, processing, and analysis protocols for tumor tissue and other desired body fluid samples must be considered. Tumor heterogeneity is a well-known phenomenon, and immunogenomic profiles can vary across both space and time. Pharmacokinetics of the drug under study should also be factored into the timing of obtaining biologic samples. Unlike in breast cancer where Ki67 is commonly employed, HNSCC studies have not coalesced on particular biomarkers, nor do standardized protocols for obtaining biomarker data or evaluating their clinical impact exist as of yet^[14,15]. Several window trials discussed here were not randomized or did not use data from control subjects, which has been known to complicate

pharmacodynamic and predictive biomarker assessment^[13]. Studies presented herein have also collected serum samples, but analytes from other body fluid samples that could serve as future “liquid biopsies^[32]” have yet to be characterized in window trials.

Given that there may be physical reduction in tumor size from the drug under study, it is important to confirm with subjects that surgery is still required as part of the study even if the tumor shrinks or disappears radiographically, and surgical margins should be based on pre-treatment tumor dimensions^[31]. Similar to biologic samples, the type, timing, protocols, and quality thresholds for radiologic tests must be carefully planned, particularly if imaging from multiple institutions are used. The window trials presented here used a variety of exams, including CT, MRI with different protocols, ¹⁸FDG-PET, and investigational PET technologies. Additionally, criteria for assessing radiologic response included those from RECIST, modified RECIST^[14], EORTC (European Organization for Research and Treatment of Cancer)^[15], the World Health Organization^[16], and others. Researchers should also be aware that pseudoprogression during immunotherapy, or an initial tumor flare due to inflammatory processes provoked by the drug, may complicate image interpretation during the short timeframe of a window study^[33,34]. This should not be confused with hyperprogression, a phenomenon of tumor growth during immunotherapy treatment experienced by a small minority of patients, which may delay curative surgery^[34,35]. Limited data are available on the optimal timing of surgery, but it is suggested that HNSCC resection should take place within a month of diagnosis^[36,37]. Treatment-related adverse events beyond hyperprogression may delay curative surgery, so investigational drugs selected for window trials should have well-characterized safety data and a tolerable safety margin. Trial stopping points based on safety events should be well-defined and monitored by an independent committee.

Finally, it is important to note that window trials cannot assess the long-term response, acquired resistance mechanisms, or safety profile for the studied treatment. Complementary study designs should be utilized to contextualize window trial results. For instance, a single window trial may provide compelling preliminary data for a full confirmatory neoadjuvant trial. The I-SPY2 TRIAL (Investigation of Serial Studies to Predict Your Therapeutic Response with Imaging and Molecular Analysis)^[38] utilizes this approach in breast cancer, with the added benefit of conducting studies on multiple agents in parallel. This technique could be applied in HNSCC, although the window trial approach is likely most effective for treatment-naïve and healthier patient populations. Biomolecular insights gained from window trials, on the other hand, could inform pathophysiology in multiple patient populations, as well as subject/agent selection for all types of clinical trial designs.

CONCLUSION

Window of opportunity studies are challenging to design and execute. Despite this, early window trials have explored the safety, pharmacodynamics, short-term efficacy, and predictive biomarkers for novel targeted therapies and immunotherapies. Window trials are a promising study design complementary to traditional clinical trials to advance understanding and treatment of HNSCC.

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Authors' contributions

All authors made substantial contributions to the conception and writing of the article.

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Original Article

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Understanding convergent signaling regulation in metastatic breast cancer cells using a bioengineered stem cell microenvironment

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Abstract

Aim: The convergence of tumorigenic and embryonic signaling pathways drives us to exploit the embryonic stem cell (ESC) microenvironment to restrict metastatic potential of cancer cells. We have previously demonstrated that bioengineered ESC microenvironments could restrict growth and metastatic potential of highly aggressive breast cancer cell (BCC). This study aims to further understand the regulation of convergent EGFR and canonical Wnt/ β -catenin signaling pathway function in triple negative metastatic BCCs using the 3D in vitro ESC microenvironment created by encapsulating ESCs in alginate hydrogel microstrands.

Methods: Co-culture with ESC-microstrands increased sensitivity to two chemotherapeutic drugs in metastatic BCCs. To test whether these changes were due to restored signaling pathway regulation in BCCs, we probed for changes in gene expression of key molecules related to the EGFR and canonical Wnt/ β -catenin signaling pathways using quantitative reverse transcription polymerase chain reaction and Western blot analysis.

Results: ESC-microstrands are able to alter the gene expression of highly aggressive BCCs at both mRNA and protein levels. These changes are indicative of a reversal of EGFR and canonical Wnt/ β -catenin signaling pathway hyperactivation following co-culture. Increased NKD2 mRNA and protein expression coinciding with dual signaling pathway inhibition within co-cultured BCCs suggests that this reversal may be attributable to restored regulation of NKD2 within these pathways.

Conclusion: ESC-microstrands are able to reverse oncogenic signaling pathway hyperactivation and restore signaling pathway regulation in metastatic BCCs. Further studies could provide insight into what role NKD2 up-regulation plays



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in BCC inhibition, leading to the development of a new targeted therapy for metastatic breast cancer.

Keywords: Metastasis, cancer cell, signaling, 3D culture, stem cells, EGFR, Wnt/ β -catenin, NKD2

INTRODUCTION

Human breast cancer is currently the highest diagnosed form of cancer and the second leading cause of cancer-related deaths in American women. Triple negative breast cancer is of the basal subtype and displays poor prognosis owing to its highly metastatic properties. Current treatments focused on eradicating breast tumors in lieu of or following local therapy include chemotherapy, hormonal therapy, and targeted therapy. Hormonal therapy is not an option for triple negative breast cancer as it does not contain hormone receptors, and there are currently no approved biological targeted therapies. Chemotherapy has proven unsuccessful because triple negative breast cancer is highly drug resistant and is associated with high toxicity^[1]. It remains a huge challenge to fully understand the mechanisms of cell migration and invasion of cancer cells, such as triple negative breast cancer cells, and to eliminate these cancer cells to prevent disease recurrence and metastasis.

Metastatic breast cancer cells have been linked to embryonic stem cells (ESCs) due to their sharing of certain similar gene signatures and convergence of tumorigenic and embryonic pathways (e.g., EGFR, Wnt/ β -catenin, and TGF- β)^[2-4]. ESC microenvironments have been exploited to restrict metastatic potential of cancer cells^[5-7]. In particular, our previous studies have shown that bioengineered 3D ESC microenvironments using alginate hydrogels have great potential to supply critical signaling molecules and reprogram highly aggressive breast cancer cells to a non-invasive phenotype^[4,8-10], including decreased cell proliferation, increased apoptosis, reduced cell migration, invasion and metastatic marker expression, reversed epithelial-mesenchymal transition (EMT), and altered cell metabolism of metastatic breast cancer cells. The engineering of unique ESC microenvironments *in vitro* which can restrict the metastatic potential of triple negative breast cancer cells (BCCs) may permit advanced study of cancer metastasis. Consequently, this could provide greater insight into the decision-making processes regarding the growth, migration, and invasion of cancer cells and its subsequent prevention.

The EGFR and canonical Wnt/ β -catenin signaling pathways control many cell properties that contribute to the metastatic phenotype. The EGFR (also known as the ErbB1/HER1) belongs to the ErbB family of tyrosine kinase receptors. Binding of ligands (e.g., EGF, TGF- α) to the EGFR initiates dimerization of the EGFR with one of the other three receptors within the same family. Dimerization activates the intracellular tyrosine kinase domain, which undergoes autophosphorylation. This causes a conformational change which exposes the activation loop and allows for protein binding and phosphorylation of downstream signaling targets^[11,12]. Phosphorylation initiates signaling cascades, such as Ras/Raf/mitogen-activated protein kinase/ERK, phosphoinositol 3 (PI3)-Akt-mechanistic target of rapamycin (mTOR), PLC- γ , JAK/signal transducers and activators of transcription (STAT), and Src kinase pathways^[11-13]. These signaling pathways affect cell growth and proliferation, differentiation, invasion, migration, apoptosis, angiogenesis, cell cycle progression, and immune function^[11,14,15]. The canonical Wnt/ β -catenin signaling pathway is initiated when one of its ligands binds to the receptor complex. This causes phosphorylation and activation of the protein dvl-1, which inhibits a destruction complex normally responsible for recruitment, phosphorylation, ubiquitylation, and proteasomal degradation of β -catenin. Signal activation and sequestration of the destruction complex to the plasma membrane prevents the phosphorylation of β -catenin, which subsequently accumulates in the cytoplasm, translocates to the nucleus, and activates transcription of target genes^[16-19]. In human breast cancer, β -catenin transactivates vimentin, a known EMT marker^[20]. β -catenin also forms a complex that binds to the cytoplasmic tail of E-cadherin, connecting it to the actin cytoskeleton^[21-24].

Cross-talk between the canonical Wnt/ β -catenin and EGFR signaling pathways is evidenced in cancer cells. For example, the EGFR signaling pathway activates the transcription of canonical Wnt/ β -catenin target genes such as cyclinD1, c-myc, and survivin. In oral and non-small cell lung cancers, there is a link between EGFR mutations and accumulation of β -catenin within the nucleus^[25,26]. The EGFR signaling pathway is turned on by Wnt ligands through the Fzd receptor, leading to cleavage of EGF-like ligands^[27,28]. EGFR signaling is transactivated in breast cancer patients after knockdown of the Wnt negative regulator SFRP1. Increased expression of Wnt1 and decreased expression of APC activates EGFR signaling in breast cancer^[28,29]. Down-regulation of β -catenin decreases levels of EGFR, STAT3 and Akt mRNA^[30]. E-cadherin regulates EGFR localization and activation^[31]. NKD2 is a negative regulator of Dvl-1, which promotes β -catenin translocation to the nucleus^[32,33]. The N-terminal region of NKD2 also contains a moiety capable of binding to precursor TGF- α (36 kDa), a ligand for the EGFR signaling pathway. This interaction facilitates the escort of precursor TGF- α to the plasma membrane, whereby it is cleaved into mature TGF- α (17 kDa), cleaved into a soluble form (6 kDa), and secreted^[29,34].

These previous studies establish a relationship between the EGFR and canonical Wnt/ β -catenin signaling pathways. They also illustrate the complexity involved in this signaling pathway cross-talk. Hyperactivation of both signaling pathways is a signature of aggressive human breast cancer. NKD2 is involved in a positive feedback loop in the EGFR signaling pathway and is a known inhibitor of the canonical Wnt/ β -catenin signaling pathway. It is likely that NKD2 acts as a “molecular switch” between these pathways in breast cancer development and/or progression. The exact mechanism for this communication remains elusive. We have established a 3D *in vitro* ESC-microstrand tumor model by encapsulating ESCs in small-diameter alginate microstrands and co-culturing with metastatic human BCCs. The creation of a 3D *in vitro* ESC-microstrand tumor model to probe signaling pathway-related molecules following restoration of signaling pathway function will provide insight into this complex process^[8,10]. This model restricts metastatic BCC proliferation, survival, metabolism, invasion, migration, and oncogene expression, as summarized in Table 1^[10]. To further understand the underlying mechanism for ESCs’ inhibitory effect on BCCs, we hypothesized that ESC-microstrands will inhibit the EGFR and canonical Wnt/ β -catenin signaling pathways in highly aggressive cancer due to restored signaling pathway regulation. To test this hypothesis, co-cultured BCCs were treated with EGFR and canonical Wnt/ β -catenin signaling pathway inhibitors. Next, co-cultured BCCs were probed at the molecular level for expression of key molecules related to the EGFR and canonical Wnt/ β -catenin signaling pathways using quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blot analysis. Changes in gene expression would reflect the ability of pluripotent signals from ESC-microstrands to reverse oncogenic signaling pathway hyperactivation and restore signaling pathway regulation in metastatic BCCs.

METHODS

Cell culture

Mouse CCE ESCs were purchased from StemCell Technologies (Vancouver, Canada) and cultured in 0.1% gelatin-coated flasks. The growth medium consisting of DMEM with 4.5 g/L D-glucose supplemented with 15% (v/v) FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin, 0.1 mmol/L MEM non-essential amino acids, 10 ng/mL murine recombinant LIF (StemCell Technologies, Vancouver, Canada), 0.1 mmol/L monothioglycerol, 2 mmol/L L-glutamine, and 1 mmol/L sodium pyruvate. Cell media was changed every day and cells were passaged every 2 to 3 days. Human MDA-MB-231 and MCF7 BCCs were purchased from ATCC (Manassas, VA). Cells were cultured in growth medium consisting of DMEM supplemented with 15% (v/v) FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin and 2 mmol/L L-glutamine. Human 3T3 fibroblasts were obtained from the NIH and cultured in growth medium containing DMEM supplemented with 15% (v/v) FBS, 100 U/mL penicillin, and 100 μ g/mL streptomycin. All cell lines were maintained at 37 °C in a humidified atmosphere containing 5% CO₂.

Table 1. Summarization of inhibitory effects of 3D ESC-microstrands on BCCs^[10]

Cancer cell property	Co-cultured BCC
Proliferation	
WST-1 assay	↓
Cell cycle analysis-G2/M population	↓
Cell cycle analysis-S population	↑
Apoptosis/necrosis	
Annexin-V FITC propidium iodide assay	↑
Cell cycle analysis-SubG1 population	↑
Cell metabolism	
Glycolysis	↓
Oxidative phosphorylation	↓
Epithelial-to-mesenchymal transition	
E-cadherin protein expression	↑
Vimentin protein expression	↓
Cell migration	↓
Invasiveness	↓

ESC: embryonic stem cell; BCC: breast cancer cell

Microfluidic synthesis of 3D ESC-microstrands by encapsulating ESCs in alginate hydrogels

A sodium alginate solution (1.5% w/v alginate dissolved in 0.9% w/v sodium chloride) containing ESCs suspended at a density of 1×10^6 cells/mL was loaded into a 3 mL syringe, which was placed in a New Era NE-1000 syringe pump (New Era, Farmingdale, NY). A 50 mmol/L CaCl_2 solution was placed in one well of a 24-well plate at a volume of 2 mL. The sodium alginate-ESC mixture in the syringe was pumped into the CaCl_2 solution at a constant rate of 0.1 mL/min. Exposing the divalent cation, Ca^{2+} , to sodium alginate cross-linked the polymer chains. This set-up consistently formed microstrands with an approximate 200 μm diameter [Figure 1A]. After microstrand formation, the CaCl_2 was carefully removed using 200 μL micropipette tips, without disturbing the microstrands, and replaced with ESC maintenance media.

Co-culture of 3D ESC-microstrands with breast cancer cells

As shown in Figure 1B, ESC-microstrands were co-cultured with 2D BCCs or fibroblasts by cutting them to a specific length, and gently adding them to the well with tweezers. On the same day that ESC-microstrands were formed, MDA-MB-231 BCCs, MCF7 BCCs, and/or 3T3 fibroblasts were seeded at a cell density of 2×10^4 cells/mL in their media. After 24 h., BCC or 3T3 media was replaced with ESC maintenance media. ESC-microstrands were then measured and cut to an appropriate length based on BCC or 3T3 cell density (35.0 mm for approximate 2×10^4 cells) and added to the wells with BCCs or 3T3 cells in ESC maintenance media. After 24, 48, or 72 h, BCCs were analyzed using various biological assays. Controls included non-co-cultured BCCs and empty alginate hydrogel microstrands. The non-co-cultured control consisted of BCCs in ESC maintenance media and the empty microstrand control included BCCs in ESC maintenance media co-cultured with empty microstrands.

Cell proliferation assay

Cell proliferation of MDA-MB-231 BCCs was measured using a Premixed WST-1 Cell Proliferation Reagent (Clontech, Mountain View, CA), per manufacturer's instruction.

Treatment with EGFR and canonical Wnt/ β -catenin signaling pathway inhibitors

MDA-MB-231 BCCs were co-cultured with ESC-microstrands for 48 h. and subsequently treated with 5, 10, or 20 $\mu\text{mol/L}$ of either the EGFR inhibitor Erlotinib, the canonical Wnt/ β -catenin inhibitor PNU74654, or both to determine how sensitive these cells were to both chemotherapeutic inhibitors. Controls included non-co-cultured BCCs and non-drug-treated BCCs. After 48 h, the sensitivity of the BCCs to drug treatment was assessed using a WST-1 cell proliferation assay per manufacturer's instructions. For the drug resistance experiments, cells were treated with chemotherapeutic drugs up to four times for 24 h each and allowed to recover for either 24 or 48 h prior to analysis.

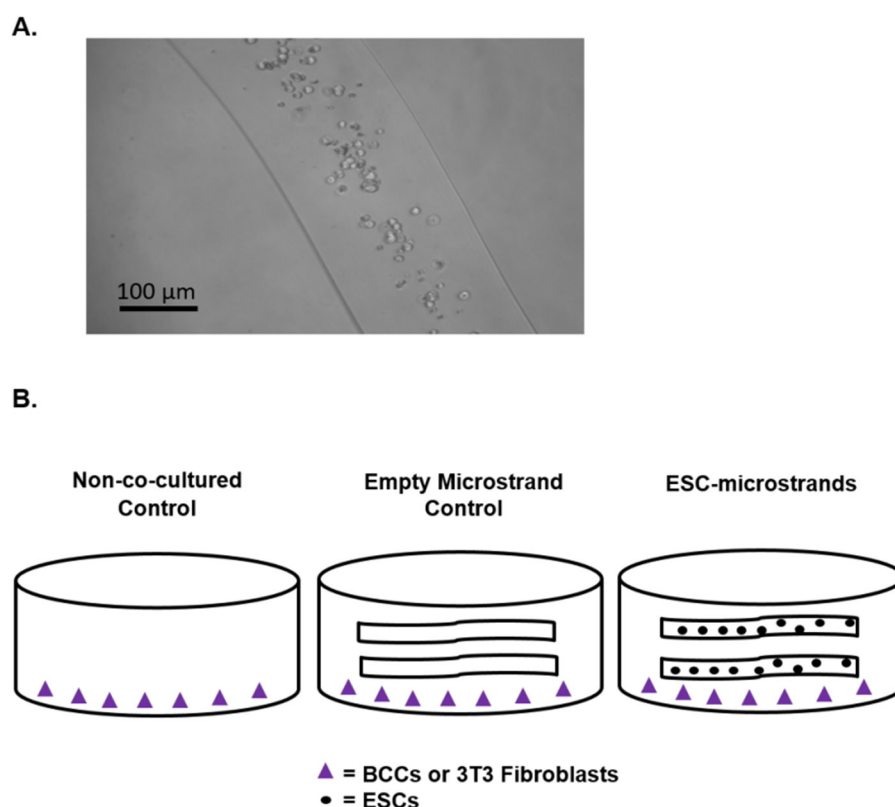


Figure 1. Schematic for co-culture of 3D embryonic stem cell (ESC)-microstrands with human breast cancer cells (BCCs). A: Optical image of mouse ESCs encapsulated in alginate hydrogel microstrands; B: experimental set-up of BCC alone (left), cultured with empty microstrands (middle), and co-cultured with 3D ESC-microstrands (right). No microstrands, empty microstrands, and 3T3 fibroblasts instead of BCCs served as experimental controls

qRT-PCR analysis

Cells were washed once with 0.5 mL of ice cold PBS. Total RNA of MDA-MB-231 BCCs was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA), per manufacturer's instruction. The RNA was of high purity with 260/280 values close to 2.0. Starting with 125 ng/mL of total RNA, cDNA was synthesized using an Omniscript first-strand cDNA synthesis kit (Invitrogen, Grand Island, NY). qRT-PCR was executed using a StepOnePlus™ Real Time PCR System (Applied Biosystem, Foster City, CA). The Mastermix contained 1.25 μL of the forward and reverse primers [Table 2], 12.5 μL of SYBR® Green PCR Master Mix (Life Technologies, Carlsbad, CA), and 5 μL of Molecular Biology Grade water (Corning, Inc., Corning, NY). Each sample was performed in triplicate and a negative control containing Molecular Biology Grade water instead of cDNA was included in every trial. The $\Delta\Delta C_t$ method was applied for quantification using β -actin as an internal control.

Western blot analysis

BCCs were washed with ice cold PBS and lysed through exposure to 0.2 mL of ice cold RIPA buffer (Sigma Aldrich) for 20 min while pipetting up and down. Cell lysates were centrifuged at 12,000 rpm for 20 min at 4 °C. The cell pellet was discarded, and the concentration of total protein was assessed using a BCA assay, per manufacturer's instructions. The protein samples were boiled for 10 min and loaded into the wells (15 μg) of a NuPage® Novex® 4%-12% Bis-Tris Protein Gel (Thermo Fisher Scientific, Waltham, MA) for separation through electrophoresis (100V for 2 h). The running buffer, 1 × NuPAGE® MOPS SDS (Thermo Fisher Scientific, Waltham, MA), contained 50 mmol/L MOPS, 50 mmol/L Tris Base, 0.1% SDS, and 1 mmol/L EDTA. Prior to loading, cell lysates were mixed with LDS sample buffer (4:1) (Pierce™ Chemical, Rockford, IL) containing 0.5 mol/L dithiothreitol (DTT) (10:1) (Invitrogen, Grand Island, NY).

Table 2. Forward and reverse primer sequences for qRT-PCR

Target	Forward sequence (5' → 3')	Reverse sequence (5' → 3')
EGFR	CTC CCA GTG CCT GAA TAC ATA AA	CCG TGG TCA TGC TCC AAT AA
β-catenin	GCT CCT TCT CTG AGT GGT AAA G	CAC CTG GTC CTC GTC ATT TAG CTT GTA
Vimentin	GAT TCA CTC CCT CTG GTT GAT AC	GGA GTG TCG GTT GTT
NKD2	AGA TAC ACA TGC CGT ACA CCA C	CGG CAG GTA GTA GCT GAA GG
TGF-α	CCC TGC GAA GAC TTG AGA TTT A	GGA GCT TGC AGA GAT GGA TTA G
GAPDH	GAT TCC ACC CAT GGC AAA TTC	GTC ATG AGT CCT TCC ACG ATA C
β-actin	AAA GAC CTG TAC GCC AAC ACA GTG CTG TCT	CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT G

qRT-PCR: quantitative reverse transcription polymerase chain reaction

Table 3. Antibodies used for western blot analysis

Target	Species	Company	Dilution/concentration
EGFR	Goat	Santa Cruz Biotechnology	1:500
E-cadherin	Rabbit	Santa Cruz Biotechnology	1:500
Vimentin	Mouse	Abcam	1:1000
TGF-α	Rabbit	Abcam	0.2 µg/mL
β-catenin	Mouse	Santa Cruz Biotechnology	1 µg/mL
NKD2	Rabbit	Abcam	1:500
β-actin	Mouse	Santa Cruz Biotechnology	1:1000
GAPDH	Mouse	Life Technologies	1 µg/mL
pERK	Mouse	Santa Cruz Biotechnology	1:500
pEGFR	Goat	Santa Cruz Biotechnology	1:500

RIPA buffer was added to give a final volume of 20 µL per well. β-actin was utilized as a loading control and 10 µL of Precision Plus Protein™ WesternC™ Standards (BioRad, Hercules, CA) was added to a well as a molecular weight reference. The proteins were wet-transferred onto a nitrocellulose membrane for 1 h at 100V. The transfer buffer consisted of 1 × Tris-glycine buffer with 20% (v/v) methanol. Prior to transfer, the nitrocellulose membrane was incubated for 10 min in 100% methanol for activation. Following wet transfer, the nitrocellulose membrane was washed three times in Tris-buffered saline and Tween 20 (TBST) in 15-min intervals and incubated for 1 h at RT in 5% (w/v) BSA diluted in TBST. The nitrocellulose membrane was incubated overnight at 4 °C in a primary antibody [Table 3], which was diluted in TBST. Three 15-min washes in TBST were performed, followed by one-hour incubation at RT in a secondary antibody diluted in TBST. Three washes were performed again in TBST and the nitrocellulose membrane was placed in Supersignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions. Signal detection was accomplished using a FluorChem E system (Protein Simple, San Jose, CA).

Statistical analysis

Data analyses were performed using a one-way ANOVA with multiple comparisons. Data were expressed as the mean ± standard deviation. A value of $P < 0.05$ was considered statistically significant. Experiments were repeated 2-3 times.

RESULTS

ESC-microstrands increase MDA-MB-231 BCC sensitivity to chemotherapeutic drugs

Highly aggressive MDA-MB-231 BCCs were treated with the chemotherapeutic drugs Erlotinib and PNU 74654, which inhibit the EGFR and canonical Wnt/β-catenin signaling pathways, respectively. For both drugs, there was a reduction in BCC viability when treated at a dose of 20 µmol/L with a 24-h recovery period [Figure 2A].

PNU 74654 decreased metastatic BCC viability more than Erlotinib, with a reduction exceeding 30%. Combining both drugs further diminished cell viability compared to individual treatment. To examine the

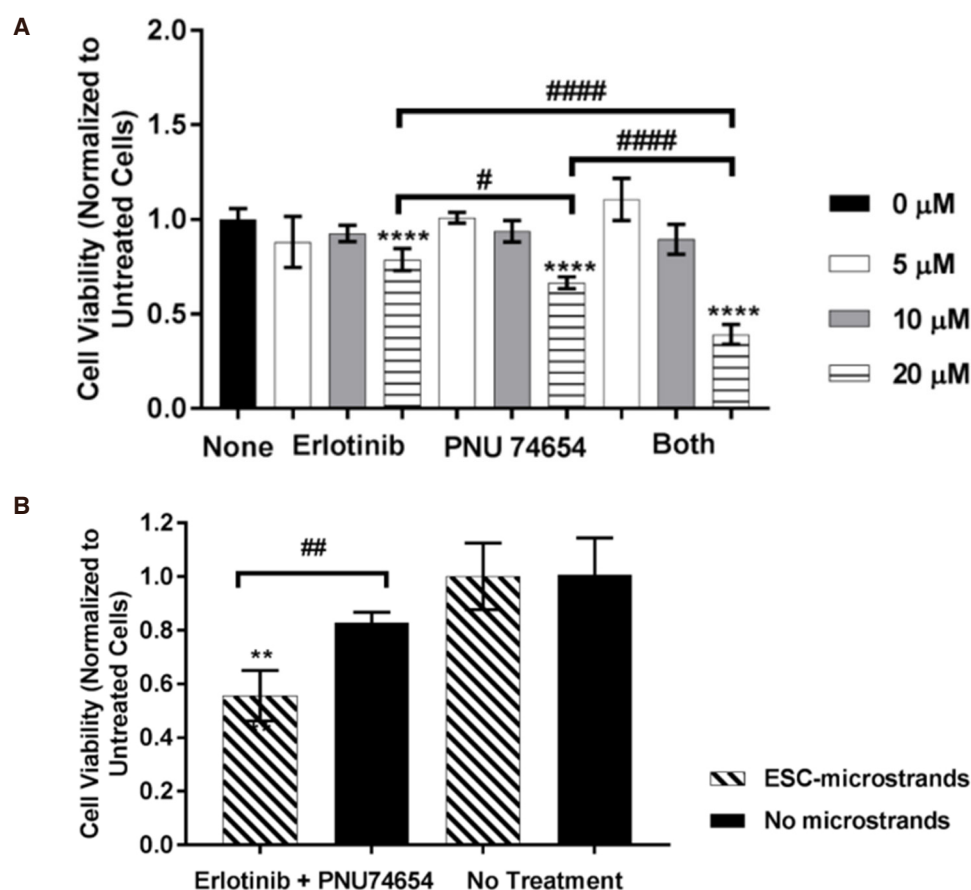


Figure 2. Exposure to embryonic stem cell (ESC)-microstrands overcomes chemotherapeutic drug resistance and reduces metastatic MDA-MB-231 cancer cell survival after drug treatment. A: MDA-MB-231 breast cancer cells (BCCs) were sensitive to the chemotherapeutic drugs Erlotinib and PNU 74654 when treated with 20 $\mu\text{mol/L}$ for 30 min, followed by a 24-h recovery period. Simultaneous treatment with Erlotinib and PNU 74654 resulted in the largest decrease in cell viability suggesting that both pathways play a role in the metastatic phenotype; B: after being co-cultured with ESC-microstrands, MDA-MB-231 BCCs exhibited significant reduction in cell viability after three cycles of treatment and recovery with both drugs at 20 $\mu\text{mol/L}$ compared to non-co-cultured BCC control. *Statistical significance compared to untreated control (* $P < 0.05$, **/ $P < 0.01$, and ****/#### $P < 0.0001$)

effect of ESC-microstrands on chemotherapeutic drug resistance, cells were treated three times with both chemotherapeutic drugs at a dose of 20 $\mu\text{mol/L}$, and viability was compared to the non-co-cultured BCCs. Three 24-h treatment and 24-h recovery cycles were performed to simulate the drug resistant state caused by multiple chemotherapeutic treatments *in vivo*. At the end of the treatments, the co-cultured BCCs were more sensitive to the chemotherapeutic drugs compared to the non-co-cultured BCCs [Figure 2B].

Co-culture with ESC-microstrands reverses oncogenic gene expression of MDA-MB-231 BCCs

To understand the inhibitory effect of exposure to ESC-microstrands on metastatic BCCs, relative mRNA expression levels of EGFR and canonical Wnt/ β -catenin signaling pathway-related molecules of MDA-MB-231 BCCs following co-culture with ESC-microstrands were examined using qRT-PCR. We have previously shown that co-culture with ESC-microstrands increases NKD2 and decreases TGF- α mRNA expression after 48 h^[10]. NKD2 is a negative regulator of the canonical Wnt/ β -catenin signaling pathway and TGF- α is a ligand that activates the EGFR signaling pathway. Similar changes in NKD2 and TGF- α gene expression following co-culture with ESC-microstrands were also observed after 72 h [Figure 3A].

The differences between BCCs co-cultured with ESC-microstrands and both the empty microstrand and non-co-cultured controls were statistically significant. For both 48 h (data not shown) and 72 h, there was a

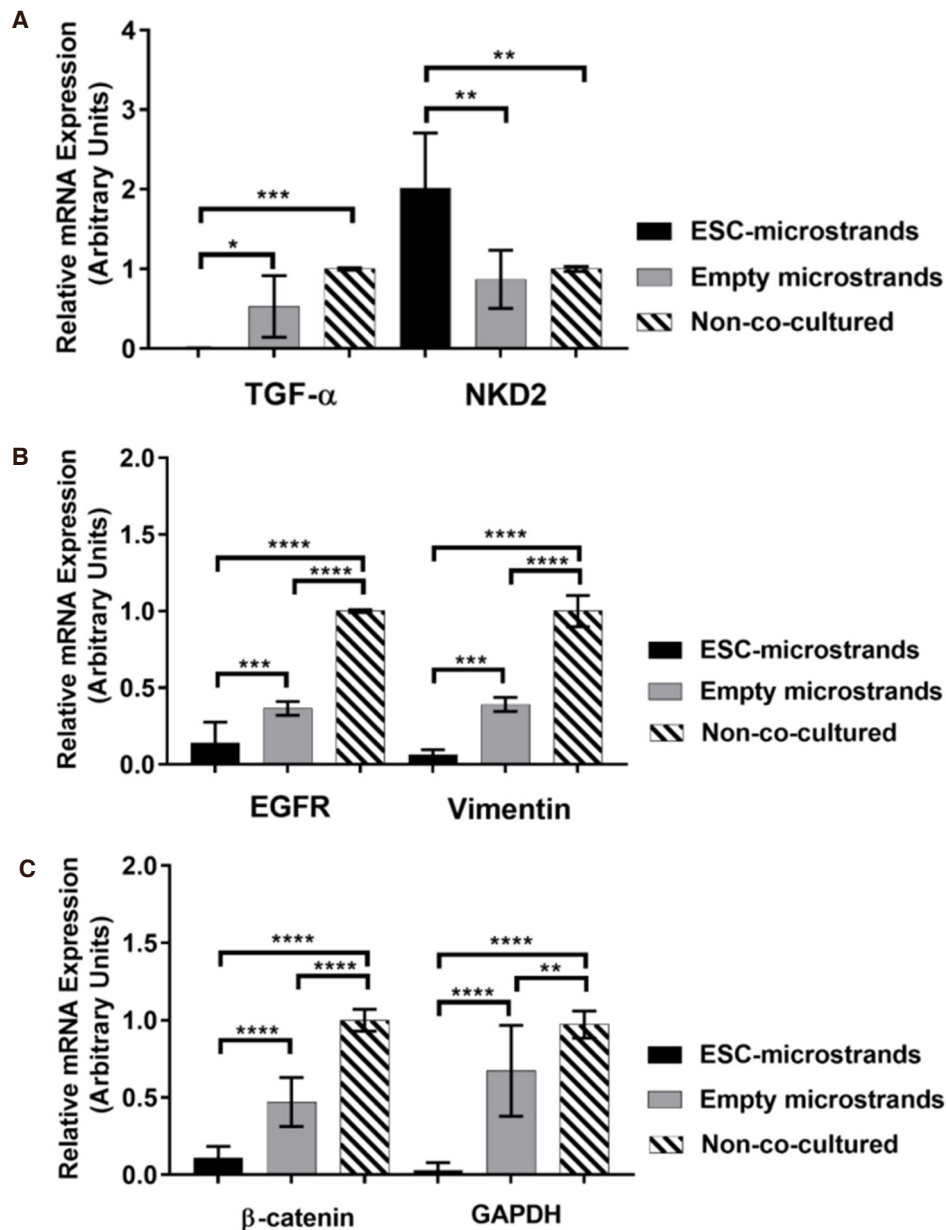


Figure 3. Reversal of gene expression of metastatic MDA-MB-231 breast cancer cells (BCCs) after exposure to embryonic stem cell (ESC)-microstrands at the transcriptional level as revealed by qRT-PCR analysis. A: After 72 h, significantly decreased TGF- α and increased NKD2 gene expression in BCCs co-cultured with ESC-microstrands compared to both empty microstrand and non-co-cultured controls; B: after 72 h, EGFR and vimentin gene expression decreased significantly compared to both controls; C: after 48 h, β -catenin and GAPDH gene expression declined in BCCs co-cultured with ESC-microstrands compared to the both controls (**** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$)

decline in both EGFR and vimentin mRNA levels in the BCCs co-cultured with ESC-microstrands [Figure 3B]. There was also a decline in expression in the empty microstrand group for both EGFR and vimentin, but it is not as extreme as for the ESC-microstrand group. Finally, both β -catenin and GAPDH mRNA levels decreased in the BCCs after co-culture with ESC-microstrands for 48 h [Figure 3C]. This decrease persisted for β -catenin, but not for GAPDH, after 72 h (data not shown).

ESC-microstrands reverse EGFR and β -catenin protein expression in metastatic BCCs

We further examined expression of EGFR, β -catenin, NKD2, and TGF- α in metastatic MDA-MB-231 BCCs at protein level after 48 h of co-culture with ESC-microstrands, in comparison with non-invasive

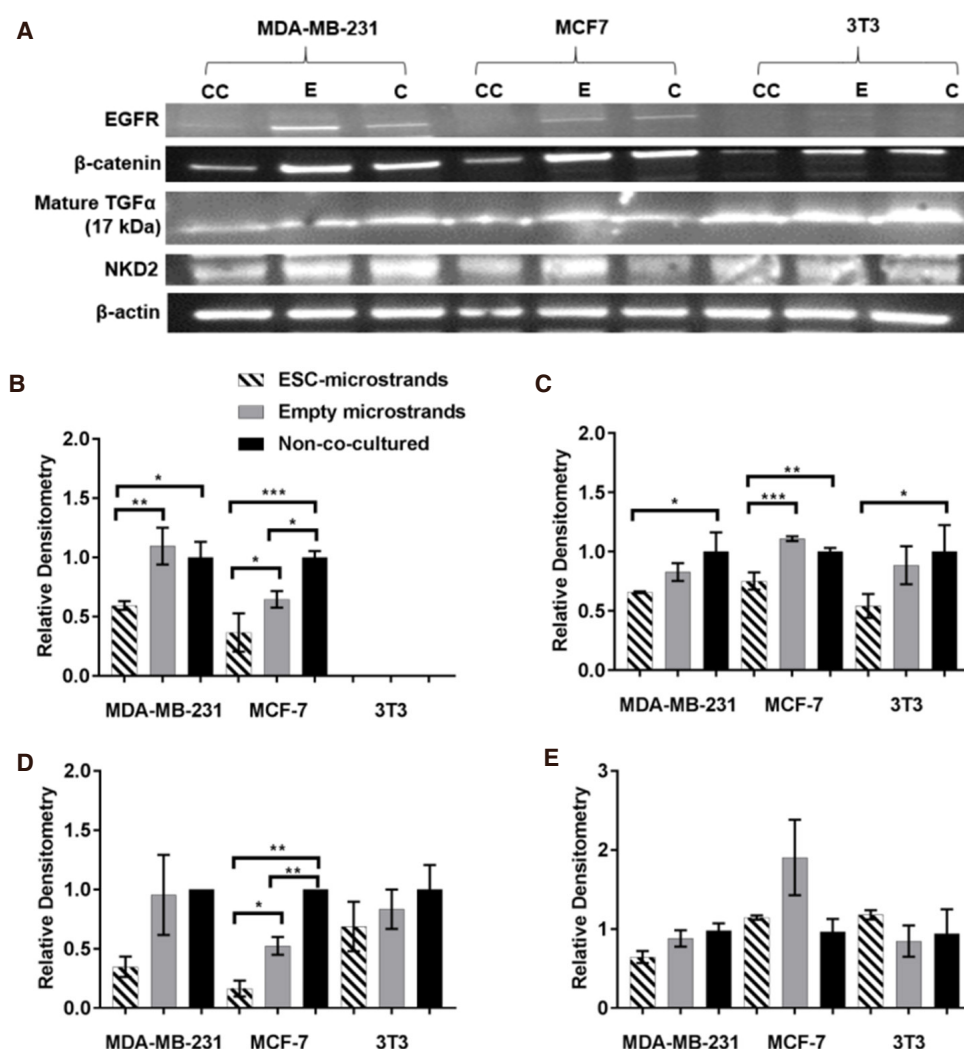


Figure 4. Protein expression of key signaling pathway molecules in metastatic MDA-MB-231 breast cancer cells (BCCs) after co-culture with embryonic stem cell (ESC)-microstrands for 48 h in comparison to non-aggressive MCF7 BCCs and 3T3 fibroblasts. Western blots show reduced EGFR, β -catenin, and mature TGF- α protein expression in the co-cultured MDA-MB-231 BCCs (CC: co-cultured with ESC-microstrands; E: co-cultured with empty microstrands; C: non-co-cultured control) (A); densitometry of protein expression of EGFR (B); β -catenin (C); mature TGF- α (D); NKD2 (E) (*** P < 0.001; ** P < 0.01; and * P < 0.05)

MCF7 BCCs and 3T3 fibroblasts [Figure 4A]. Western blot analysis of MDA-MB-231 BCCs after 48 h of co-culture with ESC-microstrands revealed a decline in EGFR protein expression compared to both empty microstrands and non-co-cultured controls [Figure 4B]. A similar trend was observed in the MCF7 BCCs, however, the reduction following co-culture with ESC-microstrands was also significantly different than the non-co-cultured control. The 3T3 fibroblasts did not express EGFR. There was also a reduction in β -catenin expression following co-culture with ESC-microstrands for all three cell types that was statistically different than the non-co-cultured control [Figure 4C]. However, only the MCF7 BCCs showed statistical significance when comparing the ESC-microstrands to the empty microstrands. TGF- α expression was decreased for both BCC types following co-culture with ESC-microstrands, but only the MCF7 BCCs showed statistical significance when compared to both controls [Figure 4D]. Finally, after 48 h, NKD2 protein expression did not significantly change following co-culture with ESC-microstrands or empty microstrands for any cell type [Figure 4E].

ESC-microstrands restore EGFR and canonical Wnt/ β -catenin signaling pathway function

Since co-culture with ESC-microstrands decreased EGFR and β -catenin expression in MCF7 and MDA-MB-231 BCCs following 48 h of co-culture, ESC-microstrands and media were removed and replaced with

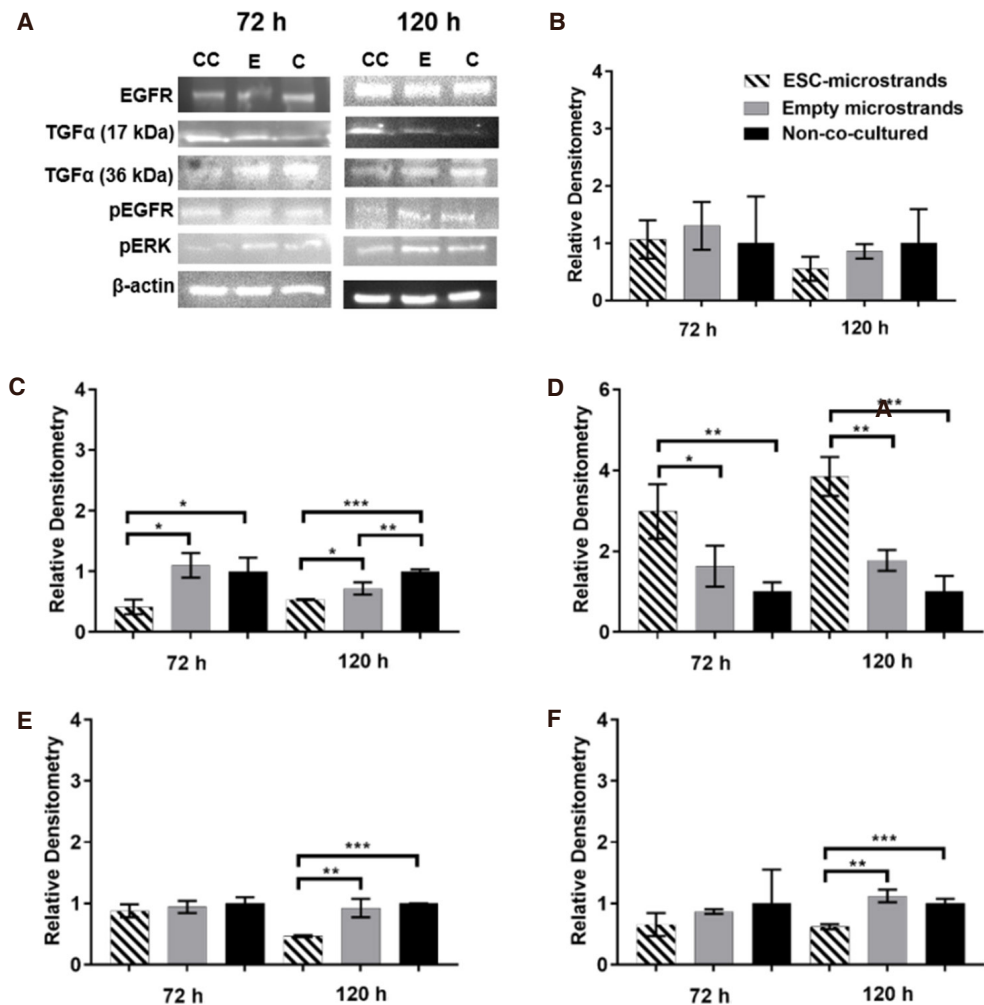


Figure 5. Western blot analysis of long-term protein expression of key EGFR signaling pathway molecules. Metastatic MDA-MB-231 breast cancer cells (BCCs) were co-cultured with embryonic stem cell (ESC)-microstrands for 48 h, followed by removing ESC-microstrands, replacing BCC media, and analyzing protein expression at 72 h and 120 h. Western blots for protein expression in metastatic MDA-MB-231 BCCs (CC: co-cultured with ESC-microstrands; E: co-cultured with empty microstrands; C: non-co-cultured control) (A); densitometry of expression of EGFR (B), precursor TGF- α (C), mature TGF- α (D), pEGFR (E), and pERK (F) (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$)

BCC media to see if there are other long-term signaling pathway-related changes in protein expression in MDA-MB-231 BCCs. The BCCs recovered for either one day or three days, which are denoted as 72 h and 120 h, respectively. Figure 5A shows the immunoblots for proteins associated with the EGFR signaling pathway. Contrasting to results for 48 h of co-culture, after 72 h, there was no change in EGFR expression in the MDA-MB-231 BCCs following co-culture with ESC-microstrands [Figure 5B]. Precursor TGF- α levels declined by 72 h [Figure 5C]. There was a statistically significant increase in mature TGF- α expression at the 72-h mark [Figure 5D], which was enhanced by 120 h. There was a decrease after 72 h in pEGFR expression [Figure 5E]. Finally, there was reduced pERK by 72-h and 120-h marks [Figure 5F].

Immunoblots for molecules related to the canonical Wnt/ β -catenin signaling pathway are provided in Figure 6A. Precursor E-cadherin was not present in any of the samples until 120 h, and for that reason, there is no band in the blot for the 72-h point. β -catenin expression was unchanged following co-culture with ESC-microstrands after 72 h, however, it was significantly reduced by 120 h compared to both controls [Figure 6B]. At 72 h and 120 h, there was an upsurge in NKD2 expression following co-culture with ESC-microstrands that was significant compared to both controls [Figure 6C]. By 120 h, there was precursor

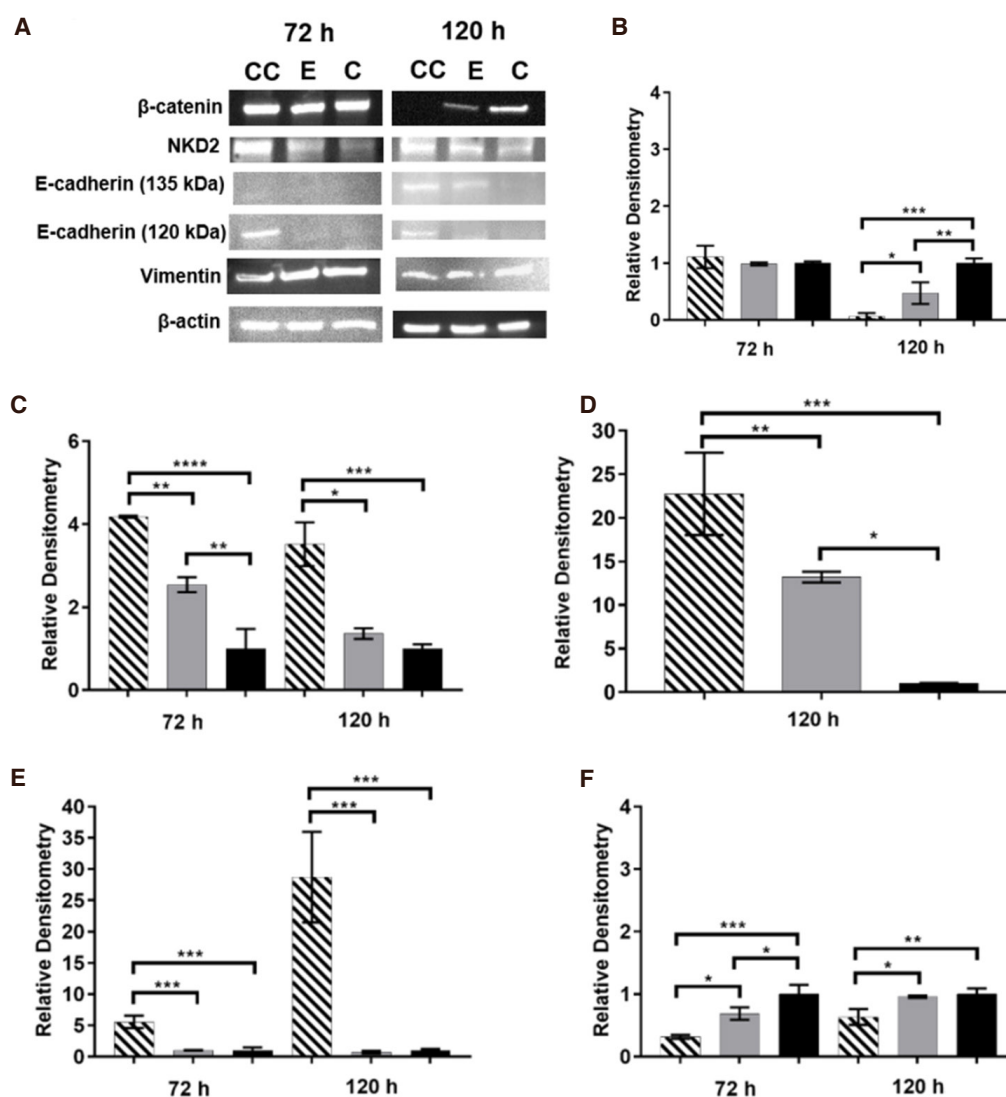


Figure 6. Western blot analysis of long-term protein expression of key canonical Wnt/β-catenin signaling pathway molecules. Metastatic MDA-MB-231 breast cancer cells (BCCs) were co-cultured with embryonic stem cell (ESC)-microstrands for 48 h, followed by removing ESC-microstrands, replacing BCC media, and analyzing protein expression at 72 h and 120 h. Western blots for protein expression in metastatic MDA-MB-231 BCCs (CC: co-cultured with ESC-microstrands; E: co-cultured with empty microstrands; C: non-co-cultured control) (A); densitometry of expression of β-catenin (B), NKD2 (C), precursor E-cadherin (D), mature E-cadherin (E), and vimentin (F) (** $P < 0.001$; * $P < 0.01$; $P < 0.05$)

E-cadherin present in the BCCs co-cultured with ESC-microstrands, and it was extremely elevated [Figure 6D]. Mature E-cadherin was expressed in the BCCs co-cultured with ESC-microstrands, but not in the non-co-cultured controls at 72 h. and 120 h [Figure 6E]. There was some expression in the empty microstrand control at 72 h, however, it was much less. Our previous work demonstrated that co-culture with ESC-microstrands decreases vimentin protein expression after 48 h^[10]. At 72 h and 120 h, vimentin protein expression was diminished in MDA-MB-231 BCCs co-cultured with ESC-microstrands [Figure 6F].

DISCUSSION

This work has demonstrated the utility of a bioengineered 3D ESC microenvironment (so-called ESC-microstrands) for the study of cross-talk of signaling pathways in cancer cells. It reveals that inhibitory effects of the ESC microenvironment on triple negative, metastatic MDA-MB-231 BCCs is attributable to restoration of EGFR and canonical Wnt/β-catenin signaling pathway regulation. Simultaneous treatment of

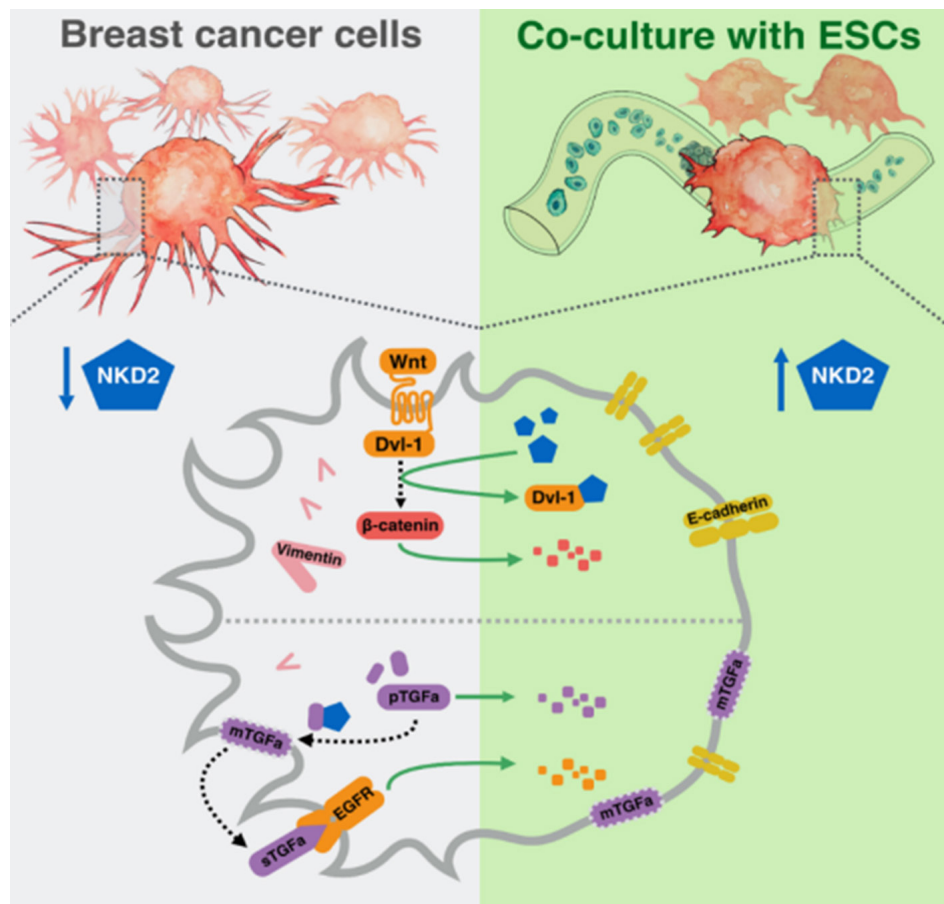


Figure 7. Schematic representation of the effects of co-culture with embryonic stem cell (ESC)-microstrands on expression of key signaling pathway molecules in MDA-MB-231 breast cancer cells (BCCs). Left Panel: BCC alone; Right Panel: co-cultured breast cancer cells with ESC-microstrands. Changes in protein and mRNA expression after co-culture with ESC-microstrands indicated inhibition of the canonical Wnt/ β -catenin (above the dash line) and EGFR (below the dash line) signaling pathways. Co-culture with ESC-microstrands causes NKD2 up-regulation at both the mRNA and protein levels, and this coincides with dual signaling pathway inhibition. It suggested restored ability of NKD2 to inhibit the canonical Wnt/ β -catenin signaling pathway and reduced ability to promote the EGFR signaling pathway following co-culture

metastatic BCCs with EGFR and canonical Wnt/ β -catenin signaling inhibitors suppresses growth more than individual treatment, suggesting that both pathways play a role in the restriction of metastatic phenotype.

The microenvironments of ESCs play a fundamental role in providing cells with appropriate signaling to induce cell proliferation, differentiation, or death. ESCs secrete soluble factors or exosomes that could reprogram malignant cancer cells to benign phenotype and suppress tumorigenesis^[35-39]. To recapitulate the native stem cell niche, researchers have exploited several bioengineering methods. Researchers have demonstrated that human ESC pluripotency is maintained when the ESCs are cultured in a 3D hyaluronic acid^[40], alginate^[41,42], or chitosan scaffold^[43]. Specifically, our lab has reported that mouse ESCs encapsulated inside aqueous alginate hydrogel microspheres remain pluripotent and restrict rat breast cancer cell proliferation and migration^[8]. Our previous work has also established that ESCs encapsulated in alginate microstrands exhibited biological effects on cell behavior of BCCs after co-culture with these ESC-microstrands, restricting highly aggressive human breast cancer proliferation, survival, invasion, migration, EMT, and metabolic activity^[10] as summarized in Table 1. This study aims to further probe whether this restriction is due to restored signaling pathway function, specifically related to the canonical Wnt/ β -catenin and EGFR signaling pathways, which are hyperactivated in human breast cancer.

Deregulation of canonical Wnt/ β -catenin and EGFR signaling is implicated in various forms of tumorigenesis causing colorectal, lung, breast, ovarian, prostate, liver, and brain cancers^[12,44]. For the canonical Wnt/ β -catenin signaling pathway, cancer also arises from overexpression of Wnt ligand genes, DNA hypermethylation of negative regulators such as p16 and E-cadherin, and overexpression of R-spondins that enhance Wnt ligand function^[23,44]. In BCC lines and primary tumors, Wnt ligands and Fzd receptors are expressed, and β -catenin is often localized within the nucleus^[44]. Inhibiting EGFR in cancer cells either directly or with cDNA transfections *in vitro* and *in vivo* causes a decrease in proliferation^[45]. Signaling pathways downstream of the EGFR are deregulated through EGFR mutations, constitutive activation, autocrine/paracrine signaling loops, enhanced ligand production, up- or down-regulation of signaling mediators, and prolonged interactions of EGFR with ErbB2 that either increase EGFR expression, activate EGFR, or prevent its deactivation^[14,46]. Activation of Rho GTPases downstream of EGFR signaling reduces E-cadherin expression and actin reorganization^[45]. Certain tumors contain mutations that delete the extracellular binding domain of the EGFR, which constitutively activates the receptor. In glioblastoma, there is frequently an in-frame deletion of exon 2-7 of the EGFR. Ligand-independent activation of the EGFR is caused by overexpression of urokinase-type plasminogen activator receptor and its association with the integrin $\alpha 5 \beta 1$ ^[11,29,47]. Prolonged interaction of ErbB2 with EGFR fosters breast CSC self-renewal through activation of the PI3K/Akt signaling cascade. Increased cellular stress and radiation have been shown to down-regulate phosphatases responsible for the EGFR signal abrogation^[48]. Downstream of the EGFR signaling pathway, the PI3K/Akt/mTOR pathway activates protein kinase B through loss of PTEN, a tumor suppressor and negative regulator of PI3K/Akt signaling. The result is stabilization of β -catenin through phosphorylation and inactivation of GSK3. As demonstrated by the previous examples, deregulated canonical Wnt/ β -catenin and EGFR signaling play an enormous role in many types of aggressive cancer, which highlights the importance of understanding their contribution to aggressive breast cancer progression. As such, our first aim was to examine whether exposure of aggressive MDA-MB-231 BCCs to ESC-microstrands increases sensitivity to chemotherapeutic drugs known to specifically target these two pathways.

Highly aggressive MDA-MB-231 BCCs were treated with the chemotherapeutic drugs Erlotinib and PNU74654. Erlotinib specifically targets the EGFR signaling pathway by binding to the ATP-binding site of the EGFR and preventing phosphorylation by its tyrosine kinase. PNU 74654 targets the canonical Wnt/ β -catenin signaling pathway by preventing the interaction of β -catenin with the transcription factor T-cell factor/lymphoid enhancer factor. The canonical Wnt/ β -catenin signaling pathway may play a larger role in MDA-MB-231 BCC aggressiveness as these cells were more sensitive to PNU74654. However, both signaling pathways are involved in the aggressive phenotype because combining both drugs significantly increases sensitivity. Co-culturing the MDA-MB-231 BCCs with ESC-microstrands prior to dual chemotherapeutic drug treatment coupled with the addition of multiple treatment and recovery periods, demonstrated that co-culture with ESC-microstrands increases BCC sensitivity to chemotherapeutic drugs.

Co-culture with ESC-microstrands reduced GAPDH mRNA expression. GAPDH is an enzyme responsible for catalyzing the sixth step of glycolysis. The original intention was to employ GAPDH as a housekeeping gene for this study, though recent works have confirmed GAPDH up-regulation in human breast cancer. A subsequent study by Maltseva *et al.*^[49] identified five reliable housekeeping genes in human breast cancer and β -actin, which was used in this study, was one of the top choices. In our model, GAPDH mRNA expression was decreased in the co-cultured MDA-MB-231 cells, which may explain their decreased glycolytic rate^[10]. The PI3K/Akt pathway lies downstream of the EGFR signaling pathway. GAPDH stabilizes activated Akt allowing oncogenic cells to circumvent caspase-independent cell death. Interestingly, Akt kinase is one of the main contributors to the Warburg effect and Akt also prevents the nuclear accumulation of GAPDH necessary for caspase-dependent cell death^[50].

In this work, inhibition of the EGFR signaling pathway following co-culture is demonstrated through reduced EGFR and TGF- α mRNA expression, increased mature TGF- α protein expression, and decreased

precursor TGF- α , pEGFR, and pERK protein expression. The reduction in EGFR protein levels in the co-culture after 48 h. is not sustained in the long-term experiment suggesting that EGFR signaling pathway inhibition in the co-culture is not entirely attributable to modulation of EGFR availability. Both soluble (6 kDa) and precursor (36 kDa) TGF- α protein levels are elevated in triple negative human breast cancer because cleavage of the precursor form is prevented and cleavage of the mature form is augmented^[51]. Possible explanations for the decrease in precursor TGF- α expression include (1) prevention of NKD2 from either interacting with precursor TGF- α and/or transporting it to the plasma membrane triggering its degradation; (2) increased cleavage of precursor TGF- α into mature TGF- α within the plasma membrane; and (3) decreased precursor TGF- α production caused by reduced TGF- α mRNA expression. The increase in NKD2 expression coinciding with decreased precursor TGF- α expression suggests that precursor TGF- α is being prevented from interacting with NKD2 and this is triggering its degradation. This would imply that decreased cleavage of mature TGF- α into its soluble form is the major contributor to the augmented mature TGF- α expression, as opposed to increased cleavage of precursor TGF- α . The EGFR signaling pathway utilizes phosphorylation cascades to activate downstream molecules that affect cell behavior. Suppression of pEGFR and pERK protein expression after 120 h indicates that EGFR signaling pathway activation is being suppressed in the co-cultured BCCs. Taken together, the changes in relative mRNA and protein expression in the co-cultured BCCs are extremely promising as they suggest that co-culture is altering the BCCs at both transcriptional and translational levels, indicating that this model can be applied to study the mechanism(s) for signaling pathway inhibition and highly aggressive BCC restriction.

The canonical Wnt/ β -catenin signaling pathway is hyperactivated in human breast cancer, and this leads to increased β -catenin expression. In this work, exposing MDA-MB-231 BCCs to ESC-microstrands decreases β -catenin mRNA and protein expression. The decline in β -catenin protein levels in western blot analysis signifies its increased ubiquitylation and degradation leading to decreased canonical Wnt/ β -catenin signaling pathway activation. Three other major signs of canonical Wnt/ β -catenin inhibition in BCCs co-cultured with ESC-microstrands are increased E-cadherin protein levels, augmented NKD2 mRNA and protein expression, and decreased vimentin mRNA and protein levels. E-cadherin is synthesized as a 135 kDa precursor that undergoes cleavage to its mature 120 kDa form^[52]. β -catenin is responsible for anchoring mature E-cadherin to the plasma membrane, and this interaction may contribute to the comparable β -catenin protein levels in the co-culture and controls after 72 h coinciding with the extreme up-regulation of mature E-cadherin in the co-cultured BCCs. Co-culture may directly prevent cytoplasmic β -catenin from translocating to the nucleus. The belief that β -catenin translocation to the nucleus involves more than its cytoplasmic concentration has gained steam, for example, Fang *et al.*^[53] assert that Akt, which is downstream of EGFR signaling pathway, promotes β -catenin nuclear translocation through phosphorylation. One possibility is that co-culture leads to decreased activated Akt, which is allowing β -catenin that has accumulated in the cytoplasm to be used for cell-cell adhesion. After 48 h, there are no detectable levels of mature or precursor E-cadherin in the MDA-MB-231 BCCs but there is mature E-cadherin expression in the MCF7 BCCs (data not shown). Interestingly, after restoring the BCC media to demonstrate the long-term effect of co-culture, there is mature and precursor E-cadherin present at the 72- and 120-h marks, respectively. NKD2 is a tumor suppressor that has a very short half-life of approximately sixty minutes and is hypermethylated in human breast cancer^[33]. The increase in NKD2 mRNA expression in the co-culture denotes a reversal of its methylation status and its increased protein expression suggests an active role in dvl-1 inhibition. Finally, the decreases in vimentin protein and mRNA expression levels in co-cultured BCCs are another sign of inhibition of this pathway because vimentin is transactivated by this pathway.

Our results showed that ESC-microstrands induced changes in gene expression of key regulators at both the mRNA and protein level in MDA-MB-231 BCCs that signified a reversal of the hyperactivated status of EGFR and canonical Wnt/ β -catenin signaling pathways and restored signaling pathway regulation present in normal non-tumorigenic cells. Specifically, decreased EGFR, pEGFR, pERK, and precursor TGF- α

coupled with augmented mature TGF- α expression indicated that the EGFR signaling pathway was being suppressed, whilst decreased vimentin and β -catenin expression coinciding with increased precursor and mature E-cadherin levels supported the notion of canonical Wnt/ β -catenin signaling pathway suppression. Importantly, these changes corresponded with an extreme up-regulation of NKD2 at both the mRNA and protein levels [Figure 7]. A possible explanation for this phenomenon is that co-culture with ESC-microstrands restores NKD2 expression in metastatic MDA-MB-231 BCCs, which enables it to inhibit the canonical Wnt/ β catenin signaling pathway, but that NKD2 is also prevented from transporting precursor TGF- α to the membrane for activation of the EGFR signaling pathway.

Altogether, we have demonstrated that exposure of triple negative BCCs to a bioengineered 3D ESC microenvironment restricts their tumorigenic, invasive, and metastatic features owing to restoration of EGFR and canonical Wnt/ β -catenin signaling pathway regulation. In particular, NKD2 could act as a “molecular switch” between these pathways in BCCs. Co-culture with ESC-microstrands has up-regulated NKD2 in triple negative, metastatic BCCs both at mRNA and protein levels. The exact role of NKD2 in this metastatic phenotype reversal will be the focus of subsequent studies. Exposure of MDA-MB-231 BCCs to ESC-microstrands may prevent NKD2 myristoylation allowing it to solely interact with Dvl-1 and thwarting precursor TGF- α transport to the plasma membrane causing its increased degradation. Future work will elucidate the mechanism leading to NKD2 preference for either EGFR or canonical Wnt/ β -catenin signaling pathway interaction allowing for the targeting of NKD2 to treat triple negative breast cancer. This work is important because it establishes that the bioengineered 3D ESC model can not only restrict triple negative breast cancer survival and metastatic potential, but can also be applied to determine the mechanism for this restriction and identify therapeutic targets to reverse metastatic disease. This is the first example of exploring the role of NKD2 in aggressive breast cancer as all other studies in literature were performed in colon cancer. In the future, we may test whether using ESC-microstrand-conditioned media could achieve the same level of restriction of cancer metastasis. Additionally, the use of a panel of triple negative metastasis breast cancer cells (e.g., MDA-MB-157, MDA-MB-468) will further validate our findings and confirm the utility of the 3D ESC model system for understanding cancer metastasis.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: Mooney B and Xie Y

Performed data acquisition and provided technical and material support: Mooney B

Created Figure 7 and revised the paper: Tian Y

Assisted in drug resistance experiments and revised the paper: Rousseau E

Availability of data and materials

Not applicable.

Financial support and sponsorship

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Original Article

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Synergistic inhibition of SCR1- and ERBB2-driven brain metastatic breast cancer cells

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Abstract

Aim: Metastasis to the brain has become a major limitation to the life expectancy and quality of life for many patients with breast cancer. Unfortunately, other than radiation and palliative treatments with trastuzumab, and pertuzumab, no effective therapy for brain metastases is currently available. This study seeks to identify novel gene targets and pharmaceutical Intervention against breast cancer brain metastasis.

Methods: The detailed methods applied to this study, including comparative RNA sequencing and bioinformatics analysis of sequence data, ingenuity pathway analysis, protein-protein interaction analysis, high throughput screening of clinical and pre-clinical drugs, cell viability and proliferation assay, toxicity and apoptosis assay using fluorescence-activated cell sorting, real-time PCR, western blotting, statistical analysis of data.

Results: The study reveals critical roles for SRC, ERBB2, PIK3CA, and GABA in the proliferation and survival of breast cancer brain metastatic (BBM) cells and showed that SRC- and ERBB2-mediated activation of PIK3-AKT/mTOR signaling regulates BBM cell survival. Selective inhibition of these candidate genes alone or in combination induces robust apoptosis in BBM cells

Conclusion: The findings of this study provide a rationale for further preclinical evaluation of SRC-targeting regimens in combination with ERBB2 inhibitors and/or GABA agonists to target breast cancer brain metastasis.

Keywords: Breast cancer, brain metastasis, human epidermal growth factor receptor 2, ERBB2, SRC, astrocytes, PI3KCA



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INTRODUCTION

Brain metastases represent a significant clinical challenge for the treatment of patients with human epidermal growth factor receptor 2 (HER2)-positive breast cancer. Although modern multimodality therapies have improved the survival of patients with primary breast cancer and systemic metastases, the overall median survival of patients with brain metastases is dismally less than one year^[1,2]. Furthermore, as the brain represents a “sanctuary site” for HER2+ metastases, and has comprised a larger proportion of relapse sites over time^[3,4]. Despite this increasing incidence, there is no current consensus on therapy for those with intracranial progression^[5,6]. Current clinical options for HER2+ brain metastases patients are limited to trastuzumab (anti-HER2 monoclonal antibody) treatment in conjunction with whole-brain radiotherapy or stereotactic radiosurgery^[7], neither of which clinically effective in treating life-threatening brain metastases that often lead to severe cognitive complications.

The poor prognosis of breast cancer brain metastatic (BBM) patients with local therapies underscores the need for better systemic treatments. Over the last few years, preclinical and clinical progress in the treatment of BBM has led to novel hypotheses for improving therapeutic outcome. The limited efficacy of trastuzumab against BM is often attributed to an inadequate penetration through the BBB^[8]. Lapatinib, a small molecule kinase inhibitor of epidermal growth factor receptor (EGFR) and HER2, was evaluated in BCBM due to its ability to better penetrate the BBB than trastuzumab^[9,10]. The success of lapatinib and capecitabine in preventing brain metastasis led to its inclusion in patients with established brain metastases. New generation ErbB family inhibitors neratinib and afatinib are more potent and specific than lapatinib, showed significant responses in limited cases of BBM^[11]. The downstream HER2 signaling inhibitors including the PI3K inhibitor BKM120 and the mTOR inhibitor everolimus were evaluated to overcome de novo or acquired resistance to anti-HER2 therapy. Indeed, targeting the HER2 family member HER3, critical for HER2 downstream signaling, enhances the efficacy of HER2-targeted therapies in preclinical models of BBM^[12,13].

Recent clinical findings described the efficacy of antibody-based therapy in BBM. Upon treatment Bevacizumab and trastuzumab-DM1 adequately accumulate in brain metastatic lesions to exert positive effect^[14-17]. Antibody-chemotherapy conjugate such as ado-trastuzumab emtansine (T-DM1) was approved for the treatment of HER2-positive breast cancer due to its higher efficacy over lapatinib and capecitabine in patients with disease progression after trastuzumab^[18]. As T-DM1 targets acquired or microenvironment-mediated activation of Her2 independent signaling pathways, this agent would be expected to be effective in such patients.

Using RNA sequencing (RNA-seq) analysis of primary HER2+ breast cancer and HER2+ breast to brain metastatic tumor resections, along with normal breast and normal brain tissues, we demonstrated that SRC- and ERBB2-mediated regulation of PI3K-AKT/mTOR signaling plays a critical role in BBM cell proliferation^[19]. Inhibition of SRC, ERBB2, and downstream kinases induced robust apoptotic cell death. In parallel experiments, we conducted a high throughput screening of 1650 clinical and preclinical drug candidates and found that activation of GABA signaling using a GABA agonist induces apoptosis in the breast to brain metastatic cells.

Interestingly, it has been shown that the SRC family of non-receptor tyrosine kinases is critical for both HER2+ and triple-negative breast cancer^[20]. SRC activation maximizes the HER2: HER3 interaction and serves as a convergent point of multiple downstream signals, including the PI3K-AKT/mTOR pathway, thus regulating cell viability^[20,21]. Similarly, activation of GABA signaling negatively regulates not only neural stem cells but also embryonic and cancer stem cells^[22-24]. GABAergic breast to brain metastatic cells rely on GABA as an energy source, and activation of GABA signaling prevents cell proliferation directly or by blocking the supply of GABA to the invading cells. This collective evidence suggests that inhibiting HER2 and simultaneous targeting SRC and/or GABA is a promising strategy for the treatment of breast cancer brain metastasis.

METHODS

Patient consent and tissue processing

De-identified archival and fresh tumor tissue samples were collected from consented patients undergoing resection of primary breast or breast to brain metastases, in accordance with a City of Hope Institutional Review Board (IRB)-approved protocol (#05091).

RNA isolation and sequencing

The flash frozen patient tissue samples were subjected to total RNA preparation using an RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions, eluted in 50 μ L of RNase/DNase-free water, and the initial concentration and purity assessed on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Prior to sequencing, RNA quality was assessed by microfluidic capillary electrophoresis using an Agilent 2100 Bioanalyzer and the RNA 6000 Nano Chip kit (Agilent Technologies, Santa Clara, CA). Sequencing libraries were prepared with the TruSeq RNA Sample Prep Kit V2 (Illumina, San Diego, CA), according to the manufacturer's protocol with minor modifications. Briefly, ribosomal RNA was removed from 500 ng of total RNA using a RiboZero kit (Illumina) and the resulting RNA was ethanol precipitated. Pellets were re-suspended in 17 μ L of Elute/Prime/Fragment Mix (Illumina) and first-strand cDNA synthesis performed using DNA polymerase I and RNase H. cDNA was end repaired, 3' end adenylated, and universal adapter ligated followed by 10 cycles of PCR using Illumina PCR Primer Cocktail and Phusion DNA polymerase (Illumina). Libraries were purified with Agencourt AMPure XP beads, validated with the Agilent 2100 Bioanalyzer, and quantified with Qubit (Life Technologies). Libraries were sequenced on an Illumina HiSeq 2500 with single-end 40-bp reads. Raw sequences were aligned to the human genome assembly (version 19, GRCh37.p13) using Tophat v2 and RefSeq gene expression levels were counted using HTseq-count. The genes expression counts were normalized using the trimmed mean of M-values method implemented in the Bioconductor package "edgeR." The differential expression analysis, clustering analysis, and pathway analysis were conducted using the DAVID online annotation tool and Ingenuity Pathway Analysis (IPA) to determine tissue-specific gene signatures and signaling pathways. The gene expression data of candidate genes was confirmed by real-time PCR.

Breast cancer brain metastasis cell cultures

HER2+ tumor samples were acquired from patients undergoing resection of the breast to brain metastases in accordance with a City of Hope IRB-approved protocol (IRB #05091). A portion of each specimen was cultured in DMEM-F12 (Life Technologies) supplemented with 10% fetal bovine serum, 1% glutamax, and 1% antibiotic-antimycotic (Life Technologies) in collagen-coated T75 flasks (Life Technologies) to derive low-passage primary cell lines COH-BBM1 (BBM1) and COH-BBM2 (BBM2).

High throughput screening of therapeutic candidates

The Lopac 1280 compound library (Sigma), DiscoveryProbe Neuronal Signaling Library (ApexBio), and clinical drugs targeting primary breast cancer and CNS tumors (Cayman Chemical) were obtained commercially. All the compounds of the two libraries are in preclinical or clinical candidates. Over 50% compounds of the library target neural disorders. The compounds were dissolved in DMSO at concentrations of 100 μ mol/L. For the initial screening, BBM1 cells were grown in 96-well plates (10000/well) and treated with the compounds at a final concentration of 1 μ mol/L ($n = 3$ per treatment). Control cells were treated with DMSO only. The viability of the cells was measured at 48 and 72 h post-treatment by using a CellTiter-Glo[®] Luminescent Cell Viability Assay kit (Promega). The compounds that suppressed BBM1 cell viability by at least 70% compared to the control were selected for secondary screening in both BBM1 and BBM2 cell lines. The compounds that showed consistent toxicity of both BBM1 and BBM2 cells over a period of 10 days were assessed for toxicity against BBM1 cells but not astrocytes.

To analyze cell type-specific toxicity, human astrocytes and BBM1 cells were grown overnight prior to treatment with 1 μ mol/L of the active compounds ($n = 6$). Control cells were treated with DMSO only. The

viability of the cells was measured at 48 and 72 h post-treatment using the CellTiter-Glo® Luminescent Cell Viability Assay kit. Compounds that showed at least two-fold greater toxicity against BBM1 cells compared to astrocytes cells were further analyzed for their concentration-dependent effects on BBM1 cells. For concentration-dependent toxicity, cells were treated with 0, 31.2, 62.5, 125, 250, 500, 1000 nmol/L final concentration of the compounds ($n = 8$). The viability was measured at 72 h post-treatment. The data normalization compared to control and IC_{50} determination was done using GraphPad Prism 7.

Real-time PCR and Western blot analysis

Total RNA from astrocytes and BBM1 and BBM2 cells was extracted using Trizol (Invitrogen) and treated with RNase-free DNase (Qiagen), according to the manufacturer's instructions. To analyze gene expression, cDNA was synthesized using an iScript reverse transcription kit (Bio-Rad). Real-time PCR quantification was conducted using gene-specific primers and SYBR Select Master Mix (Applied Biosystems) using a CFX real-time PCR system (Applied Biosystems). Control PCR reactions were conducted using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and/or actin-specific primers. PCR data was analyzed using the CFX manager (Bio-Rad).

For Western blot analysis, total cell lysates were prepared in protein lysis buffer (50 mmol/L Tris-HCl, pH 7.5; 100 mmol/L NaCl; 1% Triton X-100; 1 mmol/L EDTA; 1 mmol/L EGTA, 50 mmol/L β -glycerophosphoran, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethanesulfonyl fluoride; 2 mmol/L sodium orthovanadate, 10 μ g/mL aprotinin; 10 μ g/mL leupeptin; and 10 μ g/mL pepstatin A) by incubating cells for 20 min at 4 °C, followed by centrifugation ($15000 \times g$, 15 min, 4 °C). Protein extracts were analyzed by using antibodies specific to ERBB2; phosphorylated ERBB2 (pERBB2); Protein kinase B (AKT); phosphorylated AKT (pAKT) were obtained from Millipore; Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit, Alpha (PIK3CA), phosphorylated PIK3CA (pPIK3CA); SRC Proto-Oncogene (SRC); phosphorylated SRC (pSRC); MYC Proto-Oncogene (MYC); Mammalian Target of Rapamycin (mTOR); phosphorylated mTOR (pmTOR); procaspase3 and cleaved caspase 3 were obtained from Cell Signaling. Actin and α -tubulin antibodies were also obtained from Cell Signaling.

Apoptosis analysis

Apoptosis induction in treated and control cells was measured using Annexin V-FITC staining followed by FACS analysis. In brief, cells were treated with the compounds 24 to 48 h prior to fixing in 4% PFA. Control cells were treated with vehicle alone. The cells were immunostained with Annexin V-FITC (Invitrogen) followed by PI (Life Technology) and analyzed using BD FACS Aria Flow Cytometer (BD Biosciences). The experimental control cells were stained with IgG with or without PI.

Statistical analysis

Verification of RNA-seq data was done with real-time PCR in at least three biological replicates to confirm reproducibility and repeated at least twice. Results are expressed as the means \pm SEM. Unless otherwise stated, the two groups comparisons analyses were performed by using Mann-Whitney U tests in GraphPad Prism® 7.0 (GraphPad Software Inc., La Jolla, CA, USA). $P < 0.05$ was considered to be statistically significant.

RESULTS

Breast cancer brain metastases display a unique brain-like transcription profile

Primary HER2(ERBB2)+ breast cancer tissue (PT) and HER2+ breast to brain metastatic tissue (MT) collected from the consented patients, along with normal breast (nBreast) and normal brain (nBrain) tissues, were subjected to RNA-seq analysis. Hierarchical clustering of the normalized expression data of 20,257 transcripts revealed that, whereas PT shows transcriptome similarity with nBreast tissue, MT exhibits a brain-like transcription profile [Figure 1A and B]. IPA of the 367 MT-specific revealed ERBB2 signaling as a

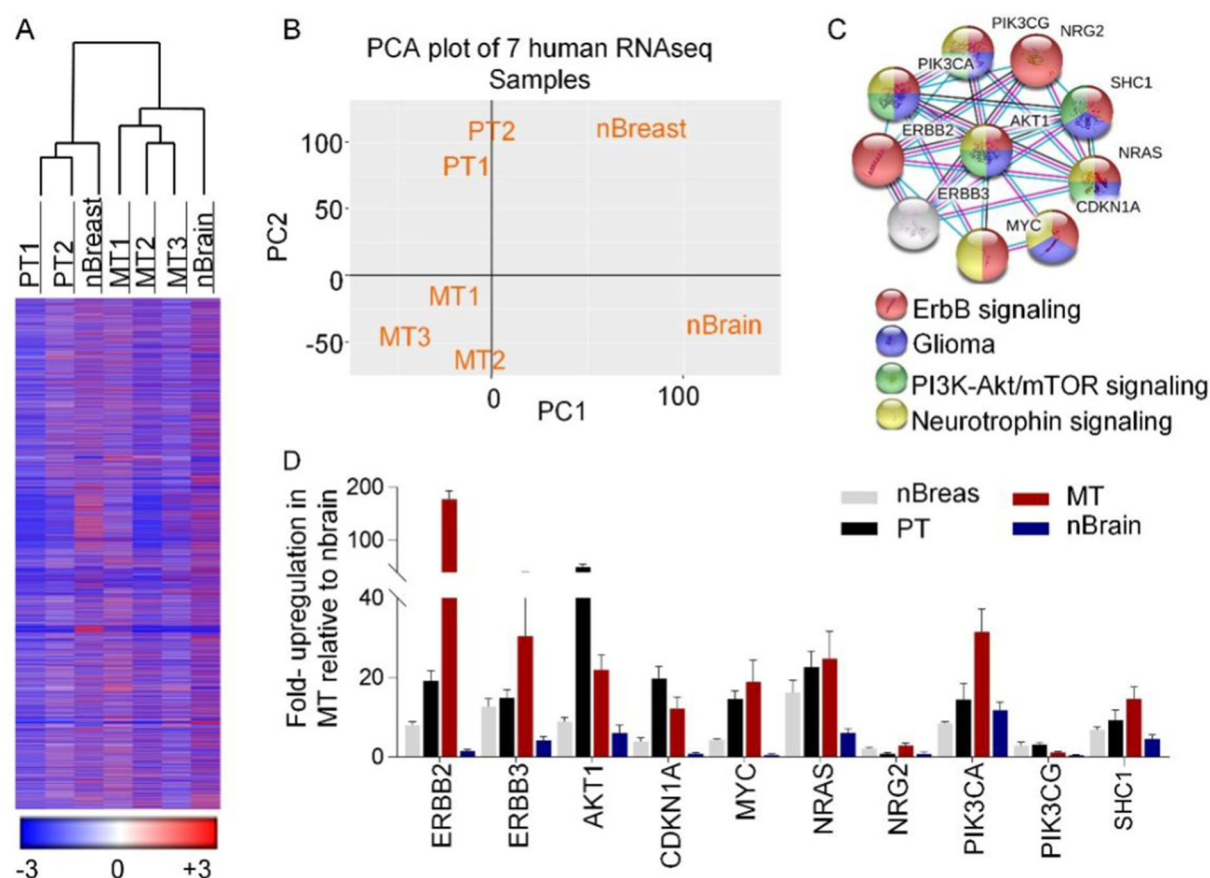


Figure 1. RNA-Seq analysis of two primary breast tumors (PT1-2), one normal breast tissue sample (nBreast), three breast to brain metastatic tumors (MT1-3), and one normal brain tissue sample (nBrain). A: Heatmap comparison of gene expression in each group, presented as log₂-normalized fragments per kilobase of transcript per million mapped reads (FPKM). Colors indicate relatively low (blue) or high (red) gene expression; B: the principal component analysis (PCA) of log₂-normalized FPKM values, showing similarities and differences between sample groups; C: protein-protein interactions of selected genes overexpressed in metastatic tissue (MT) samples compared to all other groups; D: real-time PCR quantification of genes overexpressed in different samples ($n = 3$, $P < 0.05$). Error bars indicate SEM

major canonical pathway overexpressed in MT [Supplementary Figures 1 and 2]. Protein-protein interaction analysis of the MT-specific genes revealed ERBB2/PI3K/AKT/mTOR as a potential major regulator of the breast to brain metastatic cell survival and proliferation [Figure 1C]. Similarly, KEGG pathway analysis projected ERBB2, PIK3-Akt/mTOR, and neurotrophin signaling as major pathways associated with MT-specific genes [Supplementary Figure 2].

Based on IPA, followed by protein-protein interaction and KEGG pathway analyses, we focused on a set of 10 genes that showed at least two-fold overexpression in MT tissue compared to all other groups and may be important for the proliferation of BBM cells.

To confirm the expression of these genes, we synthesized gene-specific primers and performed real-time PCR analysis of RNA extracted from MT (MT1-3), PT (PT1-2), and nBrain tissue [Figure 1D]. To further confirm the expression of these MT-specific genes, we cultured two BBM tissue-derived low-passage cell lines, BBM1 and BBM2, along with human reactive astrocytes. Both BBM cell lines showed distinct morphological differences compared to astrocytes [Figure 2A]. The real-time PCR analysis of extracted RNA showed significant ($P < 0.05$) upregulation of MT-specific genes, except for NRG2 and PIK3CG, in BBM cells compared to astrocytes [Figure 2B].

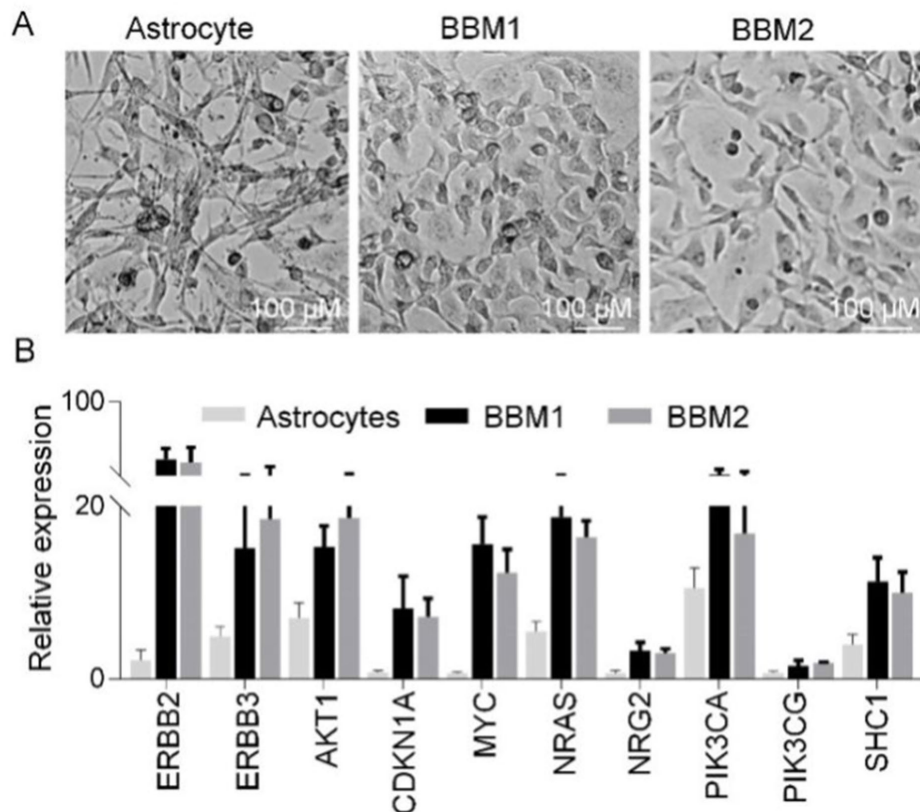


Figure 2. A: Bright field images of human Astrocytes and breast cancer brain metastasis 1 (BBM1) and 2 (BBM2) cells showing morphological differences between cell lines. Scale bars = 100 μm/L; B: real-time PCR analysis of genes enriched in Astrocytes and BBM (BBM1 and BBM2) cells ($n = 3$, $P < 0.05$). Error bars indicate SEM

Disruption of ERBB2 and the PIK3/AKT/mTOR signaling pathway induces apoptosis in BBM cells

Bioinformatics analysis of our sequence data, published data, and previous findings from our lab indicate MT-specific ERBB2-mediated activation of the PI3K-AKT/mTOR pathway [Figure 3A]. We found expression of ERBB2, PIK3CA, and MYC was 20, 8 and 4 fold, higher in HER2+ MT tissue compared to PT, indicating the potential function of the neural microenvironment in substantially increasing their expression. To confirm the function of ERBB2 on PI3K-AKT/mTOR signaling, we treated BBM1 cells with 50 nmol/L of the ERBB2 inhibitor Lapatinib or the PIK3CA inhibitor Idelalisib for 48 h. Western blot analysis of total protein extracts showed that Lapatinib and Idelalisib inhibited phosphorylation of ERBB2, PIK3CA, AKT, and mTOR [Figure 3B]. To confirm the effects of the inhibitors on cell viability, BBM1 cells were treated with various concentrations (0, 31.2, 62.5, 125, 250, 500, 1000 nmol/L) of Lapatinib and Idelalisib, as well as inhibitors of AKT (AZD5363) and mTOR phosphorylation (Rapamycin). Our analysis showed that treatment with each of the inhibitors suppressed cell viability [Figure 3C]. Lapatinib induced the highest inhibition of BBM1 cell proliferation with an IC_{50} value of 25.1 nmol/L at 72 h post-treatment.

Drug screen indicates that SRC, ERBB2, and GABA signaling pathways play critical roles in BBM1 cell proliferation

To identify novel clinical or preclinical drug candidates to target breast cancer brain metastasis, we performed a high throughput screening of 1,650 compounds. Preliminary screening was done by treating BBM1 cells with 1 μmol/L of each compound for 72 h. We identified 105 compounds that showed robust toxicity (at least 70% inhibition) against BBM1 cells [Figure 4A].

In a secondary screening, we analyzed the consistency and stability of the compounds against two HER2+ BBM cell lines (BBM1 and BBM2). Cells were treated with 50 nmol/L of each compound and analyzed over

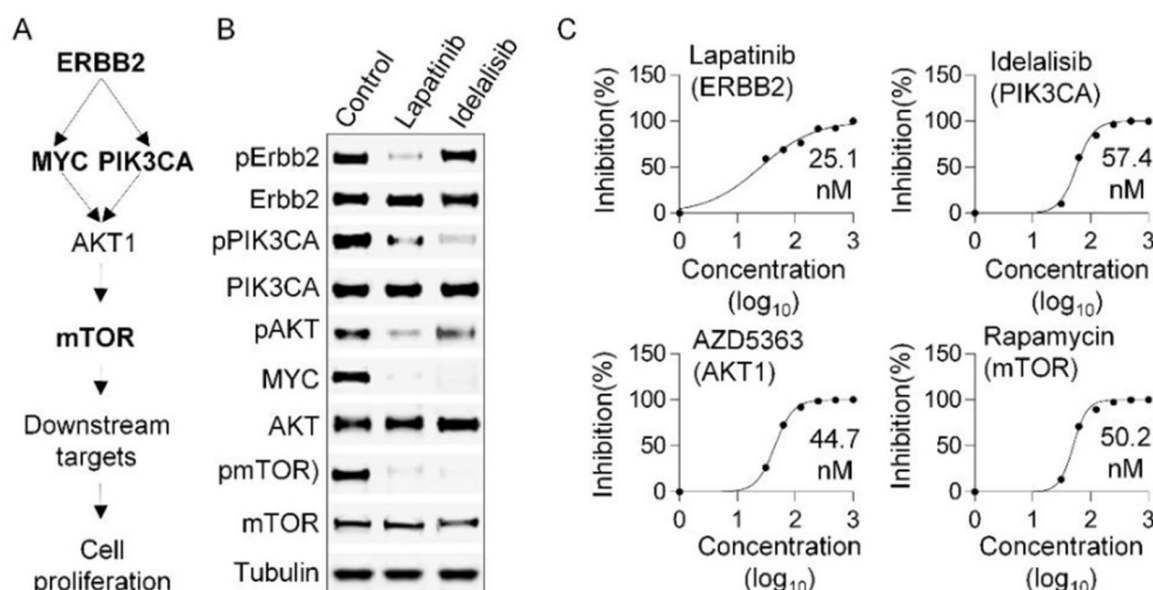


Figure 3. Disruption of ERBB2 and PI3K-AKT/mTOR signaling suppresses BBM cell survival. A: Schematic depicting the ERBB2/MYC/ AKT and PI3K-AKT/mTOR signaling pathways that affect BBM cell proliferation; B: cells were treated with ERBB2 (Lapatinib) and PI3K (Idelalisib) inhibitors at a final concentration of 100 nmol/L for 48 h. Total protein lysates from were analyzed using antibodies specific to ERBB2, pERBB2, PIK3CA, pPIK3CA, AKT, pAKT, MYC, mTOR, and pmTOR. Tubulin was used as a loading control; C: BBM1 cells were treated with pharmaceutical inhibitors of ERBB2 (Lapatinib), PI3K (Idelalisib), AKT (AZD5363), and mTOR (Rapamycin) at final concentrations 0, 31.2, 62.5, 125, 250, 500, 1000 nmol/L. Control cells were treated with DMSO only. The viability of the cells at 72 h post-treatment is shown. The IC₅₀ values of the compounds are in the bottom right of each plot

10 days. We identified 35 compounds that showed robust toxicity towards both BBM1 and BBM2 cell lines, throughout the study period [Supplementary Figure 3].

Further screening was done to identify compounds with BBM cell-specific toxicity using both BBM1 and human reactive astrocytes cells. We identified 12 compounds that were at least two-fold more toxic towards BBM1 cells than toward astrocytes [Figure 4B and C]. Finally, we analyzed concentration-dependent toxicity and identified 6 compounds that showed linear concentration-dependent suppression of BBM1 cell viability. IC₅₀ analysis revealed that Doxorubicin, a DNA-binding chemotherapeutic agent for multiple cancers, was the most toxic compound towards BBM1 cells. The SCR inhibitor (AC-93253; IC₅₀ = 85 nmol/L) and GABA receptor agonist (Isoguvacine; IC₅₀ = 99.2 nmol/L) showed greater toxicity than the Wnt agonist (CID11210285), sirtuin (SIRT) inhibitor (JDF00244), and leucine-rich repeat kinase-2 (LRRK2) inhibitor (GSK2578215A), which had IC₅₀ values ranging from 183 nmol/L to 304 nmol/L [Figure 4D]. Consistent with the drug screening data, our RNA-seq analysis indicated relatively higher expression of SRC in MT cells compared to nBrain and nBreast but not to PT [Supplementary Figure 4].

Combinatorial inhibition of ERBB2 with an SRC or PIK3CA inhibitor or a GABA agonist induces robust apoptosis in BBM1 cells

Bioinformatics analysis showed that SRC1 is an upstream regulator of ERBB2 and its targets, MYC and PIK3CA, and an activator of PI3K-AKT/mTOR signaling [Figure 5A], indicating that SRC1 potentially plays a critical role in BBM cell survival and proliferation. To confirm this function of SRC, we treated BBM1 cells with different inhibitors specific to SRC and its downstream targets for 48 h. Apoptosis analysis showed that these inhibitors induced robust apoptosis in BBM1 cells [Figure 5B]. Further confirmation of apoptosis was obtained via Western blot analysis of procaspase3 and cleaved caspase3 proteins in the treated cells [Figure 5C].

Consistent with the RNA-seq data, our compound screening results indicated that SRC-mediated activation of the PIK3-AKT/mTOR pathway plays a critical role in BBM cell survival and proliferation. To confirm the

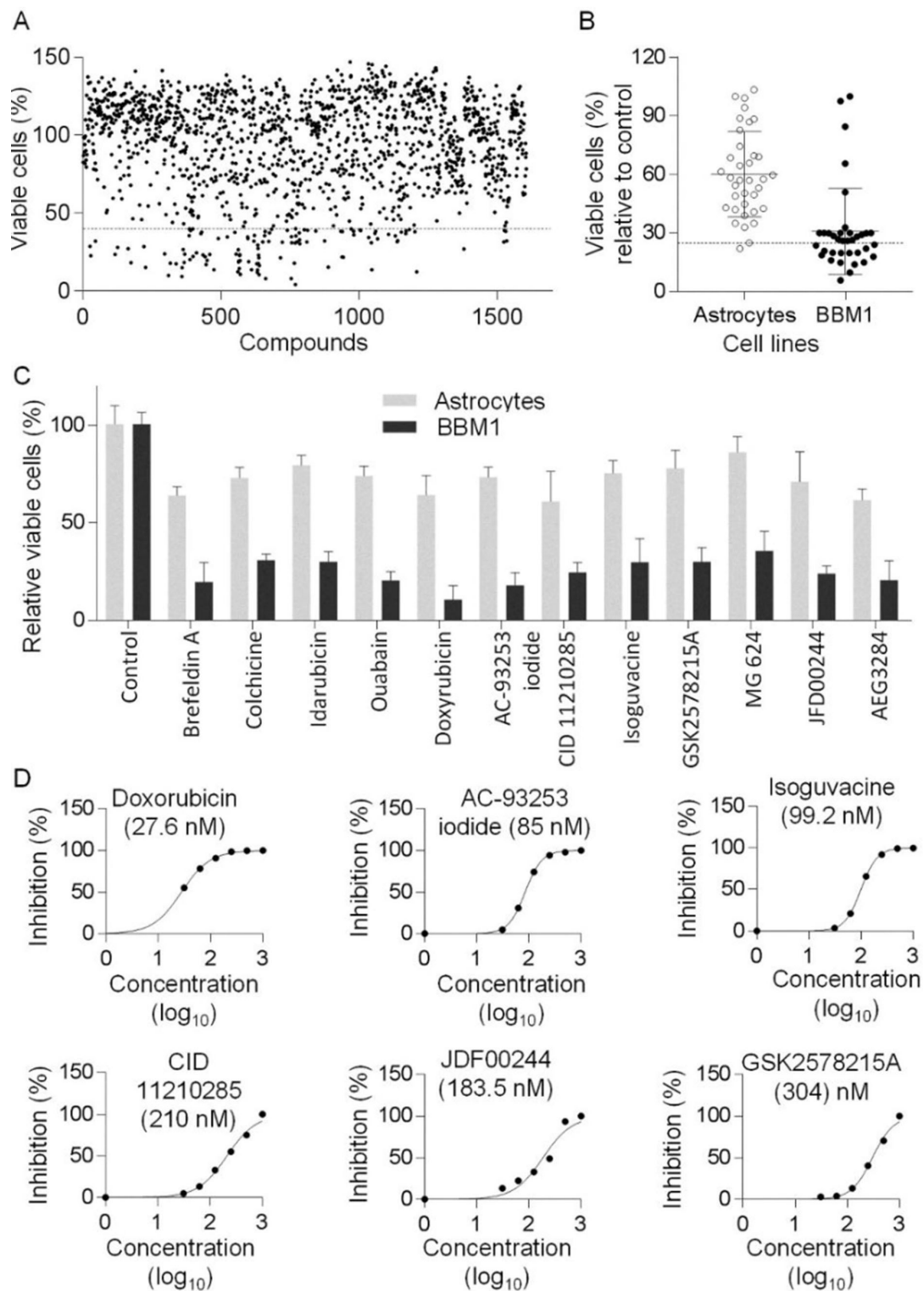


Figure 4. Screening of clinical and preclinical drugs to identify novel candidates targeting breast cancer brain metastases. A: Preliminary screening of 1,650 compounds for their efficacy against BBM1 cells. BBM1 cells were grown overnight prior to treatment with the compounds at a final concentration 1 $\mu\text{mol/L}$ for 48 or 72 h ($n = 2$). The percentages of viable cells in each treatment group relative to viable control cells treated with DMSO are shown for data collected after 72-h treatment. Compounds that suppressed viability below 70% compared to control (107 total, separated by a dashed line) were selected for secondary screening using both BBM1 and BBM2 cells lines; B, C: tertiary screening of the active compounds based on cell type-specific effects. Human astrocytes and BBM1 cells were grown overnight prior to treatment with the 35 active compounds identified in the secondary screening at a final concentration of 1 $\mu\text{mol/L}$ for 48 or 72 h ($n = 6$). The percentages of viable cells relative to viable control cells treated with DMSO are shown for data collected after 72-h treatment; C: relative viability of BBM1 cells treated with compounds (12) that reduced BBM1 cell viability by at least two-fold compared to that of astrocytes at a concentration of 1 $\mu\text{mol/L}$ for 72 h; D: concentration-dependent effects of six potent active compounds. BBM1 cells were grown overnight prior to treatment with the compounds at final concentrations ranging from 0, 31.2, 62.5, 125, 250, 500, 1000 nmol/L ($n = 8$). The viability of the cells at 72 h post-treatment is shown. The IC₅₀ values of the compounds are shown in parenthesis

SRC-mediated activation of PIK3/AKT/mTOR signaling, we treated BBM1 cells with 100 nmol/L of AC93253 for 48 h, followed by Western blot analysis of the total protein extract. Our analysis showed that inhibition of SRC leads to inhibition of PIK3CA and mTOR phosphorylation [Figure 5D]. As our analysis indicated that SRC is an upstream regulator of ERBB2 and the PIK3-AKT/mTOR pathway in BBM1 cells and that inhibition of both SRC and ERBB2 induces BBM1 cell death, we analyzed the combined effect of SRC and ERBB2 inhibitors on BBM1 cell viability. We treat the cells with 50 nmol/L of AC93253 and 50 nmol/L of Lapatinib, separately and in combination, for 48 h. Our analysis showed robust inhibition of BBM1 cells in the presence of either inhibitor alone, however, the effect was greatest in presence of both [Figure 5E]. Similar to combinatorial SRC/ERBB2 treatment, concurrent inhibition of ERBB2 and PIK3CA showed a significant additive inhibitory effect on BBM1 cell survival [Figure 5E].

We also evaluated the combinatorial treatment of ERBB2 and SRC inhibitors with a GABA agonist. BBM1 cells were treated with either 100 nmol/L of Isoguvacine alone or in combination with Lapatinib or AC93253 for 48 h. Cell viability and apoptosis analyses showed that the combination of Isoguvacine with both Lapatinib and AC93253 induced higher inhibition of cell viability compared to independent treatments [Figure 6A and B]. Our analysis showed that the expression of GABA receptors and subunits was significantly higher in nBrain compared to PT, MT, and nBreast [Figure 6C, Supplementary Figure 5].

DISCUSSION

Brain metastases are the most common and complicated central nervous system disease in adults. The incidence of brain metastases is increasing due to both improved diagnosis and increased cancer patient survival through advanced systemic treatments. Outcomes of patients remain disappointing and treatment options are limited, usually involving multimodality approaches of radiation and palliative chemotherapy. Brain metastases represent an unmet clinical challenge in caring for solid tumor, especially in breast cancer, where the incidence of brain metastases are frequent and that result in impaired quality of life.

BBMs are common in patients with the HER2-positive and TNBC breast cancer subtypes, and the natural course of BBM is strongly influenced by the biology of the primary tumor subtype. Although the biology of BBM according to tumor subtypes is still poorly understood, recent breakthroughs have been achieved in the identification of specific mediators of BBM and in the development of preclinical models for therapeutic studies. However, the use of established cell lines and comparative analysis of unrelated tissue samples often identify factors that eventually fail to represent as a key regulator in preclinical and clinical stage. To overcome this issue we obtained both primary and metastasis tissue from same individual patients (PT1/MT2 and PT2/MT2). Due to the inherent difficulty in the procurement of such tissue pairs we have used limited numbers of tissue samples. Our RNA-seq analysis revealed that ERBB2-mediated activation of PIK3CA and its downstream AKT/mTOR pathway plays a critical role in the survival and proliferation of breast to brain metastatic cells. Inhibition of ERBB2 or its downstream targets suppresses BBM cell viability to different degrees. The critical function of ERBB2 in HER2+ primary and metastatic breast cancer has been well established, and the ERBB2 inhibitor Lapatinib and trastuzumab have been used as chemotherapeutic agents targeting HER2+ breast cancer^[25-28]. In addition, our earlier studies revealed the contribution of neural factors, such as the astrocyte-secreted brain-derived neurotrophic factor, in the activation of HER2-TrkB signaling that leads to AKT1 activation and BBM1 cell proliferation^[25]. Several studies have also reported mutations and/or copy number changes in PIK3CA in hormone receptor-positive breast cancers^[29-31]. Indeed, targeting PIK3-AKT/mTOR signaling to inhibit breast cancer has been evaluated extensively, and there is phase I to III preclinical trial data demonstrating that inhibition of this pathway leads to regression of solid tumors and breast cancer^[32-37].

Recent gene expression analyses of BBM samples identified cyclooxygenase (COX)-2, EGFR ligands, and a sialyltransferase, as mediators of cancer cell passage through the BBB^[38]. In contrast to COX-2 and EGFR,

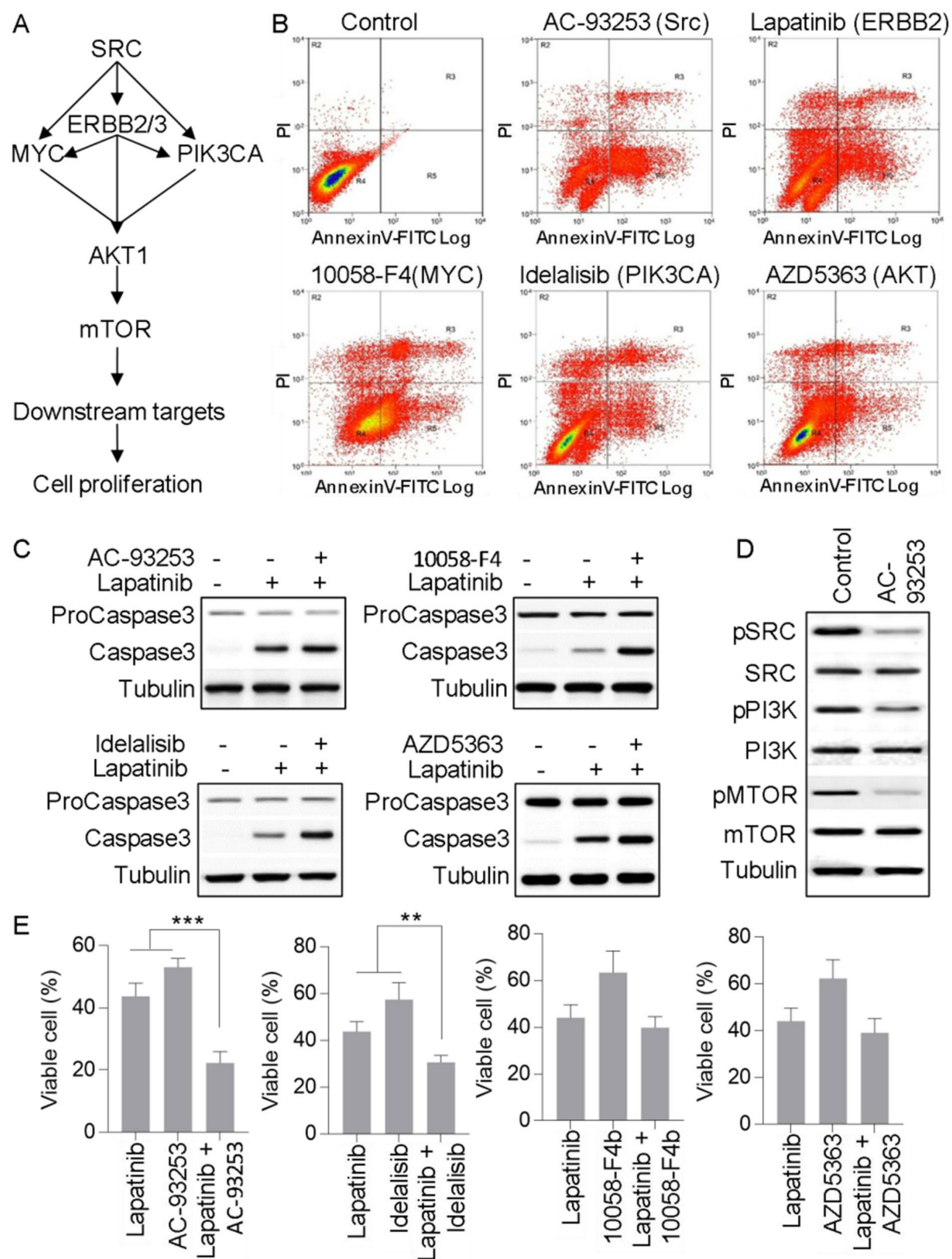


Figure 5. Inhibition of BBM1 cell proliferation by combinatorial treatment with the ERBB2 inhibitor Lapatinib and inhibitors of the PI3K-AKT/mTOR signaling pathway. **A:** Schematic depicting the functions of SRC, ERBB2, and PIK3CA in activating PI3K-AKT/mTOR signaling-mediated cell proliferation; **B:** effect of inhibition of SRC kinase, ERBB2, and PI3K-AKT/mTOR signaling on apoptosis induction in BBM1 cells. Cells were treated with inhibitors of SRC (AC-93253), ERBB2 (Lapatinib), MYC (10058-F4b), PIK3CA (Idelalisib), and AKT (AZD5363) at a final concentration of 100 nmol/L for 48 h. Control cells were treated with DMSO alone. Apoptosis induction was measured using Annexin V-FITC staining followed by FACS analysis; **C:** cells were treated with inhibitors (100 nmol/L) for 48 h, and total protein extracts were subjected to Western blot analysis using antibodies against ProCaspase3 (inactive) and cleaved Caspase3 (active). Tubulin was used as loading control; **D:** selective inhibition of the SRC kinase with AC-93253 disrupts PI3K-AKT/mTOR signaling. Cells were treated with AC-93253 (100 nmol/L) for 48 h, and total protein lysates from the cells were analyzed using antibodies specific to SRC, pSRC, PI3K, pPI3K, mTOR, and pmTOR. Tubulin was used as loading control; **E:** effect of the ERBB2 inhibitor Lapatinib alone and in combination with SRC, PIK3CA, MYC, and AKT inhibitors on BBM1 cell viability. Cells were treated with 50 nmol/L Lapatinib alone or in combination with AC-93253, Idelalisib, 10058-F4b, or AZD5363 at a final concentration of 50 nmol/L. The percentages of viable cells in each treatment group relative to viable control cells treated with DMSO are shown for data collected after 48 h. Error bars indicate SEM ($n = 3$, ** $P < 0.05$; *** $P < 0.01$)

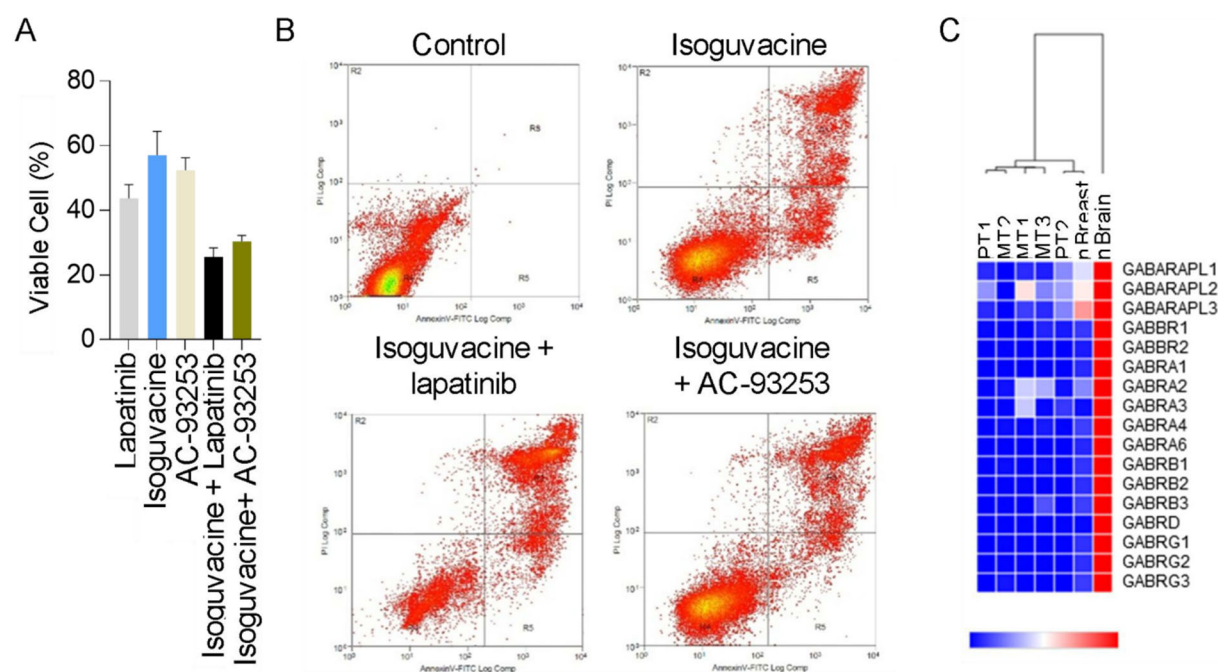


Figure 6. Inhibition of BBM1 cell proliferation upon treatment with a GABA agonist alone and in combination with ERBB2 or SRC inhibitors. A: BBM1 cells were treated with GABA agonist Isoguvacine, AC-93253, and/or Lapatinib at final concentrations of 100 nmol/L. The percentages of viable cells in each treatment group relative to viable control cells treated with DMSO are shown for data collected after 48 h ($n = 3$, $P < 0.05$). Error bars indicate SEM; B: the induction of apoptosis was measured using Annexin V-FITC staining followed by FACS analysis. Control cells were treated with DMSO alone; C: hierarchical clustering based on the expression GABA receptors and subunits in primary breast cancer (PT1-2), breast to brain metastatic tumor tissue (MT1-3), normal breast tissue (nBreast), and normal brain tissue (nBrain). Low expression (blue), high expression (red)

which are also linked to other organ metastases, aberrant expression of ST6GALNAC5 specifically mediated BBM, potentially by enhancing adhesion circulating cells to the CNS endothelium. Similarly, using larger cohort of patient samples metastasis suppressor KISS1 a prognostic marker for increased risk of breast cancer progression was identified^[39]. These concurrent genomic analysis indicates that an effective molecular target for complete remission of BBM is yet to be identified.

Supporting the RNA-seq data, our parallel screening of clinical and preclinical drug candidates identified ERBB2, PIK3CA, and their downstream targets as critical regulators of BBM cell proliferation. We identified SRC as an upstream regulator of ERBB2, PIK3CA, and MYC. Inhibition of each of SRC and ERBB2 alone or in combination with a GABA agonist-induced robust apoptosis in BBM1 cells.

The previous study also demonstrated that SRC is hyperactivated in brain-seeking breast cancer cells and that SRC activation promotes tumor cell extravasation into the brain parenchyma via permeabilization of the blood-brain barrier^[21]. Indeed, the preclinical study showed that SRC and SRC family kinases mediate intracellular signaling pathways that control key biologic/oncogenic processes in glioblastoma^[21]. In addition to neurotransmission and regulation of secretion, GABA through GABAA receptors negatively regulates proliferation of neural stem cells^[23]. GABAergic signaling and its control over proliferation have also emerged as critical in brain tumors^[40-42].

Indeed, previous studies from our group showed that human breast cancer metastases to the brain display GABAergic properties in the neural niche and metabolize GABA as an energy source^[40]. Consistent with previous findings on the inhibitory effects of GABA signaling on neural and embryonic stem cells^[43,44], our findings indicate that the attenuation of GABA signaling is potentially associated with the rapid proliferation of tumor cells in the brain. The activation of GABA signaling potentially reverses the availability of GABA as

an energy source for GABAergic BBM cells, leading to suppression of viability and proliferation. Our results indicate that combinatorial treatment with a GABA agonist and SRC or ERBB2 inhibitors is a potentially effective therapeutic approach targeting BBM. Finally, consistent with the drug screening data, aberrant Wnt signaling is a hallmark of many cancers. Dysregulation of canonical and non-canonical Wnt signaling was reported in triple-negative breast cancer^[45-47]. No previous studies have shown any relationship of SIRT and LRRK2 with HER2+ breast cancer brain metastasis. In conclusion, we have identified both molecular targets and active clinical/preclinical inhibitors to target breast cancer brain metastasis. In our future studies, we will evaluate the efficacy of these inhibitors in animal models *in vivo*.

CONCLUSION

The study reveals critical roles for SRC, ERBB2, PIK3CA, and GABA in the proliferation and survival of BBM cells and showed that SRC- and ERBB2-mediated activation of PIK3-AKT/mTOR signaling regulates BBM cell survival. Selective inhibition of these candidate genes alone or in combination induces robust apoptosis in BBM cells. In addition, the finding revealed that agonist-mediated activation of GABA signaling in combination with inhibition of SRC/ERBB2 signaling acts as an effective strategy to inhibit BBM cell proliferation. In future studies, we will analyze BBM cell-specific toxicity of the lead candidates alone or in combinations using larger numbers of BBM, primary breast cancer and glial cell lines. We will identify the candidates or combination with significantly higher BBM cell selective toxicity for preclinical evaluation using animal models. In conclusion, the findings of this study provide a rationale for further preclinical evaluation of SRC-targeting regimens in combination with ERBB2 inhibitors and/or GABA agonists to target breast cancer brain metastasis.

DECLARATIONS

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Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: Ansari SR, Jandial Z, Wu X, Liu X, Ansari KI
Performed data acquisition, as well as providing administrative, technical, and material support: Ansari SR, Jandial Z, Chen MY, Ansari KI

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

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Review

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Contribution of alternative splicing to breast cancer metastasis

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Abstract

Alternative splicing is a major contributor to transcriptome and proteome diversity in eukaryotes. Comparing to normal samples, about 30% more alternative splicing events were recently identified in 32 cancer types included in The Cancer Genome Atlas database. Some alternative splicing isoforms and their encoded proteins contribute to specific cancer hallmarks. In this review, we will discuss recent progress regarding the contributions of alternative splicing to breast cancer metastasis. We plan to dissect the role of MTDH, CD44 and their interaction with other mRNA splicing factors. We believe an in-depth understanding of the mechanism underlying the contribution of splicing to breast cancer metastasis will provide novel strategies to the management of breast cancer.

Keywords: Breast cancer, metastasis, CD44, MTDH, splicing, epithelial-mesenchymal transition

INTRODUCTION

Breast cancer is the most common type of cancer among women. Despite emerging new treatments such as PARP inhibition and immune checkpoint blockade, it remains a major challenge^[1,2] and is the primary cause of cancer mortality in women. In the majority of cases, the death from breast cancer is not due to the primary tumor per se, but rather the result of metastasis to other organs in the body^[3]. Metastasis is a multi-step process involving stromal invasion, cell migration, intravasation, anoikis resistance, extravasation and



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subsequent implantation and proliferation in sites other than the primary location^[4]. Although we have gained ample knowledge in this cellular process, an in-depth understanding at the molecular level remains to be deciphered.

Alternative splicing might be such a molecular mechanism that contributes to metastasis. It is a process whereby multiple functionally distinct transcripts are encoded from a single gene by the selective removal or retention of exons and/or introns from the maturing RNA. This process is highly regulated, involving trans-acting splicing factors and cis-acting regulatory motifs and so is susceptible to hereditary and somatic mutations. Alternative splicing is common in many eukaryote lineages. Using deep transcriptome sequencing of the human genome, over 95% of multi-exon genes were found capable of producing at least one alternatively spliced isoform^[5]. Many single-gene studies have also characterized the role of alternative splicing in various cellular processes. Disruption or dysregulation of alternative splicing has also been associated with pathological states^[6,7]. Maguire *et al.*^[8] demonstrated that spliceosomal mutations occur in a mutually exclusive manner in breast cancer and that distinct components of the spliceosome are targeted by somatic mutations in different types of breast cancer. The exact splicing pattern associated with a particular breast cancer type or stage still requires a broad characterization through molecular analysis of splicing isoforms in different patients. However, existing evidence strongly supports a pivotal role of alternative splicing in breast cancer biology and innovative tools are under development to use splicing events for diagnostic and therapeutic purposes^[9]. Shapiro *et al.*^[10] observed an epithelial-mesenchymal transition (EMT)-associated global change in alternative splicing of a number of genes that are involved in functions crucial for EMT progression, such as cell adhesion, cell motility, and cytoskeletal remodeling. Several of the splicing changes discovered *in vitro* were also found to occur in a panel of breast cancer cell lines and *in vivo* in primary human breast cancer samples. Dysregulation of alternative splicing has been increasingly recognized in cancer-related pathways. It is thus critical to investigate the functional significance of splicing regulation in the context of cancer. This review will discuss some recent progresses about the alternative splicing regulators such as CD44, heterogeneous nuclear ribonucleoprotein M (hnRNPM), SND1 and MTDH *etc.* in breast cancer metastasis.

SPlicing VARIANTS MAY ENHANCE EMT AND METASTASIS IN BREAST CANCER

Dorman *et al.*^[12] reported splicing defects in large-scale breast cancer sequencing studies. Nine hundred and eighty-eight splicing variants including exon skipping, leaky or cryptic splicing from 5,206 putative mutations were confirmed for splicing mutations in 442 Breast Cancer patients from The Cancer Genome Atlas dataset. These splicing variants were significantly increased in patients with lymph node metastasis, but not in lymph node-negative tumors. Silipo *et al.*^[13] reported that the expression profile changes of splicing factors including serine/arginine-rich splicing factor 1 (SRSF1), SRSF2, SRSF3, SRSF5, SRSF6 and SRSF10; the heterogeneous nuclear ribonucleoproteins (hnRNPs) including hnRNP A2/B1, hnRNPI, hnRNPA1 and hnRNP K; as well as eight RNA-binding proteins including HuR, Sam68, BRM5, FOX2, YB-1, PRMT6, SPF45 and PELP1 in breast cancer cells compared with normal cells, which are strongly associated with the alternative splicing pattern of many cancer-related genes despite the absence of mutations in genomic DNA. Inoue *et al.*^[14] reported aberrant splicing of CD44 gene in breast cancer, which promotes metastasis. The status of splicing factors and other splicing-related proteins in breast cancer are important to provide insights into the mechanisms that lead to breast cancer metastasis^[15]. The correlation analysis of somatic variants with alternative splicing events confirmed known trans- associations with variants in SF3B1 and U2AF1, and additional trans-acting variants (e.g., TADA1, PPP2R1A). Tumors have up to 30% more alternative splicing events than normal samples. Many tumors have thousands of alternative splicing events that are not detectable in normal samples. On average, 930 exon-exon junctions (“neojunctions”) were identified in tumors not typically found in normal tissue included in the Genotype-Tissue Expression (GTEx) project^[15]. CD44 is a cell surface protein with various isoforms that involves in motility, cell survival and proliferation

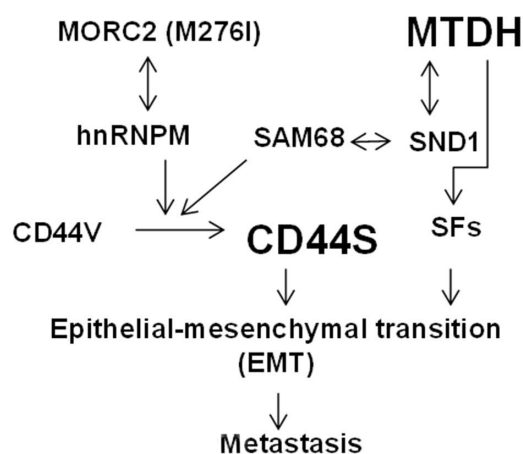


Figure 1. Mechanisms of alternative splicing of CD44 in epithelial-mesenchymal transition and breast cancer metastasis were summarized

and the formation of tumor microenvironment. Alternative splicing can produce various isoforms of CD44 with properties at different specific tissue^[16,17]. The RNA-binding protein hnRNPM was found to promote breast cancer metastasis by activating the switch of alternative splicing during EMT.

HNRNPM INCREASES CD44 ALTERNATIVE SPLICING TO ENHANCE BREAST CANCER METASTASIS

CD44 was identified as a key downstream target of hnRNPM by genome-wide deep sequencing analysis. hnRNPM is associated with increased standard form of CD44 (or CD44 standard, CD44s) in aggressive breast cancer patient specimens. Overexpressed hnRNPM competes with epithelial splicing regulatory protein 1 (ESRP1), and binds to the same cis-regulatory RNA elements of CD44 for the precisely control CD44s splice isoform switching during EMT^[18]. ESRP1 is a splicing regulator to promote an epithelial splicing program and hnRNPM is a mesenchymal splicing regulator. Harvey *et al.*^[19] reported that hnRNPM and ESRP1 co-regulate a set of cassette exon events in EMT genes associated with cell migration and cytoskeletal reorganization. Competitive binding to these cis-elements by hnRNPM and ESRP1 to antagonize alternative splicing was proposed. The expression of hnRNPM is closely correlated with invasion and metastasis of tumor cells. hnRNPM expression was upregulated in breast cancer tissues. HnRNPM and CD44s expression are positively correlated in breast cancer tissues. Cancer stem cells marker ALDH1+ was found positively associated with overexpression of CD44s and hnRNPM. High hnRNPM is associated with higher levels of CD44s, shorter overall survival and higher rates of lymph node metastases in breast cancer patients^[20].

MORC2-MUTANT M276I PROMOTES AN HNRNPM-MEDIATED CD44 ALTERNATIVE SPLICING TO ENHANCE BREAST CANCER METASTASIS

A cancer-associated Microorchidia family CW-type zinc finger 2 (MORC2) (M276I) mutant was reported to promote metastatic ability of TNBC cancer cells by enhancing interaction with hnRNPM and splicing switch of CD44 from the epithelial isoform (CD44v) to the mesenchymal isoform (CD44s) [Figure 1]. Expression of mutant MORC2 in TNBC cells increased cell migration, invasion, and lung metastasis. The M276I mutation enhanced binding of MORC2 to hnRNPM, a component of the spliceosome machinery. This interaction promoted an hnRNPM-mediated splicing switch of CD44 from CD44v to CD44s, ultimately driving EMT. Knockdown of hnRNPM reduced the binding of mutant MORC2 to CD44 pre-mRNA and reversed the mutant MORC2-induced CD44 splicing switch and EMT. As a consequence, the migratory, invasive, and lung metastatic potential of mutant MORC2-expressing cells was impaired^[21].

Table 1. List of mRNAs encoding for mRNA splicing factors identified by MTDH PAR-CLIP or HITS-CLIP

Ref Seq#	Gene	#PAR-CLIP	Ref Seq#	Gene	#PAR-CLIP
NM_006924	<i>SRSF1</i>	16	NM_004698	<i>PRPF3</i>	6
NM_003016	<i>SRSF2</i>	14	NM_014502	<i>PRPF19</i>	11
NM_003017	<i>SRSF3*</i>	7	NM_015629	<i>PRPF31</i>	3
NM_005626	<i>SRSF4</i>	8	NM_006109	<i>PRMT5</i>	7
NM_001039465	<i>SRSF5</i>	9	NM_014706	<i>SART3</i>	8
NM_006275	<i>SRSF6</i>	13	NM_018047	<i>RBM22</i>	5
NM_001031684	<i>SRSF7</i>	5	NM_012321	<i>LSM4</i>	5
NM_003769	<i>SRSF9</i>	7	NM_000344	<i>SMN1</i>	1
NM_054016	<i>SRSF10</i>	5	NM_015721	<i>GEMIN4</i>	11
NM_001190987	<i>SRSF11</i>	10	NM_015465	<i>GEMIN5</i>	4
NM_003090	<i>SNRPA1</i>	5	NM_024707	<i>GEMIN7</i>	3
NM_198216	<i>SNRPB*</i>	4	NM_024707	<i>HNRNPA1*</i>	7
NM_003093	<i>SNRPC</i>	5	NM_031157	<i>HNRNPA2B1*</i>	12
NM_006938	<i>SNRPD1</i>	3	NM_031243	<i>HNRNPA3*</i>	29
NM_004597	<i>SNRPD2*</i>	5	NM_194247	<i>HNRNPH3*</i>	10
NM_004175	<i>SNRPD3</i>	11	NM_012207	<i>HNRNPK*</i>	10
NM_003094	<i>SNRPE</i>	3	NM_002140	<i>HNRNPL*</i>	6
NM_003095	<i>SNRPF</i>	2	NM_001005335	<i>HNRPU*</i>	17
NM_003096	<i>SNRPG</i>	3	NM_004501	<i>HNRNPUL2*</i>	14
NM_022805	<i>SNRPIN</i>	4	NM_001079559	<i>PCBP1*</i>	10

*MTDH interacting mRNAs identified in both High-throughput sequencing of RNA isolated by Cross-linking immunoprecipitation (HITS-CLIP) and Photoactivatable Ribonucleoside-Enhanced Cross-linking and Immunoprecipitation (PAR-CLIP)

TDP43, CPEB2A/B AND ESRP1/RBFOX2 IN BREAST CANCER METASTASIS

Ke *et al.*^[22] reported that the loss of TDP43 (TAR DNA-binding protein 43), an important splicing regulator involved in the SRSF3 regulated unique splicing of downstream gene PAR3, promotes metastasis in TNBC. Highly expressed TDP43 is correlated with poor prognosis in TNBC. Knockdown of TDP43 inhibits SRSF3 and PAR3 mediated metastasis. Two CPEB2 splicing isoforms with or without exon 4 was reported to mediate opposing effects on cancer-related phenotypes. The CPEB2A isoform, which is produced by exclusion of exon 4 from the mature CPEB2 mRNA, inhibited tumor growth. The CPEB2B splicing isoform with the inclusion of exon 4 into the mature CPEB2 mRNA was overexpressed in aggressive forms of human breast cancer and enhanced cancer metastasis was observed^[23]. CPEB2A/B promotes the translation of two critical downstream proteins TWIST1 and HIF1a in the hypoxia/EMT pathway^[23]. Splicing factor ratio might be an index of EMT and tumor aggressiveness in breast cancer. In fact, the association of low ESRP1/RBFOX2 ratio with high risk of metastasis in early breast cancer was speculated to be a new early prognostic marker of breast cancer metastasis^[24].

RNA BINDING PROTEIN RBM47 INHIBITS BREAST CANCER METASTASIS BY REGULATING SPLICING

RNA binding motif protein 47 (RBM47) was identified as a suppressor of breast cancer metastasis through analysis of clinical breast cancer gene expression datasets, cell line models, and mutation data. Transcriptome-wide HITS-CLIP analysis revealed widespread mRNAs associated with RBM47 by binding to their introns and 3'UTRs. The dickkopf WNT signaling pathway inhibitor 1 (DKK1) is one of downstream mRNAs of RBM47. RBM47 inhibits breast cancer metastasis by increasing stability of the Wnt antagonist DKK1^[25].

NON-CODING RNA REST-003 PROMOTES BREAST CANCER METASTASIS

Non-coding RNAs (ncRNAs) RE1-silencing transcription factor (REST)-003 was reported to promote breast cancer metastasis. REST-003 is cRNAs derived from the first exon of an alternatively spliced REST transcript processed by serine/arginine repeat-related protein SRRM3. REST is a transcription factor to regulate expression of genes important for neuronal development. Interestingly, SRRM3 expression is repressed by REST^[26].

MTDH PROMOTES BREAST CANCER METASTASIS PARTIALLY BY REGULATING ALTERNATIVE SPLICING

High metadherin gene expression was highly correlated with breast cancer metastasis^[27,28]. MTDH was identified to bind to the vasculature of the lung by phage display screening cDNAs from metastatic breast carcinoma^[27]. Experimental metastasis can be inhibited by metadherin specific antibody or siRNAs. Hu et al reported that MTDH drives breast cancer metastasis to the lungs by increasing adhesion to the walls of blood vessels^[28]. Amplification of a minimal 2.9 Mb piece of chromosome 8q22 was identified in poor-prognosis breast cancers by ACE (analysis of CNAs by expression data) and fluorescence in situ hybridization (FISH) analysis. Only the enforced expression of MTDH in this amplified 8q22 region was identified to increase lung seeding after tail vein injection of the mildly metastatic breast cancer cell line MDA-MB-231^[28]. Interaction of MTDH with Staphylococcal nuclease domain-containing 1 (SND1) was independently identified by mass spectrometry (MS) by three labs^[29-31]. Overexpression of MTDH and SND1 in primary tumors is strongly associated with reduced metastasis-free survival in multiple large-scale datasets of breast cancer patients^[32,33]. SND1 acts as a novel alternative splicing regulator by interacting with SAM68 to regulate exon v5 inclusion in the CD44 mRNA splicing that promotes cancer metastasis [Figure 1]^[34-36]. Several other splicing regulators including hnRNPA0, hnRNPA2B1, hnRNPF, hnRNPA3 isoform were also identified by MS in MTDH pull-down assay^[29]. High-throughput sequencing of RNA isolated by Cross-linking immunoprecipitation (HITS-CLIP) and Photoactivatable Ribonucleoside-Enhanced Cross-linking and Immunoprecipitation (PAR-CLIP) were recently developed methods to study RNA-protein interactions^[37,38]. As shown in Table 1, mRNAs encoding for mRNA splicing regulators were identified in MTDH RNA interactome for multiple times at different sites by MTDH antibody specific PAR-CLIP and 12 splicing factors were confirmed by MTDH HITS-CLIP^[39]. Therefore, MTDH may promote breast cancer metastasis by regulating mRNA splicing through interacting with mRNAs or proteins of splicing factors.

CONCLUSION

Increased expression of mRNAs alternative splicing isoforms derived from alteration of splicing factors and MTDH expression could promote EMT and breast cancer metastasis, which provides new targets for breast cancer therapy.

DECLARATIONS

Authors' contributions

Conception and elaboration of the work: Meng X, Yang S, Zhang J, Yu H

Provided administrative, technical, and material support: Meng X, Yang S, Zhang J, Yu H

Final approval of the version: Meng X, Yang S, Zhang J, Yu H

Availability of data and materials

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

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Original Article

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PIM-1 inhibition with AZD1208 to prevent osimertinib-induced resistance in EGFR-mutation positive non-small cell lung cancer

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Abstract

Aim: The progression free survival of non-small cell lung cancer (NSCLC) patients has been doubled over the last years, but still single epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) lead to incomplete responses. Compensatory signaling pathways are activated upon single EGFR TKIs. We have shown that compounds, which inhibit these pathways, are synergistic with EGFR TKIs. Proviral integration site for Moloney murine leukemia virus (PIM) has been connected to cancer therapy resistance, being involved in receptor tyrosine kinase, signal transducer and activator of transcription 3 (STAT3) and interleukin-6 signaling. We hypothesized that combined PIM and EGFR inhibition may improve the upfront therapy of EGFR-mutation positive NSCLC.

Methods: We reviewed the literature on PIM kinases, and performed cell viability assays, gene expression analyses, and immunoblotting experiments to reveal the mechanisms of action of the PIM inhibitor (AZD1208) alone and combined with osimertinib in five EGFR-mutation positive NSCLC cell lines.

Results: Osimertinib alone induced the activation of signal transducer and activator of transcription 3 (STAT3) as well as other signaling nodes. Combined osimertinib and AZD1208 yielded moderate synergistic effects in all



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NSCLC cell lines investigated. Among the EGFR-mutation positive cell lines examined, the H1975 and PC9 cell lines had the highest PIM1 and STAT3 mRNA expression. The combination decreased the osimertinib-induced STAT3 phosphorylation.

Conclusion: This study provides a short review on PIM kinases, and shows our results of combined PIM and EGFR inhibition in EGFR-mutation positive NSCLC cell lines. The combination was moderately synergistic but decreased STAT3 phosphorylation, an important signaling node in therapy resistance.

Keywords: Non-small cell lung cancer, EGFR, PIM-1, AZD1208, tyrosine kinase inhibitors

INTRODUCTION

Activation of the epidermal growth factor receptor (EGFR) leads to downstream induction of signaling pathways, including phosphatidylinositol 3-kinase/AKT, Ras/mitogen-activated protein kinase and Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) [Figure 1]. Consequently, these pathways stimulate cell proliferation, cell motility, cell cycle progression and cell survival^[1,2]. EGFR mutations were discovered in 2004^[1]. This finding has radically changed the therapy of EGFR-mutation positive non-small cell lung cancer (NSCLC) patients, replacing standard chemotherapy with EGFR tyrosine kinase inhibitors (TKIs)^[1,3]. Treatment with first-, second-, and third-generation EGFR TKIs has significantly improved the progression free survival of NSCLC patients, harboring EGFR mutations^[4]. The third-generation EGFR TKI, osimertinib is now the standard first-line therapy of these patients^[5]. However, although initially effective, monotherapy with EGFR TKIs, including osimertinib, triggers intrinsic or acquired resistance pathways^[6-8], and the patients eventually succumb to their disease.

Signaling nodes, including signal transducer and activator of transcription 3 (STAT3) and Src/Yes-associated protein 1 (YAP1), are not abrogated or even activated upon EGFR blockade. Moreover, besides EGFR, other receptor tyrosine kinases (RTKs) are present and induce downstream signaling, independent of EGFR inhibition^[9-13]. We have demonstrated that simultaneous inhibition of EGFR, STAT3 and Src-YAP1 was highly synergistic *in vitro* and *in vivo*. Moreover, multiple RTKs and non-RTKs were up regulated at baseline or after treatment with EGFR TKIs, limiting their efficacy^[6-8]. Proviral integration site for Moloney murine leukemia virus-1 (PIM-1) is a serine/threonine kinase, involved in cell cycle progression, cell growth, cell survival and therapy resistance. PIM-1 is activated in various types of cancer, amongst which prostate^[14-16], colorectal^[15,17-19], triple negative breast cancer (TNBC)^[20,21], hematologic malignancies^[22-24], neuroblastoma^[25] and lung cancer^[26]. For this reason, PIM kinases could provide a common target for the treatment of diverse malignancies.

PIM activation is induced by changes in the tumor microenvironment, such as hypoxia, and causes resistance to antiangiogenic drugs^[15]. The combination of PIM inhibitors with antiangiogenic compounds, down regulates the expression of hypoxia inducible factor 1 (HIF1) and decreases tumor cell proliferation, the formation of tumor vasculature and the ability of the cells to metastasize^[15]. A second study has also shown the involvement of PIM kinases in therapy resistance through hypoxia. Although the authors in this study propose a HIF1-independent, nuclear factor-erythroid 2 p45-related factor 2 induced hypoxia, PIM kinase inhibitors are again the solution to prevent therapy resistance and induce cell death in different tumor types^[27].

PIM-1 is involved in RTKs, STAT3 and interleukin-6 (IL-6) signaling [Figure 1]. Specifically, STAT proteins bind to the promoter of the *PIM-1* gene, and increase PIM-1 expression. A feedback loop, in turn, regulates the expression of STAT proteins^[14,18,21,28,29]. Considering the fact that there are no approved STAT3 inhibitors, the combination of EGFR TKIs with a PIM-1 inhibitor could be of interest to block STAT3

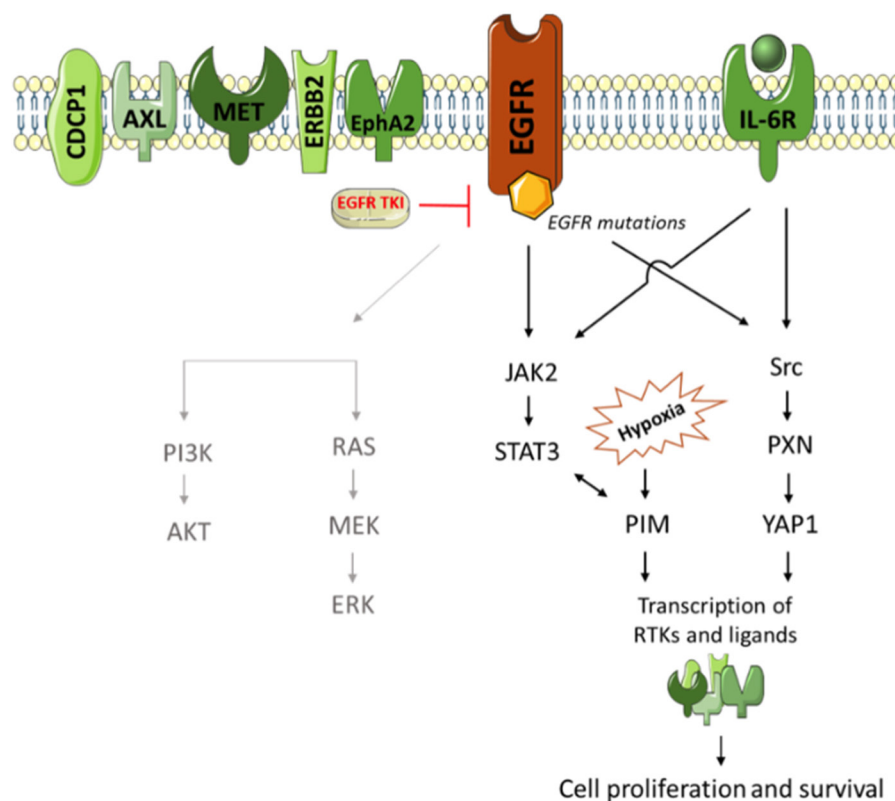


Figure 1. We present our initial literature-based model of EGFR-mutation positive NSCLC tumor cells, treated with single TKIs, and the parallel compensatory pathways that are being activated. Upon TKI treatment the downstream PI3K/AKT and RAS/MAPK pathways - in light grey - are inhibited, limiting the ability of the cells for proliferation and survival. However, our previous work has shown that this inhibition is the direct cause of the activation of the JAK2/STAT3 and Src-YAP1 pathways, and cannot abrogate coexpressed RTKs (e.g., MET, CDCP1) on the cell membrane. This in turn causes increased cell proliferation and survival, and thus leads to therapy resistance. PIM is an important signaling node in this network, potentially activated through IL-6/STAT3 and/or hypoxia. PIM was shown to be activated in multiple tumor types, and may therefore provide a druggable target for various tumor types. EGFR: epidermal growth factor receptor; NSCLC: non-small cell lung cancer; TKI: tyrosine kinase inhibitor; PI3K: phosphatidylinositol 3-kinase; MAPK: mitogen-activated protein kinase; JAK2: Janus kinase 2; STAT3: signal transducer and activator of transcription 3; YAP1: Yes-associated protein 1; RTK: receptor tyrosine kinase; PIM: proviral integration site for Moloney murine leukemia virus; IL-6R: interleukin 6 receptor; CDCP1: CUB domain-containing protein 1; EphA2: EPH receptor A2; PXN: Paxillin

activation. In prostate and gastric cancer, the efficacy of AKT inhibitors is attenuated by PIM-1 signaling, which upregulates the expression of MET, ERBB2 and other RTKs. Combined AKT and PIM-1 inhibition is synergistic in preclinical models^[16,30]. Similarly, in the activated B cell-like (ABC) subtype of diffuse large B cell lymphoma (DLBCL), the efficacy of the Bruton's tyrosine kinase inhibitor ibrutinib, was attenuated in the presence of PIM-1 mutations. In addition, chronic exposure of an ABC-DLBCL cell line to ibrutinib increased PIM-1 expression, and the combination of ibrutinib with the pan-PIM inhibitor (AZD1208) showed synergistic effects^[24]. Finally, in MET amplified NSCLC and gastric cancer cell lines, PIM-1 was identified as a mechanism of resistance to MET inhibitors. Treatment of MET inhibitor-resistant cells with PIM inhibitors restored sensitivity to MET inhibition, both in culture and in xenograft models^[26].

Several PIM inhibitors have been developed, and can be categorized into first- and next generation inhibitors. AZD1208 falls in the latter category, and inhibits all isoforms of the PIM kinase (1, 2 and 3). AZD1208 has been shown to improve outcome to radiation therapy in castrate-resistant prostate cancer cells^[14]. Moreover, PIM-1 levels were correlated to MET expression in both cell lines and patient-derived tumor samples, driving migration and invasion of cancer cells. Patient-derived tissues from a phase 1 clinical trial (ClinicalTrials.gov Identifier: NCT01489722), exploring the safety, tolerability, pharmacokinetics and efficacy of AZD1208 in acute myeloid leukemia, confirmed the mechanism of action of PIM-1 through MET regulation^[31].

The ability of AZD1208 to induce cell cycle arrest and apoptosis by inhibition of all PIM kinase isoforms indicates its potential as an anticancer treatment. Previous publications have shown that PIM inhibition augments the efficacy of targeted therapies in diverse tumor types. Therefore, dual PIM-1 and EGFR inhibition might modulate cell survival pathways and block progression and growth of cancer cells, by preventing the EGFR TKI-induced STAT3 and RTKs activation in EGFR-mutation positive NSCLC cell lines^[6-8]. This research aims to evaluate whether the pan-PIM inhibitor AZD1208 improves the efficacy of osimertinib in EGFR-mutation positive cell lines.

METHODS

Chemicals and reagents

Human lung adenocarcinoma PC-9 cells, harboring EGFR exon 19 deletion and 11-18 cells, harboring EGFR exon 21 L858R mutation were provided by F. Hoffmann-La Roche Ltd. (Basel, Switzerland), and by Dr. Mayumi Ono, (Kyushu University, Fukuoka, Japan), respectively. EGFR exon 19 deletion positive HCC4006 and HCC827 cells were purchased from the American Type Culture Collection (ATCC). The H1975 cell line, harboring both EGFR exon 21 L858R and resistant T790M mutation as well as the TNBC MBA-MB-231 cell line were purchased from ATCC. All cell lines were maintained in RPMI (Roswell Park Memorial Institute medium) 1640 supplemented with 1% penicillin/streptomycin/glutamine (Gibco) and 10% fetal bovine serum (FBS; Gibco) in a 5% CO₂ 37 °C cell culture incubator and routinely evaluated for mycoplasma contamination.

The pan-PIM inhibitor, AZD1208, and osimertinib were bought from Selleck Chemicals (Houston, TX, USA). Drugs were prepared in dimethylsulfoxide (DMSO) at a concentration of 10-100 mmol/L stock solutions and stored at -20 °C. Further dilutions were made in culture medium to final concentration before use. All antibodies used in our study, including dilution and company catalog number can be found in [Supplementary Table 1](#). All primers used can be found in [Supplementary Table 2](#).

Cell viability assay

Cells were seeded in 96-well plates at the following densities: 1.5×10^3 (PC9), 2.0×10^3 (H1975) 3.0×10^3 (HCC4006, HCC827 and 11-18), and incubated for 24 h, as previously described^[6]. For the combined treatment cells were seeded in 96-well plates at the following densities: 1×10^3 (PC9 and H1975), 1.5×10^3 (HCC827, HCC4006 and 11-18), and incubated for 24 h. Cell viability was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma Aldrich, St Louis, MO, USA). Cells were treated with serial dilutions of the drugs. For the half maximal inhibitory concentration (IC₅₀) determination, AZD1208 doses ranged from 0-200 µmol/L and treatment lasted 72 h. For the MTT viability assays with a combination of AZD1208 and osimertinib (IC₅₀s were previously obtained) drug doses were as follows: PC9, H1975, HCC4006, HCC827 cells were treated with osimertinib ranging from 0-100 nmol/L, and AZD1208 ranging from 0-100 µmol/L, or with the combination of both. The 11-18 cells were treated with osimertinib ranging from 0-200 nmol/L, and AZD1208 ranging from 0-100 µmol/L, or with the combination of both. The combined treatment lasted 96 h. After treatment incubation, 0.5 mg/mL of MTT reagent was added to the medium in the wells for 2 h at 37 °C and formazan crystals in viable cells were solubilized with 100 µL DMSO and spectrophotometrically quantified using a microplate reader (Varioskan Flash; Thermo Fisher Scientific, Waltham, MA, USA) at 565 nm of absorbance. Fractional survival was then calculated as percentage to control cells. Data of combined drug effects were subsequently analyzed by the Chou and Talalay method^[32,33]. Combination index (CI) values < 1, = 1 and > 1 indicated synergism, additive effect and antagonism, respectively.

Western blotting

For immunoblotting experiments, 1.5 million cells were seeded in T75 flasks (Starstedt, Newton, USA). The next day, PC9 and H1975 cells were treated with 24 nmol/L osimertinib, 5 µmol/L AZD1208 and

a combination of both. Untreated cells received an equivalent dose of vehicle (DMSO). After 24 h cells were washed with cold PBS and re-suspended in ice-cold radioimmunoprecipitation assay buffer (50 mmol/L Tris- hydrochloric acid in pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mmol/L sodium chloride, 1 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L sodium vanadate and 50 mmol/L sodium fluoride) containing protease inhibitor mixture (Roche Applied Science, Penzberg, Germany). Following cell lysis by sonication and centrifugation at $18620 \times g$ for 10 min at 4 °C, the resulting supernatant was collected as the total cell lysate. Briefly, the lysates containing 45 µg proteins were electrophoresed on 10% SDS-polyacrylamide gels (Life Technologies, Carlsbad, CA, USA) and transferred to polyvinylidene difluoride membranes (Bio-Rad laboratories Inc., Hercules, CA, USA). Membranes were blocked in Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE, USA). All target proteins were immunoblotted with appropriate primary and horseradish peroxidase (HRP)-conjugated secondary antibodies. Chemiluminescent (HRP-conjugated) bands were detected in a ChemiDoc MP Imaging System (Bio-Rad laboratories Inc.). β -actin was used as an internal control to confirm equal gel loading.

Quantitative real time polymerase chain reaction

RNA was isolated from cell lines using phenol-chloroform-isoamyl alcohol, followed by precipitation with isopropanol in the presence of glycogen and sodium acetate. RNA was re-suspended in water and treated with DNase I to avoid DNA contamination. Complementary DNA (cDNA) was synthesized using moloney murine leukaemia virus reverse transcriptase enzyme. cDNA was added to Taqman Universal Master Mix (Applied Biosystems, Carlsbad, CA, USA) in a 12.5 µL reaction with specific primers and probe for each gene. The primer and probe sets were designed using Primer Express 3.0 Software (Applied Biosystems) according to their Ref Seq (<http://www.ncbi.nlm.nih.gov/LocusLink>). Gene-specific primers are provided in Chemicals and reagents. Quantification of gene expression was performed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) and was calculated according to the comparative Ct method. Final results were determined as follows: $2^{-(\Delta C_t \text{ sample} - \Delta C_t \text{ calibrator})}$, where ΔC_t values of the calibrator and sample are determined by subtracting the Ct value of the target gene from the value of the endogenous gene (β -actin). Commercial RNA controls were used as calibrators (Liver and Lung; Stratagene, La Jolla, CA, USA). In all quantitative experiments, a sample was considered not evaluable when the standard deviation of the Ct values was > 0.30 in two independent analyses. As a result, the number of evaluable samples varied among the genes examined.

RESULTS

Baseline mRNA expression of EGFR-mutation positive cells and sensitivity to PIM inhibition

We first evaluated the baseline mRNA expression of *PIM*, *STAT3* and other relevant genes^[8], in our panel of five EGFR-mutation positive NSCLC cell lines. As can be seen in Figure 2A, both PC9 and H1975 cell lines had higher *PIM-1*, *STAT3* and *JAK2* mRNA expression compared to the average gene expression of all five cell lines. Hereafter, cell viability assays were performed and the IC_{50} s of single AZD1208 treatment were determined. The IC_{50} s of osimertinib have been previously calculated and reported^[6]. As expected, none of the cell lines was sensitive to single AZD1208 treatment, with IC_{50} s ranging from 17.1 to 23.0 µmol/L [Figure 2B, left panel], while all of them were sensitive to osimertinib^[6] [Figure 2B, right panel].

Combination of osimertinib plus a PIM inhibitor in EGFR-mutation positive cells

Taking into consideration our previous findings, that osimertinib alone induces the activation of resistant signaling nodes^[6], we then tried to evaluate if AZD1208 increases the effect of osimertinib on cell growth inhibition. Dual treatment revealed moderate synergistic effects, with a CoI between 0.79 and 0.85 [Figure 3A, left panel]. The lowest CoIs and therefore stronger synergism, were seen in the PC9 [CoI of 0.79; 95% confidence interval (CI): 0.66-0.92] and HCC827 (CoI of 0.79; 95%CI: 0.69-0.89) cell lines. A moderate synergism was seen in HCC4006 cells, with a CoI of 0.83 (95%CI: 0.79-0.87). The combination was less

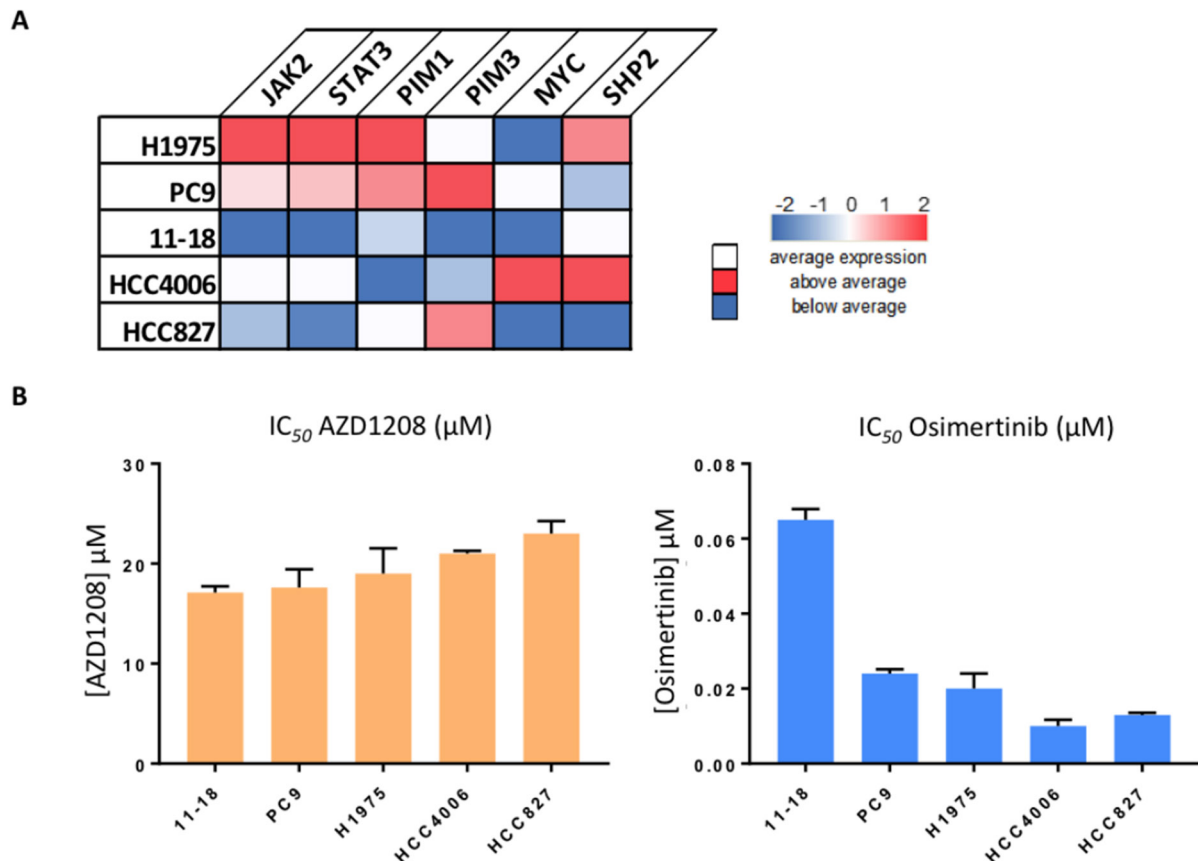


Figure 2. Baseline mRNA expression of EGFR-mutation positive cells and sensitivity to PIM inhibition. A: baseline mRNA expression of various genes in five EGFR-mutation-positive NSCLC cell lines (H1975, PC9, 11-18, HCC4006 and HCC827). Heatmap depicts gene mRNA expression (columns) in the different cell lines (rows) compared to the average mRNA expression of each gene in all cell lines. Experiments were performed in biological triplicates, and the average of these experiments are shown; B: cell viability assays were performed in all cell lines, to explore the effect of AZD1208 *in vitro*, and to determine the IC₅₀ in each cell line. Results of previously performed experiments with osimertinib in the same cell lines are also shown. Experiments were performed in biological triplicates, and the average and standard deviations are shown. EGFR: epidermal growth factor receptor; PIM: proviral integration site for Moloney murine leukemia virus; NSCLC: non-small cell lung cancer; IC₅₀: half maximal inhibitory concentration; JAK2: Janus kinase 2; STAT3: signal transducer and activator of transcription 3; SHP2 (PTPN11): tyrosine-protein phosphatase non-receptor type 11

effective, but still moderately synergistic, in the 11-18 (CoI of 0.85; 95%CI: 0.69-1.00) and H1975 (CoI of 0.85; 95%CI: 0.74-0.96) cell lines [Figure 3A, left panel].

We then tried to see the correlation between signaling nodes and cellular responses after combined treatment with osimertinib plus AZD1208. For this, we selected the PC9 and H1975 cell lines, which distinguished among the rest of the cell lines, having the highest *PIM-1*, *STAT3* and *JAK2* mRNA expression [Figure 2A]. Changes in the protein expression of RTKs, *STAT3* and other proteins after single osimertinib, AZD1208 or combined treatment were studied, using immunoblotting experiments [Figure 3B, right panel]. The results confirmed our previous findings^[6,8], as single osimertinib increased the phosphorylation of CUB-domain-containing protein 1 (CDCP1), YAP1, paxillin and *STAT3* in the PC9 and H1975 cell lines. Osimertinib abrogated or diminished EGFR and ERK phosphorylation, but it had no effect on Src phosphorylation [Figure 3B, right panel]. In contrast, single AZD1208 treatment was unable to inhibit activated EGFR and ERK, but in comparison with osimertinib, it induced at a much lower level the phosphorylation of CDCP1, YAP1, *STAT3* and paxillin. The combination of osimertinib plus AZD1208, abolished EGFR and ERK activation in both cell lines, but paradoxically, was unable to reverse - or even increased - the osimertinib-induced CDCP1 and YAP1 phosphorylation. To our positive surprise, the double

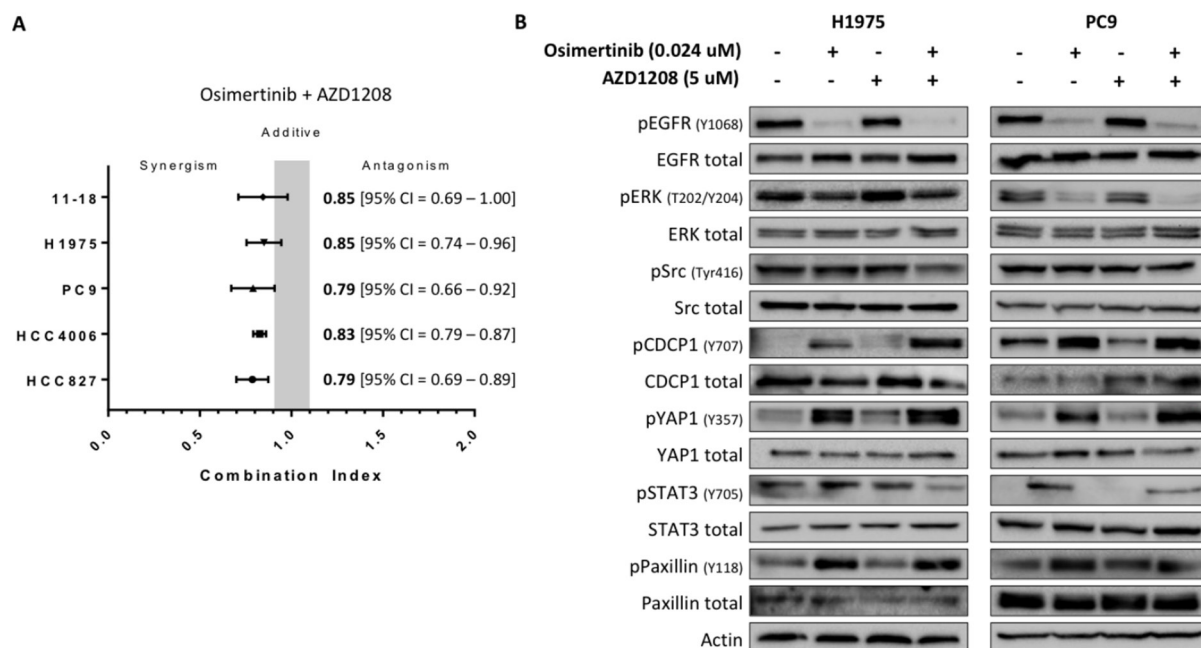


Figure 3. The effects of AZD1208 and osimertinib on RTKs and downstream components in EGFR-mutation positive NSCLC cells. A: in all cell lines, cell viability assays were performed to explore the effect of combined osimertinib and AZD1208 treatment. The obtained Col was calculated based on the Chou and Talalay method, and values < 1, = 1 and > 1 indicate synergism, additive effect and antagonism, respectively. Experiments were performed in biological triplicates, and the average and standard deviations plus 95% CIs are shown; B: two EGFR-mutation positive NSCLC cell lines (H1975 and PC9) were treated with single osimertinib (0.024 μmol/L), single AZD1208 (5 μmol/L) or with the combination. Untreated cells received an equivalent dose of vehicle (DMSO). Cell lysates were used for immunoblotting and changes in RTKs and non-RTKs upon the different treatments were investigated in the two cell lines. Experiments were performed in biological triplicates with similar results, and a representative blot is shown. RTK: receptor tyrosine kinase; EGFR: epidermal growth factor receptor; NSCLC: non-small cell lung cancer; Col: combination index; 95%CI: 95% confidence interval; DMSO: dimethylsulfoxide; CDCP1: CUB domain -containing protein 1; YAP1: yes-associated protein 1; STAT3: signal transducer and activator of transcription 3

combination reversed the osimertinib-induced STAT3 phosphorylation in both cell lines, and diminished paxillin and Src phosphorylation in PC9 and H1975 cells, respectively [Figure 3B, right panel]. Overall these data reconfirm our previous findings that, even in oncogene addicted EGFR-mutation positive cancer cells, single EGFR TKIs are insufficient^[6-8,34]. Concomitant PIM-1 inhibition may reverse some of the deleterious effects of osimertinib on the activation of oncogenic signaling pathways.

DISCUSSION

Herein, we have reviewed the available literature on PIM and we have found that this kinase is involved in multiple tumor types, including lung cancer^[26]. The survival of lung cancer patients has been improved in the last decade due to the availability of new and more effective targeted therapies. EGFR-mutation positive patients are treated with EGFR TKIs, and the last years we have seen the development and approval of three generations of these agents^[35]. However, ultimately resistance mechanisms take over and treatment options are not indefinite. Furthermore, about 20% of EGFR-mutation positive patients have intrinsic resistance to EGFR TKIs^[8,34], indicating that molecularly targeted therapy is not a one-size-fits-all approach. Therefore, defining biomarkers of resistance and designing a truly personalized therapy is of great relevance. We have previously seen, and we confirm in the present work, that EGFR TKIs, including osimertinib, induce STAT3 and Src-YAP1 activation^[6-8]. Rational double or triple combinations prevented this phenomenon and increased the efficacy of EGFR TKI monotherapy in EGFR-mutation positive models^[6-8]. Since PIM was shown to be a key player in IL-6/STAT3 signaling^[14,18,21,28,29], we hypothesized that by combining the PIM inhibitor, AZD1208 with osimertinib we may obtain similar results^[6-8].

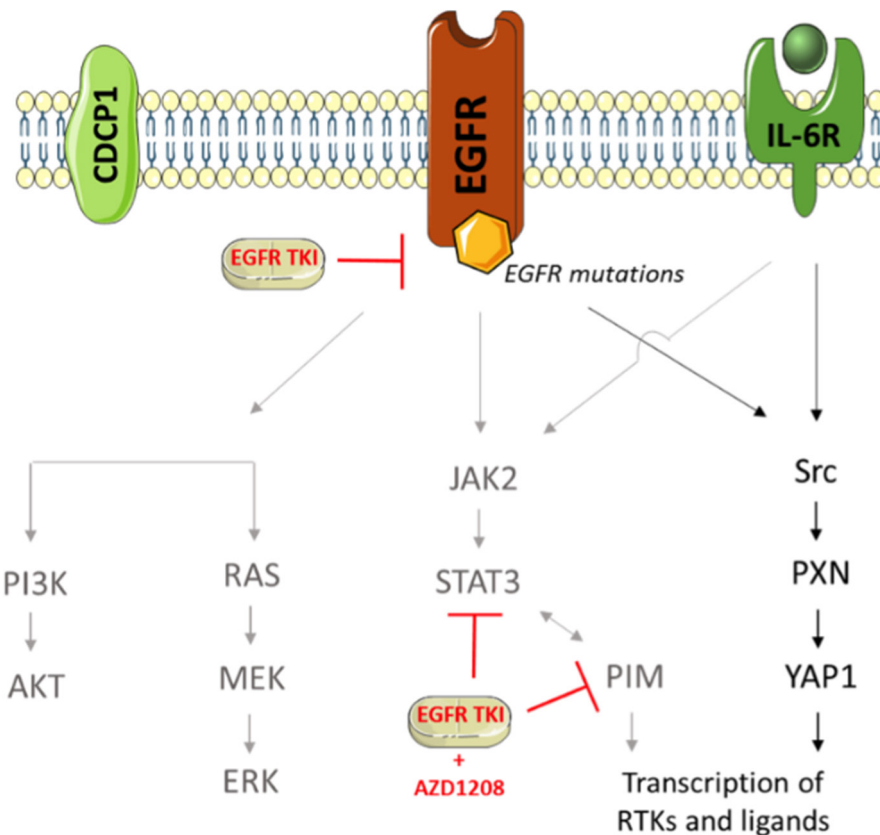


Figure 4. Obtained model of EGFR-mutation positive NSCLC tumor cells, after dual EGFR- (osimertinib) and PIM- (AZD1208) inhibition. Upon osimertinib treatment, the downstream PI3K/AKT and RAS/MAPK pathways - in light grey - are inhibited, lowering cell proliferation and survival. However, AZD1208 treatment now blocks both the activation of PIM and the activation of the JAK/STAT3 pathway. This leads to moderate synergistic effects *in vitro*. However, the Src-YAP1 pathway is still activated (PC9) or only slightly inhibited (H1975), and can therefore compensate for the blockade induced by osimertinib and AZD1208. EGFR: epidermal growth factor receptor; NSCLC: non-small cell lung cancer; PIM: proviral integration site for Moloney murine leukemia virus; PI3K: phosphatidylinositol 3-kinase; MAPK: mitogen-activated protein kinase; JAK2: Janus kinase 2; STAT3: signal transducer and activator of transcription 3; YAP1: Yes-associated protein 1; CDCP1: CUB domain-containing protein 1; IL-6R: Interleukin 6 receptor; PXN: Paxillin

As expected, single AZD1208 was not effective, but the combination of AZD1208 and osimertinib augmented the effect of each drug alone on cell viability in a moderate synergistic manner, in five EGFR-mutation positive cell lines. This synergistic effect is probably due to the AZD1208-based inhibition of resistance pathways. Our model, in Figure 4, indicates that combined EGFR and PIM inhibition does not prevent the activation of all relevant resistance signaling nodes, but it is able to decrease STAT3 phosphorylation. Since currently there are no approved STAT3 inhibitors in clinical use, PIM inhibition merits further investigation in lung cancer as well as other tumor types. AZD1208 is not in clinical development any more^[22] and we plan to extend the current work using the pan-PIM inhibitor LGH447 (PIM447). LGH447 has single agent activity, and blocks resistance pathways when combined with standard of care treatments^[22,36-38]. The agent is currently being tested in a phase I/II clinical trial (ClinicalTrials.gov Identifier, NCT01456689) for relapsed/refractory multiple myeloma, and safety and efficacy results are awaited.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: Bracht JWP, Karachaliou N, Rosell R

Performed data acquisition, as well as provided administrative, technical, and material support: Bracht JWP, Karachaliou N, Berenguer J, Fernandez-Bruno M, Filipiska M, Pedraz-Valdunciel C, Codony-Servat C, Codony-Servat J, Rosell R

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Central regulation of breast cancer growth and metastasis

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Abstract

Cancer is a systemic disease. In order to fully understand it, we must take a holistic view on how cancer interacts with its host. The brain monitors and responds to natural and aberrant signals arriving from the periphery, particularly those of metabolic or immune origin. As has been well described, a hallmark of cancer is marked disruption of metabolic and inflammatory processes. Depending on the salience and timing of these inputs, the brain responds via neural and humoral routes to alter whole-body physiology. These responses have consequences for tumor growth and metastasis, directly influencing patient quality of life and subsequent mortality. Additionally, environmental inputs such as light, diet, and stress, can promote inappropriate neural activity that benefits cancer. Here, I discuss evidence for brain-tumor interactions, with special emphasis on subcortical neuromodulator neural populations, and potential ways of harnessing this cross-talk as a novel approach for cancer treatment.

Keywords: Breast cancer, hypothalamus, immunometabolism, sympathetic nervous system, neuromodulators

INTRODUCTION

Uncovering the relationships among cancer and the physiology of its host has cemented the notion that cancer is a systemic disease. Cancer patients frequently experience systemic symptoms like depression, sleep disruption, cognitive impairment, appetite and metabolic dysfunction, and weight loss. These phenomena span different cancer types and occur independently from treatment regimens. Clinical studies consistently report that such symptoms (such as weight loss, sleep disruption, and circadian misalignment) are predictors



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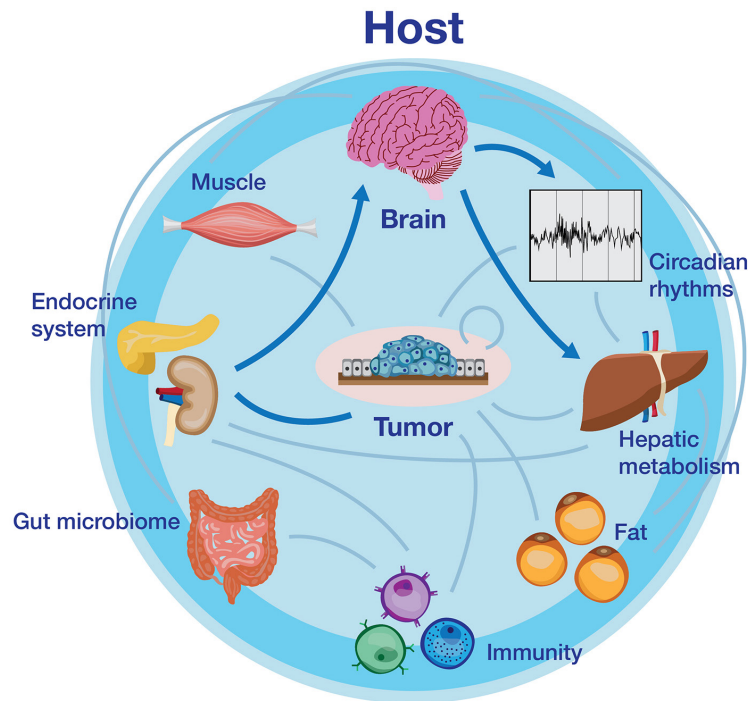


Figure 1. A simplified schematic of reciprocal tumor-host interactions. Tumors promote aberrant physiology via alterations to the immune system and secretion of metabolic “waste” which contributes to further inflammation and altered function of distal organs, including the brain. Feedback from the brain (neural or humoral) can subsequently exacerbate tumor-associated immune and metabolic changes, ultimately facilitating tumor growth, angiogenesis, metastasis, or cancer-associated co-morbidities

of poor prognoses and reduced quality of life^[1-5]. Tumors are capable of altering local macronutrient contents that modulate infiltrating immune cell function resulting in aberrant inflammation. Additionally, they secrete metabolic “waste”, which can promote inflammation and alter the function of distal organs and tissues such as the liver and brain^[6-10]. As evidence accumulates, we are learning that many of these cancer-associated co-morbidities are (at least in part) due to deregulation of normal brain function by the cancer itself, cancer treatment(s), or other factors.

Reciprocally, the host system can influence tumor growth and metastasis via immune, endocrine, and neural pathways. For example, chronic stress, which results in dysregulation of glucocorticoid and adrenergic signaling, exacerbates tumor growth and angiogenesis^[11,12]. Additionally, chronic sleep fragmentation, resulting in top-down impairments to the immune system, further promotes tumor growth^[13]. The objective of this review is to provide an up-to-date overview of cancer as a systemic disease from a basic science perspective [Figure 1]. Special focus will be given to subcortical neural populations that are sensitive to signals arriving from peripheral tissues and the environment, as well as those that send long-range projections to modulate immune or metabolic function, ultimately facilitating cancer growth and/or metastasis. Through understanding these brain-tumor interactions, potential undescribed drug or lifestyle targets will be uncovered. Additionally, these studies would open up space for existing therapies to be repurposed for effective cancer treatment (as is the case with the anti-obesity drug Metformin^[14,15]).

NEURAL CIRCUITRY DEREGULATED IN CANCER

Sleep disruption

Disruption of sleep and/or circadian rhythms in physiology and behavior are frequently observed in cancer patients. Indeed, 35%-80% of cancer patients report poor sleep quality^[16,17], as compared to 29%-32% of the general population^[18] [Table 1]. These problems may stem from the cancer itself, the stress or stigma

Table 1. Non-exhaustive list of clinical observations of systemic co-morbidities potentially influencing brain function (sleep disturbance, circadian rhythm disruption, cognitive impairment, metabolic abnormalities, microbial dysbiosis, and systemic inflammation) in patients with cancer

Systemic problem	Patient population	Methods	Primary observation	Ref.
Sleep disturbance	823 patients with cancer receiving chemotherapy	Post-hoc analysis of data from a large randomized clinical trial; Hamilton Depression Inventory used to assess sleep disturbance	36.6% ($n = 301$) of the patients with cancer reported insomnia symptoms, and 43% ($n = 362$) met the diagnostic criteria for insomnia syndrome; breast cancer had the highest number of overall insomnia complaints	[120]
	85 women with Stages I-IIIa breast cancer	actigraphy for 72 consecutive hours and filled out questionnaires (PSQI, MFSI-SF, FOSQ, FACT-B, and CES-D) on sleep, fatigue, depression, and functional outcome	women slept for ~6 h a night and napped > 1 h during the day. Sleep was disturbed and fatigue levels were high; phase-delayed circadian rhythms	[121]
	97 women with advanced breast cancer (age = 54.6 ± 9.8 years)	72 h actigraphy; sleep efficiency was determined as the ratio of total sleep time to total sleep time plus wake after sleep onset	Sleep efficiency predicted reduction in overall mortality [hazard ratio (HR), 0.96; 95% confidence interval (CI), 0.94-0.98; $P < 0.001$] at median 6 y follow-up. Remained significant (HR, 0.94; 95%CI, 0.91-0.97; $P < 0.001$) after adjusting for age, estrogen receptor status, cancer treatment, metastatic spread, cortisol levels, and depression	[3]
	40 patients (50 years, SD = 11; 53% White, 28% Asian, 19% Other) with primary breast cancer (18% Stage I, 50% Stage II, 33% Stage III) undergoing chemotherapy	Neurocognitive battery of tests including PSQI, ISI, BFI, CAD, COWAT, HVLT; actigraphy for 7 consecutive days to track arousal/sleep	Better circadian function was associated with less sleep disruption (PSQI, $r = -0.44$, $P = 0.005$) and less insomnia (ISI, $r = -0.42$, $P = 0.008$). Both subjective sleep alteration and circadian disruption were associated with levels of fatigue (BFI, all P -values < 0.05) and sleep disruption measures were strongly associated with depression and anxiety (ISI: $r = 0.51$, $P = 0.001$; PSQI: $r = 0.43$, $P = 0.005$)	[122]
Circadian rhythm disruption	389 Caucasian cases and 432 Caucasian controls	Investigated the association between an exonic length variation in a circadian gene, Period3 (Per3), and breast cancer risk using blood samples collected from a recently completed breast cancer case-control study in Connecticut	<i>Per3</i> genotype (heterozygous + homozygous 5-repeat alleles) was associated with an increased risk of breast cancer among premenopausal women (odds ratio, 1.7; 95%CI, 1.0-3.0)	[123]
	57 presurgical breast cancer patients	Daily self-reports of cancer-specific distress and avoidant coping as well as actigraphic and salivary cortisol data	Distress and avoidant coping were related to rest/activity rhythm disruption (daytime sedentariness, inconsistent rhythms). Patients with disrupted rest/activity cycles had flattened diurnal cortisol rhythms	[124]
	104 patients with metastatic breast cancer	Salivary cortisol levels assessed at study entry at 800, 1200, 1700, and 2100 hours on each of 3 consecutive days; NK cells measured using flow cytometry, activity by chromium release assay	Cortisol slope predicted survival up to 7 years later. Earlier mortality occurred among patients with relatively "flat" rhythms, indicating a lack of normal diurnal variation (Cox proportional hazards, $P = 0.0036$); associated with low counts and suppressed activity of NK cells	[4]
	43 breast cancer patients	Actigraphy, cancer-specific distress (IES, POMS), saliva samples for assessment of diurnal cortisol rhythm, cortisol awakening response (CAR), and diurnal mean. Ten potential markers of tumor progression were quantified in serum and grouped by exploratory factor analysis	Poor circadian coordination as measured by rest-activity rhythms had higher factor 1 (MMP9, TGF-beta, VEGF) scores ($R^2 = 0.160$, $P = 0.038$). Patients with elevated CAR also had higher Factor 1 scores ($R^2 = 0.293$, $P = 0.020$). These relationships appeared to be driven largely by VEGF concentrations	[2]

Cognitive Impairment	321 patients admitted to the Edmonton General Palliative Care Unit over a period of 26 months	Mini-Mental State Examination (MMSE) was used as screening tool to assess cognitive functioning and was performed on all patients at the time of admission and once to twice weekly thereafter	142 pts (44%) had abnormal MMSE scores (MMSE < 0.8) on admission, whereas 176 patients (55%) had abnormal MMSE scores at the time of death or discharge; 157 (68%) had abnormal MMSE scores prior to death; Of 124 patients with normal final MMSE scores, 64 (52%) were discharged versus 16 of 116 patients (14%) who had abnormal MMSE final scores ($P < 0.0001$)	[125]
	Meta-analysis of 23 studies on cognitive impairment in cancer patients	Articles published 1980-2012, comparing subjective and objective cognition in cancer patients treated with chemotherapy. Of 818 potentially relevant articles, 23 studies met the inclusion criteria for the current review and one article was sourced from reference lists of included studies	8/24 included studies found a significant relationship between objective and subjective measures of cognitive performance. These studies were more likely to involve breast cancer patients and to assess the relationship between memory and perceived cognitive impairment	[126]
	22 breast cancer survivors who reported cognitive impairment and who were at least 1 year post-chemotherapy treatment	Qualitative interviews, recorded, transcribed verbatim, and analyzed using a content analysis approach	6 major domains identified: short-term memory, long-term memory, speed of processing, attention and concentration, language and executive functioning; All survivors found these impairments frustrating, and some also reported these changes as detrimental to their self-confidence and social relationships	[127]
	85 women with early stage breast cancer scheduled for chemotherapy, 43 women scheduled for endocrine therapy and/or radiotherapy and 49 healthy control subjects	3-year prospective study; neuropsychological performance assessed at baseline (T1), post-chemotherapy (or 6 months) (T2) and at 18 months (T3)	No significant interactions or main effect of group after controlling for age and intelligence; reliable decline on multiple tasks was seen in 20% of chemotherapy patients, 26% of nonchemotherapy patients and 18% of controls at T2 (18%, 14 and 11%, respectively, at T3). Those who experienced treatment-induced menopause were more likely to show decline on multiple measures at T2 (OR = 2.6, 95%CI 0.823-8.266 $P = 0.086$)	[128]
Metabolic Abnormalities	265 patients with advanced breast cancer receiving palliative chemotherapy	Retrospective study; mortality was compared for diabetic and nondiabetic patients as well as for patients that presented hyperglycemia during treatment	Overall survival was greater in diabetic patients with proper metabolic control than diabetic patients with hyperglycemia. The risk of death was higher in patients with mean glucose levels > 130 mg/dL during treatment	[129]
	Meta-analysis of 20 studies (5 case-control and 15 cohort studies) that reported relative risk (RR) estimates (odds ratio, rate ratio/hazard ratio, or standardized incidence ratio) with 95%CI for the relation between diabetes (largely Type II diabetes) and breast cancer incidence	RRs were calculated using a random-effects model	All 20 studies showed that women with (vs. without) diabetes had a statistically significant 20% increased risk of breast cancer (RR, 1.20; 95%CI, 1.12-1.28). The summary estimates were similar for case-control studies (RR, 1.18; 95%CI, 1.05-1.32) and cohort studies (RR, 1.20; 95%CI, 1.11-1.30)	[130]
	Pooled individual-level data from 758,592 premenopausal women from 19 prospective cohorts	Hazard ratios (HRs) of premenopausal breast cancer in association with BMI from ages 18 through 54 years using Cox proportional hazards regression analysis. Median follow-up; 9.3 years (interquartile range, 4.9-13.5 years) per participant, with 13,082 incident cases of breast cancer	Inverse linear associations of BMI with breast cancer risk were found that were stronger for BMI at ages 18 to 24 years (HR per 5 kg/m ² [5.0-U] difference, 0.77; 95%CI, 0.73-0.80) than for BMI at ages 45 to 54 years (HR per 5.0-U difference, 0.88; 95%CI, 0.86-0.91). 4.2-fold risk gradient between the highest and lowest BMI categories (BMI ≥ 35.0 vs. < 17.0) at ages 18 to 24 years (HR, 0.24; 95%CI, 0.14-0.40)	[131]
	10,786 women ages 35-69 were recruited in a prospective study in Italy; Four matched controls were chosen for each breast cancer case ($n = 144$)	Blood samples were collected after a 12-h fast between 7:30 and 9:00 a.m.	Adjusted relative risk (RR) for the highest quartile of serum glucose vs. the lowest was 2.8 (95%CI, 1.2-6.5), and P for trend was 0.02. Insulin showed a weaker association with breast cancer, the adjusted RR of the highest quartile vs. the lowest was 1.7 (95%CI, 0.7-4.1), and P for trend was 0.14, whereas the adjusted RR of the highest quartile of IGF-I was 3.1 (95%CI, 1.1-8.6), and P for trend was 0.01	[132]

Microbial Dysbiosis	Breast tumor tissue and paired normal adjacent tissue from the same patient	Qualitative survey of breast microbiota DNA	Bacterium <i>Methylobacterium radiotolerans</i> is relatively enriched in tumor tissue, while the bacterium <i>Sphingomonas yanoikuyae</i> is relatively enriched in paired normal tissue. The relative abundances of these two bacterial species were inversely correlated in paired normal breast tissue but not in tumor tissue, indicating that dysbiosis is associated with breast cancer	[133]
	48 postmenopausal breast cancer case patients, pretreatment, vs. 48 control patients	Microbiota profiles in fecal DNA were determined by Illumina sequencing and taxonomy of 16S rRNA genes. Estrogens were quantified in urine; linear and unconditional logistic regression of microbiota α -diversity (PD_ whole tree) and UniFrac analysis of β -diversity	Estrogens correlated with α -diversity in control patients (Spearman Rho = 0.37, P = 0.009) but not case patients (Spearman Rho = 0.04, P = 0.77). Compared with control patients, case patients had statistically significantly altered microbiota composition (β -diversity, P = 0.006) and lower α -diversity (P = 0.004). Adjusted for estrogens and other covariates, odds ratio of cancer was 0.50 (95%CI, 0.30-0.85) per α -diversity tertile	[134]
	31 patients with early-stage breast cancer	Bacterial DNA was extracted from the feces; qPCR amplified, targeting 16S rRNA sequences specific to bacterial groups, and then analyzed in relation to clinical characteristics	Absolute numbers of total bacteria and three bacterial groups (<i>Firmicutes</i> , <i>Faecalibacterium prausnitzii</i> , and <i>Blautia</i>) differed significantly according to the patient's body mass index. <i>C. coccoides</i> , <i>F. prausnitzii</i> , and <i>Blautia</i> , differed significantly according to the clinical stages and the histoprognostic grades	[135]
	Eighteen patients with breast cancer (BC), 18 with uterine leiomyoma (UL), and 30 healthy women	Feces were collected on 1st admission and processed immediately; qualitative and quantitative analysis of fecal flora	Premenopausal BC patients showed increased Enterobacteriaceae (<i>E. coli</i> , log 9.7 \pm 2.1, P < 0.001); aerobic streptococci (log 7.8 \pm 2.0) and lactobacilli (log 8.0 \pm 2.8). Anaerobic bacteria were increased (P < 0.001) for clostridia (log 9 \pm 1.7), bacteroides (log 7.2 \pm 3.1), and anaerobic lactobacilli (9.1 \pm 2.5). Similar changes in menopausal samples	[136]
Systemic Inflammation	Data from the Health, Eating, Activity, and Lifestyle (HEAL) Study (a multiethnic prospective cohort study of women diagnosed with stage 0 to IIIA breast cancer) (734 total survivors)	Concentrations of CRP and SAA were measured approximately 31 months after diagnosis and tested for associations with disease-free survival (approximately 4.1 years of follow-up) and overall survival (approximately 6.9 years of follow-up)	Elevated SAA and CRP were associated with reduced overall survival, regardless of adjustment for age, tumor stage, race, and body mass index (SAA P trend < 0.0001; CRP P trend = 0.002). The HRs for SAA and CRP tertiles suggested a threshold effect on survival, rather than a dose-response relationship (highest vs. lowest tertile: SAA HR = 3.15; 95%CI, 1.73-5.65; CRP HR = 2.27; 95%CI, 1.27-4.08)	[137]
	96 patients with metastatic breast cancer. During follow-up 51 patients died of their cancer	Evaluated the value of an inflammation-based score (Glasgow Prognostic Score, GPS) in patients with metastatic breast cancer (scored on 0-2 scale)	Multivariate analysis of the GPS and treatment received, only the GPS (HR 2.26, 95%CI 1.45-3.52, P < 0.001) remained significantly associated with cancer-specific survival	[138]
	Colorectal (n = 182), gastric (n = 87), breast (n = 99), or bronchogenic (n = 404) cancer patients, who had measurements of C-reactive protein and albumin	Median survival, univariate/multivariate analyses of correlations between inflammatory markers and survival	Association between duration of survival and both log ₁₀ C-reactive protein and albumin concentrations (P < 0.0002). log ₁₀ C-reactive protein was an independent predictor of survival (P < 0.0002). When all patients were analyzed (n = 772), the hazard ratio for a 10-fold increase in C-reactive protein concentration in cancer-specific survival was 2.21 (95%CI = 1.92-2.56, P < 0.0001)	[139]
	Cross-sectional and retrospective studies. CS included 100 women undergoing mastectomy for breast cancer risk reduction (n = 10) or treatment (n = 90). Retro study was 127 women who developed metastatic breast cancer	Metabolic syndrome-associated circulating factors were compared by CLS-B status. The association between CLS of the breast and the metabolic syndrome was validated; Distant recurrence-free survival (dRFS) was compared by CLS-B status	Pts with WAT inflammation had elevated insulin, glucose, leptin, triglycerides, C-reactive protein, and IL6 and lower high-density lipoprotein cholesterol and adiponectin (P < 0.05); Compared with patients without breast WAT inflammation, the adjusted HR for dRFS was 1.83 (95%CI, 1.07-3.13) for patients with inflammation	[140]

surrounding a cancer diagnosis, different treatment regimens (e.g., chemotherapy, immunotherapy and/or radiotherapy), or additional lifestyle factors^[19]. These problems are prevalent across a variety of cancer types, with lung and breast cancer patients making up the majority of the population experiencing these symptoms^[2,20-22]. A “chicken-or-the-egg” phenomenon has emerged: poor sleep associates with elevated cancer incidence and progression, and cancer and/or cancer treatments further promote sleep disturbance^[2,3,6,23]. Due to the heterogeneity among cancer types, patient populations, treatment regimens, and lifestyle factors, it has been challenging to pin down cause and effect. This lack of knowledge prevents targeted therapies from being developed and impairs quality of life and lifespan in cancer survivors. For example, sleep disruption is associated with increased mortality in breast cancer independent of other factors like estrogen receptor status, depression, anxiety, and socioeconomic status^[3].

The hypothalamus is a critical structure for maintaining homeostasis^[24,25]. Although beyond the scope of this review, a brief discussion of its relevant circuitry is warranted to put the rest of our discussion in context. Its functions include the regulation of sleep-wake cycles, circadian rhythms, body temperature, feeding/metabolism, the stress response, and reproduction, among others. Many of these are linked to either the promotion of cancer development or its progression (as I discuss in subsequent sections). The lateral hypothalamus (LH) is a highly heterogeneous structure that serves a primary role in arousal, metabolism, and motivated behavior^[24]. A neural population that has been intensely studied in this area are those that express the excitatory neuromodulators hypocretin-1 and hypocretin-2 (aka orexin-A and -B; HO)^[26,27]. Discovered by two groups at essentially the same time^[28,29], these neurons are critical for maintaining wakefulness, as their destruction results in the sleep disorder narcolepsy^[30-33].

HO neurons project throughout the brain to participate in functions ranging from arousal and motivation, to anxiety and reproductive behavior^[27]. Importantly, they also send long range projections that modulate sympathetic outflow from the brain^[34]. Indeed, disinhibition of HO neurons in the LH can directly influence hepatic gluconeogenesis, promoting *de novo* glucose production upon stimulation^[35]. Reciprocally, HO neurons are sensitive to metabolic signals arriving from the periphery. These include hormones and other messages important in cancer regulation, including leptin, ghrelin, glucose, dietary amino acids, and changes in extracellular pH and CO₂ concentrations^[27]. Stimulation of HO neurons further activates the hypothalamic-pituitary-adrenal (HPA) axis, resulting in rapid increases in circulating glucocorticoid concentrations^[36]. Aberrant glucocorticoid rhythms are highly prevalent in breast cancer patients^[4], and their actions on the immune system may influence patient prognosis (discussed below).

Leptin, an adipokine hormone that correlates with satiety and body fat accumulation, generally inhibits HO neurons through direct and indirect pathways^[36-38]. Specifically, intermingled neurons expressing the long-form leptin receptor (LepRb) provide direct inhibitory input to HO neurons. Overexpression of leptin or its cognate receptor (Ob-R) in mammary tumors and nearby normal epithelial cells is associated with progressive and metastatic breast cancer^[39,40]. In this way, leptin overexpression may be relevant to fatigue and sleep disruption in cancer patients, through its inhibitory actions on HO neurons. Ghrelin, an orexigenic hormone produced primarily in the stomach^[41-43] has an excitatory effect on HO neurons, and inhibition of HO neural activity can prevent ghrelin-induced feeding behavior^[44,45]. Ghrelin or the activity of its catalytic enzyme ghrelin-O-acyl-transferase is frequently deregulated in cancer^[46-48], where it associates with cancer-induced cachexia. The role these and other metabolic factors play in cancer and cancer-related co-morbidities is coming into focus as the research community begins to examine them in addition to long-standing candidates from the immune system such as cytokines [e.g., interleukin (IL)-1 β , IL-6, TNF- α] and chemokines (e.g., CCL2, CXCL12).

Indeed, brain-tumor-metabolic interactions were recently tested in a mouse model of non-metastatic breast cancer^[6]. Borniger, Walker and colleagues examined sleep and whole-body metabolic changes during the

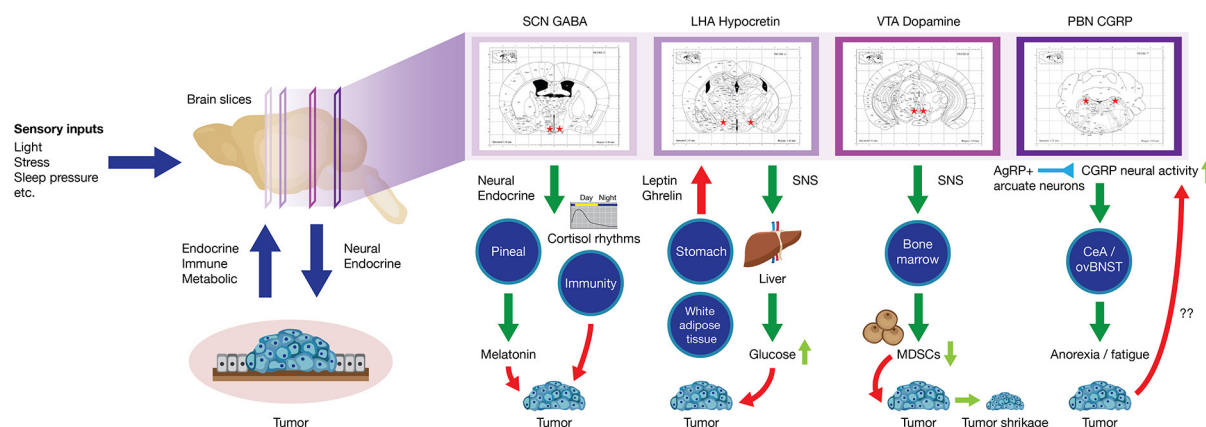


Figure 2. Highlighted pathways linking the brain and periphery in the context of cancer. Environmental (e.g., light, stress) or endogenous signals reach the brain to alter the activity of neurons involved in sleep (LHA hypocretin/orexin), circadian rhythms (SCN-GABA), reward (VTA-Dopamine), metabolism, and energy balance (Parabrachial CGRP). Aberrant activity of these cells promotes signaling in the periphery that ultimately facilitates tumor growth, angiogenesis, and invasiveness. Systems highlighted are bolded in Table 2. LHA: lateral hypothalamic area; SCN: suprachiasmatic nucleus; GABA: gamma amino butyric acid; VTA: ventral tegmental area; PBN: parabrachial nucleus; CGRP: calcitonin gene related peptide; MDSCs: myeloid derived suppressor cells; SNS: sympathetic nervous system; AgRP: agouti related peptide

course of tumor progression. They observed marked peripheral inflammation driven by the cytokine IL-6. This was associated with a shift towards hepatic gluconeogenesis over glycolysis in tandem with disrupted sleep [Figure 2]. Additionally, tumor-bearing mice had reduced circulating leptin concentrations and were hypersensitive to the orexigenic hormone ghrelin. As HO neurons are sensitive to these peripheral metabolic signals, and they are powerful regulators of wakefulness, the authors examined whether their activity was modulated by tumor growth. They noted that tumors promoted aberrant activity within HO neurons, and inhibition of their signaling (via administration of a dual HO receptor antagonist) attenuated both metabolic and sleep problems. The authors reasoned that in order for HO neurons to influence peripheral glucose metabolism, a signal must reach the liver from the brain. A potential pathway through which this could occur is the sympathetic nervous system (SNS)^[35]. Ablating the SNS with administration of 6-hydroxydopamine (6-OHDA) rescued tumor-induced metabolic deficits, supporting the idea that HO neurons modulate peripheral glucose concentrations via downstream SNS activation^[6]. Significantly more research is required to unravel the complex signaling network linking tumors in the periphery to changes in the activity of this critical neural population. However, these findings suggest that repurposing drugs targeting this system [e.g., Suvorexant (Belsomra®)] may be a novel strategy for improving sleep and metabolic health in patients with cancer.

In two mouse models of lung cancer (LLC and TC1), Hakim *et al.*^[13] demonstrated that chronic sleep fragmentation promoted tumor growth, a phenotype that was abolished in mice lacking the endotoxin receptor TLR4. TLR4 is part of a family of pattern recognition receptors that powerfully engage the innate immune system upon ligand binding. Surprisingly, the effect of sleep fragmentation on tumor progression was maintained in mice lacking TLR4 effector molecules MyD88 or TRIF, although the effect was reduced. This was the first study to causally link disrupted sleep, tumor progression, and immune deregulation. Although this approach lacks cell-type specific investigations into neural populations influenced by the sleep fragmentation protocol, it suggests one or more neural populations sensitive to this manipulation may be responsible for top-down changes to the immune system that biases the host environment to one that favors tumor growth. Recently, McAlpine *et al.*^[49] demonstrated one such pathway, where sleep disruption decreased the number of HO-expressing neurons. This led to aberrant regulation of pre-neutrophils in the bone marrow, which were found to express HO receptor 1. This change in activity promoted egress of myeloid lineage cells, which then contributed to the development of atherosclerosis. I speculate that a similar phenomenon occurs in the context of cancer^[49]. The studies discussed in this section highlight the bidirectional pathway between

sleep and cancer, where disrupted arousal influences cancer growth and aberrant neoplasia reciprocally promotes changes in sleep.

Circadian deregulation

The paired suprachiasmatic nuclei (SCN) are the primary structures responsible for setting circadian rhythms in physiology and behavior that we observe across most of the phylogenetic tree^[50-53]. The SCN receive photic input from specialized retinal ganglion cells that serve a minimal role in vision. These cells express a photosensitive protein, melanopsin, allowing them to directly sense light, and are therefore named “intrinsically photosensitive retinal ganglion cells” (ipRGCs)^[54,55]. ipRGCs transduce photic input into a neurochemical one, with axons traversing the retino-hypothalamic tract and terminating in the suprachiasmatic nucleus. Here, glutamate-mediated synaptic transmission results in downstream cyclic adenosine monophosphate (cAMP) accumulation and cAMP response element binding (CREB) phosphorylation. Phosphorylation of CREB results in it binding the promoters of the core clock genes *per* and *cry*. In a transcription-translation loop, the protein products homo or heterodimerize (e.g., PER::CRY dimers), enter the nucleus, and suppress the transcription of the positive arms of the circadian clock, the genes *arntl1* (*bmal1*) and *clock*.

This process takes approximately 24 h to complete, where light-induced gene transcription has a phase-modulatory effect on the clock. This feedback loop operates in a cell-autonomous manner throughout the body, with peripheral clocks “set” via neural and humoral routes originating from the SCN^[56,57]. Behavioral and physiological outputs controlled by the clock include sleep-wake cycles, appetite and food intake, mating and reproductive behavior, rhythms in immune function and glucocorticoid secretion, and stress responses, among others.

Chronic circadian disruption (e.g., via aberrant light exposure, genetic manipulations, or phase shifting) is repeatedly associated with spontaneous cancer occurrence in humans and multiple rodent models spanning a variety of cancer types^[2,4,21,58,59]. For example, chronic circadian disruption via repeated inversions of the light-dark cycle promotes spontaneous tumor development in a mouse model of breast cancer mimicking Li-Fraumeni syndrome^[60]. This paradigm is known to cause significant disruption of the circadian clock as well as the sleep-wake cycle. Using a transgenic approach to specifically knockdown the tumor-suppressor p53 in mammary epithelial cells (*WAP-Cre::p53^{fl/fl}*), van Dyke and colleagues demonstrated that mice undergoing the inversion protocol developed mammary tumors ~8 weeks sooner (median; 17% sooner) than their control counterparts. This was accompanied by increased body mass gain in mice experiencing circadian disruption, as well as gross increases in sleep throughout the experiment. This was the first study to demonstrate a causal role for light-induced circadian disruption in the acceleration of spontaneous breast cancer development.

In a similar study, Papagiannakopoulos and colleagues investigated the effects of environmental and genetic circadian disruption on lung tumorigenesis^[61]. Using a cre-inducible model of lung cancer [*K-ras^{LSL-G12D/+};p53^{fllox/fllox}* (*KP*) mice], the authors subjected the mice to a jet-lag circadian disruption schedule and examined tumor growth, metabolism, and proliferative capacity. Chronic jet-lag accelerated tumor growth, severity, and mortality upon cre-mediated recombination. A similar phenotype was uncovered when manipulations consisted of knocking out core clock genes (*Per2* or *Bmal1* (*Arntl1*)) in animals that develop spontaneous cancer (*Kras^{LA2/+}* mice). Tumor cells deficient in *per2* were also more proliferative in culture, and mouse embryonic fibroblasts lacking *kras* and *per2* were more sensitive to cellular transformation than their *Per2*-intact counterparts. As energy balance is powerfully regulated by circadian rhythms, they investigated cellular metabolic pathways in *Per2* deficient cells. Indeed, cells lacking this core clock gene showed a marked increase in the excretion of core energy substrates lactate, glucose, and glutamine, indicating a systemic effect of circadian disruption. Using isotope labeled glucose (U-13Cglucose) and carbon 4 (M4) labeling they demonstrated that cells with disrupted circadian clocks increased the amount of glucose loaded into the tricarboxylic acid cycle, a finding that agreed with prior reports^[62]. Finally, they investigated whether clock

gene abnormalities were found in primary patient tumors and noted that all genes (except for *clock*) were down-regulated in lung cancer samples.

In a reciprocal set of experiments to those discussed above, Masri & colleagues investigated how tumors themselves disrupt host circadian rhythms, independent of the outside environment^[10]. In a mouse model of lung adenocarcinoma, they demonstrated that tumors dysregulated the circadian expression of genes controlling immunity and metabolism in a distal organ, the liver, without affecting core components of the circadian clock. This was subsequently confirmed in an additional model of non-metastatic breast cancer, as discussed above^[6]. These changes were hypothesized to be due (in part) to tumor-induced IL-6 signaling interfering with insulin-dependent glucose uptake via a SOCS3-regulated mechanism. Experiments like those discussed above highlight the bidirectional cross-talk among the circadian system (ultimately controlled by the brain), tumors, and the host. These findings suggest that novel approaches for cancer treatment lie in the normalization of circadian rhythms via light, nutrition, or clock phase or amplitude-modulating compounds. Indeed, a flavonoid found in citrus peel, nobiletin, is a powerful clock-enhancing molecule^[63] that shows promise in the treatment of a variety of cancers^[64-66].

Melatonin

Melatonin is an indoleamine hormone produced and secreted into circulation primarily by the pineal gland in mammals, where it acts as an endogenous signal of darkness^[56,67-70]. Through a poly-synaptic pathway, the suprachiasmatic nuclei control melatonin production and secretion, rendering the concentrations of this hormone sensitive to environmental light input^[71]. Because light activates the SCN to cause downstream inhibition of the pineal gland, darkness induced disinhibition permits melatonin secretion only during the night.

Melatonin is a pleiotropic immunomodulatory molecule. Broadly, melatonin is immune-enhancing, acting as a mild anti-inflammatory agent, buffering the immune system against glucocorticoids and reactive oxidative and nitrosative stress^[72-74]. Shift work and transmeridian travel, two behaviors that strongly alter melatonin rhythms, are associated with cancer incidence. In 2007, the International Agency for Research on Cancer classified shift work with circadian disruption or chronodisruption as a probable human carcinogen^[75]. Artificial light at night (e.g., street and house lights), which inhibits pineal melatonin, is associated with increased breast cancer prevalence^[22,76,77], although the findings are not universally consistent^[78]. The mechanisms behind these trends are becoming clearer thanks to basic research.

In a clever experimental design, Blask & colleagues investigated the role of melatonin on human breast cancer xenograft tumor progression in nude rats^[79]. Blood samples were collected from healthy female volunteers during the day, night, or after 90 min exposure to bright white light at night (to putatively knockdown circulating melatonin concentrations). Melatonin deficient- (daytime or light at night collected) or sufficient blood were then perfused into the tumor xenografts. Tumors perfused with daytime or light at night-exposed blood samples showed high proliferative activity and linoleic acid uptake/metabolism, while those perfused with melatonin-rich nocturnal blood had markedly reduced proliferative activity. Additionally, exposing tumor-bearing rats to increasing intensities of artificial light dose-dependently accelerated tumor growth in tandem with knockdown of circulating melatonin. These results were the first to suggest that light at night exerts its pro-tumorigenic effects via its actions on circulating melatonin concentrations^[79]. Since the publication of this study, melatonin has been intensely investigated as an anticancer molecule, particularly in the context of breast cancer^[80,81]. Potential mechanisms for its actions have been uncovered, including anti-estrogen, angiogenic, and oxidant pathways^[82]. As an ancient and pleiotropic hormone, melatonin is not the “cleanest” anti-cancer molecule, given its distributed effects on many tissues throughout the body. However, understanding the mechanisms by which it exerts its anti-cancer effects will likely lead to novel and targeted

treatments^[83]. Additionally, due to its low toxicity and high tolerability, it may be useful as a powerful and inexpensive adjunct therapy.

Midbrain reward system

The midbrain ventral tegmental area (VTA) and neighboring substantia nigra are the primary source of all dopamine (DA) within the brain. Known for its important role in reward and motivational processing (i.e., calculating reward-prediction errors), the VTA has recently become a target for modulating cancer. Elevated concentrations of dopamine are associated with blunted tumor growth, reduced angiogenesis, and lower metastatic capacity of cancer in rats^[84]. In general, dopamine seems to inhibit cancer growth, while serotonin facilitates it^[85]. The mechanisms underlying this phenomenon are unclear, although research has started to make headway in this area. In recent years, the VTA has been linked to the modulation of both innate and adaptive immunity^[86]. Using designer receptors exclusively activated by designer drugs (DREADDs), Rolls and colleagues demonstrated that activation of VTA-DA neurons promotes monocyte/macrophage expansion and innate immune responses to *E. coli* infection. Activation of these neurons further increased the number of circulating B-cells, subsequent IgM and IgG titers in response to *E. coli*, and interferon- γ production by T-cells, suggesting enhanced adaptive immunity.

After these initial studies, they applied their findings to a mouse model of lung cancer^[87]. After injecting viruses encoding Gq-coupled DREADDs into the VTA (as previously), mice were injected with subcutaneous tumor cells (LLC or B16 cancer cells), and then given daily injections of the DREADD ligand CNO, chronically activating the VTA. Mice that were “VTA-activated” developed smaller tumors than control mice that did not express the DREADD in the VTA [Figure 2]. To examine how this signal from the brain might reach the tumor, the authors ablated the sympathetic nervous system using the neurotoxin 6-hydroxydopamine (6-OHDA; as discussed earlier). Mice that were SNS-ablated (or received a beta-adrenergic receptor antagonist) failed to reduce their tumor burden upon VTA-DA activation. They further showed that VTA activation altered norepinephrine concentrations specifically in the bone marrow, a vital immune compartment. This strongly supports the hypothesis that VTA-DA neurons alter tumor growth via SNS innervation of the bone marrow. As myeloid derived suppressor cells (MDSCs) express beta-2 adrenergic receptors and regulate tumor growth via inhibition of anti-tumor immunity, the authors examined their phenotype in response to VTA activation. DREADD-induced VTA activation reduced the number of MDSCs, suggesting that the actions of central VTA stimulation on tumor growth may be through sympathetic suppression of MDSCs. To test the role these cells played in their model, they adoptively transferred MDSCs from VTA-activated mice to control mice not expressing the Gq-coupled DREADD in the VTA. This recapitulated the anti-tumor effect of VTA-activation. This suggests that modulation of the immune system via a discrete population of neurons within the brain acts (at least in part) to suppress tumor growth via the sympathetic nervous system.

Stress - glucocorticoids and catecholamines

Glucocorticoids (primarily cortisol in humans and corticosterone in mice) are powerfully regulated by circadian rhythms, stress, metabolic state, and immune status^[88]. Their production and regulation along the HPA-axis has been known for several decades. Their role in linking psychological stress to cancer, however, has only become a subject of intense research within the 21st century^[11]. First hinted at in the 70's and 80's, psychological stress has been suspected to influence tumor growth for several decades^[89,90]. Their role in cancer associated metabolic stress is more well defined. For example, upon metabolic stress induced by cancer-related inflammation (impairments in ketogenesis), glucocorticoids can act to suppress anti-cancer immunity^[91]. This is associated with disrupted rhythms in glucocorticoid secretion, a component controlled ultimately by a crosstalk between central clocks in the SCN and ancillary oscillators in the adrenal glands^[92]. Adrenergic signaling, largely driven by activation of the SNS in the context of stress, also has immunomodulatory properties (as discussed above).

Thaker, Sood & colleagues provided empirical evidence that psychological stress can facilitate tumor growth in multiple animal models via its promotion of glucocorticoid and adrenergic signaling^[12,93]. These studies demonstrated that multiple ovarian cancer tumor cell lines (e.g., EG, SKOV3, 222, HeyA8...) enhance invasiveness when exposed to norepinephrine and/or glucocorticoids (in part) via the upregulation of matrix metalloproteinases (MMPs), critical regulators of angiogenesis and tissue remodeling. Blockade of adrenergic signaling or inhibition of MMPs prevented elevations in cell invasiveness. *In vivo* experiments demonstrated that chronic behavioral stress (restraint) increased tissue catecholamines, tumor growth, vascularization, and invasiveness in an orthotopic mouse model of ovarian cancer. These effects were driven by adrenergic signaling (through the β_2 -adrenoceptor), resulting in downstream cAMP-protein kinase A (PKA) pathway activation. This subsequently promoted the transcription of vascular endothelial growth factor and the MMPs (-2 and -9). These findings highlight adrenergic-receptor signaling as a potential target for reducing tumor angiogenesis and growth. Indeed, perioperative cyclo-oxygenase 2 and beta-adrenergic blockade was shown to improve measures of metastasis in breast cancer patients, offering a safe and effective adjuvant treatment strategy^[94].

Energy balance and feeding

Disrupted energy balance resulting in enhanced capacity to sustain proliferative growth is a hallmark of cancer^[9]. Indeed, one of the first major breakthroughs in cancer research was the discovery that tumor cells are biased towards aerobic glycolysis rather than oxidative phosphorylation to produce energy (i.e., the Warburg effect^[95-97]). Therefore, a common finding in malignant cancers is a strong upregulation of lactate and catalytic enzymes required for lactate production from pyruvate (e.g., lactate dehydrogenase)^[98-101]. Lactate normally acts to aide in glucose sensing and food intake, where it is transported into the brain via monocarboxylate transporters present on endothelial cells lining the blood-brain barrier^[102]. After entering the brain, lactate is able to interact with neurons that normally promote food intake, such as those that produce agouti-related peptide (AgRP) within the arcuate nucleus. Lactate's mechanism of action on orexigenic cells is via its effects on the adenosine monophosphate kinase/methylmalonyl CoA signaling pathway within the hypothalamus^[103]. Lactate alone, however, does not seem to be responsible for cancer-associated anorexia (discussed below)^[104].

Anorexia is a common phenomenon in cancer patients with weight loss, and even when patients attempt to eat enough to compensate, they frequently cannot maintain a healthy weight. Although significant evidence suggests that inflammatory signaling secondary to tumor growth or cancer-treatment associates with anorexia, a specific neural population and mechanism governing this common problem is lacking^[99]. An attractive candidate neural population that may underlie these traits (in part) is the calcitonin-gene-related-peptide (CGRP) expressing population of cells in the parabrachial nucleus (PBN_{CGRP}). These cells powerfully suppress appetite and promote the termination of feeding behavior^[105,106]. CGRP neurons are activated by upstream circuits that respond to cancer-associated signals, and are inhibited by those that promote feeding, including hypothalamic AgRP/neuropeptide Y neurons^[107]. These neurons are also sensitive to peripheral noxious and painful stimuli, which are other aspects of cancer progression^[108].

In a mouse model of Lewis lung carcinoma, Schwartz and colleagues investigated how peripheral tumors modulate CGRP neural activity and their role in cancer-associated anorexia/cachexia^[109]. CGRP neurons were strongly activated in tumor-bearing mice compared with controls, a phenotype typically found after ingestion of a large meal. This suggests that tumors activate cells normally responsible for meal termination and cessation of feeding behavior. Using a cre-dependent tetanus toxin transgene, they demonstrated that inactivation of these cells prevented cancer-associated anorexia/cachexia. Additionally, this manipulation normalized the activity of neurons in circuits downstream from the PBN, namely the central amygdala (CeA) and oval subnucleus of the bed nucleus of the stria terminalis (ovBNST), which may play additional roles in cancer-associated behavioral phenotypes. To control the activity of PBN_{CGRP} neurons with better temporal precision, they used Gi-coupled DREADDs to transiently inhibit these neurons in anorexic/cachexic mice. This manipulation was able to recapitulate the effects seen with their previous approach using tetanus toxin.

Another research area that is rapidly growing in scope is that of brain-gut and gut-cancer interactions. Changes in systemic microbial diversity can influence brain function, alter immune phenotypes, and dictate subsequent cancer development or a tumor's response to immunotherapy^[110,111]. In a proof-of-principle experiment, Lakritz *et al.*^[112] demonstrated that *Helicobacter hepaticus*, a pathogenic gut microbe, promotes distal breast tumorigenesis in a neutrophil-dependent manner. Cancer-prone female mice (FVB-Tg(C3-1-TAg)cJeg/JegJ) were infected with *H. hepaticus* (via gastric gavage) at 3 months of age, and then assessed for subsequent mammary tumorigenesis. Mice infected with these bacteria developed significantly more tumors than their counterpart controls that were not infected. Additionally, mammary intraepithelial neoplasias were associated with strong neutrophil invasion (myeloperoxidase staining). Chronic depletion of neutrophils (via anti-Ly6-G antibodies) prevented *H. hepaticus*-induced cancer development. These data suggest that host-microbe interactions may drive cancer in distal tissues through an immune-mediated mechanism.

CONCLUSIONS AND IMPLICATIONS

Together, the studies discussed above aim to provide an understanding of the types of inputs the brain receives, the signals it propagates, and the effects of these messages on tumor growth and metastasis. Reciprocally, tumor-induced changes in physiology are relayed to the brain via endocrine, immune, or neural signals that ultimately change the activity of discrete neural populations important for maintaining homeostasis. Resolving the “conflict of interest” between cancer and the brain will undoubtedly lead to improvements in patient quality of life and unlock a novel means for cancer treatment. A summary of these findings from basic science are presented in [Table 2](#).

In this vein, treatments targeting the circadian system (i.e., chronotherapy) have gained significant traction in recent years^[113,114]. These approaches leverage natural circadian rhythms in metabolism and detoxification systems to schedule chemotherapy or radiotherapy to coincide with times of peak effectiveness with the lowest potential for side-effects. Animal models have further demonstrated that this approach can effectively limit hepatic toxicity and the inflammatory response to chemotherapeutics^[115,116]. Artificially boosting circadian rhythms (e.g., with nobiletin) adds an additional prospective anti-cancer strategy^[64].

Alternatively, targeted stimulation of specific brain areas deregulated in cancer may help overcome resistance to more traditional treatment strategies. As discussed above, stimulation of the dopaminergic ventral tegmental area promotes tumor suppression via the sympathetic nervous system^[87]. If findings such as these translate to humans, deep brain stimulation protocols could be adapted for adjuvant cancer treatment. For example, deep brain stimulation of the subthalamic nuclei for Parkinson's disease promotes sympathetic activation in a safe and reversible manner^[117,118], a procedure that could be repurposed in the context of advanced cancer. Alternatively, biobehavioral therapies can be designed to promote positive thinking and rewarding experiences (to activate the dopaminergic system) to aide in cancer suppression. Indeed, mindfulness meditation has been demonstrated to improve mood, reduce stress, and attenuate inflammation in patients with breast cancer^[119].

As cancer drastically alters energy balance, influencing the activity of specific brain nuclei regulating metabolism and food intake (e.g., hypocretin, AgRP, POMC, CGRP neurons) represents a strategy to not only improve quality of life, but limit energy availability to the cancer. Indeed, inhibition of aberrant hypocretin/orexin signaling promotes sleep and attenuates tumor-induced metabolic abnormalities in a mouse model of breast cancer^[6]. Repurposing drugs that modify food intake and energy balance (e.g., metformin) further provides additional avenues for adjuvant cancer therapy. However, significant more research is needed to understand both (1) how the brain influences cancer-associated immune populations and (2) how the tumor communicates with the brain to deregulate homeostasis and health. Only then can we begin to manipulate this cross-talk to facilitate cancer elimination.

Table 2. Non-exhaustive list of primary animal model evidence for brain-tumor interactions regulating cancer incidence, disease progression, morbidity and mortality (see Figure 2 for more details)

Cancer type/model	Main focus	Primary findings	Ref.
67NR/4T1/4T07 syngeneic breast cancer cells (female BalbC mice; subQ/orthotopic)	Effects of peripheral tumors on central regulation of sleep and metabolism	Tumors alter leptin/ghrelin signaling, disrupting central hypocretin/orexin activity to influence glucose metabolism and sleep via the sympathetic nervous system	[6]
LL2 Lewis Lung carcinoma/B6 (male C57bl6j mice; subQ)	Dopaminergic regulation of tumor growth	Activation of VTA-dopamine neurons blunts tumor growth via sympathetic modulation of bone-marrow myeloid derived suppressor cells	[87]
p53 ^{R270H/+} WAP-Cre mutant model of Li-Fraumeni syndrome (mouse; transgenic)	Circadian disruption-induced cancer development	Chronic phase shifting accelerated spontaneous tumor growth and altered tumor phenotype	[60]
<i>N</i> -nitroso- <i>N</i> -methylurea (NMU)-induced mammary tumors (rat; chemically induced)	Effects of tumors on affective behaviors	Tumor growth is associated with central cytokine concentrations, altered glucocorticoid responses, and the development of depressive-like behavior	[141]
Colon-26 adenocarcinoma cells (mouse; SubQ)	Effects of tumors on fatigue, muscle physiology, and affective behaviors	Tumors promoted central proinflammatory cytokine production and depressive-like behavior prior to defects in muscle function, behavior rescued by SSRI	[142,143]
HeyA8, SKOV3ip1, MB-231 orthotopic human ovarian carcinoma cells (nude mice; IP)	Effects of stress on tumor development and angiogenesis	Stress-induced adrenergic signaling (cAMP->PKA) promotes tumor growth and angiogenesis	[12]
Non-metastatic methylcholanthrene-induced sarcoma (F344/NTacFBR male rats; SubQ)	Effects of inflammation on central hypocretin/orexin neurons and fatigue	Tumors reduced hypocretin/orexin transcript expression and promoted fatigue	[144]
LL2 or TC-1 lung epithelial cells (male C57Bl6 mice; subQ)	Role of sleep fragmentation (SF) on tumor growth and progression	SF accelerates tumor growth, likely through a TLR4 dependent mechanism	[13]
LL2 Lewis Lung carcinoma cells/Apc/min+ mice (male and female C57Bl6; subQ/transgenic)	Role of calcitonin-gene related peptide (CGRP) neurons in cancer-associated cachexia	Inactivation of parabrachial CGRP neurons prevents and reverses cancer-induced anorexia, fatigue, and changes in affective behavior	[109]
MADB106 breast cancer cells (outbred "hyperreactive" Wistar rats; subQ)	Role of dopaminergic system in tumor growth/metastasis	Smaller tumors, fewer metastases, and reduced angiogenesis in rats with a hyperreactive dopaminergic system	[84]
K-ras ^{LSL-G12D/+} ;p53 ^{flox/flox} (KP) or K-ras ^{LSL-G12D/+} (K) lung cancer model 129SvJ x C57bl6 mice (cre-dependent p53 deletion)	Effects of circadian disruption (environmental and genetic) on lung tumor growth and progression	Both genetic and physiologic circadian disruption accelerate tumor growth and promote c-myc upregulation and metabolic reprogramming	[61]
diethylnitrosamine-induced hepatocarcinogenesis (male Sprague-Dawley rats)	Sympathetic nervous system effect on hepatocarcinogenesis	High density of SNS bundles associated with poor prognosis, SNS activation of Kupffer cells drives inflammation	[145]
Hepatocarcinoma Morris 7288CTC cells (male buffalo rats) or steroid receptor (SR)-1+ or SR-1- MCF-7 human breast cancer xenografts (female nude rats)	Role of light and melatonin in cancer progression	Melatonin depleted blood accelerates tumor growth and metabolism compared to melatonin-rich blood from healthy women; light accelerates tumor growth in dose-dependent manner	[79]
B16 melanoma cells (male nude mice/C57bl6 D ₂ receptor-KO)	Role of peripheral dopaminergic signaling in tumor growth/angiogenesis/metastasis	6-OHDA ablation of dopaminergic nerves enhanced tumor angiogenesis and growth, likely through D ₂ -mediated mechanism	[146]
GOS Glasgow osteosarcoma and pancreatic adenocarcinoma (P03) xenographs (male B6D2F ₁ mice; subQ into flank)	Effect of suprachiasmatic nucleus lesions on tumor growth	SCN lesions drastically increased tumor size in both cancer models examined	[147]
TC-1 mouse lung cancer cells and human lung adenocarcinoma cells (male C57bl6 mice and obstructive sleep apnea patients)	Effect of sleep fragmentation on plasma exosomes and tumor growth	Chronic sleep fragmentation alters the microRNA cargo of plasma exosomes to promote tumor cell proliferation	[148]
EG, SKOV3ip1, and 222 human ovarian cancer cells (nude male mice)	Effect of stress hormones on cancer invasiveness and growth	Adrenergic and glucocorticoid signaling promotes tumor invasiveness (in part) via upregulation of MMPs	[93]

VTA: ventral tegmental area; cAMP: cyclic adenosine monophosphate; PKA: protein kinase A; 6-OHDA: 6-hydroxydopamine; MMPs: matrix metalloproteinases

DECLARATIONS

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Authors' contributions

Borniger JC contributed solely to this study.

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Not applicable.

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Conflicts of interest

The author declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Complex interaction of adiponectin-mediated pathways on cancer treatment: a novel therapeutic target

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Abstract

Obesity has the far reaching consequence on cancer pathogenesis and immune reactions. In particular, adiponectin (APN) produced by adipocytes played an important role in modulating obesity related malignancies. Via its interaction with corresponding receptors and their downstream signalling pathways, it regulates cells survival, apoptosis and cancer metastasis. Our review dissects the clinical evidence on how hypoadiponectinaemia associated with the increased risks of several cancers and the long-term prognosis and also addresses the controversies. APN also has its indirect effect on anti-cancer immune response which may influence the disease process. We also analyse the impact of APN on the immune system, the anti-tumour responses and the controversies surrounding this area. Targeting therapeutics on APN and its receptor axis represents a promising and novel anti-cancer treatment. Biological understanding of how APN and its interaction with its receptors may affect the immune reactivity. Careful strategizing the use of APN therapeutics in cancer treatment is important, as the APN receptor signalling on the immune cells can blunt anti-tumour response. Targeting APN or its receptors has an enormous implication for the treatment of cancers.

Keywords: Adiponectin, AdipoR1/R2, Cancer, signaling pathway, anti-tumor immunity, therapeutic targets

INTRODUCTION

Adipose tissue is no longer seen as only a passive energy store, but instead a complex metabolic and endocrine organ. Composed of adipocytes, nerve tissue, connective tissue matrix, stroma-vascular cells that



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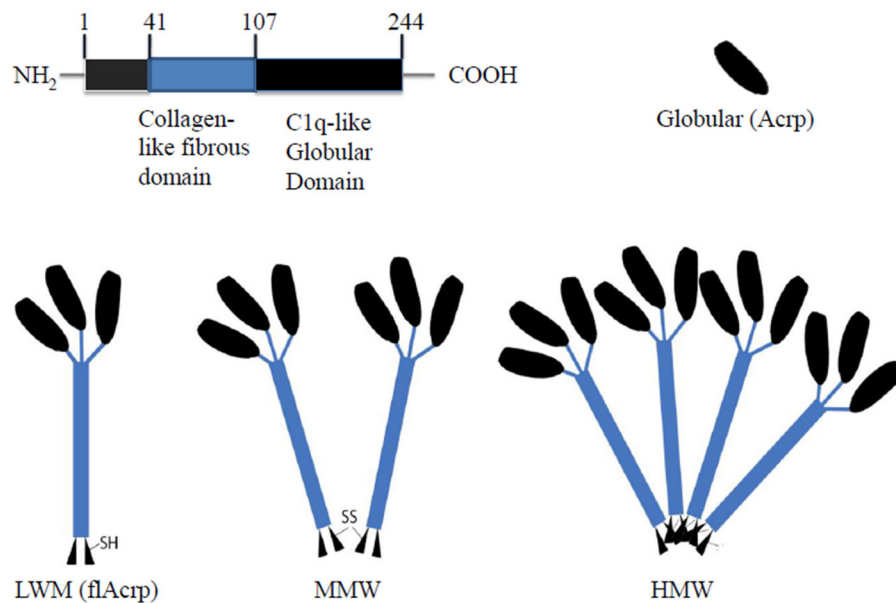


Figure 1. Structure of APN. Low molecular weight. MMW: middle molecular weight; HMW: heavy molecular weight; flAcrp: full length adiponectin; Acrp: globular adiponectin

can orchestrate the immune cells. It expresses, secretes, and responds to a number of key cellular modulators which may have a prominent role in the development and regulation of the disease process in the body^[1]. Adipocytes secrete cell signalling proteins, termed adipokines, of which adiponectin (APN) has been shown to have anti-inflammatory^[2], anti-atherogenic^[3], and anti-diabetic properties^[4,5].

With obesity being linked to approximately 20% of all cancers^[6], harnessing APN may be of great therapeutic benefit, especially with obesity-related cancer. For this to be successful, it is important to note that adipokines can have both anti-inflammatory and pro-inflammatory properties^[4]. When the homeostasis is disrupted, these molecules have the ability to cause harm, yet if we are able to counteract the imbalance, adipokines could pose an important anti-carcinogenic target for the future.

BACKGROUND OF APN AND ITS RECEPTORS

Discovery of APN and its structure

APN was first described in 1995 by Scherer *et al.*^[7] from a cDNA library containing adipocyte-specific genes. Monomeric APN is a 30-kDa glycoprotein encoded by the ADIPOQ gene on human chromosome 3q27. APNs full-length protein (flAcrp) structure was discovered three years later and structurally resembled complement protein C1q and proteins from the tumour necrosis factor (TNF) family^[8]. It consists of a N-terminal region, a hyper-variable sequence, a collagen-like fibrous domain linked to a C-terminal C1q-like globular domain [Figure 1]. Similarities between APN and C1q are seen in folding topology, intron positions, and the formation of trimers. Interactions between the collagen-like domains of three monomeric APNs form the low-molecular-weight (LMW) isoform. The LMW isoform then has the ability to form stable multimeric oligomers. Two LMW isoforms can connect via a disulphide bond to form a middle molecular weight (MMW) hexamer. With the help of post-translational modifications larger 12- or 18-mer high molecular weight (HMW) isoforms are generated^[9].

The majority of APN is produced by white adipose tissue, with lower quantities being produced from brown adipose tissue^[10]. That said, several studies have demonstrated APN being present much lower concentrations in other tissues: cerebrospinal fluid^[11], cardiomyocytes^[12], skeletal muscle^[13], liver^[14], and bone marrow^[15]. The different isoforms are found at varying concentrations throughout the body. The monomeric isoforms

are confined to adipocytes, the oligomeric isoforms are present in the circulation^[16], and the globular form (gAcrp) trimerise to form a stable complex in the circulation.

These different isoforms are thought to have varying biological effects with the HMW isoform being the most biologically active and having the highest plasma concentration. The normal plasma concentration levels of APN range from 2-30 g/mL^[17]. APN levels have been shown to be altered in many disease processes. Coronary heart disease^[18], atherosclerosis^[18], type 2 diabetes^[19], obesity^[20], insulin-resistance^[20], and many cancers are correlated with lower APN levels. Understanding how different isoforms exert effects upon various tissues may be central to understanding the pleiotropic actions of APN within the body.

APN receptors

Three receptors are known to bind with APN: AdipoR1^[19], AdipoR2^[19], and T-cadherin^[21]. AdipoR1 and AdipoR2 have an internal N-terminal region and an external C-terminal region with a central seven transmembrane domain. The topology of these receptors are extremely similar, sharing 67% of their protein sequences^[22]. However, compared to G-protein coupled receptors, their topology is completely opposite. AdipoR1 shows high-affinity binding to globular APN, and also binding to full-length APN and is highly expressed in skeletal muscle. While AdipoR2 shows intermediate-affinity binding to both forms and is predominantly expressed in the liver. More recently, T-cadherin was identified as an additional APN receptor^[21]. It is a glycosylphosphatidylinositol-anchored protein that is highly expressed in endothelial and smooth muscle cells, and has shown specific binding to MMW and HMW isoforms^[23].

APN AND CANCER

A number of cancers have been correlated with hypoadiponectinemia and altered levels of AdipoR1/R2 and T-cadherin. Therefore, APN may be a novel modifiable risk factor with uses as a prognostic or diagnostic biomarker.

Colorectal cancer

It has been long acknowledged that obesity, hyperinsulinaemia, and insulin-resistance are associated with colorectal cancer (CRC) pathogenesis. More recently it has been shown that APN levels are negatively correlated with a risk of developing CRC [Table 1]. This has been confirmed by three meta-analyses^[24-26] and multiple studies summarised in Table 1. Not only did CRC patients have lower levels of APN, but one study showed that men with high APN had a 60% reduced risk of CRC^[27]. A following study showed a similar reduced risk in CRC among men, however no link was seen in females^[28]. This highlights the point that the expression and response to APN can differ between sexes. APN also seems to have a role at the later stages of CRC and an influence on its clinicopathological characteristics. Two studies showed an inverse correlation between tumour stage/grade and APN^[29,30]. Furthermore, low pre-surgical APN levels were found in a higher proportion of relapsing patients, suggesting that APN may have potential to act an adjunctive tool to predict relapse. Mice knock-out models have suggested that interactions with AdipoR1, rather than AdipoR2, have a more protective role as only AdipoR1-KO mice demonstrated increased proliferative activity^[31].

To aid development of any therapy it is important to understand changes that occur to APN within CRC tumour cells. fAcrp can be post-translationally modified to Acrp within the tumour environment, such that the ratio between fAcrp:Acrp is higher than in the surrounding normal tissue^[32]. Local conversion to Acrp could help create a more beneficial tumour environment by functioning in an autocrine manner^[33]. Thus, not only do CRC patients often have lower circulating levels of APN, but also a different proportion of APN subtypes that benefit tumour progression.

Gastric cancer

APN levels have been shown to have an inverse correlation to gastric cancer (GC) [Table 1]. One study found that GC cells lacking AdipoR1 had higher peritoneal dissemination and lymphatic metastases than AdipoR1

Table 1. Epidemiological studies showing the relationship of serum APN and cancer

Study types	Study outcome	Ref.
Colorectal cancer - CRC		
RCC	$P < 0.001$; $P = 0.037$ 1. APN is significantly lower in CRC; 2. APN inversely correlates to tumor stage; 3. Lower APN is associated with CRC recurrence	[29]
	$P < 0.05$ 1. Lower APN; 2. No correlation visceral fat & APN in CRC or adenoma	[67]
	$P < 0.001$ - Lower APN in CRC	[68]
	1. OR = 0.0802 (0.321-1.003) for CRC risk 2. OR = 0.442 (0.189-0.946) for adenoma risk - APN is good marker for adenoma	[69]
	OR = 0.72 (0.53-0.99) for CRC risk, $P = 0.005$ - Lower APN correlates to CRC risk and APN inversely correlates to tumor grade	[30]
PCC	1. RR = 0.55 (0.35-0.86), $P = 0.02$ for men highest vs. lowest quartile 2. RR = 0.96 (0.67-1.39), $P = 0.74$ for women APN significant associated with reduced risk in men but not women RR = (0.23-0.78); P (trend) = 0.01 - Men with low APN had a higher risk of CRC	[28]
	1. RR = 0.71 (0.53-0.95), $P = 0.03$ for total APN when comparing highest vs. lowest quintile 2. RR = 0.45, (0.34-0.61), $P < 0.0001$ for non-HMW APN Total & non-HMW APN inversely correlates to CRC risk	[27]
	$P > 0.05$; OR = 0.8 (0.5-1.4) for highest vs. lowest APN quartile No significance correction between APN & CRC risk	[70]
	$P > 0.05$ - No significance association	[71]
Gastric cancer - GC		
RCC	$P = 0.0004$ - APN levels were significantly lower in Stage I cases than controls	[72]
	$P < 0.005$ - Negative correlation with pathologic findings such as tumor size, depth of invasion, tumor stage (only in undifferentiated GC)	[73]
RCC	$P > 0.005$ - No significant difference in tumor stage, localization, nodal status, lymphatic and vascular invasion	[74]
Esophageal cancer - OC		
RCC	$P < 0.05$; $P < 0.05$ - Significantly lower APN levels in ESCC & EA patients than controls EA patients had lowered APN than ESCC HR = 0.34 (0.14-0.82) - Nonlinear inverse association with risk of EA; the strongest associations were observed in 2nd tertile	[75]
	$P = 0.01$ - Serum APN was significantly lower in cases than controls	[76]
	$P = 0.802$ - APN levels were similar in various esophageal pathologies	[77]
Breast cancer - BC		
PCC	1. Adjusted OR = 0.2 (0.0-0.6); $P < 0.05$; 80% reduced risk in higher APN compared with stage I-III ^[41] 2. Adjusted OR = 0.04 (0.071-0.99) - Lower APN in early breast cancer vs. healthy controls ^[80] 3. $P = 0.04$ - 65% reduced risk in higher APN compared with stage I-III ^[40]	[40,41,80]
	1. $P < 0.005$ (for tumor size); $P < 0.05$ (for grade); > 2 cm tumor & Grade 2&3 BC cases were higher in lower tertile of serum APN ^[42] 2. $P = 0.036$ - Negative correlation with tumor size ^[43] P (trend) = 0.0270 - Inverse trend in ER/PR -ve BC (for not +ve)	[42,43]
	Adjusted HR = 0.39 (0.15-0.95) - Higher APN was associated longer BC (stage I-IIIa) survival	[44]
	Adjusted HR = 0.88 (0.81-0.96); $P = 0.03$; Lower APN was associated with a history of prior pT1mic/pT1a & higher risk of second BC in premenopausal and 12% reduction in risk of BC per unit increase of APN	[81]
	1. $P = 0.017$ ^[83] 2. OR = 0.805 (0.704-0.921); $P = 0.00$ ^[84] Lower APN was associated with nodal disease ^[83,84]	[82]
	Post-menopausal OR = 0.73 (0.55-0.98) but pre-menopausal OR = 1.30 (0.80-2.10) (all women OR = 0.89 (0.71-1.11)) Negative correlation in post-menopausal women $P = 0.43$ for linear trend - No association with risk	[83,84]
Endometrial cancer - EC		
PCC	OR = 0.56 (0.36-0.86) highest vs. lowest APN quartile Negative correlation independent of other obesity-related risk factors OR = 0.42 (0.19-0.94) comparing highest vs. lowest tertile Inverse association with risk of EC Stronger association in pre-menopausal than post-menopausal	[85]
RCC	OR = 0.52 (0.32-0.83); $P < 0.001$ - Significant negative correlation between APN level and EC risk $P < 0.001$; $P < 0.05$ - Significantly lower APN in EC patients than normal or polyps epithelium patients $P < 0.0001$ - Significantly reduced APN in cases than controls; Leptin:APN ratio correlated to post-menopausal EC risk	[86]
		[48]
		[87]
		[88]
		[89]

PCC	All Women: OR = 0.86 (0.53-1.39) and Post-menopausal: OR = 0.66 (0.29-1.50) Non-significant result (pre-diagnostic APN is not predictive of EC risk)	[50]
Pancreatic cancer - PC		
PCC	OR = 0.44 (0.23-0.82) - In never smoker, higher APN levels correlated to reduced risk of PC; OR = 1.59 (0.67-3.76) - No significance in smoker	[54]
	OR = 0.55 (0.31, 0.98); $P = 0.03$ for highest vs. lowest quintile - Higher APN were negatively correlated with PC risk	[52]
	Nonlinear association ($P < 0.01$) - Low pre-diagnostic levels of APN were associated with an elevated risk of PC	[89]
RCC	$P = 0.0035$ - Median APN levels significantly higher in PC group than controls/chronic pancreatitis group	[90]
	$P < 0.001$ - Greater APN:Leptin ration in PC patients	[91]
	OR = 2.81 (1.04-7.59) - Higher APN correlated with higher odds of PC and No association with PC stage	[53]
Renal cell carcinoma - RCC		
RCC	OR = 0.76 (0.57-1.00) $P = 0.05$ - Serum APN negatively correlated with RCC risk	[56]
	$P < 0.01$ (tumor size); $P = 0.029$ (non-metastatic vs. metastatic); $P = 0.044$ for total APN & $P = 0.041$ for HMW (non-metastatic vs. metastatic) - Strong inverse correlation was found between plasma APN levels and tumor size ^[57] , Lower APN in metastatic cases ^[57,58]	[57] [58]
	OR = 2.3 (1.1-4.6) - Higher APN was associated with RCC risk among African American males	[59]
Hepatic cancer - HC		
PCC	OR = 0.5 (0.22-1.15) for highest vs. lowest tertile for LMW APN - Higher % of LMW APN may be have a protective effect on HC	[92]
RCC	$P = 0.670$; $P = 0.752$ - No significance difference between cases and controls	[93]
Prostate cancer - PrC		
PCC	OR = 0.27 (0.07-0.87) for highest vs. lowest quintile for lethal PrC - Men with higher APN had reduced risk of HG or lethal PrC but overall no significant correlation between serum APN & PrC risk	[94]
NCC	OR = 0.86 (0.66-1.11), $P = 0.24$ for all men; OR = 0.62 (0.42-0.90), $P = 0.01$ for obese & overweight men APN levels negatively associated with PrC risk in obese & overweight men but not significant in all men	[60]
RCC	OR = 0.29 (0.10-0.89) for highest vs. lowest quartile - Reduced risk with higher APN independent of various confounders	[95]
	$P < 0.05$ - Significantly reduced APN in PrC patients	[96]
	$P < 0.001$ - APN levels in HG PrC were also significantly lower than LG or IG groups	[97]
PCC	OR = 0.87 (0.46-1.65) for highest vs. lowest tertile - No significance but the sensitivity of assays used was problematic	[98]
Lung cancer - LC		
RCC	$P < 0.0001$ - Leptin: APN ratio was significantly lower in the patients group compared to controls	[99]
	OR = 1.13 (0.80-4.97); OR = 0.25 (0.10-0.78) - Even though APN levels were not significantly different in cases than controls but they are significantly lower in advanced than limited disease stage	[64]
	$P > 0.05$ ^[100] ; OR = 2.00 (0.80-4.97), $P = 0.14$ ^[101] - No significance	[100,101]

A summary on the clinical data showing the association of serum APN with various cancers is shown. Many studies show that low levels of APN is associated with cancer risk and its progression of disease. CRC: Colorectal cancer; PCC: prospective case-control; RCC: Retrospective case-control; NCC: nested case control; OR: odd ratio; RR: relative risk; BC: breast cancer; EC: endometrial cancer; GC: gastric cancer; OC: oesophageal cancer; ESCC: esophageal squamous cell cancer; EA: esophageal adenocarcinoma; PC: pancreatic cancer; HC: liver cancer; RCC: renal cell cancer; PrC: prostate cancer; HG: high grade; LG: low grade; IG: intermediate grade

positive cells^[34]. Despite, the AdipoR1 positive group having longer survival rates, multivariate analysis indicated this was not an independent prognostic factor on GC survival. That said, it is thought AdipoR1 is the mediator of tumour growth suppression in GC and therefore could be a therapeutic target in tackling GC. One study demonstrated that rs266729, an ADIPOQ variant, may be an independent prognostic factor for non-drinking GC patients receiving surgical treatment^[35]. Similarly, APN levels have been shown to have a direct effect in GC postoperative outcomes. It was shown that the ratio of postoperative to preoperative levels of APN was the most useful predictor for postoperative infection^[36]. It is thought that reduced APN levels suggest a submaximal inflammatory response, thus predisposing to infection^[32]. Increasing APN in the weeks before surgery could be an additional method to improve surgical outcomes.

Oesophageal cancer

APN plays a significant role in the maintenance of the normal oesophageal mucosa^[32]. Lower levels of APN have been correlated with an increased risk of oesophageal cancer (OC) [Table 1]. Low tumour expression of AdipoR1 was suggested to be an independent predictor of improved overall OC survival^[37]. While, AdipoR2

expression was inversely correlated with tumour size. It was shown that low levels of LMW and high levels of HMW APN compared to the total APN ratio increased the risk of Barrett's oesophagus, which contributes to OC propagation^[38]. Looking at the dynamic changes between LMW and HMW APN levels during OC transformation could present an interesting predictive biomarker.

Breast cancer

A recent meta-analysis confirmed a link between hypoadiponectinaemia and an increased breast cancer (BC) risk^[39]. This increased risk was only found in post-menopausal women and not pre-menopausal women. Risk reduction in early BC patients with high APN levels has been reported to be from 65%-80%^[40,41]. A negative correlation has been demonstrated between APN levels and tumour size and grade^[42,43]. Interestingly, the correlation between APN and BC seems to be more prominent in oestrogen-negative and progesterone-negative BC^[44]. Therefore, it seems there may be a set group of BC patients that are most susceptible to the effects of APN and would benefit most from a potential treatment. That said, certain studies have shown no correlation between APN and BC risk^[45].

Endometrial cancer

A significant negative correlation has been found between levels of APN and endometrial cancer (EC)^[46] [Table 1]. A meta-analysis found that high levels of APN reduced the risk of EC by 53%^[47]. Whether APN levels effect pre- or post-menopausal women is unclear as studies have produced varying results^[47-49]. One proposed protective mechanism of action is that APN can reduce the number of EC cells by inhibiting proliferation and stimulating apoptosis^[49,50]. One study looked into the serum levels of the APNs isoforms and found that low levels of MMW isoform were the only independent risk factor for EC^[51]. It is unclear why this isoform in particular was most closely associated with EC, but highlights the pleiotropic effects APN exerts through its multiple isoforms.

Pancreatic cancer

The association between APN levels and pancreatic cancer (PC) is unclear from current studies [Table 1]. Some studies demonstrated a negative correlation between serum APN and PC^[52], while others showed high APN levels increasing the risk of PC^[53]. Of note, high APN levels were associated with a reduced PC risk in people that never smoked, but no significance found in smoker^[54]. PC has significant mortality associated with its diagnosis with a 5-year survival of only 9%^[55], therefore early detection using biomarkers could significantly alter treatment and prognosis. However, further prospective studies are needed to confirm APN as a PC marker.

Renal cancer

A negative correlation has been shown between serum APN and renal cancer (RCC), tumour stage, and metastasis^[56-58]. That said, one study showed how APN may exert different effects depending on ethnicity as a positive correlation was found between APN and RCC in African Americans but not Caucasians^[59].

Prostate cancer

A negative correlation between APN levels and prostate cancer (PrC) tumour grade and stage has been demonstrated in several studies [Table 1]. One study showed that the reduced risk was only found in obese and overweight men, but not all men^[60]. Furthermore, four ADIPOQ single nucleotide polymorphisms (SNP) (rs266729, rs182052, rs822391 and rs2082940) were shown to be significantly associated with PrC risk in Caucasians^[61]. Once again highlighting the differing effects of APN in different ethnicities.

Hepatic cancer

The relationship between hepatic cancer (HC) and APN levels is unclear [Table 1]. Certain studies showed that higher APN levels predicted a poorer survival with HC^[62]. APN expression pairs with tumour size but not several other parameters, therefore the rise in APN with tumour tissue may occur throughout the

process of carcinogenesis in an attempt to counteract the change and restore^[32]. Others studies suggested poor HC prognosis was associated with lower AdipoR1/R2 expression^[63]. AdipoR1 expression was correlated with the absence of vascular invasion and AdipoR2 was associated with a low stage and a lower histological grade. Although the role of APN in HC is unclear, APN may have a role pre-malignant hepatic conditions such as non-alcoholic steatohepatitis (NASH)^[32]. APN can suppress the hepatic damage caused by TNF- α in early NASH, thus highlighting an area where targeted APN treatment could help halt progression to cirrhosis and cancer.

Lung cancer

The majority of studies have not found a significant correlation between APN levels and lung cancer (LC) [Table 1]. Yet, one study demonstrated that there were lower levels of APN were present in patients with advanced disease compared to limited stage disease^[64]. AdipoR1 and AdipoR2 were only expressed in the cancerous tissue, with non-found in normal control tissue. Further studies are needed to determine a link between APN and LC.

Genetic polymorphisms

Multiple genetic polymorphisms have been associated with altered risk of malignancy in ADIPOQ, ADIPOR1, and ADIPOR2^[65]. These SNPs have been associated with a wide number of cancers and may enable screening and identification of high risk patients. This can allow for primary preventive measures, such as losing weight, diet, and exercise, as well as chemoprevention^[66]. Going forward we need to be take into consideration the interaction between various SNPs, ensure that a large and diverse group of the population are screened for more SNPs, and determine how SNPs can be applied to clinical decision making.

DIRECT EFFECT OF APN PATHWAY ON CANCER BIOLOGY

Insulin resistance and hypoadiponectinemia have been associated with multiple cancers. The binding of APN to AdipoR1/R2 triggers a number of downstream signalling cascades which have effects on cancer biology and anti-cancer immunity. Understanding these pathways can help give light to the multitude of APNs metabolic and immunological effects. These pathways are summarised in Figure 2.

Central to much of APNs actions is the AMP-activated protein kinase (AMPK) pathway^[102,103]. AMPK can act as a tumour suppressor by modulating inflammation, inducing cell-cycle arrest, and opposing metabolic changes during carcinogenesis^[104]. AMPK activation has been shown to have a beneficial effect on insulin sensitivity by increasing glucose uptake and fatty acid oxidation in muscles, and inhibiting gluconeogenesis in the liver. Interestingly, disruption of AdipoR1 reduced the AMPK activation, increased glucose production and impaired insulin resistance. While, AdipoR2 disruption enhanced insulin resistance and decreased PPAR α signalling^[20]. Downstream inhibition of the mTOR pathway contributes to AMPKs tumour suppressive qualities^[105,106]. This is due to mTORs central role as a regulator of cell growth, autophagy, and cell survival^[107]. AMPK activation has also been shown to be cytotoxic to cancer cell lines and cause apoptosis via p21 and p53 signalling^[108]. AMPK has the ability to monitor and respond to cellular energy requirements. In times of hypoxia, nutrient starvation, and redox imbalance, liver kinase B1 (LKB1) - its upstream kinase - activates AMPK. The importance of LKB1 can be seen in patients with Peutz-Jegher syndrome who are heterozygous for a *LKB1* gene mutation. Development of a second mutation in life then greatly increases the risk of multiple cancers. It is hypothesised that the loss of AMPK activation may contribute to this development of malignancy^[109]. LC and HC patients have shown evidence of LKB1 mutations and low LKB1 expression respectively^[104]. Therefore, APN mediated AMPK activation could help prognosis and disease progression in these specific cancers.

The PI3K/Akt pathway promotes cellular survival, growth, and proliferation^[110]. This signalling pathway is specifically known to be involved in gastrointestinal cancers (GI) whereby upstream mutations lead to

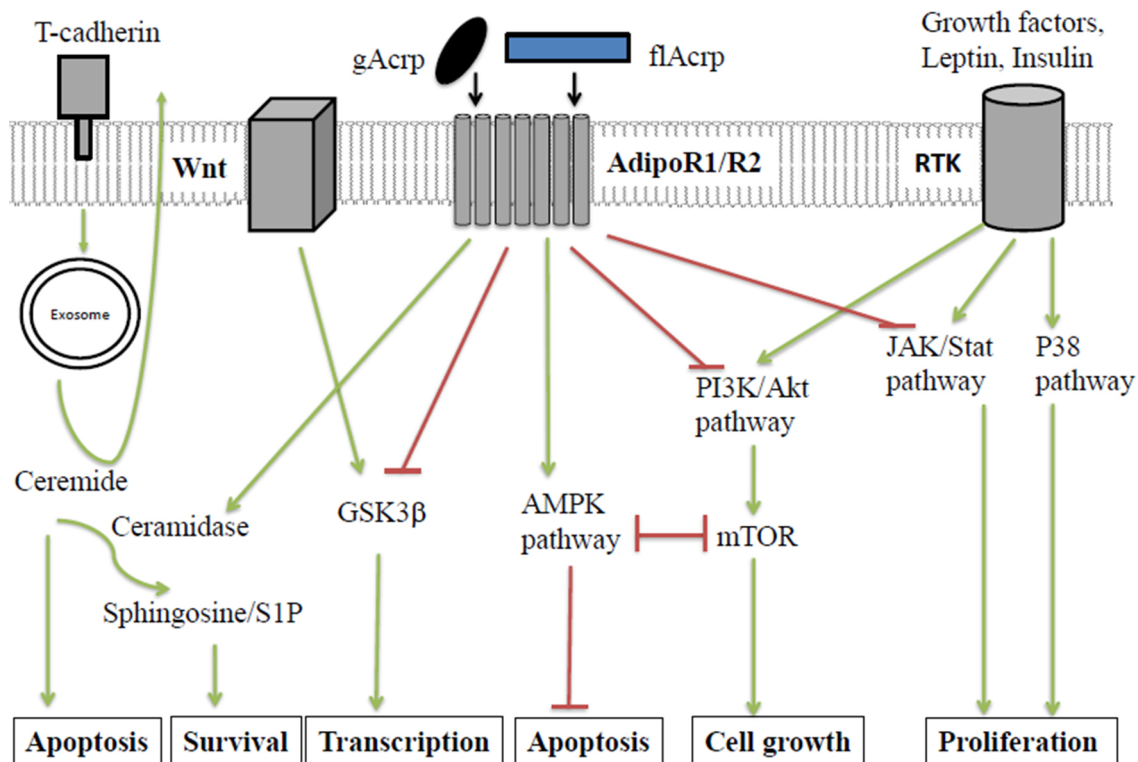


Figure 2. Direct effects of APN on epithelial cells and summary of APN's anti-cancer signaling pathways. In normal epithelial cells, APN is bound by T-cadherin and presented directly or indirectly to AdipoR1/R2 to inhibit signaling pathways activated in neoplasia. APN activates AMPK, and inhibits PI3K/AKT, mTOR, MAPK and JAK/Stat pathways, or directly affects GSK3β to suppress cancer promoting pathways. Cancer cells down-regulate T-cadherin while AdipoR1/R2 expression persists and cancer-promoting pathways prevail. One model is that ceramidase activity associated with AdipoR1/R2 weighs the balance in favor of cancer cell survival. T-cadherin expressed in the tumor vasculature promotes cancer as a pro-angiogenic factor in cooperation with APN (not shown). Green arrows represent activating pathways. Red lines represent inhibitory pathways. gAcrp: globular adiponectin; fAcrp: full length adiponectin; RTK: receptor tyrosine kinase; JAK: Janus kinases; Stat: signal transducer and activator of transcription proteins; PI3K: PI3-kinases; Akt: protein kinase B; mTOR: mammalian target of rapamycin; AMPK: 5' AMP activated protein kinase; GSK3β: glycogen synthase kinase-3 beta

its activation^[32]. Several factors related to obesity have been shown to induce this pathway and potentially result in carcinogenesis, thus making it a good target for obesity related GI cancer. APN can directly^[111] and indirectly^[112] inhibit the PI3K/Akt signalling pathway.

APN also interacts with several other pathways that are involved in carcinogenesis and the maintenance of tumour survival including: the Wnt/GSK3B/B-catenin-signalling pathway^[113], signal transducer and activator of transcription-3 (STAT-3), and mitogen activated protein kinase (MAPK). APN inhibits STAT-3 which has a role in cancer cell proliferation, invasion, and survival^[114]. STAT-3 promotes pro-oncogenic inflammatory pathways, including NF-κB and IL-6-Janus tyrosine kinase, and inhibits the STAT-1 and NF-κB mediated T helper 1 anti-tumour immune response^[114]. Both gAcrp and fAcrp have the ability to suppress STAT-3 and the associated JNK pathways in HC and PrC^[115]. APN also interacts with the MAPK which signals through cJNK, MAPKp38, and extracellular signal-regulated kinases (ERK)1/2^[40,116,117].

Multiple studies have found that AdipoR1/R2 are over-expressed in cancers. In the setting of hypoadiponectinemia it makes sense to have a compensatory upregulation of the receptors. However, this seems counter-intuitive as the cancer cells are over-expressing receptors that act to hinder their growth and proliferation. It is suggested that in a metabolically deprived tumour environment the cells metabolic needs take priority^[17]. That said, it has been shown that APN can also have proliferative effects on cancer cells^[118]. Further studies looking into how the expression of APN's receptors changes throughout tumorigenesis are important as the biological effects of APN depend on the tissue-specific expression of these receptor subtypes^[32].

One of the mechanisms by which APN prevents cancer growth is by promoting apoptosis via cytotoxic autophagy. A recent study using BC cells demonstrated that APN induces an accumulation and fusion of autophagosomes and lysosomes which leads to apoptosis. Central to this pathway is the AMPK mediated activation of the ULK1 (Unc-51 like autophagy activating kinase 1) axis which is regulated by upstream STK11/LKB1 (serine/threonine kinase 11/Liver kinase B1)^[119]. Autophagy can be cytoprotective, cytotoxic, cytostatic, or non-protective. Due to the cytoprotective role of autophagy many studies have looked to see whether inhibition of autophagy can be used in combination with chemotherapy to increase sensitivity to therapy^[120]. In contrast, this study has shown that by stimulating cytotoxic-autophagy it can enhance efficacy of chemotherapy. The dual role of autophagy in tumour suppression and promotion makes utilising this pathway therapeutically challenging and more studies are needed to determine how APN induced autophagy can be used in combination with other treatments.

The intracellular accumulation of ceramides have been associated with pathogenesis such as insulin resistance and endothelial dysfunction^[121,122]. Activation of ceramidase enhances ceramide catabolism to the anti-apoptotic sphingosine-1-phosphate^[123]. A recent study showed that APN signalling through T-cadherin, but not AdipoR1/R2, reduced cellular ceramide by enhancing exosome biogenesis and secretion^[124]. This is a novel mechanism by which APN can lower intracellular ceramide and prevent cellular damage. Unlike AdipoR1/R2, T-cadherin is usually downregulated in cancer cells and re-expression of the receptor has been associated with a better prognosis^[125]. Despite APNs anti-oncogenic pathways, dominance of the ceramidase pathway may enable over expression of AdipoR1/R2 to be pro- rather than anti-oncogenic. When assessing the overall effect of APN, it is important to consider the time at which AdipoR1/R2 are overexpressed in cancer, the concentration of serum APN, and which isoform is circulating.

INDIRECT EFFECT OF APN PATHWAY ON ANTI-CANCER IMMUNITY

Chronic inflammation in adipose tissue, mediated by adipokine signalling, and immune system dysfunction hold important roles in cancer initiation^[126]. APN has been shown to have anti-inflammatory properties through interaction with the innate and adaptive immune system [Table 2]. The majority of this is mediated by antigen-presenting cells (APC) of the innate immune system that produce anti-inflammatory cytokines and inhibit pro-inflammatory cytokines. That said, APN has also been shown to have pro-inflammatory effects [Table 2], suggesting that there may be situations where APNs effects shift from anti- to pro-inflammatory.

One of the central anti-inflammatory cytokines APN induces is IL-10 [Table 2]. The mechanism of IL-10 transcription from APN may be mediated through several pathways. One known pathway is that gAcrp exposure leads to AMPK and ERK1/2 mediated CREB transcription of IL-10^[129]. Multiple studies have demonstrated that gAcrp is the best inducer of IL-10. However, one study showed that activation of AdipoR1 by either gAcrp or fAcrp can upregulate IL-10 through the AMPK and MAPKp38 pathway [Figure 3]. IL-10 has a multitude of anti-inflammatory effects^[164], one of these may be exerted through activation of the STAT3/suppressor of cytokine signalling (SOC3) pathway. Activation of the IL-10 receptor activates this pathway which leads to downstream inhibition of NF-κB [Figure 3].

Although APN levels are generally negatively correlated with cancer risk, high expression of the APN receptors has equally been correlated with tumour progression. Activation of upregulated AdipoR1 and AdipoR2 on dendritic cells (DC) results in the arrest of DCs in an immature state and development of tolerance to tumour antigens^[117]. Each APN receptor has a separate pathway leading to inhibition of the NF-κB pathway thought to induce DC tolerance [Figure 3]. That said, suppression of the NF-κB pathway is a central anti-inflammatory mechanism that APN utilises [Table 3]. Activation of AdipoR1 results in a IL-10 dependent activation of the STAT3/SOC3 pathway and inhibition of the NF-κB pathway. A STAT3-knockout-DC study demonstrated that STAT3 is essential in developing the IL-10 dependent tolerance^[165].

Table 2. Effect of APN on the immune system

Anti-inflammatory effects	
Monocytes	Inducing anti-inflammatory cytokine; IL-10 ^[127] , IL-1RA ^[127]
Macrophages	<ol style="list-style-type: none"> 1. Inducing anti-inflammatory cytokines; IL-10^[127-138], IL-1RA^[127], IL-4^[130] 2. Suppressing pro-inflammatory cytokines; IL-1β^[128], IL-6^[131,132], TNF-α^[128,130,137-139] 3. Less cellular infiltration in disease models^[128,140] 4. Down-regulating chemokine; IP10/CXCL10^[141], I-TAC/CXCL11^[141], Mig/CXCL9^[141], CCL18 5. Inducing mannose receptors^[128] 6. Clearing early apoptotic cells via CRT pathway^[2] 7. Inhibiting MMP-12^[142] or inducing TIMP-1 due to IL-10 effect (independent of MMP-9 inhibition)^[135] 8. Inducing type 1 arginase^[128], HO-1^[130] 9. Suppressing scavenger receptors; MSR-AI^[132], class A^[139] 10. Inhibiting TF^[143] 11. Suppressing cellular maturation^[144]
Dendritic cells	<ol style="list-style-type: none"> 1. Inducing anti-inflammatory cytokine; IL-10^[127,145] & IL-1RA^[127] 2. Attenuating pro-inflammatory cytokine; IL-1β^[145], IL-8^[145], IL-12p40^[145,146], INF-γ^[145], TNF-α^[145] 3. Inhibiting positive co-stimulator (CD80^[145,146], CD86^[145,146], CD40^[145]) 4. Down-regulating HLA II^[145,146] 5. Up-regulating negative co-stimulator (PD1-PDL1^[146]) 6. Inhibiting ROS^[145] 7. Suppressing cellular maturation^[145,146]
Endothelial cells	<ol style="list-style-type: none"> 1. Inhibiting adhesion molecule, ICAM-1 (CD54)^[147], E-selectin^[148], sVEGFR1^[148] 2. Attenuating infiltration of immune cells into disease models^[141,149] 3. Inhibiting apoptosis^[18,150] 4. Suppressing pro-inflammatory cytokine; TNF-α^[18,151], IL-6^[148,152], IL-8^[153] 5. Promoting EPC function & localisation^[154]
Natural killer cells	Down-regulating TRAIL ^[155] & Fas Ligand ^[155]
T cells	<ol style="list-style-type: none"> 1. Inhibiting antigen-specific T cell proliferation^[145,146,156] 2. Inhibiting pro-inflammatory cytokine; IL-1β^[145], IL-2^[156], IL-8^[145], TNF-α^[156], INF-γ^[145,156] 3. Inducing T cell apoptosis^[156] 4. Less cellular infiltration in disease models^[141,149] 5. Promoting Th2 differentiation^[145]
Regulatory T cells	<ol style="list-style-type: none"> 1. Inducing expansion of Treg^[146] 2. Inducing T cell anergy^[145]
B cells	Inhibiting B lymphopoiesis ^[157]
Liver cells	<ol style="list-style-type: none"> 1. Inducing anti-inflammatory cytokine, IL-10^[130], IL-4^[130] 2. Inhibiting pro-inflammatory cytokines, TNF-α^[130,140], IL-1β^[140], IL-6^[140], CCL-2^[140], CXCL10^[140], ICAM-1^[140] 3. Alleviating apoptosis^[140]
Pro-inflammatory effects	
Macrophages	Only inducing transient TNF-α ^[132,133,138] , IL-6 ^[132] (subsequently suppressed by IL-10) or IL-6 (via IRS2 through unidentified APN receptor ^[158])
Dendritic cells	<ol style="list-style-type: none"> 1. Promoting positive co-stimulators; CD86^[159], CD40^[159] 2. Up-regulating HLA II^[159] 3. Inducing pro-inflammatory cytokines, IL-12^[159], IL-6^[159], IL-1β^[159], IL-23^[159] <p>*(APN suboptimal)</p>
T cells	<ol style="list-style-type: none"> 1. Activating T cells via induction of INF-γ^[160], IL-6^[160] 2. Promoting Th1 & Th17 differentiation^[159] <p>*(APN suboptimal)</p>
Liver cells	Inducing pro-inflammatory chemokine, CXCL8 ^[161]
Fibroblasts	Inducing pro-inflammatory cytokines; IL-6 ^[162,163] **
	** (fIAcrp + suboptimal)

This table summarizes the controversies surrounding the role of APN on the immune system. On balance, majorities of work support the role of APN regulating the immune system negatively. However, the caveats associated with the ability of APN to stimulating the immune system are also demonstrated (as per discussed in the text). The body of evidence on this area tends pale into insignificance comparing to the large experimental data supporting the notion of APN as an immune modulator. IRS-2: insulin receptor substrate-2; ROS: radical oxygen species; C/EBPα: CCAAT/enhancer binding protein-α; MCP-1: monocyte chemoattractant protein-1; EPC: endothelial progenitor cell; eNOS: endothelial nitric oxide synthase; IP-10: interferon (IFN)-inducible protein-10; I-TAC: IFN-inducible T cell alpha chemoattractant; Mig: monokine induced by IFNγ, M1 markers (TNF-α, IL-6, MCP-1), CRT/CD91; CRT: calreticulin; HO-1: heme oxygenase-1; TF: tissue factor; Treg: regulatory T cells

Despite STAT3 being recognised as the dominant mediator for IL-10 functions, this pathway can mediate anti-inflammatory effects in an IL-10 independent manner^[166]. This includes downstream activation of ETS family transcriptional repressor, ETV3 and a helicase family corepressor, Strawberry notch homologue 2 (SBNO2) that can inhibit NF-κB activation^[167].

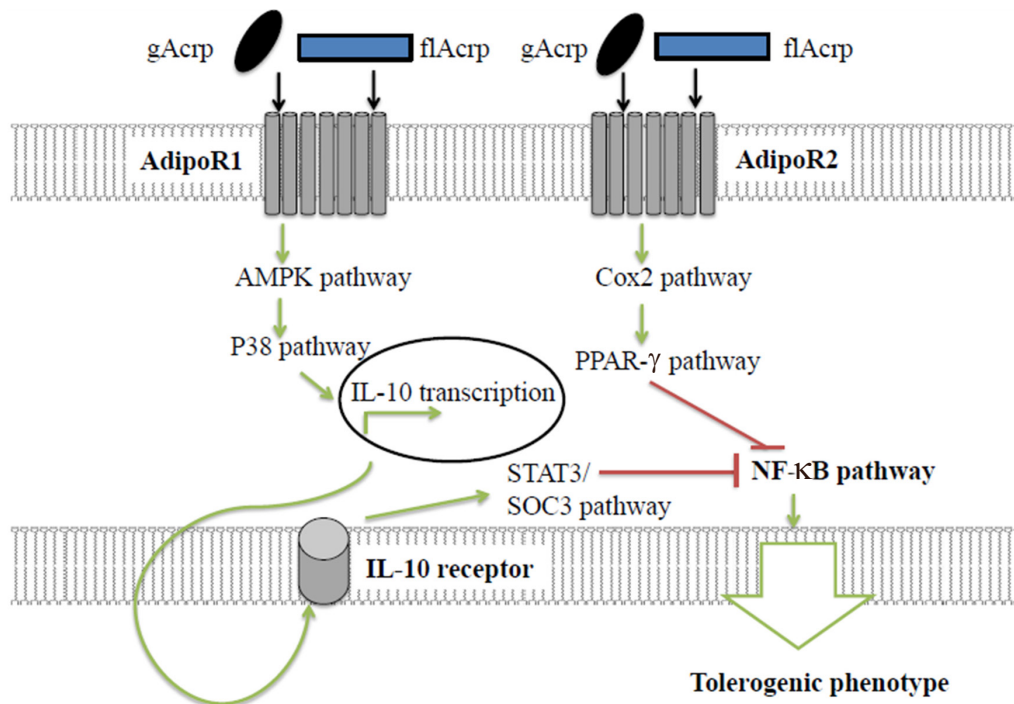


Figure 3. AdipoR1 and AdipoR2 mediated tolerance of dendritic cells. APN generates dendritic cell tolerance via two distinct pathways that converge upon NF- κ B inhibition. Green arrows represent activating pathways. Red lines represent inhibitory pathways. gAcrp: globular adiponectin; fAcrp: full length adiponectin; AMPK: 5' AMP activated protein kinase; Cox2: cyclooxygenase-2; PPAR- γ : peroxisome proliferator-activated receptor gamma; NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cells; STAT3: signal transducer and activator of transcription 3; SOC3: suppressor of cytokine signalling 3

The second distinct tolerogenic pathway is activated through AdipoR2 [Figure 3]. In AdipoR1-depleted DCs, use of IL-10 receptor antibodies had no effect on the DCs ability to induce T-cell tolerance - suggesting this is a IL-10 independent tolerance process. Instead, the COX-2 pathway upregulates peroxisome proliferator-activated receptors- γ (PPAR γ), inhibiting the NF- κ B pathway, and promoting anergic phenotypes on engaging T cells^[177]. After DCs interact with allogenic T cells the PPAR γ pathway drives Th2-cytokine production. Treating DCs with PPAR γ agonists produced similar responses^[176]. DCs with a more dominant AdipoR2 signalling produced significantly more Th2-skewing cytokine production than AdipoR1-dominant DCs^[177]. It is thought that the SOCS3 pathway may cause the smaller AdipoR1-dependent Th2-cytokine production^[177]. The role of PPAR γ is further confirmed through a PPAR γ -knockout model that prevented tolerance developing^[177]. It was thought that downstream NF- κ B effectors may contribute towards T-cell tolerance. However, inhibiting anti-NF- κ B effectors was unable to prevent DC promotion of T-cell anergy^[177]. These studies demonstrate AdipoR1/R2 pathways that enable DC-tolerance and allow cancer evade the immune system.

The role of T-cadherin and APN on the immune system is less known. One proposed mechanism is immune modulation through the enhancement of exosome biogenesis and secretion^[124]. When exomes are released from a cell, this may protect cells by modulating the immune function of recipient cells^[178], or stimulating repair of neighbouring cells^[179].

One study showed that T-cadherin is upregulated on chronic cold exposure, enabling APN to be recruited to M2 macrophage cell surface, and promote macrophage proliferation^[180]. Activation of browning and thermogenesis via this pathway may be a further mechanism that APN uses to protect from metabolic stress. Interestingly, in an AdipoR1 and AdipoR2 depleted system, APN-induced T-cell anergy was reversed, suggesting that T-cadherin mediated APN signalling is unlikely to contribute to this process^[177].

Table 3. Molecular signalling of APN on the immune system and its implication

Anti-inflammatory mechanisms	
Cells	Intracellular signalling mechanisms
Macrophages	<ol style="list-style-type: none"> 1. Suppression NF-κB pathway via inhibition of ERK1/2 & MAPK p38^[131], c-JNK & MAPKp38^[168], STAT3^[168] pathways 2. Suppression of NF-κB via activation of AMPK pathway^[128] 3. Induction of CREB-mediated transcription of IL-10 through activation of AMPK^[129,130] & ERK1/2^[129] pathways (gAcrp^[130] via AdipoR1^[130]) 4. Activating STAT4-mediated IL-4 transcription (flAcrp)^[130] 5. Induction transcriptosome resembling M2 rather than M1^[169]
Dendritic cells	<ol style="list-style-type: none"> 1. Suppression of NF-κB via activation of AMPK & MAPK p38 pathways that in turn activate IL-10-dependent STAT3 & SOCS3 pathways (through AdipoR1)^[145] 2. Suppression of NF-κB & ROS via activation of COX2 & PPARγ pathway (through AdipoR2)^[145]
Natural killer cells	Activation of AMPK to inhibit NF- κ B ^[155]
Endothelial cells	<ol style="list-style-type: none"> 1. Inhibiting ROS & apoptosis^[18,150] via activation of AMPK^[18] 2. Suppression of NF-κB via induction of C/EBP^[151], via activation of AMPK/Akt pathway (independent of ERK1/2, MAPKp38, c-JNK)^[153] 3. Activating AMPK/Akt pathway to induce eNOS^[140,170,171], activating AMPK (inhibiting ERK1/2)^[172] to inhibit hypertrophic signals, activating AMPK via T-Cadherin^[173] 4. Activating CRT/CD91-mediated COX2 activation^[174], activating COX2 to modulate pro-inflammatory cytokine production^[18] 5. Cav-1-mediated ceramidase pathway (only via AdipoR1)^[147]
T cells	Co-localization of negative costimulatory and inducing Capas ^[156]
B cells	Activation of COX2 ^[36]
Interaction with chemokines	Direct binding to chemokines to neutralize its effects (SDF-1, CCF18, MIP1a, RANTES, MCP-1 (via gAcrp) ^[157]
Liver cells	<ol style="list-style-type: none"> 1. Suppression of NF-κB pathway via inhibition of ERK1/2 and MAPK p38 pathways^[175] 2. Activating AMPK via AdipoR1^[19,20,103] & PPARα via AdipoR2^[19,20] 3. Activating STAT3 to inhibit NF-κB^[161]
Proinflammatory mechanisms	
Macrophages	<ol style="list-style-type: none"> 1. Transient activation of NF-κB via activation of ERK1/2 & Egr-1 pathways (that subsequently suppressed by its IL-10 induction)^[133,138] (gAcrp) 2. Activation of NF-κB via activation of STAT-3 (that induces IRS-2 causing IL-6) (independent of AdipoR1/R2)^[158] 3. Induction of transcriptosome mimicking pro-inflammation (no M1/M2 distinction)^[160]*
Dendritic cells	Activation of NF- κ B via PLC γ & JNK pathways ^[159] * *(APN suboptimal)
T cells	Augmenting T-bet expression via activation of MAPKp38 & STAT4 pathways ^[160] * *(APN suboptimal)
Liver cells	Activating NF- κ B via MAPKp38 & ERK1/2 pathways but is independent of AdipoR1/R2 ^[161]
Fibroblasts	Activating NF- κ B via AMPK ^{[162,163]**} , MAPK p38 ^{[162,163]**} (via AdipoR1) ^{[162]**} + ER1/2 ^{[163]**} pathways **(with flAcrp & suboptimal dose)

Summary for the molecular mechanism associated with the effect on APN in the immune system is presented here. Current evidence support many molecular pathways are triggered by the APN and its receptors to regulate various arms of the immune system. APN can negatively affect both innate and adaptive immunities. Again, some mechanism on how it can stimulate the immune system is also demonstrated. M2 markers (IL-10, arginase-1, macrophage galactose N-acetyl-galactosamine specific lectin-1). Cav-1: caveolin-1; SDF-1: stromal cell derived factor-1; MIP-1 α : macrophage-inflammatory protein-1 α ; MCP-1: monocyte chemoattractant protein-1

APN has the ability to polarise macrophages towards a M2 anti-inflammatory rather than a M1 pro-inflammatory phenotype [Figure 4]. The administration of APN stimulated expression of M2 markers and reduced expression of M1 markers in human macrophages and adipose tissue^[169]. APN mediated macrophage polarisation is thought to occur in a IL-10 dependent manner^[127,129,131,135,138,169,181,182]. The gAcrp and flAcrp polarise macrophages by using separate signalling pathways. The gAcrp stimulates CREB that results in an IL-10 mediated M2 polarisation^[129]. Whereas, flAcrp utilizes a IL-4/STAT6-dependent mechanism^[182]. One study demonstrated that flAcrp, through AdipoR2, greatly polarised macrophages to an M2 phenotype. Whereas, gAcrp signalling via the AdipoR2, was much less effective at polarising to the M2 phenotype^[182].

There is significant evidence showing that APN can suppress pro-inflammatory cytokines including TNF- α , IL-1B, IL-6 [Table 2]. Multiple studies have linked APNs anti-inflammatory effects to TNF- α suppression.

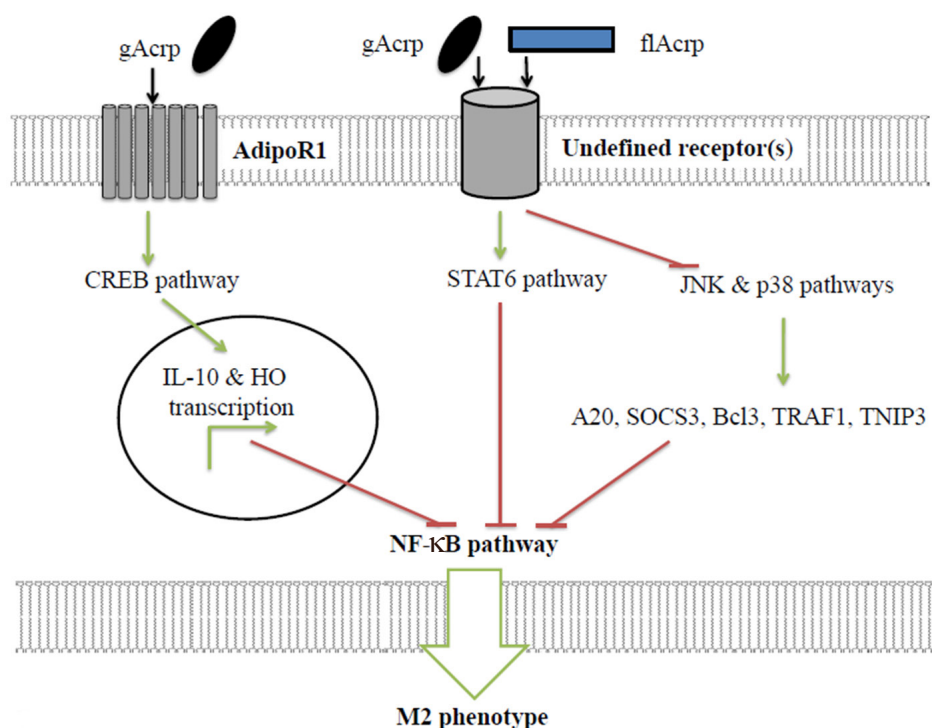


Figure 4. APN-mediated polarisation of macrophages to anti-inflammatory M2 phenotype. Through AdipoR1 and undefined receptors APN causes NF- κ B inhibition and promotion of M2 phenotype. Green arrows represent activating pathways. Red lines represent inhibitory pathways. gAcrp: globular adiponectin; fAcrp: full length adiponectin; STAT6: signal transducer and activator of transcription 6; CREB: cAMP response element-binding protein; JNK: c-Jun N-terminal kinase; SOCS3: suppressor of cytokine signalling 3; Bcl3: B-cell lymphoma 3-encoded protein; TRAF1: TNF receptor-associated factor 1; TNIP3: TNFAIP3-interacting protein 3; NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cells

APN-knockout mice showed high levels of TNF- α in both plasma and adipose tissue^[5]. APN also inhibits a TNF- α induced adhesion molecule^[183] which was found to be in high expression in APN-knockout mice^[184]. Using a reperfusion model, administration of APN reduced apoptosis and TNF- α expression via AMPK and COX-2 respectively^[18]. However, certain studies have shown that APN causes a rise in TNF- α ^[181,185]. The initial increase in TNF- α is mediated by the ERK1/2 pathway which activates early-growth response protein-1 and via a NF- κ B-dependent pathway. Despite an initial rise in TNF- α , it leads to an increased expression of IL-10, eventually shifting the system from a pro- to anti-inflammatory state^[185].

Other studies have identified further pro-inflammatory effects of APN [Table 2]. One study demonstrated that through an unidentified APN receptor, APN increased IL-6 production through NF- κ B activation^[158]. APN was again shown to activate the NF- κ B pathway via phospholipase C (PLC)- γ and the c-Jun N-terminal Kinase (JNK) pathway^[186]. This lead to DC activation and enhanced Th1 and Th17 responses. In adult rat cardiac fibroblasts, gAcrp activation of AdipoR1 induced IL-6 synthesis and release through AMPK, p38MAPK, and ERK1/2 pathways^[163]. However, through AdipoR1 signalling, A20 (zinc finger protein) and B-cell lymphoma 3-encoded protein (Bcl3) upregulation can counter-inhibit IL-6 signalling induced by APN^[117,168]. This complicates the overall picture of APN as an anti-inflammatory cytokine, but likely reflects the complex homeostatic mechanisms that are occurring. Although some evidence suggests APNs pro-inflammatory nature, the overwhelming evidence across a number of cell lines suggest to the contrary [Table 2].

An important consideration in whether APN exerts pro- or anti-inflammatory properties is the concentration that is used in studies. The physiological level of APN in a non-obese, non-diabetic patient is ~ 10 μ g/mL^[187]. When conditioning immune cells, we must take into consideration the concentration of APN used. In certain systems, when the conditioning of immune cells was done at physiological levels, the

opposite effect occurred^[188]. Therefore, the concentration of APN used for conditioning may greatly change the outcome of a study.

Many of the studies showing APN having pro-inflammatory effects used concentrations of ~10 µg/mL^[160,186]. A recent study demonstrated that approximately a plasma APN concentration of 40 µg/mL was needed to induce IL-10 production and an anti-inflammatory response^[117]. Furthermore, physical exercise can increase APN by 260% off baseline^[189]. Therefore, it may be unlikely that using a physiological level of APN would result in the desired anti-inflammatory effects due to sub-optimal signalling.

Interpreting APNs role on immune cells is further complicated as the kinetics of AdipoR1/R2 differ depending on the specific immune cell and stage of differentiation. Expression of AdipoR1/R2 occurs in 80%-90% of CD14⁺, compared to 10% of T-cells^[156]. As monocytes differentiate into macrophages AdipoR1 expression decreases, whereas AdipoR2 expression is constant^[190]. Likewise, the presence of various APN oligomers and the multitude of signalling differences make it hard to pinpoint APNs exact role. For example, differences are observed between HMW APN and gAcrp inhibition of the NF-κB pathway^[191]. HMW APN required shorter incubation period to inhibit NF-κB compared with gAcrp. While gAcrp produces a transient activation and requires a longer incubation period to inhibit the pathway^[191].

In the adaptive immune system APN has been shown to act as a negative regulator for T-cells^[156] and affect the migration of T-cells towards diseased tissue^[141,149]. Although only 10% of T-cells express AdipoR1/R2 on their surface, the majority of T-cells store these receptors within intracellular clathrin-coated vesicles. After stimulation, T-cells upregulate these receptors on the surface and APN interaction subsequently enhances apoptosis and inhibits proliferation. In APN-deficient mice, infection lead to a significantly higher number of CD137⁺ T cells^[156]. Furthermore, culturing APN-DCs with allogenic T-cells decreased T-cell proliferation and pro-inflammatory cytokine production^[146]. A rise in regulatory T-cells was also identified, providing an alternative mechanism that APN can regulate the immune system^[146]. With regard to migration, APN can inhibit expression of chemokines that act as T-cell chemoattractants in atherogenesis^[141]. Therefore, when APN levels are low in cancer, this could favour T-cell recruitment, and contribute towards adaptive immune response. Lastly, one study suggested that rather than affecting proliferation, APN directly activates CD4⁺ T-cells and increases secretion of interferon-γ and IL-6^[160]. It is thought that this inflammatory activation likely desensitises the cells to further pro-inflammatory stimuli. That said, this study uses a low concentration of APN which may influence our interpretation of the results.

FUTURE IMPLICATIONS OF INTERFERING WITH THE APN PATHWAY ON CANCER

TREATMENT

It is clear that APN has the ability to alter cancer outcomes, biology, and anti-cancer immunity. Therefore, APN has been identified as a therapeutic and preventive target for cancer therapy. The pleiotropic nature of various APN isoforms has caused trouble initially engineering an APN analogue, consequently groups decided to screen for endogenous agonists or enhancing existing APN levels.

The first APN receptor agonist screened for and produced was ADP355^[192]. ADP355 binds both AdipoR1/R2, with a greater affinity to AdipoR1^[192]. *In vivo*, this agonist inhibited orthotopic human BC xenograft growth by 31%, with an acceptable safety profile. Similar to gAcrp, ADP355 regulated signalling pathways AMPK, STAT3, PIK3/Akt, and ERK1/2. The group was able to improve agonistic activity by 5-10 fold by substituting Gly4 and Tyr7 residues with Pro and Hyp respectively^[193]. High-throughput screening identified several endogenous adiponectin ligands: AdipoR1 agonists included matairesinol, arctiin, (-)-arctigenin and gramine, while AdipoR2 agonists included parthenolide, taxifolol, deoxyschizandrin, and syringing^[194]. Further studies identified AdipoRon as a strong AdipoR1/R2 agonist at low micromolar concentrations, signalling through AMPK and PPAR-α pathways^[195].

An alternative proposed method is to augment endogenous APN levels using PPAR γ ligands^[196]. Using synthetic PPAR γ ligands, one group demonstrated that expression and secretion of APN increased in a dose and time dependent manner^[197]. Efatutazone, a selective PPAR γ agonist, showed promise on a phase I trial in metastatic solid tumours^[198], metastatic colorectal cancer^[199], and thyroid cancer patients^[200]. However, trials were terminated after phase 2 due to insufficient efficacy^[201,202].

Another PPAR γ agonist Troglitazone has gained much attention in recent years. Troglitazone has been shown to inhibit migration and invasion in human prostate cancer cells^[203], inhibit matrix metalloproteinase-9 expression and invasion of breast cancer cells^[204], and have anti-tumour effects without marked adverse effects^[205]. One study showed that Troglitazone mediated PPAR γ activation enhances human lung cell cancer cells to TRIAL-induced apoptosis^[206]. Suggesting that if used in combination with TRIAL protein it could be useful against TRIAL-resistant cancer cells. That said, phase II studies in refractory breast cancer^[207] and metastatic colorectal cancer^[208] have had disappointing results. A synthetic derivative of Troglitazone, $\Delta 2$ -Troglitazone was shown to be more potent in reducing cancer cell proliferation, however no clinical value has been found^[209].

Rosiglitazone, a PPAR γ agonist, has been shown to have beneficial effects on atherosclerosis, insulin resistance, and type 2 diabetes^[210,211]. However, a phase II trial using Rosiglitazone in liposarcoma patients concluded that this agonist was not an effective anti-tumour drug^[212]. It is important to highlight the side-effect profile of these trial drugs. Rosiglitazone has been shown to increase risk of cardiovascular disease^[213] and Troglitazone presents concerns regarding hepatotoxicity^[207]. Additional cautions should be taken with chronically elevated APN levels which can be linked to infertility and reduced bone density^[214]. Furthermore, interfering with the AdipoR1/R2 pathways can have adverse effects for anti-tumour immune response^[145], consequently a fine balance must be achieved to ensure the anti-cancer pathways are promoted.

With there being limited success with current therapeutic agents one current use for APN is as prognostic markers for cancer^[215]. Both serum APN levels, the expression of AdipoR1/R2, and SNPs could all provide avenues for prognostic and predictive molecular tests in cancer. A number of SNPs in ADIPOQ or APNs receptors have been found associated with BC, CRC, EC, GC, PC, HC, RCC, PrC, and LC^[65]. The question arises whether there would be any change in clinical decision making through screening these SNPs or whether they would give any benefit above currently used biomarkers. That said, if well targeted, these tests could help early diagnosis, aid post-operative recovery, and initiate APN modulatory therapy

It has been shown that a Mediterranean-type diet, reduction in body weight, and exercise can increase circulating APN levels^[189,216]. Furthermore, aerobic exercise was shown to increase expression of AdipoR1 and AdipoR2 in young men^[217]. Therefore, using programmes that promote weight loss and a healthy diet in parallel with new APN promoting agents may enable more favourable outcome. Similarly, new APN therapeutic agents may show more promise when being used in combination with traditional treatment by sensitising cancer cells.

A number of cancers are strongly associated with chronic inflammatory processes, for example the association between inflammatory bowel disease and CRC, or *Helicobacter pylori* infection and GC^[218]. These type of cancers could greatly benefit from the anti-inflammatory properties of APN. In cancer there is often defective inflammatory responses, the goal is to restore the normal overall host response, without undesirable side effects. However, one needs to acknowledge that by altering these metabolic pathways there is a chance of detrimental changes to anti-tumour immunity. Moreover, as carcinogenesis progresses, there are changes to the way the tumour environment harnesses the immune system, therefore the timing of treatment may greatly alter the efficacy.

When considering APN as a therapeutic agent it is important to highlight several points. Firstly, many factors influence the levels of circulating APN. Sex, age, ethnicity, and intra-abdominal fat all have

independent roles in determining APN levels^[219]. This is important to consider as a therapeutic agent may have greatly differing effects across individuals. Secondly, the pleomorphic nature of APNs isoforms mean it will be hard to replicate each isoforms action. One option would be to use recombinant APN, however this has been difficult to produce and would be expensive over the long term. The solution has been to attempt to increase endogenous APN, but we must consider whether it may be beneficial to selectively increase one isoform. Thirdly, the dosage APN used can affect the inflammatory response in a varied manner. In creating a novel agent, the dose regime must be carefully designed to ensure the desired response is seen.

CONCLUSION

Obesity related-cancers are now a recognised phenomenon. APN is the key adipokine produced by the fat cells and its role in cancer pathogenesis and disease prognosis is now slowly revealed. Hypoadiponectinaemia is often found to be associated with cancers and also affects their prognosis. Therefore, various efforts have been made to harness its therapeutic effects. It can be applied either therapeutically or prophylactically to treat various cancers. For example, identification of ADIPOQ, AdipoR1 and AdipoR2 SNPs that may confer altered risks of cancer development may enable early screening and APN level augmentation via therapeutic interventions.

Overall this field holds a huge promise, however many challenges will need to be addressed prior to its routine use. Cellular and molecular function of APN in cancer either its pathogenesis phase or advanced forms of cancer will need to be fully researched. APN may have different effect on various stages of cancers either directly or indirectly via immune system. The role of each isoform in distinct tissues and under specific conditions needs be clarified. More importantly, the molecular conditions under which APN acts as cancer suppressing or promoting and anti-inflammatory or pro-inflammatory still needs to be fully evaluated. The exact roles of APN interacting which receptors (some identified but some remained to be defined) and their downstream pathways in different cancer have largely remained elusive. Exploring an effective APN-based therapeutic and when to applied this therapeutic may be the crucial step. Understanding the complexity of APN on anti-tumour response may need to be considered when using it as a therapeutic agent.

DECLARATIONS

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Authors' contributions

Wrote and prepared most of the manuscript: Monks M

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

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Review

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Photodynamic therapy in cancer treatment - an update review

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Abstract

Cancer remains a worldwide health problem, being the disease with the highest impact on global health. Even with all the recent technological improvements, recurrence and metastasis still are the main cause of death. Since photodynamic therapy (PDT) does not compromise other treatment options and presents reduced long-term morbidity when compared with chemotherapy or radiotherapy, it appears as a promising alternative treatment for controlling malignant diseases. In this review, we set out to perform a broad up-date on PDT in cancer research and treatment, discussing how this approach has been applied and what it could add to breast cancer therapy. We covered topics going from the photochemical mechanisms involved, the different cell death mechanisms being triggered by a myriad of photosensitizers up to the more recent-on-going clinical trials.

Keywords: Photodynamic therapy, breast cancer, photosensitizers, cell death

INTRODUCTION

Cancer remains a worldwide health problem. In particular, breast cancer is the disease with the highest impact on global health. Even with all the recent technological improvements, recurrence and metastasis still are the main causes of death. In fact, the high mortality as a consequence of distant metastasis in patients remains a bottleneck for an effective treatment in clinic^[1,2]. Metastasis are characterized by a sequential and complex process during which cancer cells invade specific organs including lung, liver, brain, and bone^[3].



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Metastatic lesions are usually multiple and resistant to conventional therapies, jeopardizing successful surgical resection, chemo and radiation treatment^[4].

Light has been known to provide a therapeutic potential for several thousands of years. Over 3000 years ago, since the Ancient, Indian and Chinese civilizations it has been used for the treatment of various diseases^[5] mainly in combination with reactive chemicals, for example to treat conditions like vitiligo, psoriasis and skin cancer^[6]. After 1895 with the discovery of the phototherapy, which rendered Niels Ryberg Finsen the Nobel prize in Physiology/Medicine in 1903 in recognition of his work on the treatment of diseases, and in particular on the treatment of lupus vulgaris by means of concentrated light rays, many studies with the use of light and chemicals emerged^[7].

Photodynamic therapy (PDT) is currently being used as an alternative treatment for the control of malignant diseases^[8-10]. It is based in the uptake of a photosensitizer (PS) molecule which, upon being excited by light in a determined wavelength, reacts with oxygen and generates oxidant species (radicals, singlet oxygen, triplet species) in target tissues, leading to cell death^[11,12]. PDT cytotoxic properties have been established to be due to the oxidation of a large range of biomolecules in cells, including nucleic acids, lipids, and proteins, leading to severe alteration in cell signaling cascades or in gene expression regulation^[13,14]. Like all the newly proposed treatments, there is still place for improvements and lots of resources have been invested in this field recently. In this review, we set out to perform a broad up-date on PDT and its implication in cancer research and treatment. We have covered topics going from the photochemical mechanisms involved, the different cell death mechanisms being triggered by a myriad of photosensitizers up to the more recent reported preclinical studies and on-going clinical trials.

PHOTOCHEMICAL PRINCIPLES AND COMPONENTS OF PDT

As previously stated, PDT involves the photosensitized oxidation of biomolecules which can be separated in two mechanisms. In Type I, light energy passes from excited molecules to biomolecules through electron/hydrogen transfer (radical mechanism) in direct-contact reactions [Figure 1] and culminates in specific damage to biomolecules and in the initiation of radical chain reactions. On the other hand, in the Type II mechanism, the excitation energy is transferred to molecular oxygen ($^3\text{O}_2$), resulting in the formation of singlet oxygen ($^1\text{O}_2$), which is extremely electrophilic, being capable of causing damage to membranes, proteins and DNA [Figure 1]. Direct contact reactions usually cause more severe damage in biomolecules, but also cause photodegradation of the PS, while diffusive species are important to replenish the PS. By either mechanism, the formation of triplet excited species is the key step in terms of performance of the PS. Both tricyclic phenothiazinium salts and macrocyclic poly-pyrroles (porphyrins and derivatives) compounds generate reasonable amounts of triplets upon electronic excitation, being therefore PSs commonly used for PDT^[15]. There is no doubt that the outcome of PDT critically depends on the intrinsic efficiency of the PS. Even when the search for new PS remains mostly focused in the synthesis of compounds that produce singlet oxygen with greater efficiency, there are many factors needed to be considered including aggregation and photodegradation^[15].

Damages to proteins and membranes are of particular importance for PDT in order to optimize the cytotoxic efficiency to the process. Indeed, PSs displaying a higher degree of accumulation in cell and/or organelles membranes are usually more cytotoxic^[16,17]. The mechanism by which photosensitized oxidations on lipids cause membranes to leak out, has been recently described^[18]. In generic terms, changes in phospholipids occur due to lipid peroxidation, which are reactions that are initiated as a consequence of the formation of free radicals and singlet oxygen. After this starting point, the process becomes autocatalytic, leading to the formation of hydroperoxides and other byproducts. Figure 2 summarizes the main steps in photo-induced membrane damage. The first one usually involves the “ene” reaction between the lipid (LH)

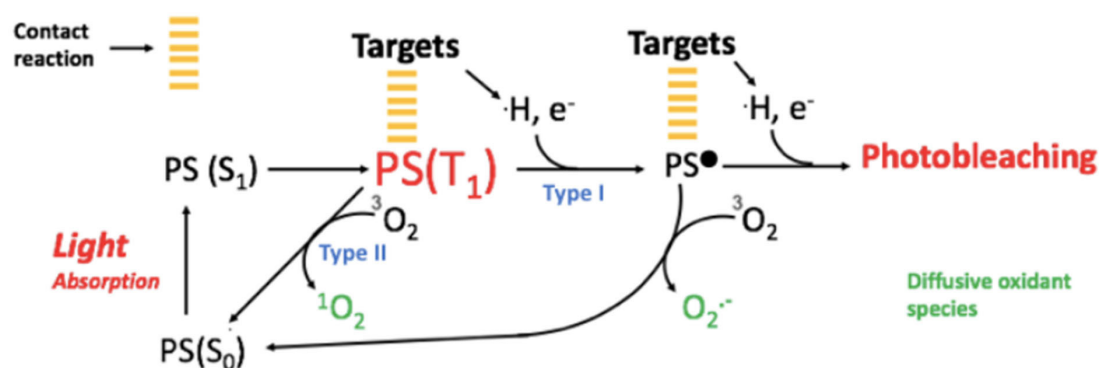


Figure 1. Mechanisms of photosensitization. The photosensitizer (PS), is a molecule capable to absorb energy from light in a specific wavelength. Once excited, the PS transits from its ground state $PS(S_0)$, to its singlet excited $PS(S_1)$ and triplet excited $PS(T_1)$ states. At this point $PS(T_1)$ can react directly with biomolecules, like proteins or lipids (targets), via Type I photochemical reaction, resulting in formation of radicals, like PS^\bullet , capable to initiate radical chain reactions. Otherwise, $PS(T_1)$ can react with molecular oxygen 3O_2 , via the Type II photochemical reaction. Both generates diffusive oxidant species like radical superoxide, $O_2^{\bullet-}$, and singlet oxygen, 1O_2 , via type I and II respectively, capable to extend the damage

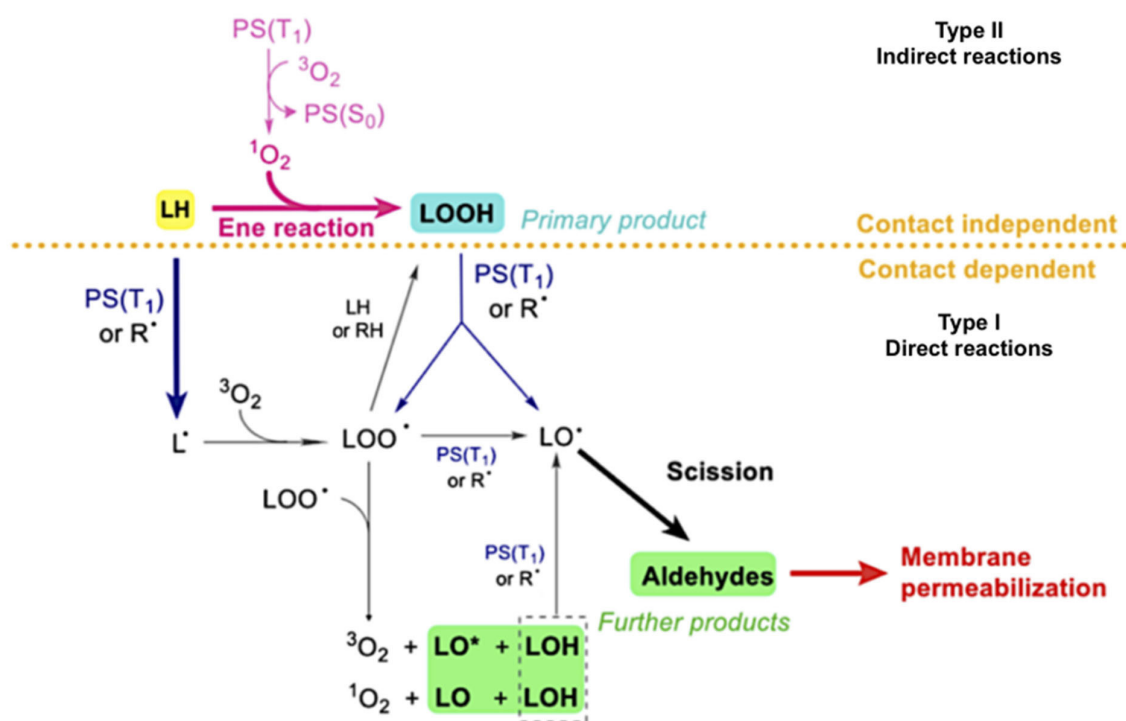


Figure 2. Mechanisms of photo-induced membrane damage. This graphical sketch represents how Type I and Type II photochemical reactions contribute to membrane leakage through lipid damage. Type I reactions lead to changes in membrane fluidity which occur as a result of direct-contact reactions between the PS triplet [$PS(T_1)$] and either the lipid double bond of LH or the LOOH. Type II reactions [$PS(T_1)$ and molecular oxygen (3O_2)] generates singlet oxygen (1O_2) which leads to the formation of hydroperoxide (LOOH) as primary product. Modified from [18]. R•: generic radical species; LH: non-oxidized lipid; L•, LOO•, LO•: lipid carbon-centered, peroxy and alkoxy radicals; LOOH, LOH, LO, LO•: lipid hydroperoxide, alcohol, ketone and excited state ketone

and singlet oxygen (formed via type II photochemical reaction between PS triplet $PS(T_1)$ and molecular oxygen) leading to the formation of hydroperoxide (LOOH). The other steps comprise direct reactions with the $PS(T_1)$ via Type I photochemical mechanism. Irreversible damage occurs with the abstraction of a hydrogen atom from an unsaturated fatty acid (LH), leading to the formation of a lipid radical (L•). This one suffers the addition of an oxygen molecule forming the peroxy radical (LOO•) which is, in turn, able to react with another LH fatty acid, initiating a new oxidation round leading to the formation of lipid hydroperoxide

(LOOH) and another L•. The propagation phase comprises the initiation of a new oxidation chain by the peroxy radical (LOO•) and the decomposition of the lipid hydroperoxides into other intermediate radicals. In light-induced reactions, formation of alkoxides is catalyzed by direct contact reactions between the triplet photosensitizer, the lipid double bond and the lipid hydroperoxide, leading to chain breakage by β -scission. This process leads to the formation of lipid truncated aldehydes and further products, which are molecules responsible for starting of the leakage process. This explains successful results as well as opens possibilities for cellular targeting strategies that will be discussed below.

RESEARCH IN PDT

Photosensitizers and photocytotoxicity mechanisms

The PS is one of the three crucial elements of PDT, apart from light and oxygen. Due to their photochemistry properties and uptake efficiency currently only a few PSs have official approval worldwide and are being used clinically. Related to cancer treatment, PS approved or in clinical trial are listed on [Table 1](#). In particular, Porphimer sodium (Photofrin), mTHPC (Foscan), NPe6 (Laserphyrin), SnEt2 (Purlytin), Visudyne (Veteporfin) and Motexafin lutetium (LuTex) are clinically or preclinically applied for breast cancer^[19-21]. These photosensitizers have shown selectivity towards tumor cells and are ideal for cellular and vascular-targeted PDT. Moreover their interference with cytoprotective molecular responses constitutes an area of growing interest^[22,23]. Other compounds such as porphyrin precursors [e.g., aminolevulinic acid (ALA)]^[24], phenothiazines (e.g., methylene blue)^[25,26], cyanines (e.g., merocyanine, indocyanine green)^[27], hypericin^[28], and xanthenes (e.g., Rose Bengal)^[29] have been considered as good candidates as PS used for PDT.

Several studies have been performed over the last decades in order to better characterize PS efficacy and selectivity. Some of them have focused on the development of agents with higher absorption wavelengths, allowing deeper penetration of illuminating sources and thus the depths at which tumor cells can be targeted, the so-called second-generation PSs. Third-generation PSs have recently emerged aiming mainly at targeting strategies, such as antibody-directed PS and PS-loaded nanocarriers. These approaches were developed in order to increase the power and efficiency of PDT and have allowed the broadening of the types of diseased tissues that could be treated^[36].

Besides the PSs photoactive capacity, which enables it as a therapeutic agent upon light activation, their autofluorescence is also an important characteristic. Indeed, PS's fluorescence confers them imaging properties to be used for detection of pre-cancer lesions and early malignancies, as well as tumor margins. Furthermore, this property can be used for the identification of remaining dysplastic tissue upon surgical tumor resections, and to monitor the progress of the PDT treatment. Thus the combination of imaging, detection and therapeutic properties confers them the characteristics of theranostic agents^[36,37]. PS 5-ALA, which is a prodrug enzymatically converted during heme synthesis to the active PS agent protoporphyrin IX, displays theranostic properties and has brought important knowledge in PDT research and treatment field. Additionally, in clinical trials 5-ALA has already shown clinical benefit in different types of tumors^[24].

PDT is considered to be involved in at least three main mechanisms of tissue destruction. The first one is the ability to directly kill cells through the action of damaging reactive chemical species generated by PS excitation. Direct phototoxic effect of PDT involves irreversible photodamage to specific targets, such as membranes and organelles^[38] as discussed in the previous section. The extent of the damage as well as the cell death mechanisms involved are dependent on the PS type, concentration, subcellular localization, energy applied and also on the intrinsic resistance characteristics of each tumor type. When perturbations of the intracellular and/or extracellular microenvironment are too intense, cells can die following a complete breakdown of the plasma membrane (necrosis morphology) or elicit specific cell death mechanisms^[39]. Additionally, PDT tumor destruction can also involve the damage of the tumor vasculature, thereby

Table 1. Photosensitizers investigated in clinical trial for cancer treatment^[15,21,23,30-35]

Photosensitizer	Chemical family	Treatment Wavelength (nm)	Cancer type	Characteristics
Porfimer sodium, HPD: hematoporphyrin derivative (Photofrin)	Porphyrin	630	Lung, esophagus, bile duct, bladder, brain, ovarian, breast skin metastases	1st generation PS Most probable intracellular localization: plasma membrane and mitochondria. Intravenous administration
5-ALA: 5-aminolevulinic acid (Levulan)	Porphyrin precursor	630	Skin, bladder, brain, esophagus	2nd generation PS Most probable intracellular localization: mitochondria Topical, oral or intravenous administration
MAL: methyl-aminolevulinate (Metvix)	Porphyrin precursor	630	Skin	2nd generation PS Most probable intracellular localization: mitochondria and ER Topical administration
h-ALA: hexylaminolevulinate (Hexvix)	Porphyrin precursor	White light	Basal cell	2nd generation PS Intracellular localization: TBD Topical administration
Veteporfin, BDP: benzoporphyrin derivative (Visudyne)	Porphyrin	690	Pancreas, breast	2nd generation PS Most probable intracellular localization: mitochondria Intravenous administration
Palladium bacteriopheophorbide, padeliporfin, WST-11 (Tookad)	Porphyrin	762	Esophagus, prostate	2nd generation PS Intracellular localization: TBD. Intravenous administration
Temoporfin, mTHPC: meso-tetrahydroxyphenylchlorine (Foscan)	Chlorin	652	Head and neck, lung, brain, bile duct, pancreas skin, breast	2nd generation PS Most probable intracellular localization: mitochondria, golgi apparatus and ER Intravenous administration
Talaporfin, mono-L-aspartyl chlorin e6, NPe6, LS11 (Laserphyrin)	Chlorin	660	Liver, colon, brain, lung, breast skin metastases	2nd generation PS Most probable intracellular localization: lysosomes Intravenous administration
HPPH: 2-(1-hexyloxyethyl)-2-devinyl pyropheophorbide-a (Photochlor)	Chlorin	665	Head and neck, esophagus, lung	2nd generation PS Most probable intracellular localization: mitochondria and/or lysosomes Intravenous administration
Rostaporfin, SnEt2: tin ethyl etiopurpurin I, or (Purlytin)	Chlorin	660	Skin, breast	2nd generation PS Most probable intracellular localization: lysosomes Intravenous administration
Fimaporfin, disulfonated tetraphenyl chlorin, TPCS2a (Amphinex)	Chlorin	633	Superficial cancers, Cholon	2nd generation PS Most probable intracellular localization: endo-lysosomal compartments Intravenous administration
Motexafin lutetium (Lutex)	Texaphyrin	732	Breast	2nd generation PS Broad intracellular localization Intravenous administration

TBD: to be determined

compromising the supply of oxygen and essential nutrients, as well as activation of the immune system, by inducing an inflammatory and an immune response against tumor cells^[23,35,40].

Cell death subroutines are strongly connected with successful therapy outcome. Even when a detailed explanation of cell death mechanisms is not the scope of this review (updated and deeper information can be assessed in^[39]), here we point some of their important features, since describing one or ones of them involved in cell toxicity constitutes a very important topic of research in the field of PDT.

At the cellular level, PDT has been shown to induce multiple cell death subroutines, that can be accidental or not [Figure 3]. Accidental cell death is an uncontrollable form of death corresponding to the physical disassembly of the plasma membrane caused by extreme physical, chemical, or mechanical cues. On the other hand, regulated cell death (RCD) results from the activation of one or more signal transduction modules, and hence can be pharmacologically or genetically modulated, at least to some extent^[39]. The RCD subroutines already related with PDT include apoptosis and different mechanisms of regulated necrosis

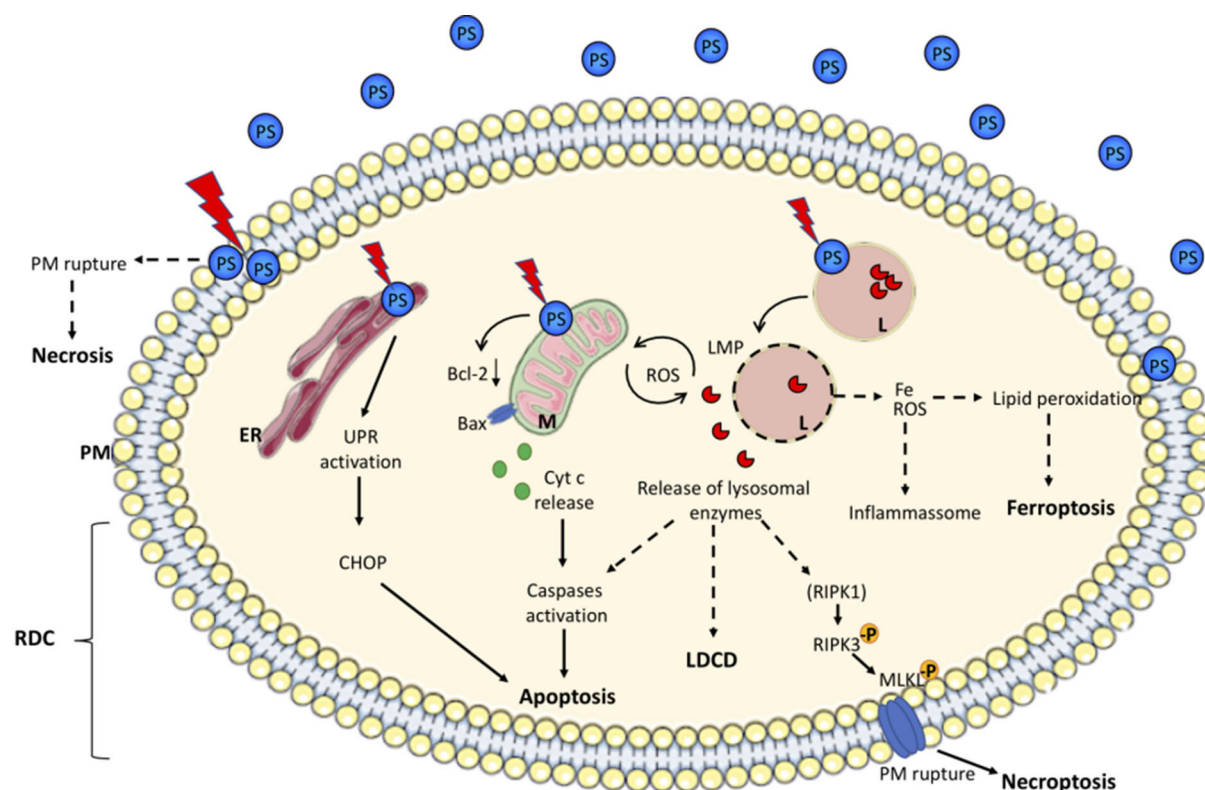


Figure 3. Overview of cell death subroutines that can be elicited by Photodynamic therapy. The most described locations of different photosensitizers (PS) are the plasma membrane (PM), endoplasmic reticulum (ER), mitochondria (M) or the lysosome (L). Depending on its localization, after activation by light (red lightningbolt) it can directly damage the PM causing unregulated necrosis or culminate in one or more regulated cell death (RCD) mechanisms. UPR: unfolded protein response; LMP: lysosome membrane permeabilization; Fe: iron; ROS: reactive oxygen species; -P: phosphate group presented in the active forms of RIPK3 and MLKL on necroptosis pathway; LDCD: lysosomal dependent cell death

(such as necroptosis and lysosome-dependent cell death)^[15,41]. Briefly, apoptosis^[42] is a type of RCD initiated by perturbations of the extracellular or intracellular microenvironment, being respectively classified as extrinsic (when signals are detected by plasma membrane receptors, and propagated by caspase-8 and precipitated by executioner caspases, mainly caspase-3) or intrinsic (demarcated by mitochondrial membrane permeabilization (MOMP), unbalance of pro and anti-apoptotic factors related with members of BCL2 family of proteins, and precipitated by the executioner caspases)^[39,43-48]. Necroptosis is a modality of RCD triggered by perturbations of extracellular or intracellular homeostasis that critically depends on phosphorylation, oligomerization and migration of MLKL (mixed lineage kinase domain-like protein) to plasma membrane, the kinase activities of RIPK3 and (at least in some settings) of RIPK1 (receptor interacting protein kinases-1 and -3, respectively)^[49]. Finally, lysosome-dependent cell death (LDCD) is a consequence of lysosomal membrane permeabilization^[50,51] and releasing of cathepsins, with optional involvement of MOMP and caspases. Indeed, one of the advantages of PDT is that this therapeutic approach has demonstrated to be able to cope with the very well described ability to bypass the several resistance mechanisms displayed by malignant cells^[26].

PS localization within or on the cell surface is critical to determining the mode of cell death induction and thus the cellular response to photodamage^[38,52,53]. Therefore, precise understanding of the preferential subcellular site of PS accumulation is important in order to determine its cytotoxic potential when used in PDT^[38,54]. PS uptake by the tumorigenic cells as well as its preferential intracellular site depends on chemical characteristics of each compound. Hydrophobic molecules can rapidly diffuse into plasmatic membranes while more polar drugs tend to be internalized via endocytosis or assisted transport by serum lipids and

proteins. After internalization, most of the PSs are localized in organelle membranes due to their common chemical core. In general, PS's cellular localization can be highly specific or quite broad, and have been reported to include the endoplasmic reticulum (ER), mitochondria, Golgi complex, lysosomes and the plasma membrane^[40,55]. To limit DNA damage and avoid the development of genetically resistant cells, PS should not accumulate in the cell nuclei^[30]. Accidental necrosis is more often observed when the PS site of action is in the plasma membrane and/or when it is activated with high energy doses^[40]. In order to suppress undesired damage to normal tissues, this effect should in general be avoided^[56]. In an organelle-specific photodamage scenario, the mechanism of autophagy is activated as a coordinated intracellular response aiming at reestablishing homeostasis. When impaired or insufficient autophagy is triggered, induction of cell death is the most common result observed^[26,38,57,58]. The best documented organelle specific cytotoxic effects of PDT are related with the photodamage of mitochondria, lysosomes and ER.

Porphyrins can present a variable localization pattern, mostly associated with plasma membranes and mitochondria. PSs that are located in the mitochondria can cause mitochondrial inner membrane permeabilization and selectively damage antiapoptotic proteins of the BCL-2 family, localized at the outer mitochondrial membrane while the proapoptotic proteins are left intact, resulting in an unbalance of pro- and anti-apoptotic players that results in caspase activation^[35]. On the other hand, some mitochondria-associated porphyrins can also activate necroptosis, as already shown in 5-ALA-PDT^[59]. A lysosome localization pattern was observed using negatively-charged porphyrins, NPe6 and the phenothiazinium methylene blue^[26,38,53]. A potential advantage of lysosome target PDT is that lysosomal damage might easily circumvent autophagic protection that can be activated in parallel of cell damage. Lysosome damaging PDT leads to LDCD^[39,51,60]. Release of cathepsins after PDT can result in cleavage of the pro-apoptotic protein Bid to a truncated form termed tBid. This product can in turn interact with mitochondria, leading to the release of cytochrome c, followed by the consequent activation of intrinsic apoptosis as already shown in NPe6-PDT^[41]. However, not only apoptosis can be activated by lysosome damage and although it has not been demonstrated as a PDT-induced mechanism yet, several parameters of regulated necrosis have been also associated with LDCD. Indeed, the degradation of caspase-8 and recruitment of necroptosis machinery^[61] or even the involvement of iron homeostasis modulation leading to an increased susceptibility to undergo ferroptosis^[62] have already been associated with lysosomal damage. Other porphyrins such as mTHPC and Hypericin, have been reported to target ER or both ER and mitochondria. Under massive photodamage in ER membranes, PDT can elicit pro-death signaling via the unfolded protein response cascade. The resulted ER stress, activates the pro-apoptotic transcription factor CHOP, which in turn mediates mitochondrial apoptosis^[63]. The induction of multiple cell death pathways is usually considered as an useful characteristic of PDT, since it increases photokilling in tumor cells harboring a particular cell death pathway resistance^[64].

PDT and immune response as a tool to deal with metastasis

The major challenge in the fight against cancer includes treatment toxicity and drug-resistance associated with incompletely tumor removal by surgical resection. In order to extend patient survival, systemic chemotherapy has become an essential part of treatment after surgery. However, therapies effective in both reducing the high mortality rate associated with the disease as well as improving patient quality of life still need to be developed^[22,35]. In an attempt to circumvent the appearance of a secondary disease, immunotherapy has been applied and developed as a tool to control cancer recurrence and metastases. There are few effective treatments for metastases, thus there is an increased interest in therapies that eliminate primary tumors and systemically activate antitumor immune responses. In this sense, PDT emerges as a potential therapy to be used alone or in combination with other approved or experimental approaches^[65] because of the recent findings indicating that PDT can also trigger systemic effects, including the reinstallation of immunosurveillance^[29,66].

Examples of immunotherapy include the use of monoclonal antibodies to block immune checkpoint activity of cancer cells, enabling anti-cancer T cell responses, or adoptive cellular therapy to prime patient's own

lymphocytes to attack cancer cells. The goal of immunotherapy is to generate a robust immune response, stimulating the endogenous cytotoxic lymphocytes to eradicate tumor cells and ultimately achieve long-term anticancer immunity^[67]. Therefore, the ideal cancer treatment should involve the direct cytotoxic action on tumor cells with potential to stimulate the immune system based on the immune-recognition of danger signals emitted by dying cells^[68]. In a typical immune response, antigens are captured by dendritic cells (DCs), which then mature and present antigenic peptides to T cells in lymph nodes, generating effector T cells that migrate towards sites of infection, inflammation or injury. IFN- γ and GM-CSF are central to the process of DC maturation and macrophage activation. DCs in turn release cytokines, like IL-1 β , IL-6, IL-12 or TNF, that shape the Natural Killer cell (NK) and T cell responses. T cells CD4+ and CD8+, together with NK cells, can receive survival signals and stimulation from IL-2, leading to full effector activities, and produce additional IFN- γ . Normal immune regulation involves cytokines like IL-10 and TGF- β to limit the activity of T cells and macrophages and reduce inflammation, terminating immune responses and protecting the host from immune-mediated damage^[67]. Some tumors appear to have somehow managed to avoid detection by the various arms of the immune system or have been able to limit the extent of immunological killing, thereby evading their eradication^[69].

The best way to reestablish an immune system response against tumors is by eliciting, therapeutically, a cancer cell death pathway that is accompanied by high immunogenicity and is possibly able to inhibit or reduce the influence of the pro-tumorigenic cytokine signaling. Over the last years, several studies demonstrated that few selected anti-cancer therapeutic approaches are able to induce a promising kind of cancer cell demise called immunogenic cell death (ICD)^[70]. The concept of ICD has been introduced to describe a cell death modality that stimulates an immune response against antigens coming from dead cells. After exposure to certain cytotoxic ICD-agents, changes in the composition of the cell surface promote the exposure or release of mediators, the so-called damage associated molecular patterns (DAMPs)^[71]. These signals are thus further recognized by DCs to stimulate the presentation of tumor antigens to T cells^[72]. It has emerged that initiating a tumor cell death modality associated with the activation of signaling pathways that release DAMPs markedly increases the immunogenicity of dying cancer cells^[73]. Up to now, the main DAMPs involved in ICD include surface-exposed calreticulin (CRT), surface-exposed heat shock protein (HSP) 70 and 90, secreted adenosine triphosphate and passively released high-mobility group box 1 (HMGB1)^[71,74]. Many conventional anticancer treatments, that include chemotherapies, radiotherapies and target therapies have had their immunogenic potential evaluated, but only a few of them have been characterized as ICD-inducers^[72]. This exclusive inducer-induction relationship exists because ICD requires, as a pre-requisite, induction of reactive oxygen species (ROS)-based ER stress, where ROS may or may not be mainly ER-directed^[75]. As a ROS-inducer therapy, the potential of PDT in stimulating ICD seems to be quite probable, and has been tested.

Preclinical studies have shown that in contrast to the effects of traditional therapies, low-dose PDT regimens can induce anti-tumor immunity, and these can be combined with high-dose PDT to achieve local tumor control with the immune suppression of distant disease^[76]. The potential mechanisms of PDT immune stimulation involve the acute inflammatory response following PDT, which might increase the presentation of tumor antigens to activate DC, and their homing in to regional and peripheral lymph nodes, thus ultimately stimulating CD8+ cytotoxic T cells and NK cells, accompanied by immune memory and the suppression of any subsequent tumor growth upon a second challenge. The degree of acute inflammation induced by PDT has been reported to be regimen dependent and to correlate with the extent of the induced vascular damage. PDT regimens that result in rapid cell death (within 1 h of treatment) and maximal tissue damage were demonstrated to cause minimal acute inflammation, presumably because of vascular shutdown, which would prevent neutrophil infiltration and systemic release of cytokines. In contrast, regimens that cause diffuse tumor damage should allow neutrophil infiltration followed by induction of expression and release of inflammatory mediators critical for enhancement of anti-tumor immunity^[77]. Ongoing studies are focused

on finding optimal PDT conditions to induce systemic immunity. Recent reports have shown that PDT can be an effective adjuvant therapy to surgery that increases the probability of long-term local disease control^[30]. Recently results from a preclinical study have also identified an anti-tumor combined PDT treatment regimen that controls primary and metastatic tumor growth and enhances anti-tumor immunity of both colon and mammary carcinomas^[76]. Recently, PDT utilizing the photosensitizer hypericin (Hyp-PDT), became the first PDT modality characterized as capable of inducing ICD^[28]. Although the immunogenic potential has not been tested for all PS yet, these recent findings with hypericin constitute an important achievement because they have allowed PDT to be classified as a therapy capable of inducing ICD. Accordingly, a recent study have shown that glycoconjugated chlorin-PDT suppressed colon rectal tumor cells (CT26) growth more efficiently in immunocompetent mice compared with immuno-deficient mice. Additionally, the exposure of the mice to PDT-treated CT26 cells was capable of protecting them against a subsequent challenge with CT26 cells and this immunogenic effect was dependent on CRT exposure in the plasma membrane and HMGB1 release, two known features of ICD^[65]. Moreover, the increased interest in PDT has actually helped in a deeper understanding of technical as well as conceptual aspects of ICD and the danger signals involved in this type of cell death. Further clinical research may perhaps point PDT as a method of vaccination against tumors^[78,79]. This clearly would be of great advantage since PDT in patients treated for primary breast cancer could also result in increasing acquired immunity against the cancer at distant sites.

Overall, accumulating evidence indicates that the therapeutic efficacy of several antineoplastic agents, including PDT, relies on their capacity to influence the tumor-host interaction, tipping the balance toward the activation of an immune response specific for malignant cells, especially for metastasize cancer.

Instrumentation, drawbacks and side effects

PDT has been shown to be effective in treating different types of cancers, especially the ones with superficial localizations, as this intervention provides a significant improvement in both the patient's quality of life^[80] and its cost-effectiveness compared to palliative surgery or palliative chemotherapy treatments^[81]. However, as happens with other therapies as well, the predominant unfavorable anatomical and microenvironment conditions have been reported to limit its efficacy and to contribute for the relatively slow transition from preclinical to clinical practice of PDT for treating these tumors^[82,83]. Adjustment of PDT parameters such as type and local concentration of the PS, light delivery and source or dosimetry in a context where there is no homogeneity is considered as one of the bigger obstacles of this therapeutic approach^[84].

The most common concern about the clinical use of PDT constitutes the limited penetration of light. However, nowadays this old idea that PDT is as a surface treatment because the application of external light may only treat superficial lesions is no longer believed. This problem is actually in the way of being solved because of the possibility of using fibers which can be placed in determined locations within the tumor site. Due to advances in fiber optics and microendoscopic technology, PDT can be used in the clinic with interstitial, endoscopic, intraoperative or laparoscopic light delivery systems. In this scenario, the laser light can be adapted into thin optical fibers for delivery of light into deeper and more difficult to access treatment sites^[85]. These fibers besides being used to deliver light, can also simultaneously act as diagnostic sensors that can gauge important PDT parameters which critically impact the therapeutic response, such as the fluence rate, PS concentration, PS photobleaching and the tissue oxygenation status^[86,87]. It is important to take into account that the vascular collapse promoted by PDT can decrease the tumor oxygenation. Since the success of the treatment relies on the preexisting ground state oxygen to produce singlet oxygen via type II photochemical reaction [Figure 1], tissue oxygenation state significantly affects the efficiency of PDT. Moreover during the treatment itself, the formation of ROS using high light fluence rates can deplete the oxygen levels in tumorigenic tissue too fast, aggravating the tumor hypoxia and hampering the treatment outcome^[40,88,89]. Thus, improvement the oxygen concentration is an important issue for PDT. In fact, adjusting the light and PS dosimetry, lowering light fluence rates or using light fractionated strategies

can prevent oxygen depletion and allow sufficient time for the replenishment of oxygen in the target tissue. However, it has already been reported that different PS affect the oxygen consumption rates differently^[90] and thus PDT outcome may vary. Altogether, these results point to the need of more studies addressing how to maintain PDT performance. An interesting study employing a combination of the fractionated PDT with a PS that could produce singlet oxygen during the dark periods^[91] has recently addressed this issue. Moreover, the presence of pre-existing hypoxic areas in tumors can be circumvented by a variety of strategies such as improving tissue oxygenation prior to PDT as well as by efficiently producing radical species via type I photochemical reactions^[88].

Regarding light source technology, researchers have been developing light delivery systems with uniform illumination, which is essential to improving light penetration and reproducibility. An example of this is the use of bulb-shaped isotropic emitters along with light detectors which have been used in hollow organs. Light dosimetry can also help to optimize the positioning of light diffusers. Furthermore, increasing the selectivity of PDT could be achieved by customizing the diffusers such as balloons and cylindrical applicators according to the form and dimensions of the target tissue^[92,93].

Additionally, recent advances in LED and diode lasers have allowed to merge their ability of output potency, enhanced portability and precision optical fiber coupling. These, non-collimated and less expensive light sources will certainly ease the translation of PDT to clinical procedures. They are usually employed due to their robustness, short bandwidth, relatively low maintenance cost and ability to be configured to the wavelength required by the PS. Lamps such as Tungsten filament lamps, metal halide lamps, powerful Xenon arc lamps and pulsed lasers are also commonly used in the PDT field^[92].

Besides the light source itself, defining the strategy of light application is of fundamental importance since different irradiation protocols using the same source can lead to different outcomes in PDT. Another drawback to be dealt with in PDT prescriptions is light dose regimens because they might also influence the host anti-tumor reactions and optimal strategies are likely case dependent^[35,85]. Therefore, a full understanding of light dosimetry is an important part of PDT. The issue of light in PDT is under careful investigation and improvements and new technologies in this field will certainly lead to optimized overall PDT efficacy and protocols^[94]. For example, one strategy to induce deep tissue phototoxicity is to perform repeated PDT or metronomic PDT (slow infusion of PS and low dose light)^[95]. Recent studies have shown that fractionated PDT might induce a high degree of necrosis deeper than PDT alone^[35,84,94]. The fact that this strategy may allow for continuous accumulation of PSs at the treatment site contributed to providing a better treatment response profile and increased the feasibility of reaching deeper tissues^[84].

In terms of adverse events associated with PDT, the most common is skin photosensitivity, especially when PSs of the first generation were systemically administered. In these cases, patients have to avoid sunlight and strong artificial light for weeks^[21]. Another side effect often reported is pain^[35]. The main mechanism behind PDT-induced pain has not yet been elucidated, but several studies have found it to be associated with the size of the treated area, PS type, lesion type, gender, age and light protocol. There are evidences suggesting that continuous activation of low levels of PS with methods using lower irradiance and possibly shorter incubation times are associated with decreased pain without loss of PDT efficacy^[96]. Additionally, the occurrence of inflammation, fever and nausea are typical but often successfully managed with medication. Tumor recurrence is often reported in clinical trials, probably due to inadequate tumor eradication. It can be a result not only to insufficient light or PS penetration, but also the presence of PDT resistant tumors. Although the causes for recurrence often lie beyond the scope of clinical trials, there is a strategy to overcome incomplete tumor elimination by PDT that is to combine two or more PSs. The idea behind this is to choose PSs that target different cellular compartments of the tumors allowing for a better overall

therapeutic outcome^[64,95,97]. Another strategy could be to associate PDT with other existing compounds used for chemotherapy. In a recent study the combined use of ferroptosis inducers and Gencitabine has shown to be able to significantly overcome the resistance to the chemotherapeutic agent displayed quite frequently in pancreatic adenocarcinomas^[98].

PDT in preclinical models of invasive cancer: an up-dated report

Most people diagnosed with cancer will require surgery as the main strategy for tumor removal with or without radiotherapy and systemic therapy. Tumor cells generally migrate from a primary tumor through the blood stream and lymphatic system and may be detected in one or more sentinel lymph nodes before spreading further to a secondary site. In the case of non-metastatic cancer, identifying cancer cells in the lymph nodes is one of the most important prognostic factors for determining the need for adjuvant radiation therapy and/or chemotherapy. Once cancer has spread to distant sites, the removal of tumor has not consistently shown a survival benefit. In these cases, surgery may help with palliative control of an ulcerated tumor on the chest wall and only unspecific broad-spectrum therapies are the currently treatment option available^[99]. Thus, effective and safe therapies for this stage of the disease are still needed.

Several *in vitro* studies have shown a synergism between PDT and ionizing radiation in killing cells^[100,101]. The combined application of low dose of radiotherapy (4Gy) and indocyanine green, with light proved to be very effective and resulted in a nearly complete reduction of survival^[101]. In a preclinical animal model, PDT following radiotherapy showed evidences of improved trabecular structure. Quantitative histological examination suggested that PDT induced increases in the bone-to-marrow ratio, due mainly to the increased formation of newly formed woven bone and an increase in osteoid formation in comparison with the situation in rats treated with radiotherapy alone^[102]. A subsequent study demonstrated that PDT appeared to improve vertebral integrity in combination with bisphosphonates or radiotherapy^[103], suggesting its potential use in adjuvant treatment of spinal metastases. These reports suggest that treatment of tumors with a combination of PS-mediated PDT and ionizing radiation could be superior to their individual use. The interaction of PDT and ionizing radiation could enhance the systemic therapeutic effect, thus reducing the radiation dose and potential side effects.

The effect of PDT combined with traditional chemotherapy for the treatment of breast cancer has also been studied and there are many examples. The treatment with appropriately combined low doses of cisplatin and indocyanine green- based PDT *in vitro* was proven to be more effective than each therapy alone^[104]. This synergistic effect is of extreme importance to be considered since cisplatin is one of the chemotherapeutics which is likely to cause severe side effects. Also the combination of mTHPC mediated PDT and 5-fluoro-2-deoxyuridine resulted in lower cell survival than the corresponding single mode treatment^[105]. Additionally, the enhanced antitumor effects between PDT and doxorubicin has already been demonstrated both *in vitro*^[106] and *in vivo*^[107] in a preclinical study on breast cancer. Although little is known about the mechanisms involving the interactions between chemotherapeutic drugs and PSs, or how they can be combined to increase cell killing and reducing side effects, all the cooperative effects between PDT and traditional chemotherapeutics cannot be neglected. Hence, PDT in combination with chemotherapy is among many strategies which have been proposed to potentiate the therapeutic outcome of low-dose chemotherapy and thus minimize side effects and acquisition of drug resistance^[35]. A great advantage of PDT is that it can be used either before or after chemotherapy, radiotherapy or surgery, without compromising these therapeutic modalities. Moreover, the adverse effects of chemotherapy or radiation do not affect sensitivity to PDT^[35]. These findings support the use of PDT, at least as an adjuvant therapy, for enhancement of anti-tumor immunity which may be capable of controlling cancer secondary disease.

Further research and more effort are required in order to allow PDT to be accepted as a suitable treatment for breast cancer. Even though, there is lot of evidence pointing that this therapeutic approach should

be considered as an antitumor treatment, the actual challenge for PDT is to translate the advances in understanding the effects in the cell-line-based and animal models studies into the clinical practice.

Clinical applications of PDT; Combination with other therapies; On-going trials

Despite the increasing number of studies with a growing number of chemical compounds and their generally increased number of favorable aspects as compared to more standard treatments, only a few PS have already been approved for clinical use and only for the treatment of a few diseases.

While the majority of PDT uses involve different types of cancer, ALA use with distinct formulations is already approved for several clinical applications, ranging from mild and moderate actinic keratosis to non-hyperkeratotic actinic keratosis. Some are also used to treat the Bowen's disease and basal cell carcinoma^[40]. Moreover, for serious ocular diseases such as age-related macular degeneration and myopic choroidal neovascularization there is a clinically approved PS called Verteporfin in current use^[108,109]. In 1993, Photofrin sodium (Photofrin) was the first PS approved for clinical use of PDT by the Canadian Health Agency for bladder cancer treatment. Later on, it was approved by the Food and Drug Administration (FDA) and also in several other countries for the treatment of various types of cancer, such as lung, esophageal, gastric, and cervical cancers as well as for cervical dysplasia^[110]. This photosensitizer is still widely used in PDT for treatment of various conditions^[111,112]. However, being a complex mixture of molecules with poor tissue selectivity and low light absorption, high concentrations of photofrin sodium are needed, which make it to persist over 2 months after the administration^[113], rendering patients photosensitive^[114]. A second-generation of PS has been then developed, with improved purity, longer wavelength absorptions and higher photosensitivity and tissue selectivity. Temoporfin was the second PS to receive approval and extensively used for treatment of advanced head and neck squamous cell carcinomas^[115,116]. Temoporfin was also tried for the treatment of breast, pancreatic and prostatic cancer^[117-121], with mixed results. For breast cancer there was response in all patients with minimal-invasive and few side effects^[119]. Similarly, for prostate cancer it appeared a safe procedure and after 8 of 10 PDT sessions, the prostate specific antigen fell by up to 67% and biopsies of treated areas revealed necrosis and fibrosis at 1-2 months^[120]. However, in patients with malignant biliary obstruction, endoscopically delivered Temoporfin-PDT caused efficient tumor necrosis and recanalization of blocked metal stents, but it showed a significant risk of complication with one leading to a fatal liver abscess and two patients developing haemobilia within 4 weeks of treatment, one of whom died with a gall bladder empyema^[118]. A mono-L-aspartyl chlorin, Talaporfin, was approved in Japan as a PDT for lung cancer but was also employed for early head and neck cancer patients and is going through the phase II trial for the treatment of colorectal neoplasms and liver metastasis^[122]. There are also other drugs approved as orphan drugs by the FDA for PDT treatment for Cutaneous T-cell lymphoma (SGX301) and for biliary tract cancer (LUZ11)^[40].

Since PDT has the potential to present a local effect, it lacks systemic adverse effects seen in other therapies. Additionally, because of its mechanism, PDT can be used in the clinic in combination with other procedures, such as radiotherapy, chemotherapy or surgery. At the present time there are a number of on-going trials using PDT with distinct PS for different types of cancer. Among all the on-going trials registered at clinicaltrials.gov (not concluded), about 50% of them use PDT alone or in combination with other therapies for at least one condition [Table 2]. So far PDT using distinct photosensitizers appears to be effective in decreasing tumor size and increasing patient survival in several of the trials, which target different kinds of tumors [Table 3]. Interestingly, the clear majority of current on-going trials for cancer are using photosensitizers that have already been approved for clinical use, mainly with Photofrin and ALA but also with Verteporfin and other still unapproved compounds.

The majority of approved PDT protocols are related to the treatment of superficial lesions of skin and luminal organs. However, due the enhancement of PS efficiency and light delivery, interstitial and intra-

Table 2. Number of on-going trials registered in clinicaltrials.gov using Photodynamic therapy for major conditions

Major condition	Number of trials
Cancer	49
Dermatological diseases	21
Mouth diseases	17
Ocular diseases	5
Cardiovascular diseases	2
Others (only 1 trial each)	6

operative approaches have been investigated for the ablation of a broad range of solid tumors^[22]. The first clinical application of PDT for breast cancer treatment was to treat skin metastases recurrence in the chest wall. The protocol tested was using Photofrin, and showed benefits in fifty percentage of the patients^[100]. Later studies with Photofrin^[123], m-THPC^[119] and Npe6^[124] supported PDT as an effective palliation treatment for chest wall recurrence in breast cancer patients. Further applications for PDT as treatment for breast cancer solid tumor and its sequelae, specifically its role in combination with adjuvant radiotherapy and chemotherapy, are being explored. Regarding the metastatic problem in breast cancer, although this systemic complication represents the most complex challenge in PDT-treatment for this type of tumor, a study conducted with fourteen patients with more than 500 truncal metastases showed that all patients presented tumor necrosis and nine of them demonstrated complete responses^[123]. Last but not least, a clinical trial of late-stage breast cancer patients treated by a local intervention using an 805 nm laser for non-invasive irradiation, indocyanine green and an immunoadjuvant (glycated chitosan) for immunological stimulation has shown that the combine therapeutic approach was safer and more efficient for treatment of metastatic breast cancer than each individual strategy^[125]. This study demonstrated that adjuvant treatment with immunotherapy following PDT may improve its effectiveness and represents a viable future treatment of tumors at distant sites, as discussed before, but still need more clinical studies.

PERSPECTIVES

There are several other new approaches being tested in “*in vitro*” and or in pre-clinical models aiming at improving the performance, the homing and delivery of PSs or including mixed formulations of chemotherapeutics in combination with PSs which appeared to show promising results up to now^[126,127]. The fast development of the field of nanostructured materials is in part responsible for the great increase of different innovative alternatives and approaches being studied. Of note is the notion that these approaches could also be used as detection and treatment alternatives at the same time supporting the idea that a better outcome could be reached by using these new multitasks tools in a near future. For example, a recent report has described the results of a pH, glutathione (GSH) and hyaluronidase (HAase) triple-responsive nanoplatform for HER2 and CD44 dual-targeted and fluorescence imaging-guided PDT/photothermal therapy (PTT) dual-therapy against HER2-overexpressed breast cancer. The nanoplatform was fabricated by functionalizing gold nanorods (GNRs) with hyaluronic acid (HA) pendant hydrazide and thiolgroups via Au-S bonds, and subsequently chemically conjugating 5-ALA, Cy7.5 and anti-HER2 antibody onto HA moiety for PDT. The final Nano platform bears fluorescent imaging and active targeting capacities, respectively. In this study the authors not only demonstrated that HER2 and CD44 receptors-mediated dual-targeting strategy significantly enhanced the cellular uptake of GNR-HA-ALA/Cy7.5-HER2 but also that the combined PDT/PTT treatment had significantly superior antitumor effect than PDT or PTT alone both in the MCF-7 cells model as well as in pre-clinical studies^[128]. Additionally, studies performed in human breast derived cell lines have shown that supersaturated hypericin (HYP) encapsulated on Pluronic® P123 (HYP/P123) micelles presented high stability and high rates of binding to cells, which resulted in their selective internalization in MCF-7, indicating their potential to permeate the membrane of these cells. Moreover, HYP/P123 micelles accumulated in mitochondria and endoplasmic reticulum organelles inducing

Table 3. Details of on-going clinical trials with the usage of PDT for handling neoplastic diseases

Indication	Photosensitizer	Study type	Estimated study completion date	Estimated enrollment (participants)	ClinicalTrials.gov Identifier
Anal Cancer	Aminolevulinic acid	Interventional	Apr/21	12	NCT02698293
Basal Cell Carcinoma	Aminolevulinic acid	Interventional	Nov/21	50	NCT03467789
Basal Cell Carcinoma	Aminolevulinic acid	Interventional	01/May/23	50	NCT03483441
Basal Cell Carcinoma	Aminolevulinic acid	Interventional	Sep/20	281	NCT02144077
	Methyl aminolevulinate				
Basal Cell Carcinoma	Methyl aminolevulinate	Interventional	01/Jun/20	20	NCT03167762
Basal Cell Carcinoma	Aminolevulinic acid	Interventional	Dec/22	117	NCT02367547
Basal Cell Neoplasms	Methyl aminolevulinate				
	Hexylaminolevulinate				
Basal Cell Carcinoma, Superficial	Aminolevulinic acid	Interventional	Aug/24	186	NCT03573401
Brain Tumor, Recurrent	Porfimer sodium	Interventional	Jun/31	30	NCT01966809
Brain Tumor, Recurrent	Porfimer sodium	Interventional	Apr/24	24	NCT01682746
Breast Cancer, Metastatic	Verteporfin	Interventional	Dec/19	15	NCT02939274
Cervical Intraepithelial Neoplasia	Aminolevulinic acid	Interventional	Nov/18	60	NCT02631863
Cholangiocarcinoma	Porfimer sodium	Interventional	22/Mar/18	55	NCT01755013
Cholangiocarcinoma	Porfimer sodium	Interventional	Jul/18	39	NCT02585856
Cholangiocarcinoma	Temoporfin	Interventional	Aug/19	20	NCT03003065
Cholangiocarcinoma	Fimaporfin	Interventional	Aug/19	55	NCT01900158
Cholangiocarcinoma, Hilar	Deuteporfin	Interventional	May/19	50	NCT02955771
Desmoid Tumors	Aminolevulinic acid	Interventional	Dec/26	140	NCT01898416
Esophageal Adenocarcinoma, Stages I, II, III	Porfimer sodium	Interventional	Dec/19	40	NCT02628665
Esophageal Squamous Cell Carcinoma, Stages I, II, III					
Esophagogastric Cancer	Palladium bacteriopheophorbide	Interventional	Apr/19	36	NCT03133650
Glioblastoma	Aminolevulinic acid	Interventional	Dec/19	10	NCT03048240
Head and Neck Carcinoma, Recurrent	Porfimer sodium	Interventional	01/Nov/22	82	NCT03727061
Head and Neck Carcinoma, Locally Advanced					
Lung Cancer	Porfimer sodium	Interventional	15/Sep/17	10	NCT03211078
Lung Cancer	Porfimer sodium	Interventional	Mar/19	10	NCT03344861
Lung Cancer, Metastatic					
Lung Cancer, Non Small Cell	Porfimer sodium	Interventional	Sep/21	66	NCT03564054
Lung Cancer, Non Small Cell	Porfimer sodium	Interventional	May/19	5	NCT02916745
Lung Metastasis					
Lung Cancer, Non-Small Cell	Porfimer sodium	Interventional	01/Aug/21	12	NCT03678350
Mesothelioma, Malignant					
Lung Cancer, Squamous Cell	Chlorin e6-PVP	Interventional	Oct/24	111	NCT03346304
Mesothelioma, Epithelioid Malignant Pleural	Porfimer sodium	Interventional	May/18	102	NCT02153229
Neurofibromatosis	Aminolevulinic acid	Interventional	Nov/18	30	NCT01682811
Neurofibromatosis	Aminolevulinic acid	Interventional	Aug/23	30	NCT02728388
Oral cancer	Aminolevulinic acid	Interventional	31/May/19	30	NCT03638622
Oral Cavity Squamous Cell Carcinoma, Stage I	HPPH*	Interventional	02/Nov/21	114	NCT03090412
Oral Cavity Squamous Cell Carcinoma, Stage II					
Pancreatic Cancer, Non-Resectable	Verteporfin	Interventional	Jan/22	15	NCT03033225
Pancreatic Cancer, Stage III	Porfimer sodium	Interventional	31/Jan/20	12	NCT01770132
Pancreas, Acinar Cell Adenocarcinoma of the					
Pancreas, Duct Cell Adenocarcinoma of the					
Peritoneal Carcinomatosis	<i>Hypericum perforatum</i> extract	Interventional	Jul/20	50	NCT02840331
Prostate Cancer, Localized	Palladium bacteriopheophorbide	Interventional	01/Mar/24	50	NCT03315754
Prostate Cancer, Recurrent	Verteporfin	Interventional	31/Dec/20	36	NCT03067051
Skin Cancer, Non-melanoma	Aminolevulinic acid	Interventional	Mar/21	20	NCT02751151
Skin Cancer, Non-melanoma	Aminolevulinic acid	Interventional	Dec/22	40	NCT02867722
Skin Cancer, Non-melanoma	Aminolevulinic acid	Interventional	30/Apr/21	20	NCT03110159
Squamous Cell Carcinoma	Aminolevulinic acid	Interventional	Jan/20	40	NCT03025724
Urothelial Carcinoma, Upper Tract	Palladium bacteriopheophorbide	Interventional	Aug/20	18	NCT03617003

Cholangiocarcinoma	Porfimer sodium	Observational	30/Dec/18	200	NCT01524146
Esophageal Adenocarcinoma, Early	any	Observational	Jan/22	400	NCT00587314
Lung Cancer	Porfimer sodium	Observational	30/Dec/24	1000	NCT01842555
Lung Cancer	any	Observational	15/Dec/23	1000	NCT03589456
Neoplastic Disease	any	Observational	Jul/21	400	NCT02159742
Prostate Cancer	any	Observational	01/Mar/20	200	NCT03492424

*HPPH stands for 2-1[Hexyloxyethyl]-2-Devinylypyropheophorbide-a

photodynamic cell death by necrosis and selective time- and dose dependent phototoxic effects on MCF-7 cells but little damage to non-tumorigenic cells. The micelles inhibited the generation of cellular colonies as well as migration of tumor cells indicating possible capacity to prevent the recurrence of breast cancer and to minimize the chances of cell spreading and thus the formation of metastases^[129].

Although PDT is based on the preferential accumulation in the tumor tissue, this selectivity is not absolute and some damage can occur to the surrounding tissue. Thus, a deeper understanding of the molecular mechanisms involved in drug delivery and specific targeting of tumors should contribute to the development of more specific technologies to deliver light and/or drugs to the tumor site and also to minimize resistance to PDT. Accordingly, the development of new PS targeting specific tumor sites have led to the modality of targeted-PDT^[130]. Another approach consists the photochemical delivery of drugs through photochemical internalization (PCI), a modified form of PDT. PCI is actually a light-controlled drug-delivery alternative in which light activation enables spatiotemporal specificity and control of the intracellular drug release^[131-133]. Moreover the potential of PCI to circumvent the resistance and increase the efficacy of a variety of anticancer agents, have been demonstrated in several tumor models including approaches to overcome PDT-resistance in breast cancer cells^[133,134]. Since identifying cellular resistance mechanisms by understanding the differences in cell response at the molecular level is of fundamental importance to improving the protocol and to increase the success of the therapy^[135], we have recently shown that breast cancer cells are differently affected by methylene blue (MB) concentrations at the same light dose in MB-PDT and that non-malignant human breast cells are significantly more resistant to the therapy compared to the malignant ones^[126]. One more example of how the use of a targeting agent would ideally improve the selectivity of PDT for the tumor tissue has been shown in a recent study. By using polyethylene glycol-coated, folate conjugated, benzoporphyrin derivative-loaded liposomes for PDT treatment of breast cancer cells, the researchers have reported that these liposomes are targeted for are greater uptake into TNBC cancer cells^[136]. Therefore, focusing on the molecular differences of cell death mechanisms induced by PDT, starting with an optimized PS choice and conditions of its delivery and activation, will certainly provide valuable clues for the development of new therapeutic strategies aiming at improving the efficacy of PDT against cancer cells^[7,15].

CONCLUSION

The challenges in fighting the disease rely on intrinsic tumor resistance properties, molecular heterogeneity, and metastasis. Considering all the information provided one can conclude that there are almost no doubts that one relevant advantage of PDT over other cancer treatments is the possibility of generating less side effects to the patients.

In summary, in this review we have explored and presented a broad up-date on the use of PDT as a therapeutic approach in the treatment of primary cancer as well as metastasis. We have covered several topics ranging from the photochemical mechanisms involved, the different cell death mechanisms being triggered by several photosensitizers up to the more recent-on-going clinical trials. Additionally, we have presented a significant amount of information underscoring the relevance of PDT as an alternative therapeutic approach capable of inducing several mechanisms of cell death, some of them simultaneously.

This capacity could be an interesting way of overcoming the problem of death resistance displayed by many tumors since one of the characteristics that is important for an alternative therapy for cancer treatment is to broaden the spectrum of cell death mechanisms being gathered in order to by-pass the different resistance mechanisms displayed by malignant cells.

Finally, even if more research is still needed in the field including the development and optimization of PS synthesis in order to increase the efficiency on specific tumor cell damage, light sources and irradiation protocols, it is evident that in the majority of the cases, PDT represents a suitable therapeutic alternative presenting several advantages over the traditional clinical approaches for cancer treatments. It is clear that PDT deserves more opportunities and investment in clinical trials. By doing so, it could be expected that the increase in the number of clinical studies with metastatic or non-metastatic cancer, in the near future, could allow the scientific and clinical community to make more robust conclusions about PDT's real translational potential to become a standard first-line therapy, either alone or in combination with other treatments, for a wide variety of tumors.

DECLARATIONS

Authors' contributions

Responsible for the paper: Baptista MS, Labriola L

Concept, design, definition of intellectual content: dos Santos AF, Baptista MS, Labriola L

Literature search, manuscript preparation and revision: dos Santos AF, de Almeida DRQ, Terra LF, Baptista MS, Labriola L

Manuscript editing: dos Santos AF, Labriola L

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Metabolic alterations and the potential for targeting metabolic pathways in the treatment of multiple myeloma

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Abstract

Metabolism is defined as the collection of complex biochemical processes that living cells use to generate energy and maintain their growth and survival. Metabolism encompasses the synthesis and breakdown of glucose, fatty acids, and amino acids; the generation of energy (ATP); and oxidative phosphorylation. In cancer cells, metabolism can be commandeered to promote tumor growth and cellular proliferation. These alterations in metabolism have emerged as an additional hallmark of various cancers. In this review we focus on metabolic alterations in multiple myeloma (MM) - a malignancy of plasma cells - including derangements in glycolysis, gluconeogenesis, the tricarboxylic acid cycle, oxidative phosphorylation, and fatty acid/amino acid synthesis and degradation. Particular focus is given to metabolic alterations that contribute to myeloma cell growth, proliferation and drug resistance. Finally, novel approaches that target metabolic pathways for the treatment of MM are discussed.

Keywords: Metabolism, alterations, multiple myeloma, treatment

MULTIPLE MYELOMA

Multiple myeloma (MM) is a malignancy of terminally differentiated plasma cells typically characterized by clonal proliferation of these plasma cells in the bone marrow. MM represents 1% of all malignancies and 18% of hematologic malignancies in the United States; accounting for an estimated 30,770 new diagnoses and 12,770 deaths in 2018 alone^[1]. Classically, MM results in the secretion of a non-functional monoclonal immunoglobulin



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(Ig) produced by the transformed plasma cells. Production of this aberrant Ig results in several of the complications associated with MM such as renal dysfunction, neuropathy, and hyperviscosity syndrome^[2,3]. However, in approximately 15%-20% of patients the abnormal plasma cells secrete only monoclonal free light chains, and in approximately 2%-3% of cases these cells secrete no monoclonal protein at all resulting in the so-called non-secretory myeloma^[4,5]. Myeloma cell growth in the bone marrow and the resultant cytokines produced by these transformed cells and/or other cells in the bone marrow microenvironment lead to the classic symptoms of active MM: osteolytic bone lesions, hypercalcemia, and anemia^[6].

The underlying epidemiology of MM remains largely undefined. Previously, exposure to ionizing radiation was thought to be a risk factor, but this was subsequently refuted in a large cohort of atomic bomb survivors in Japan^[7]. More recent data suggest a 2-3 fold increased risk for development of MM among African Americans which is thought to be related to increased rates of MGUS among this population^[8,9]. Interestingly, in contrast to most other malignancies, African Americans with MM tend to have a better prognosis compared to age-matched Caucasians with the disorder^[10]. Several meta-analyses have suggested obesity is associated with increased risk of myeloma with a relative risk ranging from 1.11-1.82^[11-14], and it has been shown that obesity significantly increases the risk of myeloma associated mortality^[15,16].

MM is primarily a disease of the elderly, with the median age at diagnosis being 69 in the United States^[1]. This population often suffers from significant co-morbidities making management of myeloma more challenging. Specifically, approximately 40% of this population meets criteria for obesity (BMI ≥ 30)^[17], and the rates of several obesity associated metabolic disorders such as diabetes and hyperlipidemia already approach 25% and 50% respectively, and continue to rise^[18,19]. Given these associations it is reasonable to wonder if these metabolic changes are significantly participating in MM pathogenesis. As this area has largely been uncharacterized, in this review we aim to highlight the metabolic changes that occur in myeloma patients, summarize how these changes are affected by myeloma directed therapy, and suggest possible interventions to enhance anti-myeloma based therapies by taking advantage of metabolic pathways which are often dysregulated in MM patients.

OVERVIEW OF METABOLISM

Glycolysis

Glycolysis is the primary process by which cells break down glucose releasing stored energy in the process which can be used to generate ATP^[20]. This process begins when membrane bound insulin receptors bind insulin resulting in autophosphorylation of the tyrosine residues. Subsequent phosphorylation of insulin receptor substrates, and activation of the PI3K and MAPK pathways promote cellular uptake of glucose^[21]. Insulin also regulates fructose 2,6-bisphosphate, a key regulator of glycolysis. In glycolysis, a single glucose molecule is phosphorylated by hexokinase (HK) to yield glucose-6-phosphate (a reaction which requires ATP), which then undergoes isomeric change by the enzyme glucose 6-phosphate isomerase to become fructose 6-phosphate. Fructose 6-phosphate is then irreversibly phosphorylated by phosphofructokinase (PFK) in a reaction that requires ATP to yield fructose 1,6-bisphosphate; this reaction is the rate-limiting and committed step of glycolysis. Fructose 1,6-bisphosphate is then cleaved by aldolase into two triose phosphates: glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Glyceraldehyde 3-phosphate is oxidized by glyceraldehyde 3-phosphate dehydrogenase using NAD⁺ as an electron donor to yield 1,3-bisphosphoglycerate (1,3-BPG). 1,3-BPG is then dephosphorylated at carbon-1 by phosphoglycerate kinase to yield 3-phosphoglycerate, and then reversibly converted to 2-phosphoglycerate by phosphoglycerate mutase. 2-phosphoglycerate undergoes a dehydration reaction catalyzed by enolase to form phosphoenolpyruvate (PEP) which is then irreversibly converted to pyruvate by pyruvate kinase (PK) in a reaction that also generates ATP^[22,23].

In eukaryotic cells, pyruvate can be reduced to produce ATP through two different pathways depending on the presence of mitochondria and appropriate blood and oxygen supply in the tissue of need: aerobic

respiration and anaerobic respiration. In aerobic respiration, pyruvate is transported into the mitochondria through a specific transporter and then decarboxylated by pyruvate dehydrogenase to produce acetyl-CoA which serves as the initial substrate for the tricarboxylic acid (TCA) cycle (see section 2 below). In anaerobic respiration, which in eukaryotes is typically limited to cells without mitochondria or poorly vascularized tissue (either endogenous or induced by states of physiologic stress), pyruvate accepts a hydrogen from NADH to produce lactate and NAD⁺. The NAD⁺ produced can then be used for glycolysis reactions or even react with TCA intermediates. Energy yield from anaerobic glycolysis is significantly reduced compared to its aerobic counterpart, however this process is more rapid and can occur in oxygen deprived tissues^[22]. Notably, some cancerous cells have been shown to preferentially undergo anaerobic respiration even under optimal conditions for aerobic respiration^[24-26]. This effect, which is thought to be due to metabolic reprogramming related to their hyperproliferative state, has been termed the “Warburg effect” after German physiologist and Nobel laureate Otto Warburg whose initial observations of increased glucose consumption coupled with increased lactate excretion from cancer cells led to the hypothesis of its existence^[27-29].

Cells have developed several mechanisms to increase or decrease glycolysis in response to metabolic needs. Furthermore, metabolic control of glycolysis occurs through various feedback mechanisms. Inhibition of insulin receptor signaling or key enzymes in the pathway (especially PFK) typically occur in fasting states or high energy states when the ratio of ATP to ADP is high. In these situations metabolism is shifted away from glycolysis and can instead result in a reciprocal induction of gluconeogenesis (see section 4 below)^[22,23]. Typically, glycolysis follows the general steps as reviewed above. However, there is some redundancy in the metabolic processes of the cell and some molecules may be synthesized in a different way and still be used in glycolysis. This can be seen in the way in which cells breakdown disaccharides like sucrose. If energy is needed, then the sucrose will be converted into fructose-6-phosphate molecules and continue through glycolysis^[22].

The TCA cycle

The TCA cycle allows for complete catabolism of organic molecules in the presence of oxygen, and results in the majority of ATP production in eukaryotes. The reactions of the TCA allow for breakdown of carbohydrates as well amino acids and fatty acids through various metabolites that can enter the pathway at different steps. For glucose metabolism the process begins with the production of acetyl Co-A in the mitochondrial matrix through oxidative decarboxylation of pyruvate in a reaction catalyzed by the pyruvate dehydrogenase complex (a complex of 3 component enzymes, 2 regulatory enzymes, and 5 coenzymes). Acetyl Co-A and oxaloacetate combine to form citrate in a condensation reaction catalyzed by citrate synthase. Citrate is then isomerized to isocitrate by aconitase which is subsequently converted to α -ketoglutarate by oxidative decarboxylation in a reaction catalyzed by isocitrate dehydrogenase. The α -ketoglutarate dehydrogenase complex catalyzes the conversion of α -ketoglutarate to succinyl Co-A and producing NADH. Succinyl Co-A is then cleaved to succinate by succinate thiokinase in a reaction that generates GTP via substrate level phosphorylation. Succinate is subsequently oxidized to fumarate by succinate dehydrogenase with FAD being reduced to FADH₂ in the process. Fumarate is then hydrated to malate in a reaction catalyzed by fumarase, with malate being oxidized to oxaloacetate by malate dehydrogenase producing another molecule NADH in the process.

There are many ways that the TCA cycle is regulated. The first regulatory step is known as the bridge reaction where pyruvate is converted to acetyl-CoA. If the cell has too many high energy molecules, then this “bridge reaction” will not occur and pyruvate will be utilized in other fashions. Other regulatory steps include the synthesis of citrate, and the oxidative decarboxylations of isocitrate and α -ketoglutarate all of which result in the production of high energy molecules (NADH, FADH₂, ATP, GTP) which then undergo oxidative phosphorylation. These steps will be inhibited if the concentration of the high energy molecules is increased. Similarly, if the concentration of low energy molecules (GDP, ADP, NAD⁺, FAD) were to accumulate then the reactions in the TCA cycle would increase^[22].

Oxidative Phosphorylation and the electron transport chain

Many of the products from glycolysis are then transported to the inner mitochondrial membrane where they donate electrons to a set of electron carriers known as the electron transport chain (ETC). As electrons transverse the ETC they lose energy and are coupled to the pumping of protons across the inner mitochondrial membrane which can subsequently be captured to drive the production of ATP through oxidative phosphorylation (OXPHOS) in a reaction catalyzed by the enzyme complex ATP synthase^[22,23].

Oxidative phosphorylation is regulated via several mechanisms. The enzymes succinate dehydrogenase and α -ketoglutarate dehydrogenase have roles in both OXPHOS and the TCA cycle, and are regulated by TCA intermediates like succinate, fumarate, and α -ketoglutarate. These two enzymes are also inhibited by high energy compounds and activated by low energy compounds. The pH of the mitochondrial matrix also regulates OXPHOS, as the ability to maintain the ETC and the subsequent proton pump is tied to maintaining a pH gradient^[22].

Gluconeogenesis

Gluconeogenesis is the synthesis of glucose, allowing the body to ensure blood sugar levels remain stable even in fasting states. The first step in gluconeogenesis is synthesizing PEP from pyruvate. This is a two-step process in which pyruvate is first converted to oxaloacetate by pyruvate carboxylase, then converted to PEP by PEP carboxykinase. PEP is converted to 2-phosphoglycerate, and subsequently to 3-phosphoglycerate, which is phosphorylated to produce 1,2-bisphosphoglycerate. Glyceraldehyde 3-phosphate is then synthesized by glyceraldehyde phosphatase dehydrogenase and converted to fructose 1,6-bisphosphate. Finally, glucose 6-phosphatase dephosphorylates glucose 6-phosphate to form glucose. Since this process is nearly the opposite of glycolysis, many of the enzymes between these two processes are the same, and gluconeogenesis is tightly regulated by many feedback mechanisms to maintain the glucose concentration and to avoid hypo/hyperglycemia. Not surprisingly then, one of the main regulators of gluconeogenesis is glucose itself; the other two main regulators are pyruvate and PEP. High concentrations of these two molecules in combination with lower levels of glucose leads to increased gluconeogenesis^[22].

Fatty acid synthesis and degradation

Fatty acid synthesis is not only important for long-term energy storage but also for structural components of cell membranes and eicosanoids. Fatty acid synthesis typically begins with a reaction between acetyl-CoA and malonyl-ACP to yield acetoacetyl-ACP and CO₂. This condensation reaction is facilitated by 3-ketoacyl ACP synthase (KAS III). Acetoacetyl-ACP subsequently undergoes reduction, hydration, and re-reduction to reduce the 3-keto group eventually yielding acyl-ACP, which can be used to initiate elongation of the fatty acid chain. This cycle continues until acyl-ACP's backbone reaches 16 or 18 carbons. After appropriate elongation acyl-CoA can be used for multiple different processes, including synthesis of glycerophospholipids and triacylglycerides, production of phosphatidate, and synthesis of phosphatidylcholine (PC). While lipogenesis can occur through the conversion of carbohydrates to acetyl-CoA, it can also take place by *de-novo* lipogenesis through the conversion of glycogen to fatty acids which typically occurs when glycogen storage is full^[22].

To release energy stored at fat, the molecule undergoes β -oxidation, or fatty acid oxidation. As fatty acids are unable to diffuse through the mitochondrial membrane, they are first converted to acylcarnitine which can enter through the carnitine antiports. Once in the matrix, acylcarnitine is converted to fatty-acyl-CoA. β -oxidation is largely the reverse reaction of lipid synthesis. Starting with acyl-SCoA there is oxidation, hydration and oxidation again to yield 3-ketoacyl-SCoA. The β -carbonyl is then cleaved by HS-CoA, resulting in a fatty Acyl-CoA molecule that now holds two less carbons than it did at the start of the cycle. Each cycle thus produces ubiquinol, NADH and acetyl-CoA which can all be used in aerobic respiration^[22].

Fatty acid synthesis and degradation are regulated largely by cellular energy dependence. Fatty acids serve as long-term energy storage molecules. During starvation where ATP production from the breakdown of glycogen cannot produce adequate amounts of energy, fatty acid degradation accelerates. On the other hand, if there are adequate supplies of ATP and glycogen storage is full, then fatty acid synthesis can occur^[23].

Amino acid synthesis and degradation

Amino acids and proteins remain a central aspect of cellular metabolism. While there have been over 300 amino acids described, only 20 are commonly found in mammalian proteins. Not surprisingly, these 20 amino acids are the only amino acids coded for by DNA^[30]. Of these, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine are termed essential as they cannot be synthesized by humans. Amino acids serve various roles in metabolism. In addition to protein synthesis, they can be used for energy production and synthesis of hormones. In extreme situations where energy is scarce and fatty acid reserves have been exhausted, protein turnover becomes a main source of energy^[22].

Oxidation of proteins typically occurs in small quantities since the ammonia/ammonium byproduct is toxic and must be transported bound to *L*-glutamine. *L*-glutamine can leave the tissue and be transported to the liver for energy production with NH₄⁺ excreted as urea. Alternatively, proteins can be metabolized by the glucose-alanine cycle. When breakdown of muscle protein yields *L*-glutamate, a reaction with pyruvate occurs yielding alanine and α -ketoglutarate. Alanine is then transported to the liver where transamination occurs resulting in glutamate which subsequently undergoes deamination releasing NH₄⁺ into the urea cycle. Pyruvate is also a byproduct of the transamination reaction and can be used in the liver for gluconeogenesis.

Integration of metabolic processes

Cellular metabolism is intertwined and tightly regulated. Glycolysis and the TCA cycle produce many byproducts that may enter the ETC for oxidative phosphorylation or can be used for other purposes. For example, the oxidation of glyceraldehyde 3-phosphate which occurs in glycolysis also results in the production of NADH and H⁺. These molecules can serve as electron donors for the ETC. Similarly, FADH₂ which is a byproduct of the reaction that converts succinate to fumarate in the TCA cycle can also serve as an electron donor in the ETC.

Anaerobic and aerobic respiration are closely linked by the use of NAD⁺ and NADH. Production of lactate during anaerobic respiration is one of the main ways in which NADH and H⁺ are oxidized back into NAD⁺ which allows for the TCA cycle to continue without any significant changes. Ultimately, glycolysis, the TCA cycle, gluconeogenesis, fatty acid metabolism, and protein synthesis are all interconnected and exist in a highly regulated and interdependent environment designed to maintain homeostasis^[31-33].

ALTERATIONS IN METABOLISM OF MULTIPLE MYELOMA

Multiple changes in metabolism occur in myeloma cells which are summarized below and in Figure 1. Understanding the metabolic alterations in MM has important implications in the care of patients with MM. It sheds new light into our understanding of MM development and progression, and allows for the assessment of predictive or prognostic biomarkers for optimal management of myeloma patients. Importantly, it provides potentially novel therapeutic targets for the treatment of MM.

Over the last decade, several technologies such as mass spectrometry, ¹H-nuclear magnetic resonance (¹H-NMR) spectroscopy and mass spectrometry, and ¹³C stable isotope resolved metabolomics (SIRM) have been used to determine metabolic alterations in monoclonal gammopathy of undetermined significance (MGUS) patients, newly diagnosed MM (NDMM) and relapsed/refractory MM (RRMM) and to compare metabolic changes in drug resistant MM cells. Steiner *et al.*^[34] used mass spectrometry to analyze 188

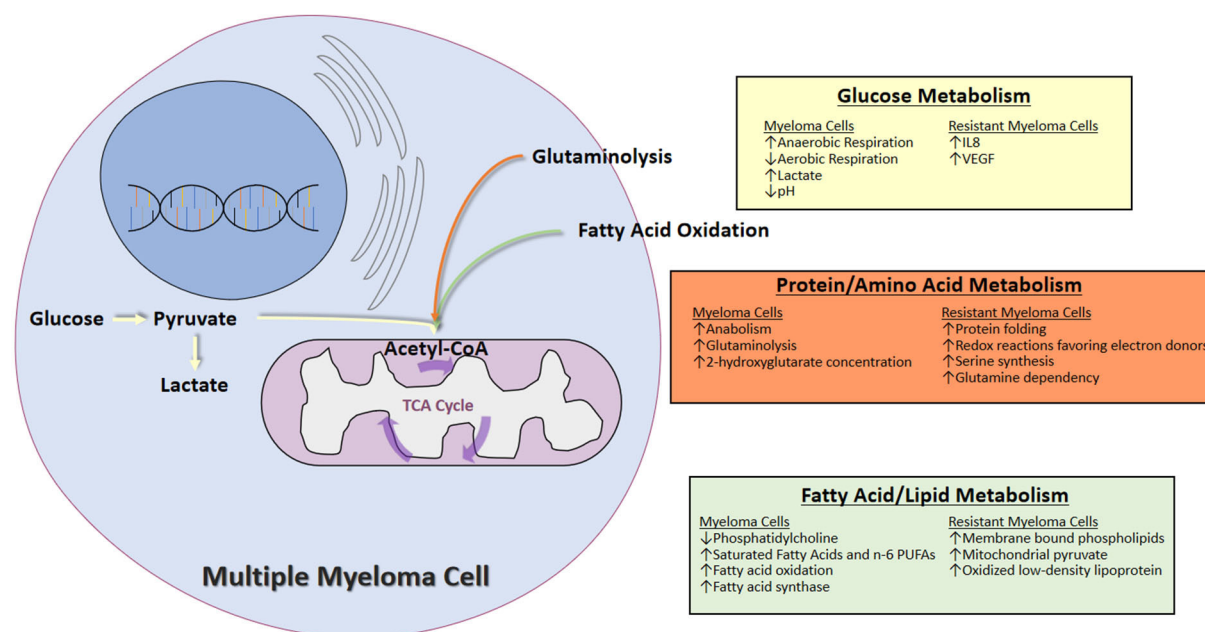


Figure 1. Summary of the metabolic changes in multiple myeloma cells and in resistant myeloma cells

endogenous metabolites in peripheral blood plasma samples of healthy controls, patients with MGUS, NDMM, or RRMM. The metabolomic profile was quite different between healthy controls and patients with MGUS, NDMM or RRMM with significant alterations in amino acid, lipid and energy metabolism. Eight plasma metabolites - free carnitine, acetylcarnitine, glutamate, asymmetric dimethylarginine (ADMA) and four PC species - were different between MGUS and NDMM patients, demonstrating that metabolic changes occur during MM development and progression^[34].

High-resolution ¹H-NMR spectroscopy and mass spectrometry provides quantitative analysis of metabolite concentrations and reproducible information with minimal sample handling. Puchades-Carrasco *et al.*^[35] performed ¹H-NMR spectroscopy metabolomic analyses of serum samples of MM patients and healthy subjects, and also compared the metabolic profiles of MM patients at the time of diagnosis and after achieving complete remission. Compared to healthy subjects, MM patients at the time of diagnosis exhibited higher levels of isoleucine, arginine, acetate, phenylalanine, and tyrosine and decreased levels of 3-hydroxybutyrate, lysine, glutamine, and some lipids. Interestingly, when myeloma patients achieved complete remission, the levels of lysine, citrate, lactate dehydrogenase (LDH), and choline went up whereas glucose level decreased in comparison to those at the time of diagnosis^[35].

A similar study using ¹H-NMR spectroscopy was performed by Ludwig *et al.*^[36] to characterize the metabolic profile within the bone marrow using filtered plasma derived from bone marrow aspirates of healthy donors and patients with MGUS or MM. This study found that the essential amino acids isoleucine and threonine were significantly decreased in the bone marrow of MGUS and MM patients as were the nucleotide-breakdown products, hypoxanthine and xanthine, suggesting an increase in anabolism. The products of arginine metabolism, creatinine and urea, were also significantly altered. The authors suggested that alterations in bone marrow metabolism are an early and consistent feature during the development of MGUS and MM^[36].

Recently, Gonsalves *et al.*^[37] performed ¹³C SIRM using U[¹³C₆]-Glucose and U[¹²C₅]-Glutamine in human MM cell lines to determine the contribution of carbon substrates from glutamine into the TCA cycle of clonal plasma cells in comparison with those from glucose. Their results suggest that glutamine is the main

contributor of carbon substrates to the TCA cycle whereas glucose is an important contributor of carbon substrates for the formation of lactate in clonal plasma cells^[37]. This was also supported by the finding that clonal bone marrow plasma cells in MM patients have higher glutamine uptake compared with the remainder of bone marrow mononuclear cells. Additionally, 2-hydroxyglutarate (2-HG) - an oncometabolite of glutaminolysis - is significantly increased in the bone marrow supernatant of MM patients compared to that of MGUS patients. Similarly, levels of 2-HG in the bone marrow supernatant or in the peripheral blood plasma of patients with smoldering multiple myeloma was associated with higher risk of progression to MM, and correlates with the percentage of bone marrow plasma cells expressing c-MYC^[37]. These results demonstrate the utility of measuring 2-HG levels in BM supernatant or peripheral blood plasma of SMM patients as a potential biomarker for disease progression and for identification of patients who may benefit from early therapeutic intervention.

To characterize the lipid profile of MM cells, Hossen *et al.*^[38] sorted single MM and normal plasma cells using flow cytometry and performed matrix-assisted laser desorption/ionization-imaging mass spectrometry. They found that PC (16:0/20:4) was significantly decreased in MM cells compared to normal plasma cells, which is likely due to the decrease of its integrated fatty acids or to increased metabolism of C_{16:0}.

The development of drug resistance is also associated with metabolic alterations in MM. Zub *et al.*^[39] performed global proteomic and transcriptomic profiling on melphalan sensitive and resistant RPMI8226 MM cell lines. Glycolysis was found to be the most significantly altered pathway. Gluconeogenesis, glutaryl-CoA degradation, isoleucine and tryptophan degradation, TCA cycle, ketolysis and ketogenesis were also significantly altered when MM cells develop resistance to melphalan. Specifically, the glycolytic and pentose phosphate pathway enzymes were up-regulated in the melphalan resistant cells, whereas the TCA cycle and ETC proteins were down-regulated. Additionally, the melphalan-resistant cells displayed increased tolerance to overall oxidative stress, but were sensitive to mitochondrial electron transport inhibitors. It was further demonstrated that lactate accumulation, Interleukin-8 and vascular endothelial growth factor (VEGF) signaling, as well as aldo-keto reductase played a role in the development of drug resistance to melphalan in MM cell lines^[39]. Similarly, in MM cell lines resistant to proteasome inhibitors (bortezomib and carfilzomib), complex proteomic changes, particularly in redox and energy metabolism were observed^[40]. Proteins involved in metabolic regulation, redox homeostasis, and protein folding and destruction were upregulated in proteasome inhibitor-resistant MM cell lines, which exhibited metabolic adaptations that favored the generation of reducing equivalents such as NADPH^[40]. The glucose metabolism was rewired in bortezomib-resistant MM cells, leading to higher activity of both the pentose phosphate pathway and serine synthesis pathway and an increased anti-oxidant capacity in bortezomib-resistant cells^[41]. Interestingly, the expression of 3-phosphoglycerate dehydrogenase (PHGDH), which catalyzes the rate-limiting step of serine synthesis, was up-regulated in bortezomib-resistant MM cells. Consistent with increased serine synthesis observed in bortezomib-resistant MM cells, serine starvation enhances the cytotoxicity of bortezomib. These data suggest that interfering with serine metabolism could be a novel strategy to improve bortezomib therapy and PHGDH could be a potential biomarker for bortezomib resistance^[41].

To identify therapeutic targets for the prevention and treatment of drug resistance, Maiso *et al.*^[42] performed metabolic profiling of multiple myeloma cells in normoxic and hypoxic conditions. It was found that in hypoxic conditions, intermediates of the TCA cycle and the ETC were reduced whereas intermediates of glycolysis were elevated. Specifically, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, pyruvate, and lactate were significantly increased in hypoxic cells. Gene expression profiling further demonstrated that the main pathways altered by hypoxic culture were those involved in glucose metabolism and the TCA cycle including key metabolic genes such as hexokinase 2 (HK2) and lactate dehydrogenase A (LDHA). Interestingly, bortezomib treatment inhibited HK2 activity but not lactate activity, suggesting LDHA may play a role in modulating drug resistance of MM cells in hypoxic conditions.

TARGETING METABOLIC PATHWAYS FOR THE TREATMENT OF MULTIPLE MYELOMA

The alterations in metabolism provide potentially therapeutic targets for the treatment of MM.

Targeting glycolysis for the treatment of MM

Glucose is the most abundant energy producing molecule in the human body and as such is broken down to produce energy for the body to maintain its regulatory functions. MM exhibits significant alterations in glucose metabolism. This was demonstrated by the fact that myelomatous bone and soft tissue lesions have elevated glucose uptake on positron emission tomography (PET) scans. Total lesion glycolysis and metabolic tumor volume, parameters measurable using ^{18}F -FDG PET CT scan, are highly associated with progression-free and overall survival and can significantly improve the prognostic value of both the GEP70 and International Staging Systems^[43]. These data suggest that targeting specific aspects of glucose metabolism may offer a novel avenue for therapy.

Like many other tumors, MM cells demonstrate enhanced glycolysis and lactate production (e.g., aerobic glycolysis) instead of proceeding through the TCA cycle. Enhanced glycolytic flux confers tumor cells a growth advantage and plays an important role in maintaining myeloma cell survival and proliferation and in inducing chemoresistance. Many genes are involved in the enhanced glycolysis seen in MM. The PI3K/AKT pathway, a cytoplasmic chemical messaging system, has been linked to increased glucose metabolism and may be part of the reason why glycolytic intermediates are upregulated in myeloma cells^[44-47]. Subsequently, it was found that a serine/threonine protein kinase, mTOR, regulated PI3K/AKT signaling^[45]. Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that is upregulated in the bone marrow microenvironment (where hypoxic conditions are standard), in myeloma cell lines, and CD138+ plasma cells isolated from MM patients^[48,49]. HIF-1 has been shown to play a key role in the accumulation of increased glycolytic metabolites in MM^[42,50,51]. HIF1- α (a subunit of HIF-1) induces transcription of several genes related to the response to hypoxia including genes that upregulate glycolytic enzymes and lactate production^[42]. HIF1- α expression is increased in drug resistant myeloma cells, and is associated with increased risk for metastatic disease and worse prognosis in various cancers^[42,50-53]. Drug resistance and disease relapse are thought to be due to the minimal residual disease cells that reside in the hypoxic bone marrow microenvironment. Maiso *et al.*^[42] showed that specific inhibition of LDHA and HIF1- α can restore drug sensitivity to anti-myeloma agents and inhibit tumor growth suggesting that targeting HIF1- α or LDHA can be used to inhibit myeloma growth and overcome drug resistance.

Pyruvate kinase M2 (PKM2) is a key factor regulating aerobic glycolysis and promoting tumor cell proliferation and survival^[54]. Recently, Gu *et al.*^[55] showed that never in mitosis gene A (NIMA)-related kinase 2 (NEK2) regulates splicing of PKM and increased the PKM2/PKM1 ratio in myeloma cells to promote aerobic glycolysis and oncogenic activity.

LDH is a key enzyme that regulates glycolysis and the conversion of pyruvate and NADH to lactate and NAD⁺ respectively^[56]. LDHA has been shown to be upregulated in MM cells by the proliferator-activator receptor-g coactivator-1 β acting on the LDHA promoter. This increase has been shown to significantly potentiate glycolysis metabolism resulting in increased cell proliferation and tumor growth^[42,57].

MM cells exhibit increased activity in glucose transporters and in key glycolytic enzymes such as HK. Most of the work targeting glucose metabolism in MM has focused on the GLUT family. Up-regulation of GLUT1, GLUT4, GLUT8, and GLUT11 has been shown to increase glycolytic metabolites in myeloma cells^[58]. Myeloma cells rely on the insulin-responsive glucose transporter GLUT4 for basal glucose consumption, maintenance of Mcl-1 expression, growth, and survival^[58]. Ritonavir is an FDA-approved HIV protease inhibitor and has a selective off-target inhibitory effect on GLUT4. Treatment with ritonavir elicits dose-dependent abrogation of both glucose uptake and proliferation on KMS11 and L363 myeloma cells^[58]. Interestingly, a subset of myeloma

cells survives glucose deprivation or ritonavir treatment likely by engagement of mitochondrial oxidative phosphorylation. Metformin is an FDA approved anti-diabetic medication that targets mitochondrial complex 1. The combination of ritonavir and metformin effectively elicited apoptosis *in vitro* in MM cell lines and primary human myeloma cells and showed anti-myeloma activity *in vivo* in a xenograft model of MM^[59]. Further analysis of the combination showed that it suppressed AKT and mTORC1 phosphorylation, and down-regulated the expression of Mcl-1 in myeloma models^[59].

Hexokinases catalyze the first irreversible step of glycolysis and play a critical role in the regulation of glycolytic activity. HK2 interacts with the voltage dependent anion channel in the outer membrane of mitochondria, where it catalyzes the conversion of glucose to glucose 6-phosphate^[60]. HK2 has been shown to be overexpressed in a variety of cancers including MM^[61,62], suggesting that HK2 could be a viable target for inhibiting the proliferation of multiple myeloma cells^[62-65]. Notably, treatment with bortezomib or vincristine, downregulated the expression of GLUT-1 and hexokinase, and induced apoptosis in OPM2 MM cells^[66].

An additional approach to targeting glucose consumption has been with the use of the novel purine analogue 8-aminoadenosine (8-NH(2)-Ado). Shanmugam *et al.*^[67] showed that treatment of the MM1S myeloma cell line with 8-NH(2)-Ado reduced glucose consumption through regulation of the GLUT4 transporter.

Targeting amino acid metabolism for the treatment of MM

In addition to MM cells being dependent on glucose, myeloma cells are also dependent on glutamine for energy equivalents through a process known as glutaminolysis. This process is especially prevalent in chemotherapy resistant myeloma cells. Bajpai *et al.*^[68] showed that when glucose metabolism is inhibited in myeloma cells exposed to various anti-myeloma agents, (bortezomib, melphalan, or carfilzomib), the resistant myeloma cells became preferentially dependent on glutamine and thus less likely to undergo apoptosis. Using glutamine as a primary energy source also affects the bone marrow microenvironment in MM patients. Specifically, the T-cells and NK cells in the tumor microenvironment are suppressed by the nutrient deprivation, hypoxia, and decreased pH that results from increased dependence on glutamine as an energy source^[69].

Glutaminolysis breaks down glutamine into alpha-ketoglutarate which then enters TCA cycle. Glutamine can be converted to glutamate, a reaction termed glutamine anaplerosis. Alpha-ketoglutarate can be further broken down to 2-HG which is associated with c-MYC overexpression^[37,70]. 2-HG is an oncometabolite, and elevated levels of 2-HG in smoldering MM patients are associated with increased risk of progression to MM^[37]. Glutamine is crucial for the survival and proliferation of certain cancer cells, and starvation of glutamine induces cancer cell death^[71]. Effenberger *et al.*^[72] recently showed that *in vitro* cultures of MM cells are dependent on glutamine for survival and that this is dependent on MYC protein expression. Notably, when these cells were treated with the glutaminase inhibitor benzophenanthridinone 968 apoptosis was induced. Additionally, MM cells show high expression of the glutamine transporters SNAT1, ASCT2, and L-type amino acid transporter 1 (LAT1); and inhibition of the ASCT2 transporter exhibits anti-myeloma activity^[73]. Furthermore, high expression of LAT1 is associated with high proliferation and poor prognosis in newly diagnosed MM patients, and predicts poor overall survival independent of the International Staging System^[74]. These data demonstrate that inhibition of glutaminolysis serves as a potential therapeutic approach in the treatment of MM^[71,72].

Targeting fatty acid metabolism for the treatment of MM

Fatty acids and lipids play important roles in the development and pathogenesis of MM through direct influence on the metabolism of myeloma cells, functioning as intercellular messengers, or acting as mediators of the pathological immunologic pathways^[75]. Analysis of plasma or erythrocyte membrane lipid

composition provides a simple, suitable model to study fatty acid metabolism. Jurczynszyn *et al.*^[75] measured the fatty acid composition of RBC membranes in MM patients and compared them with healthy controls. MM patients exhibited higher levels of saturated fatty acids and n-6 polyunsaturated fatty acids (PUFA) and lower levels of monounsaturated n-3 PUFA and trans-fatty acids, than controls. It was suggested that the fatty acid content of the RBC membrane could serve as a diagnostic and/or predictive biomarker in MM. Similarly, increased plasma levels of saturated fatty acids and n-6 PUFA were observed in MM patients compared to healthy controls, indicating increased synthesis of these fatty acids in MM patients^[76]. Recently, it has been shown that several myeloma cell lines (U266, RPMI8226, and NCI-H929) increase fatty acid oxidation as a mechanism to maintain metabolic hemostasis and viability during conditions of decreased glycolysis and increased lactate accumulation^[77-79].

Fatty acid synthase (FAS) expression was up-regulated in human myeloma cell lines as well as primary myeloma cells and contributes to myeloma cell proliferation and survival. Inhibition of fatty acid β -oxidation with etomoxir or *de novo* fatty acid synthesis with orlistat significantly reduced myeloma cell proliferation. The combination of 50 mmol/L etomoxir and 20 mmol/L orlistat resulted in an additive inhibitory effect on myeloma cell proliferation. Interestingly, the inhibitory effect was associated with reduced levels of p21 protein and phosphorylated retinoblastoma protein^[79]. These data suggest that inhibition of fatty acid metabolism provides a potential therapeutic approach in the treatment of MM, however O'Connor *et al.*^[80] recently showed that etomoxir loses its specificity at concentrations above 5 mmol/L and results in increased ROS production so these experiments must be interpreted with caution.

Obesity is associated with increased risks of myeloma incidence and mortality. To understand the molecular mechanisms by which adipocytes contribute to myeloma cell survival and progression, Bullwinkle *et al.*^[81] co-cultured MM cell lines with adipocytes harvested from normal, overweight, obese, or super obese individuals. It was found that MM cells proliferated faster, displayed increased pSTAT-3/STAT-3 signaling, and had enhanced adipocyte endothelial tube formation and cell adhesion when co-culturing with conditioned media from obese and super obese individuals.

FUTURE DIRECTIONS

Several areas of future research provide promise in the development of novel treatments for MM.

Lactate research

As the primary molecule thought to induce the hypoxic microenvironment surrounding many cancer cells, lactate is one of the most heavily researched metabolites in MM cells. In 1991 it was discovered that high serum LDH levels could identify myeloma patients that were at increased risk for early progression after treatment^[82]. Furthermore, LDH has been identified as a marker that identifies higher risk patients^[83]. The increased anaerobic respiration in the hypoxic microenvironment of myeloma cells is thought to be linked to the HIF1- α transcription factor, and leads to an increase in lactic acid production. In MM, HIF1- α activity increases glycolytic metabolites, inhibits production of TCA intermediates, and activates IL-6 which all contribute to tumor growth and survival^[84,85]. Notably, hypoxia has been shown to promote myeloma cell dissemination from the bone marrow to the peripheral blood through the downregulation of E-cadherin and the upregulation of the epithelial to mesenchymal transition proteins SNAIL, FOXC2, and TGF β 1^[86]. Hypoxic conditions also promote upregulation of VEGF leading to increased angiogenesis in a mechanism that is thought to be related to HIF1- α activation^[86-89].

Transporters of lactate are also possible metabolic targets in myeloma. The MCT family of transporters are involved in lactate transport in and out of the cell. MCT1 and MCT4 are preferentially expressed in myeloma cells. Inhibition of MCT1 with α -cyano-4-hydroxycinnamic acid led to reduced lactate incorporation and induced apoptosis in myeloma cell lines^[90,91]. Increased lactate is secreted out of MM cells through the MCT

transporters leading to an acidification of the bone marrow microenvironment. This results in inhibition of MCTs in T-cells and thus accumulation of intracellular lactate and H⁺, which-together with their reduced access to glucose by overconsuming tumor cells-leads to decreased T-cell activation^[92].

OXPHOS in multiple myeloma

Since oxidative phosphorylation is the last step in ATP synthesis for aerobic respiration it has been a particular focus of research in cancer metabolism. Given the increase in glycolysis during the progression of myeloma reviewed above, it might be predicted that OXPHOS increases as well. However, it has been found that OXPHOS activity is decreased during increased glycolysis in MM. This is thought to occur because the TCA cycle is not producing enough electron carriers to supply the OXPHOS mechanism to operate at a normal rate of ATP production^[90]. Given these conditions, energy production shifts to anaerobic respiration since other mechanisms, like ketosis, are evolutionarily reserved for starvation and extreme cases, meaning that lactate is the main source of energy in myeloma cells.

While many groups have investigated the role of metabolism in myeloma, relatively few have focused on the role of oxidative phosphorylation in MM. The complex 1 inhibitor, IACS-010759, has been tested in leukemia cells where it led to decreased proliferation and increased apoptosis in leukemia cells^[93,94]. This compound is actively being tested in clinical trials of relapsed AML (NCT02882321), and advanced solid tumors and lymphomas (NCT03291938).

Similarly, inhibitors of complex III have been explored as possible anti-cancer agents. Inhibition of complex III leads to an increase in ROS which can combat tumor proliferation and metastasis as well as induce apoptosis in MM. Previously, Arihara *et al.*^[95] demonstrated that reactive oxygen species have antimyeloma activity. Using CP-31398, an activator of p53 which also induces the formation of ROS, the investigators demonstrated decreased MM proliferation and increased apoptosis in a p53 independent fashion, and this effect was synergistic with carfilzomib. Notably, no additional hematologic toxicity was seen with the agent in animal studies^[96].

Fatty acid research

It is established that resistant MM cells can increase lipogenesis as a mechanism of chemotherapy resistance^[42,79,97]. PUFAs have previously been shown to enhance chemotherapeutic drug effects by selectively inducing apoptosis in multiple cancer cell lines^[98-103]. Specifically, administration of two PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) to four MM cells (L363, OPM-1, OPM-2 and U266) resulted in induction of apoptosis through increased caspase-3 activation, and mitochondrial membrane perturbation with no effect on normal human peripheral mononuclear cells^[104]. Similarly, studies by Dai *et al.*^[105] examined the effects of EPA and DHA on the myeloma cell lines MM1S and MM1R and found that treatment with these agents resulted in increased apoptosis which was enhanced by the addition of dexamethasone. Notably, this synergy with dexamethasone was observed in the MM1R cell line which is inherently dexamethasone resistant. As glucocorticoids are known to increase lipogenesis, and remain a mainstay of MM therapy, these data suggest that EPA and DHA may resensitize cells that have acquired resistance to glucocorticoids. Additional studies on MM cell lines showed that treatment with EPA or DHA decreased MM cell proliferation through induction of lipid peroxidation. This effect was inhibited by superoxide dismutase, or cyclooxygenase/lipoxygenase inhibitors suggesting a role for superoxides, prostaglandins, and leukotrienes in MM proliferation^[106]. Similarly, Wang *et al.*^[107] examined the expression of FAS in bone marrow from MM patients and matched healthy volunteers and found 70% of MM patients had elevated FAS while none of the healthy volunteers had detectable levels. Treatment of the FAS expressing MM cell lines U266 and RPMI8226 with the FAS inhibitor cerulenin resulted in induction of apoptosis evidenced by increased Annexin V staining suggesting FAS as a possible target of anti-myeloma therapy. These studies are admittedly preliminary but warrant additional research in this area.

Other metabolic targets

Other studies have been focused on TCA intermediates. Sanchez *et al.*^[108] were able to identify dichloroacetate (DCA) as a possible target for cancer therapy. DCA is an activator for pyruvate dehydrogenase which allows for increased production of TCA intermediates and consequently, increased OXPHOS for ATP production. Subsequently, DCA was found to inhibit glycolysis in multiple myeloma and increase sensitivity to bortezomib.

CONCLUSION

The study of metabolism's role in MM remains in its infancy, and many avenues remain unexplored. With combination therapies being the norm in myeloma management and acquired resistance to conventional therapies remaining a challenge, the potential exists for additional adjunctive therapies in MM patients. Targeting metabolic pathways is a novel area with preclinical data suggesting efficacy. Targets such as the GLUT and MCT transporters, IGF-1, FAS, and metabolites of the ETC and OXPHOS warrant additional exploration as possible novel anti-myeloma strategies, but care must be taken to minimize adverse effects when targeting ubiquitous pathways. Ideally, future preclinical and clinical studies will help to elucidate metabolism's role in myeloma development and progression, and may lead to the discovery of novel therapies for patients suffering from this disorder.

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Authors' contributions

All authors wrote the manuscript.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Stereotactic radiosurgery in the era of novel systemic therapy for lung cancer brain metastases

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Abstract

The emergence of novel systemic therapies has spurred a dramatic paradigm shift in lung cancer treatment. Research has revealed greater intracranial efficacy in targeted agents and immune checkpoint inhibitors (ICI) compared to conventional chemotherapy. Concurrently, advances in stereotactic radiosurgery (SRS) have contributed to the increased use of this highly localized, minimally-invasive treatment modality for local tumor control. In this era of precision medicine, the combination of these novel agents and SRS demands further prospective exploration - particularly as questions regarding their sequence of administration and the risk of neurotoxicity remain unanswered. Presently, although data are limited and largely retrospective, literature supports the concurrent administration of ICI and radiation, with no observed increases in immune-related adverse events or acute neurologic toxicities. In the case of patients with driver mutations, newer generations of tyrosine kinase inhibitors (TKI) display improved intracranial efficacy and are currently preferred alone upfront in patients with asymptomatic brain metastases (BM) due to lack of data. Evidence of combining TKI and SRS is limited with mixed results. In this review, we explore the evidence regarding the use of novel systemic agents and SRS for treatment of lung cancer BM. Clinical practice will continue to be refined as larger, prospective studies yield results.

Keywords: Lung cancer; stereotactic radiosurgery, brain metastasis, tyrosine kinase inhibitors, immunotherapy



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INTRODUCTION

Lung cancer remains the leading cause of cancer-related mortality^[1]. Eighty-five percent of diagnoses represent non-small cell lung cancer (NSCLC)^[2]; the remaining 10%-14% are diagnoses of small cell lung cancer. Brain metastases (BM) are diagnosed in approximately 10% of lung cancers at time of diagnosis^[3] and approximately 40%-50% will be diagnosed with BM during the course of their disease^[3,4]. BM in lung cancer are known to be associated with poor prognosis. Historically, standard treatment for BM from lung cancer involves neurosurgical resection^[5], radiotherapy and/or chemotherapy^[5,6].

Recent advances in NSCLC, including molecular analysis, matched targeted therapies and immunotherapy, has altered the standard of care. These novel approaches have shifted the paradigm in lung cancer^[7] and improved median overall survival in lung cancer patients with BM^[7,8]. Survival now ranges between 9-15 months and can be as high as 46 months in patients with favorable prognostic factors such as good performance status and epidermal growth factor receptor (EGFR) and/or anaplastic lymphoma kinase (ALK) positivity^[9]. In addition, over the past decade, standard of care in radiotherapy for BM has increasingly favored SRS^[10,11]. Precise delivery of high-dose radiation localized to the tumor results in higher local tumor control^[10] and fewer side effects such as neurocognitive damage^[11] compared to whole brain radiotherapy (WBRT).

The trifold advancement in targeted therapies, immunotherapy and SRS has revolutionized the treatment of BM in lung cancer. Employing these novel therapies, lung cancer patients are living longer, becoming more likely to develop brain metastases. Certainly, the use of these therapies - either individually or in combination - is anticipated for the treatment of lung cancer BM.

In this review, we discuss the current evidence regarding the use of SRS employed alone and in combination with novel therapies for treatment of lung cancer BM.

BRAIN METASTASIS IN LUNG CANCER LACKING A DRIVER MUTATION

SRS

SRS, typically delivered in a single fraction, serves as a key modality for delivering high-dose radiation to smaller target sites (usually < 3 cm)^[12], sparing adjacent structures from exposure and mitigating the harmful effects of radiation^[11].

For single BM, SRS has demonstrated efficacy and safety. Therefore, both surgical resection plus postoperative radiation and SRS alone are reasonable options and treatment should be individualized as comparative data is lacking^[13]. Surgery plus postoperative radiation is preferred for a single, large and symptomatic BM to allow for decompression, lower morbidity and higher local control^[13]. One small retrospective study found similar survival when comparing surgical resection vs. SRS for solitary BM^[14]. However, more local recurrence was demonstrated in the surgery group^[14].

For surgically-resected brain metastases, post-operative SRS (post-SRS) is the current standard of care. Some concerns with post-SRS include radiation necrosis and leptomeningeal disease (LMD) recurrence. The hypothesis behind LMD recurrence is the intra-operative seeding of viable tumor cells, which is supported by a study where post-SRS demonstrated higher rates of LMD compared to adjuvant WBRT^[15]. A new approach, pre-operative SRS (pre-SRS), is being evaluated as a potential method to decrease radiation necrosis and LMD. Potential benefits of pre-SRS include: (1) better local tumor control through improved delineation when contouring an intact metastasis compared to an irregularly-shaped surgical cavity; (2) reduced risk of radiation necrosis, as there is no need to treat surrounding brain tissue and the majority of the treated BM will be resected; (3) reduced risk of LMD as a result of a potential sterilizing effect via the intraoperative seeding of treated tumor cells; and (4) the potential to treat more patients, as with post-

SRS some patients are lost to follow-up. A potential disadvantage includes reduced wound healing^[15]. Asher *et al.*^[16] evaluated pre-SRS in 47 patients, demonstrating its safety and efficacy with local control rates of 85.6% at 12 months. One retrospective study evaluating pre- and post-SRS cohorts of 180 BM patients (including approximately 40% with NSCLC) showed similar rates of local recurrence and overall survival. However, pre-SRS was associated with significantly reduced rates of radiation necrosis and LMD^[17]. Clinical trials currently comparing pre-SRS and post-SRS in BM include NCT03741673 and NCT03750227.

SRS plays a major role in patients with multiple small BM (< 3 cm) or surgically inaccessible BM. Younger lung cancer patients of high Karnofsky performance status with limited BM and a low burden of extracranial disease may derive the most benefit from SRS^[10,18]. Generally, SRS use has been limited to patients with few, small, easily radio-accessible BM^[2]. Although a multi-center retrospective analysis demonstrated a survival advantage for patients treated with SRS possessing fewer than four BM ($n = 189$ for NSCLC) compared to WBRT (adjusted HR for NSCLC, 0.58; 95%CI: 0.38-0.87; $P = 0.01$)^[19], Yamamoto and colleagues recently evaluated SRS alone in 1,194 patients with up to ten lesions (largest tumor < 10 mL in volume and < 3 cm in longest diameter; total cumulative volume ≤ 15 mL)^[20]. Their group found no difference in overall survival (10.8 months) or treatment-related adverse events (9%) between patients with two to four tumors and patients with five to ten tumors^[20]. These results suggest expanding SRS for the treatment of patients with up to ten BM^[2,5]. Further data propose total BM volume as potentially more significant than the total number of BM^[10].

Adverse effects of SRS

Overall, the adverse effects of SRS are consistent with known toxicities of intracranial irradiation. Both acute (developing over weeks to months) and late-onset (developing over months to years) toxicities may result. Certainly, the risk, severity and incidence of radiation-induced toxicities is highly dependent on the site, dose, fractionation and volume of tissue irradiated along with the patient's comorbidities^[21]. Acute toxicities are uncommon and include nausea, headache, dizziness, seizure or new transient focal deficits^[22]. Patients are usually treated with a short course of glucocorticoids.

The most common delayed adverse effect of SRS treatment to BM is radiation necrosis, occurring in approximately 5%-10% of patients^[23]. However, this risk rapidly increases with increasing BM size and/or volume as well as with a history of radiation to same lesion^[21,23-25]. A study suggests employing multifractionated SRS in three to five fractions rather than single fraction SRS as a means of decreasing the risk of radiation necrosis and improving local control^[26]. The use of fractionated SRS also allows for the safe treatment of larger BM (> 3 cm)^[26,27]. Data on long-term effects of SRS on neurocognition is limited but reassuring^[25].

SRS in combination with immunotherapy

Immune checkpoint inhibitors (ICIs) have become a routine part of the treatment of advanced NSCLC lacking a driver mutation, administered with chemotherapy doublet or alone in patients with $\geq 50\%$ expression of programmed cell death-ligand 1 (PD-L1)^[8]. Most patients with advanced NSCLC and BM are eligible to receive ICIs - either alone or with chemotherapy.

Concerns to using ICIs to treat BM include: (1) pseudo-progression with the potential of symptom aggravation; and (2) steroid use for symptomatic BM which may reduce ICI activity, as demonstrated by a decreased objective brain response with the addition of steroids during ICI treatment in melanoma^[28]. Present data on ICIs for BM from advanced NSCLC is limited. Most ICI clinical trials in NSCLC excluded patients with untreated or unstable BM, yet included stable and treated BM, comprising 6%-17% of included patients^[29]. Overall, in the small subgroups of BM patients included in these trials, ICI appears safe^[29]. However, outcomes were mixed as some trials demonstrated benefit over chemotherapy and others did not^[29]. A rationale for positive response to ICIs includes the inflammatory microenvironment of BM, with

the presence of significant tumor-infiltrating lymphocytes (TILs). In a series of 116 BM specimens (including 61 from NSCLC BM), more than 50% of all specimens had dense TIL infiltration - also associated with improved survival^[30].

Radiation therapy (RT) also induces an antitumor immune response by upregulating PD-L1 and inflammatory cytokines as well as facilitating T-cell infiltration^[31]. Localized RT may induce an abscopal effect, which reflects the regression of non-irradiated metastatic lesions due to systemic anti-tumor response. The dose and fractionation of RT plays a role in its effects on the immune system. Schaeue *et al.*^[32] found that fractionated treatment with medium-sized radiation doses of 7.5 Gy per fraction yielded the best tumor control and anti-tumor immune responses. Dewan *et al.*^[33] showed that 5×6 Gy and 3×8 Gy protocols of RT were more effective in inducing immune-mediated abscopal effects than a single ablative dose of 20 Gy when combined with an anti-CTLA-4 antibody. These preclinical data suggest a better systemic anti-tumor effect with hypofractionated (e.g., fractionated SRS) than conventional RT. Most data regarding the abscopal effect are from treating systemic disease; if the same impact occurs in the treatment of BM is uncertain. Although based on small studies, there is growing evidence in favor of an abscopal effect when treating BM^[34].

RT alone is a poor inducer of immune-mediated local and abscopal responses; but, evidence suggests these responses are enhanced by combining radiation with ICI^[31,35,36]. Although data regarding the combination of ICI and radiotherapy in BM due to NSCLC are limited and mostly retrospective, data indicate that combining ICI and radiation in BM is safe with similar adverse events^[37,38] and support a concurrent administration of ICI with radiation over a sequential administration^[38]. While most studies demonstrated similar adverse events with combination ICI-radiotherapy, the role of ICI in radiation necrosis remains controversial. One retrospective study with 61% NSCLC patients showed that the incidence of symptomatic radiation necrosis after stereotactic radiation was higher in patients who received ICI - especially those with melanoma^[39]. Other retrospective studies including patients with NSCLC and BM did not report a higher incidence of radiation necrosis with combination ICI-radiotherapy^[37,38,40].

Many questions regarding the combination of ICI and RT remain unanswered including the optimal timing, the impact of steroids and neurotoxicity. Questions such as these should be investigated through prospective trials. Current clinical trials evaluating the combination of ICI and radiation therapy in NSCLC BM include NCT02978404 (Nivolumab + SRS), NCT02858869 (Pembrolizumab + SRS) and NCT02696993 (Nivolumab + SRS/WBRT and Nivolumab + Ipilimumab + SRS/WBRT). These phase I and phase II trials include other malignancies and are expected to finish by 2020.

BRAIN METASTASIS IN LUNG CANCER WITH DRIVER MUTATIONS

In NSCLC, sensitizing EGFR mutations are found in 10% of Caucasians as well as up to 50% of Asians^[41]. ALK-rearrangement is found in 2%-7%^[42] and ROS proto-oncogene 1 (ROS1) occurs in 1%-2% of patients^[43]. The incidence of BM is higher in patients with driver mutations. The rates of BM present at diagnosis is 24.2% and 23.8% in EGFR-mutated and ALK-rearranged lung cancers, respectively, and increasing to > 45% of patients at three years post-diagnosis^[44]. ROS1-rearranged NSCLC also has a high incidence of BM (36%) and is the common first site of progression^[45].

First- (erlotinib, gefinitib) and second-generation (afatinib) anti-EGFR TKI have demonstrated improved survival and brain response rates of over 50% in EGFR-mutated patients compared to EGFR wild-type patients^[5]. The third-generation anti-EGFR TKI, osimertinib, demonstrated an even higher rate of intracranial response (91% vs. 68% in patients with measurable BM lesions), a lower rate of central nervous system (CNS) progression and longer progression-free survival when compared to first generation anti-EGFR TKI in the FLAURA trial^[46,47]. Though first- and second-generation anti-EGFR TKI have activity in patients with BM, these agents have a much lower intracranial concentration as compared with osimertinib^[5]. Given

the high intracranial activity of osimertinib, it is preferred as the initial therapy for patients presenting with asymptomatic BM in EGFR-mutated NSCLC. Osimertinib has not yet been evaluated with SRS, but it is reasonable to use SRS in the case of isolated intracranial progression and continue to use osimertinib. A clinical trial is open to evaluate osimertinib with or without SRS for EGFR-mutated NSCLC with BM (NCT03497767). The trial is expected to be completed in 2022.

If osimertinib is unavailable, first- or second-generation anti-EGFR TKI can be used in patients with asymptomatic BM, but not alone. Preclinical studies have shown a sensitizing effect of radiotherapy on EGFR expression and an enhanced radiation response through the inhibition of EGFR^[48,49]. Although limited, clinical data is not yet reflective of preclinical data, but shows promise for future trials. A meta-analysis including 363 patients and another retrospective study of 351 patients with EGFR-mutated NSCLC (treated with first- and second-generation anti-EGFR TKI) suggested upfront intracranial radiation demonstrates better overall survival^[50,51]. SRS followed by anti-EGFR TKI resulted in the longest overall survival, with WBRT followed by anti-EGFR TKI demonstrating intermediate overall survival and anti-EGFR TKI followed by SRS or WBRT at intracranial progression resulting in the shortest median overall survival^[50,51]. Based on these data, for patients with asymptomatic BM started on a first- or second-generation anti-EGFR TKI, upfront SRS is appropriate - particularly given its better outcomes compared to delaying radiation. The concurrent use of anti-EGFR TKI with SRS or WBRT is more controversial, demonstrating mixed results. A phase III study found decreased overall survival with WBRT + SRS + erlotinib compared to WBRT + SRS^[52]. Another retrospective study found similar survival in patients receiving a concurrent administration of radiation + anti-EGFR TKI vs. patients receiving radiation followed by anti-EGFR TKI^[53]. At this point, due to the absence of data, we recommend stopping TKI administration during SRS treatment and resuming after completion.

ALK-directed TKIs have also demonstrated intracranial activity. In a retrospective analysis, the first-generation ALK inhibitor, crizotinib, showed an intracranial disease control rate of 56% in untreated BM and 62% in previously treated BM^[54]. Despite this intracranial efficacy, approximately 20% of patients progressing on crizotinib developed BM^[54]. Second-generation ALK-directed TKIs (ceritinib, alectinib, brigatinib) have better intracranial efficacy. Alectinib was compared to crizotinib in two phase III trials (J-ALEX and ALEX) and showed improved survival and superior CNS activity with an incidence rate of CNS progression at 12 months of 4.6% with alectinib compared to 32% with crizotinib in patients without BM at baseline^[55-57]. Brigatinib is also superior to crizotinib in the frontline setting, as seen in the ALTA trial, with an intracranial response rate of 78% with brigatinib vs. 28% with crizotinib^[58]. Crizotinib, ceritinib, alectinib and brigatinib are all approved as first-line therapies for ALK-positive advanced NSCLC. However, alectinib is preferred per National Comprehensive Cancer Network guidelines^[8]. Alectinib alone is appropriate in asymptomatic BM.

In patients who progress on crizotinib, ceritinib, alectinib or brigatinib are all appropriate as they have not been directly compared in terms of BM efficacy. In a small case series, alectinib also showed intracranial response in ceritinib-resistant patients^[59]. In the setting of alectinib-resistant disease, options include switching to lorlatinib, which has documented CNS activity in patients who have failed second-generation inhibitors^[60] or local therapy (such as SRS) if only oligometastatic disease is present^[61].

Crizotinib is the standard first-line treatment for patients with ROS1-rearranged NSCLC^[8], but as previously discussed, it has poor intracranial activity. Other approved TKIs include ceritinib and lorlatinib that demonstrate better intracranial activity^[62,63]. There are no data available on the combination of radiation therapy and TKI in ROS1-rearranged NSCLC.

In one study, patients with EGFR-mutated or ALK-rearranged NSCLC who had oligo-progression on erlotinib or crizotinib were considered for local ablative therapy to the sites of progression and continuation

of the TKI^[64]. The TKI was stopped on the days of radiation and restarted with the same dose^[64]. Twenty-five of 51 patients who progressed were deemed suitable for local therapy and most received stereotactic radiation^[64]. Ten of the 25 patients had intracranial progression^[64]. Post local ablative therapy, the median progression-free survival was six months^[64]. Minimal grade three and four adverse events were seen^[64]. Another study of ALK-rearranged NSCLC showed that combining stereotactic radiation with crizotinib is safe and can achieve durable control, although the study only included extracranial sites of progression^[65]. A retrospective study showed prolonged overall survival (49.5 months) of NSCLC patients with BM when treated with ALK-directed TKI therapy and brain radiation^[66]. In the ALEX trial, patients with previous radiation to BM had higher intracranial response rates (86% *vs.* 79%) compared with patients without prior radiotherapy^[57]. In summary, data on the safety and outcome of combining radiation with ALK-directed TKIs is limited and favors SRS over WBRT.

An ongoing clinical trial is evaluating ALK inhibitors and other targeted therapies in combination with stereotactic brain treatment in patients with stage IV oncogene-driven (EGFR, ALK, or ROS1) NSCLC (NCT02314364).

CONCLUSION

The development of novel, targeted agents and immunotherapy has advanced the systemic treatment of lung cancer. These therapeutics demonstrate far greater intracranial efficacy than conventional chemotherapy - transformative for BM treatment. However, this paradigm shift in treatment warrants the careful consideration of systemic therapy as a frontline approach. While SRS remains an important aspect of the management of BM, its role combined with novel systemic therapies is largely unclear. Limited available evidence suggests combination is safe with favorable outcomes, but the sequence of administration remains uncertain. Many clinical trials are underway that aim to further address these questions. As the results of these studies emerge, clinicians will gain further evidence-based insight into the clinical management of patients with lung cancer BM.

DECLARATIONS

Authors' contributions

Made substantial contributions to the research, writing and editing of the manuscript: All authors

Availability of data and materials

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

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Consent for publication

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Case Report

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Contralateral axillary metastasis: is surgical treatment the best option?

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Abstract

Contralateral axillary metastasis (CAM) is a rare entity normally treated as a systemic disease. Recent publications have proposed aggressive surgical treatment with benefits to the patients in terms of survival. We present a case of a 74-year-old patient with a history of unilateral breast cancer, recurrence on the ipsilateral breast and then development of a CAM. The patient was treated with aggressive surgical treatment, but she developed an early recurrence of the disease with distant metastasis. There is limited evidence of the correct management of CAM, although proposed to treat it as a loco regional disease, individualized and multidisciplinary management is the best option for these patients.

Keywords: Breast cancer, contralateral axillary metastasis, lymphatic routes blockage

INTRODUCTION

Contralateral axillary metastasis (CAM), posterior to a unilateral breast cancer, is a rare entity. The reported incidence is between 1.9% and 6% in different publications^[1,2]. Although rare, it is very clinically relevant, as lymph node metastasis is the most important prognostic factor in breast cancer. Correct identification of the primary origin of axillary metastasis is a priority; however, sometimes it can be a true challenge. There are many controversies about the pathophysiology and appropriate management of CAM because the contralateral axilla is considered a distant metastasis, thus stage IV disease, with limited therapeutic



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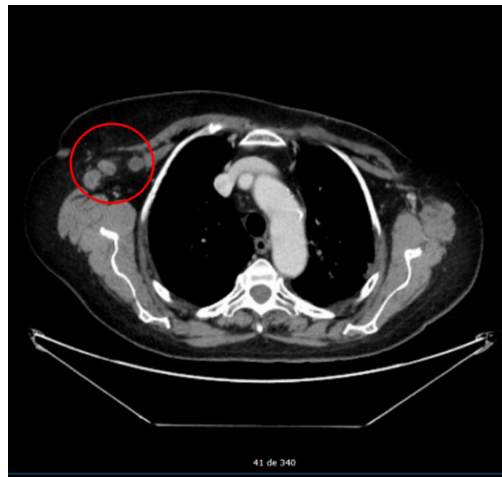


Figure 1. Computer tomography scan showing right axillary lymph node enlargement

strategies involved. Some authors have advised about a multidisciplinary and more aggressive approach to treat CAM, such as a loco regional spread breast cancer rather than distant metastasis^[3,4].

CASE REPORT

A 74-year old female, with an oncologic history of Hodgkin lymphoma (HL) stage IV B, with supra diaphragmatic and infra diaphragmatic adenopathies and bone marrow infiltration, was treated with chemotherapy twenty years ago.

The patient presented, in 2007, a left breast lesion on the upper outer quadrant (UOQ) detected by mammography. An ultrasound (US) and magnetic resonance imaging (MRI) study confirmed and classified the lesion as a BI-RADS 5. A core needle biopsy (CNB) was performed and the histopathology analysis (HA) reported an invasive ductal carcinoma (IDC), grade two, negative estrogen receptor (ER), positive progesterone receptor and negative human epidermal growth factor receptor type two (HER2/neu). A lumpectomy and sentinel lymph node biopsy (SLNB) with one step nucleic acid amplification (OSNA) technique, were performed. The SLN was negative and the size of the tumor was 1.5 cm. Classified as a stage IA, complement treatment with radiotherapy (RT) and hormonal therapy with Anastrozole was decided for five years.

In 2016, a lesion of 2 cm in the surgical scar of the left breast was identified. Although the lesion was classified as an IDC, this time it was triple-negative (TN) by immunophenotype and with a Ki-67 of 35%. A total left mastectomy and SLNB were indicated, with one positive lymph node in the OSNA analysis, thus requiring an axillary lymph node dissection (ALND). A total of 15 lymph nodes were excised without disease. Adjuvant systemic chemotherapy (ASC), with doxorubicin, cyclophosphamide, methotrexate and paclitaxel, was indicated.

The surgery was complicated with a chronic seroma, and it was managed with conservative measures for six months without success; thus, requiring surgical treatment. During the procedure, in August 2017, a suspicious lesion was observed in the surgical bed of the mastectomy. A biopsy was taken, reporting metastatic infiltration of carcinoma in the pectoralis major. A wide local excision of the pectoralis major muscle was performed and the HA reported an IDC of 3.5 cm, grade three, TN, Ki67 of 75% and free margins. With this result, the patient underwent ASC with Capecitabine plus RT.

In March 2018, suspicious right axillary lymph nodes were identified during a computer tomography scan [Figure 1]. The study, complemented with US and MRI, identified an intramammary lymph node

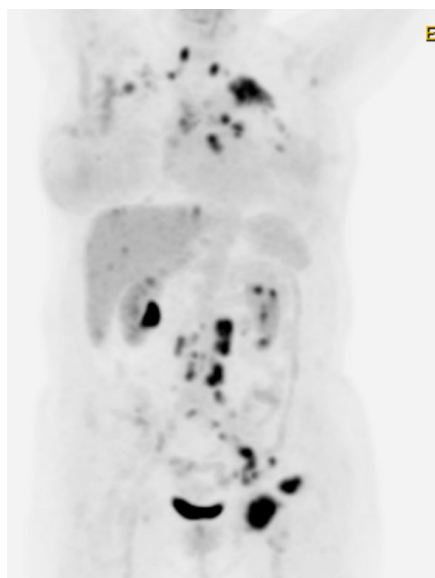


Figure 2. Positron emission tomography computer tomography showing multiple metastatic disease

in the UOQ of the right breast. Surgical treatment was determined and 43 out of 45 of the right ALND were positive, seven of them with capsular rupture. In addition, the intramammary lymph node was also positive for IDC, TN immunophenotype with a Ki67 of 63%. ASC and RT to the whole breast, axillary and supraclavicular region was decided.

During controls, in May 2018 the patient complained of a lump in the left inguinal area, when performing the physical exploration, a three cm lymph node was identified. A CNB reported metastatic carcinoma compatible with breast cancer. A positron emission tomography (PET) scan revealed multiple abnormal lymph nodes in the cervical, supraclavicular, mediastinal, retroperitoneal and inguinal regions. In addition, two bone lesions, one in the pelvis and another in the humeral bone were described as well as unspecific pleural nodules [Figure 2].

Treatment with carboplatin and gemcitabine was administered. However, a new PET revealed disease progression with new muscular, hepatic, subcutaneous and bone lesions. The patient was admitted to the hospital for severe respiratory insufficiency due to bilateral pleural effusion; finally, she died on October 10th of 2018.

DISCUSSION

One of the main explanations of CAM is aberrant drainage due to blockage of normal lymphatic routes. Perre *et al.*^[5] described this theory; they studied 23 patients with breast cancer who underwent lymphoscintigraphy before and after surgical treatment with or without radiotherapy. Only one patient presented drainage to the contralateral axilla before treatment; however, this same event presented in four patients after ALND of the opposite axilla and in one patient after radiotherapy. In a more recent publication, van der Ploeg *et al.*^[6] observed, in patients with previous ALND for breast carcinoma, contralateral axillary drainage in four out of 12 of them, during lymphoscintigraphy and SLNB. This finding becomes more relevant because two of the four involved lymph nodes were positive for metastasis. Both patients underwent ALND on the same side and reported alive and free of disease for 22 and 36 months after the procedure. In these two publications, the authors concluded that this mechanism of drainage reflects regional dissemination to the node on the direct pathway, making the “crossover metastasis” effect. However, a different outcome would be in cases of untreated breast cancer, where CAM means dissemination beyond the regional nodes.

Other risk factors for CAM have been described, including obstruction of the ipsilateral lymphatic drainage, bulky tumor in the breast, extensive tumor burden in the axilla, and as mentioned before, previous surgery and/or radiotherapy^[3,7].

There are challenges in the clinical scenario of CAM. Contralateral spread from the original tumor is not the only possibility. An occult second primary in the contralateral breast and axillary metastasis from the extramammary site should be considered^[8]. Although it is possible to have certainty of the tumor origin with a biopsy of the axillary node, to discard an occult second primary in the contralateral breast is not always an easy task. MRI has been proposed to be a good technique to identify occult primary breast carcinoma (OPBC). In a prospective analysis carried out by Buchanan *et al.*^[9], 69 patients with occult OPBC, underwent breast MRI, identifying 80% (55 of 69) of suspicious lesions and proved to be breast carcinoma in 49% of them (34 of 69). Although MRI has its limitations, due to false positive results, the authors of this study support that OPBC is a primary indication for the use of MRI.

CAM is an uncommon event in breast cancer and has a poor prognosis. AJCC 8th edition classifies CAM as stage IV disease because contralateral axillary lymph nodes are not considered a regional extent of the disease, but a distant metastasis^[10]. However, several publications have questioned this affirmation and proposed that treatment of CAM should be aggressive and multidisciplinary as it is in the case of locally advanced breast cancer, rather than treated as a distant metastasis^[3,4,8]. A systematic review analyzed 48 patients with CAM, 23 had complete follow up data during a mean time of 50.3 months with an overall survival of 82.6%. An important fact is that 92.1% of patients received surgical treatment and 88.9% systemic treatment^[11].

The patient of the case had many risks factors, like conservative surgery, radiotherapy in the left breast and an ipsilateral recurrence treated with mastectomy and SLNB. After completing ALND, 15 lymph nodes were negative for metastasis. However, CAM was identified two years later.

History of HL has been described as a risk factor for breast cancer, with an excess risk of 40% even after 40 years. The main risk factor is mantle radiotherapy, especially in young women in the “peri-pubertal” period, corresponding as the highest breast radio sensitivity period^[12,13]. The patient in our case was treated with chemotherapy exclusively and after menopause, thus we cannot assume a specific risk association.

It is important to mention that even though this patient received aggressive surgical treatment, chemotherapy and radiotherapy, the disease progressed as a systemic disease in a short period of time. This reflects the complexity of the case and that different approaches should be taken into account when treating these patients.

In conclusion, CAM are an unusual form of metastasis in unilateral breast cancers. The assumption of a disseminated disease prompt to systemic therapies rather than an aggressive surgical treatment. There are many controversies regarding the appropriate management and the prognostic influence on these patients. Unfortunately, there is poor evidence for the adequate treatment, due to the rarity of the event. An individualized and multidisciplinary approach is encouraged for these patients, so a positive impact on survival is achieved.

DECLARATIONS

Authors' contributions

Conception or design of the work: Giménez MJ, Patrón JM

Data collection: Giménez MJ, Patrón JM, Vento G, Ferrer R, Asensi J, Gavila J

Drafting, critical revision of the article, final approval of the version to be published: Giménez MJ, Patrón JM, Vento G, Bayón A, Maisto V, Bolumar I, Ferrer R, Asensi J, Gavila J, Estevan R

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Conflicts of interest

The authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Original Article

Open Access



Can microcalcifications' characteristics predict the risk of breast cancer metastasis to bone?

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Abstract

Aim: To correlate the microcalcifications' characteristics, such as morphology and elemental compositions, with the occurrence of bone metastatic lesions at 5 years from diagnosis.

Methods: In this retrospective study, we enrolled 70 patients from which we collected one breast biopsy each. From each biopsy, paraffin serial sections were obtained to perform histological classifications, immunohistochemical analyses and Energy Dispersive X-ray evaluation.

Results: Microcalcifications analysis showed a significant association between the presence of calcium crystals made of magnesium substituted hydroxyapatite and the development of bone metastasis from 5 years from diagnosis. No significant association was observed by evaluation the morphological appearance of microcalcifications. Immunohistochemical analysis displayed a significant association between the expression of bone morphogenetic proteins 2 and pentraxin-3, two osteoblast induction factors, and the formation of bone metastatic lesions.

Conclusion: Results here reported highlighted the possible use of breast microcalcifications as a negative prognostic marker of bone metastatic diseases. In particular, the association between elemental composition of breast microcalcifications and the formation of bone lesions can lay the foundation for the development of new *in vivo* diagnostic tools based on the analysis of microcalcifications and capable to predict the formation of bone metastasis.



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Keywords: Microcalcifications, breast cancer, bone metastasis, breast osteoblast-like cells, bone morphogenetic proteins 2, pentraxin-3

INTRODUCTION

Bone metastasis from breast cancer represent the main disability associated to breast cancer^[1,2]. Indeed, the occurrence of bone metastatic lesions affected the patients' quality of life by inducing pain, hypercalcemia, bone fracture and spinal compression^[2]. Also, the progression of metastatic lesions is often the cause of the patient's death^[2].

In this context, the early identification of breast cancer lesions with high propensity to form bone metastasis could improve the patient's survival allowing to clinicians to choose the more appropriate therapeutical protocol.

In the last years, several studies investigated the cellular and molecular mechanisms involved in the breast cancer osteotropism^[3-6]. In particular, our group described, for the first time, a new breast cancer cell type showing an osteoblast-like phenotype, the breast osteoblast-like cells (BOLCs)^[7]. As the real osteoblasts, it is demonstrated that these cells are capable to product calcium crystals made of hydroxyapatite (HA) of magnesium substituted hydroxyapatite (MgHAp) in a process similar to the physiological mineralization^[7]. Several molecules have been associated to the process of the formation of microcalcifications in breast cancer. Among them, deserve particular mention the bone morphogenetic proteins 2 (BMP-2) and pentraxin-3 (PTX3). BMP-2 is currently considered the most powerful osteoblast induction factor^[8], whereas it is recently demonstrated the ability of PTX3 to induce both osteoblast proliferation and activity^[9,10]. In particular, studies of Scimeca *et al.*^[7] highlighted the essential role of PTX3 in bone metabolisms founding a correlation between the impairment of PTX3 expression and the inhibition of osteoblast activity. In these reports authors suggested a direct role of PTX3 in the assembly of HA crystals. Surprisingly, the presence of BOLCs into breast cancer lesions is also associated to the development of bone metastatic lesions at five years from diagnosis. Therefore, the presence of microcalcifications made of HA or MgHAp in breast lesions could be considered as a negative prognostic factor for bone metastatic diseases as well as the BOLCs.

Starting from these considerations, the aim of this study was to correlate the microcalcifications' characteristics, such as morphology and elemental compositions, with the occurrence of bone metastatic lesions at 5 years from diagnosis.

METHODS

In this retrospective study, we enrolled 70 patients from which we collected one breast biopsy each. Our study protocol was approved by independent ethical committee. From each biopsy, paraffin serial sections were obtained to perform histological classifications, immunohistochemical analyses and energy dispersive X-ray (EDX) evaluation. Exclusion criteria were history of previously or concomitant other neoplastic diseases, autoimmune diseases, viral chronic infections (HBV, HCV, and HIV), and any antitumoral treatment received before biopsy.

Histology

After fixation in 10% buffered formalin for 24 h, breast tissues were paraffin embedded. Four μm thick sections were stained with haematoxylin-eosin (H&E)^[11].

EDX microanalysis

The EDX microanalysis is a technology that performs the elemental and chemical analysis of a sample in a transmission electron microscope. When the electron beam in an electron microscope hits a thin sample,

some atoms of the sample will be excited or ionized. When they return into their ground state, they will emit characteristic X-rays. The X-ray emission at different wavelengths may then be measured by a photon-energy-sensitive detector^[12,13].

The EDX detector system performs a simultaneous display of all mid-energy (1-20 keV) X-rays collected during any individual analysis period. Therefore, it is possible to detect those elements with N.A. > 10. The minimal detectable elemental concentration, which requires some signal averaging, is approximately 0.1 mmol/kg of dry specimen (i.e., 10 ppm), whereas spatial resolution ranges from about 10 nm to a few micrometers^[12,13].

All breast samples underwent ultrastructural microanalysis. Following to the identification of microcalcifications, six-micrometer-thick paraffin sections were embedded in Epon resin as previously described^[12]. Briefly, sections were de-paraffinized, hydrated, osmium tetroxide-fixed, dehydrated in ethanol and propylene oxide and infiltrated in Epon. The embedding capsules were positioned over areas containing microcalcifications identified by Toluidine Blue staining previously. Unstained ultra-thin sections of approximately 100-nm-thick were mounted on copper grids for microanalysis. EDX spectra of microcalcifications were acquired with a Hitachi 7100FA transmission electron microscope (Hitachi, Schaumburg, IL, USA) and an EDX detector (Thermo Scientific, Waltham, MA, USA) at an acceleration voltage of 75 KeV and magnification of 12,000. Spectra were semi-quantitatively analyzed by the Noram System Six software (Thermo Scientific, Waltham, MA, USA) using the standardless Cliff-Lorimer k-factor method^[12,13]. The EDX microanalysis apparatus was calibrated using an X-ray microanalysis standard (Micro-Analysis Consultants Ltd., Cambridgeshire, UK).

Immunohistochemistry

We employed immunohistochemical techniques to study the expression of molecules associated to microcalcifications production, PTX3 and BMP-2. Briefly, antigen retrieval was performed on 3- μ m-thick paraffin sections using EDTA citrate pH 7.8 buffers for 30 min at 95 °C. Sections were then incubated for 1 h at room temperature with the following primary antibodies diluted 1:100: BMP-2 (clone N/A; Novus Biologicals, USA) and PTX3 (clone MNB1; Abcam, UK). Reactions were revealed by HRP-DAB Detection Kit (UCS Diagnostic, Italy). To assess the background of immuno-staining we included a negative control for each reaction by incubating the sections with secondary antibodies (HRP) and detection system (DAB). Reactions have been set-up by using specific control tissues as indicated in the data sheets.

Statistical analysis

Separate χ^2 tests were used to assess the associations between morphological appearance and experimental groups and between elemental composition and experimental groups. Difference between the expression of biomarkers evaluated by immunohistochemistry were evaluated by Mann Whitney test. Immunohistochemical values were reported as mean value \pm standard error.

RESULTS

Histology

The study of H&E sections allowed us to classify breast biopsies in ductal infiltrating carcinomas according to Nottingham Histological system^[14]. Specifically, we observed 15/70 G1 infiltrating carcinomas, 38/70 G2 infiltrating carcinomas and 17/70 G3 infiltrating carcinomas. Also, based on the presence of metastatic lesions at 5 years from diagnosis, biopsies collected in the study were classified as follow: 30 infiltrating carcinomas of patients with clinical evidence of bone metastasis (BM+) (59.65 \pm 1.23 years) and 40 infiltrating carcinomas of patients without clinical evidence of bone metastasis (BM-) (57.91 \pm 0.96 years). Microcalcifications were detectable in 63.3% of BM+ and in 62.5% of BM-. From morphological point of view, we observed 63.3% of psammomabodies and 36.7% of polymorphous calcifications in BM+ and 62.5% of psammomabodies and 37.5% of polymorphous calcifications in BM- [Figure 1A-C].

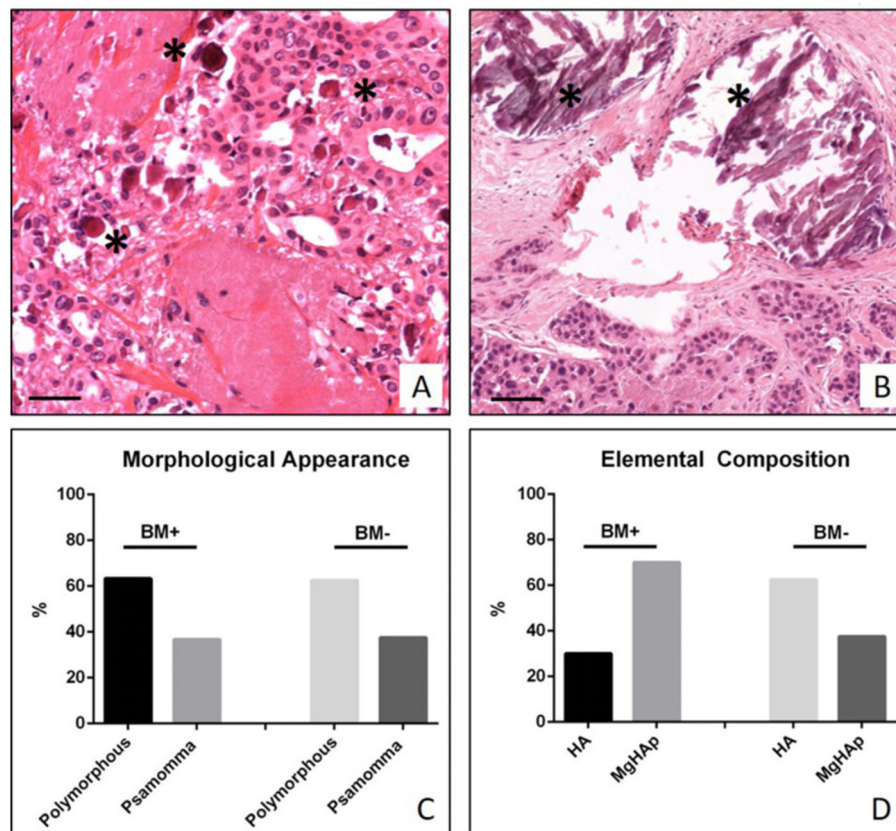


Figure 1. Study of breast microcalcifications. A: Representative image of ductal infiltrating breast carcinoma with several psammoma bodies (asterisks); B: image shows a ductal infiltrating breast carcinoma with polymorphous calcifications (asterisks). Scale bars represent 100 μ m for all images; C: graph displays the percentage of psammoma bodies and polymorphous calcifications in BM+ and BM- patients; D: graph displays the percentage of hydroxyapatite (HA) and Magnesium substituted hydroxyapatite (MgHAp) in BM+ and BM- patients

EDX microanalysis

Analysis of the elemental composition of microcalcifications revealed the presence of two form of calcium crystals in our samples: HA, and MgHAp [Figure 1D]. Statistical analysis shows a significant distribution of these types of calcifications in BM+ and BM- [Figure 1D]. In particular, calcifications of BM+ group were 70% MgHAp and 30% HA, whereas in BM- group we noted 37.5% of MgHAp and 62.5% of HA [Figure 1D].

Immunohistochemistry

We employed immunohistochemical techniques to study the expression of two osteoblast induction factors, BMP-2 and PTX3. Immunohistochemical positivity was evaluated on digital images (Iscan Coreo, Ventana, Tucson, AZ, USA) by a semi-quantitative approach. Specifically, immunoreactions for BMP-2 and PTX3 were evaluated by counting the number of positive breast infiltrating cells (out of a total of 500 in randomly selected regions). Our results showed a significant increase of BMP-2 expression in BM+ breast lesions as compared to BM- group (BM+ 205.6 ± 17.57 ; BM- 131.8 ± 14.17 ; $P = 0.030$) [Figure 2A and B]. In agreement with this, we also observed a significant increase of PTX3 expression in patients of BM+ group as compared to those of BM- (BM+ 209.0 ± 19.32 ; BM- 135.6 ± 13.10 ; $P = 0.0024$) [Figure 2C and D].

DISCUSSION

In the last years, numerous studies highlighted the role of breast microcalcifications in the patho-physiogenesis of both breast cancer occurrence and progression^[3-6,15,16]. In particular, we recently demonstrated the presence of breast cancer cells with an osteoblast phenotype (breast osteoblast-like cells-

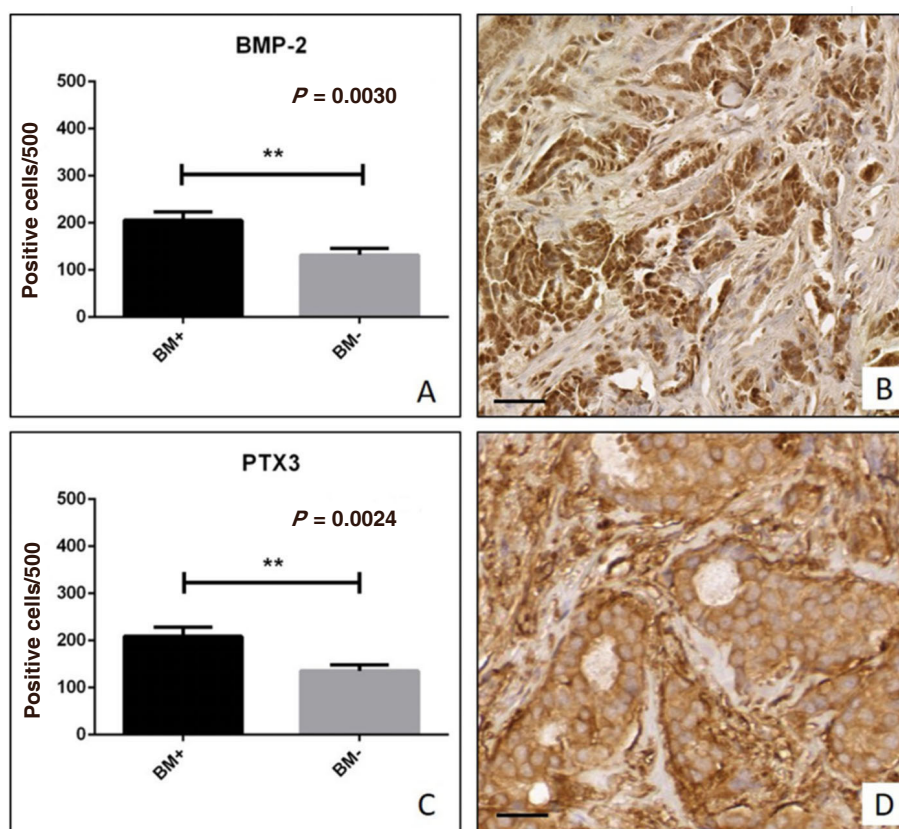


Figure 2. Immunohistochemical analysis of bone morphogenetic proteins 2 (BMP-2) and pentraxin-3 (PTX3). A: Graph shows the number of BMP-2 positive breast cancer cells in BM+ and BM- patients; B: representative image of BMP-2 expression in a ductal infiltrating breast carcinoma; C: graph shows the number of PTX3 positive breast cancer cells in BM+ and BM- patients; D: image displays the PTX3 expression in a case of ductal infiltrating breast carcinoma. Scale bars represent 100 μm for all images

BOLCs) able to product microcalcifications made of HA or MgHAp^[7]. Noteworthy, the breast cancer lesions characterized by the presence of BOLCs showed high propensity to form bone metastasis^[7]. Thus, the presence of microcalcifications in breast lesions could represent a negative prognostic marker for metastatic diseases. Starting from these considerations, the aim of this study was to correlate the microcalcifications' characteristics, such as morphology and elemental compositions, with the occurrence of bone metastatic lesions at 5 years from diagnosis. To this end, we retrospectively collected breast cancer lesions of patients with (BM+) or without (BM-) clinical evidence of bone metastatic lesions. Our results clearly indicate that elemental composition, but not morphological appearance, of breast microcalcifications can predict the presence or development of bone metastatic lesions. In particular, according with the data reported by Bonfiglio *et al.*^[15], in this study the breast cancer lesions of BM+ patients were frequently characterized by the presence calcifications made of MgHAp. Thus, the presence of MgHAp could play an active role in breast cancer progression. Indeed, as hypothesized in our previously study, the ability of HA to bind Mg can support the neoplastic progression by inhibiting the activity of DNA repair enzymes that require Mg as co-factor. These data further support the hypothesis that the bone metastatic process of breast cancer can be driven by BOLCs. Indeed, we also noted that breast cancer cells of BM+ patients acquired the capability to express two of the most important osteoblast induction factors such as BMP-2 and PTX3. BMP-2 is a member of TGF-β superfamily that which regulate numerous process in bone metabolisms^[8]. Specifically, BMP-2 is involved in both mesenchymal stem cells recruitment and differentiation into mature osteoblasts^[8]. In a recent paper, Scimeca *et al.*^[7] demonstrated the expression of BMP-2 by BOLCs. PTX3, also known as TNF-inducible gene 14 protein, is a molecule involved in several process of innate immunity^[17,18]. It is also demonstrated that PTX3 have a role in the extracellular matrix formation as well as bone formation^[9]. In

this context, the expression of BMP-2 and PTX3 in BM+ lesions can provide a scientific rationale for BOLCs development and microcalcifications formation. Also, it is known that both BMP-2 and PTX3 are involved in the EMT phenomenon. Thus, it is possible to speculate that these molecules have role both in BOLCs generation that in the production of HA or MgHAp crystals by the BOLCs themselves.

In conclusion, results here reported highlighted the possible use of breast microcalcifications as a negative prognostic marker of bone metastatic diseases. In particular, the association between elemental composition of breast microcalcifications and the formation of bone lesions can lay the foundation for the development of new *in vivo* diagnostic tools based on the analysis of microcalcifications and capable to predict the formation of bone metastasis. In this scenario, the *in vivo* analysis of elemental composition of microcalcifications by RAMAN spectroscopy could improve the clinical armamentarium available for the diagnosis and stadiation of breast cancer.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: Bonfiglio R, Scimeca M, Bonanno E

Performed data acquisition and performed administrative, technical, and material supports: Polidori A, Nazzaro C, De Silva G

Availability of data and materials

The data used to support the findings of this study are included within the article. Further details can be made available upon request.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Our study protocol was approved by independent ethical committee. Experimental procedures were carried out according to *The Code of Ethics of the World Medical Association (Declaration of Helsinki)*. Specimens were handled and carried out in accordance with the approved guidelines.

Consent for publication

Not applicable.

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Original Article

Open Access



Breast cancer metastasis to the stomach

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Abstract

Aim: This study focuses on the stomach as an unusual but not rare site of metastasis of breast cancer.

Methods: We performed a literature search on gastric metastasis from breast cancer searching for reviews from 2000 to 2018 and case reports from 2013 to 2018. We found 11 reviews and 36 case reports and we compared their findings about important aspects of gastric metastasis, such as disease free survival, overall survival, symptoms, endoscopic findings, therapy, histology, and immunohistochemistry.

Results: The incidence of stomach as site of metastasis of breast cancer ranges from 5% to 18%. Reviews and case reports reached similar conclusions about several of the aforementioned aspects: invasive lobular breast cancer (ILC) is mainly responsible for gastric metastases; disease free survival can vary greatly ranging from 0.5 months to 30 years; gastric metastases usually present with non-specific symptoms, even though five patients in case reports were asymptomatic; linitis plastica is the most common endoscopic finding; immunohistochemistry is essential for differentiating primary gastric cancer from metastasis; the preferred treatment is systemic therapy, but surgery is still an option in case of emergency; median overall survival of patients with gastric metastasis from breast cancer is 24 months.

Conclusion: Breast metastasis to the stomach should be considered in any patient suspecting gastric neoplasm previously treated for breast carcinoma, especially if the treated carcinoma was ILC.

Keywords: Gastric metastasis, breast cancer, immunohistochemistry, stomach



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INTRODUCTION

Breast cancer is the most common malignant tumor in the female population. Different histologies metastasize to different organs^[1]: ductal cancer metastasizes mainly to lungs, bones and liver, while lobular cancer has a tendency to metastasize to the gastrointestinal system^[2,3]. Gastric metastases from breast cancer are not common, but the autoptic incidence is not negligible, and varies from 5% to 18%^[3,4]. Gastric metastases are metachronous in most cases and even in patients with a known history of breast cancer it may be difficult to correlate the two diseases to a common cause, since the gastric disease often presents only several years after the treatment for the breast tumor^[5,6]. This late manifestation of the disease may lead to erroneous diagnosis in clinical practice and a correct identification of the disease becomes possible only after performing surgery, even though this approach is used only in limited cases. With this study we want to compare current clinical practice found in case reports with the results presented in recent reviews.

METHODS

We performed a literature search aimed at finding reviews published from January 1st 2000 to October 31st 2018 about gastric metastasis from breast cancer and case reports on the same argument from January 1st 2013 to October 31st 2018. The results presented in the reviews and in the case reports were compared with the aim of analyzing eventual similarity and differences in the findings discussed therein.

Characteristics of the included studies are summarized in [Table 1](#)^[7-17], whereas characteristic of patients from case reports (2013-2018) are reported in [Tables 2-4](#)^[9,14,18-51].

RESULTS

Part 1. Reviews

The search for reviews about gastric metastasis from breast cancer was carried out using the combination of words (gastric OR stomach) and (metastasis OR metastases) and (breast) for the PubMed database, using the filter for reviews. Articles written in languages other than English, articles about primary gastric cancer with breast metastasis and about primary breast cancer metastasizing in organs other than the stomach were excluded. The time span considered went from January 1st 2000 to October 31st 2018 and 11 articles fulfilling the criteria were found as a result of the search. The main aspects of the included studies are summarized in [Table 1](#).

Incidence

Nine of the articles reported the incidence of gastric metastasis from breast cancer: the incidence ranged from 0.3% to 35%, with the value 2%-18% reported in 3 articles.

Breast histology

Nine reviews reached the same conclusion that the most common histological type of breast cancer responsible for gastric metastasis is the lobular cancer. Two of the articles also reported the occurrence of metastasis associated with different types of cancer, with lobular cancer being identified as the cause of metastasis in 83% of the cases.

Disease Free survival

Nine of the reviews cited interval time from the appearance of the primary breast cancer to the presentation of gastric metastasis, however the time intervals were not specifically defined, as for example two articles reported “many years” and one article “few months to several years”. Other articles reported a median disease free survival of 5 years, but gastric metastasis may occur up to 30 years after the primary tumor.

Table 1. Summary of reviews from 2000 to 2018

Study	Incidence	DFS	Symptoms	Endoscopy	CT	Breast histology biopsy	Gastric histology	Gastric metastasis receptor	Therapy	Surgery	Palliation	Survival
Pectasides <i>et al.</i> ^[7] , 2009	0.3%-18%	Many years	Non specific: dysphagia, dyspepsia, anorexia, abdominal pain, early satiety, nausea, vomiting, bleeding	Linitis plastica, negative in 50%		Lobular 83%	Signet ring morphology	Positive CK7, GCDFFP-15, CEA, ER, PgR, Negative CK20	Chemotherapy or endocrine therapy	Only for complications, obstruction or bleeding		
Ayantude <i>et al.</i> ^[8] , 2007	7.4%-18%	Few months to several years	Non specific: nausea, vomiting, epigastric pain, anorexia, dyspepsia, bloating	Linitis plastica, normal mucosa in 30%	Thickened visceral wall, other metastasis	Lobular	Signet ring morphology	Positive CK, GCDFFP-15, CEA, ER, PgR, Negative CK20	Systemic therapy, hormonal therapy	Only for emergency situation, bypass for obstruction	Radiotherapy, brachytherapy, stenting or dilatation	
Villa Guzman <i>et al.</i> ^[9] , 2017	2%-18%	4-5 years	Inexpressive	Diffuse infiltration		Lobular	Signet ring	Positive ER, PgR, CK7, CK18, Negative HER2, CK20	Systemic therapy	Obstruction, bleeding		
Reiman <i>et al.</i> ^[10] , 2001	6%-18%		Nausea, vomiting, epigastric pain, anorexia, weight loss, dysphagia, melena, hematemesis, iron deficiency anemia, guaiac positive stools	Linitis plastica		Lobular	Signet ring		Systemic therapy, radiotherapy, endoscopy			
Critchley <i>et al.</i> ^[11] , 2011	8%-35%	7 years		Normal in 50%		Lobular			Systemic therapy, hormonal therapy	obstruction, mass effect		
Namikawa <i>et al.</i> ^[13] , 2013		50-78 months	Epigastric pain, melena, anemia, nausea, vomiting	3 patterns: localized, PET diffuse, external compression*	PET has low sensitivity				Systemic therapy	Emergency, bypass for obstruction		
Weigt <i>et al.</i> ^[12] , 2015		75.6 months	Epigastric pain, dysphagia, vomiting, gastrointestinal hemorrhage	Sub-mucosal type lesion, solitary lesions*					Systemic therapy	Obstruction, bleeding	Endoscopic hemostatic procedures, stents	
Barranco <i>et al.</i> ^[14] , 2017	2%-18%		Non specific: anorexia, dyspepsia, epigastric pain, vomiting	Linitis plastica								
Jones <i>et al.</i> ^[15] , 2007	2%-18%	Many years	Dyspepsia, anorexia, epigastric pain, early satiety, vomiting, bleeding	Linitis plastica, normal mucosa in 50%		Lobular 83%			Systemic therapy	Bypass for obstruction	Endoluminal stent, endoscopic or endovascular therapy for bleeding	28 months
Aurello <i>et al.</i> ^[16] , 2004	0.7%-15%	Majority within 5 years, 7 years, 30 y	Weight loss, nausea, vomiting, epigastric pain, early satiety	Discrete nodules, linitis plastica		Lobular			Individualized, systemic therapy	Intestinal obstruction, bleeding, perforation		2 year
Ellis <i>et al.</i> ^[17] , 2009	15%	0-369 months (84 epigastric pain, early satiety, vomiting, bleeding)	Dyspepsia, anorexia, epigastric pain, early satiety, vomiting, bleeding	Linitis plastica (lobular type); discrete nodules (ductal type)		Lobular	Negative Signet ring in 50%	ER, PgR, E-cadherin	Medical therapy			

ER: estrogen receptor; PgR: progesterone receptor; HER: human epithelial receptor; CK: cytokeratin; GCDFFP-15: gross cystic disease fluid protein 15; CEA: carcinoembryonic antigen

Table 2. Characteristics of patients

Variables	Number of patients	n (%)
Age, years		
≥ 55	34	59
< 55	24	41
Symptoms		
Yes	45	90
No	5	10
Presentation		
Synchronous	10	17
Metachronous	54	83
Endoscopy		
Linitis plastica	21	37
Ulcers	16	29
Polyp/Nodule	4	7
Other	15	27
Other metastasis		
Yes	56	81
No	13	19
Therapy		
CHT ± OT	41	69
Surgery + CHT	10	17
Surgery	7	12
RT	1	2
Overall survival		
Median	24	
CHT	23	
Surgery + CHT	91	
Surgery	45	

CHT: chemotherapy; OT: hormonal therapy; RT: radiotherapy

Table 3. Characteristics of primary breast cancer

Variables	Number of patients	n (%)
Histology		
Lobular	42	64
Ductal	16	24
Other	8	12
ER status (breast)		
Positive	34	92
Negative	3	8
PgR status (breast)		
Positive	29	80
Negative	7	20
HER2 status (breast)		
Positive	7	25
Negative	21	75

ER: estrogen receptor; PgR: progesterone receptor; HER: human epithelial receptor

Symptoms

Ten articles reported the most common presentation symptoms related to gastric metastasis: they are often non specific, with epigastric pain as the most frequently reported, followed by nausea, vomiting, dyspepsia and dysphagia. Other reported symptoms are anorexia, early satiety, weight loss, and bleeding that may manifest as hematemesis, melena or iron deficiency anemia. One article used the term “inexpressive” to describe these symptoms.

Endoscopy

All the studies reported the main endoscopic findings, and the majority of them agreed that the most common presentation is linitis plastica. Because linitis plastica is caused by the infiltration of tumor cells in submucosa, the overlying mucosa is normal, thus resulting in negative exam results in 50% of cases, as reported in two studies, or 30%, as reported by Ayantude. Two articles discussed different patterns of

Table 4. Characteristics of gastric metastasis

Variables	Number of patients	n (%)
Gastric Histology		
Biopsy	45	80
Definitive specimen	11	20
ER status (stomach)		
Positive	50	91
Negative	5	9
PgR status (stomach)		
Positive	28	61
Negative	18	39
HER2 (stomach)		
Positive	11	30
Negative	25	70
CK7 (stomach)		
Positive	21	91
Negative	2	9
GCDFP-15 (stomach)		
Positive	9	75
Negative	3	25
CK20 (stomach)		
Positive	1	5
Negative	20	95
MGB (stomach)		
Positive	6	86
Negative	1	14
GATA-3 (stomach)		
Positive	4	100
Negative	0	0
CDX-2 (stomach)		
Positive	0	0
Negative	7	100
E-cadherin		
Positive	3	37
Negative	5	63

ER: estrogen receptor; PgR: progesterone receptor; HER: human epithelial receptor; CK: cytokeratin; GCDFP-15: gross cystic disease fluid protein 15; MGB: mammoglobine

presentation, but they analyzed all gastric metastases from other primary tumors and not only the ones related to breast cancer.

Alternative instrumental tools

Other instrumental tools were not frequently cited. Ayantude and Aurello mentioned that computed tomography (CT) is commonly used to evaluate thickened visceral wall or the presence of other metastases. Namikawa observed that PET has a low sensitivity in diagnosing metastasis, but it may be useful to evaluate response to treatment in those tumors with intense FDG (F-18 fluorodeoxyglucose) uptake.

Gastric biopsy/histology

Two articles reported that gastric biopsy may be negative in 30% or even in 50% of the cases because the tumor cells infiltrate the submucosal layer and not the mucosal layer, which shows a normal histological aspect; there is thus a need for performing deeper biopsy in order to study also underlying layers, which is essential in this particular metastatic pattern. The most common reported histological pattern is signet ring cells, as cited in seven articles, which in some cases may be confused with tumor of gastric origin.

Gastric metastasis receptor

Immunohistochemistry is essential for differentiating gastric metastasis from primary tumor; positivity for estrogen and progesterone receptors in gastric metastasis is reported in seven articles, and they are the main receptors that direct the diagnosis to a secondary tumor of breast origin. Metastases of breast tumor

also show positivity for other receptors, such as CK7, CK18, GCDFP-15 and CEA, which are reported in five articles, and they are negative for CK20 and CDX2, which are usually expressed in intestinal cells, and also negative for E-cadherin and HER2, indicating a probable lobular breast cancer origin.

Therapy/Surgery/Palliation

Ten articles reported that the main therapy for gastric metastasis from breast origin is systemic therapy, either chemotherapy or hormonal therapy; surgery is reserved only in case of complications or emergency, such as bleeding, perforation or obstruction. In this latter case, a more conservative approach is advised and three articles suggest to perform a simple bypass instead of a gastrectomy. Endoscopy can prove very useful and must be considered before surgical approach in case of obstruction and bleeding to put stents or to perform dilatation; also bleeding can be managed conservatively either with endoscopic hemostatic procedures or by embolization radiologists.

Survival

Only two articles mentioned overall survival and they are approximately concordant, with Jones reporting a survival of 28 months, and Aurello reporting 2 years.

Part 2. Case reports

We also performed a review of the literature focusing on case reports of gastric metastasis from breast cancer from January 1st 2013 to October 31st 2018 because we wanted to update the aforementioned reviews with the latest current clinical practice present worldwide. We found 36 reports for a total of 69 patients. Data were not reported entirely in all the case reports, so we analyzed and compared only mentioned data. All the patients were females except one male^[43]. The median age of the patients was 58 years, with a range from 33 to 86 years. For the case reports that reported the age of the patients, 34 (59%) patients were > 55 years old, whereas 14 (41%) were < 55 years.

Breast tumor/receptor

The most common histological type of breast cancer was ILC (invasive lobular cancer), that was found in 42 cases (63%); other histological type of breast tumor were IDC (invasive ductal cancer) in 16 (24%), mixed in 2 (4%), and tubular in 6 cases (9%). In one case^[30] breast tumor was not found at instrumental research after the diagnosis of gastric metastasis. Breast cancer tumor had ER positive in 34 (92%) cases and negative in 3 (8%); PgR was positive in 29 (80%) and negative in 7 (20%), and HER2 was negative in 21 (75%) cases and positive in 7 (25%). The most common receptor status is ER+/PgR+/HER2-.

Disease free survival

Usually gastric metastases from breast tumor occur several years after primary cancer; 54 (83%) patients had metachronous disease, whereas only 10 (17%) had synchronous tumors.

Time of presentation of gastric metastasis from primary breast cancer varies from 0.5 months to 20 years later, with a median time of 61 months (about 5 years). This explains the difficulty to reconduct the gastric disease to breast tumor, because in many cases there was a long time between the manifestations of the two disease.

Symptoms

Gastric metastasis mostly presented with non specific symptoms in 45 cases, such as epigastric pain (28), nausea and/or vomiting (14), dyspepsia (3), dysphagia (3), anorexia (5), weight loss (12), hematemesis or melena (13), anemia (3). Five patients^[24,37,49] were totally asymptomatic, and diagnosis of gastric metastasis in these cases was incidental during exams in follow up. In one case^[37] elevated CEA and CA15-3 rose suspiciously for metastasis and then PET scan confirmed gastric localization.

Endoscopy

At endoscopy the most common presentation was linitis plastica, present in 21 cases (37%), whereas ulcers were found in 16 cases (28%). In three of these latter cases^[27,28,41] the first presentation was perforation, which resulted in elevated morbidity and mortality. Polyps or nodules were present in 4 patients (7%).

Gastric tumor/receptor

Histological study of gastric metastasis showed in the majority of cases adenocarcinoma with signet ring cells. In six (9%) patients initial gastric biopsies suspected a primary gastric cancer so they were submitted to surgical treatment; only after histological study of the specimen it was clear that they were metastases from breast cancer and not primary tumor of the stomach.

Immunohistotype is essential to diagnose and differentiate primary gastric cancer from metastatic tumor; the mainly studied receptors were ER, that was positive in 50 (91%) cases and negative in 5 (9%); PgR, positive in 28 (61%) cases, and negative in 18 (39%); HER2 negative in 25 (70%) and positive in 11 (30%); CK7 positive in 21 (91%) and negative in 2 (9%) cases; CK20 negative in 20 (95%) and positive in only 1 case (5%); CDX2 negative in 7 cases (100%); GCDPF-15 positive in 9 (75%) and negative in 3 cases (25%); GATA-3 positive in 4 cases, Mammoglobine positive in 6 cases (86%) and negative in 1 (14%); E-cadherin positive in 3 (37%) and negative in 5 cases (63%).

In 45 cases immunohistological study of the biopsies from gastric lesions or gastric wall were taken before starting any treatment; considering patients who underwent gastrectomy, nine^[23,26,27,28,35,42,43,45] were taken after surgical treatment whereas three^[23,28,43] were done before, but authors decided to perform surgery because they suspected primary gastric cancer or they couldn't exclude it.

In two patients^[22,43] immunohistological pattern of gastric metastasis was different from the one of primary breast cancer; in one patient^[40] pattern changed during treatment history, passing to a luminal A type and then to a triple negative.

Other metastasis

Gastric metastasis is usually part of a systemic disease and presents with other localization of malignant cells; considering all case reports, 56 patients (81%) presented with other metastases at time of diagnosis.

Therapy

The preferred treatment is systemic therapy, as chemotherapy, endocrine therapy or a combination of both of them; 41 of the patients were subjected to chemotherapy with or without hormonal therapy. Combination of surgery and chemotherapy was used in 10 patients^[23,28,29,35,37-39,41,43,45]; two of them^[28,29] were submitted to neoadjuvant chemotherapy followed by surgery in the belief of a primary gastric cancer. Surgical treatment alone was chosen in 7^[26,27,42] patients, with three^[27,42] of them in an emergency setting for bleeding and perforation. Endoscopic stenting for obstruction was used successfully in 2 patients^[19,22].

Survival

Median overall survival was 24 months, in line with data given by current literature. In some papers survival at follow up was the only data cited, and the media of these data was 28 months. Patients submitted to chemotherapy had an OS of 23 months; patients subjected to surgical treatment alone had an OS of 45 months. Those subjected to surgery plus chemotherapy had an OS of 91 months. We must consider the fact that for this latter group only two patients were considered and that they had stomach as the only site of metastasis. However, due to the paucity of number of cases considered, this data can be misleading.

Part 3. Comparison between reviews and case reports

The final results from the review and the case reports show that the breast cancer that is responsible for most of the metastasis is the lobular type (83% vs. 63%). Both summaries report that the time of presentation of

gastric metastasis is very variable, and ranges from few months to many years (20 years vs. 30 years), and that median disease free survival is 5 years.

Symptoms related to gastric metastasis are reported in both studies as non specific, such as epigastric or abdominal pain, nausea or vomiting, dysphagia, dyspepsia, anorexia, weight loss, and bleeding that may manifest as hematemesis, or melena or anemia. Interestingly, in five patients^[24,37,49] of case reports there were no symptoms and diagnosis of gastric metastasis was based on other signs, such as elevated serum markers or incidental findings at instrumental routine follow up.

Regarding endoscopic findings, the principal pattern is linitis plastica in both studies; reviews say that in 30%-50% of cases endoscopic findings are negative, but this aspect is not present in the majority of case reports.

The most common histological pattern found in gastric metastasis is adenocarcinoma with signet ring cells; this pattern can be confused with primary gastric cancer, as said in the reviews; indeed in three case reports^[37-39] preoperative findings showed this pattern on gastric biopsies and surgical treatment was performed.

Immunohistochemistry is mainly based on expression of ER and PgR. They are reported as positive in reviews, and, in the case reports, 50 (91%) tumors had ER positive and 28 (61%) had PgR positive; other receptors are HER2 and E-cadherin, whose absence is related to lobular cancer (negative in reviews, in case reports 25 (70%) tumor had HER2- and 5 (63%) had E-cadherin-); positivity for CK7, GCDPF-15 and MGB and absence of CK20 and CDX2 are also related to breast cancer origin. Systemic therapy is the treatment of choice in the reviews and even in case reports (used in 41 patients). Surgery is usually performed in case of complications such as perforation or bleeding; in three cases patients were submitted to emergency laparotomy, two^[28] for perforation and septic shock and one^[42] for bleeding and hemorrhagic shock. Main surgical intervention is gastrectomy (performed in 13 patients), whereas in one patient^[41] only resection and biopsy were performed in the setting of a perforation. Bypass, that is considered the best option according to reviews, was not even considered in case reports. Indeed obstruction can be managed conservatively by endoscopic stenting as mentioned in reviews, and this was performed in two patients^[19,22]. No embolization was performed in case reports. Median overall survival is similar (about 2 years) in the two summaries. Unfortunately reviews didn't subdivide OS by type of treatment.

DISCUSSION

Breast cancer is the most common malignancy in women and the leading cause of cancer-related death in female gender. Global incidence increased about 3.1% every year in the past 30 years, with an increase of the number of cases in Middle East, south Asia, southeast Asia, and central Latin America, and also mortality increased at an annual rate of 1.8%^[1]. Malignant proliferation may arise from ductal or lobular epithelium: the most frequent is the ductal type that includes 75%-82% of all cases^[4,52,53]. Other less frequent types are lobular carcinoma (4%-10% of all cases), phyllodes, or tubular cancer.

Metastases are possible either in ductal and lobular carcinoma, but they may develop in different organs: ductal carcinoma metastasizes more frequently to the lung, the brain and the liver, whereas lobular cancer tends to metastasize to the gastrointestinal (GI) tract, gynecologic organs, peritoneum and bones^[2,3]. Breast cancer, melanoma and lung cancer, represent the most frequent malignancies metastasizing to GI tract^[15,16,53]: the common sites of GI metastasis from breast tumor are colon and rectum (45%), stomach (28%), small intestine (19%), and esophagus (8%)^[53]. Median overall survival of patients with gastric metastasis ranges from 24 to 36 months^[53].

Incidence

Gastrointestinal metastases from breast cancer are rare, among them the stomach is the second most common site. In McLemore *et al.*^[53], 2005 study, which regarded 12,001 patients, gastric involvement represents 28% of all cases. The incidence of breast cancer metastasis to the stomach is reported to be from 0.1% to 6%, but in autopsy series the estimated incidence is found to be higher, from 5% to 18%^[3,4]. Gastrointestinal metastases are usually associated with other concurrent tumor localization: in a retrospective study lead by Taal *et al.*^[3], 1992 up to 94% of patients present with disseminated disease, that involve the skeleton (60%), liver (20%), and also the lung (18%)^[4]. In our study 81% of the patients had other metastases at time of diagnosis.

Metastasis

Gastrointestinal metastasis from breast cancer can be synchronous or more frequently metachronous, with a mean time of presentation reported to be from 6 to 7 years^[5], but in literature time can vary from 2 to 30 years^[5,6]. In our study there were 54 cases with metachronous presentation and only 10 patients with synchronous disease.

Studies report three main mechanisms of dissemination of malignant breast cells to the upper GI tract: lymphatic spread, hematogeneous route, and direct invasion from surrounding organs^[3,4,54,55]. The first type is more common in esophageal metastasis: the typical presentation is stricture or submucosal nodules with normal overlying mucosa, that makes endoscopic diagnosis difficult^[54,55]. Also the infiltration of paraesophageal lymphonodes can result in compression of the esophageal lumen with consequent intramural infiltration^[56]. Hematogenous spread is typical of esophagus, stomach or duodenum which results in stenosis or mass localized intramural or in the submucosal layer, that may become ulcerated. The most common pattern of presentation is linitis plastica-like; in this situation malignant cells are trapped from blood stream to the submucosal or subserosal layer with diffuse infiltration that cause diffuse thickening and rigidity of the organ wall^[4,54,55,57].

An important role in metastatic pathway is played by chemokines. CXCR4 and CXCR7 are the main chemokine receptor expressed in breast cancer cells involved in transendothelial migration (TEM), and they are responsible for the chemotaxis to certain target organs^[58]. CXCR4+ tumors are associated with more distant metastasis than CXCR4-tumors, even though this association is not statistically significant^[59]. Their ligands are CXCL12 and CCL21; the first is implied in modulating integrin expression, metalloproteinase production, tumor angiogenesis, tumor cell adhesion and apoptosis^[60,61]. Metalloproteinases are essential to degrade extracellular matrix to permit invasion of the cells attracted in metastatic sites by chemokines from the bloodstream^[60]. CXCL12 promotes homing of tumor cells to secondary sites and then recall endothelial stem cells for blood vessel formation and subsequent proliferation^[61].

Circulating breast tumor cells pass from blood or lymphatic stream to areas that express CXCL12, thereby metastatizing in many organs such as bone marrow, lymph nodes, liver and lung^[59,61].

An interesting hypothesis suggested by an article by J. Carlos Villa Guzman highlights the implication of inflammation response in tumorigenesis. Chronic inflammation induced by *Helicobacter Pylori* is associated with higher expression of chemokines and interleukins that may attract tumor cells to gastric or colon mucosa with subsequent proliferation and develop of metastatic disease^[9]. Even though these mechanisms are not fully understood, this hypothesis offers an interesting start point for other future studies.

Lobular breast cancer

Breast cancer metastases are more frequently related to lobular type than ductal type; in 83% of all metastases the primary breast cancer has lobular histotype^[4]. According to the study of Borst *et al.*^[62], 1993



Figure 1. Endoscopy showing localized lesion at gastric corpus. Biopsy confirmed the presence of gastric metastasis from breast cancer

the incidence of lobular carcinoma metastasis is higher in organs such as gastrointestinal system (4.5% vs. 0.2% in ductal carcinoma); gynecologic organs (4.5% vs. 0.8%); peritoneum-retroperitoneum (3.1% vs. 0.6%); bone marrow (21.2% vs. 14.4%). We found 42 cases with gastric metastasis from lobular carcinoma, whereas 16 cases were related to ductal carcinoma. The propensity of lobular breast cancer to give metastasis seems to be correlated with mutations of E-cadherin genes; the impaired function of the produced protein determines loss of adhesion among epithelial cells. Cells are initially separated from each other so that they can invade the surrounding tissue and then enter in the lymphatic system or in the bloodstream, leading to the progression of metastatic disease^[63,64].

Symptoms

Gastric metastases of breast cancer have no specific symptoms and may often be confused with primary gastric cancer or other conditions, such as effects of chemotherapy, radiotherapy, oral medications, liver metastasis or hypercalcemia^[4,57]. These symptoms include anorexia, dysphagia, feeling bloated soon after eating, bleeding (melena, hematemesis), dyspepsia, epigastric pain andretch^[65,66]. Common findings in blood tests are iron deficiency anemia or abnormal high levels of CA 15.3 that would arise the suspect for a relapse of primary breast cancer^[9,67]. Gastric metastases usually occur many years after primary breast cancer, so when the patient presents with these vague symptoms it's difficult to reconduct them to the primary causative disease. In our case reports, nine^[23,28,29,35,37,38,39,43,45] of the patients had a wrong initial diagnosis and they were submitted to surgical treatment in the suspect of a primary gastric cancer.

Diagnosis (instrumental)

Endoscopy is the main diagnostic tool when patients present with symptoms related to upper GI disease. Gastric metastases from breast cancer show three main different patterns: localized pattern (in about 18% of cases, as large ulcers and polyps [Figure 1], diffuse infiltration (57% of cases, such as linitis plastica-like with diffuse infiltration of the submucosal and seromuscular layer with a fibrotic reaction that causes narrowing lumen, rigidity, wall thickening with reduced peristalsis), and external compression (in 25% of cases)^[4,68,69]. Gastric metastases are usually localized in submucosal and seromuscular layers; in more than 50% of cases endoscopy study is negative^[70]. In one case report^[18] first histological examination of gastric biopsies was negative; after synchronous diagnosis of primary breast cancer, endoscopy was repeated and biopsies of submucosal layer were performed again, showing gastric metastasis from lobular cancer. Also in another patient^[19] initial gastric biopsies were negative, reporting only mild chronic gastritis. Endoscopic ultrasound showed thickening of the muscularis propria so biopsies were repeated focusing on this layer: muscular wall was infiltrated by malignant cell from lobular breast carcinoma.

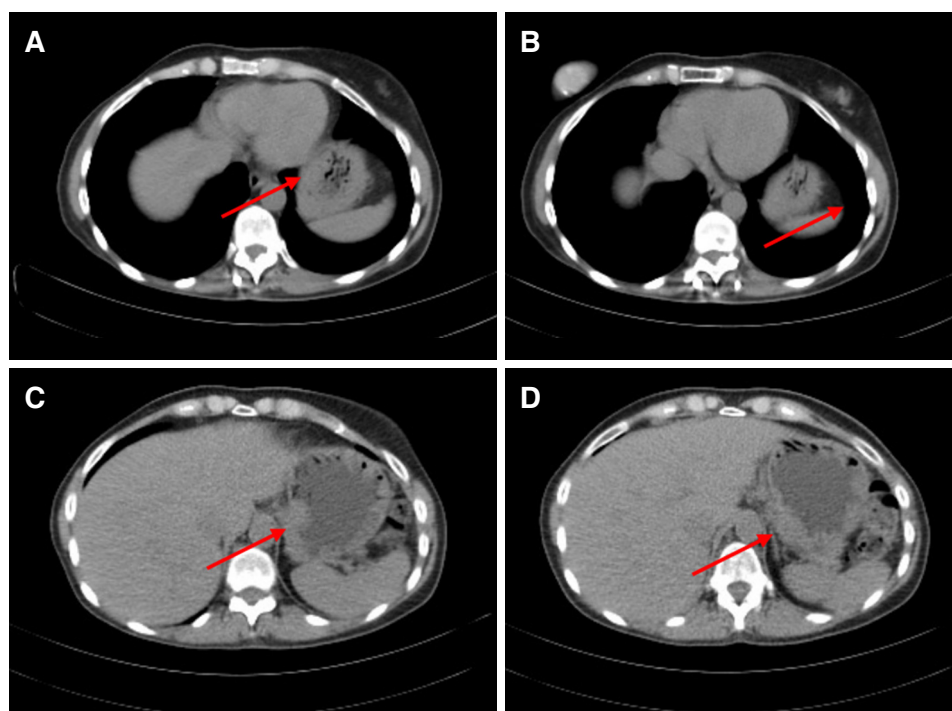


Figure 2. CT scan showing gastric wall thickening at gastric fundus (A-D)

In literature CT scan study is not mentioned as one of the main diagnostic source to establish the nature of primary cancer, but it is used to evaluate wall thickening despite a normal aspect in endoscopic study and other site of metastasis^[54] [Figure 2].

A new useful diagnostic approach to differential diagnosis is magnifying endoscopy with narrow-band imaging (ME-NBI); it shows alterations in the framework of microvessels that are characteristic of metastatic pattern in comparison to primary malignancy of the stomach^[51].

The sensitivity of PET is lower for the diagnosis of gastric cancer due to physiological absorption of F-18 fluorodeoxyglucose and involuntary movements by the gastric wall [Figure 3]; early cancers, signet-ring cell carcinoma and poorly differentiated non-solid adenocarcinoma are characterized by high false-negative rates. There are also some scenarios of non-specific FDG accumulation correlated to mucosal inflammation, as in superficial gastritis and erosive gastritis, leading to false positives^[71].

Histology

Differentiation of primary gastric cancer from gastric metastasis is crucial; from the histological point of view, the first important difference is the localization of tumor cells: mucosa is generally involved in gastric cancer, while submucosal layer is usually affected in metastatic disease^[4,15]. In gastric metastasis malignant small cells with monomorphic, round nuclei and vacuolated cytoplasm typically array in chords, named “Indian files”, and infiltrate the serosal, muscular and submucosal layer^[57].

An additional difficulty is that they share signet ring cell-like morphology, thus lobular metastasis can mimic primary gastric cancer. However breast signet-ring cell carcinoma may show some morphological differences from gastric and colonic signet-ring cell carcinoma. The first shows a single, well-circumscribed univacuolated intracytoplasmatic lumina, with a central eosinophilic inclusion, whereas the latter has an extended, globoid, and optically clear cytoplasmatic acid mucin that pushes the nuclei against the cell membrane^[72].

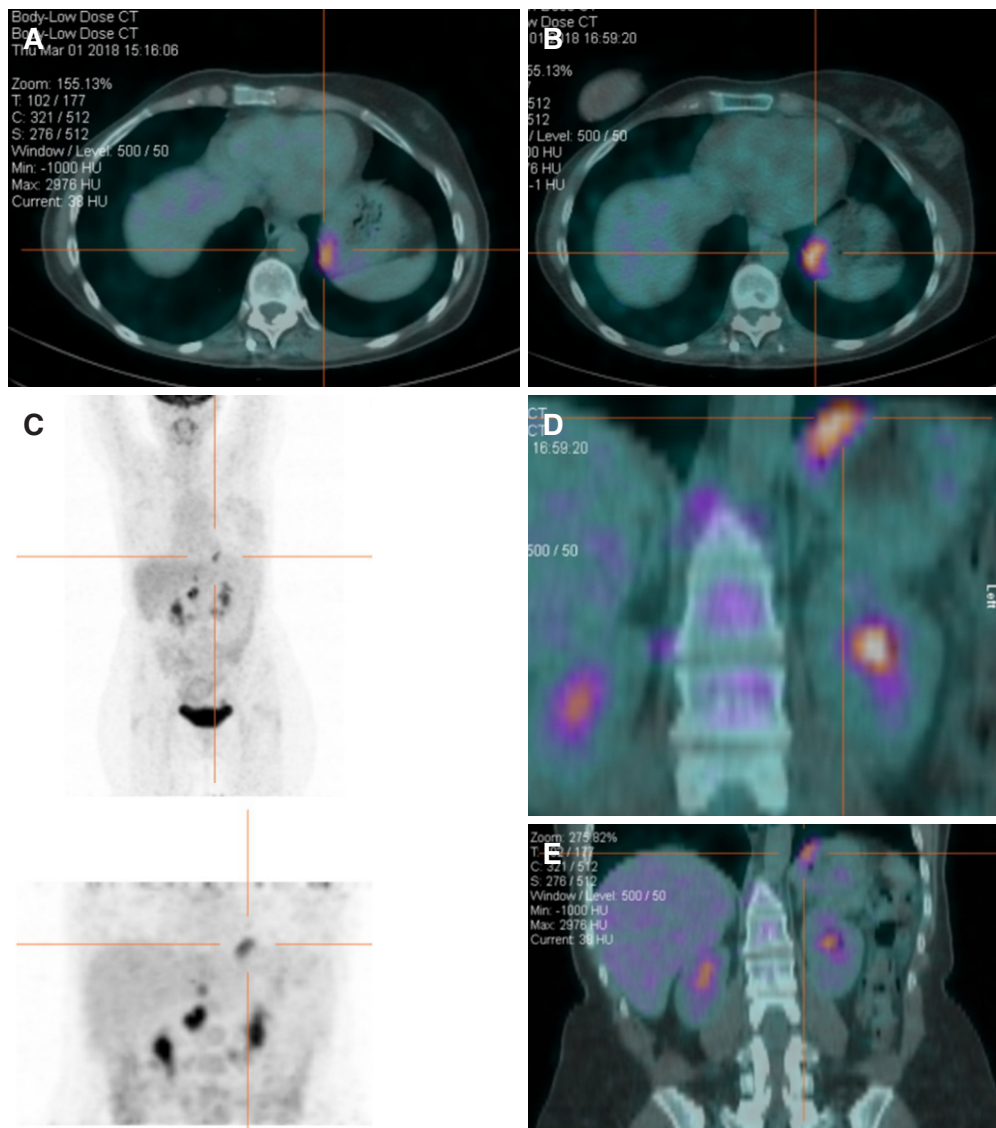


Figure 3. Pet scan showing pathological F-18fluorodeoxyglucose accumulation in the gastric fundus (A-E)

Therefore differentiation of primary gastric metastasis requires to evaluate the infiltration of the serosal, muscular and submuscular layers by cells that organize in a typical Indian file pattern^[57] with a signet ring appearance^[73].

Immunohistochemistry

Immunohistochemical analysis is the most important tool for differentiating between primary gastric cancer and gastric metastasis from breast cancer; the main markers currently employed are estrogen receptor (ER), progesterone receptor (PgR), mammaglobin (MBG), cytokeratin 7 (CK7), cytokeratin 20 (CK20), human epithelial receptor 2 (HER2), gross cystic disease fluid protein 15 (GCDFP-15), and GATA-3. A new marker is HFN4A, which is discussed below.

The expression of ER and PgR is highly indicative of breast carcinoma metastasis. Primary gastric cancer can express ER in up to 30% of cases and PgR in up to 20% of cases, but also gastric metastasis from breast carcinoma sometimes show negative ER and PgR rate even if primary breast cancer is ER and PgR positive^[66,74]. Expression of ER, PR and HER2 status can change between primary tumor and metastatic

lesions in up to 42% and this change does not correlate with an impairment of overall survival^[75]: ER and PR status are different in 14.6% and 16.7% of cases respectively, while HER2 changes in about 8.3% of cases. During treatment it can be useful to take several biopsies to modify chemotherapy or endocrine therapy in the light of ER, PR and HER2 status^[70]. Following chemotherapy, in particular anthracycline-based ones, the level of expression of ER, PR and HER2 can change^[75]; these modifications imply a change also in tumor progression and aggressiveness and a resistance to endocrine therapy or trastuzumab, producing a change in clinical strategy. In our study the male patient^[43] had different patterns between gastric metastasis and primary breast tumor (ER-/PgR- in the stomach and ER+/PgR+ in the breast), one^[22] had different expression of HER2 (HER2- in the breast, HER2+ in the stomach) and one patient^[40] showed a change in receptor status during treatment passing from a Luminal A type to a Luminal B and then to a triple negative.

Combination of CK7/CK20 is also useful to distinguish between metastasis from breast carcinoma and primary gastric tumor; CK20 is expressed in gastric, colorectal, pancreatic and in transitional cell carcinomas, but it's negative in breast cancer^[76]. The CK7+/CK20- pattern is typical of adenocarcinoma of the breast, lung, and ovary, while CK7-/CK20+ is expressed in intestinal adenocarcinoma. CDX2 is a tumor suppressor gene that is implied in intestinal cell proliferation, differentiation, adhesion and apoptosis; it's an important marker of intestinal differentiation, usually expressed in gastric cancer and in intestinal metaplasia^[77,78].

Mammoglobine (MGB) is a 93-amino acid glycoprotein and a recent marker for breast cancer with a sensitivity of 93.1%. It can be useful in diagnosis of gastric metastasis from breast tumor in combination with other receptor expression^[79,80]. GCDFP-15 is never expressed in gastrointestinal cancer, but it's found in malignant tumor from the breast, and also salivary gland, external genitals, eyelid, apocrine duct of the bronchial tubes and gynecologic adenocarcinoma^[81]. MGB is more sensitive than GCDFP-15 but has less specificity^[79]. GATA-3 is a nuclear transcription correlated to breast glandular epithelial cells and shows the highest sensitivity in comparison to MGB and GCDFP-15^[81].

Diagnosis of metastatic disease stems from the analysis of all these markers. Gastric metastases are usually positive for ER, PgR, mammoglobine, GCDFP-15, GATA-3 and CK7 and negative for CK20. Gastric cancer is normally positive for CK20, but it's usually negative for GCDFP-15, ER and PgR, even if ER and PgR expression is controversial.

HNF4A (hepatocyte nuclear factor 4 alpha) was introduced as a new marker to differentiate gastric metastasis from primary malignant tumor. HNF4A is a nuclear transcription factor correlated with invasion, metastasis and epithelial-to-mesenchymal transition, due to activation of MMP-14 and promoting tumor angiogenesis, migration and invasion^[82]. A recent Brazilian study shows that HNF4A has positive expression in all patients with primary gastric adenocarcinoma, negative in all cases of primary breast carcinoma and also negative in all gastric metastases from breast carcinoma. In combination with ER and PR expression, it exhibits high sensitivity (100%) and specificity (96%)^[83].

Therapy

Establishing the primary site of malignancy determines the right treatment. Gastric metastasis from breast cancer are considered as a systemic disease because they usually present along with other metastatic localization, therefore the appropriate therapy is systemic, such as chemotherapy or endocrine therapy, whereas for primary gastric cancer first line therapy involves surgery^[5,15,84]. Chemotherapy, hormonal therapy or a combination of both of them lead to a remission rate of 32% to 53% and a prolonged survival of 2 or 3 years^[3].

In case of metastatic disease surgery is considered in case of obstruction, bleeding or perforation of gastric wall^[85] even if more conservative treatments (endoscopic hemostatic procedures or stent) can be performed

in patients with low performance status^[12]. The surgical option must be as little invasive as possible, for example a palliative bypass can be considered in case of outlet obstruction^[15].

Many studies, such as the one conducted by McLemore *et al.*^[53], 2005 report a lack of survival benefit from surgical treatment; palliative surgery can be associated with a prolonged median survival in some cases, but other factors can affect this difference such as biased patient selection for surgical palliation. In literature, few studies show surgical resection in patients with unique localization with reported improvement in overall survival: the study published by Taal *et al.*^[4], 2000 patients with complete remission of primary breast cancer that underwent gastric resection for solitary gastric metastasis shows a survival time of 38 months to be compared with 14.38 months for patients who didn't undergo resection^[3]. This can be explained considering that for patients submitted to surgical treatment the stomach is the unique localization without other metastasis or peritoneal carcinomatosis. In these patients the disease is not as advanced as in those candidates for systemic therapy. Therefore enhanced survival is due to patient clinical conditions and not strictly related to the type of treatment. In general, surgery does not offer an increase in survival but may have role in palliation^[53]. In our study surgery was also used to perform final diagnosis; gastric biopsies were confounding, thus authors performed gastrectomy to rule out the origin of the gastric disease^[23,28,29,35,37-39,43,45].

CDH1 mutation

As mentioned before, E-cadherin is a transmembrane glycoprotein involved in calcium-dependent adhesion; when E-cadherin is mutated, this leads to loss of cell adhesion, cell migration and subsequently tumorigenesis^[86]. An important gene mutation that is both associated with breast cancer and gastric cancer is CDH1, that encodes E-cadherin. Families with CDH1 mutations have a cumulative risk of developing hereditary diffuse gastric cancer (HDGC) of 70% and 56% in males and females, whereas female members have a cumulative risk of 42% for lobular breast cancer by age 80^[87]. Other gene mutations related to breast cancer but not to gastric cancer are BRCA1, BRCA2, and TP53; both BRCA1 and TP53 are associated with invasive ductal carcinoma, BRCA2 with both ductal and lobular carcinoma while CDH1 is only associated with lobular breast carcinoma^[88]. CDH1 is a gene mutation that is mutually exclusive with BRCA1/2 germline mutations. Screening for CDH1 should be suggested to women who have a personal or a family history of a combination of diffuse gastric cancer and lobular breast cancer (with at least one diagnosed before the age of 50), bilateral lobular carcinoma diagnosed at a young age, or family history of multiple lobular carcinomas with onset before 50 years old without gastric tumour^[89]. Sometimes diagnosis of lobular breast cancer with early onset might be the first manifestation of HDGC; so in patients with a history of multiple LBCs at a young age, especially with bilateral manifestation, it's advisable to perform a test for gene mutations^[90]. When CDH1 mutation is diagnosed, therapeutic management for the stomach and the breast is quite different: usually prophylactic total gastrectomy is advised because of the high risk of DGC, whereas prophylactic mastectomy is not performed, considering various genetic penetrance for LBC, but female patients should undergo to yearly mammography and breast MRI from age 35 years onwards^[86].

In conclusion, gastric metastases are a rare but not unusual site of secundarism from breast cancer^[3,4]. They usually arise several years after diagnosis of primary tumor and sometimes this can mislead the diagnosis^[5,6]. The differential diagnosis between primary gastric cancer and gastric metastasis is crucial and made possible only by histology and immunohistological patterns. Systemic therapy is the treatment of choice because the disease is usually not only localized to the stomach but presents other concurrent metastases^[5,15,84]. Surgery still has a role in case of complications or for definitive diagnosis when preoperative biopsy is not diriment and there is still a suspicion for primary gastric cancer^[85], even though this latter case is progressively decreasing thanks to innovation in instrumental tools for diagnosis. Breast metastasis to the stomach should be considered in any patient suspected of gastric cancer previously treated for breast carcinoma, especially if the treated carcinoma was ILC.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: D'Angelo F, Rampini A, Cardella S

Performed data acquisition: Rampini A, Cardella S

Performed administrative, technical, and material support: Nigri G, Valabrega S

Data review: Antonlino L, Aurello P, Ramacciato G

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Written informed consent for publication for images used in Figures 1-3 was obtained.

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Meeting Abstracts

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Meeting abstracts of Colossal Facet Conference - 2nd World Congress on Cancer 2018 “Oncology and Cancer therapeutics in the 21st century”

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Editorial note

This Special Issue of Journal of Cancer Metastasis and Treatment is dedicated to the proceedings of the 2nd World Congress on Cancer 2018 held in Bologna (Italy) in July 23-25. The theme of the Conference Cancer-2018 was “Oncology and Cancer therapeutics in the 21st century”.

Goal of the meeting was to share the foremost updated knowledge on the biology and the novel therapeutic options in Cancer. The conference focused on the mechanisms of cancer development, on the relationship between tumor microenvironment, metabolism and Cancer progression, novel technologies for early detection, and on novel pharmacological approaches to treat cancer patients. Major topics included epigenetics, inflammation, drug resistance, cancer stem cells, autophagy, cell metabolism, experimental models, biobanking, immune response, cachexia, molecular diagnosis, biomarkers, patient clinical care and complementary phytotherapy.

This Special Issue will cover the researches at the cutting edge in the study of the biology and clinical care of Cancer presented at the Conference.

Invited Speakers, Poster presenters and participants can contribute to this special issue with either an extended abstract (no page limit), Commentary, Original articles and (mini) review articles. Inclusion of schemes, Figures and Cartoon summarizing the results is strongly encouraged. An “ad hoc” editorial team will oversee the peer review of research and review articles.

Ciro Isidoro, Guest Editor

Keywords: Autophagy, biobanking, biomarkers, cachexia, cancer metabolism, cancer therapy, cell death, drug resistance, epigenetics, metastasis



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1. Autophagy in cancer: epigenetic regulation and therapeutic opportunities

Ciro Isidoro, Chiara Vidoni, Alessandra Ferraresi, Eleonora Secomandi, Letizia Vallino

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Autophagy, lysosome-effected degradation pathway that eliminates damaged and redundant cellular self-constituents, plays a pivotal role in maintaining cell homeostasis. Autophagy is a stress-response triggered under nutrient- and energy-restricted situations, as well as in response to damaging and harmful injuries. Autophagy play an important role in preventing tumorigenic transformation by cooperating with the systems for the quality control of the proteome and of the genome. Defective autophagy may facilitate carcinogenesis, and in cancer cells autophagy may serve a survival function allowing to cope with damages induced by anti-cancer treatments or with the lack of oxygen and nutrients. The composition of the tumour environment impacts on autophagy in cancer cells. In fact, the metabolic cross-talk with stromal cells and their soluble factors, including inflammatory cytokines, can modulate the availability of nutrients, energy and growth factors that eventually modulate autophagy. Autophagy in cancer cells is also modulated epigenetically by changes of the chromatin structures and the presence of microRNA. The fact that autophagy constitutes the integrated response to all metabolic stresses and that can be epigenetically modulated offers the possibility to treat cancer through the use of autophagy-targeted epigenetic modifiers.

Biography

Ciro Isidoro is Professor of Pathology at the School of Medicine of Università del Piemonte Orientale (Novara, Italy). He received his doctoral degree in Biological Sciences from the University of Torino (Italy) and his doctoral degree in Medicine and Surgery from the University of Piemonte Orientale (Novara, Italy). He is Visiting Professor at the Faculty of Medicine, Siriraj Hospital, of Mahidol University (Bangkok, Thailand), Visiting Professor at the Department of Cell Biology of the Oklahoma City University Health Sciences Center (US), and Professeur Honoraire at the Faculté de Medecine et de Pharmacie de l'Université de Franche-Comté, Besancon (France). He is member of the Scientific board of the «Integrative Cancer Research Center of the Georgia Institute of Technology» (Atlanta, US). **Ciro Isidoro** has co-authored > 120 peer-reviewed original articles published in international journals. He serves as Co-Editor in Chief of the Journal of Traditional and Complementary Medicine and Associate Editor of Autophagy, Molecular Carcinogenesis, BMC Cancer, and other journals. His fields of expertise include the subjects “autophagy regulation in cancer” and “mechanisms of anticancer activity of dietary products”.

2. Understanding parasites in the United States

Omar M. Amin

Parasitology Center Inc, Scottsdale, AZ 85259, USA.

This Power Point presentation is based on our work at Parasitology Center, Inc. (PCI), in Scottsdale, Arizona, USA and covers the diagnosis, pathology, and treatment of human parasitic infections in the United States based on our own patient history and cases diagnosed. A brief introduction to laboratory procedures, misdiagnoses/mistreatment, and impact on public health is made. The presentation begins with a quiz which is answered at the end. A systematic treatment of protozoan, helminth (worm), and arthropod parasites follows, emphasizing epidemiology and exposure, symptoms, gross pathology, and herbal and allopathic remedies including our own anti-parasitic herbal product Freedom/Cleanse/Restore.

All topics are presented with illustrated and labeled pictures of the various kinds of parasites and their gross pathology in human tissues, when applicable. The presentation is followed by a brief discussion of case histories as tested by us at PCI as well as by a treatment of intestinal pathogenic bacteria, also tested by us, that usually cause GI symptoms similar to those caused by intestinal parasites.

3. The new oncological theory and its importance in the war with cancer

Andrei P. Kozlov

Biomedical Center, Peter the Great St.Petersburg Polytechnic University and Research Institute of Pure Biopreparation, St. Petersburg 195251, Russia.

Earlier I formulated the concept of the possible evolutionary role of tumors (A.P. Kozlov, “Evolution by Tumor Neo functionalization”, Elsevier/Academic Press, 2014). This concept suggests that heritable tumors at earlier stages of progression supply evolving multicellular organisms with extra cell masses for the expression of newly evolving genes. After expression of novel genes in tumor cells, tumors differentiate in new directions and give rise to new cell types, tissues and organs.

In the presentation, the bulk of data supporting the positive evolutionary role of tumors will be reviewed, obtained both in the lab of the author and from the literature sources. The new concept supports the possibility of sustaining a stable tumor mass as a strategy in cancer therapy.

As a result of experimental confirmation of nontrivial predictions of the new concept a new class of genes - Tumor Specifically Expressed, Evolutionarily New (TSEEN) genes - was discovered. Examples of TSEEN genes will be presented. TSEEN genes may represent the new targets in the war with cancer.

4. Discovery of a novel PRMT5 inhibitor to treat pancreatic and colorectal cancers

Tao Lu

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Recently, we discovered that protein arginine methyltransferase 5 (PRMT5) functions as a novel activator of the nuclear factor κ B (NF- κ B), a culprit in pancreatic ductal adenocarcinoma (PDAC) and colorectal cancer (CRC). In this study, we adapted the AlphaLISA technique into a PRMT5-specific high throughput screen, and discovered PR5-LL-CM01 as a novel small molecule inhibitor of NF- κ B. Treatment of PDAC and CRC cells with PR5-LL-CM01 inhibited tumor cell proliferation, anchorage-independent growth, as well as cell migration. Furthermore, PR5-LL-CM01 greatly impeded tumor growth in PDAC and CRC *in vivo* xenograft models. Importantly, PR5-LL-CM01 exhibited more potent anti-tumor effect than the commercial PRMT5 inhibitor, EPZ015666, in both PDAC and CRC. Our study has established PR5-LL-CM01 as a potential basis for novel drug development to treat PDAC and CRC in the future.

Biography

Dr. Tao Lu is a tenure-track Assistant Professor and principle investigator at Department of Pharmacology and Toxicology, and a member of Simon Cancer Center at Indiana University School of Medicine. She

obtained her Ph.D. degree from University of Toledo, School of Medicine, and finished her postdoctoral training with the world renowned scientist Dr. George Stark at Cleveland Clinic, Ohio. Her research focuses on the discovery of novel regulators of NF- κ B, particularly, on the epigenetic regulation of NF- κ B and its role in cancer therapeutics. She won multiple awards at international scientific meetings. Dr. Lu has published near 50 papers with 2 were highlighted by F1000 Prime. She currently holds 2 provisional patents regarding NF- κ B regulation and serves as the editorial board member of 8 scientific journals.

5. Using a multiplexed immunofluorescence assay to uncover an immunosuppressive mechanism of tumor-associated macrophages in the pancreatic tumor microenvironment

Anna Juncker-Jensen, Jun Fang, Judy Kuo, Mate Nagy, Qingyan Au, Eric Leones, Flora Sahafi, Raghav Padmanabhan, Nicholas Hoe, Josette William

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Background: Pancreatic ductal adenocarcinoma (PDAC) is characterized by an excessive amount of desmoplastic stroma seeded with inflammatory cells and it is one of the most aggressive forms of cancer with no current specific therapies. Tumor-associated macrophages (TAMs) are a major component of the tumor microenvironment (TME), and in most solid cancers increased TAM infiltration is associated with a poor prognosis. TAMs can be described as classically activated M1 types with pro-inflammatory antitumor functions, vs. alternatively activated M2 types with immunosuppressive pro-tumor functions. The immunosuppressive functions of M2 TAMs can be exerted through release of cytokines and growth factors as well as via direct recruitment of T regulatory cells (Tregs), a subset of lymphocytes responsible for immune tolerance of the system to the tumor. While the differentiation from M1 to M2 in PDAC has been shown to be associated with a worse prognosis^[1], not much is known about PDAC TAM polarization and its potential correlation to Treg recruitment.

Methods: For this study we have used MultiOmyx, a proprietary, multiplexing assay with similar staining characteristics as standard IHC stains but with the significant advantage that up to 60 protein biomarkers can be interrogated from a single FFPE section^[2]. MultiOmyx protein immunofluorescence assays utilize a pair of directly conjugated Cyanine dye-labeled (Cy3, Cy5) antibodies per round of staining. Each round of staining is imaged and followed by novel dye inactivation chemistry, enabling repeated rounds of staining and deactivation.

Results: Using the pan macrophage marker CD68 in combination with either M1 marker HLA-DR or M2 marker CD163 we confirmed the presence of M1 (CD68+HLA-DR+) and M2 (CD68+CD163+) populations in 9 stage IIB non-metastatic PDAC FFPE samples, the vast majority being of the M2 subtype. Moreover, we found a positive significant correlation (Pearson's correlation $P < 0.05$) between the presence of M2 TAMs and Tregs (CD3+CD4+FoxP3+), but not between M1 TAMs and Tregs. Moreover, in a spatial nearest neighbor analysis we found M2 type TAMs to be in closer proximity to Tregs compared to M1 type TAMs.

Conclusion: We demonstrate a positive significant correlation between the presence of M2 TAMs and Tregs in the TME of PDAC, suggesting a possible pathway in which TAM polarization plays an immunosuppressive function by recruiting Tregs. PDAC is one of the most aggressive forms of cancer with a 5-year survival rate below 5% and no current specific therapies. An increasing number of studies show accumulation of immune suppressor cells such as MDSCs and TAMs in PDAC patients. Hopefully,

a greater understanding of the phenotypes and functions of subsets of these cell types, will result in new cancer immunotherapy strategies for PDAC.

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6. Stereotactic radiosurgery for brain metastasis: the end of whole brain radiotherapy?

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The objective of this presentation is to evaluate the applications of stereotactic radiosurgery (SRS) alone or in association with whole brain radiotherapy (WBRT) in the management of metastatic brain disease. Special attention was given to the influence of these treatment modalities on intracranial progression, local control, functional preservation, quality of life and survival. WBRT alone or following microsurgery has been the gold standard treatment of brain metastasis. The limited disease concept of oligometastatic brain disease, opened space to the use of focal therapies, such as SRS. Furthermore, delaying neurocognitive and cerebellar deterioration related to WBRT, is a worthwhile goal in brain metastasis patients. Moreover, SRS is more effective in the treatment of tumors considered resistant to WBRT such as melanoma and renal cell carcinoma. Analysis of the available data, including prospective randomized control trials in which SRS-alone was compared with WBRT+SRS, for patients with 1-4 brain metastasis, have proved that there was no significant difference in survival, neurologic death, functional independence and quality of life between the two treatment modalities. The omission of WBRT significantly increased the intracranial progression in new sites, requiring frequent monitoring in order to detect new lesions before they became symptomatic. Level 1 data has also demonstrated better neurocognitive function in patients not submitted to WBRT. The favorable outcomes demonstrated with SRS, have made possible its application in the focal treatment of surgical cavities. Therefore, the use of the SRS alone approach for oligometastatic brain disease may allow the majority of patients to avoid WBRT.

Biography

Leonardo Frighetto is the scientific coordinator of the Stereotactic Radiosurgery Unit at Moinhos de Vento Hospital and coordinator of the Neurosurgery Residency at the Federal University of the South Frontier, in Brazil. After finishing a fellowship at the Department of Stereotactic and Functional Neurosurgery at the University of California Los Angeles, he participated in the development of one of the first Stereotactic Radiosurgery Units in Brazil. The hospital is accredited by the Joint Commission International and affiliated to Johns Hopkins Medicine International. He also served as the President of the Brazilian Society of Radiosurgery from 2012 to 2014.

7. Role of *ERCC5* polymorphism and risk of breast cancer in Thailand

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Breast cancer is a major public health problem around the world, including Thailand and it has the highest ranking among female cancer. Polymorphism of *ERCC5* gene (excision repair cross-complementary group 5 gene or *ERCC5*) was reported to associate with an increased risk of breast cancer. This study aims to investigate the relationship between *ERCC5* polymorphism and the breast cancer risk in the lower northeastern region women of Thailand. One hundred fifty samples from breast cancer patients and 122 samples from healthy control group were analysed. Genomic DNA was extracted from white blood cell of all samples. The real-time polymerase chain reaction was used to demonstrate genetic polymorphism of *ERCC5*. The results showed that the *ERCC5* rs751402 polymorphism variant AG was associated with an increased risk of breast cancer ($P < 0.05$). This study demonstrated that *ERCC5* rs751402 genotype AG was associated with breast cancer risk in the lower northeastern region women of Thailand.

8. The RNA Disruption Assay as a tool to predict pathologic complete response and improved disease-free survival in breast cancer patients after neoadjuvant chemotherapy

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We have observed that a variety of mechanistically distinct chemotherapy drugs can induce the degradation of ribosomal RNA into high molecular weight fragments in multiple tumour cell lines in a dose- and time-dependent manner - a phenomenon we term “RNA disruption”. Moreover, we have developed the RNA disruption assay (RDA) to quantify ribosomal RNA degradation in tumour cell lines and tissues. Interestingly, chemotherapy agents cannot induce RNA disruption in chemo-resistant cell lines, suggesting RDA may be useful clinically in monitoring tumour response to chemotherapy in real time. Consistent with this view, we observed that high mid-treatment tumour RNA disruption in patients with locally advanced breast cancer is associated with a pathologic complete response (pCR) and improved disease-free survival after epirubicin/docetaxel neoadjuvant chemotherapy. Moreover, we and our collaborators have recently shown that high tumour RNA disruption after only one cycle of neoadjuvant trastuzumab-based chemotherapy predicted for a post-treatment pCR in patients with Her2+ breast cancer. The BREVITY clinical trial (Breast Tumour Response Evaluation for Individualized Therapy) will soon be accruing patients in Germany, Italy, Ireland, the United States, and Canada to further assess the utility of RDA to predict response and survival after neoadjuvant chemotherapy. The patient eligibility criteria, trial design, timing of tumour biopsies, and both primary and secondary study endpoints will be discussed. RDA may enable oncologists to identify patients with chemo-resistant tumours early in neoadjuvant chemotherapy,

reducing the toxic side-effects of the ineffective regimen, and enabling patients to move to potentially more beneficial downstream or alternative treatments.

Biography

Dr. Parissenti is a professor of biochemistry at Laurentian University and the Northern Ontario School of Medicine in Sudbury, ON Canada. The RNA Disruption Assay was developed through Dr. Parissenti's collaborations with clinical researchers from the Canadian Cancer Trials Group and Rna Diagnostics, Inc. More information on RNA disruption and RDA can be obtained at <http://rnadiagnostics.com>.

9. ULLB-0005, a novel protein for cancer treatment

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Recombinant protein derived from a natural fungal protein, which has high binding specificity toward the carbohydrate antigen. The natural Amino acid sequence has been modified to make more stable and soluble protein. Modified sequence has been cloned and express in *E. coli*. The protein was purified through different column chromatography and was characterized as a single protein. Promising cytotoxicity was observed in different cancer cell line, with a good safety profile in human PBMCs with ULLB-0005. The efficacy of the Molecule as antitumor agent was assessed in respective xenograft immuno-compromised mice models *in vivo*. As expected the molecule showed strong anti-cancer activity in immune-compromised mice model in various cancers which was observed in the reduction of tumor volume. Mechanistic study showed strong apoptotic signal by modulating phosphatidyl serine externalization, mitochondrial membrane depolarization, cell cycle arrest, ultimately leading to death in cancer cells. Inhibition of proliferation and migration was observed in human endothelial cells, suggesting potential antiangiogenic effect. Single dose Pharmacokinetic study was performed for the molecule in BALB/C MICE with i.v., i.p. and i.m. route.

To elulate this novel protein as a combination with chemotherapy further studies was conducted and found that the molecule showed good synergy in *in vitro* with approved chemotherapeutic agents for Breast and Pancreatic cancer. The inhibition potential of novel protein was determined for CYP3A4, CYP2C9, CYP2D6, CYP1A2 & CYP2C19 using Cytochrome P450 inhibitor screening kit. None of these enzyme get inhibited in the presence of molecule.

Biography

Dr. Sudeep Kumar did his Post Doc in Biotech from Valencia, Spain. He has more than 20 years of experience in Biopharma. He has worked from R&D to tech transfer and manufacturing of different recombinant proteins from bacteria and mammalian cell culture. He is dealing with different regulatory agencies for approval and faced WHO, USFDA and other regulatory audits. He also actively involved with clinical research team for Preclinical and clinical trials of recombinant proteins and vaccine. He established the VLP technology platform for different vaccine in India in collaboration with Novavax. At present his major research area to develop new recombinant protein for different cancer.

10. Relevance of FoxO3a in tamoxifen resistant breast cancer

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Resistance to endocrine treatments is a major clinical challenge in the management of estrogen receptor positive (ER+) breast cancers (BC). Although multiple mechanisms leading to endocrine resistance have been proposed, the poor outcome of this subgroup of BC patients demands additional studies. Here we show that the expression of FoxO3a transcription factor is reduced in ER+ BC cells that developed resistance to Tamoxifen (Tam), TamR cells, due to its hyper-phosphorylation and degradation mediated by a hyperactive ERK1/2 pathway. On the other hand, FoxO3a silencing counteracts Tam induced growth inhibition in wtMCF-7, demonstrating that FoxO3a is a mediator of cell response to Tam.

FoxO3a re-activation in TamR/FoxO3a inducible clones, developed to express a constitutively active FoxO3a under tetracyclines control, re-established the sensitivity to the antiestrogen, inhibiting proliferation and cell cycle progression, as well as restoring Tam dependent apoptotic response.

Proteomics analysis unveiled novel interesting and potential mediators of the anti-proliferative and pro-apoptotic activity of the transcription factor, while Kaplan-Meier survival curves showed that high levels of FoxO3a transcripts strongly correlate to a positive response to Tam therapy in BC patients. Finally, FoxO3a induction by the anti-convulsant Lamotrigine (LTG), strongly reduced tumor mass in TamR-derived mouse xenografts.

Altogether, our data indicate that FoxO3a is a good prognostic factor in ER+ BC, predicting a positive response to endocrine therapy, and a key target to be exploited in combination therapy. In this context, LTG might represent a valid candidate to be used as an adjuvant to Tam therapy in patients at risk.

Biography

Catia Morelli has completed her PhD from University of Calabria, Cosenza, Italy, after a 4 years training period (1999-2003) at the Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA (USA). She is now Researcher at the Department of Pharmacy and Health and Nutritional Sciences at the University of Calabria, recently awarded as Department of Excellence by the Italian Ministry of Education, University and Research. She encounters more than 35 papers in reputed journals and she is also co-founder of NanoSiliCal Devices, a spin-off company of the University of Calabria, developing silica-based nanosystems to be employed in targeted therapy (<http://www.nanosilicaldevices.com/en/>).

11. Deciphering human B cell repertoire with next-generation sequencing technology

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Next-generation sequencing (NGS) has allowed a massive increase in capacity to sequence genomes at relatively low cost and in a short time frame. It has revolutionized multiple aspects of biological research and is actively being adopted into profiling human B cell receptor (BCR) repertoires. Several NGS platforms are currently available, with average read lengths of 75 bp to 8,500 bp and different error rates.

Using NGS, we successfully constructed database of human BCR repertoire from convalescent patients who recovered from severe fever with thrombocytopenia and middle east respiratory syndrome. Afterwards, we developed algorithms for analyzing the diversity, enrichment pattern, accumulation of somatic hyper-mutation in BCR repertoire. Through *in silico* analysis we selected clones of interest and prepared recombinant antibodies using a mammalian transient expression system. Their reactivity to viral coat proteins and virus-neutralizing capability was confirmed in *in vitro* and *in vivo* experiments.

Currently we are investigating the clinical value of BCR repertoire profiling in autoimmune disease patients including Neuromyelitis Optica and various cancers.

Biography

Junho Chung has completed his MD, PhD from Seoul National Univ. College of Medicine, South Korea. He is the professor of Seoul National Univ., in the Department for Biochemistry and Molecular Biology. He has published more than 95 papers in reputed journals and has been serving as a vice-editor-in chief of Journal of Cancer Research and Clinical Oncology.

12. Biobank in Pilsen, Czech Republic, efficient tool for cancer research and personalised treatment

Judita Kinkorová, Ondřej Topolčan

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Biobank in University Hospital (UH) Pilsen is newly established (opened in April 2017) hospital integrated biobank, based on previous high quality sample collection. Since 2000 the hospital repository started to collect and store systematically biological material of patients for research in the UH. The main diagnoses for sample collection are oncological samples of breast cancer, colorectal cancer, liver and kidney carcinoma, and prostate cancer.

To be a fully-fledged biobank the following activities have been realised: collection and storage high quality biological material (blood, serum and plasma) of oncological patients, collection management and storage of related data, implementation of ethical, social and legal issues, harmonization of IT systems, implementation of relevant requirements for providing biological material to the Czech Republic and abroad, preparation of education and training programmes and courses, active participation on BBMRI - ERIC activities, and cross-boarder collaboration in the BRoTHER project.

Currently is the biobank ready to implement aspects of personalised medicine in practice. Regarding the high quality aspects the selected long and short term stability of tumor markers are studied. Together

with the standard diagnostic procedures like biomarkers, imaging techniques, and clinical stage (multidisciplinary approach), new diagnostic algorithms based on multiparametric and multidisciplinary approach for selected diagnoses are created and brings benefits both for the patient and the hospital.

Personalized medicine approach in University Hospital is closely connected with the education programme realised at Faculty of Medicine in Pilsen Charles University.

Biography

Judita Kinkorová has completed his PhD from Academy of Science of the Czech Republic in Prague. She is the associate professor, in the Department for immunochemistry University Hospital Pilsen and a manager of biobank in University Hospital Pilsen. She has published more than 40 papers in reputed journals and has been serving as an editorial board member of EPMA Journal.

13. TM4SF5-mediated hepatic carcinogenesis

Jung Weon Lee

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Diverse functions of cancer cells including proliferation, migration and invasion critically and greatly depend on extracellular environment during their survival and metastasis. The environment consists of extracellular matrix proteins, neighboring cells, and soluble factors, including of cytokines, chemokines, and growth factors. Efficient dissemination of a cancer cell from a primary tumor mass and invasion through the environment can result in a successful metastasis. Therefore, identification of biological cancer marker(s) playing roles in diverse cancerous functions would be beneficial for clinical and therapeutic purposes, and can be performed in 2D flat, 3D cell and tissue cultures, and animal models. Transmembrane 4 L six family member 5 (TM4SF5) is a transmembrane glycoprotein of the transmembrane 4 L six family, a branch of the tetraspan(in) family, highly expressed in many types of cancers including hepatic cancer, and shown to cause epithelial-mesenchymal transition. TM4SF5 in hepatocytes is induced by TGF β 1-mediated Smad and EGFR activation and involved in liver fibrosis. Transgenic animal showed that overexpression of TM4SF5 in liver caused inflammatory, fatty, and fibrotic phenotypes, indicating that TM4SF5 expression following actions by TGF β 1 prevalent in chronic liver injury situation may lead to playing regulatory roles in functions for different metabolic pathways. Further, disease-prone TM4SF5 transgenic mice showed enhanced expressions of CD34, HIF1- α , AFU, and AFP in the livers. A small calchone compound, TSAHC and chimeric anti-TM4SF5 antibody could successfully block the TM4SF5-mediated regulation of metabolic activities, fibrosis, and xenograft tumor formation.

Biography

Dr. Jung Weon Lee is a Professor at Dept. of Pharmacy, Seoul National University (SNU), Korea. He got Ph.D. in Pharmacology, University of North Carolina at Chapel Hill, NC, USA. He had a postdoc period at Memorial Sloan-Kettering Cancer Center, NY, USA. In 2001, He came back to Korea, and in 2009 moved to Dept. of Pharmacy, SNU. His researches focus on how cellular functions occur at the molecular levels. His researches focuses on the roles of a tetraspanin, TM4SF5, in metabolic disorders, fibrosis, tumorigenesis and metastasis, and on the anti-TM4SF5 reagents to block TM4SF5-mediated diseases (Lab homepage: <http://www.snupharm.ac.kr/jwl/>).

14. The histone methyl transferase DOT1L is a key functional partner of estrogen receptor alpha for regulation of gene transcription in hormone-responsive breast cancer

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Estrogen Receptor alpha (ER α) is a key mediator of estrogen signaling in hormone-responsive breast cancer (BC) and target of endocrine therapies, where resistance to treatment is a major problem in clinical management of these patients. A better understanding of the molecular mechanisms of ER α actions in cancer may lead to identification of new therapeutic targets against endocrine therapy-resistant tumors. We recently identified the epigenetic writer DOT1L (DOT1 Like Histone Lysine Methyltransferase) as novel a nuclear partner of ER α in hormone-responsive BC cells. To investigate the functional role of this enzyme in mediating receptor actions in target cell nuclei, physical and functional ER α -DOT1L interaction on chromatin was mapped by Chromatin Immunoprecipitation coupled to Mass Spectrometry (ChIP-MS) and Sequencing (ChIP-Seq), transcriptome profiling (RNA-Seq) coupled to gene silencing and pharmacological inhibition of either protein. Cellular and functional assays were also used to evaluate the impact of the ER α -DOT1L complex in hormone-responsive BC cell functions. ChIP-MS confirmed co-recruitment of the two factors on chromatin in a multiprotein complex. Gene expression profiling and Nascent RNA-Seq before and after DOT1L pharmacological inhibition showed that this enzyme is involved in ER α -mediated transcriptional regulation of several estrogen responsive genes, including ER α itself. ChIP-Seq mapped co-occupancy of several chromatin sites by both proteins, including the ER α gene promoter itself. These results reveal that physical and functional association between ER α and DOT1L is a key molecular event in hormonal control of BC cell functions, suggesting that this enzyme might represent a potential drug target against hormone-responsive tumors.

Biography

Dr. Giovanni Nassa completed his PhD at University of Campania “Luigi Vanvitelli” and presently is fixed-term Researcher (RTDa) of General Pathology at the Department of Medicine, Surgery and Dentistry “SMS” of the University of Salerno. AIRC Fellow during the early stages of his career, he was also visiting scientist and FEBS fellow at the Institute of Biotechnology and Biomedicum of the University of Helsinki. He published more than 30 papers in international peer-review journals, with research mainly focusing on the epigenetic mechanisms underlying hormone-responsive breast cancer pathogenesis and loss of response to endocrine therapy during progression.

15. IL-4 as a potential treatment for cancer-induced muscle wasting

Paola Costelli

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Skeletal muscle wasting, one of the most relevant features of cancer cachexia, is mainly associated with marked alterations of protein turnover. Few years ago the observation that also myogenesis is impaired in

cancer hosts was reported. In particular, Pax7⁺ cells accumulate in the muscle of tumor-bearing animals without proceeding into differentiation and fusing with existing myofibers. Such impairment was proposed to depend on persistently expressed Pax7 due to NF- κ B hyperactivation. The reduced myogenesis does not depend on an intrinsic defect of muscle stem cells, since they can easily differentiate *in vitro* and can fuse with damaged myofibers in an experimental model of muscle dystrophy. However, myogenic precursors isolated from mice bearing the C26 tumor cultured in proliferation medium spontaneously differentiate to adipocytes.

To investigate the mechanisms underlying such “adipogenic drive”, mice bearing the C26 carcinoma were treated intraperitoneally with IL-4, a cytokine previously shown to inhibit adipogenesis in muscle. The results obtained show that muscle wasting was partially prevented in tumor-bearing animals receiving IL-4, in terms of both tissue weight and myofiber cross sectional area. The improved muscle phenotype is associated with reduced levels of phosphorylated ERK, in agreement with previous data. The spontaneous adipogenic differentiation is markedly reduced in primary cultures of myogenic precursors isolated from the C26 hosts treated with IL-4 in comparison to those obtained by untreated animals. This observation suggested that IL-4, by inhibiting adipogenesis, could force myogenesis. Consistently, when IL-4 is administered to tumor-bearing animals in which muscle damage was induced by injection of BaCl₂, regeneration occurred faster than in the untreated C26 hosts.

On the whole these results suggest that primary cultures of myogenic precursors isolated from tumor-bearing animals are committed to differentiate to adipocytes, but can be forced through myogenesis by adequate stimuli. By contrast, the muscle microenvironment existing *in vivo* in the tumor host is able to inhibit both myogenic and adipogenic differentiation of these precursors, that indeed accumulate in the tissue. Preliminary data suggest that the lack of IL-4 production could play a role in this regard.

16. Lenvatinib in anaplastic thyroid cancer

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The oral multitargeted tyrosine kinase inhibitor lenvatinib acts against VEGFR1-VEGFR3, FGFR1-FGFR4, PDGFR α , RET and v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT) signaling networks involved in tumor angiogenesis.

The antitumor activity of lenvatinib (1 nmol/L, 100 nmol/L, 1 μ mol/L, 10 μ mol/L, 25 μ mol/L and 50 μ mol/L) has been investigated in primary anaplastic thyroid cancer (ATC) cells, in the human cell line 8305C (undifferentiated thyroid cancer) and in an ATC-cell line (AF), *in vitro*; and *in vivo* in AF cells injected in CD nu/nu mice.

Lenvatinib significantly reduced ATC cell proliferation ($P < 0.01$, ANOVA), increasing apoptosis ($P < 0.001$, ANOVA). Furthermore, lenvatinib inhibited migration ($P < 0.01$) and invasion ($P < 0.001$) in ATC, inhibited EGFR, AKT and ERK1/2 phosphorylation and down-regulated cyclin D1 in ATC cells. Moreover, lenvatinib significantly inhibited 8305C and AF cell proliferation, increasing apoptosis.

AF cells were injected subcutaneously into CD nu/nu mice and tumor masses were evident after 20 days. Tumor growth was significantly inhibited by lenvatinib (25 mg/kg/day), as the VEGF-A expression and microvessel density in AF tumor tissues.

In conclusion, we show for the first time the antitumoral effect of lenvatinib in primary human ATC cell cultures obtained from patients. These results could open the way to the clinical use of lenvatinib in the treatment of patients with ATC.

Biography

Prof. Antonelli is currently Director of the Immuno-Endocrine Section of Internal Medicine and Associate Professor in the Department of Clinical and Experimental Medicine, University of Pisa, Azienda Ospedaliera-Universitaria Pisana, Pisa, Italy.

He graduated in Medicine cum laude at the University of Pisa in 1982, where he received the post-graduate Specialization in Endocrinology in 1985 and in Occupational Health in 1987. He received also the post-graduate Specialization in Oncology (at the University La Sapienza, Rome, Italy) in 1992.

His researches have been published in more than 320 articles on International journals (Impact Factor > 1200; H index 60).

17. Novel inducers of noncanonical and immunogenic cell death

Marc Diederich

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Apoptosis was most often considered as the most important type of cell death and numerous compounds triggering this programmed form of cell demise were explored. Over the last 10 years, other cell death modalities were also described, and proof of concept was provided that such compounds are efficiently able to target cancer cells that already became resistant to conventional apoptosis inducers. Moreover, the interplay between different cell death modalities gained interest^[1]. We became interested to assess the anticancer effect of compounds that were shown to kill cells by triggering various necrosis-related phenotypes^[2,3]. We believe that these compounds provide a more efficient therapeutic outcome. For our research we essentially investigated compounds of natural origins from both marine^[4-6] and terrestrial sources^[7-10]. Multiple forms of regulated necrosis and the in-depth elucidation of the corresponding cell signaling pathways allowed to better predict the immunogenic potential of cancer cells dying by non-canonical cell death mechanisms^[11-13]. This presentation will cover the effect of various natural and hemisynthetic compounds while describing their cell death-inducing activity leading to secretion or exposure of immunogenic cell death markers^[14-16].

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Biography

Marc Diederich earned his PhD in molecular pharmacology in 1994 from the University Henri Poincaré Nancy 1, France. After training at the University of Cincinnati, USA, he focused his research on cancer and leukemia cell signaling pathways and gene expression mechanisms triggered by natural compounds with epigenetic-, anti-inflammatory- and cell death-inducing potential. He directs the Laboratory for molecular and cellular biology of cancer (LBMCC) at Kirchberg Hospital in Luxemburg. He was appointed associate Professor of Biochemistry at the College of Pharmacy of Seoul National University in 2012. In 2017, he was tenured and promoted to full professor at SNU.

18. Co-expression of immune checkpoint genes at the cancer/antigen-presenting-cell side of the immunological synapse

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Introduction: The interface between T lymphocytes and cancer cells or antigen-presenting cells (APC), termed “the immunological synapse” (IS), comprises of both co-inhibitory and co-stimulatory checkpoint proteins that modulate the signal transmitted to T lymphocytes, leading to either activation, anergy or exhaustion. Monoclonal antibodies against checkpoint proteins (designated “checkpoint inhibitors”) have anti-neoplastic activity in several malignancies, but not all cancers and not all patients within a given cancer respond.

Methods: We analysed the tumor cancer genome atlas database for the expression of 15 checkpoint mRNAs suggested from the literature to be expressed at the cancer/APC side of the IS in 28 tumor types. The Spearman rho correlation co-efficient for the co-expression of each pair of checkpoint mRNAs was

calculated. For each cancer type, we calculated the average correlation co-efficient of any combination of 2-15 mRNAs.

Results: The number of co-expressed checkpoint genes with an average Spearman rho co-efficient of above 0.5 was significantly different between tumor types, ranging from 10 to 0. Thyroid carcinoma, testicular cancer and melanoma were the three tumors with the highest number of co-expressed checkpoint mRNAs, whereas in acute leukemia there were no co-expressed checkpoint mRNAs, as predicted. Similarly, the nature and mix of co-stimulatory and co-inhibitory checkpoint mRNAs was different between different tumor types, with each tumor exhibiting a different “network” of checkpoint co-expression with differing strengths of correlations.

Conclusion: Our bioinformatic analysis indicates that both co-inhibitory and co-stimulatory checkpoint mRNAs are co-expressed in solid malignancies in varying extents in different cancers. We propose that the differences in these “checkpoint networks” formed on the cancer side of the IS take part in determining cancer immunogenicity. It is tempting to speculate that these networks may also take part in determining the potential of a tumor type to respond to the currently-available immune checkpoint inhibitors.

Biography

Raya Leibowitz completed her PhD and her MD at the Tel-Aviv university, Israel. She is currently a senior medical oncologist (clinician-investigator) at the Sheba medical center and an assistant professor at the department of oncology, faculty of medicine, Tel-Aviv university. Her major scientific work until now (first and last authorship) was published in *Nature Medicine*, *Cancer Research*, *Annals of oncology* and *Molecular Cancer*. Among other grants, she was a recipient of the “melanoma research alliance young investigator award”, the “Gerald Kirsh” humanitarian award and the “Conquer Cancer foundation” merit award twice.

19. Sunitinib in thyroid cancer: a review of the literature

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Sunitinib is a multi-targeted tyrosine kinase inhibitor (TKI), acting on different receptors for platelet-derived growth factor receptors and vascular endothelial growth factor receptors, c-KIT, fms-related tyrosine kinase 3 and RET, leading to tumor vascularization reduction, tumor cell apoptosis, and causing tumor shrinkage.

It has been approved for the treatment of pancreatic neuroendocrine tumors, imatinib-resistant gastrointestinal stromal tumor and renal carcinoma.

In vitro studies have been conducted on the RET/PTC1 cell, showing that sunitinib is able to target the cytosolic MEK/ERK and SAPK/JNK pathways inhibiting cell proliferation and causing stimulation of sodium/iodide symporter gene expression.

Also *in vitro* and *in vivo* studies conducted on anaplastic thyroid cancer cells have shown the efficacy of sunitinib, also as first- and second-line TKI therapy in patients with advanced dedifferentiated thyroid cancer, or medullary thyroid cancer.

The most frequent adverse events are fatigue, decreases in blood cell counts (especially leukocytes), diarrhea, hand-foot skin reaction, nausea, hypertension and musculoskeletal pain. A dose of 37.5 mg/day sunitinib is well-tolerated, and effective.

To sum up, although sunitinib is promising in the therapy of differentiated thyroid carcinoma, until now no phase III studies have been published. Further prospective researches are needed to evaluate the real effectiveness of sunitinib in aggressive thyroid cancer.

Biography

Giusy Elia is graduated in Biology applied to Biomedicine in 2012 and specialized in Clinical Pathology and Clinical Biochemistry in 2017 at the University of Pisa (Italy). Her areas of interests range from Immunology, Pharmacology to Biochemistry. Her researches have been published on International journals (HI = 5).

20. Vandetanib in anaplastic thyroid cancer

Silvia Martina Ferrari¹, Poupak Fallahi², Ilaria Ruffilli¹, Giusy Elia¹, Francesca Ragusa¹, Sabrina Rosaria Paparo¹, Claudia Caruso¹, Alessandro Antonelli¹

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Anaplastic thyroid cancer (ATC) represents about 1% of thyroid cancer (TC), and it is one of the most aggressive human tumors, representing approximately 15%-40% of TC deaths. The multimodal treatment, that includes debulking, chemotherapy, and hyperfractionated accelerated external beam radiotherapy, is the most effective treatment, with a median survival of 10 months. New drugs have been recently evaluated in ATC, but until now no significant improvement of survival has been observed.

Vandetanib is an oral once-daily multi-tyrosine kinase inhibitor, inhibiting the activation of RET, EGFR, VEGFR-2, VEGFR-3, and slightly VEGFR-1, and with a potent anti-angiogenic activity.

Its antitumor activity was tested at different concentrations (1 nmol/L, 100 nmol/L, 1 µmol/L, 10 µmol/L, 25 µmol/L and 50 µmol/L) in primary ATC cells, in the 8305C continuous cell line, and in AF cells; and in 8305C cells in CD nu/nu mice.

Vandetanib decreased significantly ATC cells proliferation ($P < 0.01$, ANOVA), inducing apoptosis dose-dependently ($P < 0.001$, ANOVA), and inhibiting migration ($P < 0.01$) and invasion ($P < 0.001$). Vandetanib inhibited EGFR, AKT and ERK1/2 phosphorylation and down-regulated cyclin D1 in ATC cells.

In 8305C and AF cells, vandetanib inhibited significantly the proliferation, inducing also apoptosis. 8305C cells were injected sc in CD nu/nu mice and tumor masses became detectable 30 days after. Vandetanib (25 mg/kg/die) inhibited significantly tumor growth and VEGF-A expression and microvessel density in 8305C tumor tissues.

In conclusion, we show the antitumor and antiangiogenic activity of vandetanib in ATC, paving the way to a future clinical evaluation.

Biography

Silvia Martina Ferrari is graduated in Biological Sciences cum laude in 2002 and specialized in Clinical Pathology in 2007 at the University of Pisa (Italy). Her principal areas of expertise are autoimmune thyroid disorders, chemokines and cytokines, type 1 diabetes, systemic autoimmune disorders, HCV-associated thyroid disorders and thyroid cancer. Her researches have been published in more than 158 articles on International journals (HI = 38). She serves as an editorial board member and is Referee and Reviewer of many scientific International journals.

21. Mitochondrial dynamics and chemoresistance in ovarian cancer

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Various cellular and acellular components constitute tumor microenvironment affecting metabolism and malignant phenotypes of cancer. Hypoxia and increased level of reactive oxygen species (ROS) are frequently observed in many types of malignant tissues composed of cancer cells and surrounding tumor microenvironment, including ovarian cancer. In response to the increased level of ROS, cancer cells activate antioxidant mechanisms to counterbalance increased ROS. PGC1 α is a key molecule critical for mitochondrial biogenesis and upregulation of antioxidant enzymes. In our previous research, we reported that PGC1 α is associated with cisplatin-resistance in tumor spheres. Tumor sphere cells acquired stem cell-like properties together with increased production of ROS. ROS was shown to be important for maintaining stemness and PGC1 α expression of the tumor sphere cells in ovarian cancer. Decreased sensitivity to cisplatin was observed in both tumor sphere and PGC1 α -overexpressing cells, while silencing PGC1 α sensitized the tumor sphere cells to cisplatin. Along with upregulation of PGC1 α expression in tumor sphere cells, mitochondrial fission was increased in comparison to their parental cells. Similar to the in-vivo environment of the malignant tumor, tumor spheres exhibit hypoxia at the core. The center of tumor spheres is exposed to hypoxia like *in vivo* tumor. As the tumor sphere grows larger in size, a hypoxic gradient towards the core of the sphere is created. Next, we investigated the effect of hypoxia on mitochondrial dynamics. Mitochondria, dynamic intracellular organelles, go through incessant processes of fission/fusion and biogenesis by various stimuli in tumor microenvironment. Under hypoxic (< 1 % O₂) culture conditions, mitochondrial fission was increased together with increased resistance to cisplatin in ovarian cancer cells. Inhibition of mitochondrial fission enhanced sensitivity of cancer cells to cisplatin. Our findings suggest that PGC1 α -induced mitochondrial biogenesis and alteration of mitochondrial dynamics by hypoxia could cause the cisplatin resistance of ovarian cancer cells. The changes in mitochondrial biogenesis and dynamics of cancer cells adapted to tumor microenvironment could be a novel therapeutic target to overcome chemoresistance in ovarian cancer.

Biography

Prof. Yong Sang Song is with the Department of Obstetrics and Gynecology, College of Medicine, Seoul National University, Korea. He received MD, College of Medicine, Seoul National University, Korea from 1977 to 1983; the MS and the PhD, Postgraduate School Seoul National University, Korea. His major interests are molecular mechanisms of tumors, especially the role of tumor microenvironment in cancer cell metabolism and chemoresistance and precision medicine in ovarian cancer. He is particularly interested in the impacts of components of tumor microenvironment in ovarian cancer progression. He has published more than 300 papers in the science citation index journals.

22. A strategy for the prevention of thyroid cancer with a focus on the experience of managing radio-induced thyroid cancer after the Chernobyl Accident

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One of the lessons learned from Chernobyl is the dramatic increase in incidence of thyroid cancer in Belarus. However the incidence of thyroid cancer has been steadily increasing during the last 30 years worldwide most markedly in the Republic of Korea, France, Italy, Australia, and USA. Analyzing this problem, some scientists have recently identified this epidemic of thyroid cancer as “overdiagnosis” and “overtreatment”. On the other hand, overall incidence-based mortality for thyroid cancer in USA from 1994 to 2013 substantially increased (1.1% per year), and it increased even faster for patients diagnosed with advanced-stage papillary thyroid cancer (2.9% per year). These findings are consistent with a true increase in the occurrence of thyroid cancer. The main factors contributing to this increase continue to be debated. The experience of managing radio-induced thyroid cancer after the Chernobyl Accident has shown that radiation exposure and the usage of drinking water with a high concentration of nitrates were significantly associated with high incidence of thyroid cancer. Better understanding of the combined impact mechanism of radiation and nitrates exposure and maybe other endocrine disruptors will give us more opportunities to develop new ways of thyroid cancer preventing.

The rising incidence and optimization of management of nodular goiter are also actual problems for Belarus. Surgery for treating of low-risk thyroid papillary microcarcinomas and nodular goiter might be an overtreatment and it is important to develop a less traumatic modalities. To ablate small thyroid nodules (less than 2 cm in diameter), we started to use new originally developed low-cost device for bipolar radiofrequency ablation - «Thyrablator» designed as a sterile disposable needle for the fine needle biopsy (20-gauge). The first experience showed a promising efficacy and safety of new device - «Thyrablator» as a minimally invasive treatment of thyroid lesions.

More studies are needed to better verify the causes of the epidemic of thyroid cancer in the world and to concentrate our efforts on the prevention and development of new minimally invasive approaches for the treatment of nodular goiter and early forms of thyroid cancer.

23. Reducing PD-L1 expression with epigenetic modifiers enhances immunotherapy

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Histone deacetylases (HDACs), were originally described in a limited context as histone modifiers. New evidence has demonstrated that HDACs are also involved in a diverse range of cellular processes that are

not restricted to the chromatin environment, such as the regulation of the cell cycle/apoptosis and, more recently, a modulator of immune response. However, much remains unknown about the mechanism of action of HDACs and their roles in the immune-biology of cancer. The non-specific nature of pan-HDAC inhibitors results in a narrow therapeutic window of use, limiting the dose and duration due to toxicity. Our group has focused in one specific HDAC, HDAC6, and shown that both the genetic abrogation and pharmacological inhibition of this HDAC modulates the expression of a variety of immune-regulatory proteins in the tumor microenvironment, including PD-L1, PD-L2, MHC class I, B7-H4 and TRAIL-R1. We have previously demonstrated that both pharmacological inhibition and/or genetic abrogation of HDAC6 plays a critical role in the immune check point blockade by down-regulating the expression of PD-L1 and other check-point modulators such as PD-L2, B7-H4, *etc.* Moreover, we have also observed that *in vivo* inhibition of HDAC6 reduces tumor growth in B16 and SM1 murine melanoma models within syngeneic immunocompetent hosts. Additionally, we have found that the combination of low doses of the HDAC6i Nexturastat A and checkpoint immune blockade therapies, including anti-PD-1 and anti-CTLA4, result in an important improvement in anti-tumor responses in our murine model as evidenced by the reduction of tumor growth when compared to treatment with individual stand-alone agents and the improved modulation of various immune markers. Our studies have also demonstrated that tumors treated with stand-alone check point inhibitor treatments, specifically anti-PD-1, results in a substantial increase in the production of IFN γ and IL-2, resulting in the upregulation of PD-L1 and other critical checkpoints in the immune blockade. Similar levels of IFN γ and IL-2 were found in the combination subject groups, however, the levels of PD-L1 and PD-L2 were more comparable to the non-treated group. Overall, our evidence suggests that HDAC6i has the potential for use as an adjuvant in ongoing therapeutic options involving the immune check-point blockade.

24. Trisenox disrupts MDM2-DAXX-HAUSP complex, degrades MDM2, and activates p53 in acute leukemia cells

Paul Bernard Tchounwou, Sanjay Kumar

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Trisenox (TX) has been successfully used in the treatment of both de novo and relapsed acute promyelocytic leukemia (APL) patients. It inhibits APL cells growth efficiently through cell cycle arrest and apoptosis, however exact molecular mechanisms of action poorly understood. In present study, we found a new target of TX action that involves the activation of p53 and p21 expression through association with death domain-associated protein (DAXX), disruption of MDM2-DAXX-HAUSP complex and degradation of MDM2 in APL cells. TX-induced stress signal is transmitted by protein kinase (ATM & ATR) and phosphorylation of CHK1 & CHK2 at Ser 345 and Thr68 residues leading to complex disruption and accumulation of p53 in APL cells. TX-induced p53 caused cell cycle arrest by regulating expression of p21, cyclins and cyclin dependent kinases proteins and forcing cells to undergo apoptosis by apoptotic proteins expression modulation, mitochondrial membrane depolarization leading to caspase 3 activation. Our immunoprecipitation studies also showed that the complex molecules were well associated in APL cells, and TX disrupted their associations leading to accumulation of p53. We further studied the functional role of p53 in disruption and expression of complex molecules in p53-knock down APL cells using lentiviral shRNA approach. Taken together, our findings showed that TX activates p53, through association of DAXX, disruption of MDM2-DAXX-HAUSP complex, MDM2 degradation in APL cells leading to cell cycle arrest and apoptosis. This novel target of TX activity may be useful for designing new APL drugs.

Biography

Dr. Paul B. Tchounwou is Associate Dean of the CSET, Presidential Distinguished Professor, and Director of the NIH-funded RCMI-Center for Environmental Health. He is an internationally recognized biomedical scientist whose research focuses on the mechanisms of action of xenobiotic compounds and cancer therapeutic drugs. He is author of 228 peer-reviewed publications and 507 scientific presentations. He is the Editor-in-Chief of Environmental Toxicology (New York, USA) and of International Journal of Environmental Research and Public Health (Basel, Switzerland). He has received several awards for excellence including the 2003 Millennium Award for Research Excellence conferred by the White House (Washington DC).

25. The potential role of milk's amino acid- and miRNA signaling in the pathogenesis of prostate cancer

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Epidemiological studies reported a dose relationship between cow's milk intake and prostate cancer (PCa). Lactose intolerance protects against the development of common cancers and a lower incidence of lactose intolerance in comparison to the general population has been reported in PCa patients. Daily milk consumption during adolescence in comparison to less frequent milk consumption increased the risk of advanced PCa in adulthood by 3.2. An increased risk for PCa has also been observed for patients with a history of severe acne, which is another milk-induced disease, pointing to common underlying signaling pathways. Several components of milk such as dairy protein, calcium, galactose, miRNAs, androgen precursors and estrogens derived from high-yield dairy cows have been implicated to play a role in milk-induced PCa. We provide translational evidence, that milk's essential amino acids via enhancing the secretion of insulin and insulin-like growth factor 1 result in AKT-stimulated mouse double minute 2-mediated degradation of the tumor suppressor p53. Moreover, milk exosomal miRNA-125b, which survives pasteurization, may directly repress TP53 mRNA. Milk-derived miRNA-148a via depletion of DNA methyltransferase 1 and hypomethylation of cancer-germline genes may promote epithelial-mesenchymal transition and cancer stem cells. Although boiling and ultraheat treatment of milk eliminates milk's miRNA signaling, milk's amino acid signaling is not affected by thermal procedures. We recommend that patients with severe acne vulgaris, adults with a history of severe acne, as well as individuals with a family history of PCa should refrain from pasteurized cow's milk to prevent PCa.

Biography

After his medical studies at the University of Münster, Germany, Bodo C. Melnik was visiting scientist at the University of California San Francisco from 1982-1984. From 1984-1990, he was resident at the Department of Dermatology, University of Düsseldorf. He is currently Associate Professor at the Department of Dermatology, Environmental Medicine and Health Theory, University of Osnabrück, Germany, member of the European Society for Dermatological Research and coeditor of the Journal of Translational Research. His research interests are milk signaling and milk microRNA signal transduction in diseases of civilization such as acne and prostate cancer.

26. Human-enzyme mediated, systemic depletion of amino acids for cancer prevention and treatment

John DiGiovanni

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Significant differences exist between the metabolism of normal and malignant cells. We have recently evaluated two potential therapeutic enzymes that degrade critical amino acids required for tumor growth. These engineered human enzymes, one that degrades cystine/cysteine [Cyst(e)inase] and one that degrades methionine (Methioninase), are currently under investigation for anticancer activity with promising preclinical results for prostate cancer as well as several other cancers. Depletion of extracellular cystine/cysteine leads to depletion of intracellular cysteine, decreased levels of intracellular glutathione (GSH) and increases in intracellular ROS leading to activation of cellular signaling pathways and cancer cell death. Depletion of extracellular methionine leads to reductions in intracellular *L*-methionine, *S*-adenosylmethionine and polyamines as well as reduced levels of cysteine and GSH and cancer cell death. Both enzymes given i.p. significantly reduced serum levels of their respective amino acid targets and significantly inhibited tumor growth *in vivo*. These and other studies on the mechanisms associated with their potential anticancer activity will be presented. In addition, studies are underway to identify agents that would synergize with one or both of these enzymes for enhanced therapeutic efficacy. Promising results have been obtained through targeting parallel antioxidant defense pathways and mitochondrial metabolism and these results will also be presented. Research supported by NIH NCI grant CA189623.

Biography

John DiGiovanni received his BS degree in Pharmacy and his PhD degree in Pharmacology from the University of Washington, Seattle, Washington. He did his postdoctoral work at the McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI in carcinogenesis and cancer biology. Dr. DiGiovanni joined the University of Texas at Austin (UT Austin) in January of 2010 and is Professor in the Division of Pharmacology and Toxicology, College of Pharmacy. He holds the Coulter R. Sublett Endowed Chair in Pharmacy. He also has adjunct appointments in the Department of Nutritional Sciences (College of Natural Sciences) and the Department of Pediatrics (Dell Medical School). In addition, Dr. DiGiovanni is Director of the Center for Molecular Carcinogenesis and Toxicology (CMCT) at UT Austin and is Associate Director for Basic Research in the LiveSTRONG Cancer Institutes, Dell Medical School at UT Austin. He is the Editor-in-Chief for the journal Molecular Carcinogenesis. Dr. DiGiovanni has published more than 250 research articles in prestigious peer-reviewed journals and more than 50 invited reviews/book chapters.

27. Cancer stem cells and miRNA in osteosarcoma

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In the last years, several studies have identified the presence inside many types of tumors of a subpopulation of stem-like cells, a biologically unique subset of cells, which are probably involved in tumor progression and in the resistance to the antineoplastic treatments. These cells are defined as cancer stem cells (CSCs) and their uncomplete eradication represents a possible explanation as why many therapies seem to have an initial effect, but the patient later relapses. On the other hand, there are a lot of researches about the role of microRNAs (miRNAs) in several cancerous process (i.e., growth, invasion and metastases) and in the maintenance of stemness. On the base of this, we have studied the presence of CSCs inside the primary and aggressive bone malignancy which primarily affecting children and young adults, the osteosarcoma (OS). In this study, we have shown not only the presence of the CSCs population inside different high grade types of OS (i.e., telangiectatic OS, small cell OS and conventional OS), but also the preliminary results about the possibility to find a profile of miRNA expression, as “miRNA codes”, which can be analogue to all the analyzed subtypes of OS. These results could be important to identify a common molecular code among the three highly malignant forms of OS that could be used in the design of new and more effective treatments against the highly aggressive types of OS.

Biography

2011 she has obtained her Master's Degree in Biology at the University of Florence, Florence, Italy. 2016 she has completed her PhD from University of Florence, Florence, Italy, in the field on pathophysiology of bone. 2016-present she is a Post-Doc researcher, at the Department of Surgery and Translational Medicine, University of Florence, Florence, Italy. 2013-present she's carrying on researches on primary bone cancers (i.e., osteosarcoma, Ewing's sarcoma and giant cell tumor). Her interest are focused on cancer stem cells and on miRNAs involved in these bone diseases. She has published several papers in reputed journals and she has attended national and international meetings on this topic.

28. Vitamin E δ -tocotrienol induces apoptosis in prostate cancer cells through activation of the ER stress-autophagy axis and rewiring of mitochondrial metabolism

Fabrizio Fontana, Marina Montagnani Marelli, Michela Raimondi, Roberta Manuela Moretti, Monica Marzagalli, Raffaella Longo, Maurizio Crestani, Patrizia Limonta

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Prostate cancer is androgen dependent in its early stage and responds to the androgen deprivation therapy; however, it often progresses to the castration-resistant prostate cancer (CRPC) stage in which cancer cells become resistant to therapies. Preliminary experiments performed in the authors' laboratory demonstrate that vitamin E-derivative δ -tocotrienol (δ -TT) exerts a proapoptotic activity in CRPC cells while sparing normal prostate epithelial cells. These studies were performed to investigate the molecular mechanisms underlying this activity: (1) in CRPC cells, δ -TT induces endoplasmic reticulum (ER) stress (expression of ER stress markers such as BiP, PERK, p-eIF2 α , IRE1 α , ATF4 and CHOP) and autophagy (expression of autophagy markers such as LC3- II and p62). Using the ER stress inhibitors salubrinal and 4-PBA and the autophagy flux inhibitors 3-methyladenine and chloroquine, we confirmed that the effect of δ -TT is

mediated by both these mechanisms. Moreover, treatment of CRPC cells with salubrinal impairs δ -TT-induced LC3-II expression, demonstrating that this compound triggers the ER stress-autophagy axis; (2) in CRPC cells, the expression levels of the proteins of the respiratory chain complexes are increased compared to non-tumoral cells, supporting a reprogramming of the OXPHOS system. In these cells, δ -TT: decreases the expression levels of the proteins of the respiratory chain complexes, specifically of complex I; decreases the mitochondrial activity as well as the mitochondrial activity/mass ratio; decreases both ATP production and oxygen consumption. These data demonstrate that δ -TT exerts a significant proapoptotic activity in CRPC cells through activation of the ER stress-autophagy axis and rewiring of mitochondrial metabolism.

Biography

Patrizia Limonta has completed her post-graduate degrees in Pharmacology and in Experimental Endocrinology. She is full professor of Applied Biology at the Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Milano, Italy. She has published more than 90 papers in reputed journals and has been serving as an editorial board member of Frontiers in Endocrinology (Cancer Endocrinology Section), Recent Patents in Anticancer Drug Discovery, Oncology Letters, Clinical Cancer Drugs.

29. Targeting metabolic dysfunction in cancer cells by combinatorial treatment with natural compounds

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Natural compounds ingested through the diet can modulate key molecular signaling, inhibit cancer cell proliferation and induce apoptosis. According to the World Health Organization, an increased consumption of natural compounds could prevent about one-third of all cancer deaths. Libraries of natural compounds incorporate phytochemicals characterized by vast molecular diversity and are essential to identify combinations that synergistically contribute to improving treatment outcome. Given the very large number of possible combinations, high-throughput screening methods are paramount to identify the most efficacious compound combinations. In this study, we used high-throughput screening to test the outcome of natural compound combinations on prostate cancer. The *in vitro* screen identified ursolic acid, curcumin and resveratrol as top-hits. These compounds were then administered via the diet to a mouse allograft model of prostate cancer. Combinations of the compounds greatly improved outcome, *in vivo*. This outcome was, at least in part due to the modulation of glutamine metabolism, as determined via untargeted metabolomics and metabolic flux analysis, induced by compound combinations. Moreover, the combinations affected levels of ASCT2 and activation of STAT3, mTORC1 and AMPK. Overall, the high throughput approach is effective for identifying combinations of natural compounds that can synergistically contribute to chemoprevention and therapeutic outcome.

30. The effects of water extract of *Ruta graveolens* on glioblastoma and melanoma cells is selective and depends on REST/NRSF expression

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Heterogeneity and recurrent relapse of tumors, drugs resistance and the lack of selectivity of current chemotherapy calls for new drugs.

Here we report that RGWE is able to kill a number of glioblastoma (GBM) cell lines and cancer stem cells as shown by MTT and trypan blue assay. In particular, we analyzed U87MG, U138, C6 cell lines as well as 3 cancer stem cells originated from patients affected by GBM. RGWE, differently to temozolomide and cisplatin, does not show any toxic activity towards differentiated neurons. Interestingly, differently to GBM cells responsive to RGWE, T98G GBM cell line, unresponsive to RGWE, do not express the transcription factor REST/NRSF, encoding a zinc finger protein that function as a master regulator of neural cell differentiation in normal physiological conditions. NRSF is highly expressed in embryonic stem cells but reduced rapidly in neuron progenitors and maintained at very low levels after differentiation. In contrast, in neuroepithelial tumors, high levels of NRSF are expressed in medulloblastomas, neuroblastomas and multiform glioblastoma and are correlated with the proliferation and severity of these tumors. We observed that down regulation of REST/NRSF in U87MG and C6 cells by means of siRNA, prevents RGWE cytotoxic effects.

We also found that human melanoma cell lines, HNCB and A375 expressing high and low levels of REST/NRSF respectively, respond differently to RGWE. HNCB (REST^{high}) cells die upon RGWE administration whereas A375 (REST^{low}) cells are unresponsive. RGWE stimulation is able to affect also cell migration as shown by wound assay. Upon siRNA-mediated REST down-regulation, HNCB cells show unresponsiveness to RGWE's effects.

Finally, we evaluated the effects of RGWE in the tube formation assay on Matrigel, an angiogenic assay. We found that RGWE is able to impair tubule network formation when administered to human endothelial cells, HUVEC.

31. A natural agent bitter melon exerts strong efficacy against pancreatic cancer in combination with gemcitabine in patient derived xenografts

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Pancreatic cancer (PanC) remains the 4th leading cause of cancer related-deaths in U.S. resulting in a dismal survival rate of < 5%. This generates a critical need for identifying novel non-toxic agents aimed at

effective PanC management with minimal patient distress. Bitter melon (*Momordica charantia*), a dietary agent, is actively being examined for its anti-cancer efficacy against a panel of malignancies. Recent data from our group highlights the anti-PanC potential of bitter melon juice (BMJ); its role in PanC-cancer stem cell (CSC) self-renewal/ kinetics and gemcitabine (GEM) resistance in PanC cells. Combinations for BMJ and GEM *in vitro* also showed synergism in AsPC1 and synergistic effect in BxPC3 PanC cells. As patient-derived xenografts are gaining importance, we next assessed the efficacy of BMJ and GEM alone, or in combination; tumor growth was significantly inhibited in all treated groups with GEM and Combo displaying comparable efficacies followed by BMJ. Notably, tumor regrowth determination with subsequent treatment washout period showed maximum tumor regrowth in GEM group comparable to controls; however, BMJ consistently showed a prolonged inhibitory effect and Combo group remained close to BMJ, suggesting no apparent antagonistic interference. In mechanistic evaluations, in tumor tissues, minimal expression levels of Ki67 and VEGF were observed in BMJ and Combo groups by study end compared to controls and GEM groups. Together, these results show strong BMJ efficacy as a single agent as well as being a perfect combinatorial candidate with GEM for PanC management and treatment. Supported by NCI R01 grant CA195708.

Biography

Rajesh Agarwal completed his Ph.D. from Lucknow University, India in 1981. He is currently Professor and Vice Chairman, Department of Pharmaceutical Sciences, University of Colorado. He has over 360 peer-reviewed publications and several book chapters, has been an invited speaker across the globe, has over three hundred presentations in national and international scientific meetings, is an active member of several National Institutes of Health, USA grant review committee, and is an editorial board member of several lead cancer journals.

32. The mitochondrial-targeted compound SS-31 improves cachexia by increasing muscle energetics

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Cachexia is a complex syndrome that frequently complicates the management of cancer patients^[1]. This syndrome is characterized by progressive wasting of body mass, mainly due to the depletion of both adipose and muscle tissue^[2]. Together with increased protein degradation, also mitochondrial impairment likely contributes to muscle wasting^[3]. Indeed, alterations of mitochondrial morphology and function have been observed in the skeletal muscle of tumor-bearing animals with cachexia^[3].

The aim of the present study was to investigate if selective targeting of mitochondria with SS-31 could exert positive effects on muscle wasting in mice bearing the C26 tumor treated or not with chemotherapy (oxaliplatin+5-fluorouracil; C26 and C26 OXFU, respectively).

Both C26 hosts and C26 OXFU mice showed anorexia, body weight loss and decreased muscle mass and strength. In the C26 hosts, SS-31 partially prevented body wasting, anorexia and muscle mass. In the

skeletal muscle of both C26 and C26 OXFU animals, mitochondrial markers such as PGC-1 α , cytochrome c and SDHA were reduced and these alterations were not corrected by SS-31. In the C26 OXFU mice the reduced expression of mitochondrial markers was also associated with impaired muscle oxidative capacity and ATP content. These latter were improved by SS-31 in the C26 hosts but not in the C26 OXFU mice. Muscle wasting and mitochondrial impairments were associated with reduced protein synthesis in the C26 hosts. Consistently with improved muscle energetics, SS-31 administration partially corrected muscle protein synthesis in C26-bearing mice.

Overall, SS-31 administration could contribute to correct muscle wasting and to improve muscle energetics in tumor-bearing animals, suggesting that targeting mitochondria could be part of a multimodal therapy against cancer cachexia. In order to increase the translational value of the results, further analysis and treatment optimization are needed in order to demonstrate the effectiveness of SS-31 in C26-bearing mice treated with chemotherapy.

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33. Resveratrol counteracts the pro-invasive activity of lysophosphatidic acid in ovarian cancer cells by rescuing autophagy and down-regulating the hedgehog pathway

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Ovarian cancer emerges as a highly aggressive metastatic disease characterized by a high grade of lethality and it remains the most lethal gynecologic cancer with a five-year survival rate of about 30%-40%. Cancer progression is facilitated by pro-invasive factors released by the tumour microenvironment and modulating the epithelial-to-mesenchymal transition (EMT).

Lysophosphatidic acid (LPA), a bioactive phospholipid highly secreted in the ascitic fluid and plasma of ovarian cancer patients, stimulates the growth and promotes the tissue invasion of cancer cells. Resveratrol (RV), a polyphenol found in grapes and in a variety of natural products, is attracting the interest of many researchers thanks to its several anti-cancer properties.

Here we show that LPA induces the EMT through induction of the Hedgehog pathway and concomitant inhibition of autophagy in the cancer cells at the migration front. We found that RV and LPA regulate in an

opposite fashion the expression of BMI-1, a Polycomb transcriptional repressor belonging to the Hedgehog pathway, involved in cancer cell stemness and metastasis. Interestingly, BMI-1 silencing restores autophagy and halts ovarian cancer cell migration. Our data indicate that there is a functional cross-talk between autophagy and Hedgehog/EMT/cell migration processes and we propose BMI-1 as the joining mediator.

In conclusion, our findings indicate that RV elicits its anti-tumor effect through induction of autophagy and down-regulation of BMI-1, and that this process is sufficient to inhibit cancer cell migration and invasion.

34. Effects of HDAC inhibitors on glioblastoma cells

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Background: Glioblastoma Multiforme (GBM), a high-grade glioma (WHO grade IV), is the most aggressive form of brain cancer. Treatment options for GBM involving a combination of surgery, chemotherapy and radiation resulted in a poor survival outcome. Epigenetic mechanisms are increasingly implicated in GBM pathogenesis. Unlike genetic mutations, epigenetic changes are reversible and can be targeted by drugs. We evaluated whether different histone deacetylase Inhibitors (HDACis) are able to affect migration, invasion and vasculogenic mimicry in GBM cells.

Methods: We tested Varinostat (SAHA) and Trichostatin A (TSA) as inhibitors of class I and II HDACs, Entinostat (MS275) as selective inhibitor of class I HDACs (specifically of HDAC 1 and 3) and MC1568 as selective HDAC class II inhibitor. Tube Assay was used to evaluate the ability of HDAC is to interfere with vessels formation by human GBM U87MG and rat glioma C6 cell lines and by cancer stem cells (CSCs) isolated from human GBMs. U87MG directional cell migration and cell invasion have been assessed by Boyden chamber assay and using xCELLigence Real-Time Cell Analyzer. Trypan Blue exclusion test and MTT assay were employed to assess cell viability and cell proliferation.

Results: Our data show that sublethal doses of HDACis significantly decrease U87MG directional cell migration and that MS275 HDACi is able to impair U87MG matrigel invasion. Finally, we observed that HDACis inhibit significantly vasculogenic mimicry in U87MG, C6 and CSCs cells.

Conclusion: GBM depends on vascular networks to supply blood, oxygen, and nutrients. Tumor blood vessels can either be formed from pre-existing blood vessels (neo-angiogenesis) or from tumor cells (vasculogenic mimicry) due to a process of epithelial-mesenchymal transition; vascular mimicry provides a mechanism whereby GBM could escape anti-angiogenic therapies. Our results suggest that HDACis may be promising candidates for blocking vascular mimicry.

35. Resveratrol and Halofuginone Counteract TGF- β -Induced activation and proliferation of stromal fibroblasts by Up-regulating Autophagy

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Cancer can be viewed as a wound that never heals. In this respect, keloids can be considered as the “benign” counterparts of cutaneous tumors. Keloids are pathological scars derived from an altered skin wound healing process and are characterized by hyper-activation and abnormal proliferation of stromal fibroblasts. Among the several pro-inflammatory cytokines involved in the pathogenesis of keloids, transforming growth factor- β (TGF- β) plays a crucial role in myofibroblast proliferation and differentiation. Resveratrol (RV), a naturally polyphenolic compound, is a strong autophagy inducer and inhibits oxidation, inflammation and collagen I deposition. Halofuginone (HF) is a synthetic halogenated derivate of febrifugine, well known for its anti-fibrotic therapeutic potential.

In the present work, we focused our attention on the role of autophagy in keloid formation and progression. We found that RV and HF counteract TGF- β -induced migration of keloid fibroblasts and, in parallel, an increase of autophagic flux is detected, suggesting autophagy as a putative mechanism responsible for slowing down cell motility. Moreover, HF reduces the number of Ki67+ cells, decreasing the proliferative capacity of keloid fibroblasts. Both RV and HF attenuate α -SMA and COL1 synthesis and limit the contraction capability of myofibroblasts within the collagen matrix, even in co-treatment with TGF- β .

36. The possible mechanism of the antioxidants on the antitumor effect of bortezomib in melanoma and myeloma cell lines

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Bortezomib is a proteasome inhibitor chemotherapeutic agent used to treat multiple myeloma; recently studies offer it as a promising drug to treat melanoma. Bortezomib-induced peripheral neuropathy (BIPN) is a dose-limiting side-effect, that can be treated with antioxidants, e.g., alpha-lipoic acid and thiamine.

The objective of our experiments was: to verify the cytotoxicity of bortezomib; to test and compare the influence of the antioxidants on the antitumor effect of bortezomib in melanoma and myeloma.

The cells were U266 myeloma, WM35 primary and A2058 metastatic melanoma. The concentrations were: (1) bortezomib: 20, 100 and 300 ng/mL; (2) alpha-lipoic acid: 10 and 100 μ g/mL (3) thiamine: 150 and 300 nmol/L. Impedimetry (xCELLigence SP) was used to evaluate the cytotoxicity. To study the cell cycle profile, Cell-ClockTM, a redox dye was applied and evaluated by ImageJ. Apoptosis was analyzed by flow cytometry after annexinV assay and phospho-p53 immunostaining.

Bortezomib was cytotoxic and blocked U266 cells in the G1 phase. Alpha-lipoic acid enhanced the cytotoxicity of bortezomib (20 ng/mL) in U266 cells, but counteracted bortezomib in melanoma cells.

Thiamine (300 nmol/L) with bortezomib antagonized the antitumor effect of bortezomib on myeloma cells. In every cell, bortezomib was apoptotic, which effect was decreased in melanoma by the co-treatments.

A good correlation between the apoptotic and the cell cycle arresting effect of bortezomib as well as the antagonism of the antioxidants was found. In conclusion, the combination therapy of bortezomib and a low dose of antioxidants could be offered for the treatment of BIPN in multiple myeloma.

Biography

Angéla Takács received the Doctor of Pharmacy degree from the Semmelweis University in 2017. Currently, she is a PhD student in the Modern Trends in Pharmaceutical Scientific Research Program at the Department of Genetics Cell- and Immunobiology, Semmelweis University. She is working on projects in connection with targeted tumor therapy.

37. Profiling of the transcripts and microRNA in ovarian cancer cells subjected to fasting or to the caloric restriction mimetic Resveratrol

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The lack of nutrients and of growth factors has a great impact on the metabolic pathways involved in cancer cell proliferation. In this condition, cancer cells activate autophagy as a pro-survival pathway and eventually exit the cell cycle to undergo a dormant state. Presently, the potential benefit of nutrient starvation in the prevention and treatment of cancer is under clinical consideration. Resveratrol (RV), a dietary polyphenol acting as a protein (caloric) restriction mimetic, could substitute for amino acid starvation. Here we report on the changes of the global transcriptome in ovarian cancer cells subjected to amino acid starvation or to the protein-caloric restriction mimetic RV. Based on the Gene Ontology analysis, the mRNAs expressed in the two treatments affected different biological functions. The number of transcripts positively impinging on the autophagy pathway was higher in RV-treated than in starved cancer cells. Our data support the view that RV treatment can be more effective than and can substitute for nutrient starvation to induce a dormant-like state in cancer cells.

38. Resveratrol abrogates IL-6 - induced ovarian cancer cell motility by limiting glucose availability and up-regulating autophagy

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In this work, we investigated whether glucose uptake plays a role in ovarian cancer cell migration. IL-6, a cytokine released by cancer-associated fibroblasts and cancer cells, promotes ovarian cancer metastasis. We have previously found that Resveratrol (RV), a naturally polyphenolic compound, counteracts IL-6-induced cancer cell migration, through the epigenetic up-regulation of autophagy. Here, we show that IL-6 stimulates glucose-uptake along with cell migration. We found that in the presence of glucose, IL-6-induced mitochondrial oxidative stress with production of anion superoxide and inactivation of ATG4, the cysteine protease that processes LC3 needed for autophagosome formation. RV reduced glucose uptake, prevented the formation of anion superoxide, rescued ATG4 function and increased the level of autophagosome formation. RV-mediated up-regulation of autophagy was more pronounced at the migration front, leading to a reduced cancer cell motility. These data indicate that IL-6 promotes ovarian cancer cell migration by promoting glucose uptake which is metabolized to produce mitochondrial oxidative stress that eventually inhibits autophagy, and RV may counteract this action by limiting the glucose uptake and the production of anion superoxide.

39. Warty Carcinoma of the uterine cervix - do we know it

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Introduction: Warty carcinoma of the uterine cervix is a rare subtype of squamous cell carcinoma that usually occurs in women over 48-50 years. Warty carcinoma is not as aggressive as the other subtypes, and has a better prognosis. It is also a slow-growing tumor. The prognosis depends on a set of several factors that include: the size of the tumor and the extent of its invasion, stage of cancer, FIGO grade, involvement of the regional lymph nodes, whether the tumor is occurring for the first time, or is a recurrent tumor, response to treatment.

Aim: The aim of the study was to explore the prognosis of this type of cervical cancer.

Methods: For ten year period (2008-2017) in the Clinic of Gynecologic oncology at the UMHAT - Pleven, Bulgaria were operated 714 cases with Carcinoma of the uterine cervix and 14 of which were histologically diagnosed as a Warty carcinoma. Radical abdominal hysterectomy was performed on all patients with pelvic lymph node dissection with postoperative telegamma therapy. Patients were investigated by retro- and prospective analysis for overall and recurrence-free survival rate.

Results: Warty carcinoma was 1.94% of all cervical carcinomas, treated in our clinic. The mean age of the patients was 48 years (29 to 72 years) and 57% of them were peri- and postmenopausal and 43% were in women under the age of 45. According to the FIGO staging systems for cervical cancer, patients were staged as follows: in stage IB1 - 43% and in stage IB2 - 57%. In 29% the tumor size was under 2 cm, in 14%

the size was 2-4 cm and in 57% the size of the local tumor is above 4 cm. Never the less the high percentage of locally advanced process (patients with tumor above 4 cm), only in one case there was local spreading of the tumor to the uterine cavity and in one case there lymph node metastasis. Lymphovascular space invasion was not seen in any patient. Our patients are free of tumor recurrence or occurrence of symptoms for a period of 9.4 years (112 months), to 4 months after the surgical procedure.

Conclusion: Warty carcinoma of the cervix has good clinical prognosis and it is possible to rethink for not so aggressive surgical treatment and even for fertility sparing surgery when it is necessary.

40. Peptide based targeted drug delivery of mono- and dual-drug conjugates to prostate cancer cells

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Targeted drug delivery (TDD) systems are becoming important in cancer therapy for the specific delivery of cytotoxic drugs into the target cells. This strategy bypasses the disadvantages of traditional chemotherapy such as non-specific toxicity and development of drug resistance and may also reduce drug doses. TDD systems commonly consist of a targeting moiety (such as an antibody, protein or peptide) chemically conjugated to a linker which has been coupled to a cytotoxic compound. In order for targeted nanoparticles to be effective therapeutics, a number of design issues need to be considered. This presentation will discuss selection of appropriate peptides derived from phage display libraries as targeting moieties, focusing on our results in the development of peptide-drug-conjugates (PDCs) for prostate cancer therapy. Based on our previous studies in TDD systems, we defined a series of phage selection criteria which enabled the choice of candidate peptides for testing *in vitro* and *in vivo*. Using the selection criteria, two lead peptides, P10 and P12 were used to build mono- and dual-drug PDCs which were shown to be target specific and effective against a variety of prostate cancer cells. These results demonstrate several important principles in the tailored design and selection of both the peptides and cytotoxic drugs suitable for incorporation into effective PDCs. We are now using these principles to design PDCs for other cancers.

41. Pregnancy-associated breast cancer is it always possible to keep the pregnancy

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Introduction: Pregnancy-associated breast cancer can occur in any moment during gestation, lactation, or even within 1 year after delivery. The incidence of that cancer is 1:3000 to 1:10000 pregnancies. The diagnosing process is often hard due to the pregnancy-related physiological changes of the breast.

Case presentation: We present the case of a 41-year old woman, diagnosed with breast cancer during pregnancy, in 20th week of gestation; the cancer was found due to presence of multiple pathological fractures of the spinal cord.

Discussion: Despite the fact that treating breast cancer during pregnancy is done following the same principals as the management of breast cancer in non-pregnant patients taking into account that the aim of the treatment is to provide the best curative treatment with minimal or no harm to the fetus maximizing the gestational period and ensure safe delivery of the fetus, is not always easy to keep the pregnancy.

Conclusion: In conclusion, with the increasing birth age for women, PABC will be a more common disease. We have to focus our thinking on it. Elective termination of pregnancy has not proven to improve the outcome in breast cancer. Therefore, it is not routinely recommended. The decision to terminate has to be individualized based on the oncologic situation and maternal concerns.

Biography

Vasileva PP has completed her graduation from Medical University-Pleven, Bulgaria in 2011. Since 2012 he has been working in Clinic of Obstetrics and gynecology, University Hospital “Dr.Georgi Stranski”-Pleven, Bulgaria. She has several publications in various magazines on different topics. She has published more than 10 papers in reputed journals.

42. Mild exercise training partially prevents muscle wasting induced by tumor and chemotherapy

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Cachexia is a frequent feature in cancer patients. Its occurrence is associated with reduced tolerance to anti-cancer treatments, impaired survival and bad quality of life. This syndrome is mainly characterized by loss of body weight, depletion of muscle mass and metabolic alterations^[1]. The loss of muscle mass and function is also associated with autophagy deregulation and mitochondrial impairments^[1] that could be exacerbated by anti-cancer treatments^[2]. In this regard, exercise could represent a promising non-pharmacological approach to correct muscle wasting in cancer cachexia^[3]. The main goal of the present study was to investigate if mild exercise could improve muscle wasting in mice bearing the C26 tumor, in the presence or in the absence of chemotherapy treatment [oxaliplatin+5-fluorouracil (OXFU)]. Although chemotherapy increased the lifespan of C26-bearing mice, it resulted in muscle wasting more severe than that observed in the untreated C26 hosts. Such wasting pattern was associated with increased levels of molecules accepted as markers of autophagy and mitophagy, while the expression of markers of mitochondrial content and dynamics decreased. Consistently, C26 OXFU mice showed impaired muscle oxidative metabolism (SDH total activity) and reduced protein synthesis. Moderate exercise in OXFU-treated C26-bearing mice exerted beneficial effects on the loss of both muscle mass and function, partially restoring the expression of markers of autophagy and mitophagy, increasing mitochondrial content and dynamics and improving muscle oxidative capacity.

On the whole, chemotherapy administration could contribute to muscle wasting in cancer patients, enhancing the alterations of metabolism due to tumor growth. In this regard, moderate exercise may support cancer patients under chemotherapy regimen preserving muscle mass and function.

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43. Mouse mammary carcinomas secrete extracellular vesicles - *in vitro* and *in vivo* study

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Many types of cells including cancer cells secrete extracellular vesicles (EVs). EVs are small vesicles released by donor cells that can be taken up by recipient cells. EVs contain receptor proteins, proteolytic enzymes, miRNAs, and mRNAs which are transferred into the target cells, and then affect various cell functions. EVs are categorized depending upon where in the cells they originate: vesicles that are derived from multivesicular bodies are referred to as exosomes (50-100 nm in diameter) and those from the plasma membranes as microvesicles (500-1000 nm in diameter). There are numerous *in vitro* studies, however, little is known about EVs *in vivo*. Here we show the chemical and ultrastructural features of EVs derived from cultured mouse mammary carcinoma cells and their tumors in mice formed upon inoculation. BJMC338 tumors show a low metastatic propensity, while BJMC3879 tumors show a high metastatic propensity, especially to lymph nodes and lungs. Both EVs contain premature vascular endothelial cell growth factor C, and inoculated tumors demonstrated that EVs were in their lumen and surrounding connective tissue. Many exosomes were accumulated in the cytoplasm, Golgi complex and r-ER of tumor cells. These observations suggest that mouse mammary carcinoma cells in tumors secrete EVs.

44. Epithelial splicing regulatory protein 1 expression is an unfavorable prognostic factor in prostate cancer patients

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The objective of this study was to evaluate the role of epithelial splicing regulatory protein 1 (ESRP1) expression in predicting prognosis and disease progression in a large cohort of PC patients with long-

term follow-up. The ESRP1 participates in the epithelial-mesenchymal transition, a necessary prelude to disease progression in prostate cancer (PC). The prognostic role of ESRP1 has been studied in various human primary tumor tissues and has been described to be tumor-specific. However, little is known about the prognostic value of ESRP1 in PC. A preliminary investigation into the clinical significance of ESRP1 was conducted using The Cancer Genome Atlas PC dataset. Tissue microarrays of radical prostatectomy specimens from 514 PC patients at the National Cancer Center of Korea were immunohistochemically stained for ESRP1. PC samples were grouped into high and low expression of ESRP1 based on the immunohistochemistry results. The median follow-up period was 91.2 months. The immunohistochemistry was interpreted semi-quantitatively using *H*-score, defined by the intensity (0, 1, 2 and 3), and the percentage of expressed areas (0-100%). The prognostic significance of ESRP1 expression was analyzed using the Cox proportional-hazards model ($P < 0.05$). After adjusting for clinicopathological variables, high expression of ESRP1 was significantly associated with worse biochemical recurrence-free survival [hazard ratio (HR): 1.37; 95% confidence interval (CI): 1.02-1.83; $P = 0.037$] and shorter cancer-specific survival (HR: 3.43; 95% CI: 1.12-10.54; $P = 0.031$). PC patients with high expression of ESRP1 appear to have increased risk of biochemical recurrence and cancer-specific death. The expression of ESRP1 in patients with poor survival outcomes indicates that there may be an opportunity to predict response to androgen deprivation therapy based on ESRP1 expression.

Biography

Dr. Kang Hyun Lee has completed his MD and PhD from Seoul National University, College of Medicine, Seoul, Korea. He is a Tenure Chief Scientist in the Division of Precision Medicine and Specialist in Center for Prostate cancer, National Cancer Center of Korea. He is also the Emeritus Professor of GCSP and he has published more than 130 papers in academic journals and had been serving as 6th President of National Cancer Center of Korea.

45. Gene-specific methylation as a molecular biomarker in gastric cancer

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Despite the fact that overall rates of gastric cancer (GC) continue to decline worldwide, the majority of patients are still diagnosed with advanced disease in Western countries. New strategies for early diagnosis and new therapeutic methods in GC need to be explored. Epigenetic control using inhibitors of DNA methylation, such as 5-aza-2'-deoxycytidine (also known as Decitabine), may offer new possibilities in GC therapy. Our research group previously identified 86 differentially expressed genes (DEGs) by microarray analysis comparing GC cell lines treated with Decitabine and non-treated cells. Among the upregulated

DEGs identified by this methodology, *NRN1* was selected for further analyzes. This study aimed to evaluate, compare and correlate *NRN1* mRNA and methylation levels of GC and adjacent non tumor samples from patients with primary gastric adenocarcinoma. The mRNA level was assessed by quantitative reverse transcription PCR. DNA methylation analysis was assessed by Ion Torrent PGM sequencer. A total of 12 CpG sites were evaluated in *NRN1* promoter region. GC samples showed a significant increased DNA methylation at five specific CpG sites of *NRN1* promoter region compared with adjacent non tumor samples ($P < 0.05$), besides no difference in *NRN1* mRNA levels between these groups. At this time, no significant correlation between mRNA and methylation levels, as well as, significant clinicopathological associations were observed. Although different mechanisms may be involved in gene regulation, *NRN1* methylation seems to play an important role in gastric carcinogenesis. This study is still in progress and new samples and other gene regions will be incorporated in our analyzes.

Biography

Fernanda Wisnieski has completed her PhD at Federal University of São Paulo (UNIFESP), São Paulo, Brazil. Currently she is a postdoctoral researcher at Genetics Division of UNIFESP. She has experience in the area of Human and Medical Genetics, working mainly on genetics and epigenetics aspects of gastric cancer clinical samples and cell lines models. Since the end of her doctorate degree she has published 15 papers in the area.

46. A semi-comprehensive analysis of gene amplification in breast cancers using multiplex ligation-dependent probe amplification and fluorescence in situ hybridization

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Gene amplification is a common event in breast cancer. The aim of the present study was to determine the amplification status of 22 genes that are reportedly frequently amplified in breast cancers. A total of 320 formalin-fixed and paraffin-embedded invasive ductal cancer tissues were screened by multiple ligation-dependent probe amplification, and 885 genes with “gain” or “amplified” status were further examined for the respective gene amplification by fluorescence *in situ* hybridization (FISH). In the FISH analysis, 116 of 320 tumors (36%) displayed gene amplification of at least 1 of the 22 genes. The frequencies of gene amplification were as follows: *ESR1* (5 cases, 1.5%), *EGFR* (one case, 0.3%), *ZNF703* (25 cases, 7.8%), *FGFR1* (24 cases, 7.5%), *ADAM9* (17 cases, 5.3%), *IKBKB* (10 cases, 3.1%), *PRDM1* (5 cases, 1.5%), *MTDH* (15 cases, 4.7%), *MYC* (29 cases, 9.0%), *CCND1* (39 cases, 12%), *C11ORF30* (19 cases, 5.9%), *CDH1* (1 case, 0.3%), *CPD* (9 cases, 2.8%), *MED1* (25 cases, 7.8%), *ERBB2* (35 cases, 11%), *CDC6* (14 cases, 4.3%), *TOP2A* (12 cases, 3.8%), *MAPT* (1 case, 0.3%), *PPM1D* (10 cases, 3%), *BIRC5* (6 cases, 1.9%), *CCNE1* (3 cases, 0.9%), and *AURKA* (1 case, 0.3%). In addition to the frequent co-amplifications of genes closely located on the same chromosome region, such as 8p11, 8q24, 11q13, or 17q12-21, co-amplification of genes located in different chromosomes was also found. The co-amplification of *ZNF703/FGFR1* (8p11) and *CCND1* (11q13) in single cells was found in 11 tumors, occurring in the same amplicons in 5. The co-amplification of *ZNF703/FGFR1* and *ERBB2* (17q12-21) in single tumor nuclei but different amplicons was observed in 11 cases, and that of *CCND1* and *ERBB2* was noted in 5 cases. The co-amplification of *MYC* (8q24) with *CCND1* or *ERBB2* in single nuclei was found in three and two tumors, respectively. The amplifications of *ESR1* and *ERBB2* as well as *EGFR* and *ERBB2* were found in one tumor each, although in different cancer cells. Amplified genes are attractive potential targets for therapy, so a precise and feasible analysis of the gene amplification status is clinically valuable.

47. Spinless metal isotopes are able to suppress DNA Polymerase Beta in WERI/Y79 retinoblastoma cells: beyond the cytostatic effect

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Nuclear spin possessing isotopes (^{25}Mg , ^{43}Ca , ^{67}Zn) promote the marked magnetic isotope effects (MIE) on DNA Polymerase Beta (DNAPolB) in *ex vivo* survived human retinoblastoma (RB) cells. In *N*-ethyl-melamide chase experiment, the RB in situ catalytic activity of DNAPolB has been selectively estimated as a function of MIE. A resulted enzyme function breakdown leads to a sharp decrease of cancer cell viability. This study a paramagnetic chemotherapy path is all about.

Review

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Targeting autophagy with small molecules for cancer therapy

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Abstract

Autophagy is a conserved lysosomal-dependent catabolic process that maintains the cellular homeostasis by recycling misfolded proteins and damaged organelles. It involves a series of ordered events (initiation, nucleation, elongation, lysosomal fusion and degradation) that are tightly regulated/controlled by diverse cell signals and stress. It is like a double-edged sword that can play either a protective or destructive role in cancer, by pro-survival or apoptotic cues. Recently, modulating autophagy by pharmacological agents has become an attractive strategy to treat cancer. Currently, a number of small molecules that inhibit autophagy initiation (e.g., ULK kinase inhibitors), nucleation (e.g., Vps34 inhibitors), elongation (e.g., ATG4 inhibitors) and lysosome fusion (e.g., chloroquine, hydroxyl chloroquine, *etc.*) are reported in pre-clinical and clinical study. Also a number of small molecules reported to induce autophagy by targeting mammalian target of rapamycin (e.g., rapamycin analogs) or adenosine 5'-monophosphate-activated protein kinase (e.g., sulforaphane). The study results suggest that many potential "druggable" targets exist in the autophagy pathway that could be harnessed for developing new cancer therapeutics. In this review, we discuss the reported autophagy modulators (inhibitors and inducers), their molecular mode of action and their applications in cancer therapy.

Keywords: Autophagy, cancer, ATG, ULK inhibitor, Vps34 inhibitor, mammalian target of rapamycin inhibitors, lysosome fusion inhibitors

INTRODUCTION

Autophagy is a natural cellular process that occurs for the maintenance of cellular homeostasis. During autophagy, catabolic degradation occurs to recycle unnecessary, dysfunctional cellular components,



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Table 1. Characteristics of three types of autophagy

Sl. No.	Type	Mechanism	Biological effects	Markers
1	Macroautophagy (commonly referred as autophagy)	Autophagosome formation	Cell killing and survival	Beclin1, Atg5, Atg12, LC3-I and LC3-II
2	Microautophagy	Vacuole invagination process	Cell killing and survival	Hsc70 multi-vesicular bodies and multi-vesicular lysosomes
3	Chaperon mediated autophagy	Receptor mediated process	Selective cell killing, T-cell activation	LAMP-2A, Hsc70

damaged organelles and protein aggregates^[1]. It also removes intrusive pathogens to protect our body from various infectious diseases and other disorders including cancer^[2,3]. The autophagic process occurs when the cells are in stress conditions such as starvation or hypoxia. Based on these cues, the cytoplasmic contents (cargo) get sequestered within the autophagosome and then fuse with lysosome for cargo degradation. Three forms of autophagy have been reported: (1) chaperon mediated autophagy; (2) microautophagy; and (3) macroautophagy^[4] [Table 1].

The type and mode of action differs from one another based on how and what target cargoes are subjected to lysosomal degradation. In chaperone mediated autophagy, the substrate with the targeting motif KFERQ is recognized by the chaperone Hsc70, and moves the individual protein substrate to lysosome degradation^[5]. While, microautophagy is responsible for basal degradation of the cytoplasmic content by the direct invagination into lysosome^[6]. On the other hand, macroautophagy, an ubiquitous pathway in eukaryotic cells, starts with the formation of double membrane structures called autophagosomes in which cargo sequestration occurs^[3]. The early autophagosome is formed from components derived from endoplasmic reticulum, which acquires V-ATPase and LAMP to become late autophagosome^[7,8]. Finally, the late autophagosome fuses with the pre-existing lysosomes to become the autolysosome^[9]. The autolysosome contains unrecognizable cytoplasmic materials as they are in degradation and recycling process^[10]. The multi-step process of macroautophagy (here onwards referred to as autophagy; Figure 1) is regulated by autophagy related genes, which were originally found in autophagy defective yeast mutants.

Autophagy is initiated through diverse signaling pathways in response to major stress response and plays a pro-survival role by nutrient recycling. The stress factors include low cellular energy levels, amino acid deprivation, growth factor withdrawal, hypoxia conditions, ER stress, oxidative stress, organelle damage and infection^[11]. Over a period of stress, the cells employ autophagy process either to move contents or to degrade harmful components such as damaged mitochondria or invading pathogens through the process of lysosomal degradation^[12]. Aberration in autophagy process has been implicated in a wide range of diseases including neurodegenerative disorders that involve the accumulation of pathogenic proteins, inflammatory disorders and cancer^[4,13]. The following sections discuss various signaling pathways involved in the autophagy process, their role in cancer and other diseases; also small molecule inhibitors that target the autophagy process that are useful for cancer therapy are detailed along with the mode of interaction with their targets, if known.

AUTOPHAGY PROCESS

Initiation step

Autophagy is a multi-step process involving, initiation, nucleation, elongation/expansion and closure steps^[14]. The initiation of phagophore formation is governed by multi-protein complex known as ULK complex (Unc-51-like kinase 1, FIP200, ATG101 and ATG13), that integrates upstream nutrient and energy status and thereby initiates the process of autophagy [Figure 2]. Each protein of the ULK complex has a unique role; ULK1, a serine-threonine protein kinase, plays a key role in the scaffold formation of ULK1-ATG13-FIP200 complex^[15]; ATG13, an adaptor protein mediates interaction between ULK1 and FIP200, and directly binds to LC3 as well. Another protein, ATG101 which is a subunit of ULK complex recruits downstream Atg proteins that are essential for autophagy^[16,17].

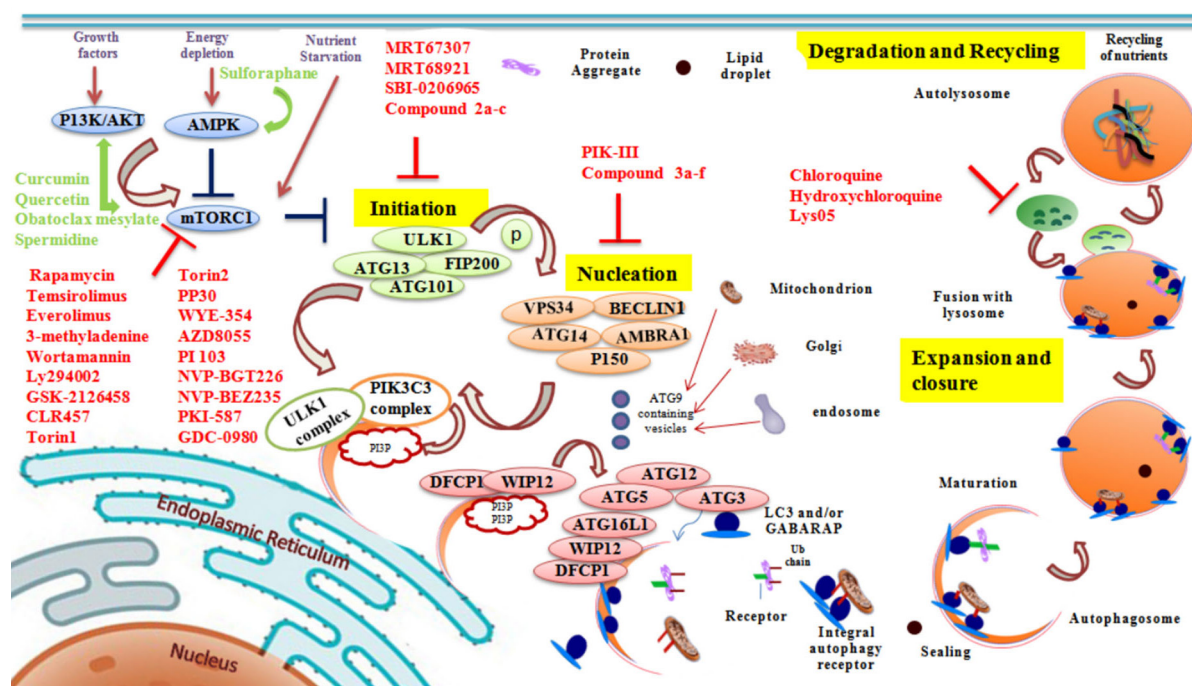


Figure 1. Cartoon representation of pathways involved in autophagy and the chemical inhibitors/inducers of the process. Autophagy involves 4 steps: (1) initiation; (2) nucleation; (3) expansion and closure; and (4) degradation and recycling. Small molecule modulators that affect different steps of autophagy are shown in red color (inhibitors) and green color (inducers)

Initiation step of autophagy process in normal nutrient conditions (e.g., sensed by the levels of growth factors, amino acids and glucose) is regulated by a negative autophagy regulator mammalian target of rapamycin (mTOR) that phosphorylates two subunits, ULK1 and Atg13 of ULK complex. The complex of mTOR that consists of mTORC1, Raptor and MLST8 as subunits directly binds ULK1 protein and thereby leads to ULK complex dissociation^[18]. Upon nutrient starvation, mTORC1 dissociates from ULK complex and hence ULK1 gets activated and phosphorylates ATG13, ATG101 and FIP200 in order to initiate the phagophore formation [Figure 3]. Investigations suggest that phosphorylation of ULK1 plays a vital role in the regulation of autophagy initiation process and at least 30 phosphorylation sites have been reported. However, the molecular basis and the molecules involved in ULK1 phosphorylation are yet to be completely unraveled^[18].

In contrast to mTORC1, adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) indirectly activates ULK complex by phosphorylating TSC2 and raptor. Recently, AMPK has also been shown to directly interact with and phosphorylate ULK1 in a nutrient-dependent manner. Several phosphorylation sites, including Ser555, Ser637 and Ser757 were reported. AMPK phosphorylation site Ser555 is thought to recruit phospho-binding protein to the ULK complex^[19]. During glucose starvation, AMPK targeting phosphorylation sites on ULK1 are triggered and contributes to ULK1 activation [Figure 2]. AKT is a serine/threonine kinase that acts as a sensor of growth factor levels in the cell and is activated in nutrient rich condition. Upon activation, AKT phosphorylates Ser9 of GSK3, which acts as inhibitory cue for GSK3. Dephosphorylation of Ser9 activates GSK3 and eventually the activated GSK3 phosphorylates TIP60 at Ser86^[20]. Further, TIP60 acetylates ULK1 and thereby increases the kinase activity of ULK1 [Figure 2]. Finally, accumulation of activated ULK complex initiates the phagophore formation^[20].

Nucleation step

Phagophore formation starts with the nucleation step of autophagy process, where protein subunits including Vps34, Beclin1, Ambra1, ATG14 and p150 coordinate with each other and form a nucleation complex^[21].

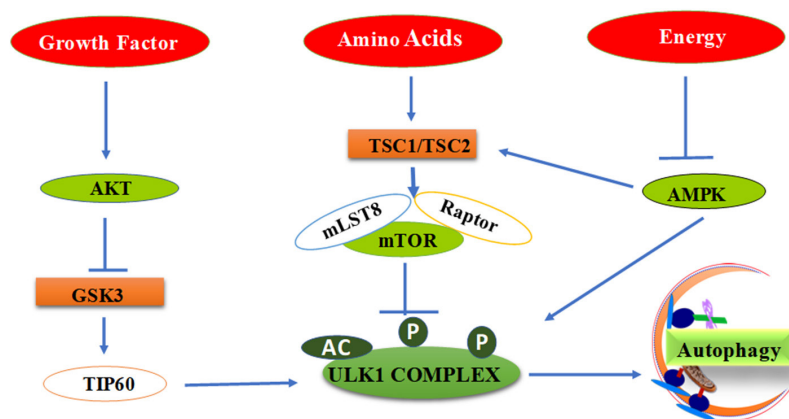


Figure 2. Diagrammatic representation of various signals leading to the formation of autophagy initiation - ULK complex

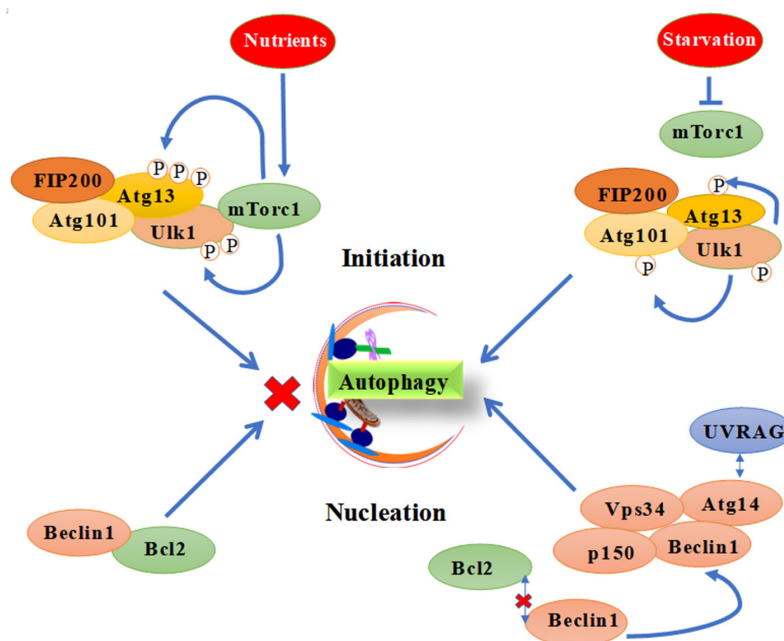


Figure 3. Schematic representation of molecules involved in the (1) initiation; and (2) nucleation process leading to the autophagosome formation during autophagy

During nucleation, vesicular sorting protein 34 and its enzymatic product phosphatidylinositol-3-phosphate, an essential component play a vital role in recruiting other autophagy protein subunits such as WIPI-1, DFCP1, ATG5 and LC3. The effectors WIPI-1, WIPI-2 and DFCP1, binds to PI3P via WD repeats and FYVE domains, respectively^[21,22]. In nucleation process, Vps34 associates with the phagophore membrane via p150 [Figure 3] that is anchored by myristic acid. Beclin-1 is the third important component for phospholipid kinase activity. Activity of Beclin-1 is affected by many different binding partners. Beclin1 dissociates from the anti-apoptotic factor Bcl2, which leads to the activation of Vps34. The association of beclin1 with Vps34 is stabilized by the other two components - UV radiation-associated resistance gene and beclin-1-associated autophagy related key regulator^[23].

Elongation step

In elongation step, the maturation of the autophagosome takes place with the help of two ubiquitin like conjugation system [Figure 4]. In the early step of autophagosome maturation, Atg12 is activated by Atg7

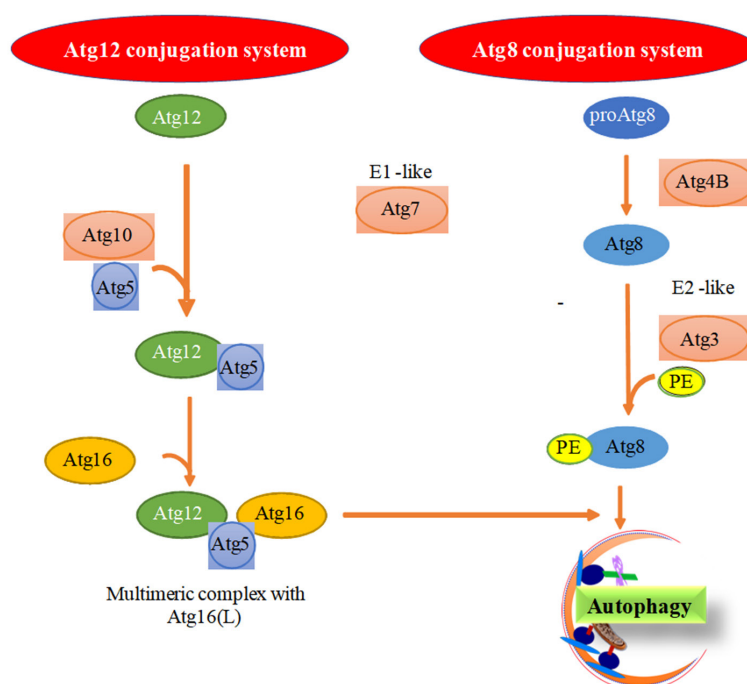


Figure 4. Two ubiquitin like conjugation system is involved in the autophagophore elongation/maturation process

and transferred to Atg10 thereby forming a covalent linkage with Atg5^[24]. This Atg12-Atg5 conjugate complexes with Atg16L and forms autophagy elongation complex. Carboxy terminal Gly residue of Atg12 is involved in the formation of thioester linkage with the active site Cys residue of Atg5 and Atg10, and also involved in amide linkage with Lys residue of Atg5^[25]. Elongation complex forms a dimer which provides a site for LC3 lipidation, a process required for association of LC3 with autophagosome membrane^[26]. Although, Atg12 does not possess similarity with ubiquitin, it forms an ubiquitin-like fold and is involved in autophagy elongation step. For the autophagosome maturation, LC3 lipidation is very essential and acts as a second ubiquitin-like conjugation. This conjugation occurs in a series of reactions including proLC3-I cleavage by Atg4B, LC3-I activation by Atg7, transfer to Atg3 and finally conjugation with PE. Like Atg12, carboxy terminal Gly of LC3 is involved in thioester linkage with Cys residues of Atg7 and Atg3, and an amide linkage with PE^[27]. These reactions are similar in LC3 homologues GABARAP, GATE-16 and mAtg8L. Completion of these maturation steps leads to autophagosome-lysosome fusion^[28] and then degradation of the cargo.

Fusion and degradation step

Degradation and recycling of cellular components is a central function of all living cells to meet cell demands. In final stage of autophagy, matured autophagosome fuses with multivesicular endosomes and lysosomes. Degradation of cytosolic components is not a random process and thus several proteins such as Vps34/SKD1 and Rab11 involvement is necessary to accomplish autophagosome-lysosome fusion process^[29]. A recent study has reported that components of HOPS complex (homotypic fusion and protein sorting) plays a major role in the formation of autophagosome-endosome fusion. Moreover, dysfunction or absence of subunits of the ESCRT III complex and proteins required for biogenesis of endosomes severely affects the fusion process^[30]. The fusion of inner membrane of autophagosome delivers the cytosolic proteins to lysosomes, where hydrolysis takes place to complete the degradation of the cargo.

ROLE OF AUTOPHAGY IN VARIOUS DISEASES

Dysregulation in the autophagy process results in various diseases. Defects or deregulation is especially important in cancer, ageing related disease, neurodegenerative diseases and lysosomal storage diseases^[2].

In aging process, the functional role of autophagy is expulsion of aggregated protein which increases the lifespan; in the case of defective autophagy process, the formation of vacuoles and improper fusion of vacuoles with the lysosomes results in impaired protein flux^[31]. In infectious disease, the functional role of autophagy is to remove the bacterial and viral pathogens through sequestration in autophagic vacuoles and then degradation. This provides immunity against pathogens^[32]; while a defective autophagy process provides a conducive environment for pathogens. In lysosomal storage disorders, the removal of lysosomal stock such as fatty acids, cholesterol are defective and an increased number of autophagosomes and reduced organelle turnover occur^[33]. In the case of neurodegeneration disorders, the neuronal protein aggregates are removed by autophagic process; while, in the defective process the protein aggregates accumulate in neurons leading to neurodegeneration^[34].

Role of autophagy in cancer

Autophagy is a complex process that responds to a variety of stressful environments such as nutrient deprivation, abnormal protein accumulation and damaged organelle, and thereby maintains the cellular homeostasis^[35]. Autophagy plays a cyto-protective role by clearing the damaged organelles, misfolded proteins and ROS, thus confining the genomic instability and aberrant mutations that ultimately leads to cancer. Consequently, autophagy machinery can be defined as cell survival mechanism in normal and as death mechanism in cancer cells.

However, deregulation of autophagy has been reported in a variety of diseases including cancer. Many reports have shown that autophagy plays dual role in cancer development. In early stage of cancer, autophagy suppresses/abolishes tumor formation by clearing the damaged proteins and organelles, and thereby induces cell death; whereas in advanced cancers, the stress mediated properties of autophagy has been hijacked by tumor cells to meet their increased metabolic requirements that are indispensable for tumor survival and rapid proliferation. Hence, autophagy has been reported as a tumor promoter in advanced cancers. Additionally, the regulation of autophagy through diverse signaling mechanisms can contribute upregulation/downregulation of tumor suppressor/oncogenes, and that can lead to inhibition/induction of cancer development^[36,37]. For example, negative regulation of tumor suppressor genes through different signaling mechanisms (i.e., mTOR, AMPK, *etc.*) could induce autophagy and suppression of cancer initiation; whereas, activation of oncogenes could lead to inhibition of autophagy and promotion of cancer development.

It has been widely reported that autophagy modulates cancer growth and development, and this depends on cancer type, stage, and genetic context. A basal level of autophagy is considered as a cancer suppressive mechanism in normal cells. However, abnormal levels of autophagy in stressful conditions (i.e., Hypoxia, ROS, *etc.*) lead to inhibition of break down of damaged organelles and proteins, and subsequent cancer development. Nonetheless, it has been reported that mutation in autophagy related proteins leads to tumor suppression/promotion in a variety of cancers. For example, BECN1 related proteins (e.g., BIF-1 *etc.*) have been found abnormal/absent in gastric and colorectal cancer^[38,39]. Further, mutation in UVRAG protein reported to reduce autophagy, and consequent colorectal cancer development^[40]. On the other hand, an unexpected high basal-level of autophagy has been reported in several types of RAS-activated cancers (e.g., pancreatic cancers) and inhibition of autophagy in these cancers hinders the tumor formation^[41].

In order to identify alterations of different genes that are involved in autophagy signaling pathways, we have analyzed 1,087 cancer patient samples data from Cbioportal database (<http://www.cbioportal.org/>). Through this data analysis, we have noticed that mTOR gene shows high alterations with 12% (altered/profiled ratio = 128/1,087) and PIK3C3 shows 9% (altered/profiled ratio = 97/1,087) alterations. Further we also observed the alterations of other genes including ULK1 (5%), UVRAG (5%), beclin1 (2.7%), ATG4B (4%), ATG16L1 (2.1%), ATG5 (4%) and ATG12 (2.8%). Altogether, these studies explain that cell transformation and deregulation of many signaling pathways are connected directly or indirectly with autophagy modulation. These evidences,

suggest that autophagy has a dual role in cancer and is dependent on biological factors such as the driving oncogene, tumor suppressor involved and tumor type. Hence, autophagy is considered as a double-edged sword, by both protecting from and promoting cancer^[42,43].

Autophagy acts as a tumor suppressor during tumorigenesis

Autophagy is widely documented as a tumor suppressive mechanism, as its deregulation leads to genomic instability, aberrant mutations, tumor formation and metastasis^[44]. Primarily, the role of beclin1 in autophagy has been studied extensively. For example, mice having monoallelic deletion of beclin1 gene induce tumor formation. As is evident, the allelic loss of beclin1 was found in 40%-75% in breast, ovarian, and prostate cancers^[44,45]. It is well documented that Beclin-1 promotes autophagy by binding to Vps34 via its conserved domain that was reported essential for tumor suppression. Recently, phosphorylation of multiple tyrosine residues of beclin1 has also been observed, which leads to a decrease in the activity of beclin1/PI3KC3 complex and thereby the reduction of autophagy^[46].

The reduction of beclin1 protein levels is also reported in many brain cancers. A study conducted to investigate beclin1 mRNA expression in different histotypes of brain tumors reported the expression levels vary based on the type of tumor. After examining mRNA expression in 212 primary brain tumors, the study identified low expression in most high-grade ependymal neoplasms, astrocytic and atypical meningiomas; whereas, high expression in low-grade tumors and medulloblastomas^[47]. Additionally, monoallelic deletion/mutations in UVRAG protein and decreased expression levels of Bif-1 were also reported in colon, gastric, breast, prostate and bladder cancers^[38]. The reported results clearly indicate that autophagy related protein Beclin-1 and its regulators (i.e., UVRAG and Bif-1) mediate tumor suppression.

Further, deregulation of several proteins of PI3K/Akt pathway were also reported to impair autophagy mechanism and can lead to tumorigenesis. For example, phosphatase and tensin homolog protein was reported to inhibit Akt survival pathway, and thereby induces autophagy mechanism. However, a mutation in PTEN gene leads to constitutive activation of Akt and inhibition of autophagy, leading to cancer formation^[44,48]. Furthermore, the accumulations of p62 aggregates were reported to cause several cancers owing to impaired autophagy mechanism. Another study by Kang *et.al.*^[49], identified the frameshift mutations with mononucleotide repeats in ATG genes in gastric and colorectal carcinomas. Further, this study suggested that these mutations are associated with cancer progression by autophagy deregulation. The study investigated the expression of BNIP3 (Bcl-2/adenovirus E1B 19 kDa-interacting protein), a key regulator of mitochondrial autophagy in breast cancer and reported that BNIP3 expression is significantly lost in invasive breast cancers and suggested that breast cancers cells shows high proliferation with low BNIP3 expression. Thus, collectively these evidences suggest that autophagy plays essential role in tumor suppression and conversely its deregulation leads to cancer.

Autophagy acts as a cell survival mechanism in cancer cells

Genome-wide screening studies show that many genes are involved in the regulation of autophagy either through suppression or enhancement of autophagy^[50]. High throughput analyses also contribute to the understanding of autophagy regulation at protein level and in terms of protein-protein interactions. Further, research in yeast and animals suggests that stress-induced autophagy under nutrient-limiting condition promotes cell survival by influencing the bioenergetics of the cell. A study conducted in human prostate cancer cells PC3 and LNCaP, and breast cancer cells MCF7 shows that autophagy acts as a survival mechanism in hypoxic tumor cells. Hypoxia inducible factor1, a positive regulator of autophagy enhances tumor metabolism and metastasis^[51], and thereby limits the radiation and chemotherapy. Hypoxia inducible factor1 is involved in the induction of BNIP3 and BNIP3L which disrupts beclin1-bcl2 complex, and releases beclin1 to induce autophagy. Further, BNIP3 induced autophagy acts as an adaptive survival mechanism in hypoxia tumors^[52]. *In vitro* and *in vivo* studies also reported that autophagy acts as survival mechanism in squamous cell carcinoma by protecting endoG-mediated apoptosis^[3].

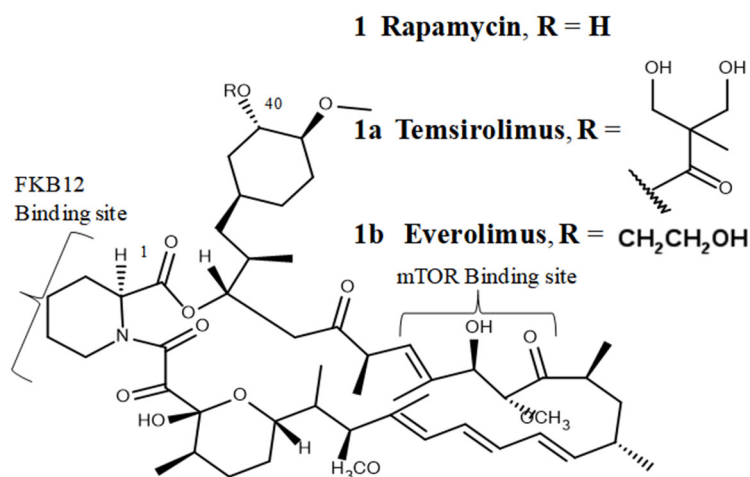


Figure 5. Mammalian target of rapamycin (mTOR) inhibitors

Additionally, it has been reported that RAS-activating mutations induce high basal-level of autophagy, and consequently assist in the development of lung, colon, and pancreatic cancers; hence inhibition of autophagy in these cancers hinders the tumor growth^[44,48,53]. Genetic studies carried on mice also disclosed that deletion of autophagic gene FIP200 inhibits the cell growth in mammary tumors^[41]. Moreover, mutations in BRAF protein reported to induce high levels of autophagy in CNS tumor^[53], melanoma^[54] and thyroid cancers^[45]; while, inhibition BRAF lead to impaired autophagy and decreased cell proliferation and cancer growth. All together, these studies suggest that inhibition of autophagy could be an appropriate strategy for the treatment of cancer and targeting the autophagy pathway with small molecules would be fruitful.

AUTOPHAGY MODULATORS FOR CANCER THERAPY

mTOR inhibitors

The mTOR, a member of PI3K family, is critical for serving as a primary regulator of cell growth, proliferation, metabolism and survival^[55]. The catalytic subunit of both mTOR1 and mTOR2 complex is involved in many oncogenic signaling pathways. The hyperactive characteristic of mTOR in many human cancers led to target this protein kinase as therapeutic target. Therefore, inhibiting mTOR has gained much attention in anti-cancer therapy. Rapamycin [Figure 5] with two binding moieties is the first generation inhibitor of mTOR. In order to form ternary complex, one binding moiety of rapamycin binds with FKBP12 and the other binds with mTOR^[56]. Initially, rapamycin was recognized as immunosuppressant which blocks T-cell activation and later on the anti-cancer activity was documented. Several rapalogs were generated by replacing C-40-O with different moieties and among them Temsirolimus is the first to get FDA approval for cancer treatment^[57]. Recent studies have shown that rapamycin can also act as a cytostatic agent, slowing or arresting the growth of various cancer cell lines.

Pan-PI3K inhibitors

PI3K is an essential subunit of PI3K-AKT-mTOR pathway involved in cell proliferation and survival; it is a well known protein kinase for the induction of autophagy. In several cancers including diffuse intrinsic pontine glioma, glioblastoma, paediatric high-grade glioma, breast cancer and cutaneous melanoma, over activation of this pathway has been observed and hence inhibition of PI3K has become an important target in several cancers^[58]. PI3K inhibitors including 3-methyladenine (3-MA) and wortmannin [Figure 6] are well characterized as autophagy inhibitors based on their inhibitory effect on the autophagy induction. Besides inhibiting PI3K with an IC₅₀ of 60 µmol/L, 3-MA has also been reported as inhibitor of Vps34 with an IC₅₀ 25 µmol/L. Cell culture studies revealed that 3-MA suppresses cell migration and leads to cancer cell death

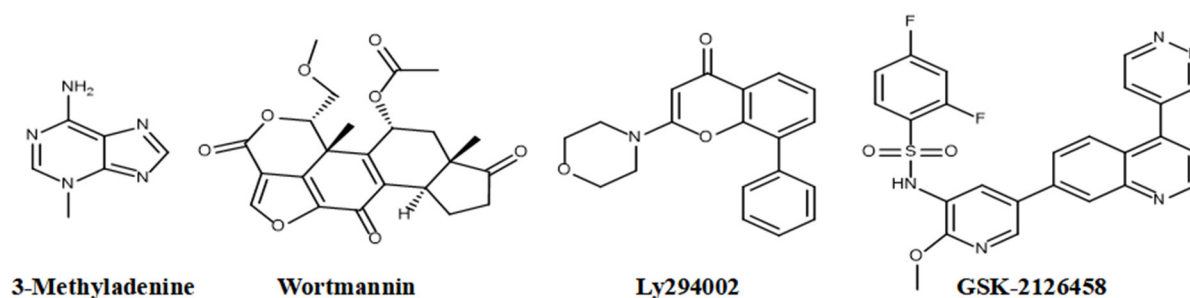


Figure 6. Pan-PI3K inhibitors

under normal as well as starvation conditions^[56]. Surprisingly, prolonged treatment (upto 9h) with 3-MA has shown autophagy flux promotion by increasing the autophagic markers levels such as LC3 protein^[59].

Wortmannin, a fungal metabolite is a selective, irreversible and potent inhibitor of PI3K that inhibits autophagic sequestration. It has been demonstrated that the lower concentration (nanomolar) of wortmannin potently and specifically inhibits PI3K; whereas, higher concentrations can inhibit the ataxia telangiectasia gene-related DNA-dependent protein kinase. At physiological pH (6-8.5), wortmannin compete with ATP and ATP analogs binds to PI3K, this suggests that wortmannin binds in the substrate binding site of PI3K. More importantly, site directed mutagenesis studies shows that Lys802 is essential to form nucleophilic interaction^[60]. These observations of wortmannin interactions with PI3K provide the molecular basis for designing better inhibitors for PI3K kinase family proteins to treat cancer via autophagy inhibition.

Ly294002, an inhibitor of PI3K class family proteins is derived from the flavonoid quercetin. Ly294002 is not completely selective for PI3K family proteins, and additionally act on other unrelated proteins and lipid kinases^[61]. Ly294002 binds at the ATP binding site and is more stable in solution than wortmannin. More importantly, this compound shows its inhibitory effect with IC_{50} values of 0.5, 0.97 and 0.7 $\mu\text{mol/L}$ for PI3K α , β and δ targets, respectively^[61,62]. To improve the selectivity and specificity, many Ly294002 analog were synthesized; SF1126 a prodrug of Ly294002 entered into clinical trial but was recently halted.

CLR457, a potential inhibitor of all PI3K isoforms is an orally bioavailable inhibitor with antineoplastic activity. It has been extensively characterized by *in vitro* biochemical methods and *in vivo* tumor xenograft studies^[63]. Dose limiting toxicity studies show that CLR457 potently inhibited PI3K isoforms including p110 α (IC_{50} = 89 nmol/L), p110 β (IC_{50} = 56 nmol/L), p110 δ (IC_{50} = 39 nmol/L) and p110 γ (IC_{50} = 230 nmol/L). However, the characteristics such as poor tolerability and limited antitumor activity of CLR457 resulted in the termination of clinical development.

Recently, omipalisib (GSK2126458) is presented as an autophagy inhibitor that specifically binds to PI3K in PI3K/mTOR signaling pathway. It directly targets Akt phosphorylation by PI3K and reverse phosphorylation of Akt by mTOR. It is an orally bioavailable dual ATP-competitive inhibitor of PI3K and mTOR with high potency^[64]. The indirect inhibition of Akt by omipalisib induces cytotoxicity and promotes autophagic cell death at 0.5 $\mu\text{mol/L}$ dose. Further, investigations carried out to know whether the mechanism of cell death occurs through autophagy or apoptosis reported that there is no significant difference in treated and untreated cells when apoptosis markers were used^[65]. Cell culture studies reported that GSK2126458 arrests cell cycle at G1 phase and affects proliferation of several cell lines such as breast cancer cell lines T47D and BT474 with IC_{50} values of 3 and 2.4 nmol/L, respectively. Currently, omipalisib is in clinical trials for idiopathic pulmonary fibrosis and solid tumors. The difficulties in PI3K inhibitors such as lower solubility of wortmannin and broad spectrum inhibition of Ly294002 and limited antitumor activity of CLR457, further necessitate the identification of new inhibitors for PI3K.

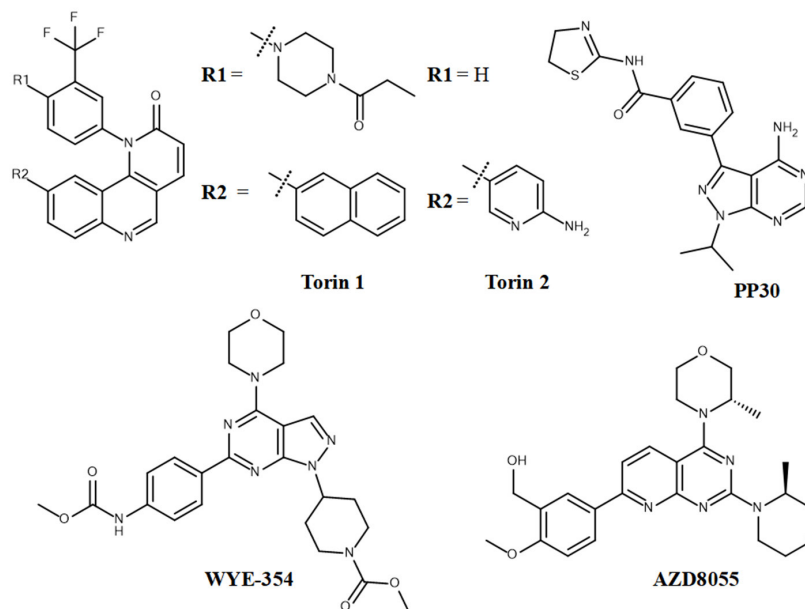


Figure 7. Pan-mTORC inhibitors

Pan-mTORC inhibitors

Activation of mTORC1 and mTORC2 is important in many cancers. Compounds with ATP competitive mechanism inhibiting both mTORC1 and mTORC2 offer better alternative to rapalogs. Torin 1 [Figure 7], is a commercially available autophagy inhibitor that is selective and highly potent ATP-competitive inhibitor of both mTORC1 and mTORC2. Torin 1 shows more efficacy towards blocking the phosphorylation of mTORC1 and mTORC2, when compared with rapamycin a well-known classical mTOR allosteric inhibitor. *In vitro* kinase assay reveals that Torin 1 has an IC_{50} of 3 nmol/L, 3 μ mol/L, 1.8 μ mol/L and 1 μ mol/L for mTOR, hVps34, PI3K- α and DNA-PK, respectively. The results show that Torin 1 is more selective for mTOR inhibition over other kinases^[60]. Second generation ATP-competitive inhibitor, Torin 2 is a potent and selective inhibitor of mTOR with better pharmacokinetics profile to overcome the limitations of Torin 1. *In vitro* studies revealed that Torin 2 reduced cell proliferation in several cancer cell lines and exhibited combinatorial response with AZD6244, an inhibitor of MEK kinase in the molar ratio of 1:50. Torin 2 shows inhibition of several PI3KK family proteins including mTOR, ATM, ATR and DNA-PKs with an IC_{50} value of less than 10 nmol/L^[66]; whereas, Torin 1 inhibits only mTOR, ATR and DNA-PK. Further, effects of Torin 2 in autophagosome formation were examined and found to induce autophagy^[66].

AZD8055 is another novel ATP-competitive inhibitor of mTOR kinase (IC_{50} of 0.8 nmol/L) that shows approximately 1000-fold selectivity against other kinases^[67]. Remarkably, in xenografts studies, AZD8055 shows substantial growth inhibition and suggests that AZD8055 can be a potent therapeutic drug in many human cancer treatments^[67]. AZD8055 treated acute myeloid leukemia cells have shown significant decrease in cell cycle progression and cell proliferation in blast phase. More interestingly, AZD8055 treatment results in decreased growth of leukemic progenitors but not normal immature CD34+ cells^[68]. Another independent study also revealed that AZD8055 treated chronic lymphoid leukemia (CLL) cells show significant reduction in CLL cell proliferation and increase in apoptosis. Currently, this inhibitor is in phase 1 clinical trials.

WYE-354, another heterocyclic compound is a powerful dual ATP-competitive kinase inhibitor that selectively blocks mTORC1/2 activity with an IC_{50} value of 4.3 nmol/L. Although it displays a weak inhibition of PI3K α , it has no inhibitory effect on other proteins/kinases. *In vitro* analysis revealed that WYE-354 potently blocks several cancer cell proliferation including LNCap, A498, MDA-MB-231, MDA-MB-361, MDA-MB-468 and HCT116 cell lines. Mechanistically, WYE-354 arrests cell cycle at G1 phase and induces

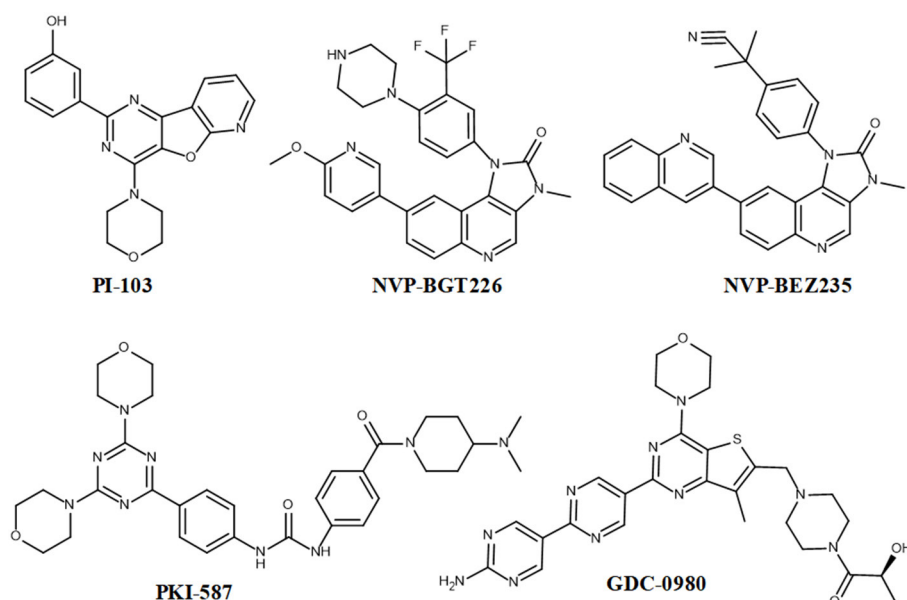


Figure 8. Dual PI3K/mTOR inhibitors

apoptosis by activation of caspase cascade. Autophagy inhibition by shRNA and autophagy inhibitors such as bafilomycin A1 and 3-MA, notably sensitized WYE-354 mediated anti-colon cancer cell activity. *In vivo* studies, further witnessed that WYE-354 administration in severe combined immunodeficient mice inhibited growth of xenografts^[69]. PP30 is an adenine-mimetic pyrazolopyrimidine scaffold compound that selectively inhibits mTORC1 (IC_{50} = 8 nmol/L) and mTORC2 (IC_{50} = 80 nmol/L).

PI3K/mTOR inhibitors

mTOR shares high sequence homology with the hinge region of PI3K, as they belong to the same family of phosphatidylinositol 3-kinase. Hence, several small molecules target both mTOR and PI3K simultaneously. PI-103 [Figure 8] belongs to pyridofuroprymidine class of compounds and is a multi-target inhibitor that inhibits PI3K and mTOR. Studies using human leukemia cell lines including MV4-11, OCI-AML3 and MOLM14 clearly indicated that PI-103 treatment arrested the cell cycle at G1 phase and eventually reduced the cell proliferation in these cells. The effects of PI-103 in AML patient samples have shown that 82% reduction of AML progenitor clonogenicity. The significant increase in apoptosis is also observed in blast cells when treated with 1.0 μ mol/L of PI-103. On the other hand, PI3K/Akt and mTOR inhibition has also been shown when the AML blast cells treated with RAD001 and IC87114 (RAD + IC); but the mechanism of antiproliferative effect is yet to be elucidated. At the same time, this study also reported that inhibitory effect of PI-103 is not much higher than that of RAD + IC in AML blast cells^[70].

PKI-587, a dual ATP competitive inhibitor of known therapeutic targets PI3K (PI3K- α and PI3K- γ) and mTOR is an orally bioavailable inhibitor^[56]. It shows potent inhibitory efficacies of PI3K- α , PI3K- γ and mTOR with IC_{50} values of 0.4, 5.4 and 1.6 nmol/L, respectively. PKI-587 not only inhibits wild type PI3K, but also exhibits inhibitory activity against most commonly occurring mutants including H1047R and E545K of PI3K with an IC_{50} value of 0.6 nmol/L for both forms. In *in vitro*, PKI-587 has exhibited excellent antitumor activity in over 50 human cancer cell lines^[71]. In *in vivo*, PKI-587 showed inhibition of tumor growth in MDA-MB-261, BT474, HCT116, H1975 and U87MG xenograft models, when administrated intravenously.

A novel inhibitor apitolisib, also known as RG7422/GDC-0980 is an orally available dual PI3K and mTOR inhibitor with excellent pharmaceutical and pharmacokinetics properties. The GDC-0980 inhibition of PI3K and mTOR overexpression has shown significant reduction in tumor cell growth by inducing apoptosis. In

cell free assays, GDC-0980 treatment results in the inhibition of class 1 PI3K isoforms α , β , δ and γ at low nanomolar IC_{50} values of 5, 27, 7 and 14 nmol/L, respectively and mTOR inhibition with K_i value of 17 nmol/L. These preclinical data show high potency and selectivity of GDC-0980 inhibitory activity. However, GDC-0980 has less effectiveness than everolimus in metastatic renal cell carcinoma^[72].

NVP-BGT226, a potent orally bioavailable dual inhibitor of PI3K and mTOR signaling pathways, blocks cell cycle at G0/G1 phase and induces autophagy and apoptosis. It is shown that NVP-BGT226 suppresses the growth of primary myeloma and common myeloma cell lines at nanomolar concentrations. Specifically, NVP-BGT226 inhibits PI3K α , β and γ isoforms with IC_{50} values of 4, 63 and 38 nmol/L, respectively. The analysis of NVP-BGT226 effects in hepatocellular carcinoma (HCC) shows cell growth and proliferation inhibition with potent cytotoxic activity. Hence, the capabilities of NVP-BGT226 in targeting PI3K and mTOR may represent it as an anticancer agent in HCC^[73]. NVP-BEZ235, an imidazo[4,5-c]quinoline derivative, is a dual kinase inhibitor of PI3K and mTOR that binds to the ATP binding site and halts cell cycle at G1 phase. When the compound is given orally to animal models, it displayed disease stasis of human cancers^[74]. Although co-crystallization studies of this compound with targets are ongoing, docking studies revealed that NVP-BEZ235 forms H-bond with ATP binding cleft residues including Val851, Asp933, and Ser774 of PI3K α homology model. This compound has entered phase I clinical trials for the treatment of breast cancer, advanced solid tumors and Cowden syndrome.

Unc-51-like kinases inhibitors

ULK belong to serine/threonine kinase family proteins, and play a crucial role in autophagy regulation^[34]. Humans contain four ULK kinases including ULK1, ULK2, ULK3 and ULK4. Among them, ULK1 is well studied and it is utmost important for autophagy initiation. Under nutrient deprivation, ULK1 is activated by several up-stream signals (e.g., AMPK, *etc.*) and then initiates autophagy process by recruiting various other proteins (i.e., FIP200, ATG101 and ATG13 for ULK complex) to the on-site of autophagy initiation. Thus, ULK1 and its associated proteins (i.e., ULK complex) play essential roles in cell survival mechanism under nutrient deficiency^[75]. However, disruption of ULK1 and its associated protein complex lead to autophagy inhibition and cell death. As cancer cells generate energy and nutrients through autophagy mechanism and eventually help in cell survival and tumor progression, disruption of ULK1 function by developing small molecule inhibitors has become an attractive approach to treat cancer^[75,76]. As a proof of concept, few ULK1 inhibitors have been reported in the literature [Figure 9].

MRT67307 and MRT68921 are two closely related derivatives with different substitution pattern on the pyrimidine ring. MRT67307 inhibits ULK1 and ULK2 with an IC_{50} of 45 and 38 nmol/L, respectively. This compound led to the identification of MRT68921, that has 15-fold improved inhibition of ULK1 (IC_{50} = 2.9 nmol/L) and 30-fold improved inhibition of ULK2 (IC_{50} = 1.1 nmol/L), when compared with MRT67307. Studies using MRT67307 and MRT68921 in MEFs cells show that these compounds are able to block autophagic flux. In addition, MRT68921 treated cells show significant increase in SQSTM1 level and decrease in LC3-2/LC3-1 ratio. These results suggested that MRT68921 treatment efficiently inhibits ULK-mediated autophagy^[77]. However, the molecular basis of this block remains to be elucidated. SBI-0206965 is a potent and specific inhibitor of ULK1 with an IC_{50} of 108 nmol/L and also selectively inhibits ULK2 with IC_{50} of 711 nmol/L, but with less efficacy when compared with ULK1. Inhibition of ULK1 in NSCLC cells results in anti-proliferative effect^[51]. Specifically, SBI-0206965 suppresses phosphorylation events in cells that are mediated by ULK1. SBI-0206965 at 10 μ mol/L concentration shows high selectivity and inhibits only 10 out of 456 kinases.

In 2015, Lazarus *et al.*^[75] reported the first crystal structure of ULK1 in complex with multiple inhibitors. The structure consists of an N-terminal kinase domain, a serine-proline rich region, and a C-terminal interacting domain. They used a standard 32 P-ATP radioactive assay to screen a collection of 746 compounds against ULK1 that led to the identification of several pyrazole aminoquinazolines as ULK1 inhibitors. For example,

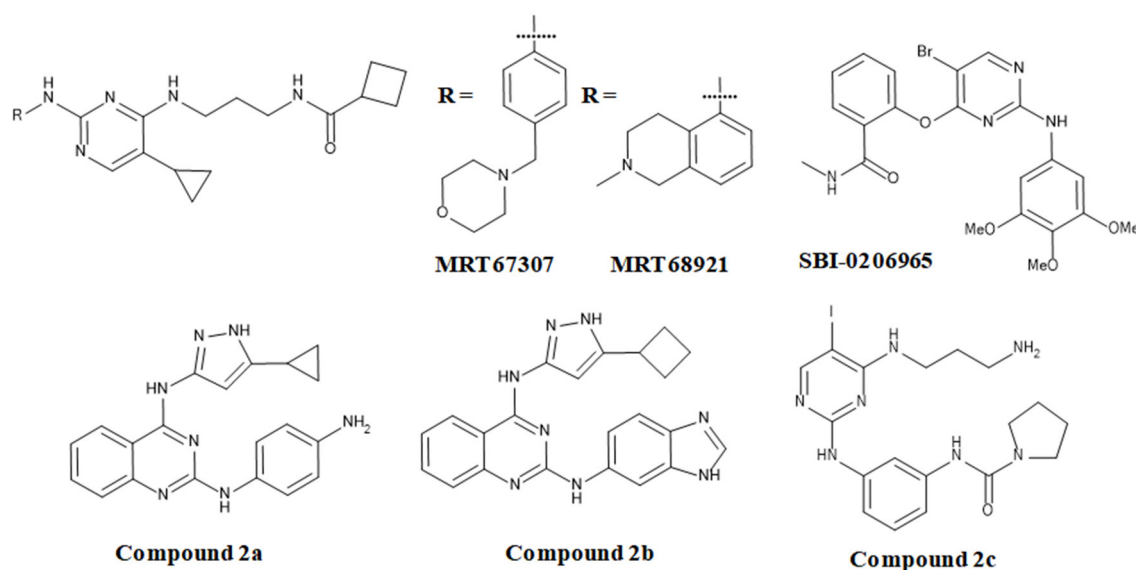


Figure 9. ULK1 inhibitors

the identified compound 2a showed dose dependent inhibition with IC_{50} of 160 nmol/L when re-tested in *in vitro* assay. Further, co-crystallization of this compound with ULK1 demonstrated that compound 2a [Figure 10] bound in the ATP binding site by making hinge region interaction with its amino pyrazole core group. Moreover, they observed that the aniline moiety made contacts with Asp165 of DFG motif, and the cyclopropyl substituent moiety fitted into the pocket close to the gate keeper residue methionine. Quinazoline ring interacts through position 6 and 7 with the backbone of kinase. In conformity with this steric obstruction, no compounds with substituents at these positions showed activity against ULK1.

Further, modifications made to these series of compounds led to the identification of compound 2b with improved potency with an IC_{50} of 8 nmol/L^[56]. However, the co-crystallization of this compound in similar condition (as of compound 2a) with ULK1 produced different space groups that indicate compound 2b produced conformational changes in the kinase domain, which led to the improved activity. Detailed analysis revealed that there were major changes in the conformation of interlobe loop, the side chain of Asp165 (DFG loop residue) and methionine of gatekeeper residue^[75]. Testing of compound 2b against a small panel of kinases showed non-selectivity, suggesting the need for improving the selectivity and potency against ULK1.

Vacuolar protein sorting 34 inhibitors

Vps34 is a lipid kinase that belongs to subgroup of class III PI3K family protein. The major function of this family protein is to phosphorylate the 3-hydroxyl group of inositol ring of phosphatidylinositol (PtdIns) lipid substrates to generate PtdIns3P^[22,79,80]. Vps43 interacts with multiple protein subunits and forms Vps34 complex I (i.e., Vps34, Vps15, Beclin 1, and Atg14L) that precisely catalyzes the phosphorylation of PtdIns to PtdIns3P and anchors with intracellular membranes. Further, the membrane-bound PtdIns3P interacts with proteins that contains FYVE, PX, or WD40 domain and involves in vesicle trafficking and autophagy mechanism. Thus, Vps34 complex is essential for initiation/induction of autophagy during nutrient deprivation through regulation of mTOR pathway. Therefore, the disruption of Vps34 complex leads to autophagy inhibition and cancer cell death. Hence, Vps34 has become an attractive drug target for cancer therapy and a number of inhibitors have been reported in the literature, and few compounds have entered into clinical studies.

Recently, Dowdle *et al.*^[81], identified a hit containing bisaminopyrimidine core as a potent and selective Vps34 kinase inhibitor using high-throughput screening of compound libraries. Optimization of this

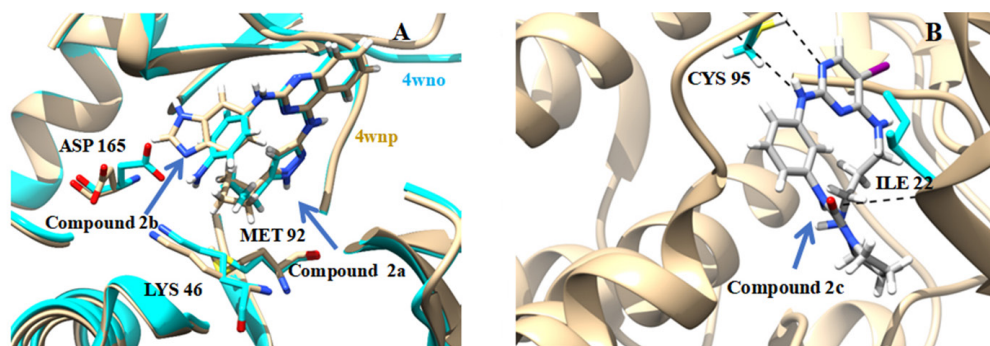
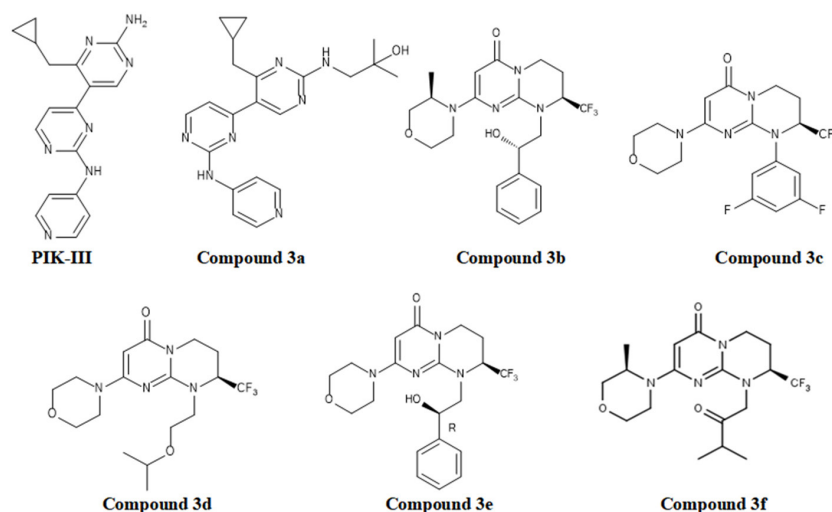


Figure 10. A: Superimposition of the binding mode of compound 2a (PDB ID: 4WNO) and compound 2b (PDB ID: 4WNP) in ULK1; B: binding mode of compound 2c (PDB ID: 5CI7) in ULK1. Hydrogen bond interactions are represented in black dotted lines. Compound 2c with a diaminopropyl group, a closely related pyrimidine analog of MRT67307, showed dose dependent inhibition with an IC₅₀ of 120 nmol/L. Co-crystallization of compound 2c with ULK1 [Figure 10B] showed that it has similar orientation with the diaminopropyl group occupying a similar space as that of quinazoline of compound 2a. The main difference in the kinase is the movement in the β sheet in the N-terminal lobe with Gly23 towards the inhibitor, which allows Ile22 to twist away from the bulky diaminopropyl substituent on the pyrimidine. The other difference is the gatekeeper methionine moves towards iodine group, to adopt a suitable dipole-dipole interaction. A flexible region involving Ile22 was required to pack above the aminopropyl group^[78]

compound led to the identification of 4-aminopyridine-containing PIK-III inhibitor [Figure 11] with improved potency and selectivity^[81]. Additional biochemical testing and profiling of this compound showed that PIK-III is at least 100-fold selective for Vps34 as compared to related kinases including PI3K α , mTOR, and additional 44 protein kinases. Moreover, they also demonstrated that PIK-III inhibited the co-localization and distribution of PtdIns3P specific lipid binding domain (FYVE) fused with GFP with an IC₅₀ of 55 nmol/L concentration and that is > 10,000 times more potent than the non-selective Vps34 inhibitor 3-MA.

Co-crystallization of PIK-III with human Vps34 kinase [Figure 12A] revealed that the overall structural geometry was comparable to that of PI3K γ and *Drosophila melanogaster* Vps34 structures^[81]. Further, analysis revealed that the structure appears like a typical lipid and protein kinase structure that has a relatively narrow active site with hydrophobic pocket appropriate for binding co-planar aromatic compounds. Binding mode of PIK-III to Vps34 structure demonstrated that the cyclopropyl group occupied the hydrophobic pocket that was formed with side chains of Phe612, Pro618 and Phe684. Two hydrogen bond interactions were observed between PIK-III acceptor/donor and backbone amide & carbonyl oxygen of Ile685. In addition, solvent mediated hydrogen bond network bridges were observed between aminopyrimidine moiety of PIK-III and side chains of Asp671 and Asp644. Superimposition of Vps34 and PI3K α active site revealed that in both the structures the hydrophobic cavity was enclosed/covered with P-loop residues, however their relative orientations were quite different with respect to their hinge regions. In Vps34 it is relatively displaced towards hinge region, whereas in PI3K α structure it is wider and proximal to hinge region. In Vps34 structure, the relative orientation of Phe612 has significant role as it allows cyclopropyl group to fit into the hydrophobic pocket to acquire optimal interaction with hinge region. Whereas, in PI3K α structure the corresponding phenylalanine was replaced with methionine residue and it doesn't allow the cyclopropyl group to fit into the pocket. Thus, this structural difference makes an ideal tool for developing selective inhibitors of Vps34 and also to explicitly measure pharmacological consequences of VPS34 inhibition *in vivo*.

Further, docking and structure-activity relationship (SAR) study led to the identification of compound 3a with greater potency and improved metabolic stability providing an excellent candidate for *in vivo* pharmacokinetics evaluation^[80]. Compound 3a showed exceptionally selective activity over other lipid and protein kinases (> 100-fold against more than 280 kinases evaluated, except TAK1 and PI3K δ). Co-crystallization of compound 3a with Vps34 [Figure 12] revealed that its binding mode is similar to that

**Figure 11.** Vps34 inhibitors

of PIK-III. Moreover, compound 3a prevented the degradation of various autophagy substrates (p62, NCOA4, NBR1, NDPS2 and FTH1) as that of PIK-III. Pharmacokinetics profile of compound 3a revealed that it is rapidly absorbed and showed moderate mean systemic clearance (30 mL/min/kg) with good oral bioavailability ($F\% = 47$). Oral administration of compound 3a to RKO colon cancer bearing mice at 50 mg/kg twice a day for 7 days showed time-dependent accumulation of LC3-II with reduced autophagy capacity without reduction of tumor volume. Hence, additional studies with long term drug administration need to be evaluated.

Pasquier *et al.*^[82], reported a series of tetrahydropyrimidopyrimidinone derivatives [Figure 11] as Vps34 inhibitors using a cell based high-throughput phenotype screening campaigning. The reported compounds comprise/contain a pyrimidinone moiety with a morpholine group as hinge binder and shown to have Vps43 inhibition in kinase profiling assay panel. However, these compounds also showed cross-reactivity with class I PIKs (isoforms α , β , δ , and γ) and at a lower concentration level, with mTOR. Further, evaluation of these compounds activity against Vps34 using GFP-FYVE cell based assay revealed that compound 3b shows higher cellular potency compared to other compounds^[82]. Hence, compound 3b was selected as advanced hit for back screening. Additional screening of tetrahydropyrimidopyrimidinone derivatives and SAR studies focused on potency and selectivity over lipid kinases. Substitution at 1-position of the pyrimidinone scaffold led to the identification of compounds 3c, 3d and 3e [Figure 11] with enhanced Vps34 enzymatic potency, substantial GFP-FYVE cellular potency, attractive ligand efficiency (LE) and ligand lipophilicity efficiency (LLE) values. Moreover, these compounds also showed favorable *in vitro* ADME properties and sensible microsomal stability.

X-ray co-crystal structure of compound 3c [Figure 12C], 3d and 3e with human Vps34 demonstrated that all the compounds adapted a DFG-in conformation and were involved in hinge region interaction via oxygen atom of the morpholine ring. Moreover, this moiety was also involved in making promising vander Waals interactions with adjacent residues. The aromatic ring of pyrimidinone moiety was stacked in between Ile634 and Ile760 side chains, whereas carbonyl function of this moiety was involved in making hydrogen bond interaction with catalytic Ly636 side chain, and water mediated H-bond interactions with Asp644 and Tyr670 side chains. The (2S)-trifluoromethyl group of tetrahydropyrimidine ring was pointed towards the hydrophobic pocket under the P-loop residues Phe612 to Ala619. Compounds 3c-e show difference only at their N-substituent that points towards the exit of the ATP binding site. Further, modification of hinge binder moiety led to improved selectivity, and replacing the trifluoromethyl moiety on

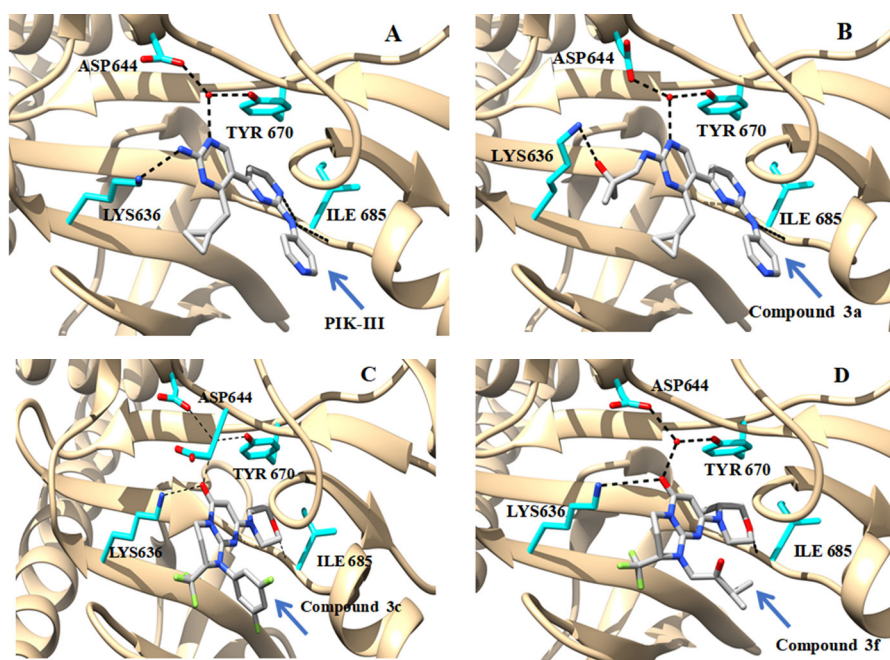


Figure 12. 3D binding mode and interaction of PIK-III (PDB ID: 4PH4) (A), compound 3a (PDB ID: 5ENN) (B), compound 3c (PDB ID: 4UWF) (C) and compound 3f (PDB ID: 4UWL) (D) with Vps34. Hydrogen and water mediated hydrogen bonds are represented in black dotted lines

tetrahydropyrimidinone ring gained Vps34 potency and lost selectivity against other lipid kinases. From the final step of optimization, compound 3f was selected as Vps34 inhibitor for *in vivo* pharmacological study.

In vitro studies revealed that compound 3f displayed IC_{50} values of 2 and 82 nmol/L on Vps34 enzymatic assay and GFP-FYVE cellular assay, respectively. Biophysical characterization using surface plasmon resonance and isothermal titration calorimetry demonstrated that the compound 3f has a binding affinity (K_D) of 2.59 and 2.7 nmol/L, respectively. Moreover, compound 3f showed good physiochemical/drug-like properties (such as LE = 0.41, and LLE = 6.22). Further, co-crystallization of compound 3f with Vps34 [Figure 12D] revealed that methyl group of morpholine moiety points towards Met682. Like compound 3e, water mediated hydrogen bonds were observed between carbonyl oxygen atom and Asp761 of Vps43. Thus, this binding orientation provided enhanced Vps34 selectivity and potency. *In vivo* pharmacokinetics (PK) profiles of compound 3f disclosed a good oral bioavailability (F% = 85) with maximal plasma concentration observed at 0.5 h and reasonable systemic clearance. Moreover, PK/PD experiments using GFP-FYVE H1299 tumors xenografted in SCID mice revealed that compound 3f had sustainable inhibition (> 80%) of granular staining, and a dose dependent target modulation.

Lysomotrophic agents

Chloroquine [Figure 13], a widely used inhibitor of autophagy which inhibits last stage of autophagy is initially discovered to treat malaria and inflammatory diseases. Although, the mode of action of bafilomycin A1 and lysosomal protease inhibitors were well characterized, mode of action of chloroquine still remains unclear. However, it is believed that chloroquine inhibits autophagic flux through rising pH and thereby inactivates lysosomal hydrolases^[83]. Currently, chloroquine (CQ) and hydroxychloroquine (HCQ) are being investigated as autophagy modulator in Phase II/III trials for cancer therapy^[84]. Recent *in vitro* and *in vivo* studies revealed that chloroquine affects the endo-lysosomal system and golgi complex thereby modulates autophagic flux through decreasing autophagosome-lysosome fusion^[85]. This study invalidated lysosomal degradation function of chloroquine.

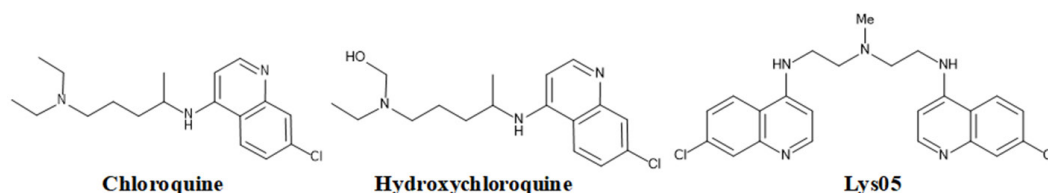


Figure 13. Lysomotrophic inhibitors

HCQ, a derivative of CQ is a 4-aminoquinoline that has antimalarial and anti-inflammatory activities; it is currently being investigated as the inhibitor for autophagy. Several clinical trials of HCQ in combination with other anti-cancer drugs (e.g., temozolomide, bortezomib, temsirolimus, vorinostat, doxorubicin, *etc.*) showed partial response and stable disease outcome for various cancers (melanoma, colorectal cancer, myeloma and renal cell carcinoma)^[86]. As a drug, this basic compound alkalinizes acidic environment of lysosomes and thereby prevents autophagosome-lysosome fusion. HCQ is proved to be threefold less toxic than CQ and can augment the cytotoxicity of a number of chemotherapies and targeted therapies. A recent meta-analysis of clinical trials of CQ and HCQ concluded that their use in cancer patients has better treatment response, when used in combination with existing anti-cancer therapy^[87].

Based on the 4-aminoquinoline core structure of CQ and HCQ, Lys05 was designed; it was more potent in *in vitro* and *in vivo* as a single agent. The increased activity of Lys05 was due to the bivalent aminoquinoline rings, C7-chlorine and a short triamine linker. Lys05 trihydrochloride is water soluble and shows potent anti-tumor activity in several human cancer cell lines as a single agent. Intermittent high dose or chronic daily dosing of Lys05 at lower doses have shown early blockage of autophagy in melanoma and colon cancer xenograft models^[88]. Comparative study in cancer cells also revealed that HCQ at 100 $\mu\text{mol/L}$ cannot show complete deacidification of endovascular compartment; whereas 50 $\mu\text{mol/L}$ of Lys05 has shown complete deacidification. Further, in mice models Lys05 at high dose (80 mg/kg, i.p.) causes Paneth cell dysfunction with loss of lysozyme biosynthesis and bowel pseudo-obstruction^[88,89]. Studies evidenced that high dose of Lys05 is associated with intestinal toxicity and it has been also observed that high dose of HCQ also causes low grade nausea and constipation in patients. Lys05 is a new lysosomal inhibitor that has a potential to be further developed as a drug for cancer treatment.

Autophagy inducers

In addition to the above agents/compounds that were designed to modulate the autophagy process by specifically interacting with targets in autophagy pathways, there are other agents that induce autophagy. Particularly, naturally occurring compounds have multiple modes of action and targets different pathways. Some of them induce autophagy by targeting autophagy pathway signaling molecules and are discussed in this section [Figure 14].

Curcumin

Curcumin, a natural compound of golden spice turmeric shows numerous activities including anti-inflammatory, antimicrobial, antioxidant, hypoglycemic and wound healing activities. Considering these activities, curcumin has been investigated in many clinical conditions such as multiple myeloma, breast cancer and non small cell lung cancer. Although, it has proven efficacy in several clinical aspects, curcumin has therapeutic limitations due to rapid systemic elimination, rapid metabolism and poor absorption. Curcumin showed anticancer effects by sensitizing chemotherapy and radiation therapy^[90]. In gastric cancer cell lines SGC-7901 and BGC-823, curcumin significantly inhibited cell proliferation by exhibiting autophagy induction. The studies have reported that curcumin induces autophagy by its dual functionality in up-regulation of p53 and p21, and down-regulation of PI3K/Akt/mTOR signaling pathways^[91]. Another recent investigation also reported that curcumin induces autophagy in human pancreatic cancer cell lines^[91].

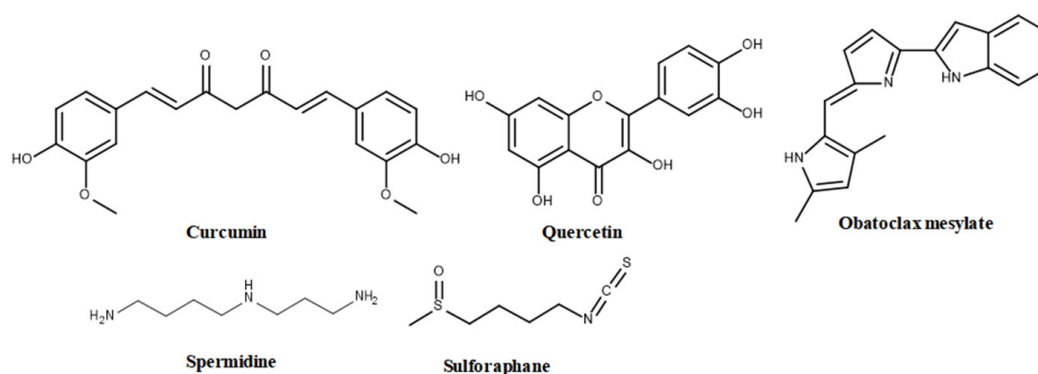


Figure 14. Autophagy inducers

Curcumin treatment of human lung adenocarcinoma cell line A549 showed autophagy induction by means of increased AMPK phosphorylation^[92].

Quercetin

Quercetin, a well-known cancer therapeutic agent and autophagy modulator is abundantly present in vegetables and fruits, and suppress tumor proliferation^[93]. It has been shown that quercetin suppresses Akt/mTOR and Bcl-2 signaling pathways and activates LC3, ERK and caspase. Studies in human neuroglioma cells U87 and *in vivo* studies revealed that quercetin nanoparticles significantly inhibit neuroglioma cell proliferation by inducing autophagy and apoptosis. Cell proliferation assay of neuroglioma cells showed that quercetin nanoparticles treatment with 10-50 µg/mL gradually reduced cell viability^[94]. A recent study reports that quercetin modulate p-STAT3/Bcl2 pathway and there by induces protective autophagy in ovarian cancer^[95].

Obatoclax mesylate

Obatoclax, a small molecule indole bipyrrole compound is an inhibitor of Bcl2 family proteins and exhibited anticancer activity in acute lymphoblastic leukemia^[96]. In addition to its involvement in apoptosis, obatoclax has also been involved in triggering cell death via autophagy induction^[97]. Primarily, this compound plays a key role in stimulation of necrosome assembly on autophagosome membrane to induce autophagy. Co-immunoprecipitation studies indicated that obatoclax enhances the interaction between Atg5 and necrosome components including FAD, RIP and RIP3. The same study investigated the role of obatoclax with 200 nmol/L concentration in rhabdomyosarcoma cells to unravel whether cell death occurs via apoptosis or autophagy and noticed little DNA fragmentation when the most of cell lost their viability.

Spermidine

Spermidine is a polyamine present in most of the mammalian cells and has important roles in several cellular activities under physiological conditions. It is well known for its longevity-promoting activity in association with autophagy enhancement. A study conducted in differentiated rat pheochromocytoma PC12 and human embryonic kidney 293 cells, revealed the possible role of spermidine in autophagy. After treating the cells with spermidine, the cell death analysis and caspase activity assays indicated that spermidine restricts neuronal injury via inhibiting caspase-3 dependent Beclin1 cleavage. Eventually, this mechanism elevates dysregulated autophagy flux levels^[98]. Very recent immunoprecipitation and immunohistochemistry studies in human chondrocytes and *in vivo* study conducted in mice reported that the cells treated with spermidine (100 nmol/L) showed autophagy activation and promoted chondrogenesis^[98]. However, spermidine role in cancer is still controversial as some studies reported that spermidine do not induce tumorigenesis^[99], whereas some studies established that spermidine synthase inhibitors show little cell growth reduction in cancer cell lines^[100].

Sulforaphane

Sulforaphane, an organic isothiocyanate compound derived from glucosinolates shows both cytoprotective and cytotoxic activities. It activates nuclear factor E2-related factor 2 signaling that elevates the expression of antioxidant response proteins in oxidative stress. A study in murine osteosarcoma cells reported that sulforaphane induces apoptosis through cell cycle arrest and inhibits tumor cell growth^[101]. It has also been reported that the treatment of sulforaphane initiates various cellular processes in human prostate cancer^[102]. To investigate whether sulforaphane is reducing the cell growth or encourages cell death, a study was conducted in human lens cell line and reported that sulforaphane reduced the cell viability in human lens cell line and promotes cell death. The study also revealed that sulforaphane promotes ER stress and autophagy induction via MAPK signaling^[103], and longer treatment with sulforaphane has shown significant decrease in AMPK phosphorylation at thr-172^[104] in human prostate cancer cells.

FDA approved drugs with autophagy modulation activity

In addition to the above discussed autophagy modulators, few FDA approved drugs (for different indications) are reported to have additional autophagy modulator activities [Table 2]. This section is intended to list out these drugs and provide brief information and mode of autophagy modulation by these drugs. Some of these drugs are already used as anticancer agents, while some are used for other indications. These data suggests that some of the approved anticancer drug may be partly working through autophagy modulation. Moreover, understanding the exact molecular mode of action of these drugs could help to repurpose these drugs for cancer therapy in the future.

Temozolamide, an alkylating agent is a FDA approved drug for the treatment of glioblastoma multiforme in combination with radiotherapy^[105]. Recent studies have investigated the role of temozolamide in autophagy modulation and reported that it induces autophagy in glioblastoma cancer cells through EGFR independent manner^[106]. Temozolamide also showed cytotoxicity in PI3K/AKT/mTOR pathway inhibited adenoma cells^[107]. Gefitinib which targets tyrosine kinase activity of EGFR and binds competitively to the ATP binding site is a FDA approved drug for treating the patients with non-small cell lung cancer (NSCLC)^[108]. Gefitinib induces autophagy in lung cancer cells through blocking of PI3K/AKT/mTOR pathway^[109] and also exhibited autophagy induction in combination with clarithromycin in NSCLC cells^[110].

Metformin, a biguanide antihyperglycemic agent is FDA approved for treating non-insulin-dependent diabetes mellitus. It controls glucose level by means of decreasing hepatic glucose production and also by increasing insulin-mediated glucose uptake. It is reported that metformin is involved in autophagy induction by AMPK dependent manner. Several cancer models have shown significant growth inhibition upon metformin treatment^[111]. A study also reported that metformin promotes autophagy and selectively inhibits esophageal squamous cell carcinoma cell growth by down regulating STAT3 signaling^[112]. Studies on human multiple myeloma cells show that metformin inhibits the cell proliferation by promoting autophagy and cell cycle arrest and this study suggested metformin dual repression of mTORC1 and mTORC2 via AMPK activation^[113].

Bortezomib is a proteasome inhibitor that specifically inhibits nuclear factor kappaB and is a FDA approved drug to treat multiple myeloma^[114]. Bortezomib has shown anticancer activities in several human cancers including prostate cancer, colon cancer, ovarian cancer and breast cancer. Studies have explored the possible role of Bortezomib in autophagy and found that it promotes cancer cell death through blockage of autophagic flux in ERK phosphorylation dependent manner^[115]. Sodium phenylbutyrate is a chemical chaperon that inhibits histone deacetylase and is FDA approved drug to treat urea cycle disorders. Sodium phenylbutyrate is also under clinical investigation in several human diseases including hemoglobinopathies, motor neuron diseases, cancer and cystic fibrosis^[115]. Sodium phenylbutyrate has been shown to reduce dithiothreitol or tunicamycin induced autophagy^[116].

Table 2. FDA approved drugs for various human diseases that are reported to have autophagy modulating activity

Sl. No.	Drug	Mode of action	FDA approved use	Nature of the modulator
1	Temozolomide	Alkylating agent	Glioblastoma multiforme	Autophagy inducer
2	Rapamycin	Inhibits mTOR	Transplant rejection	Autophagy inducer
3	Metformin	Activates AMPK	Type 2 Diabetes	Autophagy inducer
4	Gefitinib	PI3K/AKT/mTOR pathway	Metastatic non-small cell lung cancer (NSCLC)	Autophagy inducer
5	Bortezomib	Activates AMPK	Multiple myeloma	Autophagy inhibitor
6	Sodium Phenylbutyrate	Improves cathepsin D and B activities and lysosomal-autophagic function	Urea cycle disorders and acute promyelocytic leukemia	Autophagy inhibitor
7	Carbamazepine	Reduces inositol and Ins (1,4,5)P3 levels	Epilepsy	Autophagy inducer
8	Verapamil	Inhibits Ca ²⁺ channel lowers intracytosolic Ca ²⁺ levels	Hypertension	Autophagy inducer
9	Rilmenidine	Reduces cAMP levels	Hypertension	Autophagy inducer
10	Choloroquine and Hydroxychloroquine	Inhibits lysosomal function	Malaria, Lupus and rheumatoid arthritis	Autophagy inhibitor
11	Pantoprazole	Proton pump inhibitor, increased endosomal pH	Erosive esophagitis	Autophagy inhibitor
12	Celecoxib	Inhibits cyclooxygenase 2	Rheumatoid arthritis and osteoarthritis	Autophagy inhibitor

Carbamazepine is a FDA approved anticonvulsant drug for the treatment of epilepsy. Evidences indicate that carbamazepine diminishes hepatocellular death in autophagy dependent manner. A study on SW480 colon cancer cell lines revealed that carbamazepine decreases β -Catenin and VEGF levels, resulting in antitumor activity^[117]. Verapamil and Rilmenidine are well known drugs for the treatment of hypertension and now these drugs are under investigation to unravel their role as autophagy modulators in cancer. Autophagy induction has been observed upon treatment with verapamil in COLO 205 cells with cytoprotective activity^[118]. It has been observed that Rilmenidine promotes autophagy in an mTOR independent manner, however the reduction in disease progression has not been observed^[119].

Pantoprazole is a protein pump inhibitor used to treat certain esophagus and stomach related problems. Various studies reported that pantoprazole sensitized cancer cells to anticancer drugs by suppressing autophagy induction in time- and dose-dependent manner^[120-122]. Celecoxib is a nonsteroidal anti-inflammatory drug that is FDA approved for Rheumatoid arthritis and osteoarthritis treatment. Recent studies have evaluated celecoxib activities in cancer and reported its antitumor effects in solid tumors. Also autophagy reduced significantly upon treatment with celecoxib in imatinib resistant chronic myeloid leukemia cells^[123]. All together, although these drugs have proven to be useful in various human diseases, currently many studies are investigating their impact in cancer though autophagy modulation.

CONCLUSION

Autophagy is a conserved cellular process that is essential for the cells to cope-up with adverse conditions, such as the lack of nutrients or bacterial/viral infections. In such conditions, autophagy plays a pro-survival role by recycling the cellular components by lysosomal degradation and also helps in the removal of pathogenic organism by sequestration and degradation. Because of its central role in maintaining cellular homeostasis, alterations in the autophagic process through aberrant signaling has been linked to several disease states such as cancer and neurodegeneration, among others. In cancer, autophagy has the role of a double edge sword, with tumor suppressing activity during the initial stages of cancer development and pro-survival effects in the later stages of cancer development.

The relevance of autophagy modulation in cancer is increasingly appreciated and many therapeutic targets involved in autophagy process have been identified. In this review, we highlighted the role of promising autophagy signaling pathway/biomarkers that could be modulated for cancer therapy. Further, we also

reviewed and analyzed the available autophagy inducers/inhibitors. Except for the mTOR targeting rapalogs, no other autophagy targeting agents are approved in the clinics for cancer treatment. Currently, chloroquine and hydroxychloroquine are being clinically tested as autophagy modulators either alone or in combination with other anti-cancer therapeutics (e.g., temozolomide, bortezomib, temsirolimus, vorinostat, doxorubicin, *etc.*). Eventhough hydroxychloroquine shows partial response and improves treatment outcomes in various cancers such as melanoma, colorectal cancer, myeloma and renal cell carcinoma, dose related toxicity may preclude its widespread use as autophagy modulator. However, this may change soon as several groups are developing molecules to various drug targets such as ULK and Vps34 within the autophagy signaling pathway. Developing selective autophagy modulators with minimal cross-talk with other targets will be challenging and crucial for making autophagy modulation as a successful strategy for cancer therapy. Moreover, patient selection based on the alteration in the autophagy signaling pathways could pave way for better treatment outcomes for autophagy modulators.

The importance of autophagy related research can be fathomed from the ever increasing number of publications (90 articles in the year 2000, 2050 in 2010 and 6700 articles in 2018; scopus data) and the fact that 2016 Nobel prize in Physiology or Medicine was awarded to Prof. Yoshinori Ohsumi for his research on Autophagy in yeast. Eventhough, we have made big strides in understanding the autophagy process, the complete molecular machinery of autophagy, the signaling process involved and their roles in various disease conditions are not yet completely elucidated in humans. Future research should hold promise as well as provide insights in these directions for identifying better treatment for cancer and other diseases.

DECLARATIONS

Authors' contributions

Collected the literature and wrote the article: Kondapuram SK, Sarvagalla S

Collected the literature, corrected the article and approved: Coumar MS

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All authors declared that there are no conflicts of interest.

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Review

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Peripheral biomarkers for pediatric brain tumors: current advancements and future challenges

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Abstract

Circulating biomarkers - nucleic acids, proteins, and metabolites - have been used in several adult oncologic processes to affect early detection, measure response to treatment, and offer prognostic information. The identification and validation of biomarkers for pediatric brain tumors, however, has been meager by comparison. Early detection and serial screening of pediatric brain tumors has the potential to improve outcomes by allowing for rapid therapeutic interventions and more targeted therapies. This is particular resonant for pediatric brain tumors where treatment success is heavily dependent on early surgical intervention. This highlights the need for biomarker development in pediatric neuro-oncology. The authors reviewed current circulating biomarker targets in various biofluid reservoirs and discuss the current barriers to biomarker development in pediatric neuro-oncology patients.

Keywords: Pediatric, brain tumor, biomarker, miRNA, biofluid, overview

INTRODUCTION

Pediatric brain tumors are the leading, oncologic cause of death in children under 10 in North America. Roughly 4,600 children are diagnosed with brain tumors every year in the United States, and maximal, safe surgical resection remains the primary treatment modality^[1-3]. Five-year survivals can vary by as much as 70% with some of the most common pediatric brain tumors, depending on a surgeon's ability to achieve complete safe resection of the mass^[4-8]. This makes early diagnosis and intervention, potentially, critical to



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ensuring favorable outcomes, as surgical resection is generally more successful when tumors are smaller, confined to a single location, and less entwined with critical neural structures.

Despite a general awareness of the importance of establishing a prompt diagnosis for pediatric brain tumor care, delay in time to diagnosis is common with pediatric brain tumor patients, as children have a unique capacity to tolerate intracranial volume and pressure changes with minimal outward symptoms^[9]. Additionally, the immature nature of the nervous system in infants makes clinical screening for brain tumor-related symptoms challenging. Widespread radiographic screening of children, be it with computed tomography or magnetic resonance imaging, is impractical due to the risks of radiation exposure to the developing central nervous system, neurotoxic effects of early anesthetic exposure, and prohibitive diagnostic costs^[10-12].

As such, a minimally invasive, reliable, and cheaply implemented screening tool has become a great clinical need for physicians and researchers seeking to improve early tumor detection, develop minimally invasive methods for molecular stratification of tumors, and allow for reliable post-treatment screening in pediatric brain tumor populations. Numerous permutations exist for researchers seeking to discover and validate novel biomarkers, as potential tumor markers need to be examined in different biofluid reservoirs and with different analytic techniques. Each variation in biomarker sampling and analysis exposes different advantages and limitations that researchers and clinicians must be cognizant of as they search for accurate and realistically deployable brain tumor biomarker assays. The authors will review here the history of biomarker development for pediatric brain tumors to date, the limitations and advantages of current target biofluids, promising biomarkers for the most prevalent pediatric brain tumors, and the major limitations of biomarker development in pediatric populations.

Historical biomarkers

Attempts to acquire objective evidence of a tumor from extra-tumor biofluids date back at least 100 years, beginning with cerebrospinal fluid (CSF) cytology. Tumor cytology is the oldest and most well-understood method for detecting and differentiating pediatric brain tumors via a biofluid^[13]. The use of cytology to diagnose tumors in a minimally invasive manner dates back to the 19th century^[14]. The process for acquiring CSF samples for cytologic analysis has the benefit of being relatively simple to perform and widely available in most regions of the world. In order to acquire a CSF sample, a lumbar puncture is performed with a sterile 20-22-gauge needle at or below the L2/L3 interlaminar space. Ventricular CSF samples have been used, as well, for CSF cytology; but the sensitivity of samples acquired from cerebral ventricular punctures is significantly lower than samples drawn from the lumbar cistern, likely due to sedimentation of tumor cells into the dependent lumbar cistern^[15]. CSF samples are then fixed and processed for light microscopic evaluation or flow cytometry. CSF cytology can provide pathologic diagnoses in many oncologic cases. Immunohistochemical assays are also possible, making the cytologic analysis of CSF somewhat comparable to tissue biopsy in the range of qualitative information it can provide. However, CSF cytology has a low sensitivity (45%), unless performed repeatedly on the same patient, making it an inaccurate and impractical brain tumor screening modality by itself^[16,17].

Arguably, modern pediatric intracranial tumor serum biomarker assay development began in the 1960s and 70s in the field of germ cell tumor diagnostics. Research with immunoperoxidase staining of testis germ cell tumors revealed excesses in alpha-fetoprotein (AFP) and beta human chorionic gonadotropin (βHCG) production within the tissues of some tumor types^[18]. Immunostaining and radioimmunoassay techniques quickly proliferated for these compounds; and by the mid-1970s, pathologists were able to utilize AFP, βHCG, as well as other proteins, to distinguish endodermal sinus tumors, choriocarcinomas, dysgerminomas, and teratomas on immunohistochemical review and via serum-based tests.

In 1979, Jeffrey Allen, MD and colleagues at Memorial Sloan-Kettering applied serum and CSF AFP/ β HCG radioimmunoassays for use in a group of 6 pediatric patients with biopsy proven intracranial germ cell tumors. This study was not powered to allow for statistical discrimination of the results, but it demonstrated, within individual cases, high correlations between serum AFP and CSF β HCG levels and the presence of intracranial germ cell tumors. Furthermore, those patients with elevated AFP or β HCG assay levels had complete normalization of their serum and CSF levels concurrent with successful tumor radiographic response to therapy^[19]. This work has been expanded upon numerous times since, and the correlations found in Allen *et al.*^[19]'s paper have proven to be robust within larger cohorts. AFP and β HCG serum and CSF assays have now become routine components of intracranial germ cell tumor diagnostics, in some cases obviating the need for tissue sampling entirely^[20-23].

The story of AFP/ β HCG serum and CSF assays is a rare success story in the realm of clinical oncologic biomarkers for pediatric brain tumor patients, and one that has, frustratingly, not been matched since. No other intracranial pediatric tumor has a widely accepted diagnostic peripheral biomarker capable - in select circumstances - of replacing tissue biopsy in the clinical evaluation algorithm^[23]. Even for intracranial germ cell tumors, AFP/ β HCG serum and CSF assays can provide false negatives, and research continues as we seek ever more reliable biofluid-based biomarkers.

CSF biomarkers

The most studied and fruitful biofluid target for pediatric oncologic biomarkers is CSF. A normally low-protein, relatively acellular biofluid^[24], CSF is a natural choice for mining biomarker data for central nervous system (CNS) pathologies as it lies in direct contact with the whole of the CNS's pial and ependymal surfaces. Cells, both pathologic and normal, shed proteins and nucleic acids, be they naked or within extracellular vesicles, into the extracellular spaces. These compounds and vesicles will accumulate within the local biofluid compartments. In the case of the CNS, intravascular spaces, parenchymal interstitial spaces, and the intraventricular/subarachnoid spaces represent the major extracellular compartments where biomarkers can collect. With diffusion into the intravascular spaces being limited by the blood brain barrier (BBB)^[25], the CSF and interstitial spaces, theoretically, should be the most rich in cytologic, genetic, and protein markers of tumor growth^[16]. Most CSF based biomarker studies have focused on detecting: (1) tumor cells; (2) cell-free tumor DNA; (3) non-coding RNA; or (4) tumor-related metabolites. The authors have already discussed CSF cytology (i.e., detecting tumor cells) in the previous section, so we will only focus on the last three biomarker sources going forward.

Cell-free DNA

Circulating, cell-free tumor DNA (cfDNA) analysis employs several techniques to identify specific genetic sequences or screen for arrays of genetic sequences within biofluids. Methods for detection of genomic alterations in cfDNA are mainly PCR-based assays: real-time PCR, methylation-specific PCR, droplet digital PCR, and next-generation sequencing (NGS). The latter two techniques have particularly improved the sensitivity of cfDNA detection compared to older PCR-based assays^[26]. While not specifically confined to use in the CSF, it has been most successful at correlating with brain tumor pathologies when examined in the CSF, due to the fact that non-CSF biofluids typically contain a very small percentage ($\leq 1\%$) of brain tumor genetic material in a sea of normal tissue genetic material^[27]. Within CSF, cfDNA has been shown to detect the presence of a CNS neoplasm at rates significantly higher than CSF cytologic analysis^[28], as well as identify clinically relevant mutations, even when present at strikingly low frequencies ($1:1 \times 10^7$), through the use of NGS techniques like duplex sequencing^[29].

CSF cfDNA has been shown in several studies of adult intracranial tumor patients to exceed CSF cytologic detection levels when specific mutations are screened for (EGFR for glioblastoma, KRAS for non-small cell lung cancer, BRAF p. V600E for melanoma, *etc.*)^[27,28,30,31]. Among pediatric brain tumor patients,

this biomarker has been only sparsely examined and almost exclusively in CSF. Despite cfDNA's limited utilization, to date, in pediatric brain tumor biomarker development, the authors feel that it bears special discussion, due to its potential as a low-risk diagnostic for midline gliomas.

Midline gliomas represent a one of the most difficult groups of pediatric primary brain tumors to treat. Characterized by a H3K27M histone mutation^[32], these tumors are generally aggressive and often not safely surgically accessible^[33], making it difficult for clinicians to acquire biopsies. This limits the amount of molecular information available to direct therapy and inform prognosis, and it elevates the clinical need for a safe, reliable method of extracting molecular information from these tumor patients.

Using cfDNA isolated from CSF samples from pediatric brain tumor patients, researchers have made significant progress towards the detection of diffuse midline glioma histone mutations important for prognostication. A study conducted by Huang *et al.*^[34] that reviewed CSF samples from 6 pediatric patients with diffuse midline gliomas found that the H3F3A mutation could be isolated or detected in up to 83% of patients, with a specificity of 100%. This methodology has, subsequently, been confirmed in a larger cohort of 48 patients in a multi-institutional study. In that study by Panditharatna *et al.*^[35], the authors found that the H3 p. K27M mutations in CSF strongly correlated with the presence of a diffuse midline glioma with the same H3K27M mutation. The amount of detectable genetic material also correlated with the response of that tumor to radiation therapy. The accuracy of the H3 p. K27M cfDNA was 87% in this study. Interestingly, Panditharatna's group also looked at the presence of the H3 p. K27M mutant genetic material in matched serum samples, as well. They reported comparable sensitivity and sensitivity results with serum as compared to CSF, though with markedly lower quantities of detectable cfDNA^[35].

CSF-based cfDNA does have important limitations that have yet to be overcome. While sensitivity for intracranial tumor detection with CSF-based cfDNA is superior to CSF cytology in general, its sensitivity for primary CNS tumors seems to be much less than for extra-CNS neoplasm. In a study of 53 adult patients with intracranial tumors, 62.5% of patients with metastatic CNS tumors had detectable tumor-specific mutant cfDNA in the CSF, while only 50% of patients with primary CNS neoplasms had detectable cfDNA in the CSF^[27]. cfDNA detection in the CSF may be limited by anatomic factors, such as tumor location relative to CSF cisterns. A study of 35 pediatric and adult patients with primary brain tumors found that 86% of tumors abutting a CSF cistern had detectable cfDNA in the CSF, but none of the tumors with entirely parenchymal locations had detectable CSF cfDNA^[36]. Tumor biology may also limit detection as some authors have noted low to absent rates of cfDNA detection even for some ependymomas with extensive surface contact with CSF^[37].

Non-coding RNA

Non-coding RNA, predominantly miRNA, are small (18-25 nucleotides) RNA that function to regulate mRNA pre- and post-translationally. miRNA form from a precursor RNA (pri-miRNA) that is cleaved by the RNase III nuclear enzyme Drosha into a 60-70 nucleotide intermediate known as pre-miRNA, which is then exported from the nucleus via Exportin-5. Once in the cytoplasm, pre-miRNA is cleaved again by Dicer, another RNase III enzyme, into miRNA, which then complexes with a transactivation-responsive RNA binding protein and Argonaute to form an RNA-induced silencing complex^[38]. This miRNA-enzyme complex is the active unit at the end of miRNA biogenesis, and it is capable of recognizing complementary mRNA and (1) inhibiting translation initiation via cap-40S association; (2) inhibiting translation initiation via 40S-AUG-60S association; (3) inhibiting elongation; (4) causing premature ribosome drop-off; (5) causing cotranslational protein degradation; (6) causing sequestration of mRNA in P-bodies; (7) causing increased mRNA degradation; (8) increasing mRNA cleavage; or (9) effecting transcriptional inhibition by interaction with promoter sequences or increased methylation of promoters^[39].

There are currently > 1,900 known human miRNA (miRbase.org) regulating at least 30% of human genes^[40]. Table 1 lists some of the more prominent miRNA implicated in pediatric brain tumor biology^[41-43]. These

Table 1. Summary of commonly implicated miRNA alterations in pediatric brain tumors

MiRNA	Tumor types	Regulation	Potential actions	Ref.
MIR-15B	Glioma	Upregulated	Modulates multi-drug resistance and cell migration	[16,47]
MIR-21	Glioma	Upregulated	Targets PTEN, PDCD4, Bcl2 and other tumor suppressor genes.	[42,47,48]
	Medulloblastoma	Upregulated		
	Ependymoma	Upregulated		
MIR-23A	Glioma	Upregulated	May regulate cell migration and apoptosis.	[44]
MIR-34A	Ependymoma	Upregulated	Regulates apoptosis through SIRT1	[42]
MIR-124A	Medulloblastoma	Downregulated	Modulates glycolysis and cell cycle through CDK6	[43,49]
MIR-125B	Glioma	Upregulated	SHH pathway modulation and apoptosis	[16,47-49]
	Medulloblastoma	Downregulated		
MIR-128A	Medulloblastoma	Upregulated	Tumor growth and apoptosis via MYC and Bcl2	[43]
MIR-146B	Glioma	Upregulated	Suppresses stemness and migration	[44]
MIR-518B	Glioma	Upregulated	Tumor suppressor acting through PDGFRB	[50]
	Ependymoma	Downregulated		

molecules are secreted into the extracellular spaces, predominantly in microvesicles, apoptotic bodies, and exosomes. Within these vesicles, the miRNA are protected from degradation by endogenous RNases, making them an unusually stable transcript compared to other forms of RNA. Furthermore, research has shown that extracellular vesicles from tumor patients tend to be particularly enriched with tumor-related miRNA^[44,45], and these extracellular vesicles can induced oncogenic protein expression changes in adjacent, normal cells^[46].

A myriad of miRNA have been detected in the CSF of adult brain tumor patients, most prominently miR-21, miR-15b, miR-125b, and miR-223^[16,44,48,49]. These markers individually or in combination with other miRNA have demonstrated high levels of specificity and sensitivity for gliomas and medulloblastomas^[47,49]. However, there are no published articles at the time of this review analyzing the miRNA in the CSF of pediatric glioma, embryonal, or ependymal tumor patients in isolation from adult populations. The composition and clinical applicability of miRNA in the CSF of pediatric brain tumor patients remains a significant knowledge gap, at present, in the field of pediatric neuro-oncology.

Tumor metabolites and proteins

Aberrations in tumor metabolism and cell signaling pathways relative to normal tissues tends to lead to an accumulation of well-characterized small molecules that can be used to predict the presence of a malignancy; and in certain instances, even predict the molecular signature of that malignancy^[51]. Dyscrasias have been identified in all of the major metabolic cycles employed by cancer cells, including glucose metabolism, the pentose phosphate pathway, amino acid metabolism, and fatty acid metabolism, as well as many proliferative signaling pathways, such as the PI3K/AKT and WNT/ β -catenin pathways^[8,16]. Lactate, isocitrate, citrate, and D-2-hydroxyglutarate are metabolites known to accumulate in gliomas utilizing aerobic glycolysis as their dominant ATP source^[52]. D-2-hydroxyglutarate, in particular, has been shown to be elevated in the CSF of patients with IDH1 mutant gliomas, and its detection in the CSF may be a surrogate for this prognostically important mutation^[53].

As with CSF-based miRNA, very little research has been done examining the use of CSF metabolites to diagnose, track, and molecularly subtype pediatric brain tumors. What work has been done revolves around monoamine metabolism in pediatric brain tumors. In 1987, Bostrom and Mirkin looked at the concentrations of homovanillic acid (HVA), hydroxymethoxyphenylethyleneglycol (MHPG), and vanillylmandelic acid in the CSF of adult and pediatric patients with leukemia, glial, neuroectodermal, or retinoblastoma tumor histories. In their study, MHPG and VMA were significantly elevated among patients with primary CNS tumors or neuroblastoma cranial metastases, suggesting that these metabolites increased in response to disruption of the BBB or mass effect within the CNS^[54]. This work was followed in 2014 by a study by Varela *et al.*^[55] of 22 pediatric patients with posterior fossa astrocytomas, medulloblastomas,

and one case of an ependymoma. Ventricular CSF samples and serum samples were acquired and levels of MHPG, HVA, and 5-hydroxyindoleacetic acid were measured. The authors found that MHPG levels were significantly higher (19.4%) in astrocytoma patients versus medulloblastoma patients. However, none of the measured monoamine metabolites significantly varied from age-matched controls in this small study^[55].

Like tumor metabolite CSF biomarkers, most protein biomarkers investigated in CSF are part of well-defined aberrant tumor cell signaling pathways. One of the most well studied family of proteins influencing proliferation and differentiation in pediatric brain tumors is the insulin-like growth factor (IGF) family of mitogens, binding proteins, and receptors. The IGF mitogens are primarily synthesized in the liver and circulate in conjunction with any of 7 IGF binding proteins (IGFBP) to promote CNS development, among other functions. They have been implicated in the proliferation and formation of medulloblastomas and ependymomas in *in vitro* and murine models^[56,57], and IGF-1 levels have been shown to correlate with an individual's propensity for developing cancer^[58].

In a study of 35 CSF samples from pediatric patients, mostly medulloblastoma patients, IGFBP-3 levels were found to be significantly elevated in the CSF of medulloblastoma patients compared to control or ependymoma patients. This finding was comparable to mRNA levels for IGFBP-3 in the tumors themselves. The study did have several limitations that tempered the confidence that could be assigned to the results. The levels of IGFBP were overall highly correlated with total CSF protein levels, as has been the case with other CSF-based protein biomarkers for brain tumors^[59,60], suggesting that IGFBP may just be an indicator of BBB disruption, rather than a specifically oncologic biomarker. The authors did attempt to normalize IGFBP-3 levels to total CSF protein levels, and the significance of the finding remained. Another issue with the study was that correlation with tumor treatment could not be assessed, as follow-up CSF samples in all patients were taken within 2 weeks of surgical resection, potentially contaminating the samples with tumor cell slough and physiologic changes related to general anesthesia and surgery^[61]. Even so, this study suggests that the IGF signaling pathway may be leveraged in the future to identify and track disease in pediatric patients with medulloblastoma.

Another paper by Desiderio *et al.*^[62] examined changes in the CSF proteome of 14 pediatric brain tumor patients and 5 controls from the time of surgical resection and at 6 days post-surgery via top down liquid chromatography-mass spectrometry. The authors found a non-significant trend towards decreased LVV-h7 and VV-h7 hemorphins in the CSF of pediatric patients with brain tumors. In Desiderio *et al.*^[62]'s cohort, the LVV-h7 and VV-h7 protein suppression reversed in the same patients if they underwent a gross total resection of their tumor and had no metastatic tumor burden. These findings have yet to be verified in a follow-up study, and the results included CSF samples within the window of surgical contamination^[62].

A similar whole-CSF proteome approach was utilized by Saratsis *et al.*^[63] to search for biomarkers predictive of diffuse intrinsic pontine gliomas. In this study, 55% of patients with diffuse intrinsic pontine gliomas demonstrated elevated levels of cyclophilin A in their CSF, while no patients with alternate supratentorial gliomas or control patients had meaningfully detectable levels of the same protein. Those patients with elevated cyclophilin A levels also showed a greater tendency towards radiographic evidence of necrosis and rapid tumor progression, suggesting that cyclophilin A may be indicative of more aggressive forms of diffuse intrinsic pontine glioma^[63].

Lastly, a study of 33 pediatric medulloblastoma patients and 25 age matched controls in a multi-centered study of prostaglandin D2 synthase (PGD2S), a highly abundant glycoprotein found in CSF, found a 6-fold reduction in PGD2S levels in medulloblastoma patients compared to controls. This was a highly significant finding ($P < 0.00001$). The authors theorized that this may be related to a host response to the tumor, as it has been described in other brain tumor patients and in some demyelinating diseases. PGD2S CSF levels

remained suppressed after tumor patients went into remission, so it is unlikely that this protein would be useful for longitudinal screening of pediatric brain tumor patients^[64].

Limitations to CSF-based biomarkers

While relatively minimally invasive, accessing the lumbar cisterns in order to collect CSF samples is still a more difficult affair than phlebotomy, saliva collection, or urine sampling. The procedures involved in CSF sampling - lumbar puncture or ventricular puncture - require skilled, advanced practice medical personnel with knowledge of basic anatomy and sterile technique. In the case of pediatric patients, sedation is sometimes required in order to perform a lumbar puncture, and it is mandatory for ventricular access procedures. These technical difficulties currently limit the availability of patient CSF as a routine diagnostic medium and will likely frustrate attempts to use CSF-based biomarker assays for screening large populations of pediatric patients. This limits the availability of patient CSF as a routine diagnostic medium.

As has been stated earlier, CSF may also be an inappropriate source of biomarker molecules for more parenchymal based tumors, where tumor cells do not make direct contact with ependymal or pial surfaces and are, therefore, less likely to release genetic material and small molecules into CSF. In reality, 70% of pediatric brain tumors will arise in or near the 4th cerebral ventricle, making this potential limitation applicable to a minority of cases^[65].

In the same vein, though, the tendency of pediatric brain tumors to arise near the 4th ventricle in the posterior fossa makes lumbar puncture more perilous for patients, as drainage of CSF from the lumbar cistern, rarely, can create a pressure gradient across the infratentorial compartment in the setting of a large mass lesion. These pressure gradients, in severe cases, can lead to brainstem and cerebellar herniation through the foramen magnum, causing significant neural injury and even death^[66]. For this reason, routine lumbar punctures for the purposes of oncologic screening may not be deemed safe in many pediatric brain tumor patients.

Serum biomarkers

While CSF can be problematic to acquire, more peripheral biofluids, such as serum, urine, and saliva, can and are easily acquired from pediatric patients in ambulatory settings every day. In this sense, they are far more practical sources for pediatric brain tumor biomarker discovery than CSF; but discoveries in these media must be approached cautiously, as biomarkers in these biofluids are subject to dilution and filtration at multiple points between synthesis and their collection sites, sometimes greatly compromising the fidelity of the observed biomarker concentration^[67-69].

That being said, serum remains the most successful source of clinically relevant biomarkers in practice today. Examples of serum biomarkers in clinical use include prostate specific antigen, AFP, β HCG, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, and carcinoembryonic antigen^[70,71]. As is the case in adult oncology, most research into pediatric brain tumor peripherally circulating biomarkers has focused on protein biomarkers, similar to those just listed, but miRNA are also beginning to show promise as potentially specific and easily measured diagnostics for detecting, diagnosing, and tracking pediatric brain tumors. Due to their relatively rapid turnover in the peripheral blood stream, DNA and RNA based biomarkers are particularly well suited for gauging tumor response to therapy^[72].

Non-coding RNA

As with CSF, serum miRNA and other forms of non-coding RNA have been targeted for potential diagnostic and screening purposes in neuro-oncology. miR-21, miR-125b, miR-128, miR-15b, and miR-182 have all been implicated in adult glioma studies of miRNA signatures within patient sera^[73]. Serum miR-21 and miR-15b, in particular, have been shown to strongly predict glial tumors in adult patient populations, with combined

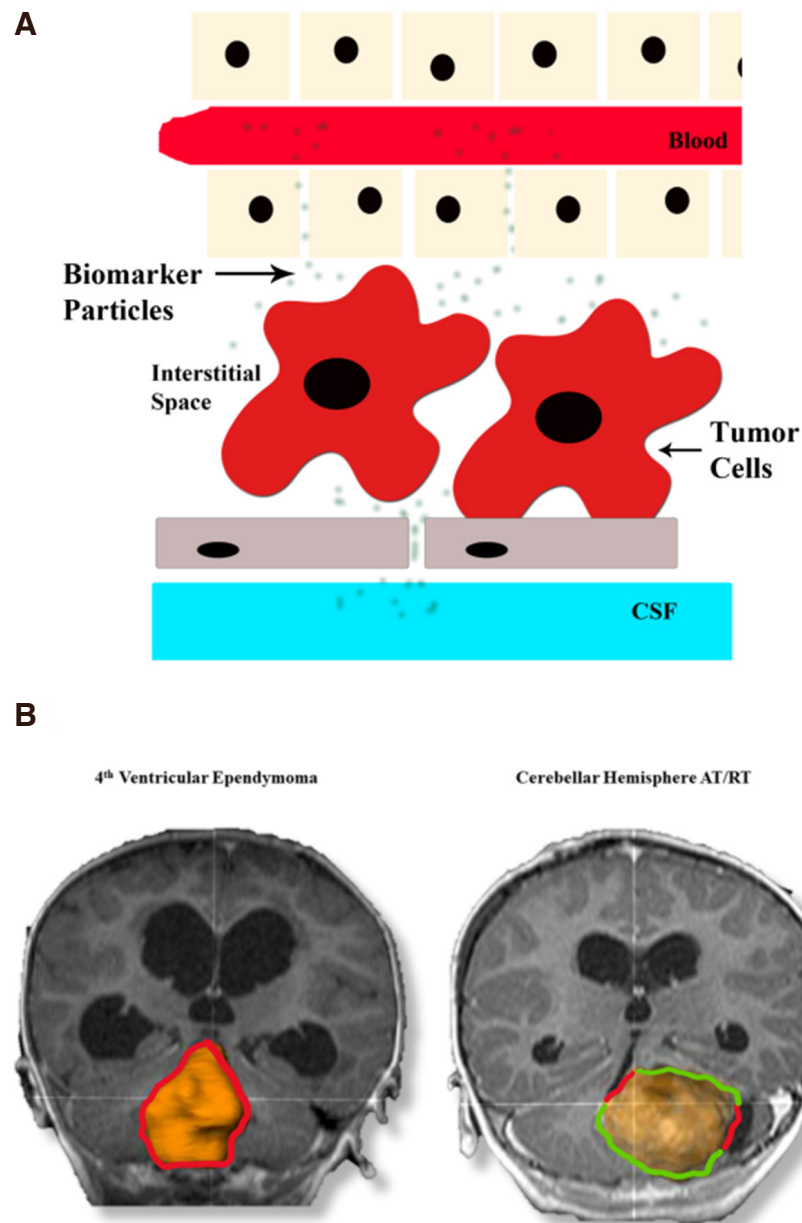


Figure 1. A: Schematic of diffusion of tumor nucleic acids, proteins, metabolites, and microvesicles into various extracellular compartments; B: Representative MRI images of an intraventricular (left) and mainly parenchymal (right) tumor with cerebrospinal fluid (CSF) contact points denoted in red and parenchymal contact points denoted in green

sensitivities and specificities of 100% in a series of 112 patients with gliomas, non-small cell lung cancer, and controls^[74]. Numerous studies examining serum miRNA signatures in gastric cancer, hepatocellular carcinoma, lung cancer, and breast cancer have also demonstrated the potentially powerful predictive value of serum miRNA in screening, risk-stratifying, and tracking cancer patients^[75-79].

In the realm of pediatric neuro-oncology, however, serum miRNA have only begun to be leveraged for tumor detection and screening. The first study to examine the potential utility of serum miRNA in detecting and screening pediatric brain tumors was done by this author and their colleagues (Bookland *et al.*^[41]) in a small cohort of pediatric juvenile pilocytic astrocytoma, ependymoma, and control patients. The authors identified four miRNA (miR-21, miR-15b, miR-23a, and miR-146b) that accurately predicted the presence, tumor nodule size, and response to therapy of juvenile pilocytic astrocytoma patients with a sensitivity of

86% and specificity of 100%. In particular, levels of miR-15b, miR-21, and miR-23a correlated strongly with tumor nodule volume (miR-15b, $R^2 = 0.86$; miR-21 $R^2 = 0.92$; miR-23a, $R^2 = 0.86$) and returned to normal levels within 24 h of gross total tumor resection. Interestingly, patients with tumors situated more deeply within the brain parenchyma demonstrated higher ratios of miRNA to tumor nodule volume than those more peripherally situated^[41]. These results further support the notion that oncologic miRNA serum levels may be affected by tumor anatomic location, just has been speculated with CSF biomarkers [Figure 1].

This pilot study evaluating the potential utility of serum miRNA as a biomarker for pediatric brain tumors is encouraging, but this initial work also highlighted significant barriers to serum miRNA development, including: (1) normalization of miRNA profiles; (2) low miRNA yields compared to tissue or CSF sources; (3) and a poor understanding of relative contributions tumor and host tissues make to the final serum miRNA signatures^[41,71]. One significant example of these challenges can be demonstrated in observing the effect that general anesthesia has on select serum miRNA levels. miR-125b, which has been implicated as an oncomir in gliomas and medulloblastomas^[80,81], is suppressed in humans and rodents after exposure to general anesthetic agents^[41,82]. Yet, few miRNA biomarker studies have controlled for the effects of anesthesia miRNA levels. Indeed, controlling for anesthesia in pediatric clinical trials is an ethically and technically complicated matter that adds a layer of complexity to serum miRNA interpretation of which clinical researchers must be cognizant. This example highlights the importance of developing a thorough understanding of the interplay between oncologically-relevant miRNA and the normal functions they regulate so that confounding host and external forces acting on miRNA of interest can be controlled.

Protein/small molecule

As mentioned previously, protein and small molecule serum biomarkers have been extensively studied, and they represent, perhaps, the most significant cohort of potential biomarkers for pediatric brain tumors. An exhaustive list of all proteins and small molecules implicated in pediatric brain tumor diagnostics and prognostics is beyond the scope of this brief review, but we will touch on some of the more widely reported biomarkers in the literature.

Osteopontin is an extracellular matrix protein expressed in a wide variety of tissues and involved in mineralization, immune modulation, cell migration, and anti-apoptosis^[83]. It has been shown to be overexpressed in atypical teratoid/rhabdoid tumors (AT/RT), as well as several other CNS tumors. In a series of 39 pediatric patients with AT/RT, medulloblastoma, epilepsy, or hydrocephalus, serum and CSF levels of osteopontin were shown to be significantly elevated in AT/RT patients versus medulloblastoma patients (~2:1 in serum) and versus control patients (~4:1 in serum). Serum osteopontin levels also correlated with AT/RT response to therapy, and higher serum levels were associated with a more malignant disease course^[84]. This work raises the possibility that osteopontin may be a highly specific and reliable biomarker for risk-stratifying AT/RT patients and gauging their response to therapy.

Another protein involved in a variety of carcinogenic signaling pathways, including proliferation, immunomodulation, migration, anti-apoptosis, and chemotherapy resistance, is metallothionein. Different isomers of metallothionein have been implicated *in vitro* in inducing chemoresistance in neuroblastoma, melanoma, rhabdomyosarcoma, and medulloblastoma cell lines^[85-87]. A group of researchers, using differential pulse voltammetry to measure serum metallothionein levels, attempted to exploit metallothionein's association with oncogenesis to see if it could be reliably detected at supernormal levels in pediatric solid tumor patients, including in 10 medulloblastoma patients and 4 ependymoma patients. All 38 pediatric solid tumor patients in this study had serum levels of metallothionein roughly 6-8-fold higher than adult controls ($P < 0.05$)^[88]. This work strongly suggests that serum metallothionein may be useful as a non-specific oncologic biomarker in pediatric patients, though additional work is needed to confirm the results with age-matched controls.

A larger study of 106 pediatric patients with brain tumors, including gliomas, medulloblastomas, and ependymomas, studied the relationship between serum levels of the pro-angiogenic growth factors and pediatric brain tumors. In this study, serum vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) levels were measured at presentation and following surgical resection or debulking of the tumor. While the authors of the study found no statistically significant changes in bFGF levels between groups, serum VEGF was 16.4% higher ($P = 0.05$) in pediatric brain tumor patients compared to controls. Interestingly, subset analysis of only the glioma patients showed that serum VEGF levels were 16.4% lower ($P < 0.05$) than controls^[89]. This is a curious result given glioblastoma and other high-grade glial tumors are known to overexpress VEGF^[90]. The authors also found no statistically significant difference in serum VEGF or bFGF levels in any of the post-surgical patients, regardless of the degree of tumor resection achieved^[89]. These results are, in the end, difficult to reconcile; and this may reflect the extremely diverse collection of pediatric brain tumors included.

Behrends *et al.*^[91] in a study of 40 pediatric cancer patients, including 10 brain tumor patients, employed a less conventional method to identify humoral targets for biomarker development. In contrast to other biomarker development projects that rely on a priori knowledge of cell signaling proteins known to be abnormal in the disease process of interest, Behrend's group used SEREX technology to identify potential humoral targets, in some cases finding cancer antigens with no described function. The group utilized autologous sera and serially screened these sera against autologous cDNA expression libraries in 4 pediatric medulloblastoma patients. From this, the group identified 15 antigens. Humoral responses to these 15 antigens were then tested in the 40 pediatric cancer patients, as well as in 40 pediatric controls. Antibodies were found to 5 of the 15 antigens exclusively in pediatric cancer patients. The authors noted, though, that the humoral responses to these antigens was not uniform. Only 2-3 out of 5 medulloblastomas had detectable antibodies to any one antigen, and the humoral responses varied over time depending on response of the tumor to therapy. The authors also point out that humoral responses may change, as well, due to mutations in some tumors that support immune evasion^[91]. Taken in balance, SEREX screening for humoral biomarker targets is an innovative technique for identifying novel pediatric brain tumor biomarkers, but clinical application is likely to be limited by the marked variability in humoral response across patients.

Limitations to serum biomarkers in pediatric brain tumor patients

While familiar and easily accessible, serum sources for biomarkers do have significant drawbacks. As a peripheral biofluid, the milieu of serum proteins and genetic material is affected by every organ system in the human body. Even large amounts of oncologic biomarkers can easily be diluted out by normal serum components^[35]. Additionally, it is difficult to know what sources - host or tumor - are driving the targeted biomarker concentration levels. Studies have shown that even epigenetic factors, such as a change in diet, can significantly alter measured levels of some serum biomarkers for adult cancers^[38]. As with CSF, the location and biology of pediatric brain tumors also seems to play a part in serum levels of some presumptive biomarkers, with certain tumor-related proteins being undetectable outside of the BBB^[61,63].

Urine

Urine is a filtrate of serum via the glomeruli, and it represents a particularly easy and well-studied biofluid to obtain from children. Excreted oncologic biomarkers such as metanephrine and VMA have been employed clinically for decades as routine components in the work-up of several catecholamine producing solid tumors, including pheochromocytomas and neuroblastomas in both children and adults^[92].

A paper by Pricola Fehnel *et al.*^[93] in 2016 examined the use of urinary bFGF, MMP13 and TIMP3 levels as biomarkers for juvenile pilocytic astrocytomas. The study followed 21 astrocytoma, 17 medulloblastoma, and 21 control pediatric patients with serial urine samples pre-tumor treatment and post-tumor treatment. The authors also examined bFGF and TIMP3 levels in primary juvenile pilocytic astrocytoma cell line conditioned media. They found a significant elevation in bFGF and TIMP3 urine concentrations among the

astrocytoma patients, with a combined sensitivity and specificity of 100% and 95%, respectively. Conditioned media from cultured primary astrocytoma cells also showed relative enrichment of bFGF and TIMP3, concordant with the findings in patient urine samples. Among 9 patient samples where pre- and post-treatment imaging was available, bFGF and TIMP3 levels also significantly correlated with tumor volume changes on imaging^[93].

Another, earlier project from the same group looked at VEGF, MMP2, and MMP9 in urine and CSF samples from a cohort of 28 brain tumor patients ($n = 11$ pediatric, $n = 17$ adult) and 23 control patients. Urinary concentrations of VEGF, MMP2 and MMP9 were all significantly increased within the tumor patient cohort; and urinary VEGF and MMP2, in particular, had excellent sensitivity and specificities for the presence of a brain tumor. VEGF had 95.2% sensitivity and 89.5% specificity, while MMP2 demonstrated 82.1% sensitivity and 95.7% specificity. Both of these proteins were undetectable in the urine of 5 patients for whom follow-up imaging demonstrated complete resolution of their tumors^[94].

Both of these studies are very encouraging for the prospect of developing a clinically deployable and accurate laboratory assay for pediatric brain tumors. However, as with all biomarker sources, urine has special limitations as a diagnostic media that have still not completely been addressed in either of the aforementioned studies. Nolen *et al.*^[69] laid out in an extensive analysis of over 200 potential urinary biomarkers the effects of normalization methods, population variability, and temporal variability on measured biomarker concentrations. The authors of this study found that, while urine total protein had the smallest impact on biomarker variability, none of the normalization methods tested (urine creatinine, urine albumin, urine B2M) was clearly superior to the other. Additionally, the authors observed significant intra- and inter-day variability in urine biomarker concentrations; in most cases, the coefficients of variation exceeded 50%^[69]. These findings should prompt caution when interpreting urinary biomarker data, as robust differences in biomarker concentrations between diseased and normal states will be needed to consistently overcome such high levels of biomarker variability. Even so, the work by Pricola Fehnel *et al.*^[93] suggests that such high levels discrimination may indeed exist for some pediatric brain tumor urinary biomarkers.

Challenges of developing biomarkers from pediatric populations

Compared to the adult neuro-oncology world, the development of circulating biomarker candidates for pediatric brain tumor patients is still in its infancy. This is partially due to a significant statistical barrier to research within pediatric brain tumor patient populations. There are an estimated 4,600 new pediatric brain tumor diagnoses each year in the United States of America^[1]. This compares to an average of 22,172 new adult primary brain tumor diagnoses per year^[95]. As these statistics demonstrate, pediatric brain tumor cases are relatively rare, and individual institutions often do not have enough volume to organize large scale studies on their own. Multi-centered trials and novel screening assays are needed in order to create study populations with enough power to generate meaningful conclusions.

Another issue is the lack of comparative data across biofluids and pediatric brain tumor clinical stages for individual biomarker candidates. As mentioned in this review article, there exist significant theoretical and practical limitations to each biofluid currently targeted for biomarker discovery. Additionally, there is mounting evidence that tumor stage and location relative to the biofluid of interest can alter biomarker yields^[36,41,96]. A large study examining one or more biomarkers in multiple biofluids serially across early and late stages of brain tumor therapy will be needed to answer the question of which biofluid is most suitable for diagnosing and screening which pediatric brain tumors. This is a daunting ask given the difficulties researchers face accruing and maintaining large population pediatric brain tumor studies. A study by Pages *et al.*^[97] is currently underway examining the cfDNA in the CSF, serum, and urine of 192 pediatric brain tumor patients, but results are not available as of the production of this manuscript. It is hoped that this study and others to come like it may help to define the effect of biofluid choice on biomarker sensitivity and specificity for pediatric brain tumors.

Table 2. Summary of select works identifying circulating biomarkers for pediatric brain tumors

Source	Marker(s)	Method	Tumor type	Finding	Ref.
CSF	Type 1 collagen IGFBP4 Procollagen C-endopeptidase enhance 1 Glial cell-line derived neurotrophic factor receptor $\alpha 2$ Inter-alpha-trypsin inhibitor heavy chain 4 Neural proliferation and differentiation control protein-1	LC-MS/MS, RPPA, ELISA	Mixed Brain Tumors	Protein signatures may discriminate metastatic tumors from controls	[98]
CSF	H3F3A /HISTIH313 mutations	Sanger sequencing (DNA isolation, nested PCR) of cfDNA	Diffuse midline glioma	Feasibility and specificity of H3K27M detection in DNA from CSF	[34]
CSF	Prostaglandin D2 synthase	Proteomics MALDI-TOF/ELISA	Medulloblastoma	PGD2S ↓	[64]
CSF	Apolipoprotein A-II	SELDI-TOF mass spectrometry	Brain tumor	ApoAII level ↑	[59]
CSF	LVV VV-hemorphin-7	Proteomics LC-MS	Mixed Brain Tumors	LVV-/VV-h7 ↓ in tumor patients	[62]
CSF	c-Tau	ELISA	Medulloblastoma Ependymoma Astrocytoma	c-Tau ↑	[99]
CSF	CyclophilinA Dimethylarginase 1	Proteomics, mass spectrometry	DIPG	↑ cCypA and DDAH1	[63]
CSF	cfDNA	Genome-wide sequencing	High Grade Glioma JPA Medulloblastoma Ependymoma	cfDNA detected in 74% of tumor patient CSF	[36]
CSF, serum	Human chorionic gonadotropin	Enzyme immunoassay	Germinoma	HCG ↑ in patients with recurrence events	[22]
CSF, plasma	IGF, IGFBPs, IGFs	Microarray, RIA, western	Ependymoma Medulloblastoma	IGFBP-2/IGFBP-3 ↑ tumor patients	[61]
CSF, plasma	Osteopontin	ELISA, qPCR	Medulloblastoma AT/RT	Osteopontin ↑ in AT/RT recurrence	[84]
CSF, serum	AFP beta-hCG	Immunoenzyme	Germinoma NGGCT	↑ AFP in serum ↑ bHCG in CSF	[20]
CSF, serum	miR-371a-3p miR-372-3p miR-373-3p miR-367-3p	qRT-PCR	Germ cell tumors	miRNA ↑	[100]
CSF, plasma	H3K27M-mutant cfDNA	Digital drople PCR	Diffuse midline glioma	H3K27M mutant cfDNA correlates with treatment response	[35]
Blood/serum	ERCC2 methylation	Illumina Beadchip	Medulloblastoma	ERCC2 promoter methylation ↑ correlates with poor prognosis	[101]
Serum	Metallothionein	Differential pulse voltametry	Medulloblastoma Ependymoma	↑ in cancer patient's serum	[88]
Blood/serum	miRNA profile	qPCR	JPA	↑miR21, mir15b, miR23, miR146b correlated with tumor mural nodule size	[41]
Serum	VEGF bFGF	ELISA	High grade glioma	VEGF ↑	[89]
Urine, CSF	MMP-2 MMP-9 MMP-NGAL VEGF	ELISA	JPA	MMP-2, MMP-9, MMP-NGAL, VEGF levels ↑	[94]
Urine	bFGF TIMP3 MMP-13	ELISA	JPA	bFGF, TIMP3 ↑ MMP-13 ↓	[93]
Urine	Netrin-1	ELISA	Medulloblastoma	Netrin-1 ↑ in metastatic medulloblastoma	[102]

CSF: cerebrospinal fluid; cfDNA: cell-free tumor DNA; IGF: insulin-like growth factor; IGFBPs: IGF binding proteins; VEGF: vascular endothelial growth factor; bFGF: basic fibroblast growth factor

CONCLUSION

Pediatric brain tumors remain the most significant oncologic cause of death among children, and pediatric brain tumor diagnosis and longitudinal screening is heavily reliant on MRI - a very limited and expensive screening tool. The creation of reliable and easily deployed biomarker-based assays could dramatically improve pediatric brain tumor detection. Early detection could, in turn, improve current care by allowing for early surgery. Patient therapy could also be tailored based on biomarker-directed tumor risk stratification.

While there are currently many circulating biomarker candidates in CSF, serum, and urine, all the studies to date are hobbled by small cohort size and often mixed populations of tumor types [Table 2]. Follow-up multi-centered studies have failed to materialize for most biomarkers, leaving the validity of virtually all of these candidate pediatric brain tumor biomarkers in doubt. Hopefully, with continued interest, time, and collaboration, these current research needs will be met.

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Authors' contributions

Made substantial contributions to conception and design of the review and performed data collection: Bookland MJ

Performed data acquisition, as well as provided administrative, technical, and material support: Kolmakova A

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All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

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Review

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Sensitive and specific detection of circulating tumor cells promotes precision medicine for cancer

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Abstract

Circulating tumor cells (CTCs) have the potential to provide genetic information for heterogeneous tumors, which may be useful for monitoring disease progression and developing personalized therapies. However, the isolation of CTCs for molecular analysis is challenging due to their extreme rarity and phenotypic heterogeneity, which hinders the transformation of CTCs into traditional clinical applications. In order to achieve clinically significant CTC detection, devices utilizing novel microfluidics and nanotechnology have been developed to achieve high sensitivity and specificity capture of CTCs. In this review, we discuss these newly developed devices for CTC capture and molecular characterization for early diagnosis and determining ideal treatment regimen to better manage these cancers clinically. In addition, the potential prognostic values of CTCs as treatment guidelines and that ultimately contribute to realize personalized treatment are also discussed.

Keywords: Circulating tumor cells, sensitivity, treatment, precision medicine



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INTRODUCTION

The main cause of cancer-related mortality is cancer metastasis. During this greatly complicated and multi-stage disseminative process, tumor cells (the seeds) detach from primary roots, shed into blood and lymph circulation, undergo the immune attack and shear stress, travel to preferable metastasis soil, and eventually seed and proliferate to develop metastases. On their way to the potential organs, these circulating tumor cells (CTCs) undergo epithelial-mesenchymal transition (EMT)^[1], thereby resulting in enhanced motility and migratory ability that facilitates vasculature invasion. Upon reaching a suitable niche, the CTCs undergo mesenchymal-epithelial transition (MET), subsequently reacquiring the stem cell properties and reactivating proliferative capability to colonize at metastatic sites^[2]. In order to prevent and surveil the development of metastasis disease, especially metastatic carcinoma, the detection and characterization of CTCs are of great interest to scientists. CTCs were first detected in cancer patient in 1869 by Australian physician named Thomas Ashworth. In the past couple of decades, numerous studies have suggested that the presence of CTCs in the blood of cancer patients has the clinical potential as a noninvasive diagnosis marker and a prognosis indicator known as a “liquid biopsy” to replace traditional invasive biopsy, whilst also facilitating technical advances for detection of CTCs.

CTC analysis has a variety of clinical applications, including real-time non-invasive monitoring of CTCs as biomarkers for new cancer therapies as well as identifying new potential therapeutic targets that directly inhibit cancer metastasis. Although the potential applications of CTC analysis appear to be very promising, due to the rarity (one CTC per billion hematologic cells) and heterogeneity (e.g., differences in morphology and gene expression) of CTCs in the blood of cancer patients, there are few commercially available techniques for clinical use. High sensitivity and specificity of CTC detection methods thus have a great impact on improving patient outcomes. Therefore, currently available technologies for CTC detection have become increasingly more sensitive and reliable, with the goal of early cancer detection and thus successful cancer treatment. An important new direction in this field is the development of devices and materials that provide information beyond CTC enumeration. Integrated devices allow for the separation of heterogeneous CTCs to facilitate a more in-depth characterization of these cells (e.g., phenotypic and molecular profiling) to develop a personalized treatment plan. Nanomaterials and microfluidic-based nanotechnologies may be the most promising strategies for implementing ideal CTC capture devices to replace traditional tools, primarily relying on their small size, high throughput capacity and large surface-to-volume ratio to solve the problem of CTC heterogeneity^[3]. In this review, we will provide an overview of current CTC isolation strategies and molecular characterization with brief insights into the potential clinical implications of CTC capture and characterization.

SENSITIVE CTC ISOLATION METHODS

CTCs may have the potential to predict the disease progression in patients with early-stage or advanced cancer, even before the formation of primary tumor. However, the extreme rarity of CTCs in blood poses a challenge for detecting CTCs from blood; for example, one study indicated that only 1.43% of 350 metastasis cancer patients had ≥ 500 CTCs/7.5 mL blood^[4]. Inability to draw large volume of blood from patients highlights the need for improved CTC isolation methods to achieve sensitive and specific CTC detection in small sample volumes. CTC isolation methods have been developed based on either biological (surface antigen, cytoplasmic protein, invasion capacity, *et al.*) or physical (size, density, deformability and charge, *et al.*) properties of tumor cells. We discuss the most popular technologies and latest advances in the following sections [Figure 1].

Detection of CTCs based on their biological properties

Immunomagnetic beads-based isolation

The most widely used enrichment method is a positive selection method based on the epithelial cell adhesion molecule (EpCAM) antibodies^[5-7]. So far, CellSearch System (Menarini Silicon Biosystems, Italy) is the first and also the only one being up to the standard of US Food and Drug Administration (FDA),

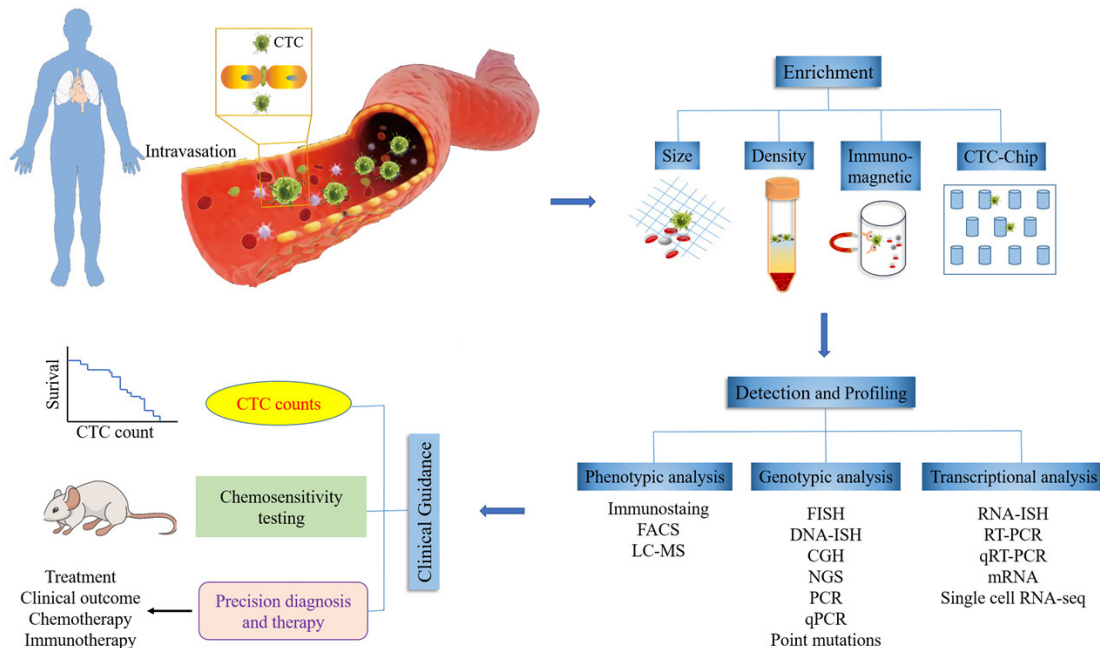


Figure 1. Illustration of current applications of CTC technologies. The CTCs exit the primary tumor and intravasate into the bloodstream. CTCs are enriched through various CTC isolation technologies such as size, density, immunomagnetic and CTC-Chip. Detection methods are utilized to detect CTCs based on phenotypic, genotypic and transcriptional analysis. The clinical applications of isolated and detected CTCs. The CTC count is associated with the potential of patient's survival. CTCs can be good chemotherapy monitoring markers for predicting drug sensitivity/resistance in preclinical and clinical settings. CTC: circulating tumor cells; FACS: fluorescence activated cell sorting; LC-MS: liquid chromatograph-mass spectrometry; FISH: fluorescence in situ hybridization; CGH: comparative genomic hybridization; NGS: next-generation sequencing; PCR: polymerase chain reaction; qRT-PCR: quantitative reverse transcription-polymerase chain reaction

which consists of the CellTracks Autoprep and the CellSearch Epithelial Cell kit, integrating EpCAM based immunomagnetically enrichment, 4',2-diamidino-2-phenylindole (DAPI) based cell nuclei staining, CD45-Allophycocyan specified leukocyte negative selection and cytokeratin 8,18,19-Phycoerythrin specified epithelial cells positive selection into an objective indicator (EpCAM+, DAPI+, CD45-, cytokeratin+) of CTC counts. In 2004, it was cleared for monitoring the outcome of therapies and optimizing clinical decision for breast cancer; later, it was also cleared for use in prostate and colorectal cancers. Through the CellSearch, which has become the benchmark for all other CTCs isolation methods, CTC counts have been associated with prognosis for progression-free survival (PFS) and overall survival (OS) in these three kinds of metastatic cancer^[8-10].

Although clinical correlations have been identified, methods for large scale isolation of CTCs from peripheral blood are lacking, therefore, efforts have been focused on improving the isolation sensitivity and efficiency. Talasaz *et al.*^[11], reported a magnetic sweeping device (MagSweeper, Stanford University, Stanford) consisting of a nonadherent plastic sheath covered magnetic rod with anti-EpCAM antibody functionalized beads, allowing for a ~60% capture efficiency to target cells and a purity of 100% for HLA-A2 cells. Ephesia technology integrated anti-EpCAM functionalized self-assembled magnetic beads with microfluidics, demonstrating a capture efficiency > 94%^[12]. Similar immunomagnetic platforms [Figure 2A] also included the Magnetic Sifter with magnetic pores incorporated into a microfluidic chip^[13]. Moreover, compared with CellSearch system, Adna Test (Qiagen, Hannover), a highly specific immunomagnetic cell-isolation system, with its improved antibody cocktails provided an effective approach to increase the efficiency of CTCs capture and complements the CellSearch for detection of CTCs^[14]. Mayo *et al.*^[15] utilized MACS cell separation platform (Miltenyi Biotec) based on a mixture of cytokeratin (CK) coated magnetic beads to isolate CTCs in lung cancer patients.

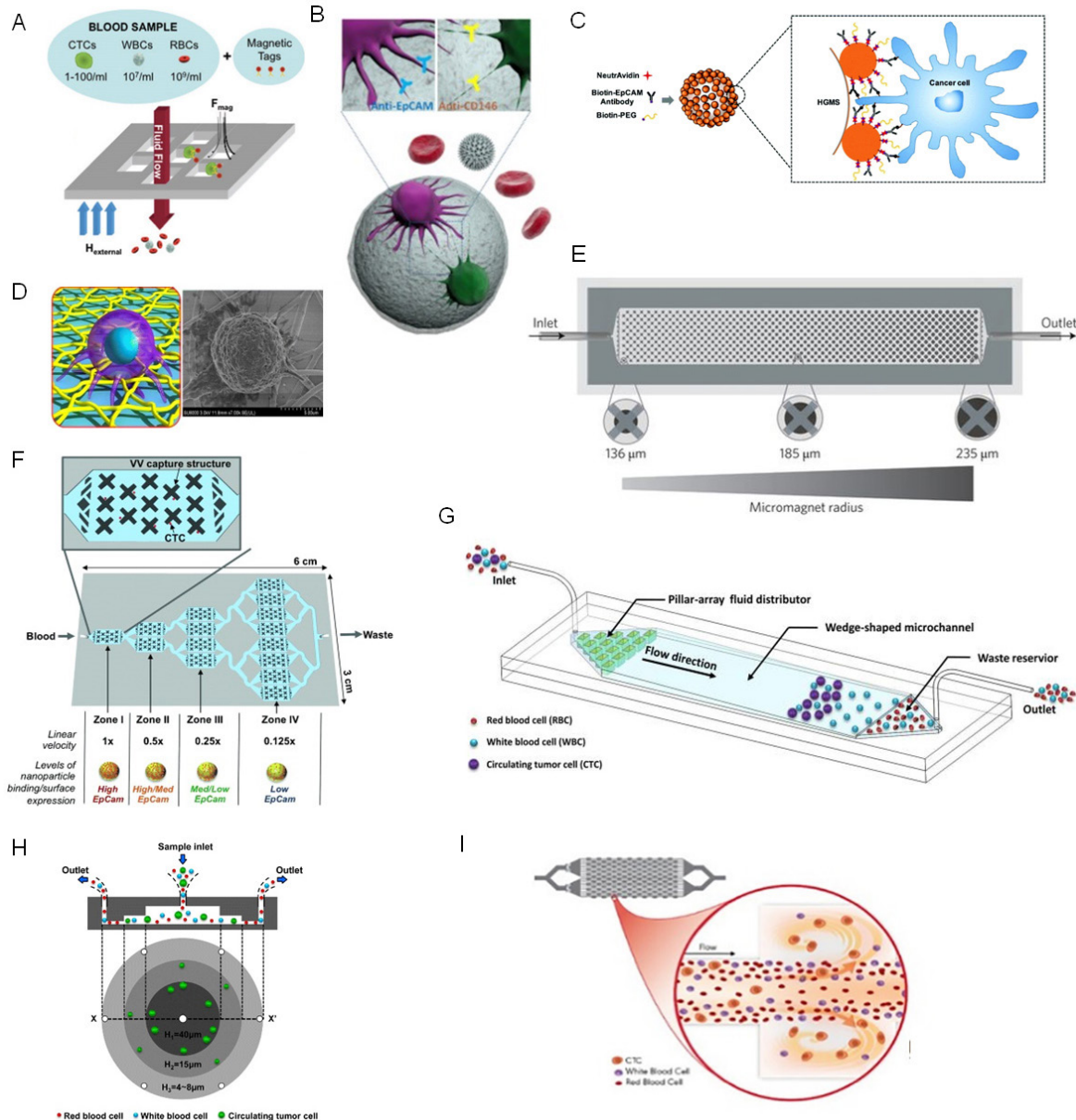


Figure 2. Strategies for CTC enrichment. A: A magnetic sifter device for CTC isolation when a magnetic field is applied. Magnetically labeled CTCs are captured at the edges of the pores, while unlabeled cells pass through the pores under fluid flow; B: dual antibodies (anti-EpCAM and anti-CD146) and biodegradable gelatin nanoparticle-coated microbeads for the capture of mesenchymal CTCs; C: a microfluidic device embedded a wedge-shaped microchamber for cell separation based on multiple biophysical properties; D: a 3D bionic cytosensor with PLGA nanofibers for CTC capture; E: vortex technology exploited for CTC isolating; F: a multizone velocity valley device for isolating heterogeneous CTCs in four different regions of varying linear velocities; G: the hollow glass microspheres with nanotopographical structures (NSHGMS) for excellent CTC isolation; H: a microfluidic device embedded a pyramid-shaped microchamber for size-based CTC separation; I: a MagRC approach for separating and in-line profiling of heterogeneous CTCs. A: Copyright Royal Society of Chemistry, 2013. Reproduced with permission from reference^[13]; B: Copyright Ivyspring International Publisher, 2013. Reproduced with permission from reference^[27]; C: Copyright Royal Society of Chemistry, 2018. Reproduced with permission from reference^[42]; D: Copyright BioMed Central, 2018. Reproduced with permission from reference^[43]; E: Copyright Nature, 2017. Reproduced with permission from reference^[45]; F: Copyright Wiley, 2015. Reproduced with permission from reference^[46]; G: Copyright Institute Of Electrical And Electronics Engineers, 2018. Reproduced with permission from reference^[52]; H: Copyright Springer, 2018. Reproduced with permission from reference^[53]; I: Copyright Wiley, 2018. Reproduced with permission from reference^[54]

However, a number of studies have shown that the levels of CTCs estimated by EpCAM-based methods including CellSearch, is uncorrelated with prognosis in patients with some types of carcinomas. Most of the evidence attributes this inconsistency to the large degree of heterogeneity in CTCs. Specifically, CTCs might undergo full (or partial) EMT during dissemination, resulting in several phenotypes including epithelial, mesenchymal or hybrid (epithelial/mesenchymal) CTCs. These subpopulations of cells may insufficiently bind to antibodies, thereby evading detection^[16,17]. Therefore, a lack of sensitive and specific biomarkers still hinders the isolation and detection of CTCs. Recent studies provide some probabilities. Here are some examples of successful markers. Glycan sialyl-Tn (STn) is often associated with cancer metastasis and expressed in metastatic colorectal and bladder tumors. Neves *et al.*^[18] fabricated a STn affinity-based microfluidic device for specifically isolating STn+ CTCs, following an enzyme-based method to recover viable CTCs for downstream analyses. It showed greatly higher isolation efficiency from the blood of patients with advanced bladder and colorectal cancers. Plastin3 (PLS3) is expressed in metastatic cancer cells but absent in normal cells^[19]. Similarly, telomerase which is expressed at high levels in almost all the cancer cells, but not in normal cells, plays an important role in cancer immortality by replenishing chromosome ends^[20]. Green fluorescent protein (GFP) fused adenoviral was employed as a probe to target telomerase in cancer cells, and this strategy was applied to detect and isolate CTCs in Non-Small Cell Lung Cancer (NSCLC) to evaluate response to radiation therapy and to potentially detect recurrence and progression of disease. Oncofetal chondroitin sulfate (ofCS) is expressed in both epithelial and mesenchymal tumor cells, as well as the cells that have undergone EMT, suggesting that it may be an ideal candidate for isolating and analyzing CTCs^[21,22]. Agerbæk *et al.*^[23] employed recombinant VAR2CA (rVAR2) to efficiently target ofCS expressed CTCs from patients with hepatic, prostate, pancreatic or lung cancer, allowing for isolation of a larger and more diverse population of CTCs compared to anti-EpCAM-antibody approaches. More recently, Ding *et al.*^[24] detected Folate receptor (FR) positive CTCs in peripheral blood from 200 patients with lung adenocarcinoma, and further determined that FR+ CTC number could be used for screening solitary pulmonary nodules (SPNs) in patients and diagnosing early-stage lung cancer with sensitivity of 70.2% and specificity of 79.3%. Meanwhile, more specific biomarker for specific subgroup of CTCs is of interest. Cyclooxygenase-2 (COX-2) has been implicated in transforming growth factor- β (TGF- β 1) mediated EMT progress^[25] and has a higher level of expression in subpopulations of mesenchymal CTCs correlated with distant metastases^[26]. These results suggested FR might be a novel biomarker for isolation and therapy targets. A subpopulation of tumor cells can express cluster of differentiation 146 (CD146) during EMT process, during which EpCAM expression is reduced. Therefore, Huang *et al.*^[27] [Figure 2B] designed dual antibodies (anti-EpCAM and anti-CD146) and biodegradable gelatin nanoparticle-coated microbeads to improve the capture of mesenchymal CTCs, achieving high efficiency (> 80%) and high cell viability (92.5%).

All aforementioned *ex vivo* detection systems require substantial quantities of blood. The GILUPI CellCollector (NANOMEDIZIN), approved by Conformite Europeenne in 2012, is another commercial EpCAM positive based selection device and is the first developed *in vivo* CTC isolation system to overcome the limitations of blood sample volume^[28-30].

Except those EpCAM-based positive selection, CD45 negative selection is applied to deplete the CD45+ cells, mostly using RosetteSep system (Stem Cell Technology, Vancouver), and to analyze the EpCAM-negative CTCs in combination with EPISPOT (Epithelial Immunospot assay, France). Ramirez *et al.*^[31] first evaluated the EPISPOT assay on a large cohort of metastatic breast cancer patients with a positive rate of 59% compared with the 48% positive rate using CellSearch, demonstrating its clinical prognostic relevance.

Microfluidic and nanotechnology-based CTC devices

Microfluidic devices enable efficient processing of complex blood samples with minimal damage to target cells. Owing to the synergistic benefits of the microfluidic devices and immunomagnetic separation, microchip-based immunomagnetic technologies are also commonly used for CTC detection. The most representative

microfluidic device based on anti-EpCAM for CTCs isolation is a microscope slide sized CTC-Chip with a mass of geometrically distributed microposts coated with anti-EpCAM^[32]. A 98% viability of captured CTCs was reported with minimal preprocessing and low flow stress^[33]. Ozkumur *et al.*^[34] developed an automated platform, termed “CTC-iChip”, combining the strengths of microfluidics and the benefits of magnetic-based cell isolation for single-cell separation. This CTC-iChip was able to detect the *EML4-ALK* gene fusion in lung cancer, suggesting that it could be a promising tool for clinical diagnosis. Then Stott *et al.*^[35] developed a microvortex-generating herringbone (HB)-Chip for effective capture of CTCs. The micromixer device was fabricated to enhance the cell-surface interaction. Subsequently, improvement was achieved by employing nanostructured substrates and chaotic micromixers, increasing the recovery rate up to 95%^[36].

For the sake of increasing the sensitivity of capturing exceedingly rare CTCs, many efforts have been made to fabricate nanostructures into the microfluidics to increase the interaction between ligands and cells; such devices include electropolymerized polymer nanodots^[37], electrospun TiO₂ nanofiber^[38], and silicon nanowires^[39-41]. More recently, Dong *et al.*^[42] [Figure 2C] utilized a nanotopographical surface (^{NS}HGMS), based on the CTC isolation technology of anti-EpCAM antibody modified Self-floating hollow glass microspheres (HGMS), to achieve excellent capture performance (93.6% ± 4.9% efficiency and 30 cells/mL detection limit in 20 min). A preferable method was based on a combination of advantages of different approaches. Wu *et al.*^[43] [Figure 2D] tactfully fabricated a 3D bionic cytosensor with electrospun polymers (PLGA) nanofibers crosswise stacked on Ni micropillars for better CTC filopodia climbing, subsequently coupled with immuno-selection by anti-EpCAM quantum dots, demonstrating a sensitive detection range and limit of 10¹-10⁵ cells/mL and 8 cells/mL, respectively, as well as a recovery range of 93.5%-105%.

However, fabricating these nanoscale substrates is time-consuming. Sheng *et al.*^[44] developed a microfluidic device combined with DNA aptamer modified gold nanoparticles (AuNPs) to enhance the capture performance without elaborate establishment for nanostructure. When compared with aptamer on the surface alone, the binding efficiency of AuNPs-aptamer showed a 39-fold increase and the capture efficiency rose from 49% to 92%. In order to profile the dynamic phenotypes of rare CTCs, Poudineh *et al.*^[45] [Figure 2E] fabricated a magnetic nanoparticles-enabled ranking cytometry (MagRC) approach to separate and in-line profile heterogeneous CTCs based on the longitudinal profile of magnetic field gradients. They demonstrated that this device was capable of profiling CTCs with higher sensitivity at a single-cell resolution in unprocessed blood from cancer patients compared to other previously developed magnetic sorting techniques. Similarly, an immunomagnetic nanoparticle-mediated binning and profiling approach [Figure 2F] was developed to separate CTCs with different phenotypes based on the differential expression of surface markers^[46]. The CTC subpopulations could be spatially sorted in different compartments of a fluidic chip, providing a powerful means to sort heterogeneous CTCs and investigate EMT in patient CTCs.

Detection of CTCs based on their physical properties

CTCs undergo cellular processes (EMT, MET, *et al.*) during dissemination, resulting in a number of phenotypes. Thus, it is important to determine which CTC fractions possess greater metastatic potential and/or stronger resistance to immune surveillance and medical treatment. In this case, CTCs would have better prognostic and therapeutic values. The aforementioned methods depend on specific markers of interest for isolation; however, subpopulations of CTCs lacking the markers may be unintentionally overlooked. Therefore, additional methods that could serve as complements to protein markers are urgently needed.

Size-based CTC isolation

Alternative methods that isolate CTCs dependent on physical properties have been developed to replace or complement the antibody-based isolation methods. Most of the CTCs are believed to be larger than normal blood cells (leukocytes, erythrocytes). And pores with ≈ 8 μm in diameter have been shown to be appropriate for CTC detainment.

Thanks to various advantages, such as retention of cell morphology, antigen independence, and high sensitivity and specificity, membranous filter devices, for example, isolation by size of epithelial tumor cells (ISET) (Rarecells Diagnostics, Paris, France), have caught more attention in CTC researches. In a comparative study, Bai *et al.*^[47] estimated the clinical effect of CTCs by using CellSearch system and ISET devices among patients with renal cell carcinoma (RCC), discovering that ISET was more appropriate for RCC patients. CTCBIOPSY (Wuhan YZY Medical Science and Technology Co., Ltd., China) is a commercial one-step ISET device which could complete automatic detection and identification within 10 min^[48]. The Parsortix technology (ANGLE plc) incorporated a microscope slide sized cassette for CTC separation based on cell size and compressibility^[49]. Owing to the excellent capture performance and the advantage of easy retrieval of viable CTCs for downstream analysis, the FDA clearance process of this device for diagnose is underway. The ClearCell FX system (Clearbridge BioMedics, Singapore), one of the first automated cell separation and retrieval systems, is a new label-free and size-based technology with extremely high recovery rates by dean flow fractionation^[50]. These devices, together with other similar size exclusion platforms, such as ScreenCell (ScreenCell, France) based on microporous membrane filter^[51], CellSieve™ (Creatv Microtech), and MetaCell (Ostrava, Czech Republic), constitute the next generation label-free CTCs enrichment technologies, demonstrating CTC isolation and detection with high efficiency, purity and viability.

Moreover, size difference can be combined with other physical features to improve capture yield. For example, a recent wedge-shaped microfluidic device [Figure 2G] based on the difference in size, as well as rigidity and nuclear/cytoplasmic ratio between CTCs and normal blood cells, was fabricated to enhance CTC isolation, exhibiting excellent capture performance with $\geq 85\%$ capture efficiency^[52]. Similarly, benefiting from those multiple biophysical properties, Liu *et al.*^[53] [Figure 2H] developed a pyramid-shaped microchamber to achieve a more than 85% capture efficiency and a 93% recovery yield. In addition, vortex technology has been exploited and validated for isolating CTCs based on differences in size, shape and deformability by inertial microfluidics and laminar micro-vortices. VTX-1 liquid biopsy system [Figure 2I] was developed for fully automated isolation and enumeration of CTCs with either high recovery mode or high purity mode in the vortex microfluidic chip^[54].

Density-based CTC isolation

The density of nucleated CTCs lies between plasma and red blood cells, and within the scope of white blood cells. Quantitative buffy coat analysis by centrifuging for separation was established by Stephen C. Wardlaw in 1983^[55]. AccuCyte separation based on this principle is the first step of the commercial RareCyte Platform (RareCyte, Inc. Seattle)^[56], coupled with fluorescence analyzing (CyteFinder system) and picking (CytePiker) to count and retrieve cells for downstream single-cell characterization, overcoming the limitation of capture methods which are dependent on sizes that might miss the small sized-CTCs and immunomarkers that might not be expressed on some subpopulations. Some commercial density gradient solutions, such as Ficoll-Paque (GE Healthcare) and Percoll (GE Healthcare), provide simple-to-use and inexpensive methods for separating CTCs in the mononucleocyte layer from granulocytes and erythrocytes. OncoQuick (Greiner Bio-One/Hexal Gentech, Germany) consists of a sterile tube with a porous barrier inserted above separation medium, allowing the simple, rapid and highly efficient enrichment of CTCs through density-based centrifugation and size-based separation.

Dielectrophoresis based CTC isolation

The overlap of size or density between CTCs and normal cells may affect the efficiency of these size-/density-based approaches. Electrical properties of CTCs have been applied to discriminate them from other normal cells using dielectrophoresis (DEP). Based on conventional DEP devices, microchips are used to manipulate electric fields to achieve higher capture efficiency and recovery rate. Nguyen *et al.*^[57] fabricated a microchip to guide target lung CTCs to sensing electrodes by DEP and hydrodynamic forces, achieving a LOD of 3 cells and an efficiency over 90% at 50 kHz electric field intensity within 10 minutes. The commercial

DEPArray TM cartridge (Menarini Silicon Biosystems, Italy) that contains an array of electrodes embedded with detection sensors is based on the same principle for isolating target cells for subsequent analysis.

MOLECULAR CHARACTERIZATION OF CTCs

Not all the CTCs are detectable and not all detected CTCs have the potential for metastases, indicating that CTC enumeration alone may not be an effective marker of progressive disease. A commonly used chemotherapy agent, isosfamide, was reported to decrease the number of lung cancer nodules but also increase CTC frequency in a pre-clinical model of osteosarcoma^[58]. Although a large number of clinical trials have suggested that CTC presence is associated with poor survival in patients with some metastatic cancers^[59]. Their characterization, including phenotyping and genotyping, could lead to a better understanding of heterogeneity of metastatic tumor and further facilitate the management of patients for individualized treatment.

Protein analysis of CTCs

Most CTC detection assays are compatible with identification systems for enumeration and follow-up characterization. The most common procedure consists of morphological analysis (size, shape, nuclear cytoplasmic ratio, cell shrinkage), immunohistochemical analysis^[4] [Figure 3A], and fluorescence immunocytochemistry (ICC). There are various markers that are useful for ICC analysis. For instance, DAPI (nuclear counterstaining), pan-keratin (positive marker), and CD45 (negative marker) are applied in the CellSearch system. Fluorescence channels and specific antibodies are now accessible for users to define the detection of more established markers, for example, epidermal growth factor receptor (EGFR)^[60], androgen receptor (AR)^[61], folate receptor (FR)^[62], and several EMT-related markers (such as vimentin) etc., which are not only the identification markers but also therapy-associated targets. Furthermore, the ELISPOT assay combines cell culture and an immunospot test to quantitatively and qualitatively detect single viable cells expressing-cancer associated marker proteins.

One of the more reliable analytical approaches is protein profiling of captured CTCs, which can shed light on the roles of CTCs in tumor metastases and disease progression. At present, proteomic analyses mostly rely on mass spectrometry and the sensitivity of this method is improved with appropriate sample preparation and targeted cell enrichment. High-resolution porous layer open tube based liquid chromatograph-mass spectrometry (PLOT-LC-MS) lead to identification of approximately 4000 proteins of 100-200 MCF-7 cells with zeptomole detection sensitivity. Recently, Zhu *et al.*^[63] incorporated the nanodroplet processing platform (nanoPOTS) with ultrasensitive LC-MS, allowing identification of 1500-3000 proteins from 10-140 cells. He subsequently combined CD45 negative selection and laser capture microdissection-based purification with nanoPOTS-LC-MS for studying protein expression of rare or single CTCs, identifying 164 and 607 protein groups of 1 and 5 spiked LNCaP cells, respectively^[64].

Because of these analytical advances, a wealth of information regarding disease metastasis and progression is being discovered. For example, a recent report about profiling of single live CTC protease activity demonstrated the increased expression of matrix metalloproteases (MMPs) secreted by CTCs relative to normal cells is capable of triggering proteolytic processes that assist in invasion and immune evasion^[65].

Gene analysis of CTCs

Fluorescence *in situ* hybridization (FISH) technology is highly commercial and easily accessible for analysis of genetic alterations (deletion, insertion, translocation and rearrangement) at the chromosomal level, and is widely used in CTC identification and characterization. For example, RNA-ISH has been utilized for detection of onco-miRNA (such as miRNA-21)^[66], while DNA-ISH has been used for quantifying copy number of cancer-related genes (such as HER2/*neu* gene)^[13]. Based on an optimized FISH method, Frithiof *et al.*^[67] [Figure 3B] performed a CellSearch-based CTC separation assay to quantitatively measure HER-2

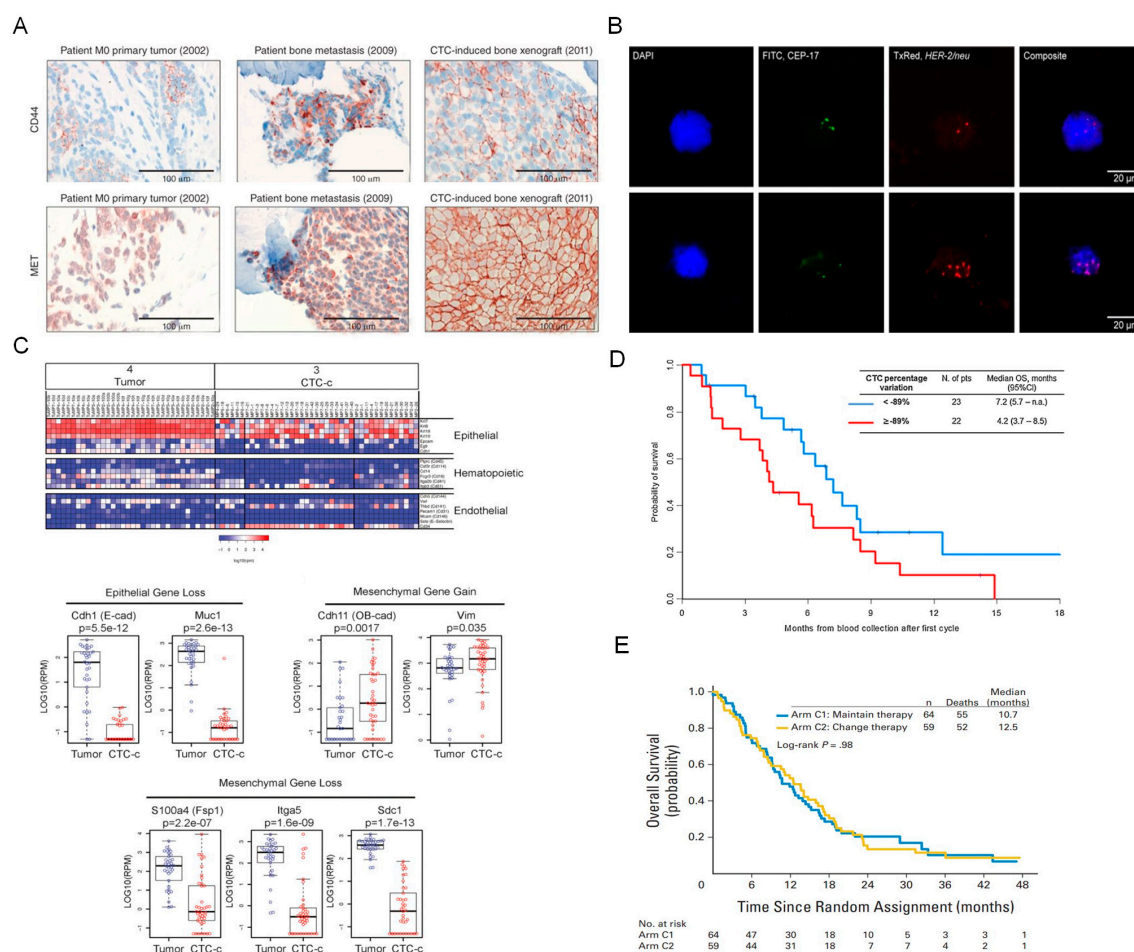


Figure 3. Molecular profiling and clinical application of CTCs. A: Immunohistochemical analysis of the expression of CD44 and MET in primary tumor (M0, nonmetastatic stage), bone metastasis and CTC-induced bone xenograft after transplantation of sorted CTCs; B: FISH images of a patient with metastatic breast cancer have no detectable HER-2 amplification (top panel), and the lower panel is HER-2 amplified CTC; C: the expression heatmap of epithelial, hematopoietic, and endothelial markers in primary tumors and classical epithelial CTCs (CTC-c). Epithelial and mesenchymal genes differentially expressed in CTCs vs. tumors; D: Kaplan-Meier curves of overall survival according to CTC change after one cycle of chemotherapy; E: overall survival (OS) and PFS in patients with metastatic breast cancer for whom therapy failed to reduce CTCs at first follow-up (approximately 21 days after first dose of chemotherapy), or randomly assigned to maintain the original chemotherapy (arm C1) or to switch to an alternative chemotherapy (arm C2). A: Copyright Nature Publishing Group, 2013. Reproduced with permission from reference^[41]; B: Copyright Dove Medical Press, 2016. Reproduced with permission from reference^[67]; C: Copyright Cell Press, 2014. Reproduced with permission from reference^[75]; D: Copyright Elsevier, 2014. Reproduced with permission from reference^[79]; E: Copyright American Society of Clinical, 2014. Reproduced with permission from reference^[80]

amplification in breast cancer CTCs. They validated that FISH was superior to protein evaluation of *HER-2* status in predicting breast cancer patients' response to *HER-2* targeted immunotherapy and found that one in six patients underwent CTC *HER-2* amplification during the treatment of metastatic disease.

Hybridization analyses, much like ICC analyses, are limited by the availability of both antibodies and microscope filters. Multiple polymerase chain reaction (PCR) targeting associated RNA and DNA for detecting assorted genetic mutations may overcome these limitations. Real-time PCR (RT-PCR) can be used to determine the differences of gene expression between CTCs and normal cells. And quantitative reverse transcription PCR (qRT-PCR) takes advantage of a reverse transcriptase reaction to convert RNA into cDNA before regular RT-PCR. Global or specific gene expression profiling of CTCs has been generated by real-time reverse transcription-PCR analysis, providing insights about mechanism of cancer and development of novel diagnostic biomarkers and therapeutic targets^[68]. These technologies following CTC isolation has been used for identification of various gene markers, such as ALDH1 (stem cell marker), phosphoinositide kinase-3 (PI3K α), TWIST1, TP53, and Akt2

(stem cell markers)^[69]. Mostert *et al.*^[70] performed a CTC isolation assay and mRNA expression profiling using the CellSearch technique in 142 metastatic colorectal cancer (mCRC) patients. They measured 95 mRNAs by RT-qPCR and found that 34 CTC-specific mRNAs were higher in patients with ≥ 3 CTCs compared with healthy donors. This CTC-specific gene panel for mCRC patients, such as *KRT19*, *KRT20* and *AGR2*, may aid in characterizing how CTCs with different expression profiles contribute to malignancy, thereby furthering the realization of individualized cancer treatment. Campton *et al.*^[71] developed a comprehensive and sensitive platform, named as AccuCyte®-CyteFinder® system, for identification and characterization of individual CTCs. Using the whole genome amplification (WGA) product, they confirmed that the TP53 gene, which is known to contain the R175H mutation in SKBR3, enables personalized, molecularly-guided cancer treatment.

Ampli11 TM (Menarini Silicon Biosystem), a product developed for single-cell WGA, can be used to amplify DNA for downstream genotyping analysis, including comparative genomic hybridization (CGH) and next-generation sequencing (NGS). Upon its development in 1992, CGH technology opened a new avenue in genomic investigation and, more particularly, in cancer gene analysis. In the past, CGH was applied for analysis of tumor tissues, but many studies have suggested that data from primary tumors alone is insufficient. Array CGH, which exploits ordered arrays of genomic DNA sequences, is widely used for analysis of CTCs, including identification of genomic alterations which include insertion/deletion, single-nucleotide variations, copy number variations (CNVs)^[72]; identification of candidate oncogenes or tumor suppressors; identification of novel biomarkers involved in metastasis, cancer progression and therapy response; and identification of subgroups of CTCs^[73]. High-throughput NGS is another strong technology to analyze heterogeneity of CTCs and reveal the mechanisms of metastasis, which might be the Achilles' heel in disease progression. Bertucci *et al.*^[74] utilized aCGH and NGS to compare DNA copy number and mutational profiles of 365 cancer-related genes between primary tumors and metastases and discovered a degree of divergence for actionable driver genes that might be extremely relevant with cancer metastasis. Profiting from whole genome amplification technology, the limited amount of single-cell genomic DNA sample can be amplified indistinguishable for sequencing.

Single-cell RNA sequencing was used to detect the heterogeneity of CTCs, unveiling the mechanism of drug resistance of androgen receptor (AR) inhibitors in prostate cancer^[61]. The technique was also used to identify conduct a transcriptomic analysis in pancreatic CTCs, finding increased expression of stromal-derived extracellular matrix (ECM) proteins, which facilitate cell migration and invasiveness, in CTCs from mice and humans with pancreatic cancer^[75] [Figure 3C].

Single-cell exome sequencing of isolated CTCs from cancer patient, revealed insertion/deletion and single-nucleotide variation in CTCs after whole genome amplification. The results showed cancer-type specific CNVs that are reproducible within cells of the same patient, or even between patients with the same type of cancer^[76].

CLINICAL IMPLICATIONS

Prognostic and diagnostic value of CTCs

Several studies have highlighted the correlation between CTC burden and treatment effect, indicating the prognostic value for patients receiving chemotherapy or surgery and the potential of surveillance of disease recurrence or metastasis [Table 1]. Several years after chemotherapy, patients with high-risk breast cancer with elevated CTC counts in their peripheral blood were reported to have worst survival prospect^[77]. Similarly, patients with colorectal cancer with elevated CTC counts were more likely to have recurrence after 3 years of curative resection, showing the relation between post-operative CTCs and poor prognosis^[78]. The prognostic value of CTCs was demonstrated by Nicola *et al.*^[79] [Figure 3D] in 60 patients with extensive SCLC. After assessment with the CellSearch system, the group isolated and analyzed CTCs in 90% (54/60)

of patients at baseline and demonstrated that CTC count was significantly associated with the number of organs involved. A reduction in CTC count of more than 89% after chemotherapy significantly improved the prognosis accuracy and was associated with a better outcome. They concluded that only the change of CTC count after the first chemotherapy cycle provided clinically relevant information. However, there were still some clinical trials (such as: gov NCT00382018) that failed to observe improved PFS or OS for cancer patients with decreased CTCs after therapies^[80] [Figure 3E]. Compared to either CTCs levels or cancer specific antigen levels alone, the combination of both two biomarkers provided a notably better predictive indicator for patients with advanced cervical cancer^[81].

Paired CTCs are correlated strongly with origin tumor cells, thus characterization of this subset of genes of CTCs might help to predict primary tumor origin. And CTCs detached from different parts within same tumor or even from different tumors are originally heterogeneous in nature. Gene expression profiling of CTCs from patients with different metastatic cancers showed the different but unique gene expression patterns of those cancer types, providing novel noninvasive diagnostic tools^[68] and essential information for personalized treatment.

On the other hand, CTCs undergoing certain transitions, such as EMT and MET, might generate new genetic alterations which are absent in the primary tumor, but related with potential distant tumor. Characterization of these subgroups of CTCs might assist in localizing specific distant metastatic sites. Additionally, some subgroup of CTCs may harbor changes that are undetectable in the tumor of origin, but are related to drug-resistance and management of treatment. Revealing the qualitative and quantitative divergence between the CTCs and the primary tumor or within CTCs by genotyping and phenotypic analysis is crucial for future studies of individualized medicine in metastatic disease. However, some changes present in CTCs homogeneously take place within primary tumor, suggesting CTCs, to some extent, might be a noninvasive and real-time indicator for following cancer progression and monitoring therapeutic response.

CTCs predict therapy outcome and provide personalized therapeutic targets

CTC enumeration can be a good marker for predicting drug sensitivity/resistance in preclinical and clinical settings. CTC quantity reasonably correlates well with clinical and instrumental tumor response. To investigate the clinical significance of CTCs in predicting the tumor response to chemotherapy, Wu *et al.*^[82] detected CTCs at baseline and during chemotherapy in 453 eligible lung cancer patients, indicating that disease control rate (DCR) of CTC-negative patients was significantly higher than that of CTC-positive patients; more importantly, patients also showed higher OS. The CTC status has been reported to be related to prognosis and is altered in response to chemotherapy in many other tumor types, such as colorectal cancer^[10], breast cancer^[83], osteosarcoma^[58]. Smerage *et al.*^[80] confirmed the prognostic significance of CTCs in patients with metastatic breast cancer, demonstrating that an increased number of CTCs was associated with poor prognosis. Early conversion to alternate cytotoxic therapies was ineffective in prolonging OS in patients with increased CTCs after receiving 21 days of first-line chemotherapy. For this population, a more effective treatment than standard chemotherapy is needed.

CTC characterization can generate predictions of drug potency and therapeutic efficacy before or during treatment according to analysis of targeted protein expression and signaling pathway activity. The development of immune check point inhibitors for cancer therapy, for example, anti-PD-L1, have achieved much success due to increased efficacy and decreased toxicity^[84]. However, PD-L1 expression detection for prediction of therapeutic response using tumor biopsies prior to treatment is invasive and insufficiently precise to guide treatment planning, resulting in some cancer patients being treated with an expensive but ineffective and toxic therapy. Instead, serial monitoring of patients treated with immune checkpoint inhibitors showed that a decrease of CTC score correlated with improved PFS and OS, and further demonstrated that RNA-based CTCs score during the immunotherapy has the potential to be a predictive biomarker for immunotherapeutic outcome^[85].

Table 1. Presence of CTCs and clinical outcome in cancer patients

Tumor type	Technique	Patient number	CTC cut-off	Patient Ratio	Baseline		Treatment		PFS		Post/During Therapy		Ref.
					P value	HR (95%CI)	P value	OS	HR (95%CI)	P value	HR (95%CI)	P value	
IBC (stage III)	CellSearch	63	≥1 CTC/ 7.5 mL	27%	NR	NR	NR	NR	4.22 (1.67-10.67)	log-rank P = 0.005	1.53 (0.41-5.79)	log-rank P = 0.54	[99]
Breast Cancer	CellSearch	1,574	≥2 CTC/ 7.5 mL	12.6%	< 0.001	3.93 (2.81-5.45)	< 0.001	NR	NR	NR	NR	NR	[83]
MBC	CellSearch	1,944	≥5 CTC/ 7.5 mL	46.9%	< 0.0001	2.78 (2.42-3.19)	< 0.0001	NR	NR	NR	NR	NR	[100]
MBC	CellSearch	595	≥5 CTC/ 7.5 mL	53.6%	NR	NR	NR	NR	1.94 (1.52-2.47)	< 0.001	2.13 (1.63-2.79)	< 0.001	[80]
CRC (stage IV)	qRT-PCR	50	NR/7.5 mL	26%	0.001	2.96 (1.14-6.81)	0.003	CT	2.49 (1.12-5.11)	0.013	3.58 (1.59-8.07)	0.002	[101]
mCRC	Immunofluorescence	75	≥2 CTC/ 7.5 mL	28%	0.61	1.97 (0.89-4.37)	0.09	CT	0.92 (0.30-2.78)	0.88	3.55 (1.1-11.5)	0.03	[102]
NSCLC (stage III/IV)	CellSearch	59	≥2 CTC/ 7.5 mL	32%	0.01	5.32 (1.62-17.50)	0.006	CT	NR	NR	3.07 (1.33-7.05)	0.008	[103]
NSCLC (stage IIIB/IV)	CellSearch	43	≥5 CTC/ 7.5 mL	23.2%	0.16	3.1 (1.2-8.2)	0.016	First-line CT	4.3 (1.2-14.4)	0.016	2.9 (0.7-11.4)	0.11	[104]
SCLC	CellSearch	83	≥5 CTC/ 7.5 mL	60%	0.001	3.4 (1.8-6.3)	< 0.001	CT	1.9 (1-3.5)	0.05	NR	NR	[105]
SCLC	CellSearch	56	≥5 CTC/ 7.5 mL	50%	< 0.001	0.3 (0.2-0.6)	0.001	CT	0.7 (0.3-1.7)	0.486	0.4 (0.2-0.9)	0.034	[106]
AGC	CellSearch	106	≥3 CTC/ 7.5 mL	41%	NR	NR	NR	CT	2.152 (1.11-4.16)	0.022	3.463 (1.82-6.58)	< 0.001	[107]
AGC	FACS-ICC	24	> 2 CTC/ 8 mL	67%	0.133	2.7 (0.82-8.89)	0.101	drug exposure	3.9 (1.13-12.7)	0.031	4.8 (1.37-16.8)	0.014	[108]
GGEA	CellSearch	106	≥2 CTC/ 7.5 mL	46%	NR	1.8 (1.2-2.9)	NR	no treatment	4.8 (2.4-9.7)	NR	3.1 (1.5-6.3)	NR	[109]
GGEA	qRT-PCR	62	all marker (+) vs. others	69.4%	0.000	4.1 (1.7-9.5)	0.001	CT	3.84 (1.6-9.3)	0.003	6.5 (2.0-21.3)	0.002	[110]
GEC	IsoFlux platform	43	>17 CTC/ 7.5 mL	46.5%	NR	4.4 (1.7-11.7)	0.0013	NR	NR	NR	NR	NR	[111]
EC	CellSearch	22	> 2 CTC/ 7.5 mL	37.5%	1.06	0.52	0.23	NR	NR	NR	NR	NR	[112]
BC	CellSearch	1,087	>1 CTC/ 7.5 mL	20.6%	Log-rank P < 0.001	(pre+/post+) 10.57 (3.92-28.56)	Log-rank P < 0.001	Drug and adjuvant CT	(pre-/post+) 2.49 (1.51-4.10)	Log-rank P < 0.00	7.38 (3.19-17.08)	Log-rank P < 0.001	[77]
CRC (stage I-III)	ISETdevice-CTCBIOPSY	138	>1 CTC/ 2.5 mL	79%	0.153	NR	NR	surgery	2.82 (1.39-5.75)	0.004	NR	NR	[78]
Cervical cancer	NEIm-FISH	99	3 CTCs/ 3.2 mL	45.5%	NR	NR	NR	radiotherapy	2.425 (1.131-4.477)	0.005	NR	NR	[81]
Lung cancer	CellSearch/CD45-FISH	453	56%	NR	< 0.001	3.43 (2.21-5.33)	< 0.001	CT	3.78 (2.33-6.13)	< 0.001	3.16 (2.23-4.48)	< 0.001	[82]
MM	Digital CTC Assay	49	48	NR	0.95	0.46 (0.14-1.52)	0.20	ICIT	0.17 (0.05-0.62)	0.008	0.12 (0.02-0.91)	0.04	[85]

IBC: inflammatory breast cancer; MBC: metastatic breast cancer; CRC: colorectal cancer, NSCLC: non-small-cell lung cancer, SCLC: small-cell lung cancer; GC: gastric cancer; GGEA: gastric and gastroesophageal adenocarcinomas; GEC: gastroesophageal cancer; EC: esophagogastric cancer; MM: metastatic melanoma; HR: hazard ratios; CI: confidence interval; PFS: progression-free survival; OS: overall survival; CT: chemotherapy; NR: not reported; ICIT: immune checkpoint inhibition therapy

Beyond enumeration alone, CTCs could provide crucial information of tumor malignancy via molecular characterization, leading to better treatment monitoring and molecular-/cancer cell-targeted therapies. The genotypic changes in CTCs provided the best suitable targeted therapy and enabled assessment treatment regimen efficacy over time. Epidermal growth factor receptor (EGFR) on the CTC surface has been verified as extremely significant in the process of tumor growth and progression. EGFR inhibitors (HER2 inhibitors, tyrosine kinase inhibitors, TKI and monoclonal antibodies) have been licensed for treatment of cancers caused by EGFR up-regulation, such as NSCL, breast, renal cell, squamous cell, colon and pancreatic cancers. However, in some cases, EGFR-targeted inhibitors are not effective due to the emergence of drug-resistance mutations. Mutation screening analysis of EGFR in CTCs may provide an explanation for drug-resistance mechanism and also reveal possibilities for diagnostic and therapeutic interventions^[60]. Inhibitors of other therapeutic molecular targets including mTOR, such as temsirolimus, and phosphoinositide 3-kinase (PI3K), such as ZSTK474, LY294002, have shown to have anti-proliferation in clinical trials^[86,87]. Most cell populations of the immune system play an important role in survival and seeding, or even enhancing the growth of tumorigenic subpopulations of CTCs. The molecular characterization of CTCs might assist in unveiling intercellular interaction mechanisms and providing potential therapeutic targets. For instance, the extracellular surface interacting protein, PD-L1, is one such target that is currently generating much interest^[84]. In consideration of costs and toxicity of anti-PD-L1 therapy, predictive biomarkers able to distinguish responders from non-responders are in urgent demand. Real-time CTC analysis provides significant information on drug resistance^[88]. In addition, CTCs are now regarded as a new cellular therapeutic target. Photodynamic therapy was used to selectively kill GFP-expressing CTCs by energy transfer between expressed GFP and pre-accumulated rose bengal (RB) in cells, demonstrating that clearance of CTCs could reduce metastasis and extend survival^[89].

CONCLUSION AND OUTLOOK

The potential clinical value of CTCs has been established. Advances in CTC isolation and molecular characterization offer the possibility for early detection and diagnosis, improve the satisfaction of therapies, as well as expand our knowledge about underlying mechanisms of cancer dissemination and progression.

Although the tremendous technical advances in CTC isolation and detection make it possible to analyze extremely rare CTCs, there are still many hurdles. First, a criterion to standardize different kinds of detection assays is urgently needed in clinical applications. Secondly, while the emergence of new predictive biomarkers leads to clearer recognition about tumor metastases and disease progression, novel targets for prognosis and treatment need to be further validated and standardized. The next frontier of CTCs detection lies in thorough characterization, which might rely on developing single-cell multi-omic technologies, including genomics, proteomics, transcriptomics etc.. Finally, research findings provide arguments in favor of the hypothesis that only a subpopulation (metastasis-initiating cells, MICs) of CTCs in patient blood is responsible for initiating carcinoma metastasis. A majority of cancer cells may never develop into metastatic phase, but instead maintain a dormant state or die from the anoikis, immune attacks and physical shear stress in the vasculature. However, our understanding about the requirements for CTCs being activated from latency into overt metastases is far from complete. Apart from CTCs, other noninvasive “liquid biopsies” might provide more supplementary information, including some cell-free components such as circulating tumor DNA (ctDNA)^[90], microRNAs (miRNA), exosomes, as well as long-coding RNA (lncRNA)^[91-94]. Recently, due to the success in immunotherapy of cancers, immune checkpoint blocker programmed death-ligand 1 (PD-L1), whose expression in CTCs correlate with tumor status^[95], has gained interest as a potential independent prognostic marker for PFS and OS^[96], extending the spectrum of noninvasive liquid biopsies^[97]. And, CTC detection may also have the potential to monitor the efficacy of anti-PD-L1 therapy^[98]. Comprehensive and systemic liquid biopsy analyses may contribute to thorough understanding of metastatic malignancy and better management of cancer patients.

DECLARATIONS

Authors' contributions

Huang QQ and Chen XX contributed equally to this work.

Availability of data and materials

Not applicable.

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Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Breast cancer, metastasis, and the microenvironment: disabling the tumor cell-to-stroma communication network

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Abstract

Breast cancer (BC) is the leading cause of cancer-related deaths in women worldwide. However, the majority of cancer mortalities can be attributed to cancer cell metastasis to distal organs/tissues rather than the primary tumor mass itself. The microenvironment surrounding the main tumor mass, as well as its final migration destination, plays a crucial role in the survival, growth, proliferation, and progression of BC. Intercellular stromal cells and components of the microenvironment surrounding a tumor comprise a nurturing cubicle that provides a communication network of cross-talk and signaling between the tumor cells and the extracellular matrix (ECM) and interstitial cells. This network connection enables the tumor cells to engage in metastatic-associated activities such as cell adhesion, invasiveness, mobility, migration, cell shape change, cell-to-cell contact, and basement membrane degradation. An untapped therapeutic approach that might disable the communication network between cancer and stromal cells could possibly aid in providing this unmet need in treating metastatic disease. The intravenous administration of select protein-derived peptides to patients might have the potential to occupy, saturate, and block receptors and binding proteins at the interstitial/ECM communication interface with tumors.

Keywords: Alpha-fetoprotein, cancer, metastasis, breast, microenvironment, interstitium, extra-cellular matrix, stroma

INTRODUCTION

Breast cancer (BC) and its metastasis are worldwide public health issues causing financial, economic, social, and personal lifestyle problems in addition to their high mortality rate. An increased metastatic potential in



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BC patients adds to their ultimate demise in both young and older women^[1]. However, there exists an innate flaw in BC cell survival and metastasis regarding its dependence on the microenvironment that surrounds the malignant tumor^[2]. This dependence presents a novel opportunity into an untapped therapeutic potential for BC patients with metastatic disease. The BC tumor mass maintains an intimate relationship with its microenvironment via a 2-way interchange of cross-talk and signaling communication cues between the two entities; this establishes a network-dependent connection^[3]. When tumor growth outpaces its blood and nutrient supplies, cells detach from the primary mass seeking out new fertile “nesting” grounds. The process of cell detachment, migration, and dissemination is the hallmark of metastases with its widespread cell dispersion throughout the body; this results in an increased lethality more than the primary BC tumor itself. In 1889, Stephen Paget^[4] proposed a “seed and soil” hypothesis in which break-away tumor cells seek out tissue/organ sites which are beneficial to resume new tumor growth in sites resembling their original microenvironments. Thus, migratory tumor cells seek out secondary nurturing destinations in which tumor cells grow unabated in distal target organs such as liver, lungs, bone marrow, and brain.

The tumor cells themselves first undergo an epithelial-to-mesenchymal transition that transforms the cell's phenotypic identity to adopt a migratory and invasive behavior^[5]. The dispersed migrating BC cells are attracted toward distal organ/tissue destinations under the influence of chemoattractant signals that determine their migratory gradient patterns and behavior. The chemotactic signals originate from a subfamily of secreted cytokines termed chemokines, most notably the CXCL12 ligand together with its cognate cell surface receptor, CXCR4. Such a 2-member complex is present on both tumor cells and stromal cells. Together, this chemokine/receptor complex forms an axis that can regulate cancer cell growth, proliferation, chemoresistance, angiogenesis, and metastasis^[6]. Hence, both the directive and the objective of the present report are to address the cancer cell-to-microenvironment interaction as a potential therapeutic intervention strategy targeted toward disrupting the communication signaling network both at primary tumor and its metastatic sites.

THE TUMOR MICRO-ENVIRONMENT COMPONENTS

The micro-environment surrounding the primary tumor mass and that encompassing the metastatic destination site have similar shared communication networks. The tumor cell-to-microenvironment connection serves as a signaling bridge between tumor cells and interstitial stromal components which sponsor two-way communication systems. Such signaling activities include nutrient supply, angiogenesis, cell adhesion, migration, cell-to-cell contact, adherens cell junction maintenance, invasiveness, and basement membrane proteolysis^[7]. Non-cellular constituents of the interstitium consists of multiple and diverse components such as: (1) metabolic by-products; (2) exosomes and microvesicles; (3) cell secreted and cell-surface proteins, co-factors and enzymes; (4) extracellular matrix components; (5) signaling molecules; and (6) endocrine and exocrine secretions such as cytokines. The non-malignant cell populations residing in the extracellular spaces and matrices consist of: (1) capillary and lymphatic cells of vessels and ducts; (2) extracellular matrix (ECM) cells; (3) stromal cells; (4) mesenchymal stem cells; (5) fibroblasts; (6) pericytes; (7) immune-associated cells (lymphocytes); (8) macrophages; (9) myofibroblasts; and (10) endothelial cells^[3,8,9]. Some of these constituent cells express and secrete various proteins involved in growth, cell movements, apoptosis, membrane proteolysis, and integrin and growth factor signaling. Thus, the cellular and non-cellular components of the institutional and ECM spaces represent an untapped source of potential molecular and cellular targets for cancer therapy. These will be addressed below.

CELL SIGNALING IN THE MICROENVIRONMENT

The tumor-to-microenvironment intracellular spaces offer a little-recognized cluster of molecular signaling targets with no place to hide. Hematologic and solid tumors are known to interact through microenvironments via cell surface chemokine receptors and their cognate ligands. As discussed above, the

major components in the extracellular signaling arena are the chemokine receptor complexes present on both tumor and ECM cells, which activate diverse intracellular signaling transduction events as discussed above^[10]. Such intracellular signaling systems can activate a variety of pathways such as ERK1/2, p38, JAK/STAT, SAPK/JNK, NF- κ B, mTOR, PI3K-AKT, and BT-kinases^[11]. Chemokine receptors, such as CXCR4 and CXCR7, represent members of the seven-transmembrane domain G-protein coupled receptor family that transmit intracellular activation of processes such as calcium flux, chemotaxis, transcription, cell cycle transition, and cell survival^[13]. The disruption, blockage, or dysregulation of chemokine receptors and associated proteins could lead to aberrant off-target signaling and de-sensitization of the chemokine receptor transactivation apparatus.

At present, the increasing insight and appreciation of the functional role of the microenvironment to cancer growth and metastasis is prompting further research to more fully elucidate and investigate the tumor cell-to-stroma cell dependency. The identification of metastasis-associated protein targets, both cell-bound and secreted, which occupy the extra-cellular matrix and spaces are key targets for disabling the communication lines between the tumor and the interstitium. The protein constituents which aid and abet this signaling complex display properties associated with processes such as cell adherence, cell-to-cell contact, invasion, immigration, matrix proteolysis, growth factor and receptor regulation, and ECM remodeling and restructuring. For example, a recent report has described the existence of a cancer cell-to-stromal macrophage EGF/CSF-1 paracrine signaling loop in the invasive spread of a primary rat mammary tumor [Figure 1]. The invasive response was inhibited by blocking the EGF and CSF receptors and/or macrophage function demonstrating that tumor migration invasiveness was dependent on the paracrine loop.

PROTEIN CONSTITUENTS OF THE TUMOR MICRO-ENVIRONMENT

The intrinsic pressure driving the metastatic process of tumor cells is the need for additional nurturing factors such as new blood vasculature and increased nutrients for the ever-expanding volume of the primary tumor cell mass. The disseminated tumor cells take advantage of the metastasis-associated proteins already residing in and on the cells of the ECM and interstitial spaces. These metastasis-associated proteins, so crucial for break-away tumor cells, are presented in the discourse listed below and in Table 1.

Cell adherence and cell-to-cell contact family proteins

Cell adherence and contact processes can be attributed to a gene superfamily termed the cadherins which consist of protocadherins, cadherins, desmocollins, desmogleins, contactins, and connexins^[14] [Table 1]. Such proteins function in cell adhesion to other cells, and to ECM constituents, and to formation of adherin junctions between cells. Cadherin family members display calcium (Ca^{2+}) binding repeat domains in their intrinsic polypeptide structure^[15]. Different cadherin subfamily members can act in concert to join cells together in both homotypic and heterotypic types of attachments. Some family members (protocadherin Cad-13) serve to stabilize and maintain cell connections during oxidative stress, neural cell development, neurite outgrowth, cell signaling, invasion, and migration^[16]. Other family members are involved in cell survival, potassium channel transmission, maintaining the microvasculature, KRAS signaling, and transmembrane activities. The PECAM, connexin, and contactin proteins play roles in gap junction maintenance, platelet adherence, and aged white blood cell removal and destruction^[17].

ECM family proteins

The ECM family of proteins include the integrins, zinc matrix metalloproteases (MMPs), A distinguishing and metalloproteinase (ADAM) family, collagenases, gelatinases, and annexins^[18] [Table 1]. The MMPs are synthesized as nonactive precursor proteins which require proteolytic cleavage exposing catalytic hemopexin domains utilized for collagen degradation. The MMPs play key roles in development, metabolic disorders and diseases, immune and autoimmune disorders, and in cancer. In comparison, the ADAM family members function as transmembrane-anchored proteins resembling snake venom disintegrins which are

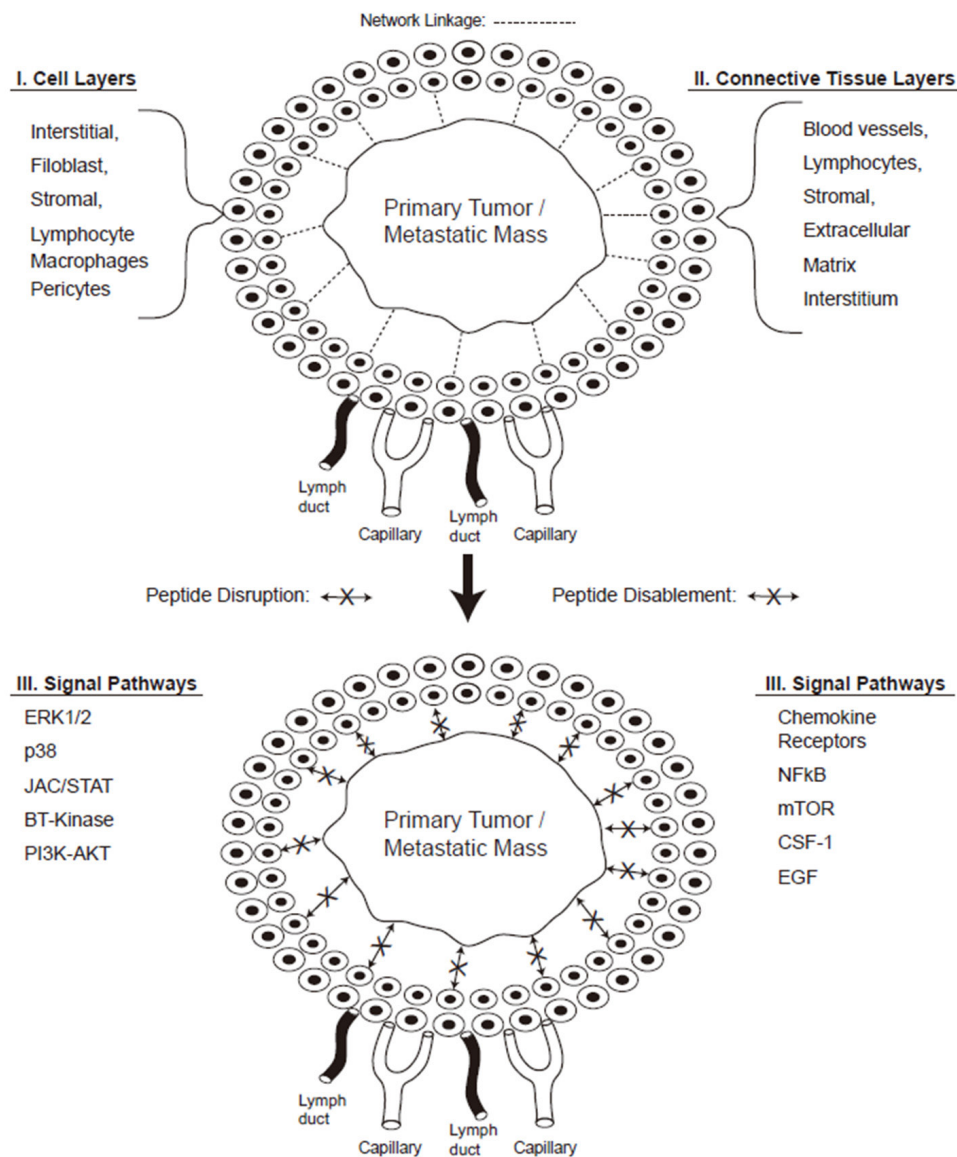


Figure 1. Peptide disruption of tumor to stroma communication. The peptide disruption of the tumor-to-stromal cell communication network is displayed together with the connective tissue layers, cell components, and signaling pathways. Note that the top half of the diagram displays the communication network connection indicated by the dashed lines (---) between tumor/stromal cells; while the bottom second half demonstrates the blocked linkage (←---X→) due to peptide disruption of tumor-to-stroma communication (see text for references and Ref.[45])

involved in cell mobility, migration, adhesion, platelet aggregation, and cell-to-cell contact; such processes occur during reproduction, neurogenesis, muscle development, and tumorigenesis^[19]. The collagenases and gelatinases are family members involved in tissue remodeling, bone mineralization, reproduction, autoimmune diseases, inflammation, and cancer development^[20]. The integrins are composed of alpha/beta chain proteins that participate in cell adhesion/contact and ECM-to-cell membrane inside-out signaling^[21]. Finally, annexins are Ca^{2+} and phospholipid binding proteins that serve in blood coagulation processes^[22].

The growth factor receptor family proteins

The growth factor receptors associated with the tumor microenvironment and metastatic sites are largely G-coupled seven-transmembrane receptors containing kinase domains required for intracellular signaling and cross-talk between adjacent pathways^[23] [Table 1]. These receptors bind growth factors such as fibroblast

Table 1. Selected metastasis-associated protein constituents of the tumor microenvironment are listed as four major functional groups. Such proteins are involved in cell activities such as adherence, mobility, growth, migration, invasiveness, angiogenesis, signaling, and proteolytic degradation

Group-I proteins: cell adherence and cell-to-cell contact
1. Protocadherin-Beta-1 (Q9Y5F3)
2. Cadherin-6 (AAH00019)
3. Cadherin-13 (NP_001248)
4. Cadherin-22 (CAB51587)
5. Contactin-3 (N_P387504)
6. Neural Cell Adhesion Molecule (NP_000606)
7. Platelet Endothelial Adhesion Molecule (NP_000433)
8. Connexin GJA5 (AAH13313)
9. Neuratropic tyrosine kinase R-3 (NP_001012338)
Group-II proteins: matrix metalloproteases (MMP) and ADAMS
1. MMP-2 (ABD38929)
2. MMP-9 (CAC10459)
3. MMP-10 (AAH02591)
4. MMP-13 (AAH74808)
5. ADAM-22 (NP_068368)
6. Integrin Alpha-6 (AAH50585)
7. Integrin Alpha-2 (NP_002194)
8. Annexin-A8 (P13928)
9. Collagen IV, Alpha-3 (CAA56335)
Group-III proteins: growth factor receptors - cell surface
1. Fibroblast Growth Factor Receptor (P22455)
2. G-protein Coupled Receptor-54 (QT69F8)
3. Interleukin-8 Receptor (NP_001548)
4. FMS-like Tyrosine Kinase Receptor (AAB23636)
5. Laminin Receptor (AAC50652)
6. TSH-associated Receptor (AAD31568)
7. Somatostatin Receptor-2 (NP_001041)
8. Ephrin Receptor-2B (CA122899)
9. C-Met Hepatocyte Growth Factor Receptor (NP_000236)
Group-IV proteins: growth factors and regulators
1. Transforming Growth Factor B-1 (NP_000651)
2. Vascular Endothelial Growth Factor (CAC19516)
3. Retinoblastoma-Assoc. Protein-1 (NP_000312)
4. p53 Protein Tumor Cell Antigen (NP_000537)
5. Tyrosine Protein Phosphatase Non-Receptor-7 (NP_002823)
6. C-terminal (CTB) Binding Protein (AAD14597)
7. NME-Nucleoside-Disphosphate Kinase (CAG46912)
8. Metastasis Suppressor Protein MTSS-1 (AAF15947)

See Table 3 for Metastasis-associated functional activities, text, and Ref.[14-31]. Parenthesis indicates National Center for Bioinformatics (NCBI) Accession number. ADAM: A disintegrin and metalloproteinase gene family

growth factor, laminin, somatostatin, ephrin, IL-8, and KISSIR^[24]. The receptor-mediated activities encompass angiogenesis, mitogenesis, growth enhancement, differentiation, kinase function, cancer genesis and maintenance. These receptors can further activate, influence, and regulate cell cycle transitions, mitogenesis, cell motility, migration, and metastasis. Some receptors contain tyrosine kinase domains that enhance or influence growth in multiple tissues including the blood vasculature. The somatostatin receptors regulate the release of various hormone and secreted proteins, while the ephrin receptors are involved in tissue formation, cell migration and contact, stem cell differentiation, and cancer growth^[25,26]. Finally, the G-coupled receptor-54 functions as a receptor for the KISS gene product, a cancer metastatic suppressor protein which when mutated, induces chemotaxis and invasion in numerous cancer cells. Thus, the metastasis-associated growth factor receptors are heavily involved in cell-to-ECM adhesion, invasion, migration, cancer growth and metastatic activities.

Growth factor and regulator family members

The microenvironment and metastasis-associated proteins encompass a wide range of growth factors and kinase enzymes that include transforming growth factor, vascular endothelial growth factor, MTSS1, p53 cell

Table 2. Selected examples of naturally occurring protein-derived peptides (small proteins) are displayed together with their amino acid sequence numbers and protein of origin

Name of peptide	Number of amino acids in sequence	Host protein of origin (pre-protein)	Peptide biological activity, function
1. Angiostatin	184	Plasminogen	Blocks blood vessel growth
2. Endostatin	330	Collagen Type XVIII	Angiogenesis inhibitor
3. Vasostatin	180	Calreticulin	Angiogenesis inhibitor
4. Constatin	150	Collagen Type IV	Inhibits endothelial cell growth
5. Tenacin	220	Fibronectin Repeat Domain	Anti-adhesion Function
6. Fragment of GHRH	15	GHRH	Stimulates Lipogenesis
7. Angiotension II	8	Angiotensinogen 22 Globulin	Increases Vasopressin Production
8. Bradykinin	9	Kininogen Precursor	Contracts Smooth Muscle
9. Oxytocin	9	Oxytocin Precursor	Uterine Contracting Hormone
10. Endothelin	21	Pre-Pro-Endothelial	Potent Vasoconstrictor
11. TRH	3	242 Amino Acid Polypeptide Precursor	Governs Release of TSH
12. GIP*	34	Alpha-fetoprotein polypeptide (609 amino acids)	Suppresses growth of hormone and non-hormone dependent growth and proliferation (normal and cancer growth)
GIP Sub-fragments:*			
(1) P149a	12	AFP AA#464-475	Inhibit platelet aggregation and blood factors
(2) P149b	14	AFP AA#476-488	Blocks metastasis-assoc. proteins and cancer growth
(3) P149c	8	AFP AA#489-496	Inhibits estrogen-dependent growth

Note that many peptides are derived from blood and extra-cellular matrix proteins. Naturally-occurring peptides can number in the hundreds of amino acids, while synthetic peptides are limited to 50 amino acids or less. *Synthetic peptides. AA: amino acid; AFP: alpha fetoprotein; GIP: growth inhibitory peptide; P149a: AAs; LSEDKLLACGEG (12 mer); P149b: AAs; AADIIIGHLCIRHE (14 mer); P149c: AAs; EMPVNPNG (also known as AFPep) (8-mer; both linear and cyclic). Data extracted from Ref.[33-38]. GHRH: growth hormone releasing hormone; TRH: thyroid releasing hormone; GIP: growth inhibitory peptide; TSH: thyrotrophin stimulating hormone

tumor antigen (phosphoprotein-53), tyrosine (tyr) protein-phosphate non-receptor type-7 protein, among others^[28,29] [Table 1]. These proteins function in activities such as angiogenesis, tumorigenesis, metastasis, cancer proliferation, signal transduction, and cell cycle progression. Some of these proteins contain kinase enzyme domains which are contained in their polypeptide structure; other proteins act as regulators of cell cycle transition and tumor growth. The p53 cell antigen is a phospho-protein, which in the non-mutated state, normally function as tumor suppressors that protect the genome. The tyr (tyrosine) protein phosphatase non-receptor-7 is a signaling molecule involved in cell transformation, cell cycle transition, growth, and proliferation both in non-cancer and in malignant cells^[30]. This latter protein further interacts with lymphokine-secretory cells and exhibits MAP kinase activity. Another family member of this group can phosphorylate the C-terminal end of the SRC family kinases, while the NDPK protein is involved with cell growth, proliferation, development, signal transduction, and G-protein coupled receptor activities^[16]. Finally, the MTSS1 protein functions, in its non-mutated state, as a metastasis suppressor protein that contains an actin binding (cytoskeletal) segment involved in cell migration^[16].

Naturally-occurring protein-derived fragments

There exists an extensive reserve of natural protein-derived peptide segments encrypted within the polypeptide structure of circulating blood proteins and ECM/interstitial cell-secreted proteins^[31,32]. The proteome of both humans and other mammals contain precursors and preproteins that when cleaved by proteolysis, release peptide fragments with diverse biological activities. Although not widely mentioned, this containment fraction of active peptide fragments are derived from naturally-occurring sources such as blood proteins, growth factors, hormones, clotting factors, ECM proteins, and angiogenic factors. Such peptide fragments constitute segments from precursor derived proproteins or preproteins which are produced following proteolytic enzyme degradation (i.e., trypsin); they can also be produced by cleavage in the laboratory using agents such as cyanogen bromide. Some of nature's most potent anti-angiogenic peptides are derived from abundant plasma proteins, growth factors, and ECM and stromal proteins^[33,36] [Table 2]. Such peptide fragments are garnering attention in the biomedical literature due to their functioning in signal transduction, receptor cross-talk, growth regulation, vasodilation/vasoconstriction, hormone release, blood clotting and inflammation^[16]. Some peptide fragments can be cleaved from the amino-terminal

Table 3. Selected cell adherence and cell-to-cell contact protein superfamily members are listed with their functional activities together with their third domain alpha-fetoprotein binding/interaction sites

Selected Protein Name	Functional Activities	AFP Amino Acid Sequence Numbers	AFP Amino Acid Sequence**
Cell adherence and cell-to-cell contact proteins			
(1) Cadherin-6	Calcium dependent cell adhesions and connections	AA#285-292 AA#500-507	FQTENPLE CTSSYANR
(2) Cadherin-22	Cell adhesion, K ⁺ channel-associated	AA#481-488 AA#516-524	LGHLCIRH* VDETYVPP
(3) Connexin GJA5	Transmembrane GAP Junction Protein	AA#413-421 AA#529-537	KRSCGLFQ DKFIFHKD
Matrix metallo proteases and ADAM family proteins			
(4) MMP-9	Degrades ECM proteins, cell migration	AA#409-417 AA#444-452	GALAKRSC SELMAITR
(5) ADAM-22	Cell-to-cell and matrix interaction	AA#429-437 AA#481-489	NAFLVAYT IGHLCIRH*
(6) Integrin alpha-2	An interacting protein for laminin, collagen and fibronectin	AA#433-441 AA#485-493	VAYTKKAP CIRHEMTP*
Growth factor receptors - cell surface			
(7) Fibroblast growth factor receptor	Regulate cell growth, proliferation	AA#401-408 AA#477-485	LQKYIQES ADIIIGHL*
(8) Ephrin receptor	Receptor for cell migration and angiogenesis	AA#453-461 AA#477-485	KMAATAAT ADIIIGHL*
(9) G-protein coupled receptor-54	Receptor for metastasis kiss peptide-4	AA#481-489 AA#500-507	IGHLCIRH* CTSSYANR
Grow factors and regulators			
(10) Vascular endothelial grow factor	Stimulates vascular permeability for angiogenesis	AA#477-485 AA#497-505	ADIIIGHL QKLISKTR
(11) Metastasis suppressor protein MTSS1	Tumor metastasis; acts to bind actin	AA#425-433 AA#444-452	YYLQNAFL SELMAITR
(12) Tyrosine phosphate non-receptor type-7	Regulates cell growth and cell cycle	AA#421-429 AA#477-485	KLGEYYLQ ADIIIGHL

**AFP amino acid sequence (single letter code); *growth inhibitory peptide (GIP) sequences. AFP: alpha-fetoprotein; ADAM: A disintegrin and metalloproteinase protein family; MMP: matrix metalloproteinases; Data extracted from Ref.[16,31,37-39]

or carboxy-terminal ends of various proteins, while others can be exposed following a conformational change in the proteins' tertiary structure. Such a conformational change in a protein can be induced by environmental events such as stress, shock, oxidative state changes, osmotic pressure, pH, and high ligand concentrations^[31,32]. Once identified in nature, single peptide fragments can be synthesized in the laboratory.

Examples of protein-derived fragments from the amino- or carboxy- ends of natural large proteins encompass a growing list of candidates. Such fragments include: angiotensin from angiotensinogen; endothelin from pre-pro-endothelin; bradykinin from kininogen, and others [Table 2]. Some examples of internal peptides encrypted within the chain of a protein include: Tenacin-C from fibronectin-III, angiostatin from plasmin, and endostatin from collagen-III [Table 2]. The chemically-synthesized peptides are usually restricted to less than 50 amino acids (AA) due to technical synthesis limitations, while naturally produced peptides can exceed 100 AAs. An example of a conformationally-exposed peptide segment from Table 2 is the growth-inhibitory peptide (GIP) derived from the full-length alpha-fetoprotein (AFP) molecule^[37-40]. The GIP segment is uncovered on the AFP molecule following exposure of the protein to high concentrations of estrogens, fatty acids, and growth factors. The GIP-exposed form of AFP is a transitory (molten globular) form which can refold back to its natural tertiary structure following removal of the high ligand concentrations. The encrypted GIP segment on AFP is normally concealed within a hydrophobic cleft of the tertiary-folded AFP molecule. Moreover, GIP and its sub-fragments have been chemically synthesized, purified, and characterized in multiple assays of biological activities including anti-cancer growth and metastasis inhibition in both *in vitro* and *in vivo* models^[37,38].

In the AFP derived GIP fragment example, a normal growth enhancing AFP molecule is converted to a growth inhibitory full-length protein capable of blocking normal and cancer growth and metastasis. Aside from GIP, other protein-derived peptide sites on the AFP third domain have since been identified and mapped; some such peptide segments have been found to bind and/or interact with other proteins such as metastasis-associated family members of the ECM, interstitial, and stromal proteins of tumor microenvironments^[16].

Third-domain derived AFP peptide-interactions with ECM proteins

As displayed in Table 2, the third domain of AFP provides an example of naturally occurring protein-derived peptides segments with biological activities of significance. The AFP third domain houses multiple peptide segments (8-10 or more amino acids) that have been identified by “in silico” prediction tools and molecular modeling; these were verified by in vitro cell-based assays and the AFP-derived peptides were found to bind various proteins^[16,41,42]. Recent examples of confirmed protein-to-peptide binding interactions between AFP peptides and natural proteins include the scavenger receptor proteins, immune dendritic cells, mucin proteins, retinoic acid receptors, and metastasis-associated proteins in both cancer and non-malignant models^[42]. Logic would dictate that disruption and/or interference of cross-talk and signaling pathways between tumor cells and their surrounding proteins (both free and cell bound) located in the microenvironment might serve to hamper or obstruct this crucial linkage. As discussed above, the tumor microenvironment contains 4 or more protein families of metastasis-associated proteins relate to tumor cell detachment, adhesion, contact, and migration via the ECM. By use of the AFP receptor and protein binding third domain peptides as displayed in Table 3, it can be noted that such peptides might be capable of disabling and/or disrupting communication lines between the tumor primary mass and/or metastasis nesting sites. The identities of these potential protein-to-AFP peptide interaction sites with the metastasis-associated proteins from tumor micro-environments are addressed below.

Interaction of metastasis-associated proteins with AFP-derived peptides

It has previously reported that metastasis-associated proteins are capable of binding and/or interacting with AFP-derived peptides of the third domain^[16,42]. In that report, members of the cell adhesion/contact protein family, such as cadherins, contactins and connexins were identified “in silico” to interact with AFP derived peptide sites on the amino-terminal half of domain-3, AA#401-500. The same was true for the ECM-associated proteins of the MMP and ADAM family proteins being clustered from AA# 409-480; moreover, these results showed interaction sites with AFP AA# 504-558 in the carboxy end of domain-3. The third group of interacting proteins, the growth factor receptors, demonstrated two cluster interaction sites on AFP-3D; the first group at AA#s 433-477 and the second grouping at AA# 512-550. Finally, the last class of AFP interacting proteins, the growth factors and regulators, consisted of two distinct interaction regions; the first extending from AA# 413-487, while the second encompassed sites at AFP AA#s 508-522. It can be concluded from these data that metastasis-related proteins are potentially capable of interaction with various AFP peptide segments on both the 1st and 2nd halves of the AFP domain-3^[16].

CONCLUSION

It can be concluded from the above discourse that both naturally occurring protein-derived and synthetic peptides, ranging from 8-50 AA or larger, might be candidates capable of disabling tumor-to-microenvironment communications. Such network connections are essential for supplying blood vascular and nutrient supplies from the ECM to the tumor, critical locations required for tumor survival and subsequent metastasis. The microenvironmental compartments surrounding tumors are required for successful cell detachment from the primary mass and taking advantage of migration and adhesion factors already present in the extracellular and stromal cell areas.

The present study further highlighted the many natural protein-derived peptides in human beings, whose activities involved cell adhesion, mobility, contact, angiogenesis, blood clotting, and tumorigenesis. In

some instances, the derived peptide displayed an opposite action from its host protein such as angiogenic inhibition versus enhancement of new blood vessel growth. In another type of protein-derived peptide, the amino acid segment is not cleaved from the preproprotein, but rather exposed following a conformational change of the tertiary folded polypeptide. As an example, metabolic stresses of excessive ligand concentrations can temporarily convert the growth enhancing AFP molecule into a growth inhibitory protein. The transitory AFP molecule then refolds back to its tertiary form, and full length AFP once again displays the property of growth enhancement.

In the future, it might be feasible to administer short peptides to metastatic patients by injection, infusion, and osmotic pumps, or via sublingual routes to patients in early and/or late metastatic disease. Such peptides are also capable of down-regulating the expression of metastasis-associated proteins as previously described for GIP^[44]. Peptides are short half-life molecules with good targeting properties, and adequate target binding (loading/off-loading) affinities^[43]. Natural or synthesized peptides might potentially serve as treatment adducts in combination with next generation therapeutic cancer drugs. Peptide binding, occupation, down-regulation, and saturation of ECM proteins in the interstitium may possibly serve to disable and sever the primary tumor and/or metastatic nesting sites from their ECM communication networks.

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Mizejewski GJ solely contributed to the article.

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Original Article

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Cancer stem cells in liver metastasis from colon adenocarcinoma express components of the renin-angiotensin system

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Abstract

Aim: We have recently identified a cancer stem cell (CSC) subpopulations within the tumor nests (TNs) and another within the peritumoral stroma (PTS) in liver metastasis from colon adenocarcinoma (LMCA). This study investigated the expression of components of the renin-angiotensin (RAS): pro-renin receptor (PRR), angiotensin converting enzyme (ACE), and angiotensin II receptor 1 (AT1R) and angiotensin II receptor 2 (AT2R) in LMCA.

Methods: 3,3-Diaminobenzidine (DAB) immunohistochemical (IHC) staining was performed on 16 LMCA samples for PRR, ACE, AT1R and AT2R. Immunofluorescence (IF) IHC staining was performed to investigate co-expression of these components of the RAS with SOX2 or OCT4. NanoString analysis ($n = 6$) and Western blotting (WB, $n = 3$) were performed on snap-frozen LMCA samples to confirm mRNA and protein expression, respectively.

Results: DAB IHC staining showed the expression of PRR, ACE, AT1R and AT2R within all LMCA samples. NanoString analysis and WB confirmed gene and protein expression of these components of the RAS. IF IHC staining demonstrated expression of PRR, AT1R and AT2R by the SOX2⁺ CSCs within the TNs and the OCT4⁺ CSCs within the PTS. ACE was expressed on the endothelium of the microvessels within the PTS.



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Conclusion: These finding suggests the CSCs within LMCA maybe a novel therapeutic target by manipulation of the RAS.

Keywords: Cancer stem cells, colorectal, colon, cancer, adenocarcinoma, liver, metastasis, renin-angiotensin system

INTRODUCTION

Colorectal cancer (CRC) accounts for 9.7% of all cancers with approximately 1.5 million cases worldwide in 2015^[1], including 3158 cases in New Zealand^[2]. It is the second most common cause of cancer death in the United Kingdom^[3] and New Zealand^[2].

The pathogenesis of CRC relates to a complex interaction between genetic predisposition and lifestyle factors^[1]. There are hereditary and non-hereditary CRC sub-types, although the majority are sporadic and result from somatic mutation in response to environmental factors^[1]. A key molecular pathway in the development of about 80% of CRC cases is the adenocarcinoma sequence, in which an accumulation of genetic mutations, particularly in the adenomatous polyposis coli pathway leads to carcinogenesis^[4]. Diets high in animal fat and red meat, physical inactivity and excess body weight, heavy alcohol consumption and smoking are known modifiable risk factors for CRC^[5].

The liver is commonly the first metastatic site for CRC, and may be the only site of spread in up to 30%-40% of patients with advanced disease^[6]. 20%-25% of CRC patients have detectable synchronous liver metastases at the time of diagnosis, and another 40%-50% of patients will subsequently develop metachronous liver metastasis within three years of resection of the primary tumor^[7].

The current concept of CRC proposes that the primary mode of tumor spread is via the portal system, and hence resection of isolated liver metastasis from CRC adenocarcinoma may be curative^[6]. However, the observation of circulating CRC cancer stem cells (CSCs)^[8] infers that the assumption of the liver being the most suitable site for metastasis remains to be determined. The median survival of liver metastasis from CRC adenocarcinoma without treatment is less than 12 months from presentation, with improved prognosis for patients with isolated liver metastasis^[9]. Approximately 20%-30% of patients with liver metastasis from CRC adenocarcinoma have disease confined to the liver and is therefore potentially operable^[10] with a five-year survival of 25%-44%^[7,11]. There has been recent focus on pre-operative neo-adjuvant chemotherapy to improve disease-free and overall survival by downsizing metastatic liver disease to increase operability and treat systemic disease^[12].

The CSC concept of cancer proposes that a subpopulation of cells within a cancer that possesses the properties of embryonic stem cells (ESCs) are the driving force for carcinogenesis due to their innate ability to promote angiogenesis, local invasion, distant metastasis, and resistance to apoptosis^[13]. Although the origin of CSCs remains to be elucidated, they are distinguishable from the cancer cell population by their expression of ESC markers, well researched in many cancer types including oral cavity squamous cell carcinoma (OCSCC) affecting different sub-sites^[14-16] and glioblastoma (GB)^[17]. Increased expression of ESC markers has been associated with increased tumor size, local invasion and metastasis^[18], worsening prognosis, treatment resistance^[19] and risk of local recurrence or metastasis^[20].

We have recently demonstrated the presence of three CSC subpopulations within liver metastasis from colon adenocarcinoma (LMCA): a SOX2⁺/NANOG⁺/KLF4⁺/c-MYC⁺/OCT⁻ subpopulation within the tumor nests (TNs), and a SOX2⁺/NANOG⁺/KLF4⁺/c-MYC⁺/OCT4⁻ CSC subpopulation and a SOX2⁺/NANOG⁺/KLF4⁺/c-MYC⁺/OCT⁺ CSC subpopulation within the peri-tumoral stroma (PTS)^[21].

The renin-angiotensin system (RAS) is a hormonal system that is classically known for regulation of cardiovascular homeostasis and blood pressure^[22]. A key component of the RAS is angiotensinogen which

is physiologically released from the liver into the circulation and is converted to angiotensin I (ATI) by renin - the active form of the proenzyme, pro-renin. The receptor for both renin and pro-renin is pro-renin receptor (PRR)^[23]. ATI is then converted to angiotensin II (ATII) by angiotensin converting enzyme (ACE). The effects of ATII are mediated through its receptors, angiotensin II receptor 1 (ATIR1) and angiotensin II receptor 2 (ATIR2)^[24].

Recent studies show that the RAS promotes tumor growth at a local tissue level by modulating angiogenesis, tumor cell proliferation, immune responses and extracellular matrix formation^[24]. Patients who are administered RAS modulators have a reduced incidence of CRC as well as lower rate of developing distant metastasis from CRC^[25,26].

CSCs in a number of cancer types, including OCS^[16,27,28] and GB^[17] have been shown to express components of the RAS. Hence CSCs may be a novel therapeutic target by modulation of the RAS. A recent meta-analysis reports an average 20% reduced risk of metastasis and improved survival from cancer including CRC, in patients who are administered RAS modulators^[29].

Currently there are few publications showing the presence of the components of the RAS in LMCA. This study aimed to investigate the expression of the components of the RAS: PRR, ACE, ATIR1, and ATIR2 within LMCA, in relation to the three CSC subpopulations we have recently identified^[21], using 3,3-diaminobenzidine (DAB) and immunofluorescence (IF) immunohistochemical (IHC) staining, Western blotting (WB), and NanoString mRNA expression analysis.

METHODS

Tissue samples

LMCA tissue samples from 16 male patients aged 50-80 (mean 65) years including those in our previous study^[21] were sourced from the Gillies McIndoe Research Institute Tissue Bank and used for this study which was approved by the Central Health and Disabilities Ethics Committee. Informed written consent was obtained from all participants.

Histochemical and immunohistochemical staining

Hematoxylin and eosin (H&E) staining was carried out on 4 µm-thick formalin-fixed paraffin-embedded sections of 16 LMCA tissue samples to confirm the presence of the tumor on the slides by an anatomical pathologist (HDB). DAB IHC staining was then performed on these sections using the Leica Bond Rx auto-stainer (Leica, Nussloch, Germany) with primary antibodies for PRR (1:2000; cat# AB40790, Abcam, Cambridge, MA, USA), ACE (1:100; cat# MAB4399, AbD Serotec, Oxford, UK), ATIR1 (1:300; cat# AB9391, Abcam), and ATIR2 (1:2000; cat# NBP1-77368, Novus Biologicals, Littleton, CO, USA), OCT4 (1:30; cat# MRQ-10, Cell Marque), SOX2 (1:200; cat# PA1-094, Thermo Fisher Scientific, Rockford, IL, USA). Antibodies were diluted in Bond primary antibody diluent (Leica). All DAB IHC-stained slides were mounted in Surgipath Micromount (Leica, Nussloch, Germany). To confirm co-expression of two proteins, two representative samples of LMCA from the original cohort of 16 patients used for DAB IHC staining, underwent IF IHC staining. Vectafluor Excel anti-mouse 488 (ready-to-use; cat# VEDK2488, Vector Laboratories, Burlingame, CA, USA) and Alexa Fluor anti-rabbit 594 (1:500; cat# A21207, Life Technologies, Carlsbad, CA, USA) were used to detect the combinations. All IF IHC-stained slides were mounted in Vecta Shield Hardset mounting medium with 4, 6-diamino-2-phenylindone (Vector Laboratories). All antibodies were diluted in Bond primary diluent (Leica). All DAB and IF IHC staining was performed using the Leica Bond Rx auto-stainer (Leica), as previously described^[16,28,30].

Image analysis

The DAB IHC-stained slides were viewed and the images were captured using an Olympus BX53 light microscope fitted with an Olympus DP21 digital camera (Olympus, Tokyo, Japan). An Olympus FV1200

confocal laser-scanning microscope (Olympus) was used for IF IHC-stained slides and the images were processed with CellSens Dimension 1.11 software using the 2D deconvolution algorithm (Olympus).

NanoString mRNA expression analysis

Six snap-frozen LMCA tissue samples from the original cohort of 16 patients included for DAB IHC staining were used to isolate total RNA. RNA was extracted using the MagJET RNA Kit and KingFisher Duo (ThermoFisher Scientific) protocol and quantitated by the NanoDrop 2000 Spectrophotometer (Thermo Scientific) and Qubit (Thermo Scientific). mRNA was assayed by New Zealand Genomics Ltd (Dunedin, NZ), using the NanoString nCounter Gene Expression Assay (NanoString Technologies, Seattle, WA, USA). Probes for the genes were designed and synthesized by NanoString Technologies and are PRR (ATP6AP2, NM_005765.2), ACE (CD143, NM_000789.2), ATIIR1 (AGTR1, NM_000685.3), and ATIIR2 (AGTR2, NM_000686.3). Raw data were analyzed by nSolver software (NanoString Technologies) using standard settings and were normalized against the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as routinely performed in our laboratory^[16,28].

Western blotting

Total protein was extracted from three snap-frozen LMCA tissue samples from the original cohort of 16 patients used for DAB IHC staining, resolved by SDS-PAGE, and transferred to a PVDF membrane as previously described^[31], and probed using the following antibodies: PRR (1:500; cat# ab40790, Abcam), ATIIR1 (1:500; cat# sc-1173, Santa Cruz, CA, USA), ATIIR2 (1:1000; cat# ab92445, Abcam), ACE (1:200; cat# sc-12184, Santa Cruz), and β -actin (1:500; cat# ab8229, Abcam). Secondary and tertiary antibodies were goat anti-rabbit HRP (1:2000; cat# ab6721, Abcam) for PRR and ATIIR1, rabbit anti-goat SuperclonalTM biotin conjugate (1:5000; cat# A27013, Thermo Fisher) and goat anti-rabbit SuperclonalTM biotin conjugate (1:5000; cat# A27035, Thermo Fisher) followed by PierceTM Streptavidin Poly-HRP (1:5000, cat# 21140, Thermo Fisher) for ACE and ATIIR2, respectively, and chicken anti-goat Alexa Fluor 647 (1:2000; cat# A21244, Life Technologies) for β -actin. Clarity Western ECL (cat# 1705061, Bio-Rad) was used as the substrate for HRP bands and the Chemi Doc MP Imaging System (Bio-Rad) and ImageLab 5.0 software (Bio-Rad) were used for detection and analysis, as previously used^[16,28]. Positive controls were snap-frozen human placenta tissue for PRR and ATIIR1, snap-frozen mouse lung tissue for ACE, and a recombinant ATIIR2 protein (cat# H00000186-P01, Novus Biologicals) for ATIIR2. Matched mouse (1:500; cat# ab18443, Abcam) and rabbit (1:500; cat# ab171870, Abcam) isotype controls were used as appropriate negative controls.

Statistical analysis

Statistical analysis of the NanoString mRNA data was performed using *t* test (SPSS v 24).

RESULTS

Histology and DAB IHC staining

H&E staining confirmed the presence of LMCA on the slides for each of the 16 tissue samples. DAB IHC staining demonstrated the expression of PRR [Figure 1A, brown] which was localized to cells within the TNs, cells within the PTS and the endothelium of the microvessels within the PTS. ACE [Figure 1B, brown] was expressed on the luminal surface of the TNs and weakly on the endothelium of the microvessels within the PTS. Strong cytoplasmic expression of ATIIR1 [Figure 1C, brown] was present on the cells within the TNs, the cells within the PTS and the endothelium of the microvessels within the PTS. ATIIR2 [Figure 1D, brown] was also expressed by the cells within the TNs, and weakly on the endothelium of the microvessels within the PTS.

The expected expression patterns of positive controls for PRR [Supplementary Figure 1A, brown], ACE [Supplementary Figure 1B, brown], ATIIR1 [Supplementary Figure 1C, brown], and ATIIR2 [Supplementary Figure 1D, brown] were demonstrated in human placenta, kidney, liver and kidney,

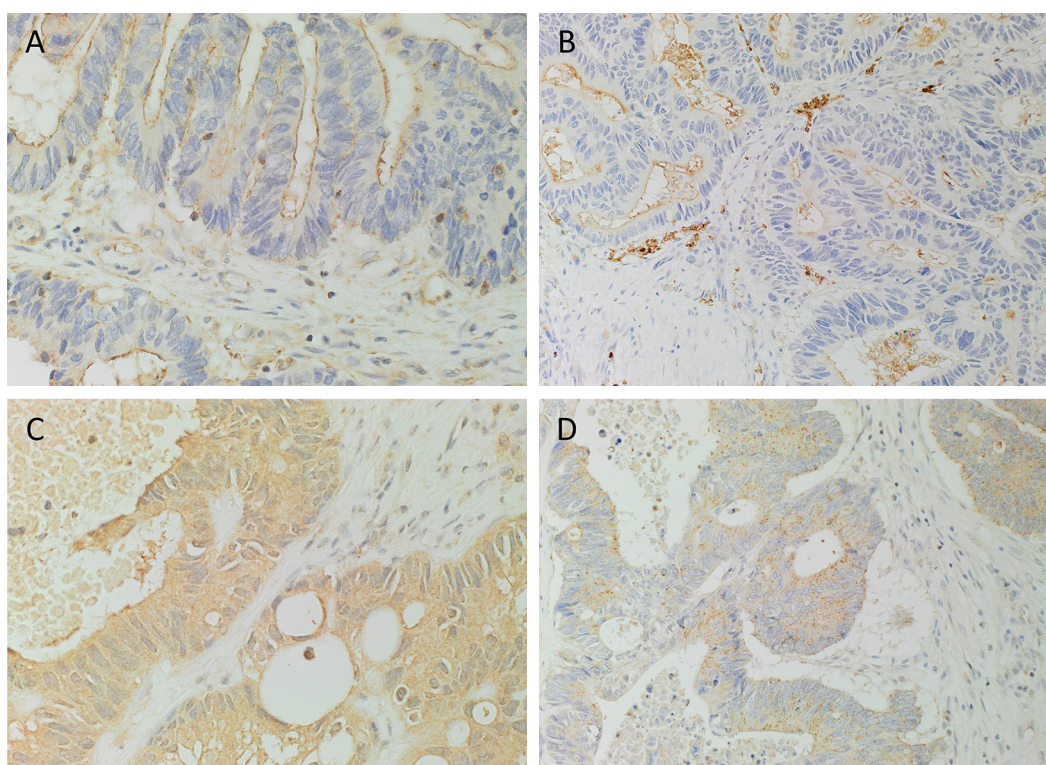


Figure 1. 3,3-Diaminobenzidine immunohistochemical-stained sections of liver metastases from colon adenocarcinoma demonstrating the expression of pro-renin receptor [(A), brown] localized to cells within the tumor nests (TNs), the cells within the peritumoral stroma (PTS) and the endothelium of the microvessels within the PTS. Angiotensin converting enzyme [(B), brown] was expressed on the luminal surface of the TNs and weakly on the endothelium of the microvessels within the PTS. Angiotensin II receptor 1 (ATIIR1) [(C), brown] demonstrated strong cytoplasmic expression on cells within the TNs, the endothelium of the microvessels and the cells within the PTS. ATIIR2 [(D), brown] was also expressed on the cells within the TNs and weakly on the endothelium of the microvessels within the PTS. Nuclei were counter-stained with hematoxylin [(A-D), blue]. Original magnification: 400×

respectively. The negative controls on LMCA tissue samples, stained using an IgG isotype [Supplementary Figure 1E], demonstrated the specificity of the primary antibodies used.

IF IHC staining

IF IHC staining was employed to investigate the expression of components of RAS by the CSC subpopulations within LMCA by co-staining with ESC markers OCT4 and SOX2. PRR [Figure 2A, red] was expressed by the cells within the TNs and the cells within the PTS, regardless of whether they were OCT4⁺ [Figure 2A, green] or OCT4⁻ [Figure 2A]. ACE [Figure 2B, green] was expressed on the luminal surface of the cells within the TNs as well as the endothelium of the microvessels within the PTS, which also expressed SOX2 [Figure 2B, red]. ATIIR1 [Figure 2C, green] was demonstrated in cells within the TNs, the endothelium of the microvessels within the PTS [Figure 2C] and the cells within the PTS [Figure 2C], which also expressed SOX2 [Figure 2C, red]. A similar expression pattern was seen for ATIIR2 [Figure 2D, red] in the cells within the TNs and the cells within of the PTS whether they expressed OCT4 [Figure 2D, green] or not [Figure 2D].

Split images of the stains presented in Figure 2 are shown in [Supplementary Figure 2]. Minimal staining was present on the negative control [Supplementary Figure 2I], confirming the specificity of the primary antibodies used.

NanoString mRNA expression analysis

NanoString mRNA expression analysis of PRR, ACE, ATIIR1, and ATIIR2 on six snap-frozen LMCA tissue samples of the original cohort of 16 patients used for DAB IHC staining, normalized against the

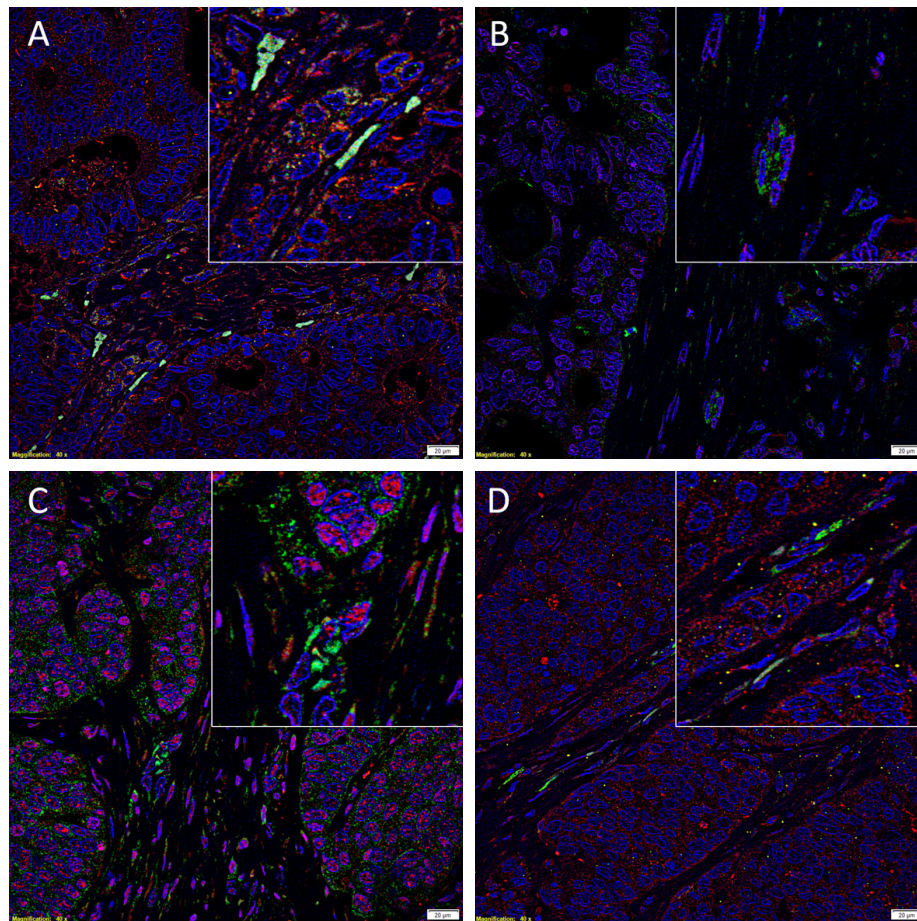


Figure 2. Representative immunofluorescence immunohistochemical-stained sections of liver metastasis from colon adenocarcinoma demonstrating the expression of pro-renin receptor [(A), red] by the cells within the tumor nests (TNs), the cells within the peritumoral stroma (PTS) regardless of whether they were OCT4⁺ [(A), green] or OCT4⁻ (A). Angiotensin converting enzyme [(B), green] was expressed on the endothelium of the microvessels within the PTS, which also expressed SOX2 [(B), green]. Angiotensin II receptor 1 (ATIIR1) [(C), green] was demonstrated on cells within the TNs, the endothelium of the microvessels within the PTS (C) and the cells within the PTS (C), which also expressed SOX2 [(C), red]. A similar expression pattern was seen for ATIIR2 [(D), red] in the cells within the TNs and the cells within of the PTS whether they expressed OCT4 [(D), green] or not (D). All slides were counter-stained with 4',6'-diamino-2-phenylindole [(A-D), blue]. Scale bars: 20 μ m; insert: 400 \times magnification

housekeeping gene GUSB, confirmed the presence of PRR, ACE and ATIIR1 mRNA in all six samples and ATIIR2 in one sample [Figure 3], with ATIIR2 mRNA expression at a significantly lower level in all six samples compared to the other genes ($P < 0.05$).

Western blotting

WB on the three snap-frozen LMCA tissue samples demonstrated the presence of bands at the expected molecular weight for PRR, ATIIR1, ATIIR2 and ACE [Figure 4]. PRR was detected in all three samples at 22 kDa [Figure 4A], with the specificity of the antibody confirmed using a recombinant PRR protein (cat# ab153053, Abcam). ATIIR1 was detected in all three samples at the expected molecular weight of 41 kDa [Figure 4B], and ATIIR2 was detected in two out of three samples at 50kDa [Figure 4C], consistent with ATIIR2 variants from adrenal glands and from ATIIR2 proteins with high glycosylation^[32]. ACE was detected at a low abundance in all three samples at the expected molecular weight of 194 kDa [Figure 4D]. This is supported by detection of ACE using the same antibody in different tissues^[33]. β -actin confirmed approximate equivalent protein loading for all LMCA samples examined [Figure 4E]. The isotype controls are presented in Supplementary Figure 3.

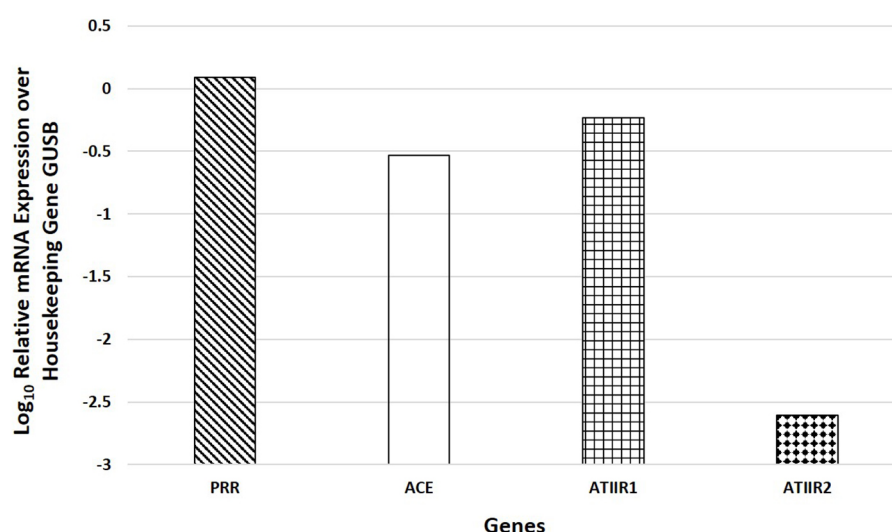


Figure 3. NanoString mRNA expression analysis of pro-renin receptor (PRR), angiotensin converting enzyme (ACE), angiotensin II receptor 1 (ATIIR1), and angiotensin II receptor 2 (ATIIR2) on six snap-frozen samples of liver metastasis from colon adenocarcinoma normalized against the housekeeping gene GUSB, confirmed the presence of PRR, ACE and ATIIR1 mRNA in all six samples and ATIIR2 in one sample, with ATIIR2 mRNA levels at a significantly lower level within all six samples compared to the other genes ($P < 0.05$)

DISCUSSION

The classical RAS is an endocrine system that is crucial in regulating blood pressure and fluid homeostasis through its effects on the kidney, cardiovascular, and central nervous systems^[24]. Physiologically angiotensinogen is cleaved by renin to form ATI. ACE then cleaves ATI to form ATII. ATII is the main effector of the RAS that mediates its effects by binding with its receptors ATIIR1 and ATIIR2^[24]. Recent literature suggests a critical role for the RAS in cancer growth and metastasis^[24]. There is increasing appreciation of the complex interplay between the RAS and other pathways including IGF, VEGF, and potential bypass loops consisting of cathepsins B, D and G^[22,34]. These related pathways are implicated in a number of biological processes such as angiogenesis, cell migration, tumorigenesis and hematopoiesis which may create a conducive environment for carcinogenesis by promoting the proliferation of CSCs^[22].

More specifically to LMCA, a report by Heinzerling *et al.*^[26] shows the use of ACE inhibitors independently predicts less likelihood of patients with stage II CRC to develop distant metastasis. Using a murine model of CRC liver metastasis, Wen *et al.*^[35] show that administration of captopril, an ACE inhibitor, reduces proliferation of the CRC cells. These coupled with a recent review, highlighting the role of ACE inhibitors and ATIIR1 blockers in reducing of the incidence of CRC and the development of distant metastasis from CRC^[25]. Taken together it is exciting to speculate that the expression of components of the RAS by the CSCs in LMCA is crucial in the maintenance and proliferation of these CSCs, although this remains the topic of future investigation.

We have recently demonstrated three subpopulations of CSCs within LMCA, including an OCT4⁺ subpopulation, and an OCT4⁻ subpopulation within the PTS and the TNs, suggesting the presence of a CSC hierarchy^[21]. In this study we have demonstrated the expression components of the RAS: PRR, ACE and ATIIR1 and ATIIR2 by these CSC subpopulations within LMCA. It is interesting that ATIIR2 was detected by DAB IHC staining in all 16 samples, but only two out of the three samples by WB and in only one sample by NanoString mRNA analysis, possibly due to sampling bias.

The novel findings in this study mirror similar findings in OCSCC affecting the oral tongue^[28], buccal mucosa^[27] and lip^[16], and GB^[17]. The expression of ACE on the endothelium of the microvessels within the

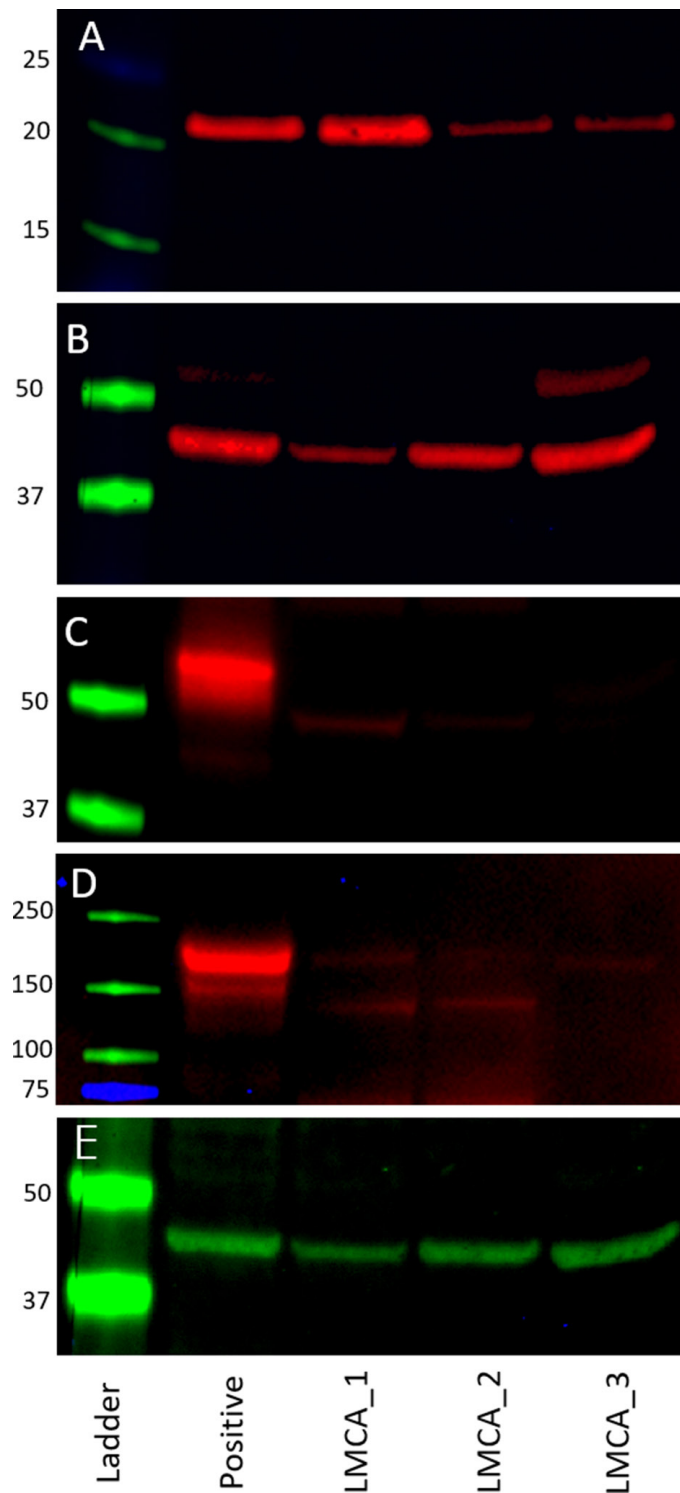


Figure 4. Western blot images of total protein extracted from snap-frozen samples of liver metastasis from colon adenocarcinoma (LMCA) from three patients demonstrating the presence of pro-renin receptor (PRR) in all three samples at 22 kDa (A), with the specificity of the antibody confirmed using a recombinant PRR protein. Angiotensin II receptor 1 was detected in all three samples at the expected molecular weight of 41 kDa (B); angiotensin II receptor 2 was detected in two out of three samples at 50 kDa (C); angiotensin converting enzyme was detected at a low abundance in all three samples at the expected molecular weight of 194 kDa (D); and β -actin confirmed approximate equivalent protein loading for all LMCA samples examined (E)

PTS suggests a role in regulating epithelial-to-mesenchymal transition by influencing angiogenesis^[16,24], although this requires further investigation.

It is exciting to speculate that CSCs may be a novel therapeutic target by modulation of the RAS using existing medications such as aliskiren which targets renin, β -blockers which inhibit the production of prorenin and hence decrease renin levels, ACE inhibitors and ATII receptor blockers^[36-38]. A recent meta-analysis on the use of ACE inhibitors and ATIIR1 blockers demonstrate an average 20% improved survival or reduction of recurrence in different types of cancers, including primary CRC^[29], suggesting a crucial role of these drugs in tumorigenesis, potentially through modulation on the RAS, although this requires further investigation.

Cathepsins B, D and G are proteases that constitute bypass loops for the RAS^[22] and have been implicated in tumorigenesis and metastasis in CRC due to their ability to degrade extra-cellular matrix. We have shown the expression of cathepsin B and cathepsin D by the CSC subpopulations within oral tongue squamous cell carcinoma^[27], GB^[39] and more recently LMCA^[34], implying the presence of bypass loops for the RAS.

The novel finding of the expression of the components of RAS and cathepsin B and cathepsin D by the CSC sub-populations within LMCA, provides insights into possible more effective modulation of the RAS in targeting the CSCs. Further studies including a larger sample size and *in vivo* functional experiments are needed to explore the functional role of the RAS.

In conclusion, the novel finding of the expression of PRR, ATIIR1 and ATIIR2 on the CSCs within the TNs and CSCs within the PTS, and the expression of ACE in the endothelium of the microvessels in LMCA suggests the CSCs may be a novel therapeutic target by manipulation of the RAS.

DECLARATIONS

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Authors' contributions

Formulated the study hypothesis: Tan ST, Itinteang T

Designed the study: Wickremesekera SK, Tan ST, Itinteang T

Interpreted the DAB and IF IHC data: Narayanan A, Brasch HD, Tan ST, Itinteang T

Interpreted the NanoString mRNA expression data: Narayanan A, Wickremesekera SK, Tan ST, Itinteang T

Performed the WB analysis and interpreted the results: van Schaijik B

Performed the statistical analysis: Marsh RW

Drafted the manuscript: Narayanan A, Tan ST, Itinteang T

Availability of data and materials

Data supporting the findings of this study can be obtained by contacting the corresponding author.

Financial support and sponsorship

None.

Conflicts of interest

The authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

This study was carried out with the approval of the Central Health and Disability Ethics Committee (Ref. 15/CEN/106) with written informed consent from all subjects in accordance with The Declaration of Helsinki.

Consent for publication

Not applicable.

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Original Article

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The molecular interaction of ADAMTS-1 and fibulin-1 and its potential contribution to breast cancer biology

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Abstract

Aim: Fibulins and ADAMTSs are two families of extracellular matrix proteins implicated in key functional and pathological processes. The fact that the fibulin-1 and ADAMTS-1 proteins interact raises new questions about the roles of these extracellular matrix proteins in modulating tumor progression. Herein, we described the functional implications of the interaction between fibulin-1 and ADAMTS-1 on the behavior of breast cancer cell lines.

Methods: Fibulin-1 and ADAMTS-1 were exogenously expressed in MCF-7 and MDA-MB-231 cell lines to assay the effect of their interaction in cellular properties.

Results: ADAMTS-1 expression exacerbates tumor effects in terms of proliferation, invasion and mammosphere formation. In contrast, the simultaneous expression of ADAMTS-1 and fibulin-1 impairs these effects. The analysis of the expression of both proteins in human breast cancer tissue arrays provides new insights into the complex roles of fibulin-1 and ADAMTS-1 in this type of tumor.



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Conclusion: Our results suggest that the interaction between ADAMTS-1 and fibulin-1 induces a pronounced anti-tumoral effect.

Keywords: ADAMTS-1, fibulin-1, cell migration, cell proliferation, breast cancer, MCF-7, MDA-MB-231

INTRODUCTION

The extracellular environment is a dynamic niche in which multiple molecular interactions occur that govern cell fate. Several families of extracellular proteins function in this molecular dance, influencing cell properties and thus regulating either physiological or pathological processes^[1,2]. In addition, stromal cells are considered especially important in the extracellular environment as they induce extracellular modifications that can promote dysfunction in processes such as in cancer progression^[3].

In general, proteases are considered to be responsible for altering tissues and initiating the extracellular remodeling of tissues. Among proteases, members of the ADAMTS family have been widely implicated in different steps of cancer development. The ADAMTS family consists of 19 members, all of which have several domains, including a metalloprotease, a disintegrin and a variable number of thrombospondin motifs, which allow them to interact with different components of the extracellular matrix (ECM)^[4]. ADAMTS-1 was the first identified member of this family of matrix metalloproteases due to its association with inflammation processes^[5]. Since its discovery, ADAMTS-1 has been described to participate in several other processes, such as organogenesis, vessel formation and ovulation^[6-8]. The participation of ADAMTS-1 in cancer is underlined by its implication in some of the most important features of cancer development and progression^[9]. Thus, ADAMTS-1 has positive effects on cell survival, invasion and migration processes and, in general, does not participate in cell proliferation^[10,11]. Furthermore, ADAMTS-1 promotes angiogenesis in breast xenografts, but it is also able to inhibit angiogenesis in lung and hepatic metastasis as well as in other cell-based angiogenic experiments^[11,12].

Several studies provide conflicting associations of ADAMTS-1 with breast cancer; ADAMTS-1 has been reported to be both a pro- and anti-tumorigenic factor^[10,13-15]. This dual function of ADAMTS-1 depends on the conditions in which the ADAMTS-1 pathway is functioning, i.e., microRNAs such as miR-365 or peroxisome proliferator-activated receptor (PPAR δ)^[16,17]. The tumor promotion or tumor inhibition properties associated with ADAMTS-1 can also depend on the fragmentation of ADAMTS-1 or the interactions of ADAMTS-1 with other components of the ECM^[18]. One of the known partners of ADAMTS-1 in the ECM is fibulin-1; the interaction of these two proteins was described as a result of a yeast two-hybrid screen for potential interactions of ADAMTS-1. As a result of this interaction, the proteolytic activity of ADAMTS-1 towards aggrecan is increased; thus, fibulin-1 is considered a cofactor of ADAMTS-1 aggrecanase activity^[19].

Fibulin-1 was the first identified member of the fibulin family of matrix proteins^[20]. The members of this family each contain a fibulin-like domain at the carboxy terminal of the protein. The fibulin family consists of seven members of different lengths that contain different motifs with varying functions, such as anaphylatoxin domains (fibulin-1 and fibulin-2), epidermal growth factor (EGF)-like modules (fibulin-3 and fibulin-4), thrombospondin-like and von Willebrand factor domains (fibulin-6) or a sushi domain (fibulin-7)^[21]. In particular, fibulin-1 and fibulin-2 are closely related and characterized by the presence of anaphylatoxin and calcium-binding EGF (cbEGF) modules. However, only fibulin-2 contains two additional modules: cysteine (cys)-rich and cys-free domains. In addition, fibulin-1, fibulin-2, fibulin-3, fibulin-4 and fibulin-5 are considered elastogenic fibulins since they are important components of elastic fibers that are particularly abundant in the dermis, lungs and arterial walls^[21,22].

The roles of fibulins in tumor development have been widely discussed, and they show both pro- and anti-tumor activities^[23]. In particular, fibulin-1 has been described to be overexpressed in various human cancers and associated with processes such as invasion, migration, and *in vivo* tumor growth^[24-26]. The role of fibulin-1 in breast cancer depends on multiple factors. For example, four alternative forms of fibulin-1 have been described (fibulin-1A, fibulin-1B, fibulin-1C and fibulin-1D), which differ at the carboxy terminal^[27]. Fibulin-1D acts as a product of a tumor suppressor gene, whereas fibulin-1C (lacks only 21 amino acids of the fibulin-like domain) behaves as an oncogenic protein^[25,28]. Furthermore, the identification of fibulin-1 localization or fibulin-1-expressing cells are also important metrics since fibulin-1 is one of the genes specifically expressed in normal fibroblasts but not in breast cancer-associated fibroblasts^[29]. The epigenetic downregulation of fibulin-1 has also been described in various cancers, such as gastric cancer, colorectal cancer, hepatocellular carcinoma, and bladder cancer, but this downregulation has not been described in breast cancer^[30-33].

Herein, we describe the effects of the ADAMTS-1/fibulin-1 interaction on breast cancer cell properties related to cancer progression and development. We found that cell proliferation, invasion and migration are susceptible to being affected by this interaction as well as the capacity of tumorsphere formation, which reflects the capability of cancer cells to form tumor focus. Finally, we detected very low levels of ADAMTS-1 expression and high levels of fibulin-1 expression in a human tissue array from breast cancer patients, which can be considered a deviation towards a cancerous phenotype.

METHODS

Cells lines and cell culture conditions

Human MDA-MB-231 and murine MCF-7 breast cancer cell lines were kindly provided by Dr. Carlos López-Otín (University of Oviedo, Spain). Cells were routinely maintained in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 50 µg/mL streptomycin and 100 U/mL penicillin (Life Technologies). For transfection experiments, vectors containing the full-length human cDNAs for fibulin-1 (kindly provided by Dr. Tatako Sasaki, Oita University, Japan) and Flag-tagged ADAMTS-1 (Origene) were transfected into cells at 75% confluence using TransIT-X2 (Mirus) as recommended by the manufacturer. Cells transfected with an empty vector were used as a control. Stably expressing transfectants were selected with 500 µg/mL G418 (Sigma-Aldrich) and used for all the experiments.

Western blotting and immunoprecipitation

For western blot analysis, proteins were resolved by 10% polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and subsequently probed with the indicated antibodies. Primary antibodies for the detection of ADAMTS-1 were purchased from Sigma-Aldrich (FLAG M2, for immunoprecipitation) and Bioss (bs-1208R-A488, for immunohistochemistry and western blotting), and those used for the detection of fibulin-1 were obtained from Novus (NBP1-84725, for immunohistochemistry) and Santa Cruz Biotechnology (H-190, for western blotting).

The anti-actin antibody used as the load control was purchased from Sigma-Aldrich (AC-15). Immunoreactive proteins were visualized using HRP-peroxidase-labeled anti-rabbit or anti-mouse secondary antibodies and an electrochemiluminescence (ECL) detection system (Pierce). For immunoprecipitation, MCF-7 double-transfected cells (ADAMTS-1 and fibulin-1) were resuspended in lysis buffer (100 mM Tris-HCl at pH 7.4, 150 mmol/L NaCl, 10 mmol/L EDTA, 1% desoxycholic acid, 1% Triton X-100, and 0.1% SDS containing one complete protease inhibitor cocktail (one tablet/50 mL buffer; Roche Molecular Biochemicals) and incubated for 2 h on ice with eventual stirring. Cell debris was removed by centrifugation in a bench-top centrifuge for 15 min at 4 °C. Protein concentration was quantified using the BCA Protein Assay Kit from Pierce Technology. For immunoprecipitation, 1 mg of protein extract was incubated with anti-FLAG M2 affinity gel

(Sigma-Aldrich) for 16 h at 4 °C following the manufacturer's instructions. After three washes in lysis buffer, the immunoprecipitates were resolved by western blotting.

Invasion assays

In vitro invasion potential was evaluated using 24-well Matrigel-coated invasion chambers with an 8- μ m pore size (BD Biosciences). For MCF-7 cells, 5×10^5 cells were allowed to migrate for 96 h using 10% fetal bovine serum as a chemoattractant. Cells that reached the lower surface were stained with crystal violet. At least three independent experiments were performed in triplicate for each condition. Cells were counted in eight randomly selected microscopic fields. In the case of MDA-MB-231 cells, invasion was evaluated after 24 h.

Migration assays

The migratory capacity of cells on the ECM components fibronectin, laminin I, and type-I collagen was examined using the RadiusTM 24-Well Cell Migration Assay kit (Cell Biolabs) following the manufacturer's instructions. Briefly, 5×10^5 cells were seeded per well, and migration was monitored by time-lapse microscopy using a Zeiss Axio Observer Microscopy. Experiments were performed in triplicate, and the covered area was quantified at different times using ImageJ. For MCF-7 cells, the results were obtained after 24 h of migration. In the case of MDA-MB-231 cells, we used a barrier-migration assay over a period of 24 h and the same ECM components^[34].

Cell proliferation determination by Ki-67 staining

Cell proliferation was estimated by the quantification of Ki-67-positive nuclei in MCF-7 and MDA-MB-231 cell cultures, including controls and the different transfectants. Cells were fixed with 4% paraformaldehyde, blocked with 10% fetal bovine serum and incubated overnight with an anti-Ki67 antibody (Santa Cruz Biotechnologies). After three washes in phosphate buffered saline (PBS), the cells were incubated with a secondary Alexa 546-conjugated antibody (Life Technologies) for 1 h. In all samples, DAPI (100 ng/mL) was added to visualize the DNA in the cell nucleus. Images were obtained using a fluorescence microscope (Axiovert). After quantification, the data were plotted as an average of Ki-67-positive nuclei in relation to the total number of nuclei per microscopic field.

Mammosphere cultures

A total of 4×10^4 MCF-7 cells were plated in 6-well ultralow attachment plates (Costar) and grown in MammoCult Basal Medium (Stem Cell Research) supplemented with 10% MammoCult Proliferation Supplement, 4 μ g/mL heparin and 0.5 μ g/mL hydrocortisone. After 7 days, mammospheres were collected and enzymatically dissociated as previously described^[35]. The individual dissociated cells were cultured in 96-well ultralow attachment plates at a density of 20 cells/well. Mammosphere formation was microscopically monitored daily to ensure that the mammospheres were derived from single cells and not from aggregates. The number of mammospheres was determined after 7 days of culture.

Human breast cancer tissue array

A breast cancer tissue array containing samples from different tumor stages was employed to evaluate ADAMTS1 and FBLN1 expression in human breast cancer samples. The tissue array was obtained from the Institute of Oncology of Asturias Tumour Bank. Written informed consent was obtained from all patients prior to sample collection. The study was approved by the appropriate institutional review board according to national and EU guidelines.

Slides were processed for indirect peroxidase immunohistochemistry as follows: sections were deparaffinized, rehydrated and then rinsed in PBS containing 1% Tween-20. For the detection of fibulin-1 and ADAMTS-1, the sections were heated in an Envision FLEX target retrieval solution at high pH and 80 °C for 20 min and then incubated for 20 min at room temperature in the same solution. Endogenous peroxidase activity (3% H₂O₂) and nonspecific binding (33% fetal calf serum) were blocked, and the sections were incubated overnight at 4 °C with primary antibodies diluted 1:100. We used labeled polymer-HRP (ready for use)

antibodies from DAKO as the secondary antibodies. 3-3'-Diaminobenzidine was employed as a chromogen. Selected slides were counterstained with hematoxylin to ascertain structural details. As immunostaining controls, representative sections were processed in the same way as described above using nonimmune rabbit or mouse sera instead of the primary antibodies or by omitting the primary antibodies during the incubation. Furthermore, when available, additional controls were carried out using specifically preabsorbed antisera.

Fibulin-1 immunostaining intensity was semiquantitatively evaluated by two independent observers directly under the microscope using ten randomly selected fields (20x objective and a 20x ocular). The intensity of the immunoreaction was evaluated as strong (+++), moderate (++), weak (+) or negative (-).

Survival analysis

To assess the effect of ADAMTS-1 and fibulin-1 on breast cancer prognosis, survival probability was determined using the data available at www.kmplot.com^[36]. The results are presented as Kaplan-Meier long-rank test survival plots.

Statistical analysis

Statistical analysis was carried out using Microsoft Office software. Data are presented as mean \pm SE. The occurrence of significant differences was determined with the Student-Welch *t* test. *P* values under 0.05 were considered statistically significant (**P* < 0.05, ***P* < 0.01, ****P* < 0.005).

RESULTS

ADAMTS-1 and fibulin-1 interact in breast cancer cell lines

Fibulin-1 has been described as an ADAMTS-1 cofactor because of its proteolytic activity towards aggrecan degradation^[19]. Fibulin-1 is not an ADAMTS-1 substrate; instead, it is an interacting partner that can affect cancer-associated properties such as the migration, invasion and proliferation of breast cancer cell lines. Thus, we used two common human breast cancer cell lines, MCF-7 and MDA-MB-231. First, we quantified the endogenous expression of both proteins in cell extracts obtained from both cell lines [Figure 1]. No detectable levels of fibulin-1 or ADAMTS-1 were visualized by western blotting in either the MCF-7 or the MDA-MB-231 cell extracts [Figure 1A]. Thus, we transfected both cell lines with the corresponding cDNAs (ADAMTS-1, fibulin-1 or both simultaneously) to obtain transfectants expressing one of the proteins or both proteins together. For control purposes, we used an empty vector as a negative control [Figure 1A, control]. Once we obtained all the transfectants, we tried to probe the *in vivo* interaction of ADAMTS-1 and fibulin-1 in MCF-7 extracts by immunoprecipitation. To this end, we took advantage of the Flag epitope present in the exogenously expressed ADAMTS-1 protein. Fibulin-1 western blotting following Flag (ADAMTS-1) immunoprecipitation showed a 100 kDa reactive band only in extracts from the ADAMTS-1/fibulin-1 double-transfected cells [Figure 1B, top], which demonstrated the existence of this interaction in the MCF-7 double transfectants [Figure 1B]. To probe the presence of ADAMTS-1 in the same immunoprecipitate, we carried out a Flag western blot that showed the presence of a 100 kDa reactive band that corresponded to the size of ADAMTS-1 [Figure 2, bottom].

Effect of the fibulin-1/ADAMTS-1 interaction on invasion properties

After we demonstrated the fibulin-1/ADAMTS-1 interaction in breast cancer cells, we decided to explore the functional consequences of this event by first checking the effects of this interaction on the invasion properties of both cell lines. To address invasion properties, we used Matrigel-coated invasion chambers [Figure 2], and we observed that the fibulin-1/ADAMTS-1 interaction resulted in a significant reduction in the invasion capability of both cell lines. Thus, the invasive potential of the double transfectants of both the MDA-MB-231 and the MCF-7 cell lines was decreased compared with that of the control cell lines (MDA-MB-231: average of 112 double transfectants cells/field vs. 240 control cells/field; MCF-7: 65 double

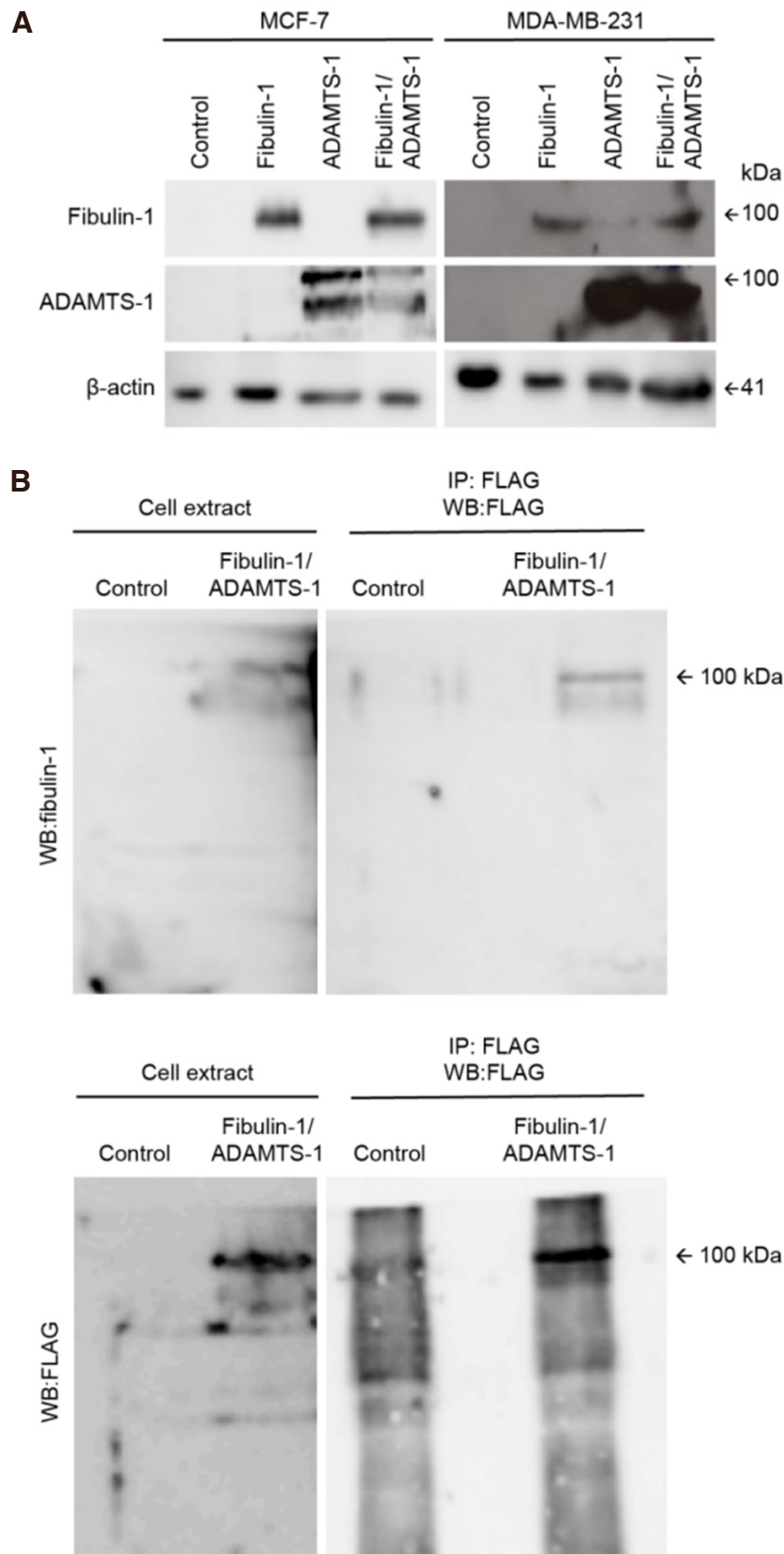


Figure 1. Selection of MCF-7 and MDA-MB-231 stable transfectants and coimmunoprecipitation of fibulin-1 with ADAMTS-1. A: Western blot analysis of MCF-7 and MDA-MB-231 expressing exogenous fibulin-1, ADAMTS-1 or both proteins simultaneously (Fibulin-1/ADAMTS-1). Control, cells transfected with an empty vector. Actin was used as a loading control. Molecular weight marker is indicated on the right. Independent gels were performed for each western blot; B: immunoprecipitation of MCF-7 control cell extracts or extracts from cells expressing fibulin-1 and ADAMTS-1 (Fibulin-1/ADAMTS-1) using an anti-FLAG M2 affinity gel. IP, immunoprecipitated. Top, detection with an anti-fibulin-1 antibody. Bottom, detection with an anti-FLAG (ADAMTS-1) antibody. Intervening irrelevant lanes are not shown

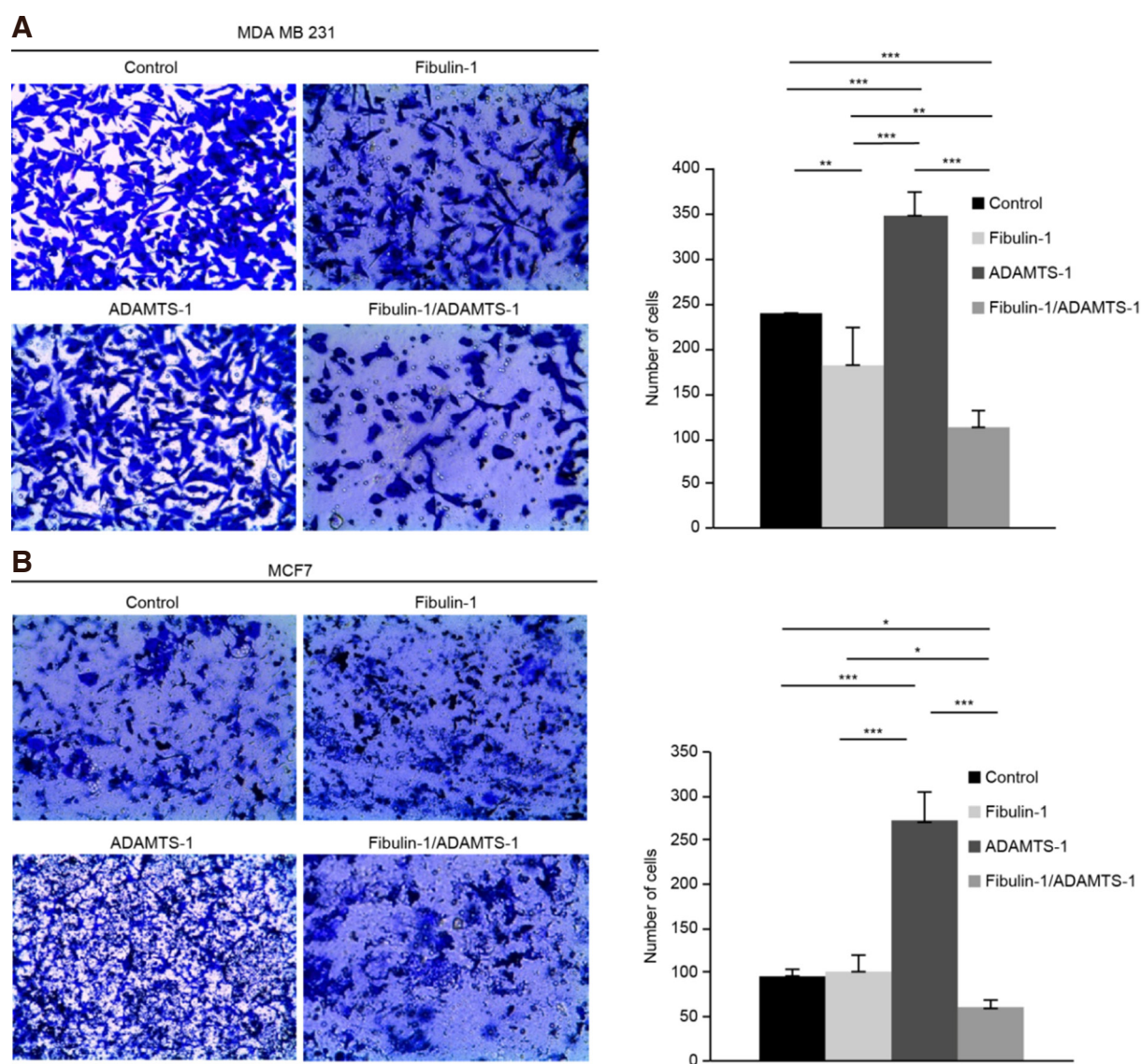


Figure 2. Coexpression of ADAMTS-1 and fibulin-1 reduces the invasion properties of the breast cancer cell lines MCF-7 and MDA-MB-231. Cell invasion assay using Matrigel-coated invasion chambers. A: representative microscopic pictures of MDA-MB-231 cells expressing exogenous fibulin-1, ADAMTS-1 or both proteins simultaneously. Cells transfected with an empty vector were used as a control. Cells that reached the lower surface were counted and are graphically shown; B: representative microscopic pictures of MCF-7 cells expressing exogenous fibulin-1, ADAMTS-1 or both proteins simultaneously. Cells transfected with an empty vector were used as a control. Cells that reached the lower surface were counted and graphically represented. P values under 0.05 were considered statistically significant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$)

transfectants cells/field vs. 90 control cells/field). The expression of fibulin-1 alone had the ability to reduce the invasiveness of MDA-MB-231 cells (an average of 175 vs. 240 cells/field) but not the invasiveness of MCF-7 cells. In contrast, and in agreement with the previously described tumor-promoting properties of ADAMTS-1, the invasion capabilities of the ADAMTS-1 transfectants of both cell lines showed an important increase compared with those of the control cell lines (averages of 340 vs. 240 cells/field for the MDA-MB-231 cell line and 275 vs. 90 cells/field in MCF-7 cells).

Effect on the migration properties of the fibulin-1/ADAMTS-1 interaction

Next, we analyzed the migration potential of both cell lines over two components of the extracellular matrix, type-I collagen and fibronectin. After 24 h, we measured the covered area and graphed the results for a better interpretation of the results. In all cases, substrates and cell lines, we observed that the migration properties

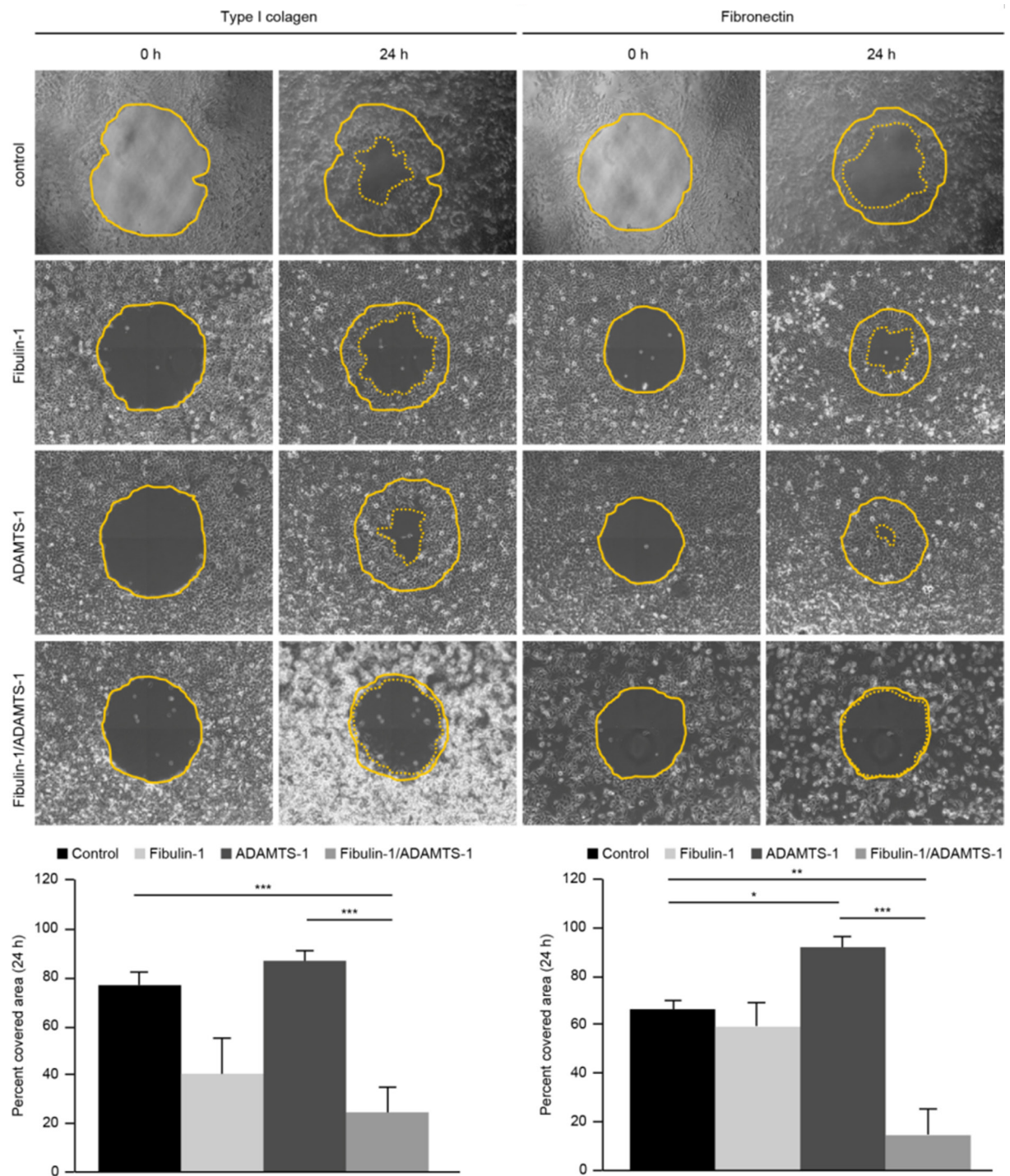


Figure 3. Coexpression of ADAMTS-1 and fibulin-1 reduces the migration properties of the breast cancer cell line MCF-7 over type-I collagen and fibronectin. Top, Representative images of the migration of MCF-7 transfectants on wells coated with type-I collagen and fibronectin using the RadiusTM 24-Well Cell Migration Assay kit (Cell Biolabs). MCF-7 cells transfected with an empty vector were used as a control. Pictures of the initial (t: 0 h) and final (t: 24 h) times are included. The starting point is indicated with a thin dotted line, and the final point is indicated with a thick dotted line. Bottom, graphical representation of percentages of the area covered after 24 h (from three independent experiments)

of the double transfectants (fibulin-1 and ADAMTS-1) were significantly compromised compared with those of their respective controls. We observed that the control MCF-7 cells were able to cover 79% of the area on type-1 collagen-coated dishes, whereas the double transfectants only were able to cover 25% of the area. In

the case of fibronectin, the control cells could cover 65% of the dish area, and the double transfectants could cover 18% of the dish area [Figure 3]. The data for the control and double transfectants cells of the MDA-MB-231 cell line were somewhat similar; the control cells covered 90% of the areas of the type-1 collagen- and fibronectin-coated plates, while the double transfectants covered 35% and 25% of the type-1 collagen- and fibronectin-coated plates, respectively [Supplementary Figure 1]. The expression of ADAMTS-1 alone also showed significant effects on migration only over the fibronectin substrate compared with the control cells and double transfectants of the MCF-7 and MDA-MB-231 cell lines [Figure 3 and Supplementary Figure 1, respectively]. The overexpression of fibulin-1 alone showed significant effects compared with the double-transfected and ADAMTS-1-overexpressing MDA-MB-231 cells over fibronectin and type-1 collagen [Supplementary Figure 1].

Effects of the fibulin-1/ADAMTS-1 interaction on cellular proliferation

Ki-67 nuclear staining is a good marker to measure the proliferative state of any given cell line. In this study, we used Ki-67 nuclear staining to evaluate the proliferative properties of all the transfectants generated. In both cell lines, MDA-MB-231 and MCF-7, ADAMTS-1 overexpression increased cell proliferation, as shown in Figure 4. The number of Ki-67-positive nuclei in the ADAMTS-1-expressing and control cells of the MDA-MB-231 cell line was 63 and 48, respectively, whereas that in the ADAMTS-1-expressing and control cells of the MCF-7 cell line was 82 and 64, respectively. On the other hand, fibulin-1 overexpression was able to significantly reduce proliferation in only the MCF-7 cells; 49 and 64 Ki-67-positive nuclei were detected in the fibulin-overexpressing and control cells, respectively. When ADAMTS-1 and fibulin-1 were transfected together, a reduction in the cell proliferation of both cell lines was observed. Specifically, in MDA-MB-231 cells, 35 and 48 Ki-67-positive nuclei were detected in the double transfectants and control cells, respectively, whereas in MCF-7 cells, 45 and 64 Ki-67-positive nuclei were detected in the double transfectants and control cells, respectively [Figure 4].

Effects of the fibulin-1/ADAMTS-1 interaction on mammosphere formation

One way of mimicking a tissue environment using 3D cell culture *in vitro* is through the formation of mammospheres, allowing the quantification of the size of these structures as well as the self-renewal capacity of the cell. In this context, we were able to examine the influence of fibulin-1 and ADAMTS-1 on the mammosphere formation of breast cancer cells. The fibulin-1/ADAMTS-1 interaction resulted in fewer and smaller mammospheres than did the other three combinations [Figure 5]. Furthermore, significant differences in mammosphere forming units (MFUs) were observed among all possible combinations. It is remarkable that the ability to develop MFUs was almost completely abolished when fibulin-1 and ADAMTS-1 were coexpressed (no MFUs were detected). Compared with the control cells, cells expressing fibulin-1 also exhibited a diminished ability to develop MFUs (11 MFUs vs. 4 MFUs). In contrast, ADAMTS-1 expression slightly increased this capacity. Taken together, our data indicate that fibulin-1 and ADAMTS-1 affect the ability of this cell line to form mammospheres.

Analysis of ADAMTS-1 expression in breast cancer tissue samples

We performed an immunohistochemical analysis of the expression of ADAMTS-1 and fibulin-1 in a tissue array containing 69 breast cancer tissue samples, including 18 ductal carcinomas, 3 poorly differentiated ductal carcinomas, 12 moderately differentiated ductal carcinomas, 1 well-differentiated ductal carcinoma, 20 lobular carcinomas, 6 mucoid carcinomas, 3 colloid carcinomas, 3 apocrine carcinomas, and 3 medullary atypical carcinomas [Supplementary Table 1]. Overall, using serial slides, the results indicated no colocalization of the proteins regardless of the tumor type or stage [Figure 6]. Consistently, ADAMTS-1 immunoreactivity was almost absent or expressed at residual levels in all types of carcinoma samples [Figure 6]. On the other hand, positive immunostaining for fibulin-1 was distributed in both the tumor cells and the stromal spaces with the exception of the colloid carcinoma, in which fibulin-1 expression was restricted to the tumor cells [Figure 6, Top].

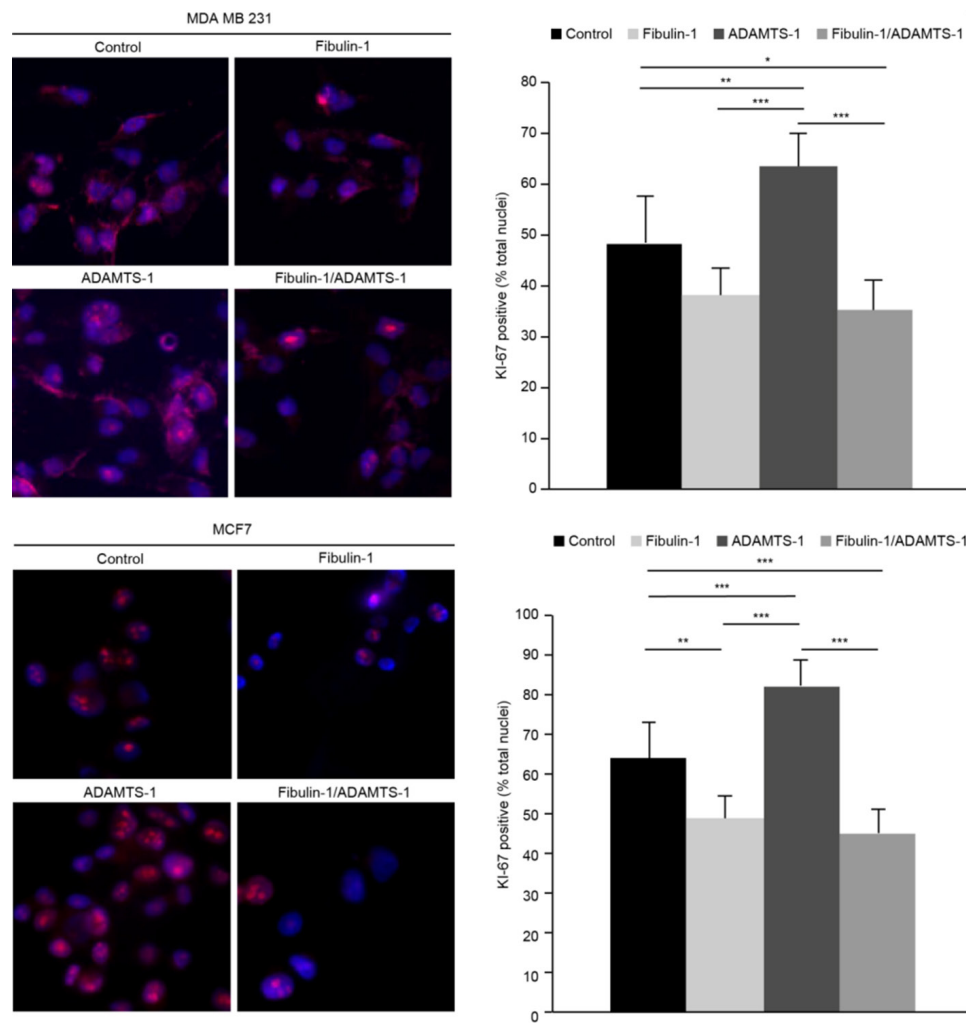


Figure 4. Coexpression of ADAMTS-1 and fibulin-1 reduces the proliferation of the breast cancer cell lines MCF-7 and MDA-MB-231. The proliferative state of breast cancer cell lines was measured by quantifying the proliferative nuclear marker Ki-67 by immunocytochemistry. Top Left: representative images of Ki-67 staining in the indicated MDA-MB-231 transfectants; bar, 10 μ m. Top Right: quantification of Ki-67-positive nuclei (as a percentage of total nuclei labeled by DAPI staining). Bottom Left, representative images of Ki-67 staining in the indicated MCF-7 transfectants; bar, 10 μ m. Bottom Right, quantification of Ki-67-positive nuclei (as a percentage of total nuclei labeled by DAPI staining)

We next assessed the association of ADAMTS1 and FBLN1 expression with clinical outcome using data available at www.kmplot.com. Overall survival Kaplan-Meier analysis revealed that the high expression of FBLN1 and ADAMTS1 correlated with the best prognosis for breast cancer patients (Figure 6, Bottom; measured as a mean of expression of both genes, $P < 0.001$). Similarly, high levels of FBLN1 can be considered a marker for good breast cancer prognosis ($P < 0.005$). On the other hand, this analysis also showed no influence of ADAMTS1 expression level on overall survival rate [Figure 6, Bottom]. We also evaluated progression-free survival and metastasis-free survival and found no significant differences in any of the cases analyzed (each gene alone or both together; data not shown). These data suggest that factors other than these two genes could modulate the pro- or anti-tumor activities of both fibulin-1 and ADAMTS-1.

Taken together, our results suggest that the inhibitory effect on the invasion, migration or mammosphere formation processes exhibited by breast cancer cell models cannot be fully extended to breast cancer tissue samples. Therefore, it cannot be ruled out that other factors can influence tumor cell behavior, such as the presence of protease inhibitors or the generation of bioactive fragments due to proteolytic activities. Additionally, the presence of fibulins other than fibulin-1, such as fibulin-5, can influence context-

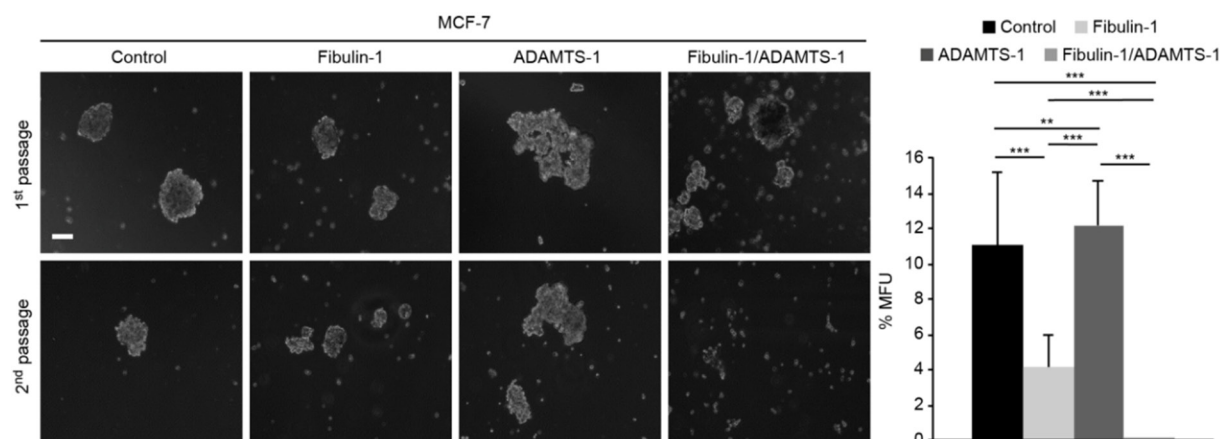


Figure 5. Coexpression of ADAMTS-1 and fibulin-1 abolishes mammosphere formation. Left: representative images of the mammospheres derived from MCF-7 transfectants in two consecutive generations. Control, MCF-7 cells transfected with an empty vector. Top row, mammosphere appearance before dissociation. Bottom row, mammosphere appearance 6 days after dissociation. Right: mammospheres were dissociated and passaged at a density of 20 cell/well in 96-well plates, and MFUs were counted and calculated as a percentage of the mammospheres formed from the number of cells seeded. White bar: 100 μ m

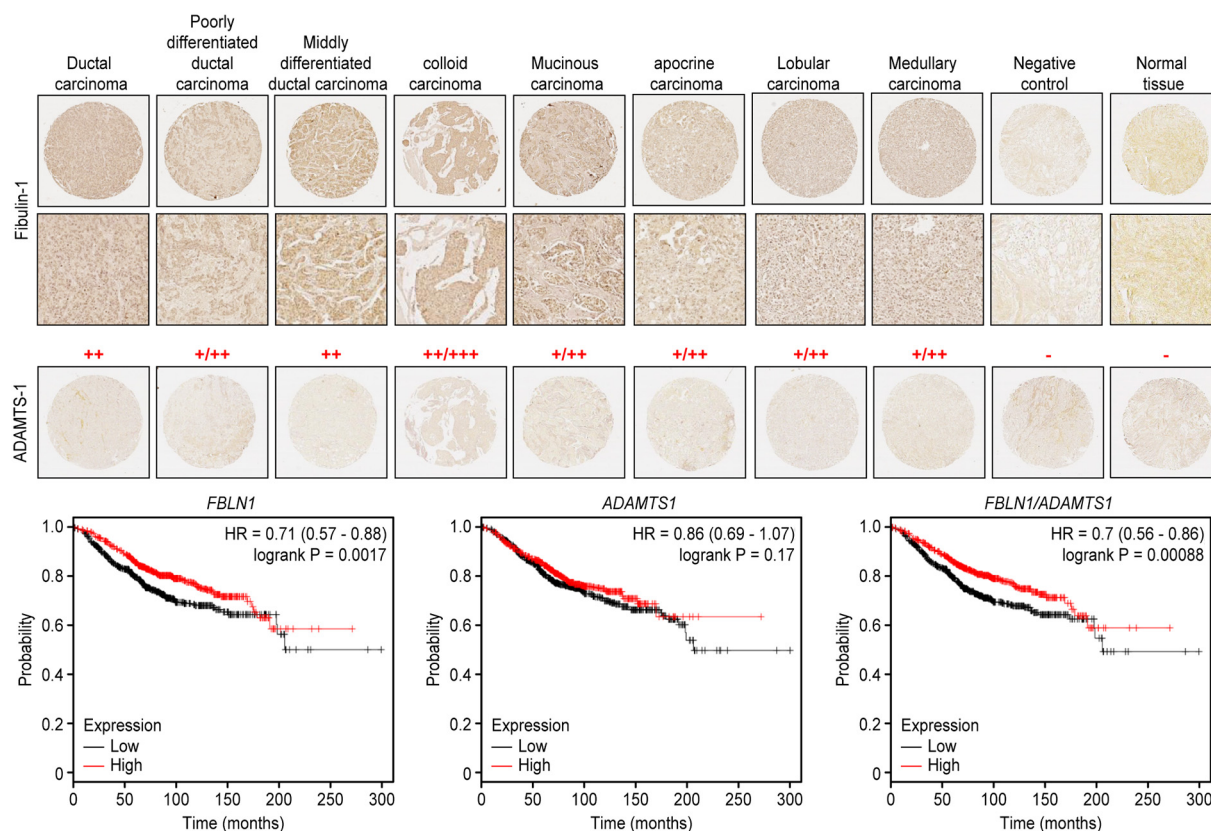


Figure 6. Different patterns of ADAMTS-1 and fibulin-1 expression in a breast cancer tissue array. Top: representative images of tissues used for the detection of ADAMTS-1 and fibulin-1 in different stages of breast carcinoma. Top two lines: fibulin-1 pattern at $\times 4$ and $\times 20$ magnifications. Bottom line: ADAMTS-1 pattern at $\times 4$ magnification. Negative controls were performed by omitting primary antibodies. The intensity of fibulin-1 immunostaining is indicated as strong (+++), moderate (++) or negative (-). Bottom: Kaplan-Meier overall survival plots for FBLN1, ADAMTS1 and FBLN1/ADAMTS1

specific effects on breast cancer cells^[37]. In this sense, fibulin-5 inhibits these same cellular properties; it was detected in a tissue array analysis of breast cancer samples and was inversely correlated with Ki-67 immunostaining^[38].

DISCUSSION

ECM proteins can be considered as important players in tissue homeostasis as it is considered the cellular component. Interactions among different components of the ECM have crucial roles in normal and pathological processes such as bone remodeling or tumor development^[1,39]. The components of the ECM that contain multiple motifs within the same protein unit are good candidates to establish various interactions and build bridge connections between cells and other components of the ECM. Such is the case for the members of two extracellular families of proteins, the fibulins and ADAMTSs^[4,21]. Members of both families have been associated with cancer development and progression and have been reported on many occasions to interact with other components of the ECM, acting as pro- or anti-tumor agents^[18,23].

Fibulin-1 has been previously described to be an ADAMTS-1 cofactor and to stimulate the proteolytic activity of ADAMTS-1 towards aggrecan and versican^[19,40]. Herein, we have been able to describe the significance of this fibulin-1/ADAMTS-1 interaction in the proliferation, migration and invasion properties of breast cancer cell lines. Overall, our data support the idea that ADAMTS-1 is a pro-tumor agent in breast cancer, whereas fibulin-1 is able to block this effect and acts as an anti-tumor agent. We have been able to show that the presence of both proteins reduces the migration and invasion properties of two breast cancer cell lines, MCF-7 and MDA-MB-231. The participation of ADAMTS-1 in cancer has been associated with mammary tumor growth and progression to metastasis in the MMTV-PyMT model in an *Adamts1*-/- background^[10], whereas it has also been reported to be a tumor suppressor gene through proteolysis of nidogen-1 and nidogen-2 in an HEK293T tumor xenograft assay^[41]. In our study, ADAMTS-1 expression was not detected by tissue array analysis in samples of different stages of breast cancer. Additionally, the Kaplan-Meier plots reflect the absence of an influence of ADAMTS-1 on expected patient overall survival. However, the ectopic expression of ADAMTS-1 in breast cancer cell lines increased the tumorigenic potential of these cell lines in terms of proliferation, migration, invasion and mammosphere formation. Initially, breast cancer profiles showed the downregulation of ADAMTS-1 in malignant tumors compared to normal tissues^[42].

However, other studies indicated the participation of ADAMTS-1 in aiding breast cancer development^[10,11,13,43]. Thus, the participation of ADAMTS-1 in breast cancer development and progression might depend on substrates it is able to act upon, such as versican to facilitate spreading of tumor cells or semaphorin 3C, which is known to promote metastatic potential of breast cancer cells^[10,44]. Interestingly, *ADAMTS1* was the third most overexpressed gene in highly metastatic MDA-MB-231 clones, and the knockdown of ADAMTS-1 in these clones regressed disease spread and reduced secondary tumor burden^[45,46]. The effect of this protein might also be dependent on which cell type produces and secretes ADAMTS-1, similar to the paracrine effect of breast cancer cells over cancer-associated fibroblasts of the vicinity^[15]. Finally, the impact of ADAMTS-1 might also depend on other ECM proteins that interact and bind with ADAMTS-1 and, as result, are able to modulate ADAMTS-1 activity.

The fact that fibulin-1 has been described as an ADAMTS-1 cofactor able to modulate ADAMTS-1 activity further implies the roles of both proteins in tumor development.

Fibulin-1 can interact with various ECM components, including known ADAMTS-1 substrates, such as nidogen-1 and versican^[2,21,27]. Fibulin-1 has also been implicated in cellular transformation and, similar to ADAMTS-1, can behave as both a tumorigenic factor or tumor suppressor depending on the tissue environment^[23]. Our results showed that the ectopic expression of fibulin-1 in breast cancer cell lines was able to block the tumorigenic properties of these cell lines. Furthermore, fibulin-1 expression was able to dramatically block these properties even in the presence of ADAMTS-1. Similar properties have also been described in our laboratory in the interaction of two related components of the ECM, fibulin-2 and ADAMTS-12. In this interaction, the expression of ADAMTS-12 alone exerted pro-tumoral activities, whereas the coexpression of both ADAMTS-12 and fibulin-2 showed an important anti-tumoral effect^[47].

It is noteworthy that fibulin-1 expression was initially associated with improved survival in patients with a lymphoid infiltrate at the tumor site^[48]. Similarly, the Kaplan-Meier analysis showed better prognosis for patients with high levels of fibulin-1 than for those with low fibulin-1 expression. In fact, the low expression of fibulin-1 has been described in various tumor types, such as gastric cancer, bladder cancer, colorectal cancer and hepatocellular carcinoma. In all these cases, low expression correlates with the hypermethylation of the FBLN1 promoter and can be used as a prognosis marker^[30-33,49]. Thus, our data are in accordance with all these studies in which it seems feasible that the presence of fibulin-1 might have a prevalent anti-tumor activity. In our immunohistochemical analysis, we could detect the presence of only fibulin-1; no ADAMTS-1 was observed. From our cellular experiments, it is clear that the presence of both proteins has a pronounced effect on the tumorigenic properties of both cell lines. The downregulation of ADAMTS-1 might be a mechanism of escape towards tumor progression, avoiding binding with fibulin-1 and thus eliminating the anti-tumoral properties of their interaction.

In summary, the interactions between members of both families of ECM proteins, fibulins and ADAMTSs, seem to be important for cancer development, as is the case for ADAMTS-12/fibulin-2 and ADAMTS-1/fibulin-1. Furthermore, we have also been able to demonstrate the cleavage of fibulin-2 by ADAMTS-4 and ADAMTS-5, which increased the tumorigenic potential of breast cancer cell lines^[50]. Therefore, it is tempting to speculate the existence of similar mechanisms that control fibulin-1 participation in breast cancer development and its interaction with other ECM components, a hypothesis that warrant further study.

DECLARATIONS

Acknowledgments

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Authors' contributions

Made substantial contributions to the conception and design of the study and performed data analysis and interpretation: Mohamed Y, Fontanil T, Cal S, Obaya AJ

Performed data acquisition: Cobo T, Cobo JL

Provided technical support: García-Suárez O

Provided administrative support and performed histological interpretation: Vega JA, Cobo J

Wrote the manuscript: Mohamed Y, Cal S, Obaya AJ

Availability of data and materials

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Conflicts of interest

All authors declared that there are not conflicts of interest.

Ethical approval and consent to participate

The study methodologies conformed to the standards set by the Declaration of Helsinki and were approved by the Ethics Committee of Clinical Investigation of the Hospital Universitario de Asturias (HUCA).

Consent for publication

Not applicable.

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Review

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ESR1 alterations and metastasis in estrogen receptor positive breast cancer

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Abstract

Endocrine therapy is essential for the treatment of patients with estrogen receptor positive (ER+) breast cancer, however, resistance and the development of metastatic disease is common. Understanding how ER+ breast cancer metastasizes is critical since the major cause of death in breast cancer is metastasis to distant organs. Results from many studies suggest dysregulation of the estrogen receptor alpha gene (*ESR1*) contributes to therapeutic resistance and metastatic biology. This review covers both pre-clinical and clinical evidence on the spectrum of *ESR1* alterations including amplification, point mutations, and genomic rearrangement events driving treatment resistance and metastatic potential of ER+ breast cancer. Importantly, we describe how these *ESR1* alterations may provide therapeutic opportunities to improve outcomes in patients with lethal, metastatic breast cancer.

Keywords: Endocrine therapy resistance, ESR1 fusions, ESR1 mutations, breast cancer, metastasis

INTRODUCTION

Breast cancer is one of the leading cancer-related causes of death worldwide with more than one million new cases and more than 450,000 deaths per year according to the World Health Organization. About 70% of diagnosed cases express estrogen receptor alpha (ER)^[1], where ER signaling is the defining and driving event contributing to tumor growth and disease progression in these ER+ breast tumors.



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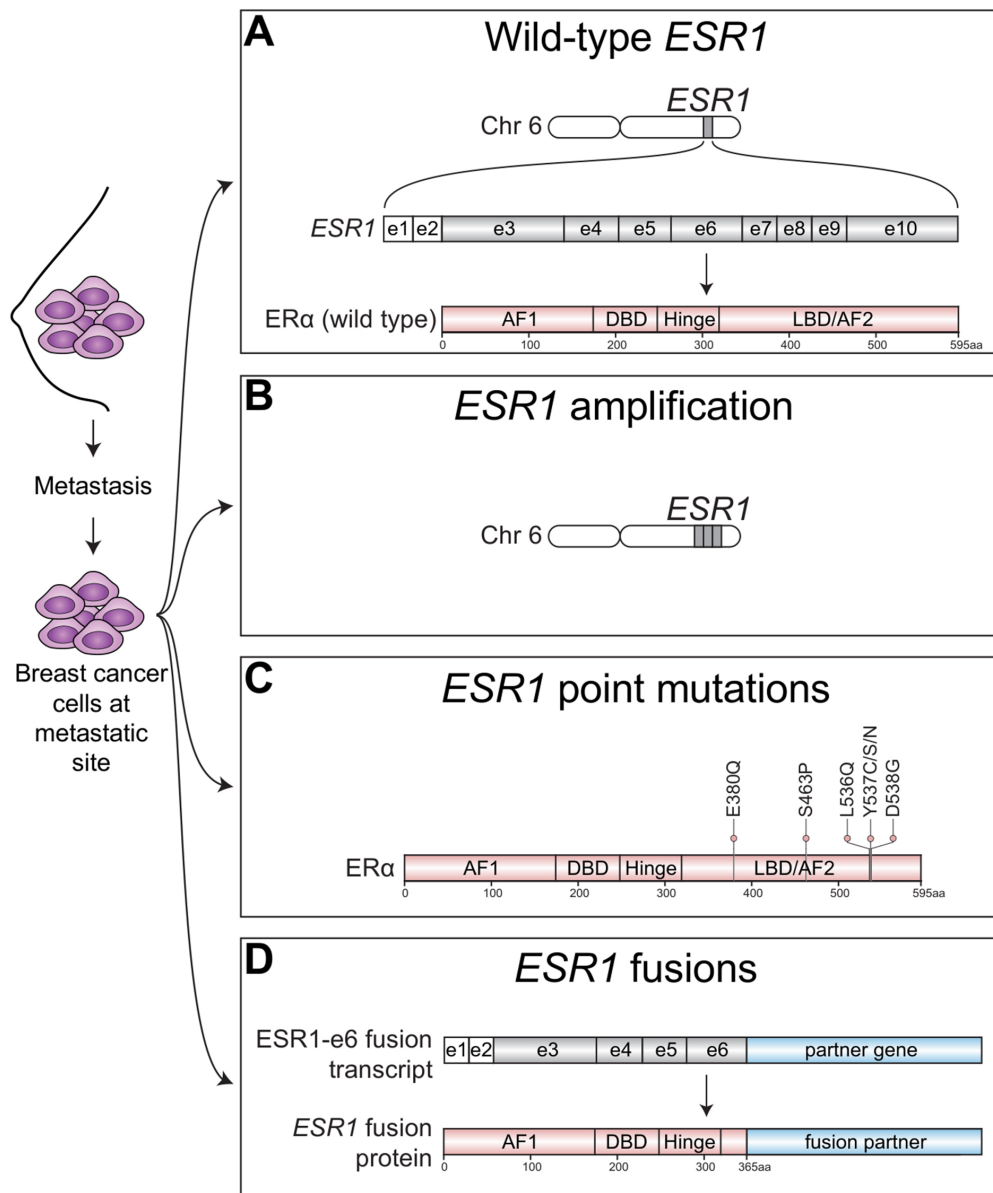


Figure 1. Spectrum of *ESR1* alterations found in metastatic ER+ breast cancer. ER+ breast cancer cells that have spread beyond the breast to metastatic sites have been found to express wild-type *ESR1* or harbor a variety of *ESR1* alterations. A: Metastatic tumors can express wild-type estrogen receptor alpha protein (ERα), which is encoded by the estrogen receptor alpha gene (*ESR1*) located on chromosome (chr) 6. *ESR1* transcripts are generated from 2 non-coding exons (e) depicted by white boxes and 8 coding exons depicted by gray shaded boxes; B: Metastatic ER+ tumors may also harbor amplification of *ESR1* resulting in multiple copies of *ESR1* and increased ER protein expression; C: Point mutations that cluster within the ligand-binding domain (LBD) of *ESR1* that confer constitutive ligand-independent activation of *ESR1* mutants have also been well-described in metastatic ER+ breast tumors, especially those which had been extensively pretreated with AIs; D: Emerging studies have now identified structural rearrangements involving *ESR1* that generate in-frame *ESR1* fusion transcripts. In-frame fusion transcripts that retain the first 6 exons of *ESR1* (*ESR1*-e6) produce stable *ESR1* fusion proteins have been shown to be transcriptionally active and drive endocrine therapy resistance and metastasis in ER+ breast cancer. AF1: activation function 1 domain; DBD: DNA-binding domain; AF2: activation function 2 domain; aa: amino acid

ER is a transcription factor consisting of various functional domains encoded by *ESR1* located on chromosome 6 [Figure 1A]. *ESR1* transcripts are generated by 2 non-coding and 8 exons that specifies protein-coding domains. The N-terminal activation function 1 (AF1) domain functions in a hormone-independent manner and is post-translationally modified by phosphorylation events that increase transcriptional and pathogenic activity^[2-5]. The DNA-binding domain (DBD) contains two zinc finger motifs responsible for binding to estrogen response element (ERE) DNA sequences within the enhancers

and promoters of ER target genes. The C-terminal domains include the ligand-binding domain (LBD) and ligand-dependent activation function 2 (AF2) domain required for dimerization and transactivation. The LBD is required not only for estrogenic ligands but is also the domain that controls responses to anti-estrogen antagonists. The hinge domain contains the nuclear localization sequence and connects the activity from the ligand-independent AF1 and ligand-dependent AF2 together to fully promote activation of ER^[6].

Standard-of-care endocrine therapies that target ER itself include selective estrogen receptor modulators (SERMs), such as tamoxifen, and selective estrogen receptor degraders (SERDs), such as fulvestrant, that bind to the LBD. Aromatase inhibitors (AIs), such as letrozole, anastrozole, and exemestane, block the production of estrogens from androgens resulting in lower levels of circulating estrogen in the body. Despite the success of these agents in reducing relapse rates when given prophylactically after breast surgery and chemotherapy (adjuvant treatment), endocrine therapy resistance and the development of lethal metastatic disease is common and a major clinical problem. A major clinical feature of the disease is the long-term persistence of disseminated tumor cells despite endocrine therapy, with relapse risk continuing for decades after diagnosis^[7]. The etiology of endocrine therapy resistance is complex and tremendous efforts have been made to uncover diverse mechanisms^[8].

Downstream signaling events from aberrantly activated growth factor receptor tyrosine kinases (RTKs) such as epidermal growth factor receptor (EGFR) and HER2 (*ERBB2*) have been shown to phosphorylate and increase ER transcriptional activity in a hormone-independent manner^[9]. ER+ tumors that exhibit *ERBB2* amplification have reduced ER expression, reduced sensitivity to ER targeted therapies, and poor outcomes^[10]. Nonetheless, co-targeting ER+/HER2+ breast cancer has been clinically successful. Experimental models have extended these ideas to other RTKs that are expressed by ER+ breast cancer. Interestingly, these investigations revealed a non-genomic or transcription-independent function of ER in association with EGFR^[11] and insulin-like growth factor receptor (IGF1-R)^[12] at the plasma membrane. However, clinical trials testing the use of EGFR inhibitors in endocrine treatment resistant ER+ breast cancer have produced modest or negative results^[13] suggesting that further insight into underlying mechanisms for RTKs and ER interactions are required for successful translation of this aspect of ER function.

Since *PIK3CA* is the most frequently mutated gene in ER+ breast cancer^[14], targeting components of the PI3K-AKT-mTOR pathway has also been described to treat endocrine refractory disease. Preclinical models demonstrated enhanced activation of the PI3K pathway in long-term estrogen deprived (LTED) ER+ breast cancer cells and a negative feedback system by which PI3K inhibition increases ER activity, potentially explaining the effectiveness of combinatorial mTOR and ER inhibition^[15]. The use of an mTOR inhibitor, everolimus, in combination with endocrine therapy, significantly improves progression-free survival (PFS) for patients failing previous endocrine therapies^[16], although side effects are severe and stratification of patients for this treatment is essential. Treatment with a pan-PI3K inhibitor, buparlisib, in combination with fulvestrant increased PFS with compared to fulvestrant alone in patients with ER+ locally advanced or metastatic breast cancer (BELLE-3 clinical trial)^[17]. Greater benefit was observed in patients treated with buparlisib harboring *PIK3CA* mutations. However, significant toxicities in buparlisib treated patients have stopped further clinical trials of the drug in this setting. In contrast to pan-PI3K inhibitors, alpelisib, an agent that specifically targets the alpha isoform of *PIK3CA*, has been shown to overcome the toxicities associated with pan-PI3K treatment. Apelisib greatly improved PFS in patients when given in combination with fulvestrant to patients with endocrine-refractory, advanced ER+ breast cancer harboring *PIK3CA* mutations^[18]. There was no significant benefit to PFS in patients with non-*PIK3CA* mutant tumors suggesting that *PIK3CA* status is a potential biomarker to predict response to PI3K inhibition. Results from studies also further suggest that targeting specific mutant isoforms of PI3K reduces toxicities leading to increased tolerability and therefore can be given for a longer duration compared to other pan-PI3K agents such as buparlisib.

Dysregulation of cell cycle components is common in ER+ breast cancer, particularly the Cyclin D-CDK4/6-Rb axis in the luminal B subtype^[19]. This includes amplification of Cyclin D1 (*CCND1*), gene copy gain of *CDK4* and loss of negative regulators such as p16 and p18 (*CDKN2A* and *CDKN2C*)^[19]. Together with downstream activity from tyrosine kinase growth factor signaling described earlier, these events promote phosphorylation of Rb and resistance to endocrine therapy^[20]. CDK4/6 inhibitors such as palbociclib and ribociclib, are now FDA approved for use in combination with endocrine therapy to treat advanced stage ER+ disease. Other studies are now examining the use of such inhibitors to treat early stage ER+ disease in both neoadjuvant and adjuvant settings (ClinicalTrials.gov identifiers for PALLET NCT02296801 and PALLAS NCT02513394, respectively). Some trials have already reported promising results in the neoadjuvant setting^[21].

In addition to metastatic breast tumors expressing wild-type ER [Figure 1A], alterations in *ESR1* itself, such as *ESR1* amplifications have been identified in metastatic ER+ disease^[22] [Figure 1B]. Other *ESR1* alterations found in endocrine therapy resistant breast tumors include point mutations in the ligand-binding domain (LBD)^[23] [Figure 1C] that confer constitutive hormone-independent activation of ER and are now a well-described mutational mechanism identified in up to 40% of metastatic breast cancer cases^[24]. These are especially enriched in tumors pretreated with aromatase inhibitors^[25]. Emerging evidence now suggests that chromosomal rearrangement events involving *ESR1* are yet another *ESR1* mutational mechanism driving endocrine therapy resistance and metastatic disease progression [Figure 1D]. Hereon, we focus on the spectrum of *ESR1* aberrations underlying treatment resistance and metastasis in ER+ breast cancer.

ESR1 AMPLIFICATION

The copy number increase of a confined area of a chromosome is defined as gene amplification/gain [Figure 1B] which may result in protein overexpression of the amplified gene therefore driving tumor biology. For example, *ERBB2* amplification^[26] and fibroblast growth factor receptor 1 gene (*FGFR1*) amplification^[27] are drivers of therapeutic resistance and poor prognosis in ER+ breast cancer. The discovery of *ESR1* gene amplifications in 1990^[28] sparked intense interest in investigating the role of this mutational event to be a potential driver of endocrine therapy resistance and recurrent disease in ER+ breast tumors.

Incidence of *ESR1* amplifications in ER+ breast cancer

ESR1 amplification is found in up to 30% of ER+ breast tumors^[22,28-37] depending on the detection method and scoring systems^[38]. A study by Holst *et al.*^[29] that analyzed over 2,000 breast tumors, showed that 20.6% of tumors harbored *ESR1* amplifications and 14% showed *ESR1* copy number gain by using fluorescence in situ hybridization (FISH) method and validated by quantitative PCR^[29]. Nearly all *ESR1* amplified tumors in these samples also expressed high levels of ER protein by immunohistochemistry. Additional analysis from precancerous ductal and lobular carcinoma in situ (DCIS and LCIS) breast tumors showed over one-third of these samples also harbored *ESR1* amplifications suggesting that *ESR1* amplifications present in early-stage breast cancer may drive disease progression. Two other independent studies that also used FISH, both showed that *ESR1* amplification frequency is between 20%-22%^[34-35], consistent with Holst *et al.*^[29]. In contrast, other studies by Brown *et al.*^[30], Horlings *et al.*^[31], Reis-Filho *et al.*^[32], and Vincent-Salomon *et al.*^[33], have shown a much lower frequency of *ESR1* amplifications, in which *ESR1* amplification or gain was less than 5% by using array comparative genomic hybridization (aCGH) and validated by FISH by the majority of these studies. Another study which used a multiplex ligation-dependent probe amplification (MLPA) approach to analyze 104 invasive breast cancers identified 16% of samples harbored *ESR1* amplifications consisting of low level gains^[36]. A variation in the frequency of *ESR1* amplification found among metastatic breast samples has also been reported. A seminal study from Jeselsohn *et al.*^[37] examined *ESR1* amplification in the metastatic setting using next generation sequencing approaches. They reported the frequency of *ESR1* amplification in ER+ tumors at less than 2% in both the primary and metastatic setting^[37]. Using NanoString sequencing approaches, a recent study reported that 13% of ER+ metastatic breast tumors harbored *ESR1* amplifications. Interestingly, the authors found an enrichment of *ESR1* amplifications in bone metastatic

samples, suggesting that *ESR1* amplification may underlie organ-specific metastatic behavior of ER+ breast cancers^[39].

Correlation between *ESR1* amplification, protein expression, and clinical significance

Many studies show positive correlation between *ESR1* amplification and ER protein expression suggesting that amplification may lead to production of elevated levels of oncogenic ER protein^[28,29,34,35]. Interestingly, studies have shown that *ESR1* amplification in a subset of ER+ breast cancers were associated with tamoxifen resistance and poor prognosis^[40,41]. In contrast, contradicting studies have identified *ESR1* amplification as an indicator of longer disease-free survival and increased sensitivity to tamoxifen treatment^[35,42]. These conflicting results suggest that more dedicated studies will be required to fully understand the clinical implications of *ESR1* amplifications. Results from other studies have identified *ESR1* amplification in benign and early-stage breast cancer and is associated with endocrine therapy resistance. Discovery of *ESR1* amplifications in benign papillomas and early-stage breast cancer such as ductal hyperplasias suggests that *ESR1* amplifications may play a role in the tumor initiation process since high expression of ER in benign breast cells is associated with higher breast cancer risk^[29,43,44], but these findings still require further validation. The insignificant difference of *ESR1* amplification between invasive and non-invasive breast cancers suggests that *ESR1* copy number alteration might not be used as a key predictive marker for invasion and metastasis, however its enrichment in recurrent disease, especially after endocrine therapy treatment, suggests that it likely plays a role in intrinsic and/or acquired resistance to endocrine therapy and metastatic disease progression^[45-48].

Although the use of endocrine agents that block estrogen production (AIs) or block ER function (SERM/SERD) are front-line therapies to treat metastatic ER+ breast cancer, the use of high-dose estrogens has also been reported to be effective. This approach was first described over 70 years ago before the discovery of anti-estrogens to treat advanced breast cancer^[49]. More recently, a study reported a breast cancer patient harboring an *ESR1* amplification showed tumor regression in a liver metastasis after receiving estradiol treatment as a primary therapy^[50]. Another study using a patient-derived xenograft (PDX) model harboring an *ESR1* amplification derived from a patient with endocrine-refractory disease demonstrated that tumor growth was suppressed with estradiol treatment^[47]. These results were corroborated in an independent study using a LTED ER+ MCF7 breast cancer cell model system in which such cells acquire *ESR1* amplification during long term estrogen deprivation showed an apoptotic response upon estradiol treatment^[48]. Collectively, these studies suggest a role for *ESR1* amplification in driving endocrine therapy resistance and metastasis and that treating *ESR1* amplified tumors with intermediate doses of estradiol (6 mg daily) is an option for some patients.

The presence of *ESR1* amplification in some breast cancers is undeniable. However, a clear link between the presence of *ESR1* amplifications in breast tumors and endocrine therapy resistance and metastasis remains to be shown. Deeper multi-dimensional characterization of relapsed and/or metastatic breast tumors at the RNA, DNA, and protein levels may aid to better understand its prognostic value. Therefore, more studies will be required to better understand the functional and therapeutic significance of *ESR1* amplifications in driving endocrine therapy resistance and metastasis.

***CYP19A1* amplification**

While *ESR1* amplification has been an intense area of investigation underlying endocrine therapy resistance as described above, a study focusing on genomic aberrations of the drug target of AIs, aromatase (*CYP19A1*), has deepened our understanding of endocrine-refractory ER+ breast tumors. Copy number alterations in the gene encoding aromatase, *CYP19A1*, also has been shown to promote resistance to AIs in patients with metastatic ER+ breast cancer. While *CYP19A1* amplification is very rare in primary untreated ER+ breast cancers, Magnani *et al.*^[51] found that 21.5% of AI-refractory relapsed tumors to harbor *CYP19A1* amplification, suggesting that *CYP19A1* amplification is an acquired endocrine therapy resistance

mechanism^[51]. This study also revealed that both *CYP19A1* and *ESR1* were frequently co-amplified in AI treated patients, further suggesting that these two amplification events may function collaboratively. To better understand the role of *CYP19A1* amplification and endocrine therapy resistance, a LTED MCF7 ER+ breast cancer cell model was used which was found to acquire copy number alterations around the *CYP19A1* locus compared to parental cells MCF7 cells^[51]. Elevated levels of both *CYP19A1* mRNA and CYP19A1 protein were observed in *CYP19A1* amplified LTED cells compared to parental cells. The functional consequences of *CYP19A1* amplification in the LTED cells were increased aromatase activity, enhanced ER recruitment to regulatory regions on DNA of target genes and their transcriptional activation leading to reduced sensitivity to AI treatment^[51]. These results suggest that *CYP19A1* amplification, in addition to *ESR1* amplification, could potentially represent biomarkers of endocrine therapy resistance. More studies are needed to validate these findings in more patient datasets. Furthermore, deeper studies focusing on how these amplification events contribute to the metastatic behavior of endocrine-refractory ER+ breast tumors are needed. These results highlight the possibility that response to standard-of-care endocrine therapies are not only as a consequence of *ESR1* amplification but may also be critically dependent on the status of the target genes of endocrine therapies themselves.

***ESR1* POINT MUTATIONS**

When patients with ER+ breast cancer relapse, up to 15% have lost ER expression and therefore targeting ER in this population is likely to be ineffective, although false negative ER results are a concern if the ER analysis was conducted on bone biopsies exposed to acid formalin, or if the analysis was conducted on samples prone to degradation such as cells detected in pleural fluid. The remaining 85% of patients may initially benefit from first-line endocrine therapy, but metastatic disease progression due to acquired resistance is inevitable. One well-established mechanism explaining this relentless pattern of acquired endocrine therapy resistance is the acquisition of activating point mutations that cluster within the ligand-binding domain (LBD) of *ESR1* [Figure 1C]. Substitution of tyrosine at position 537 to serine (Y537S) in the LBD of *ESR1* was first reported to confer constitutive, ligand-independent activity of ER in experimental breast cancer models^[52]. However, such mutations were not known to occur in human tumors until Fuqua *et al.*^[23] reported that estrogen-independent activation could be driven by another Y537 substitution, Y537N, that was identified in a metastatic sample from a breast cancer patient who experienced disease progression on hormonal therapy. This study also showed that Y537N was able to drive resistance to tamoxifen in experimental models.

Frequent *ESR1* point mutations in endocrine-refractory, metastatic ER+ breast cancer

Advances in sequencing technologies have allowed more sensitive detection and thus insights into the landscape of *ESR1* LBD point mutations in both primary and metastatic ER+ breast tumors. Three *ESR1* mutations, Y537S, Y537N, and D538G were identified by next-generation sequencing in 14 out of 80 patient samples with endocrine-refractory, metastatic ER+ breast cancer^[53]. Notably, all breast tumors from patients that were found to harbor *ESR1* LBD point mutations were treated with AIs. Interestingly, these alterations were not detected in matched primary samples and were also not detected in separate large sets of treatment naïve patients. Analysis of an independent ER negative (ER-) cohort also failed to detect any *ESR1* point mutations in the LBD^[53]. Although *ESR1* mutations were found in 3% of primary samples in this population, alterations in Y537 and D538 residues of *ESR1* were enriched in patients treated extensively with AIs^[53]. These results suggest that these *ESR1* LBD mutations are acquired, or detected, in patients after treatment with endocrine therapy.

In addition to Y537 alterations, frequent amino acid substitution of aspartate 538 to glutamate (D538G) was identified in liver metastases from 5 out of 13 metastatic ER+ breast samples^[54]. Another study which enrolled 11 metastatic ER+ breast cancer patients with exposure to serial endocrine therapies, identified that over half of these patient's metastatic samples harbored *ESR1* mutations localized in the LBD, that included Y537S, Y537C, Y537N, D538G, and L536Q mutations^[55]. Further evidence for the recurrent presence of Y537 and D538 mutations in the LBD of *ESR1* was shown in 9 out of 76 metastatic samples from patients with ER+

disease^[37]. One patient from this study acquired a tyrosine substitution to cysteine mutation (Y537C) at the metastatic site, which was not detected prior to treatment^[37]. Taken together, these studies indicate the most frequent *ESR1* LBD point mutations are those affecting Y537 and D538 residues. Furthermore, the presence of *ESR1* point mutations predominately appear in late-stage breast cancer patients that have been treated with multiple lines of endocrine therapies but rarely in treatment naïve cases. This strongly suggests a role for *ESR1* point mutations in acquired endocrine resistance and metastasis.

Although formalin-fixed paraffin-embedded tumor specimens are widely used for next generation sequencing to capture *ESR1* mutations used by studies as described above^[37,53,54], collection of plasma circulating DNA to detect *ESR1* mutations by droplet digital PCR (ddPCR) have now been implemented in several clinical trials^[56-59]. Such “liquid biopsies” have shown that collecting circulating DNA samples maintains the genomic landscape of the primary tumor suggesting that less invasive detection methods may efficiently identify *ESR1* point mutations once the disease has become resistant to treatment and/or has become metastatic. Interestingly, Y537 and D538 substitutions were identified in 7% of ER+ primary tumors using ddPCR, which may lead us to review the conclusion that *ESR1* point mutations rarely exist in primary tumor, towards the idea that rare *ESR1* mutant sub-clones exist in primary breast tumors that become selected for over time^[60].

Experimental models of *ESR1* point mutations

Several preclinical breast cancer models harboring *ESR1* LBD point mutations have been generated, providing research platforms to characterize the functional, transcriptional, and pharmacological properties of these mutations. ER point mutant proteins have been overexpressed by transfecting^[37,53,54] or transducing lentiviral vectors^[55,61] encoding *ESR1* mutant constructs into various ER+ breast cancer cell line models. The growth promoting properties of *ESR1* mutant expressing cell line models have shown that *ESR1* LBD mutants drive hormone-independent proliferation that is resistant to tamoxifen treatment^[23,37,47,53,54]. Although fulvestrant efficiently inhibited the growth of point mutation bearing cells in a dose-dependent manner, growth was not reversed to levels of wild-type *ESR1* expressing cells^[37,47].

Since the expression of exogenous *ESR1* variant transcripts encoded by expression vectors is often initiated from non-endogenous human promoters that drive very high expression of constructs, it is unlikely to mimic the expression levels in human breast tumors harboring *ESR1* point mutations. To more accurately recapitulate tumor-related *ESR1* mutational events, CRISPR/Cas9 approaches have been utilized to knock in *ESR1* mutated sequences into ER+ breast cancer cells^[62,63]. Both heterozygous and homozygous knock-in models have been shown to mediate resistance to endocrine therapies^[62,63].

Transcriptional properties of *ESR1* mutations in the LBD include their ability to drive constitutive hormone-independent transcriptional activation and enhance cell proliferation^[23,37,47,53-55]. Human embryonic kidney 293T cells transfected with Y537C, Y537N, and D538G mutant constructs strongly activate an ERE-luciferase reporter in a ligand-independent manner compared to wild-type ER. Luciferase activity was unaffected by clinically relevant doses of tamoxifen and fulvestrant, however, high doses of these agents blocked *ESR1* mutant driven ERE-luciferase reporter activity^[37,53-55]. These *ESR1* point mutations have also been shown to drive estrogen-independent activation of ER target genes in ER+ breast cancer cells^[37,53,54]. The recruitment of ESR1-Y537S mutant to ER target genes and their expression driven by the mutant were further validated by ChIP-seq and RNA-seq^[62].

ESR1 mutant-driven estrogen-independent tumor growth was also validated in both ER+ cell xenografts and patient-derived xenograft (PDX) models^[47,53]. A PDX harboring ESR1-Y537S, WHIM20, has been generated from a patient with endocrine-refractory metastatic ER+ breast cancer that retains genomic features of the human counterpart^[47]. This WHIM20 PDX model demonstrated estrogen-independent tumor growth^[47].

Despite such in-depth studies of transcriptional and growth-promoting properties endowed by *ESR1* LBD point mutations, the role of such mutations in driving cell invasion and tumor metastasis is underexplored. A scratch wound assay was performed on Y537S and D538G mutant expressing MCF7 cells to examine cell motility which showed enhanced cell migration under hormone-deprived conditions driven by these *ESR1* mutants^[54,61]. A recent study sheds light on ER mutant-driven metastatic biology, showing a remarkable enrichment of metastasis-associated gene sets in *ESR1* mutant cells^[64]. Consequently, Y537S and D538G mutant expressing MCF7 cells developed metastases after survival surgery to remove primary tumors in xenograft models. The Y537S mutant greatly potentiated both tumor growth and metastasis compared to D538G mutant^[64].

Mechanisms and therapeutic vulnerabilities of breast cancers harboring *ESR1* point mutations

Structural analysis has revealed that the formation of hydrogen bonds between S537 or G538 and D351 located within helix 12 of *ESR1* LBD confers an agonist conformation to *ESR1* mutant proteins^[53]. In wild-type ER, the binding of ligand alters the position of helix 12 into an open pocket, favoring recruitment of transcriptional coactivators such as p160 family members that include SRC-3, and histone acetylases CBP and p300. In contrast, tamoxifen results in disposition of helix 12 that hinders coactivators binding and results in recruitment of corepressors such as N-CoR/SMRT^[65]. The substitution of D538 to glycine mimics the active conformation of wild-type ER bound by estrogen^[54].

To better understand the consequences of coactivator recruitment to mutant ER proteins, a proteomic profiling approach was used and revealed enhanced recruitment of transcriptional coactivators, histone H3 lysine 4 (H3K4) methyltransferase KMT2D/2C complex, as well as steroid receptor coactivators (SRCs), to ERE-bound *ESR1*-Y537S and *ESR1*-D538G mutants compared to ERE-bound wild-type ER^[66]. Genetic inhibition of SRC-3 in HeLa cells expressing *ESR1*-Y537S and *ESR1*-D538G significantly suppressed activity of an ERE-luciferase reporter. Pharmacological inhibition using a pan-SRC inhibitor, SI-1, also suppressed transcriptional activation in *ESR1* mutant expressing HeLa cell lines and blocked cell proliferation in ER+ breast cancer cells stably expressing *ESR1*-Y537S and *ESR1*-D538G. Using a PDX naturally harboring the *ESR1*-Y537S mutation (WHIM20), treatment with an improved pan-SRC inhibitor, SI-2, suppressed growth *in vivo*. Suppression of WHIM20 tumor growth was even greater when SI-2 was administered in combination with an oral SERD, AZD9496, compared to either single agent alone, suggesting that targeting coactivator recruitment in combination with endocrine therapy could be a promising therapeutic strategy for breast tumors harboring *ESR1* LBD mutants such as Y537S and D538G^[66]. Another study identified that the transcription factor TFIIH was also recruited by the *ESR1*-Y537S mutant^[62]. Phosphorylation of Ser118 was found to be mediated by TFIIH kinase, cyclin-dependent kinase (CDK) 7 and subsequent *ESR1*-Y537S driven cell proliferation was suppressed by a CDK7 inhibitor, THZ1^[62]. These results suggest that CDK7 may represent another target that is associated with *ESR1* mutant proteins for therapeutic intervention.

Targeting non-genomic signaling pathways activated by *ESR1* mutants has also been investigated. As discussed above, interactions between ER with RTKs such as EGFR, HER2, and IGF1-R can activate downstream kinases. This results in phosphorylation of multiple transcriptional factors, including ER, and coregulators leading to changes in gene expression in a hormone-independent manner^[67]. A recent study demonstrated that IGF1 signaling was the most activated pathway in *ESR1* mutant MCF7 cells^[61]. IGF1 stimulation lead to increased phosphorylation of both IGF1-R β and insulin receptor substrate-1 (pIRS-1). Treatment with an IGF1-R β inhibitor (GSK183705A) monotherapy was able to block Y537S-driven cell motility and combinatorial treatment with tamoxifen abrogated transcriptional activity and cell growth driven by Y537S, Y537N, and D538G mutants^[61]. These results suggest that targeting non-genomic signaling pathways activated by *ESR1* mutants may be an additional therapeutic strategy to block *ESR1* mutant driven breast tumors.

Fulvestrant is used to treat metastatic ER+ breast cancer patients who have developed resistance to AI and tamoxifen. In preclinical models, transcriptional activity and cell proliferation of *ESR1* LBD mutant cells

are partially sensitive to fulvestrant, requiring higher doses of fulvestrant compared to controls^[37,47,63]. Moreover, fulvestrant did not completely block transcriptional activity nor cell proliferation compared to control cells expressing wild-type *ESR1*. Of note, *ESR1* mutants showed differential responses to fulvestrant. Y537S required the highest dose to completely block transcriptional activity and cell proliferation compared to other mutants, D538G, E380Q and S463P^[63]. Using an MCF7 xenograft model, *ESR1* mutants also showed differential responses to fulvestrant. Tumor growth of E380Q, S463P and D538G expressing tumors were significantly reduced while Y537S tumors showed resistance to treatment^[63]. Given the inconvenience and poor bioavailability of intramuscular fulvestrant injections, second-generation SERDs, such as AZD9496, that can be orally administered have been tested and showed anti-proliferative ability in endocrine resistant experimental models cell xenograft models^[63,68]. AZD9496 which has improved bioavailability compared to fulvestrant, was able to provide greater suppression of tumor growth in the Y537S MCF7 xenograft model and in a D538G PDX model compared to fulvestrant treatment^[63]. A phase I clinical trial with AZD9496 in extensively pretreated advanced ER+ breast cancer patients has recently been completed with promising results, providing disease stabilization to the study cohort^[69]. These results suggest that newer generation SERDs with improved bioavailability could be an attractive therapeutic option to treat endocrine-refractory breast tumors driven by *ESR1* mutations.

Treatment of late-stage ER+ breast cancer patients with CDK4/6 inhibitors in combination with endocrine therapy has been tremendously successful. CDK4/6 inhibitors have also been tested in PDX breast cancer models harboring *ESR1* point mutations. Wardell *et al.*^[70] reported the suppressive effects of a CDK4/6 inhibitor, palbociclib, on endocrine-refractory PDX tumors as long as the downstream target retinoblastoma (Rb) protein was expressed. Used as monotherapy or in combination with a hybrid SERM/SERD, bazedoxifene, palbociclib suppressed tumor growth of a WHIM20 PDX tumor harboring an *ESR1*-Y537S mutant. In contrast, palbociclib was ineffective in inhibiting the growth of WHIM43, a PDX naturally bearing *ESR1*-D538G mutant due to the lack of Rb protein expression, suggesting that Rb is a determinant of CDK4/6 treatment response. CDK4/6 inhibitors also showed favorable therapeutic effects in treatment-resistant ER+ patients harboring *ESR1* point mutations^[59].

Currently, screening of *ESR1* point mutations have not been used as biomarkers to predict response to therapy in the clinic. Wild-type ER, human epidermal growth factor receptor 2 (HER2), and progesterone receptor (PR), are histopathological markers that guide therapeutic selection. In clinical management of metastatic ER+ breast cancer, SERDs, such as fulvestrant is used for patients with resistance to AIs and tamoxifen without regard for *ESR1* mutation status. An analysis of BOLERO-2, a phase III clinical trial that enrolled ER+ breast cancer patients with locally advanced or metastatic disease whom progressed on AI, evaluated the prevalence of the two most frequent *ESR1* point mutations, Y537S and D538G and their effects on patient outcomes in ER+ metastatic patients^[56]. Having either one or two of these mutations was associated with decreased overall survival. In the PALOMA-3 clinical trial which enrolled ER+ breast cancer patients with advanced, endocrine refractory disease, palbociclib combined with fulvestrant led to longer PFS than fulvestrant alone^[59,71]. 69% of patients from the PALOMA-3 were analyzed for *ESR1* mutation status, which showed that 25% of these cases harbored *ESR1* mutations consisting mainly of Y537S, Y537N, D538G, and E380Q mutations^[59]. However, palbociclib was found to provide equal benefit regardless of *ESR1* mutation status. Although these studies indicate that the presence of *ESR1* mutations may predict poor outcomes, they also highlight the need for more analyses of studies investigating the predictive value of *ESR1* mutation status and response to therapy once the disease has become endocrine therapy resistant.

The development of sequencing technologies and the various models to recapitulate *ESR1* mutant bearing tumors allow insightful studies into the landscape and targeted therapies of activating point mutations in the *ESR1* LBD. Further studies are needed to address the use of *ESR1* mutations as predictive biomarkers to stratify patient subsets and predict *ESR1* mutation specific therapeutic vulnerabilities.

***ESR1* structural rearrangements and *ESR1* fusions**

In contrast to well-studied *ESR1* point mutations, structural rearrangements involving *ESR1* are understudied. A variety of *ESR1* gene fusion transcripts have been identified in luminal breast tumors^[72,73]. Analysis of RNA-seq data from 990 primary TCGA breast samples revealed that 21 of these tumors (2.1%), all of the luminal B subtype, contained recurrent fusion transcripts involving the first two non-coding exons of *ESR1* fused to various C-termini sequences from the coiled-coil domain containing 170 gene, *CCDC170* (*ESR1-e2>CCDC170*)^[73]. These fusion transcripts do not provide sufficient coding sequences to generate chimeric ER fusion proteins but instead generate truncated forms of *CCDC170* proteins (Δ *CCDC170*). Exogenous expression of Δ *CCDC170* in ER+ breast cancer cells led to enhanced growth and reduced sensitivity to tamoxifen^[73] suggesting a role for *ESR1-e2>CCDC170* in endocrine therapy resistance. Another independent study that examined early stage and non-metastatic ER+ breast samples also identified two *ESR1-e2>CCDC170* fusion transcripts as well as *ESR1-e2>C6orf211* and another fusion containing the first 6 exons of *ESR1* fused to *AKAP12* (*ESR1-e6>AKAP12*)^[72]. These *ESR1* fusions were identified in 4 out of 62 surgical samples (6.5%) that were resistant to letrozole aromatase inhibitor treatment 10-21 days post treatment as defined by Ki67 labeling^[74], suggesting a higher frequency for these *ESR1* fusions gene events in endocrine-refractory tumors compared to primary, untreated samples. However, detailed functional characterization and evidence demonstrating a causal role for *ESR1* fusions in endocrine therapy resistance has been lacking and the incidence of *ESR1* fusions from late-stage ER+ breast cancer still remains unclear. Furthermore, therapeutic strategies to treat *ESR1* translocated tumors remains poorly understood.

Using a PDX model to better understand endocrine therapy resistance, we previously reported a somatic gain-of-function event in the form of a chromosomal translocation identified in a patient presenting with aggressive endocrine therapy resistant, metastatic ER+ disease. This translocation produced an in-frame fusion gene consisting of exons 1-6 of *ESR1* (*ESR1-e6*) and the C-terminus of the Hippo pathway coactivator gene, *YAP1* (*ESR1-e6>YAP1*), thereby generating a stable *ESR1* fusion protein that was a highly active constitutive transcription factor^[47] [Figure 1D]. Our group more recently discovered another in-frame *ESR1* fusion gene involving the protocadherin 11 X-linked gene, *PCDH11X* (*ESR1-e6>PCDH11X*) provided by inter-chromosomal translocation that also produced stable *ESR1* fusion protein identified in a patient with endocrine-refractory, metastatic ER+ breast cancer^[75]. In both *ESR1-e6>YAP1* and *ESR1-e6>PCDH11X* fusions, the LBD of *ESR1* is replaced with in-frame sequences from another gene, and therefore the drug binding domain that endocrine therapies recognize is absent. These two fusions promoted endocrine therapy resistant cell proliferation and constitutively activated ER target genes. Interestingly, both fusions also upregulated an epithelial-to-mesenchymal transition (EMT)-like transcriptional signature, induced cell motility, and increased lung metastatic frequency^[75]. These results suggest that *ESR1* fusions are able to drive not only endocrine therapy resistance, but also drive metastasis, linking these two lethal processes together.

Importantly, *ESR1* fusion-driven growth could be suppressed by CDK4/6 inhibition. This suggests that targeting downstream kinases of ER could be a potential therapeutic strategy to treat *ESR1* translocated tumors and further suggests that *ESR1* fusion status may be a potential biomarker to stratify patients to CDK4/6 inhibitor therapy. To further explore therapeutic strategies to target *ESR1* fusions, a collaborative study was performed to examine interacting proteins with *ESR1* fusion transcriptional complexes^[66]. Results from that study showed enhanced recruitment of 26S proteasomal subunits to *ESR1-e6>YAP1* driving transcriptional activation and cell proliferation. Subsequent pharmacological inhibition with a broad-spectrum proteasome inhibitor, MG132, blocked *ESR1-e6>YAP1*-mediated activation of an ERE-luciferase reporter. Furthermore, bortezomib, a specific 26S proteasome inhibitor in phase II clinical trial used to treat endocrine-refractory, metastatic ER+ breast cancer in combination with fulvestrant^[76] suppressed growth driven by *ESR1-e6>YAP1*. Taken together, these results suggest that downstream ER kinases such as CDK4/6 as well as transcriptional coregulators such as the 26S proteasome are attractive therapeutic targets to treat *ESR1* fusion positive, metastatic breast tumors.

Additional in-frame *ESR1* translocations with diverse partner genes have now been identified in late-stage, endocrine-refractory, ER+ metastatic cases. These include *ESR1-e6>DAB2*, *ESR1-e6>GYG1*, and *ESR1-e6>SOX9*^[77]. Like the *ESR1-e6>YAP1* and *ESR1-e6>PCDH11X* fusions, the *ESR1-e6>DAB2* and *ESR1-e6>GYG1* fusions produce stable *ESR1* fusion proteins and all three were able to drive hormone-independent activation of a ERE-luciferase reporter^[77]. Remarkably, these *ESR1* fusions all follow a pattern preserving the first six exons of *ESR1*, containing the N-terminal DNA binding domain fused in-frame to C-terminal partner genes, thus excluding the LBD in *ESR1* [Figure 1D]. Therefore, these additional *ESR1* fusion proteins likely drive pan-endocrine therapy resistance like our previously discovered *ESR1-e6>YAP1* and *ESR1-e6>PCDH11X* fusions^[75]. The functional and therapeutic significance of these additional *ESR1* fusions are the focus of ongoing investigation by our group and others.

In contrast to transcriptionally active *ESR1* fusions, we also identified an in-frame *ESR1-e6* fusion, *ESR1-e6>NOP2* in a treatment naïve primary breast tumor that was transcriptionally inactive despite producing stable *ESR1* fusion protein^[75]. *ESR1-e6>NOP2* did not promote endocrine therapy resistant growth and was found to bind relatively few sites in a genome-wide DNA binding assay, potentially explaining the weak functional activity measured by our experimental systems. In addition, out-of-frame *ESR1* fusions identified in primary tumors preserving diverse exons of *ESR1* gene, *ESR1-e3*, *ESR1-e4*, *ESR1-e5*, and *ESR1-e6* did not facilitate estrogen-independent proliferation^[75]. More studies are required to fully understand the contribution of transcriptionally inactive in-frame and out-of-frame *ESR1* fusions in breast cancer.

ESR1 fusion structural studies revealed that driver *ESR1* fusions from metastatic patients follow the same fusion pattern containing the first 6 exons of *ESR1* (*ESR1-e6*) fused to C-termini of diverse gene partners suggesting this pattern is strongly connected to endocrine therapy resistant, metastatic ER+ breast tumors. The observation of a highly consistent and recurrent *ESR1* breakpoint, together with the promiscuity of *ESR1* for a variety of fusion partners is certainly interesting. In prostate cancer, recurrent fusions involving promoter regions of an androgen regulated gene, transmembrane protease serine 2 gene (*TMPRSS2*) fused to coding sequences of erythrovirus E26 gene (*ETS*) family members have been identified in more than 50% of prostate cancer cases^[78]. Androgen receptor (AR) signaling has been shown to bring the androgen regulated gene *TMPRSS2* and the *ERG* gene in close proximity in prostate cancer cell line models^[79]. Androgen signaling also generates DNA damage in the form of double strand breaks (DSBs) at sites of *TMPRSS2-ERG* genomic breakpoints. These DSBs have been shown to be mediated by the class II topoisomerase beta, TOP2B, which is recruited to AR, inducing DSBs^[80]. *TMPRSS2-ERG* gene fusions can then arise from dysfunction of mechanisms to repair DSBs, such as homologous recombination (HR) pathway and the error-prone non-homologous end-joining (NHEJ) pathway. AR-mediated DSBs in prostate cancer may provide clues to the recurrent *ESR1* breakpoints for *ESR1* fusions seen in breast cancer. Recruitment of TOP2B to ER and subsequent DSBs have been shown to occur at regulatory regions of ER target genes as a consequence of ER-mediated transcriptional activation^[81]. Since regulatory regions of *ESR1* itself has also been shown to be bound by ER^[82], transcription-induced DSBs by ER, coupled with dysregulation of DSB repair mechanisms may contribute to the highly recurrent *ESR1* breakpoints. Although none of the fusion partners from endocrine-refractory, metastatic disease observed in our studies are known ER targets, additional studies are needed to better understand the diversity of preferred *ESR1* partner genes.

ESR1 fusions that contain the first six exons of *ESR1* fused in-frame to partner genes are almost exclusively observed in endocrine therapy resistant, metastatic ER+ breast cancer, with the exception of *ESR1-e6>NOP2*, as described above, likely suggesting a role in driving disease pathogenesis. However, very few functionally significant *ESR1* fusions have been studied to date and therefore *ESR1* fusion events remains an understudied form of somatic mutation in breast cancer. The incidence of *ESR1* fusions is also still not well understood, especially in the metastatic setting, but the studies discussed here collectively suggest *ESR1* fusions to be present in at least 1% of metastatic breast cancer cases^[77], with the actual frequency likely to

be higher as more studies on *ESR1* fusions emerge. Additional studies on *ESR1* fusions will further support the causal role *ESR1* fusions and have significant diagnostic and clinical implications since pathogenic *ESR1* fusions could be used as biomarkers to stratify patients for individualized healthcare in ER+ breast cancer. Therapeutic vulnerabilities from *ESR1* translocated tumors could be an alternative to chemotherapy in patients with rapidly progressing, endocrine therapy resistant disease.

CONCLUSION

Endocrine therapy resistance and metastasis in ER+ breast cancer patients remain significant clinical problems. This review has focused on studies describing a spectrum of *ESR1* alterations including amplification, point-mutations, and structural rearrangements in endocrine-refractory, metastatic ER+ breast cancer cases. Results from these studies have provided insights into the underlying mechanisms that contribute to endocrine therapy resistance and metastasis.

Amplification of the *ESR1* locus results in overexpression of oncogenic ER protein in the breast and potentially reducing sensitivity of *ESR1* amplified breast tumors to endocrine therapies and therefore likely leads to disease progression and metastasis. Point mutations in the LBD of *ESR1*, the most common of which are Y537S and D538G, confer an agonist confirmation to such *ESR1* mutant proteins resulting in constitutively active mutant ER transcription factors that lead to activation of ER target genes in a hormone-independent manner while also promoting activation of metastasis-associated genes^[64]. The finding that ER LBD mutant proteins are constitutively active in an estrogen-independent manner suggest that therapeutic strategies which work by blocking estrogen production, such as ovarian ablation and treatment with AIs, are likely to be ineffective in breast tumors harboring *ESR1* point mutations. Indeed, a significant proportion of *ESR1* LBD point mutations were identified in metastatic tumors that were extensively treated with AIs, suggesting that such mutations may be enriched in breast tumors upon AI treatment^[53]. *ESR1*-Y537S and *ESR1*-D538G are partially sensitive to fulvestrant^[37,47,63], and newer oral SERDs that have better bioavailability compared to fulvestrant, such as AZD9496, have shown promising results in treating tumor growth driven by *ESR1* LBD point mutants in experimental models^[66]. Although fulvestrant is used exclusively in the metastatic setting for ER+ disease, treating primary breast tumors upfront with fulvestrant or more potent SERDs like AZD9496 may reduce the incidence of disease driven by *ESR1* LBD point mutations.

Despite the potential effectiveness of fulvestrant in targeting ER proteins with point mutations in the LBD, it is completely ineffective against ER fusion proteins generated from in-frame *ESR1* fusion transcripts arising from *ESR1* translocations^[75]. These *ESR1* fusions transcripts, *ESR1*-e6>YAP1 and *ESR1*-e6>PCDH11X, were identified in patients with metastatic ER+ breast tumors that were pan-endocrine therapy resistant^[75]. Both fusions retain the first 6 exons of *ESR1* fused in-frame to C-terminal sequences of the partner gene but lack exons encoding the LBD, rendering these fusions insensitive to all endocrine therapies that target the LBD, including fulvestrant and most likely AZD9496. These *ESR1* fusions were found to generate hyperactive *ESR1* fusion proteins that not only drive endocrine therapy resistant growth, but also play a role in the metastatic process, reprogramming the ER cistrome to drive EMT and metastasis to lung^[75]. Despite the lack of an *ESR1* LBD, blocking signaling downstream of *ESR1* fusions with a CDK4/6 inhibitor, palbociclib, suppressed *ESR1* fusion-driven growth at primary and metastatic sites in experimental models^[75]. Similar to *ESR1* point mutations, *ESR1* fusion formation is likely a mechanism of acquired endocrine therapy resistance. To date, *ESR1* fusion transcripts that produce stable *ESR1* fusion proteins have only been detected in metastatic breast tumors resistant to multiple lines of endocrine therapies. This suggests that *ESR1* fusions may be enriched in tumors from the selective pressure of endocrine treatment. Since the *ESR1*-e6>YAP1 and *ESR1*-e6>PCDH11X fusions were identified from a small cohort of late-stage ER+ patients, more RNA-seq data from primary and late-stage, treatment-refractory tumors are clearly required, particularly with longer sequencing reads, which increase fusion gene detection sensitivity to better understand the incidence of *ESR1* fusions in both primary and metastatic breast cancer.

The underlying mechanism of how *ESR1* fusions arise remains unclear. However, as mentioned earlier, DSBs mediated by recruitment of TOP2B to ER transcriptional complexes may contribute to formation of *ESR1* fusion genes, and therefore TOP2B could potentially be an attractive therapeutic target to prevent the formation of *ESR1* fusion events. More studies are required to test this hypothesis. Daunorubicin, an FDA-approved chemotherapeutic drug indicated for treating leukemia, targets TOP2B, however, this agent is very toxic. Developing less toxic agents that target TOP2B may represent a therapeutic strategy to prevent *ESR1* translocation events and deserves further study in the context of ER+ breast cancer.

Therapeutic targeting these aberrant forms of ER have shown promise in pre-clinical experimental models with more studies required to translate such findings to the clinic. Collectively, these studies deepen our understanding of how *ESR1* alterations trigger breast cancer to become lethal metastatic disease and will guide development of therapeutic strategies to treat a subset of patients with tumors that contains these *ESR1* alterations.

DECLARATIONS

Author's contributions

Made substantial contributions to conception: Lei JT, Gou X, Seker S

Provided initial drafts of the work: Lei JT, Gou X, Seker S

Revising the work critically for important intellectual content: Lei JT, Ellis MJ

Final approval of the version: Lei JT, Ellis MJ

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Conflicts of interest

Ellis MJ received consulting fees from Abbvie, Sermonix, Pfizer, AstraZeneca, Celgene, NanoString, Puma, and Novartis, and is an equity stockholder, consultant, and Board Director member of BioClassifier, and inventor on a patent for the Breast PAM50 assay.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Stem cells, immortality, and the evolution of metastatic properties in breast cancer: telomere maintenance mechanisms and metastatic evolution

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Abstract

Breast cancer is the most significant cause of cancer-related death in women around the world. The vast majority of breast cancer-associated mortality stems from metastasis, which remains an incurable disease state. Metastasis results from evolution of clones that possess the insidious properties required for dissemination and colonization of distant organs. These clonal populations are descended from breast cancer stem cells (CSCs), which are also responsible for their prolonged maintenance and continued evolution. Telomeres impose a lifespan on cells that can be extended when they are actively elongated, as occurs in CSCs. Thus, changes in telomere structure serve to promote the survival of CSCs and subsequent metastatic evolution. The selection of telomere maintenance mechanism (TMM) has important consequences not only for CSC survival and evolution, but also for their coordination of various signaling pathways that choreograph the metastatic cascade. Targeting the telomere maintenance machinery may therefore provide a boon to the treatment of metastatic breast cancer. Here we review the two major TMMs and the roles they play in the development of stem and metastatic breast cancer cells. We also highlight current and future approaches to targeting these mechanisms in clinical settings to alleviate metastatic breast cancers.

Keywords: Breast cancer, cancer evolution, cancer stem cells, metastasis, telomerase



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INTRODUCTION

Breast cancer is the most common malignancy and most frequent cause of cancer-related death in women globally^[1]. The vast majority of breast cancer-related morbidity and mortality can be ascribed to metastasis, which occurs in ~30 percent of cases and underlies ~90 percent of breast cancer deaths^[2,3]. Metastasis is a multistage cascade that commences when cancer cells migrate from their primary tumor of origin and undergo hematogenous dissemination that terminates in the seeding and colonization of distant organs^[4]. This so-called “invasion-metastasis cascade” serves as an evolutionary bottleneck that requires disseminated tumor cells (DTCs) to: (1) activate migratory and invasive programs; (2) survive within the vasculature in an anchorage-independent manner; (3) interact with other circulating cells to facilitate survival and extravasation; and (4) coordinate tissue-specific signaling inputs to persist in unfamiliar microenvironments^[5-7]. Thus, metastasis can be viewed as a process of clonal selection whereby a heterogeneous primary tumor gives rise to subpopulations that are fit to traverse the invasion-metastasis cascade. Following tissue colonization, these disseminated subclones retain growth-permissive features of the original primary tumor and undergo further evolution and clonal expansion within metastatic microenvironments^[8,9].

Metastatic evolution occurs via a number of distinct yet spatiotemporally overlapping mechanisms, including linear and parallel progression of monophyletic or polyphyletic founder clones^[10]. Cancer stem cells (CSCs) are fundamental components of tumors that enable the maintenance of emergent clonal populations yielded by evolutionary forces^[11-13]. CSCs are operationally defined by their self-renewal and tumor-initiating capacities; that is, a single CSC can recapitulate a tumor in its entirety, including a stable CSC pool^[14]. Historically, stochastic clonal evolution was believed to be mutually exclusive with a tumor developmental hierarchy built upon a stem cell population^[15,16]. More recent evidence suggests that there is a relationship between tumor evolution and CSCs that manifests through at least two mechanisms. First, the CSC population itself becomes highly heterogeneous during tumor development, indicating that CSCs are directly subjected to selective pressures^[17,18]. Second, non-stem cancer cells that define unique genetic and epigenetic lineages can be reprogrammed into CSCs^[19,20]. Thus, the plasticity that exists within and between stem and non-stem cancer cells provides a bidirectional route to engender clones that harbor distinctive properties, including the ability to metastasize. Of note, the functional significance of CSC evolution in the development and progression of multiple malignancies has been extensively documented^[21-23].

Numerous pathways that exert control over the metastatic propensity of cancer cells do so by regulating the production or function of CSCs. For instance, Wnt/ β -catenin signaling in both the primary tumor and metastatic microenvironments enhances breast CSC self-renewal and metastatic colonization^[24,25]. Likewise, inhibiting Wnt signaling abrogates metastatic outgrowth by depleting the CSC population^[26,27]. Similarly, vascular endothelial growth factor (VEGF) activates stem programs in breast cancer cells via VEGF receptor (VEGFR)-and neuropilin (NRP)-dependent cascades^[28,29]. VEGF can additionally push breast CSCs to undergo endothelial-like differentiation, thereby promoting tumor vascularization and cancer cell dissemination^[30]. The NF- κ B transcription factor pathway also acts as a critical regulator of breast CSC function^[31]. In particular, microenvironmental stimuli from resident stromal cells, extracellular matrix components, and the local immune milieu activate NF- κ B signaling to sustain CSC development^[25,32,33]. As a result, NF- κ B inhibitors demonstrate potent activity against breast CSCs^[34]. Related to these events, CSC expansion is associated with the epithelial-mesenchymal transition (EMT), a process whereby epithelial cells lose their intrinsic polarity and markers of differentiation and adopt features of mesenchymal cells, including enhanced migration and invasiveness^[35,36]. Key transcription factors that orchestrate EMT in breast cancer, such as Snail, Slug, and Twist1, simultaneously play a role in the acquisition of stem-like traits^[37]. Importantly, both Wnt/ β -catenin and NF- κ B signaling exert direct transcriptional control over these EMT-associated factors^[36,38]. Furthermore, EMT induces upregulation of VEGF, which bolsters the activities of β -catenin and NF- κ B and promotes angiogenesis to support CSC self-renewal and permit dissemination^[39-41]. In short, breast CSC survival and maturation are determined by a confluence of cell-intrinsic and microenvironment-derived signals that are transduced through parallel EMT-dependent and -independent circuits.

CSCs, like embryonic and tissue stem cells, possess replicative immortality^[42], a process achieved in part by activating telomere maintenance mechanisms (TMMs)^[43,44]. As outlined below, TMMs function within a network that unites cellular immortalization with processes, including EMT, that drive the development and outgrowth of metastatic cells. Telomeres, therefore, serve as essential mediators of CSC maintenance and consequent metastatic evolution. In addition, the results detailed below implicate telomere homeostasis as an attractive target for novel therapeutics to treat metastatic breast cancer.

TELOMERES AND TELOMERE DYNAMICS IN CSCS AND METASTATIC CELLS

Telomeres are nucleoprotein complexes located at the ends of linear chromosomes that safeguard against chromosomal instability and the loss of genetic information during cell division^[45]. In humans, the DNA component of telomeres is composed of tandem (TTAGGG)_n repeats with a 3' single-stranded overhang that invades telomeric duplex DNA to form a protective loop^[46]. These DNA regions are coated with proteins that collectively constitute the shelterin complex. Shelterin proteins serve to shield telomeres from illicit activation of DNA damage responses (DDR); they also maintain genome integrity and recruit factors responsible for regulating telomere length^[47,48]. In somatic (i.e., non-immortalized) cells, telomeres shorten during iterative rounds of cell division. To combat this event, stem cells and cancer cells maintain their telomeres using one of two TMMs: telomerase or alternative lengthening of telomeres (ALT). Telomerase is a reverse transcriptase enzyme composed of an RNA moiety (TERC, also known as TR) that provides a template for telomeric DNA synthesis and a protein moiety (TERT) that facilitates telomerase recruitment and carries out its polymerase activity^[49]. In contrast, ALT relies upon homology-directed, recombination-dependent synthesis of nascent telomeric DNA^[50]. ALT requires transient deprotection of telomeres coupled to activation of a DDR that is accompanied by telomere extension in a manner similar to break-induced DNA synthesis^[51,52]. DDR activation occurs in response to alterations in telomeric and subtelomeric chromatin structure that are brought about by loss of the chromatin remodelers ATRX and DAXX^[53,54]. Notably, evidence of each of these mechanisms has been found in breast cancer and can be correlated with specific histologic subtypes or disease stages^[55,56]. These findings support the idea that TMM identity may impact breast cancer progression, including the onset of metastasis.

While TMM acquisition has been identified as a feature of both stem and non-stem cancer cells, these processes play an essential role in preferentially sustaining the CSC population^[42]. By virtue of their replicative immortality, CSCs function as progenitors that exist over a sufficient timescale for evolution to take place. Remarkably, telomere shortening appears to be a primary driving force underlying tumor evolution. Telomere shortening precedes TMM activation^[57], which allows for the formation of critically short telomeres that cannot be adequately capped by shelterin. Cells interpret these short telomeres as free DNA ends, which are temporarily repaired by chromosome end-to-end fusions that ultimately induce breakage-fusion-bridge (BFB) cycles^[58,59]. BFB cycling leads to complex genomic rearrangements including deletions, non-reciprocal translocations, and formation of dicentric or circular chromosomes^[60]. Telomere catastrophe may also yield chromosomal instability that is resolved via chromothripsis or other forms of chromoanagenesis, an event termed telomere crisis^[61,62]. Breast cancer-initiating cells can harbor both short telomeres and telomerase activity^[41], consistent with the model that telomere shortening instigates genomic instability and CSC evolution while telomere elongation maintains emergent CSC subpopulations [Figure 1]. Evidence identifying ALT in breast CSCs has not yet been found. However, ALT has a stem cell origin^[63], while ALT activity has been observed in non-breast CSCs^[44,63,64]. Future studies examining TMMs in breast CSCs and their connection to genome architecture and tumor heterogeneity will be of great value.

Telomere maintenance proteins have been heavily implicated in many of the central signaling pathways in metastasis^[65] [Figure 1]. For instance, TERT is capable of regulating Wnt target genes by forming a transcriptional co-activation complex with β -catenin^[66]. In addition, TERT directly regulates NF- κ B-dependent gene expression by binding to the NF- κ B p65 subunit at the promoters of target genes^[67,68].

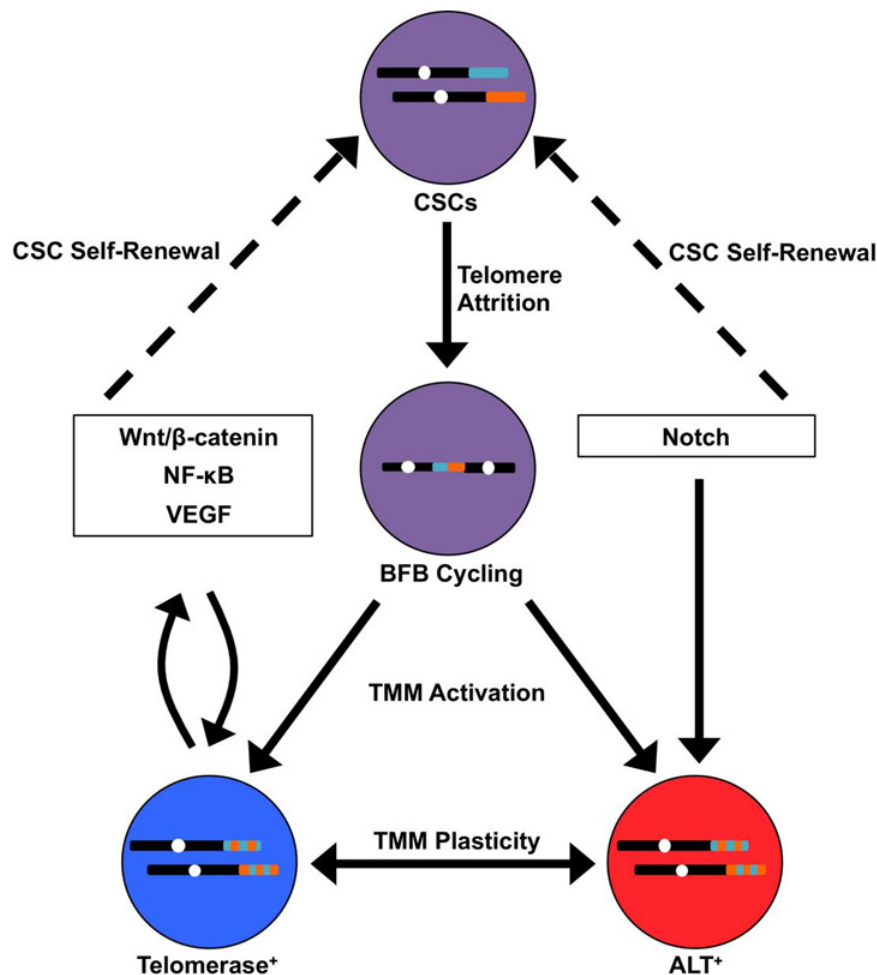


Figure 1. Telomere-centric model of breast cancer stem cell (CSC) biogenesis and metastatic evolution. CSCs (purple) harboring telomeres of a given length (shown for two different chromosomes in teal and orange) undergo telomere attrition as a by-product of self-renewal. This ultimately yields critically short telomeres that are temporarily repaired by chromosome end-to-end fusions, resulting in breakage-fusion-bridge (BFB) cycling (represented by dicentric chromosome). BFB cycling or chromoanagenesis (not shown) cause widespread chromosomal instability (represented by dual-colored telomeres) and the acquisition of new genetic features, including those that are advantageous for metastasis. At the same time, telomere maintenance mechanisms (TMMs) are activated in these new clonal populations, which are defined in part by their reliance on telomerase (blue) or ALT (red). In addition, TMMs exhibit a degree of plasticity, such that TMM identity may interconvert between telomerase and ALT. TMM selection is influenced by signaling pathways that simultaneously promote CSC propagation (dashed arrows). In turn, telomere maintenance proteins directly regulate these signaling pathways, establishing reciprocal feedback loops that coordinate TMM activation and CSC maintenance

Each of these pathways exerts reciprocal control over TERT^[69,70], thereby preserving TMM identity and CSC phenotype. Of note, TERT can also bind to the VEGF promoter to stimulate VEGF expression and neoangiogenesis^[71]. Other transcriptional regulators of TERT, such as c-Myc, further serve to induce EMT and stemness in breast cancer cells^[72-74]. Compared to telomerase, ALT is less well-characterized at a molecular level; therefore, our understanding of its role in EMT and breast cancer metastasis is presently incomplete. Nevertheless, ALT is most often associated with tumors of mesenchymal origin^[75], indicating a possible role for ALT in EMT. Accordingly, carcinoma cells exhibiting telomerase dysfunction were driven to adopt a mesenchymal stem-like phenotype, which was accompanied by activation of ALT and the formation of metastatic tumors^[76,77]. In breast cancer cells, TERT expression is mutually exclusive with the mesenchymal state^[78]. Similarly to TERT, the expression of ALT-associated proteins, such as the Bloom syndrome protein (BLM), is governed by signaling pathways, such as Notch, that are responsible for CSC fate specification and self-renewal^[79,80]. Given these findings, it is paramount that future studies explore the significance of the relationship between stemness and telomere plasticity in breast cancer progression.

TELOMERE-DIRECTED THERAPIES FOR METASTATIC BREAST CANCER: CURRENT AND FUTURE PERSPECTIVES

The functions of telomerase in tumorigenesis have been rigorously interrogated over the last several decades, as has the potential to target telomerase therapeutically^[65,81]. The telomerase inhibitors BIBR1532 and GRN163L (also known as Imetelstat) display high efficacy in depleting the CSC pool and disrupting breast cancer metastasis^[82-85]. Indeed, Imetelstat was assessed in a Phase I clinical trial for recurrent or metastatic breast cancer, although the trial was suspended due to dose-limiting toxicity^[81]. In addition to such toxicity concerns, the success of telomerase inhibitors in clinical trials has thus far been moderated by the inherent complexity of telomere homeostasis. First, telomere shortening-induced senescence can be bypassed in the absence of functional p53 or other components of the DDR machinery^[86]. Second, the critically short telomeres and chromosomal instability associated with telomere crisis are disproportionately associated with metastasis^[87,88]. Thus, the evolution of DTCs that underlie metastatic disease may be enhanced unwittingly by therapies that promote telomere shortening. Despite these challenges, telomerase remains an appealing therapeutic objective in need of innovative targeting approaches in which these evolutionary considerations are taken into account.

Emerging telomerase-targeting strategies include cytotoxic small molecules that act as substrates for telomerase as well as anti-telomerase immunotherapies^[89-92]. Current immunotherapeutic platforms are primarily centered on telomerase peptide or dendritic cell vaccines, which can be engineered to elicit either CD4+ or CD8+ T cell antitumor responses^[93]. These strategies are being assessed in diverse preclinical settings, including breast cancer. Indeed, the telomerase peptide vaccine Vx-001 is progressing through clinical trials for advanced solid tumors^[90]. More recent investigations have examined the feasibility of adoptive transfer of anti-telomerase chimeric antigen receptor (CAR) T cells for treating triple-negative breast cancer^[94]. Future studies into the generalizability of anti-telomerase CAR T cell therapy to other breast cancer subtypes, as well as the efficacy of these diverse immunotherapeutic approaches in clinical settings will be of tremendous value.

Although the functions of specific ALT-associated proteins have been elucidated, their utility as therapeutic targets for ALT-driven cancers has only recently been investigated. For example, the DNA damage-responsive kinase ataxia-telangiectasia and Rad3-related (ATR) is activated secondary to depletion of ATRX, which leads to persistent retention of replication protein A (RPA) at telomeres and generation of a recombinogenic substrate. Inhibition of ATR, in turn, triggers apoptosis of ALT-positive cells^[95]. BLM, a RecQ DNA helicase, unwinds telomeric G-quadruplex structures and coordinates 5'→3' end resection during telomere recombination^[96,97]. Accordingly, a recently-developed small molecule inhibitor of BLM may possess great potential as an anticancer agent against ALT-driven tumors^[98]. Finally, topoisomerase III α (Topo III α) associates with BLM and regulates the topology of telomeric recombination intermediates. Interestingly, genetic inactivation of Topo III α selectively reduces the survival of ALT-positive compared to telomerase-positive cells^[99]. Moreover, telomerase activity is enhanced in the surviving fraction of Topo III α -deficient cells^[100], suggesting that telomerase activation provides a pathway for chemoresistance. Thus, targeting TMMs may best be achieved using a multidrug regimen consisting of multiple anti-TMM agents or an anti-TMM agent in combination with chemotherapy or other targeted agents^[101]. The effectiveness of these therapeutic modalities in eliminating breast CSCs and in treating metastatic breast cancers remain intriguing and important open questions.

CONCLUSION

By overseeing multiple pathways that promote breast cancer stemness, EMT, and metastasis, telomeres function as critical nodes in the nexus between cellular immortalization, tumor evolution, and disease progression. The selection of TMM likely exhibits a high degree of plasticity in different tumor cell types

or across disparate stages of breast cancer development, including metastasis. Indeed, TMM selection may itself be subject to evolutionary dynamic forces. In addition, the plasticity inherent in TMM identity has far-reaching prognostic and therapeutic implications. Tumors driven by distinct TMMs may show sensitivity or resistance to specific treatments, which has substantial impact on patient survival. Moreover, different subpopulations within a single tumor (e.g., stem vs. non-stem cells) may be reliant upon unique TMMs. Such TMM heterogeneity may beget residual, resistant clones that underlie disease recurrence. In the future, gaining a deeper understanding of telomeres and the pathways controlled by the telomere machinery will provide immense insight into the origin, progression, and eradication of one of the world's deadliest cancers.

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Authors' contributions

Conception and study design: Robinson NJ, Schiemann WP

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Conflicts of interest

All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

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Review

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Prognostic and predictive role of hyponatremia in cancer patients

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Abstract

Hyponatremia is the most frequent electrolyte disorder encountered in hospitalized patients. Several studies have demonstrated that hyponatremia is a negative prognostic factor in different clinical scenarios. Noteworthy, not only severe and acute hyponatremia has been associated with an increased risk of mortality, but also moderate or even mild chronic hyponatremia may increase the risk of death. This has been demonstrated in different categories of patients, including cancer patients. There is growing evidence that both progression free survival and overall survival are significantly reduced in cancer patients with hyponatremia compared to patients with normonatremia. One important, and still open, question is whether the worse outcome associated with hyponatremia in cancer patients is directly attributable to the electrolyte disorder itself or might be a sign of the severity of the underlying disease. With regard to this point, some basic research studies suggested that low sodium concentration stimulates per se cancer cells proliferation and invasiveness. Recent clinical evidence appears to indicate that the correction of hyponatremia is an independent and favourable prognostic factor in cancer patients. Admittedly, robust confirmatory data from clinical practice are needed, in order to validate the hypothesis that cancer patients may die for hyponatremia and not just with hyponatremia.

Keywords: Hyponatremia, cancer, mortality, prognosis, vasopressin

INTRODUCTION

Hyponatremia, which is the most frequent electrolyte disorder in hospitalized patients^[1], frequently occurs also in cancer patients at different stages of disease. For instance, a recent retrospective study, performed



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at a large University Hospital in Germany in inpatients receiving specialist palliative care, showed that the prevalence of hyponatremia was 38.7% (e.g., 275 out of 710 patients) and that low serum sodium concentration $[\text{Na}^+]$ was associated with symptom severity^[2]. Several studies have demonstrated that hyponatremia is a negative prognostic factor in different clinical scenarios. Noteworthy, not only severe and acute hyponatremia has been associated with an increased risk of mortality, but there is evidence that also moderate or even mild chronic hyponatremia may increase the risk of death. This has been shown for instance in a large cohort study, which included more than 50,000 inpatients at a teaching academic medical center. This study showed that mildly reduced serum $[\text{Na}^+]$ was associated with an increased in-hospital mortality. In addition, each mmol/L reduction of serum $[\text{Na}^+]$ increased the risk of death by 2.3%^[3]. Among different clinical settings, hyponatremia has been associated with an increased risk of death in patients with heart failure^[4-6], acute myocardial infarction^[7,8], pneumonia^[9], cirrhosis^[10,11], renal failure^[12], pulmonary embolism^[13], intracerebral hemorrhage^[14], in the elderly^[15], in intensive care patients^[16], in patients undergoing surgery^[17] or cardiovascular procedures^[18]. An extensive meta-analysis, including more than 850,000 patients, of whom 17.4% with hyponatremia, confirmed an increased mortality rate associated with reduced serum $[\text{Na}^+]$ ^[19].

The literature addressing cancer patients indicated that hyponatremia is a negative prognostic factor also in this subset of patients. This chapter will review the published data, so far, with the exception of patients with lung cancer, which is the specific topic of another chapter.

HYPONATREMIA AS A PROGNOSTIC AND PREDICTIVE FACTOR IN CANCER PATIENTS

Ten years ago Waikar *et al.*^[7] published the results of a prospective cohort study, conducted in two teaching hospitals in Boston, MS, in which the association between serum $[\text{Na}^+]$ and mortality was investigated. The main outcome measures were in-hospital 1-year and 5-year mortality of patients with hyponatremia vs those with normal serum $[\text{Na}^+]$. Patients with hyponatremia had higher in-hospital mortality rates either at 1 year and at 5 years, even when serum $[\text{Na}^+]$ was only slightly reduced (130-134 mmol/L). Among the different clinical subgroups that were analyzed, the risk of in-hospital mortality was significantly higher in hyponatremic patients with metastatic cancer [hazard ratio (HR) 2.05, 95% confidence interval (CI): 1.67-2.53] compared to normonatremic patients.

Shortly after, another study addressed predictors of inpatients mortality in an acute palliative care unit at the M. D. Anderson Cancer Center of the University of Texas. Of the 500 cancer patients admitted, 124 (25%) died^[20]. Hyponatremia was one of the multiple predictors of a negative outcome (HR 3.02, 95%CI: 1.76-5.17, $P < 0.001$), together with younger age, hypernatremia, high blood urea nitrogen, high heart rate, high respiration rate, and supplemental oxygen use.

A retrospective analysis of patients admitted at the same Cancer Center during a three months period in 2006, aiming to correlate hyponatremia with 90-day mortality, was performed^[21]. Serum $[\text{Na}^+]$ was categorized into four groups, e.g., normonatremia (serum $[\text{Na}^+]$ 135-147 mmol/L), mild (130-134 mmol/L), moderate (120-129 mmol/L), and severe (< 120 mmol/L) hyponatremia. In all hyponatremic groups the risk of mortality at 90 days was higher than in normonatremic patients (HR for mild, moderate, and severe hyponatremia: 2.04 (95%CI: 1.42-2.91; $P < 0.01$); 4.74 (95%CI: 3.21-7.01; $P < 0.01$), and 3.46 (95%CI: 1.05-11.44; $P = 0.04$), respectively. Although the authors recognized limitations to the study (observational, retrospective study, inability to adjust for all comorbid conditions), and the fact that it has not been proven that hyponatremia correction can reduce mortality, these data further confirmed the clear relationship between hyponatremia and increased mortality in cancer patients.

An Italian study performed in Ancona analyzed 105 consecutive cancer patients, affected by gastrointestinal, lung, breast, female genital tract, renal, brain tumors, and sarcoma, hospitalized during a 6 month period^[22].

Median overall survival (OS) from the day of hospitalization was 50 days. Hyponatremic patients had a significantly reduced OS ($P = 0.0255$) compared to normonatremic patients. In addition, the authors reported that the presence of metastases was associated with a reduced OS ($P = 0.0418$). Similar results were obtained by Castillo *et al.*^[23], who analyzed a population of patients with lymphoma, breast cancer, colorectal cancer, small cell and non-small cell lung cancer. Hyponatremia was negatively associated with OS in all types of tumor, but the highest HRs were found in lymphoma (HR 4.5, $P < 0.01$) and in breast cancer (HR 3.7, $P < 0.1$).

In a retrospective cohort study, performed in a single center, 204 cancer patients affected by hyponatremia secondary to the syndrome of inappropriate anti-diuresis (SIAD), which is the most common cause of hyponatremia in oncology, were selected. Malignancies included lymphoma, leukemia, colorectal, breast, lung, pancreas, prostate, head and neck, bladder, esophagus, gastric cancer. About 75% of patients had malignancy-associated SIAD, whereas in roughly 25% SIAD was due to other etiologies (e.g., medications, pulmonary infections, pain or nausea). The authors found that patients with malignancy-associated SIAD had a significantly shorter median survival (58 days vs. 910 days, $P < 0.001$). The authors hypothesized that the cause of SIAD in cancer patients might represent a useful prognostic factor^[24].

A correlation between hyponatremia and a negative outcome was found also in patients with terminal cancer. A retrospective observational study conducted in a tertiary hospital palliative care unit in the Republic of Korea reported that, in addition to serum C-reactive protein (HR 1.22; $P < 0.001$) and Palliative Performance Scale (HR 0.69, $P < 0.001$), serum $[\text{Na}^+] \leq 125$ mmol/L was associated with a reduced survival time (HR = 1.91; $P < 0.001$) among 576 terminally ill patients (pancreatic/hepatobiliary, gastric, colorectal, lung cancer)^[25].

In a retrospective study performed at a University Hospital in China, among patients affected by nasopharyngeal carcinoma, glioma or oral cancer, that had been subjected to radiotherapy and developed radiation-induced brain necrosis, the risk of progression (i.e., increase of edema area $\geq 25\%$ on the MRI) was three-fold higher in patients with hyponatremia compared to those with normonatremia. Thus, the authors claimed that hyponatremia may be considered as a potential predictor for the progression of radiation-induced brain necrosis and recommended that hyponatremia is appropriately managed also in these patients^[26].

Other authors addressed their attention to a different subset of cancer patients, i.e., those who develop spontaneous tumor lysis syndrome, that induces acute uric acid nephropathy. This is a rare, yet potentially fatal complication in cancer patients, and it is important to determine the relevance of potential prognostic predictors. Although only 12 patients developed this condition among the 1,073 patients admitted with acute renal failure to a single hospital during a period of four years, hyponatremia and hypoalbuminemia on the first day of admission were the best predictors of poor prognosis^[27].

An interesting retrospective cohort study performed in a primary care setting in Copenhagen, Denmark, examined another issue, i.e., the predictive role of hyponatremia on the subsequent development of cancer. This type of analysis is facilitated in countries, like Denmark, which have a National Patient Registry. The authors showed that there is a level-dependent increased risk to develop cancer in patients with hyponatremia. The cumulative incidence increased in patients with mild (serum $[\text{Na}^+] 130\text{--}135$ mmol/L), moderate (125–129 mmol/L) or severe hyponatremia (< 125 mmol/L) [HR 1.32 (95%CI: 1.26–1.39), 1.31 (95%CI: 1.17–1.47), 1.77 (95%CI: 1.39–2.24), respectively] at 12 months, compared to normonatremic subjects. When different cancers types were analyzed separately, this finding was confirmed in head and neck, pulmonary and gastrointestinal cancer^[28].

Some studies on the role of hyponatremia in cancer have addressed specific cancer types. With regard to renal cell carcinoma (RCC), 212 newly diagnosed patients with localized RCC undergoing nephrectomy were

recruited in a study aiming to examine the prognostic value of hematologic and biochemical parameters, and other tumor-related factors. Multivariate analyses showed that preoperative serum $[\text{Na}^+]$ was an independent predictor of OS and disease-free survival, when considered either as a continuous variable or when patients were grouped based on a cut-off of serum $[\text{Na}^+]$ of 139 mmol/L (median value). The estimates of 5-year OS were 67.6% (95%CI: 54.2-80.9) and 44.3% (95%CI: 32.8-55.8) for patients with serum $[\text{Na}^+]$ above or below 139 mmol/L respectively^[29]. A Danish study addressed patients with metastatic RCC, divided into two independent cohorts of 120 patients/each. In each cohort 20% and 14% of patients had hyponatremia. Patients with hyponatremia at baseline had a median OS of 5.5 and 4.8 months in the two cohorts, whereas patients with normonatremia at baseline had a median OS of 18.6 and 16.9 months, respectively^[30]. In multivariate analysis, hyponatremia proved to be an independent prognostic factor (HR 1.90, 95%CI: 1.1-3.2, $P = 0.014$), together with increased neutrophils (HR 1.75, CI: 1.1-2.8, $P = 0.018$), lactate dehydrogenase > 1.5 ULN (HR 2.09, 95%CI: 1.3-3.3, $P = 0.002$), and number of metastatic sites ($+3$) (HR 1.92, 95%CI: 1.3-2.9, $P = 0.003$). Finally, hyponatremia was significantly associated with lack of response to treatment in both cohorts. A more recent Japanese study confirmed that hyponatremia appears to be a powerful prognostic predictor in patients with metastatic RCC treated with tyrosine-kinase inhibitors following radical nephrectomy^[31]. In this study both progression-free survival (PFS) and OS were significantly lower in hyponatremic patients (median 10.0 and 20.9 months, respectively) than in normonatremic patients (median 28.4 and 38.5 months, respectively).

Reduced serum $[\text{Na}^+]$ was indicated as a negative prognostic marker also in patients with gastric cancer and bone marrow metastases [HR 4.57; 95%CI: 1.99-10.52; $P < 0.001$]^[32] and in patients with hepatocellular carcinoma^[33].

WHY IS HYPONATREMIA ASSOCIATED WITH A WORSE OUTCOME IN CANCER PATIENTS?

Clinical data clearly indicate that hyponatremia may be viewed as a predictor of a negative outcome in cancer patients. Admittedly, one open question is whether the reduced PFS and OS, described by a number of publications, is directly attributable to the electrolyte disorder itself or might be a sign of the severity of the underlying disease. With regard to this point, a Belgian prospective study on hyponatremia in cancer patients, published almost 20 years ago, suggested that the increase mortality rate observed in cancer patients with hyponatremia (19.5% vs 6.3% in normonatremic patients) was not apparently due to reduced serum $[\text{Na}^+]$, thus indicating that hyponatremia was to be considered as a marker of general debility in advanced disease^[34]. A retrospective review (entitled “Mortality and serum sodium: do patients die from or with hyponatremia?”) of the medical records of patients admitted to a teaching hospital in Rochester, NY, for different pathologies and who died after developing hyponatremia, also suggested that the main determinant of the observed deaths was likely the severity of the underlying illness rather than the degree of hyponatremia^[35]. Similarly, a retrospective study that included patients diagnosed with SIAD in a community hospital in Israel showed that long-term survival was determined by SIAD etiology rather than by hyponatremia severity^[36].

Recently, a study performed at two teaching hospitals in Italy and one teaching hospital in England demonstrated that serum $[\text{Na}^+]$ normalization during first-line therapy is an independent prognostic factor for OS and PFS in patients with non-small cell lung cancer^[37]. Similarly, normalization of serum $[\text{Na}^+]$ was associated with a better prognosis in patients with metastatic RCC treated with everolimus^[38]. These findings are in agreement with the results of a meta-analysis that analyzed articles reporting the outcome of patients with different diseases in which hyponatremia had been corrected. The meta-analysis indicated that any improvement of hyponatremia was associated with a reduced risk of overall mortality [HR = 0.57 (0.40-0.81)]^[39].

Anyway, this issue is still a matter of debate and prospective randomized studies in cancer patients with hyponatremia, aiming to evaluate whether correction of hyponatremia can counteract the progression of the disease, are necessary.

Interestingly, some basic research studies have evaluated whether a microenvironment with a low $[Na^+]$ may affect cell proliferation and invasion ability. A study performed in prostate cancer cells *in vitro* demonstrated for instance that the exposure to a slightly hypertonic milieu induced a dormant state. Cell dormancy represents a limiting step of the metastatic process by preventing the proliferation of isolated cells outside the primary tumor. Conversely, the authors demonstrated that in the presence of a hypotonic milieu, obtained for instance by reducing $[Na^+]$ in the culture medium, cell cloning significantly increased^[40]. Another study showed that low $[Na^+]$ reduced neuroblastoma cell adhesion and increased invasion ability^[41]. A micro-array analysis was performed, in order to analyze the gene expression pattern of cells exposed to low $[Na^+]$ compared to normal $[Na^+]$. Among the genes that had different expression levels in the presence of reduced $[Na^+]$, the heme-oxygenase 1 gene (HMOX-1), a marker of oxidative stress, was the gene with the greatest variation. In fact, HMOX-1 gene expression showed a 200-fold increase in cells exposed to low $[Na^+]$. Immunocytochemistry for HMOX-1 protein confirmed these results. It is known that oxidative stress favors carcinogenesis, cancer growth and invasion, angiogenesis, and overall creates a permissive environment for cancer cells^[42]. Accordingly, selective inhibition of HMOX-1 has been proposed as a therapeutic target for cancer treatment^[43]. Although additional studies are needed in order to confirm these data, in view of the above described results from basic research it can be hypothesized that also *in vivo* low $[Na^+]$ might promote cancer cell progression through the same mechanisms. If so, then we might probably say that patients die not only with hyponatremia, but also for hyponatremia.

CONCLUSION

In recent years evidence indicating that hyponatremia is a predictor of a worse outcome in cancer patients has accumulated. There is emerging evidence, mainly from basic research studies at this time, that hyponatremia may by itself increase the risk of mortality of patients, rather than being a simple bystander of the progression of the disease. However, robust confirmatory data from clinical practice are certainly needed in order to definitively validate the hypothesis that cancer patients may die for hyponatremia.

Meanwhile, the author is truly convinced that it is worth to correct hyponatremia in patients, including cancer patients, because of the overall beneficial effect on the quality of life, which includes clinical improvement, such as amelioration of neurocognitive and motor performance, reduced length of stay in the hospital and re-admission probability^[44-46].

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The author contributed solely to the article.

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The author declares that there are no conflicts of interest.

Ethical approval and consent to participate

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Review

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The lncRNA BORG: a novel inducer of TNBC metastasis, chemoresistance, and disease recurrence

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Abstract

Although greater than 90% of breast cancer-related mortality can be attributed to metastases, the molecular mechanisms underpinning the dissemination of primary breast tumor cells and their ability to establish malignant lesions in distant tissues remain incompletely understood. Genomic and transcriptomic analyses identified a class of transcripts called long noncoding RNA (lncRNA), which interact both directly and indirectly with key components of gene regulatory networks to alter cell proliferation, invasion, and metastasis. We identified a pro-metastatic lncRNA BMP/OP-Responsive Gene (BORG) whose aberrant expression promotes metastatic relapse by reactivating proliferative programs in dormant disseminated tumor cells (DTCs). BORG expression is broadly and strongly induced by environmental and chemotherapeutic stresses, a transcriptional response that facilitates the survival of DTCs. Transcriptomic reprogramming in response to BORG resulted in robust signaling via survival and viability pathways, as well as decreased activation of cell death pathways. As such, BORG expression acts as a (1) marker capable of predicting which breast cancer patients are predisposed to develop secondary metastatic lesions; and (2) unique therapeutic target to maximize chemosensitivity of DTCs. Here we review the molecular and cellular factors that contribute to the pathophysiological activities of BORG during its regulation of breast cancer metastasis, chemoresistance, and disease recurrence.



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Keywords: BORG, chemoresistance, disease recurrence, dormancy, long noncoding RNA, metastasis, triple-negative breast cancer

INTRODUCTION

With over 250,000 newly diagnosed cases in 2017, breast cancer is the most commonly diagnosed malignancy among U.S. women^[1]. Although recent advances in treatment have produced a moderate decline in the mortality rate associated with breast cancer^[2], this disease nevertheless still plagues women and is the second leading cause of cancer-related death, resulting in over 40,000 deaths each year^[1]. The clinical management of breast cancer is complicated by its manifestation as an exceptionally heterogeneous disease. In fact, the degree of molecular and histopathologic variation demonstrated by breast cancers necessitates its classification not as a single, uniform disease, but rather as one that is composed of a diverse collection of diseases that possess varying clinical prognoses and require distinct treatment strategies. Integral to this heterogeneity are the receptors for the ovarian steroid hormones estrogen (ER) and progesterone (PR), and for the membrane-associated tyrosine kinase HER2/ErbB-2. Moreover, the expression or lack thereof of these receptors dictates therapeutic schemes and disease-free progression^[3,4]. Likewise, the multiple various permutations in the expression patterns of these receptors exemplify the heterogeneity of the disease and necessitate the tailoring of specific treatments to each individual patient. In fact, clinicopathologic detection for the expression of these receptors remains an advantageous trait, as they represent some of the most consistently predictive and actionable molecular targets in all of oncology^[5]. As such, the largest clinical burden associated with breast cancer stems from a subset of patients whose tumors fail to express ER, PR, and HER2, lesions known as triple-negative breast cancer (TNBC). This genetically distinct breast cancer subtype constitutes ~15%-20% of all diagnosed breast cancers^[6] and portends the worst overall survival rates of all breast cancer subtypes, an untoward trait that reflects their extreme propensity to relapse within 5 years of initial diagnosis and treatment^[7]. The molecular features that underlie the development, metastasis, and relapse of TNBCs remain to be fully elucidated. Recently, a novel intergenic lncRNA known as BMP/OP-Responsive Gene (BORG) was identified as being a prominent driver of these tumorigenic activities in TNBCs. Here we highlight the pathophysiology associated with aberrant BORG expression in TNBCs, as well as discuss clinical implications of BORG and its potential for therapeutic targeting to alleviate metastatic disease.

Breast cancer metastasis

Despite immense efforts undertaken to characterize the molecular complexity of primary breast cancers, the lethality associated with all subtypes of breast cancer is attributed primarily to the dissemination and colonization of distant tissues^[8,9], an untoward clinical event that results in dismal median survival rates of ~1 year. Moreover, the finding of metastatic TNBC is essentially a fatal diagnosis regardless of the chemotherapeutic intervention deployed to combat this disease^[10]. Despite decades of intense investigation, a complete understanding of the molecular forces vital to metastasis remains incomplete, a knowledge gap that continues to hinder the development of therapeutics capable of specifically targeting and alleviating metastatic lesions. Nonetheless, fundamental steps taken by malignant cells to facilitate their dissemination from the primary tumor site to distant tissues for colonization have been identified and are called the metastatic cascade^[11]. The events that comprise this multi-step process include: (1) local invasion and migration of malignant cells into the stroma surrounding the primary lesion; (2) intravasation into the vasculature or lymphatic system to permit transit through these circulatory routes; (3) vascular stasis and subsequent extravasation into parenchymal tissues at distant sites of colonization; (4) survival and micrometastasis formation; and (5) overt growth and metastasis formation^[12]. Interestingly, the earliest phases of metastatic progression (i.e., dissociation and emigration from site of origin via the vasculature) represent the barriers that are most easily surmounted by primary lesions, as evidenced by the fact that tumors readily liberate thousands upon thousands of cells into the circulation each day^[13,14]. However,

despite the relative abundance of tumor cells traversing the vascular systems of some patients, the process of metastasis remains a supreme inefficient event. Indeed, animal modeling studies estimate that only ~0.01% of circulating tumor cells are capable of initiating some form of metastatic outgrowth^[15], and as such, it is the late stages of metastatic progression that ultimately dictate the competency of disseminated cells in establishing overt metastases within the metastatic niche^[12].

Dormancy: a metastatic bottleneck

The acquisition of metastatic phenotypes was originally believed to be an evolutionary consequence of advanced, late-stage disease. However, recent findings indicate that carcinoma cells comprising primary breast tumors do in fact enter the circulation months-to-years prior to the point at which the primary tumor becomes symptomatic and diagnosable^[16]. As such, a large proportion of breast cancer patients already harbor disseminated tumor cells (DTCs) at their time of diagnosis^[11,17,18]. Indeed, DTCs are readily detected in the bone marrow of 30%-40% of patients diagnosed with early-stage breast cancer, an event that portends a significantly worse prognosis as compared to patients whose bone marrow is free from DTCs at the time of diagnosis^[19]. Interestingly, a large fraction of these DTCs initially remain clinically asymptomatic due in part to their acquisition of dormancy-associated phenotypes upon arrival to the micrometastatic niche^[12].

Although diverse genetic and epigenetic analyses have begun to reveal the molecular landscape that characterizes metastatic breast cancers, only recently have these investigations been directed at and tailored for DTCs and their reactivation of proliferative programs during metastatic relapse. As such, a unified definition of dormancy remains elusive. At a cellular level, current models suggest that metastatic cells often undergo proliferative arrest upon arriving to a micrometastatic niche, a phenomenon believed to reflect the initial maladaptation of DTCs to foreign stromal environments^[20]. Indeed, the stromal characteristics that impact DTC dormancy are multifactorial and encompass a diverse array of immunomodulatory and vascular endothelial cell signals (e.g., cytokine milieu, rigidity of the microenvironment, presence of active immunosurveillance^[17,21,22]) that converge on niche-localized DTCs. In doing so, these unique microenvironment signals may prove to be inhospitable to DTCs, thereby provoking cellular stress and the initiation of apoptosis as these cells struggle to cope and respond to foreign junctional and adhesive signaling networks^[23-25]. Consequently, newly established micrometastatic lesions fail to propagate and expand due to: (1) deficiencies to initiate and progress through the cell cycle; and (2) propensity to undergo apoptosis at a pace that equals or exceeds the proliferative rate of DTCs. Despite these barriers, a subset of DTCs remain viable and poised to reactivate proliferative programs that result in metastatic relapse years-to-decades after implementation of initial treatments, such as chemotherapy and radical primary tumor resection^[26,27]. Clinically, extended periods of metastatic dormancy is evidenced by growth modeling studies performed on over 1,000 breast cancer patients. In doing so, two discrete peaks that define the probability of metastatic recurrence were identified: (1) one correlating with a model of continuous, slow growth of metastatic cells; and (2) one corroborating the principle that the majority of delayed relapses are indeed the result of a temporary period of dormancy prior to reactivation of proliferation programs^[28]. Indeed, it is this second peak that poses the greatest threat to breast cancer patients, with ~62% of breast cancer deaths occurring 5-20 years after initial diagnosis^[27]. Taken together, these findings reveal that dormant DTCs play a pivotal role in the majority of breast cancer-associated mortality, a feature that cements them as one of the most clinically relevant targets in all of oncology.

MODELS OF METASTATIC DORMANCY IN BREAST CANCER

In vitro and *in vivo* models of dormancy represent critical tools for investigating the molecular mediators that impact dormant states. However, the establishment of such models pose significant challenges, as the growth and propagation of dormant cell lines is, by definition, inherently impractical. Likewise, the size and sparsity of dormant micrometastases makes their identification highly burdensome. Nonetheless, accepted models of breast cancer dormancy do in fact exist. One particularly powerful model of metastatic dormancy

is the murine D2.HAN series, which consists of two cell lines that display distinct metastatic properties, namely the dormant D2.OR cell line and the highly metastatic D2.A1 cell line^[29]. These cell lines were derived from a premalignant murine hyperplastic alveolar nodule implanted into the cleared mammary fat pad of *BALB/c* mice, resulting in spontaneous tumors that were subsequently classified by alternations in their surface glycoprotein composition as determined by retention to the lectin, peanut agglutinin^[29].

Accordingly, dormant D2.OR cells exhibit high affinity for peanut agglutinin and produce slow growing tumors that are incapable of forming disseminated tumors in either spontaneous or experimental metastasis models. In stark contrast, metastatic D2.A1 cells exhibit low affinity for peanut agglutinin and produce fast growing tumors that metastasize aggressively^[30]. Importantly, *in vivo* videomicroscopy reveals that these D2.HAN derivatives show no differences in the mechanism, timing, and proportion of cells capable of extravasating into the lungs of mice, indicating that post-extravasation events underlie their vastly disparate abilities to undergo metastatic outgrowth^[31]. Despite their inability to form secondary tumors, a large proportion of disseminated D2.OR cells readily survive the process of extravasation (i.e., ~80% at 3 weeks and ~50% at 11 weeks) and remain viable despite their non-proliferative phenotype (i.e., Ki-67-negative)^[32]. Collectively, these cellular and functional features form the crux of the dormancy-associated phenotypes exhibited by D2.OR cells *in vivo*, thus establishing them as a valuable model for studying the molecular mechanisms underlying metastatic dormancy^[33-35].

lncRNAs in breast cancer

Elucidating the molecular determinants of breast cancer metastasis, especially those associated with DTC acquisition of and eventual emergence from dormancy, remains a critical undertaking that is essential to the future development of therapies capable of targeting DTCs. Recently, a surprising class of molecules called long noncoding RNAs (lncRNAs) have been identified and function as potent contributors to the malignant properties of breast cancer cells. Broadly, these transcripts are defined as RNA molecules that are > 200 nucleotides in length and lack an open reading frame capable of producing a functional protein^[36]. lncRNAs were originally believed to possess negligible function and exist primarily as “transcriptional noise” originating from illegitimate regulatory DNA elements. However, it is now clear that lncRNA expression patterns transpire in a highly specific cell- and tissue-dependent manner^[37,38]. Importantly, noncoding RNA molecules do not require protein-coding capacity to act as powerful determinants of cell fate. Indeed, lncRNAs harbor immense intrinsic functionality within the course of cellular homeostasis and disease formation. For instance, the developmental complexity of organisms correlates more closely with the extent and diversity of the noncoding genome rather than with the collective composition of protein-coding genes^[39-41].

In light of the dynamic and diverse functions attributed to lncRNAs, it is unsurprising that malignant cells, including those arising from the breast, have hijacked lncRNAs to directly and indirectly alter their proliferative, invasive, and metastatic ability^[42-44]. As such, several lncRNAs have been shown to modify critical breast cancer-associated molecular pathways in a manner that transcends hormone receptor status, frequently driving the development and progression of TNBCs^[45]. Similarly, ER-associated signaling pathways also regulate the expression of lncRNAs^[46], including HOTAIR, whose promoter contains several estrogen response elements^[47].

Moreover, induction of HOTAIR promotes the growth and metastasis of breast cancers via widespread epigenetic reprogramming^[48].

BORG

Although numerous lncRNAs have been linked to breast cancer tumorigenesis and metastasis^[49], the intergenic lncRNA BORG has recently emerged as a unique and formidable regulator of the metastatic competence and survival of breast cancer cells. Originally discovered in murine C2C12 myoblast cells treated with BMP2 or BMP7, BORG is a spliced and polyadenylated ~2.8 kb transcript that shows no

evidence of an open reading frame and carries multiple conserved repeat sequence elements of unclear significance^[50]. Collectively, these features implicate BORG as a lncRNA, whose primary sequence has been subject to several functional analyses. For instance, BORG houses several novel pentamer motifs that are essential in facilitating its strict residence in the nucleus, representing the first demonstration of sequence-based determinants operant in dictating the subcellular localization of lncRNAs^[51]. Indeed, the nuclear localization of lncRNAs directly impacts their ability to elicit widespread alterations in transcriptional networks by: (1) localizing transcription factors to specific genomic loci^[52-54]; and (2) exerting gross changes in the nuclear architecture of cells^[55]. Likewise, BORG oversees a host of cellular functions that are readily harnessed by breast cancer cells to enhance their tumorigenic behaviors. As will be discussed in the succeeding sections, these BORG-dependent events play an essential role in promoting breast cancer cell proliferation, chemoresistance, and survival.

Control of proliferation

In undertaking a combination of *in silico* and cell biological analyses, we recently determined that the expression of BORG directly correlates with aggressive breast cancer phenotypes, and with their metastatic competence and recurrence. Specifically, BORG liberates D2.OR cells from a state of dormancy in 3D-organotypic cultures by conferring a proliferative shift in the cell cycle from G0/G1 to G2/S^[56]. Importantly, this proliferative stimulus is sufficient to enable BORG-expressing D2.OR cells to form overt metastases in the lungs of BALB/c mice^[56]. Interestingly, the mitogenic properties of BORG are highly context-dependent, as they only emerge in D2.OR cells propagated in microenvironments that mimic primary and/or metastatic tumor sites (e.g., 3D-cultures). Such context-dependent activities of BORG imply that this lncRNA confers malleable phenotypes to DTCs, thus compelling them to activate adaptive signaling programs that enable their survival and outgrowth within diverse metastatic niches.

BORG as a manipulator of protein function

In searching for mechanistic insights into how BORG induces DTCs to escape from dormancy, we performed mass spectrometry analyses on proteins captured by the pulldown of biotinylated, anti-sense BORG transcripts. These analyses identified the E3 SUMO ligase TRIM28 (KAP1) as a strong binding partner of BORG^[57]. TRIM28 functions as a transcriptional co-repressor and scaffolding protein for histone and DNA modifying enzymes that enhance breast cancer cell proliferation, doing so in part by suppressing the transcription of senescence promoting genes, especially *p21* and *Gadd45a*^[58-61]. Interestingly, elevating BORG expression in D2.OR cells to levels that approximate those detected in their metastatic D2.A1 counterparts dramatically downregulated the expression *p21* and *Gadd45a*, indicating that BORG may rely upon TRIM28 to confer proliferative states to dormant DTCs. Indeed, CRISPR/Cas9-mediated knockout of TRIM28 restores a dormant phenotype in BORG-expressing D2.OR cells, as does expression of mutant BORG transcripts that can no longer bind TRIM28^[57]. Thus, the oncogenic activities of BORG depend upon its physical interaction with TRIM28, an event that serves as a proliferative stimulus to dormant DTCs.

The diverse range of functions elicited by lncRNAs is thought to be promoted by their unique structural diversity. Their inherent length and nucleic acid structure allow the formation of flexible, complex secondary and higher order structures that facilitate their interactions with macromolecular complexes^[40]. Indeed, lncRNAs can acquire behaviors analogous to ligands, as their binding to proteins can trigger conformational changes and/or modify protein: protein interactions that dramatically impact protein activation states^[62]. Accordingly, BORG enhances the function of TRIM28 to inhibit transcription by regulating the pause and release of RNA Polymerase II (Pol II)^[63,64]. For instance, heterologous expression of mutant BORG transcripts that retain their capacity to bind TRIM28 remain competent to elicit Pol II promoter pausing at the *p21* and *Gadd45a* loci, whereas those BORG mutants incapable of binding TRIM28 fail to impact the pausing index of Pol II at these loci^[57]. Furthermore, widespread evidence indicates that lncRNAs can function as molecular scaffolds for proteins, thereby: (1) tethering cooperative proteins together to enhance their functions; or (2) localizing RNA-protein complexes to specific genomic regions through base-pair and

tertiary structure interactions with DNA^[41,65]. Along these lines, chromatin immunoprecipitation assays reveal that BORG enhances the binding of TRIM28 to specific genomic loci^[57]. Thus, BORG promotes the metastatic outgrowth of dormant DTCs in part through its ability to promote the localization and transcriptional repressive activity of TRIM28. Although BORG directly modifies the ability of TRIM28 to suppress the expression of *p21* and *gadd45a* in dormant DTCs, it should be noted that TRIM28 also exerts widespread alterations in the transcriptomes of a multitude of cell types^[63,64,66]. To gain additional insight into the repertoire of transcriptional events coupled to TRIM28 in DTCs, we performed RNA-seq and microarray-based transcriptomic analyses on parental and BORG-expressing D2.OR organoids propagated in 3D-cultures. To assess the specific impact of TRIM28 on these dormancy-associated phenotypes (i.e., parental dormancy *vs.* BORG-mediated outgrowth), we also rendered these cells deficient in TRIM28 expression. Unsurprisingly, BORG-expressing D2.OR cells harbored a transcriptional signature that deviated significantly from its parental and TRIM28-deficient counterparts. Moreover, cellular network analyses revealed specific BORG- and TRIM28-dependent transcriptional patterns that were significantly enriched for proliferative and pro-metastatic signatures^[57]. Collectively, these findings establish BORG as the only known lncRNA that functions in modifying the activity and cellular localization of a transcriptional regulator (i.e., TRIM28) to confer genome-wide transcriptomic alterations that compel the reactivation of proliferative programs in dormant DTCs.

Chemoresistance

The development of therapeutic resistances continues to hamper the prolonged efficacy of standard-of-care treatment regimens. Moreover, these clinical challenges are compounded by the fact that the underlying mechanisms responsible for targeted and chemotherapeutic resistance are immensely diverse. Nonetheless, malignant breast cancer cells regularly rely on the malleable intrinsic state of cancer cells, which enables their adaptation to cytotoxic cellular stresses in order to maintain viability^[67] in a manner that most closely follows the paradigm of acquired resistance^[68]. Indeed, the plasticity underlying the appearance of chemoresistance is naturally permissive and reflects alterations in the epigenome. Moreover, these events are bolstered by defects in the ability of DTCs to maintain genome integrity that arise in response to aberrant cell cycle checkpoints and DNA repair mechanisms, and to increased rates of proliferation. Additionally, interactions between DTCs and the tumor microenvironment induce unique *de novo* mechanisms of therapeutic resistance, as cell adhesion networks (e.g., integrins) activate a specialized survival program known as “cell adhesion-mediated” drug resistance that elicit DTC insensitivity to numerous treatment regimens^[69,70]. As such, the stromal composition of the metastatic microenvironment creates a natural sanctuary for DTCs to survive therapeutic insults.

Interestingly, dormant DTCs have long been recognized for their inherent resistance to commonly used chemotherapeutic drugs^[11]. These resistant traits naturally stem from the quiescent phenotype of dormant cells, which effectively abolishes the clinical utility of chemotherapeutics and cytotoxic agents that target metabolically active and dividing cells^[71]. Moreover, dormant cells preferentially upregulate signaling pathways associated with cell survival, a trait stemming from their allocation of metabolic resources away from cell cycle progression as a means to remain viable in the face of environmental stressors (i.e., chemotherapy)^[72]. Accordingly, chemotherapeutic treatment can select for a subset of dormant cells that are enriched for pro-survival pathways and multidrug resistance, implying that cytotoxic insults can select for a population of cells that are exceedingly equipped to instigate post-therapy relapse^[73,74]. Importantly, we recently determined that BORG plays a central role in driving the development of chemoresistance in TNBCs (see below).

BORG: a novel inducer of chemoresistance

In addition to possessing enhanced proliferative abilities, BORG-expressing D2.OR cells also exhibit: (1) extensive upregulation of pro-survival and viability pathways; and (2) widespread downregulation of cell

Table 1. BORG-associated signaling pathways

Upstream inducers	BORG-complexes	Downstream effectors	Cellular response	Ref.
BMP; OP-1; TGF- β	BORG	Alkaline phosphatase	Osteoblast Differentiation	[50]
NF- κ B	BORG	NF- κ B	Cellular Stress Response/Cell survival	[56,84,85]
ATM ^{*,#}	BORG:TRIM28	P21 [*]	Proliferation	[57,58]
ATM ^{*,#}	BORG:TRIM28	Gadd45a [*]	Proliferation	[57,58]
-	BORG:TRIM28	P38/MAPK [*]	Proliferation	[57]
-	BORG:TRIM28	ERK1/2	Proliferation	[57]
Hypoxia/chemostresses	BORG:RPA1	RPA2 & RPA3 [#]	DNA Damage Response	[56,80]
Hypoxia/chemostresses	BORG:RPA1	BRCA2 [#]	DNA Damage Response	[56,81-83]
Hypoxia/chemostresses	BORG:RPA1	XPA [#]	DNA Damage Response	[56,81-83]
Hypoxia/chemostresses	BORG:RPA1	P53 [#]	DNA Damage Response	[56,81-83]

*Indicate a downregulation in protein expression; [#]represent proteins hypothesized to mediate BMP/OP-Responsive Gene (BORG) action based on findings published in the literature. BORG plays essential functions in regulating breast cancer proliferation, metastasis, and chemoresistance. The specific effectors of BORG that activate cells proliferation and survival systems are annotated above, as are the BORG:effector complexes operant in mediating these events

death pathways^[56]. Moreover, BORG expression is highly responsive to metabolic stresses such as hypoxia and nutrient deprivation, as well as to treatment with a wide panel of chemotherapeutic drugs, including doxorubicin, hydroxyurea, docetaxel, 5-fluorouracil, and 6-thioguanine^[56]. These cellular attributes of BORG are consistent with its ability to enhance metastatic outgrowth and disease recurrence. Indeed, comet assays demonstrate that heterologous expression of BORG dramatically mitigates the extent of double-stranded DNA breaks experienced by D2.OR cells in response to doxorubicin exposure. Moreover, these genoprotective features of BORG rely upon its binding to the single-strand DNA-binding protein, RPA1, which functions as an essential molecule in the repair of DNA damage^[75,76]. Interestingly, the capacity of BORG to induce resistance to doxorubicin appears to supersede its ability to enhance cell cycle progression, an event that could potentially render BORG-expressing cells more sensitive to the cytotoxic activities of doxorubicin^[77,78]. As such, the genoprotective effects of BORG are essential in establishing the foundation operant in mediating deadly relapse in patients with metastatic breast cancers. Moreover, as lncRNAs typically function as molecular scaffolds that facilitate the formation of protein complexes, particularly in the nucleus^[79], it is tempting to speculate that BORG promotes the interaction of RPA1 with proteins critical to its repair of DNA, including additional subunits RPA2 and RPA3^[80], as well as other DNA-repair associated proteins, such as BRCA2, XPA, and p53^[81-83] [Table 1].

BORG and NF- κ B: a feed-forward loop to chemoresistance

Cellular network analyses of parental and BORG-expressing D2.OR derivatives demonstrate significant enrichment of a hallmark gene signature that correlates with the induction of NF- κ B activity^[56]. Accordingly, NF- κ B reporter assays show enhanced activation of NF- κ B signaling in BORG-expressing D2.OR cells as compared to their parental counterparts. In light of the longstanding association between NF- κ B activation and the initiation of pro-survival and cellular stress responses^[84,85], we further explored the connections between NF- κ B and BORG in eliciting DTC resistance to chemotherapy. In doing so, we inactivated the NF- κ B pathway via several complementary strategies, including: (1) stable expression of a dominant-negative form of I κ B α ; (2) CRISPR/Cas9-mediated knockout of the BORG-responsive gene NEMO/IKK γ ; or (3) administration of small molecule inhibitors to IKK β . In all cases, inhibiting NF- κ B signaling in BORG-expressing D2.OR cells restored their sensitivity to doxorubicin both *in vitro* and *in vivo*, and to apoptosis induced by hypoxia- and nutrient deprivation^[56]. It should be noted that while BORG requires ample expression of RPA1 to protect against doxorubicin-induced DNA damage and subsequent apoptosis, this event appears to be dissociated from the ability of BORG to activate NF- κ B signaling. Indeed, CRISPR/Cas9-mediated disruption of RPA1 expression had no effect on the ability of BORG to activate NF- κ B signaling^[56]. Thus, the chemoresistant phenotypes afforded by BORG-RPA1 interactions act independently of the NF- κ B signaling axis.

Interestingly, the promoter region of the BORG locus contains multiple stretches of nucleotides that exhibit sequence homology to the consensus DNA-binding sequence for NF- κ B. Accordingly, NF- κ B readily bound to the BORG promoter in BORG-expressing cells, thereby identifying a novel feed-forward loop whereby the activation of NF- κ B (i.e., through chemotherapeutic and environmental stressors^[86-89]) leads to enhanced BORG expression, which further promotes the induction NF- κ B responsive genes. Along these lines, expression of a dominant-negative I κ B α in TNBCs prevented their expression of BORG following exposure to doxorubicin, and to environmental stresses, such as hypoxia and nutrient deprivation^[56]. Collectively, these findings implicate BORG as a unique lncRNA that is capable of promoting a NF- κ B feed-forward signaling loop that effectively links metastasis-associated cellular stresses to a coordinated signaling program that engenders the survival of disseminated TNBCs.

BORG and breast cancer stem cells

Breast cancer stem cells (BCSCs) are malignant cells capable of tumor initiation, self-renewal, and differentiation into a heterogeneous group of cancer cells that reflect those present in the original primary breast tumor; they are also associated with the acquisition of metastatic and chemoresistant phenotypes^[90-93], particularly upon their colonization of foreign tissue microenvironments^[94]. BCSCs typically divide asymmetrically to create: (1) a single progenitor cell capable of differentiating into a variety of functionally diverse cancer cell types; and (2) a single BCSC that can expand and undergo continual self-renew. Attempts to characterize BCSCs has been hampered by a relative lack of universal markers for BCSCs^[95,96]. Indeed, BCSCs have been linked to the expression of several cell surface proteins, such as CD133 and CD44⁺/CD24⁻, and to the intracellular protein, ALDH1^[97-99]. Recent findings have associated the expression of lncRNAs with the generation and expansion of BCSCs. For instance, the lncRNAs ROR, HOTAIR, and Hh all induce the expression of transcription factors that regulate “stemness”, such as SOX2 and OCT4; they also impact the initiation of EMT programs^[100-103], which elicit the selection, expansion, and self-renewal of BCSCs^[104]. The role of BCSCs in regulating metastasis, chemoresistance, and survival signaling are reminiscent of the features attributed to aberrant BORG expression, suggesting that BORG may also regulate the behaviors of BCSCs. Accordingly, BORG expression is elevated significantly in BCSCs as compared to non-BCSCs populations; it also enhances the mammosphere forming activity of human and murine breast cancer cells (Gooding *et al.*^[56,57], unpublished observation). Thus, future studies need to determine precisely how BORG impacts the transcriptomic and epigenetic landscapes of breast cancer cells to impart BCSC characteristics coupled to metastatic progression and disease recurrence.

DISCUSSION AND CLINICAL IMPLICATIONS OF BORG

Metastasis is an exceedingly complicated process, whereby tumor cells must undergo coordinated efforts to successfully disperse from primary tumors, emigrate to distant tissues, and survive and colonize foreign microenvironments. Even for highly transformed cells, traversing the metastatic cascade is immensely challenging, with the vast majority of disseminated cells unable to form overt metastases^[15]. The inefficiencies characterizing metastasis are predominantly attributed to the cellular stresses associated with unfamiliar metastatic microenvironments, forces that obstruct both the survival and outgrowth of DTCs. Accordingly, BORG has emerged as a potent and unique lncRNA that is poised to enhance breast cancer metastasis by altering both sides of this equation, i.e., providing both proliferative and pro-survival stimuli to DTCs.

From a potential clinical perspective, aberrant BORG expression is most frequently associated with TNBC/basal-like breast cancers, as determined by scrutinizing several publicly available RNA-seq datasets. Moreover, we detected significant elevations of BORG in metastatic human patient-derived xenograft tissue samples, and in CNS metastases and their matched primary tumors from which these metastatic foci derived. In all cases, malignant tissues clearly express increased levels of BORG as compared to normal human mammary epithelial cells^[56,57]. Thus, these findings support the hypothesis that primary

malignancies that house high levels of BORG will disperse aggressive breast cancer cells that are predisposed to establishing clinically-relevant, chemoresistant secondary lesions. Indeed, TNBC patients who succumbed to metastatic relapse within 5 years of initial diagnosis and treatment possessed primary tumors that contained significantly higher levels of BORG compared to primary tumors derived from TNBC patients who remained disease-free for at least 5 years post-treatment^[57]. Taken together, these intriguing findings suggest that quantifying BORG expression in primary tumors could offer important insights into predicting the natural and clinical course of breast disease within TNBC patients.

The correlative finding that BORG expression largely aligns with the overall malignant propensity of breast cancer cells fails to address the mechanisms and signaling systems ultimately coupled to its upregulation in developing mammary tumors. Indeed, it has been proposed that lncRNAs evolved as a means to assist in maintaining cellular homeostasis in response to a wide variety of pathophysiologic conditions^[105]. Accordingly, we show that BORG expression is similarly influenced by a number of cellular stressors, including chemotherapeutic insult, nutrient deprivation, and hypoxia^[56], as well as in response to heat shock (Gooding *et al.*^[56] and Valadkhan *et al.*^[105], unpublished observation). Moreover, NF- κ B is critical in linking the responsiveness of BORG to these environmental stressors^[56], as NF- κ B activation has long been tied to the survival of cells confronted with a host of intrinsic and extrinsic stressors^[84,85,106]. It therefore stands to reason that increased flux through the NF- κ B pathway stemming from exposure to noxious stimuli may serve as the initial impetus in triggering the aforementioned NF- κ B feed-forward loop, wherein NF- κ B-induced upregulation of BORG propagates expression of NF- κ B-responsive gene expression patterns^[56].

Tumor progression and metastatic competence are thought to rely heavily upon the intrinsic plasticity of malignant cells, which facilitates their adaptation to harsh foreign microenvironments in order to maintain viability^[67,107]. Because BORG is largely regulated by extracellular paracrine factors (e.g., TGF- β , BMP-2, and BMP-7^[50,56]) and the environmental cues, we surmise that BORG acts as a context-dependent, transcriptional rheostat for disseminated breast cancer cells, thereby dictating their engagement of proliferative and pro-survival pathways. Indeed, when faced with environmental or therapeutic stresses, the induction of BORG in malignant cells orchestrates a transcriptional signature that provokes aggressive tumorigenic states that ensures for their survival. As such, preventing BORG expression or impeding the activation of its downstream targets represents an innovative and potentially impactful strategy to target metastatic breast cancers and drive them into an apoptosis-prone state.

Current mechanistic insights into the regulation of BORG and its downstream effectors reveal that this lncRNA is uniquely poised to promote the metastasis of breast cancer cells. Indeed, BORG clearly exerts a pro-metastatic effect at both the primary and metastatic sites of tumor growth. For example, a subset of breast cancer patients may harbor primary tumor cells that have gained the expression of BORG (BORG^{hi}) as a result of environmental stresses associated with a growing primary tumor (i.e., hypoxia and nutrient deprivation; Figure 1). Such tumors are prone to shedding BORG^{hi} cells into the circulation that disseminate to distant tissues, wherein they exploit the proliferative and pro-survival effects of BORG to overcome the hostile metastatic microenvironment and form overt metastases [Figure 1]. Accordingly, and as noted above, TNBC patients who succumbed to metastatic relapse within 5 years of initial diagnosis and treatment possessed primary tumors that contained significantly higher levels of BORG compared to primary tumors derived from TNBC patients who remained disease-free for at least 5 years post-treatment. Furthermore, breast cancer cells can emigrate from the primary tumor at very early stages of tumor development^[18]. As such, these early disseminated breast cancer cells are likely to originate from a lesion experiencing little hypoxic or metabolic stress and are therefore more prone to harboring low levels of BORG (BORG^{lo}). Although still capable of disseminating to distant tissues, these BORG^{lo} cells are predicted to struggle within their foreign microenvironments, resulting in their undergoing cell death or retreating into a state of metastatic dormancy. Nonetheless, stromal paracrine signals (e.g., TGF- β , BMP2, or BMP7), as well as the

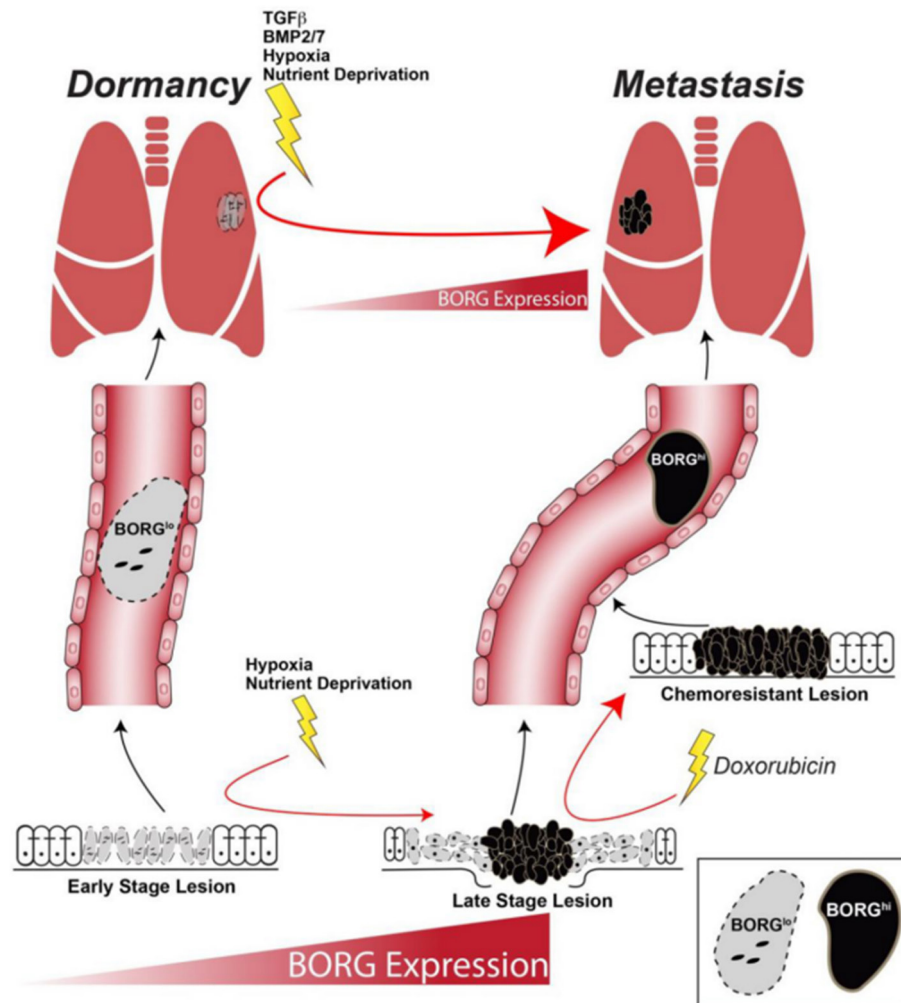


Figure 1. BORG is a potent facilitator of breast cancer metastasis. Breast cancer cells can disseminate at very early stages of development. Due to the benign microenvironment associated with these lesions, these cells are likely to harbor low levels of BORG (BORG^{lo}) expression and are prone to establishing dormant lesions in metastatic tissues. Nonetheless, stromal factors and environmental stressors in the metastatic microenvironment can induce BORG expression, thereby compelling these dormant cells to reinstate proliferative programs. The progression of primary tumors is associated with a hypoxic environment and stark competition for nutrients. Such stresses can enhance BORG expression in a subset of cells (BORG^{hi}). These BORG^{hi} cells can disseminate to distant tissues where they exploit the proliferative and survival advantages afforded by BORG to produce overt metastases. Cytotoxic chemotherapeutic treatment is effective against BORG^{lo} cells, but BORG^{hi} cells show resistance to such therapies and are the foundation for chemoresistant, residual disease that can eventually metastasize to distant tissues. BORG: BMP/OP-Responsive Gene

hypoxic and metabolic stresses associated with the metastatic microenvironment, can lead to the induction of BORG expression within these dormant DTCs, thereby activating proliferative programs and survival signaling to promote their metastatic outgrowth [Figure 1].

Finally, neoadjuvant or adjuvant treatment of primary breast cancers with chemotherapeutic agents (e.g., doxorubicin) will selectively kill BORG^{lo} cells that are not inherently resistant to the cytotoxic effects of these agents. The residual, chemoresistant BORG^{hi} cells can subsequently contribute to the recurrence of metastatic or primary lesions that are insensitive to standard-of-care therapies [Figure 1]. These diverse cellular outcomes downstream of BORG establish this lncRNA as an essential driver of breast cancer metastasis and highlight the potential utility derived from therapeutically targeting BORG or its effectors as a novel means to alleviate the metastatic outgrowth and recurrence of disseminated TNBCs.

DECLARATIONS

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Author's contributions

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Conflicts of interest

All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Original Article

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Training and evaluation of a knowledge-based model for automated treatment planning of multiple brain metastases

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Abstract

Aim: Volumetric modulated arc therapy (VMAT) has been utilized to plan and treat multiple cranial metastases using a single isocenter due to its ability to provide steep dose gradients around targets as well as low doses to critical structures. VMAT treatment is delivered in a much shorter time compared to using a single isocenter for the treatment of each lesion. However, there is a need to develop methods to reduce the treatment planning time for these cases while also standardizing the plan quality. In this work we demonstrate the use of RapidPlan, which is knowledge-based treatment (KBP) planning software to plan multiple cranial SRS cases.

Methods: The 66 patient plans with 125 lesions (range 1-4, median 1) were used to train a model. In addition, the model was validated using 10 cases that were previously treated and chosen randomly. The clinical plans were compared to plans generated by RapidPlan for target coverage and critical organ dose.

Results: Coverage to the target volume, gradient index, conformity index and minimum dose to the target showed no significant difference between the original clinical plan vs. the plan generated by KBP. A comparison of doses to the critical organs namely the brainstem, brain, chiasm, eyes, optic nerves and lenses showed no significant difference. Target dose homogeneity was slightly better with the clinical plan, however this difference was also statistically insignificant.



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Conclusion: This work demonstrates that KBP can be trained and efficiently utilized to help not only speed up the planning process but also help standardize the treatment plan quality.

Keywords: Brain metastases, radiotherapy, volumetric modulated arc therapy, knowledge-based planning, stereotactic, radiosurgery

INTRODUCTION

Volumetric modulated arc therapy (VMAT) is a radiation treatment planning and delivery technique that has been investigated and clinically applied for all disease sites including malignancies of the head and neck, thorax, abdomen and pelvis, and compared to static field intensity modulated radiation therapy (IMRT), VMAT has been shown to reduce treatment time and monitor units (MU), making it an attractive radiotherapy delivery technique^[1]. Recent reports have established VMAT as a treatment technique for delivering cranial stereotactic radiosurgery (SRS)^[2-4] providing optimal dose distributions while improving the efficiency of treatment delivery. Conventionally, SRS uses one isocenter per lesion and requires multiple isocenters for multiple lesions. This not only prolongs the delivery time reducing patient throughput but is also uncomfortable for the patient. The use of a single isocenter to treat multiple cranial metastases has been accomplished using VMAT producing highly conformal dose distributions while reducing treatment times. Target coverage and dose gradients produced by this technique have been shown to be comparable to Gamma Knife-based SRS^[5,6]. After the initial feasibility studies, reports have been presented outlining further refinement of the procedure, dosimetric indices as well as plan quality parameters for single isocenter VMAT in radiotherapy treatment of multiple cranial metastases^[7,8]. Depending on the complexity of the case however, planning for these cases can be very time consuming and reports have expressed the need to shorten the planning time, thereby aiming to improve the efficiency.

Knowledge-based planning (KBP) is a new paradigm in treatment planning and is a shift towards the direction of automating the planning process^[9,10]. KBP utilizes the dose distributions of prior plans to build a model that can predict the same for new patients. KBP has been effectively utilized to generate high quality treatment plans with consistency utilizing IMRT or VMAT for malignancies at various sites including prostate, lung, liver, head and neck as well as intracranial stereotactic radiotherapy^[11-15]. Although comprehensive studies describing prediction methodologies that make precise estimations of SRS plan quality metrics have been made using in-house systems^[15], clinical application of a commercial KBP system for treatment planning of multiple intracranial lesions with VMAT using a single isocenter has yet to be performed. Recently RapidPlan (TM), which is commercial KBP software from Varian Medical Systems, Palo Alto CA has become available for clinical use. In this study we train a knowledge-based model using RapidPlan for multiple cranial lesions treated at our institution with VMAT. We then retrospectively apply this model to compare the results of previously planned and treated cases.

METHODS

Treatment planning

VMAT plans were generated for 91 patients with a total of 139 lesions (range 1-4, median 1) in Eclipse V13.6 (Varian Medical Systems, Palo Alto, CA USA) from June 2017 to November 2018. Each case was planned with a single isocenter using 3 to 6 arcs. The location of the isocenter was based on the geometric center of the lesions to be treated. The arcs used were non-coplanar. Their distribution in terms of full arcs vs. partial arcs, couch angles, collimator angles were such that they best covered the group of lesions to be treated within that single isocenter. The arcs were chosen to avoid situations where there were two targets in the same leaf track in order to avoid excess dose to the brain. The algorithm used for optimization with VMAT was the progressive resolution optimizer and dose calculation was performed with analytical anisotropic

algorithm using a 1 mm dose calculation grid size. X-ray energy used was 6 flattening filter free (FFF) and dose rate for planning was 1400 MU/min. Contouring of the gross tumor volume (GTV) and the critical structures such as the brain, brainstem, chiasm, optic nerves and tracts, eyes and lenses closely followed previously published guidelines^[7,8]. At our institution, no margin is used to convert GTV to planning target volume (PTV). The range of the PTV was from 0.1-7 cm³. The gradient index (GI) was defined as the ratio of volume covered by the 50% isodose line to that covered by the 100% isodose^[16]. The conformity index (CI) and the homogeneity index (HI) were chosen for plan evaluation. The CI was defined as the ratio of the volume covered by the 100% isodose to the volume covered by the PTV. The HI was taken as the maximum PTV dose to the prescription dose. Dose prescribed in a single fraction was 20 Gy, 18 Gy or 16 Gy and was decided based on the size and volume of the lesion and its proximity to critical organs. The constraints and strategy for optimization was similar to that previously published^[7,8,18,19]. The dose constraints used for planning are shown in [Table 1](#).

KBP with RapidPlan

RapidPlan is a treatment planning application developed by Varian medicalsystems that utilizes a knowledge-based approach. Previously accepted clinical plans are taken from which data are extracted which include the volumes of the OARs and PTVs, percentage of the overlap volume for each OAR with the target, percentage of the OAR volume that is out of the field for each OAR, prescription dose, structure dose-volume histogram (DVH) and geometry based expected DVH for each OAR. The geometry based expected dose is a measure of dose received by a portion of an OAR if only the patient anatomy and desired target dose are to be considered. Principle component analysis is conducted on this extracted data and the principle components are used to build a DVH estimation model^[17]. When a treatment plan is to be generated for a new patient, the RapidPlan model will create DVH estimates for the OARs based on the anatomy for that particular case. These DVH estimates will then be used as part of the objectives for optimization to achieve the dosimetric goals for that patient.

Model training

To create the RapidPlan DVH estimation model, we selected 66 patients with 125 lesions (range 1-4, median 1). The model was trained for multiple target dose levels, namely high risk, intermediate risk and a low risk PTV. These levels of risk for the PTVs were matched accordingly in both the training and validation. For all single lesion cases, the plan had only 1 target, which was matched to high dose level. If the plan had 2 targets that were prescribed to the same dose level, then they both were matched to the highest dose level. However, if they went to different dose levels, the target receiving higher prescription was matched to the higher dose level and the one receiving the next dose level was matched to the intermediate dose. Similarly if a plan had 3 targets, each prescribed to different dose levels, the targets were matched correspondingly, i.e., high to high, intermediate to intermediate and low to low. However if they went to 2 different dose levels, the target(s) with the highest prescription dose would be matched to high and the target(s) with the next dose level would be matched to intermediate dose level. Similarly if all the 3 went to the same dose level, all of them would be matched to high dose. This methodology of matching is recommended by the training software. The OARs included for training were brain, brainstem, chiasm, optic nerves, eyes and lenses. The training process consisted of identifying the geometric outliers and the dosimetric outliers. The geometric outliers are typically cases where the PTV and/or OAR volumes, shapes and overlaps differ substantially from the majority of the training set. Dosimetric outliers are cases where the clinical DVH differs substantially from the estimated DVH. Geometric outliers can be identified using regression plots, which illustrate the correlation between the best prediction of the DVH and the most likely geometric parameter that would be responsible for that DVH such as the volume of the structure or the overlap with the target or a combination of both. Geometric outliers are points that are typically identified as points that fall far away from the regression line or that are substantially isolated from it. Similarly, dosimetric outliers can be identified using something called residual plots that correlate the best estimation of the DVH to the actual clinical DVH for

Table 1. Table summarizing dosimetric criteria

Structure	Parameter	Objective
PTV	D_{max} (%)	$\leq 125\%^*$
	D_{min} (%)	$\geq 90\%^*$
	V_{100} (%)	$\geq 98\%^*$
Brainstem	D_{max} (Gy)	$\leq 13\text{ Gy}^*$
Brain	$V_{7\text{Gy}}$ (%)	$\leq 5\%^*$
	$V_{12\text{Gy}}$ (cm ³)	$\leq 8\text{ cm}^3^*$
	$V_{10\text{Gy}}$ (cm ³)	$\leq 10\text{ cm}^3^*$
Optics	D_{max} (Gy)	$\leq 8\text{ Gy}^*$
Lens	D_{max} (Gy)	$\leq 8\text{ Gy}^*$

*Signifies used as limiting constraints, while the others are used as guidelines. D_{max} (%) is the maximum dose to the structure in %, D_{min} (%) is the minimum dose to the structure in %, V_{100} (%) is the volume of the structure in % that receives 100% of the prescription dose. D_{max} (Gy) is the maximum dose to the structure in Gy and $V_{x\text{Gy}}$ (%) is the % volume of the structure receiving X Gy, and $V_{y\text{Gy}}$ (cm³) is the absolute volume of the structure receiving Y Gy. At our institution, the maximum dose constraint to the brainstem is used only if target overlaps with the brainstem. PTV: planning target volume

the training case. A case is considered to be a dosimetric outlier when the clinical DVH differs substantially from the DVH estimated by the model. By removing the geometric and dosimetric outliers, and re-training the model, the ability of the model to estimate the DVHs is measured. A minimum of 20 patients/cases are required to successfully train the model.

Optimization objectives

Once the model has been trained and reviewed, optimization objectives are added to the model. It is critical to mention at this point that these estimation models can predict achievable OAR DVHs for individual patient anatomy; however optimization objectives are required to achieve these dosimetric goals. These dose-volume objectives and the priorities are estimated by the model for the selected structure and are automatically loaded when the model is selected to create a plan for a new patient.

Model validation

Ten patients were chosen for validation. Among the 10 patients, 6 had 1 lesion each, 2 had 2 lesions each, 1 had 3 lesions and 1 had 4 lesions. The validation process consists of comparing the clinical DVH to that obtained from the plan using the model and then evaluating the plan for clinical acceptability. The dosimetric parameters extracted with respect to target coverage were maximum dose to the PTV, minimum dose to the PTV, volume of the PTV covered by 100% of the prescription dose, i.e., PTV V_{100} , GI, CI and HI, all of which have been defined earlier. With respect to the critical organs, the dosimetric parameters noted were the maximum dose to the brainstem, brain, chiasm, optic nerve, eyes and lenses. The $V_7\text{ Gy}$, $V_{12}\text{ Gy}$ and $V_{10}\text{ Gy}$ to the brain were also noted. Significance testing between the dosimetric results of the clinical plan vs. the plan generated by the model, i.e., the validation plan was performed by using the Wilcoxon sign rank test, which is a non-parametric test for matched pairs of data.

RESULTS

Although 91 patients with a total of 139 lesions were planned, we could only use 66 of them with a total of 125 lesions due to limitations in Eclipse V 13.6 as some of the target volumes were too small to be extracted. Comparison of dosimetric parameters for target coverage between the original clinical plan and the corresponding knowledge-based validation plan using RapidPlan are shown in Table 2. Similarly in Table 3, is the comparison of the dosimetric parameters for OAR sparing between the original clinical plan and the validation plan. In Table 4, for the validation cases, the diameter and volume of each lesion treated in a single radiotherapy session is indicated. For multiple lesion cases, i.e., 2.3 and 4 lesions, the total volume of all lesions treated as well as the maximum diameter is indicated. In Table 5, is shown the distribution of the lesions to the various risk levels. Each validation plan was generated within 30 minutes without human

Table 2. Comparison of dosimetric parameters for target coverage between the original clinical plan and the corresponding knowledge based plan, i.e., Rapidplan. Wilcoxon sign-rank test shows that the dosimetric results with respect to target coverage show no statistically significant difference between the two planning techniques

Structure	Parameter	Clinical plan	KBP	P value
PTV	D_{max} (%) = HI	118% ± 3.4%	120% ± 2.6%	0.05
	D_{min} (%)	97% ± 2.8%	97% ± 3.3%	0.86
	V_{100} (%)	100% ± 0.8%	100% ± 1.1%	1.00
	CI	1.5 ± 0.2	1.5 ± 0.2	1.00
	GI	4 ± 0.6	4.3 ± 0.7	< 0.01

CI: conformity index; GI: gradient index; HI: homogeneity index; KBP: knowledge based plan; PTV: planning target volume

Table 3. Comparison of dosimetric parameters for critical organs between the original clinical plan and the corresponding knowledge based plan, i.e., Rapidplan. Wilcoxon sign-rank test shows that the dosimetric results with respect to target coverage show no statistically significant difference between the two planning techniques

Structure	Parameter	Clinical plan	KBP	P value
Brainstem	D_{max} (Gy)	2.2 ± 3.7	2.2 ± 3.5	1.00
Brain	D_{max} (Gy)	20.8 ± 1	21.2 ± 1	0.02
	V_{7Gy} (%)	0.7% ± 0.8%	0.7% ± 0.8%	1.00
	V_{12Gy} (cm ³)	3.5 ± 2.8	4 ± 3.4	0.13
	V_{10Gy} (cm ³)	5.2 ± 3.9	5.9 ± 4.9	0.16
	Mean (Gy)	0.6 ± 0.3	0.7 ± 0.4	0.06
Chiasm	D_{max} (Gy)	0.5 ± 0.5	0.5 ± 0.5	0.63
Eye	D_{max} (Gy)	0.3 ± 0.5	0.4 ± 0.5	0.30
Optic nerve	D_{max} (Gy)	0.3 ± 0.4	0.3 ± 0.4	0.38
Lens	D_{max} (Gy)	0.2 ± 0.2	0.2 ± 0.2	0.13

CI: conformity index; GI: gradient index; KBP: knowledge based plan

Table 4. Table showing the diameter and volume of each lesion treated in a single radiotherapy session for each of the validation cases. For multiple lesion case i.e., 2,3 and 4 lesions, the total volume of all lesions treated as well as the maximum diameter is indicated

Case	No. of lesions	Diameter (cm)	Volume (cm ³)
Case 1	1	1	3.9
Case 2	1	0.5	0.4
Case 3	1	0.4	0.2
Case 4	1	0.4	0.3
Case 5	1	0.9	2.7
Case 6	1	0.9	3.2
Case 7	2	0.5	0.5
Case 8	2	0.4	0.3
Case 9	3	0.7	1.7
Case 10	4	0.6	0.7

Table 5. Table showing the distribution of lesions to the various risk levels

Case	No. of lesions	Prescription (Gy)	Dose level assignment
Case 1	1	20 Gy	High dose level
Case 2	1	18 Gy	High dose level
Case 3	1	18 Gy	High dose level
Case 4	1	18 Gy	High dose level
Case 5	1	18 Gy	High dose level
Case 6	1	18 Gy	High dose level
Case 7	2	All 18 Gy	High dose level
Case 8	2	All 18 Gy	High dose level
Case 9	3	All 18 Gy	High dose level
Case 10	4	All 18 Gy	High dose level

intervention. A comparison of the dose distributions between the clinical plan and the KBP plan for a single lesion, 2 lesion and a 4 lesion case are shown in [Figures 1-3](#) respectively. A comparison of the DVHs for the

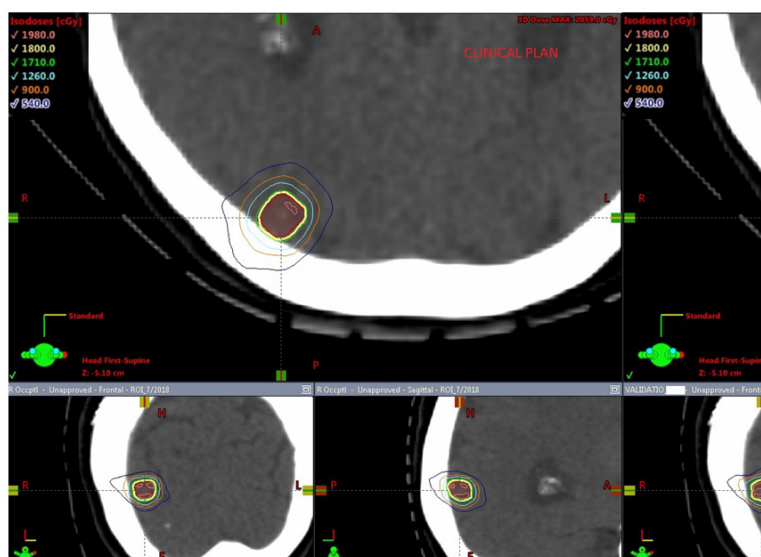


Figure 1. Comparison of dose distribution in the axial, coronal and sagittal views for a clinical plan vs. a RapidPlan for a single lesion case

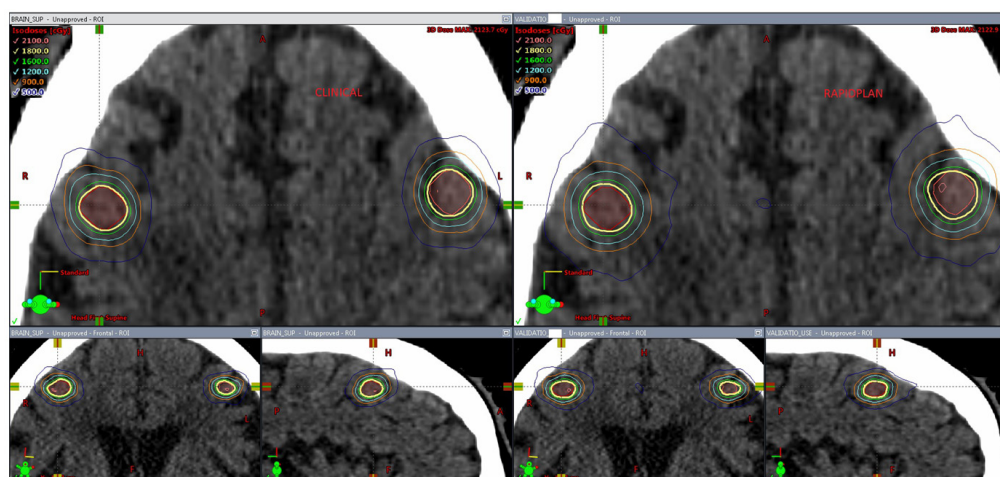


Figure 2. Comparison of dose distribution in the axial, coronal and sagittal views for a clinical plan vs. a RapidPlan for a 2 lesion case



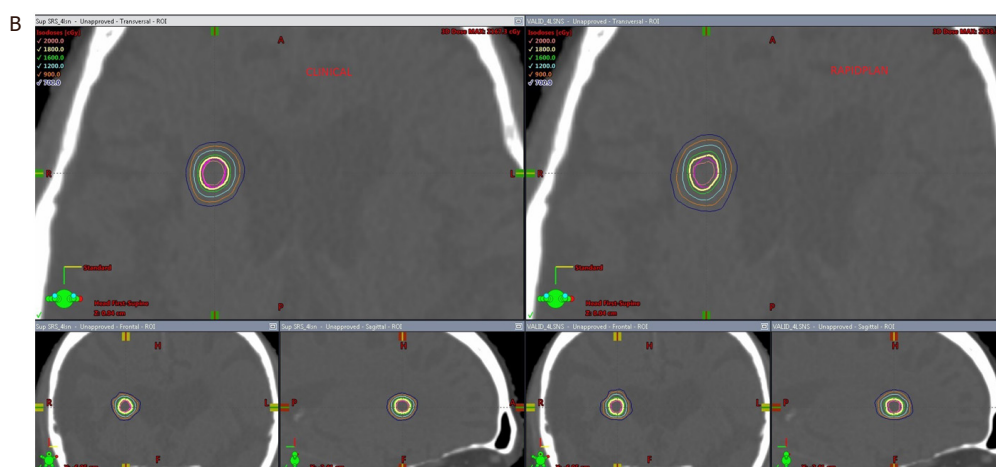


Figure 3. A: Comparison of dose distribution in the axial, coronal and sagittal views for a clinical plan vs. a RapidPlan for a 4 lesion case (first 3 lesions seen in this figure); B: comparison of dose distribution in the axial, coronal and sagittal views for a clinical plan vs. a RapidPlan for a 4 lesion case (4th lesion seen in this figure)

PTV and the critical organs for the clinical plan vs. the RapidPlan for one of the single lesion cases is shown in [Figure 4](#).

PTV coverage

The PTV coverage in terms of PTV V100, the minimum dose to the PTV showed no statistically significant difference between the clinical plan vs. the RapidPlan. Both the clinical and the RapidPlan showed no statistically significant difference in the CI. The GI was slightly higher with the RapidPlan compared to the clinical plan. The maximum dose to the PTV was higher by 2% using KBP. However these hotspots were retained within the target volume.

OAR sparing

Dose constraints for the brainstem, brain, chiasm, optic nerves, eyes and lenses were all achieved as per [Table 1](#) for both the clinical plan as well as the KBP. No statistically significant differences were seen in the dosimetric parameters to the majority of these critical structures.

Monitor units (MU) and calculated treatment time

The total MU for the original clinical plan on average were $5,215 \pm 924$, while with RapidPlan were 5503 ± 1208 . This difference was not found to be statistically significant ($P = 0.5$).

DISCUSSION

Studies have shown that LINAC based radiosurgery plans using VMAT can produce target coverage and dose fall-off in the high dose area similar to Gamma-Knife plans^[5,6]. Single isocenter cranial VMAT radiosurgery technique can produce with the major advantage being improvement in clinical efficiency. The use of FFF beams with a high dose rate of delivery at 1400 MU/min has further contributed towards to this goal. As the single isocenter VMAT technique replaces the use of multiple isocenter techniques for multiple targets, there is also a need to improve the efficiency of clinical treatment planning for these cases. Although the concept of knowledge-based planning with in-house systems has been applied to predict plan quality metrics in intracranial SRS^[12], our study has demonstrated the use of RapidPlan, which is a commercial system for automated planning of intracranial SRS. Our results indicate that both the clinical as well as the validation plan (RapidPlan) showed no significant difference with respect to target coverage, conformity index, gradient index, homogeneity as well as critical organ sparing.

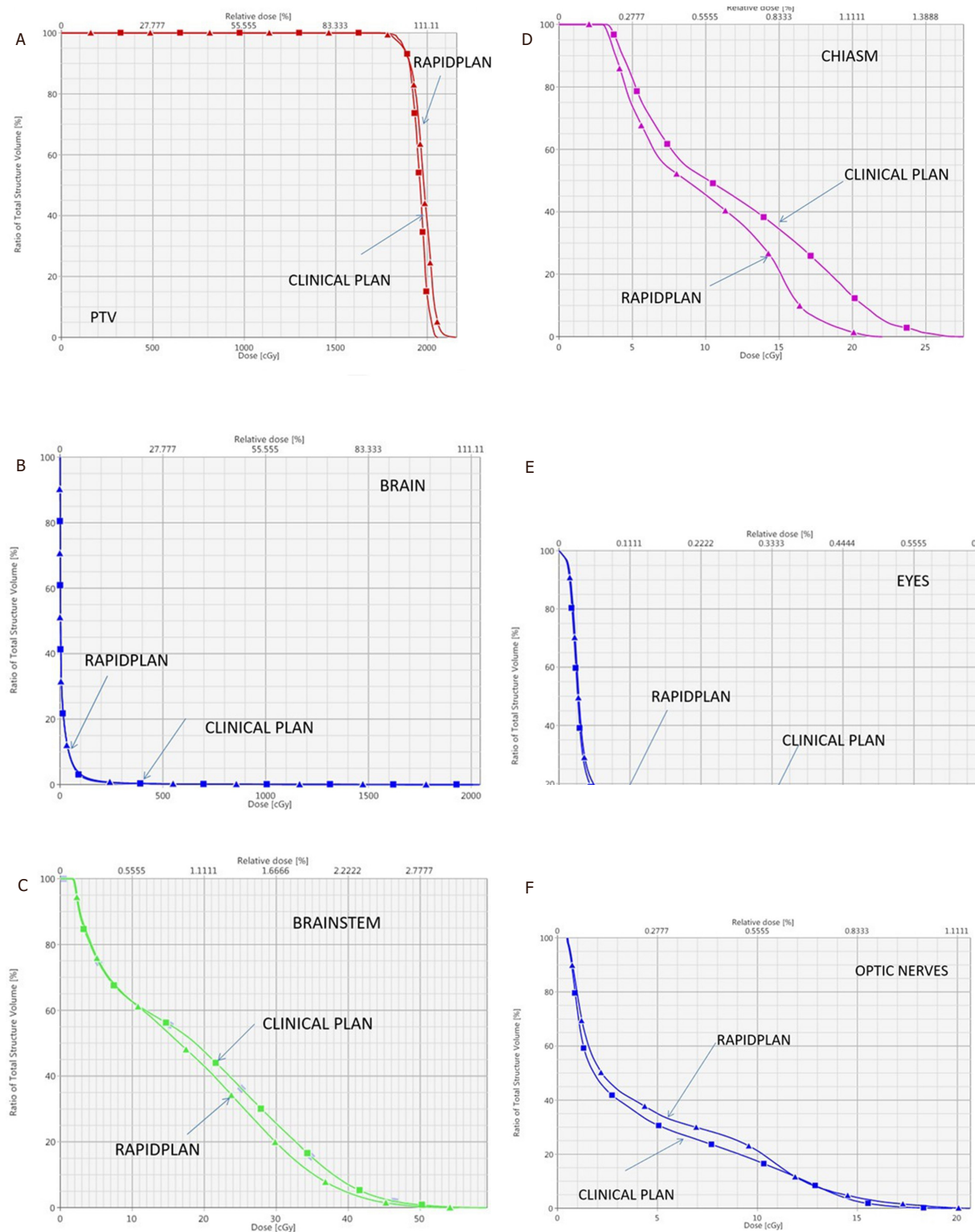


Figure 4. A: DVH comparison of target coverage for clinical plan vs. RapidPlan for the single lesion case shown in Figure 1; B: DVH comparison for the brain between the clinical plan vs. RapidPlan for the single lesion case of Figure 1; C: DVH comparison for the brainstem between the clinical plan vs. RapidPlan for the single lesion case of Figure 1; D: DVH comparison for the chiasm between the clinical plan vs. RapidPlan for the single lesion case of Figure 1; E: DVH comparison for the eyes between the clinical plan vs. RapidPlan for the single lesion case of Figure 1; F: DVH comparison for the optic nerves between the clinical plan vs. RapidPlan for the single lesion case of Figure 1. DVH: dose-volume histogram

All the cases for training and validation were chosen without any pre-selection criteria. The limitation of this study is that it was trained and tested only with a maximum of 4 lesions per case. This was because in the cohort of patients that were treated, there were only 3 cases that had > 4 lesions, which essentially is not enough to train a model. As we treat and acquire data on more patients that have > 4 lesions, these can be incorporated into the model. Another limitation of this study is that the cases used for training were of smaller volume and were typically peripheral and far away from critical organs as well as from each other. This model therefore cannot be used for lesions that are overlapping with critical organs or for lesions in close proximity of each other.

The plans were slightly more inhomogeneous compared to the original clinical plans. This could be due to the fact that we used a line objective for the DVH in addition to the maximum point dose objective for the critical organ. Although the DVH for the PTV shows a longer tail, the dose inhomogeneity is contained within the target. We anticipate that with more training cases, the dosimetric results will improve. The GI was also slightly higher with the KBP plans than the recommended value of < 4. However the plans were dosimetrically very similar. Moreover, when a typical clinical plan would take at least 2 to 3 h to complete, the RapidPlan gave a clinically acceptable result in under 30 min.

The quality of the treatment plan generated by RapidPlan can only be as good as that of the treatment plans used to generate the DVH estimation model. Other technologies such as Hyperarc® are available from Varian Medical Systems that can automate SRS planning, however, they are currently unavailable with RapidPlan. This study demonstrates the feasibility of using RapidPlan as it pertains to a limited number of lesions (≤ 4). Going forward, we plan on expanding its training and application to > 4 lesions. In the future we plan to expand this to > 4 lesions and perform a comparison with plans generated using Hyperarc.

In conclusion, we have developed an efficient method for treatment planning of multiple cranial SRS lesions using VMAT and a single isocenter. This is a step not only towards a reducing the treatment planning time but also providing the planner a guide on the achievable dose distribution for the given case, thereby helping to standardize the quality of the treatment plans.

DECLARATIONS

Authors' contributions

Built the model, studied design, performed the dosimetric and statistical analysis: Dumane VA

Planned the clinical cases that were used for training and testing while building the model: Tseng TC

Designed the database used for obtaining patient treated with SRS using VMAT on Eclipse planning system: Sheu RD

Provided input towards study design and performed statistical analysis: Lo YC

Contributed towards writing the manuscript: Dumane VA, Tseng TC, Sheu RD, Lo YC, Gupta V, Saitta A, Rosenzweig KE, Green S

Participated in the study design, contouring of all the target volumes and critical organs, reviewing plans: Green S

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Autophagy in breast cancer metastatic dormancy: tumor suppressing or tumor promoting functions?

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Abstract

Breast cancer is the second leading cause of cancer-associated death in women in the United States, with more than 90% of those deaths attributed to metastasis. Breast cancer metastasis is incurable and possesses few treatment options and a poor overall prognosis due in part to confounding metastatic attributes, particularly the acquisition of dormancy-associated phenotypes. Dormant disseminated tumor cells can persist for years-to-decades before recurring as highly aggressive, secondary lesions. Dormancy-associated phenotypes are exhibited by breast cancer stem cells (BCSCs), which undergo tumor initiation and unlimited self-renewal. In addition to their specialized abilities to circumvent chemotherapeutic insults, BCSCs also upregulate autophagy during metastatic dormancy as a means to survive in nutrient poor conditions and environmental stress. As such, therapeutic targeting of autophagy is actively being pursued as an attractive strategy to alleviate metastatic disease and the recurrence of dormant BCSCs. Here we review the molecular and cellular features of autophagy, as well as its paradoxical role in both suppressing and promoting mammary tumor development and metastatic progression. Finally, we highlight the clinical challenges associated with therapeutic targeting of autophagy in metastatic breast cancers.

Keywords: Autophagy, breast cancer, cancer stem cells, metastatic dormancy, metastatic relapse

INTRODUCTION

Breast cancer is the second deadliest malignancy in women, accounting for nearly 41,000 deaths in the United States in 2018^[1]. More than 90% of the deaths attributed to breast cancer are caused by metastasis, a disease state associated with poor prognosis and little-to-no effective treatment options^[2]. Indeed, while initial treatment of breast cancers can be effective and achieve remission, an estimated 30% of lymph



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node-negative and 70% of lymph node-positive breast cancer patients will eventually relapse 5-20 years following initial diagnosis^[3,4]. The period of time between clinical remission and relapse can be attributed to dormancy, a process whereby disseminated tumor cells (DTCs) enter a non-proliferative state coupled with the activation of cellular stress programs^[5]. Even in the earliest stages of mammary tumor development, breast cancer cells are actively shed from the growing tumor and traverse the metastatic cascade before colonizing distant metastatic sites^[6,7]. These solitary micrometastases can persist in distant organs for years or even decades before emerging as recurrent metastatic tumors. Indeed, experimental evidence and *in silico* modeling indicate that dormant DTCs exist in a quiescent state as opposed to one that reflects a balance between cell proliferation and apoptosis^[8-12]. Dormant cells upregulate pro-survival factors and are inherently chemoresistant given their non-proliferative state. As such, treatment with currently available therapeutics does little to limit the population of dormant cells in breast cancer patients. In fact, ~62% of breast cancer-associated deaths occur 5 years following diagnosis^[13]. As such, the clinical detection and treatment of these recurrent metastases remains challenging due to: (1) difficulties in detecting growing lesions years or decades following remission; (2) limited treatment options that are effective against metastatic disease^[14,15]. Despite the fact that systemic relapse following a period metastatic dormancy remains a large unmet clinical burden, the precise mechanism(s) that enable dormant metastatic lesions to reactivate proliferative programs and recur remains incomplete^[3]. Here we highlight the importance of breast cancer stem cells (BCSCs) and their reliance upon autophagy to govern the activation and eventual emergence from metastatic dormancy, as well as clinical implications of targeting autophagy therapeutically as a means to alleviate metastatic disease.

BCSCS AND METASTATIC DORMANCY: A ROUTE TO EVADE DETECTION AND THERAPEUTIC ELIMINATION

Recent evidence suggests that DTCs endowed with the ability to survive metastatic dormancy and initiate recurrent metastatic lesions are BCSCs^[16-18], which undergo unlimited self-renewal and contribute to tumor initiation^[19]. Likewise, genomic analyses of primary and relapsed metastatic breast cancers reveal numerous common driver mutations shared between primary and metastatic tumor lesions in a given patient. As such, these common mutational landscapes implicate the presence of a common malignant cell of origin and support the notion that disseminated BCSCs initiate recurrent metastatic lesions years or decades following clinical remission^[20-23]. This process reflects the ability of BCSCs to adopt dormancy-associated phenotypes through several malleable events, including modulation of E-cadherin and lncRNA expression^[24,25]. Equally important facets of metastatic relapse are the capacity of BCSCs to evade immune surveillance and resist therapeutic interventions aimed at eradicating residual disease. Amongst the pro-survival strategies activated by BCSCs are: (1) upregulated expression of ATP-binding cassette transporters that mediate cellular efflux of chemotherapeutic agents^[26-28]; (2) increased production of Interleukin-4 (IL-4) to suppress apoptosis^[29]; (3) enhanced generation of reactive oxygen species in response to radiation^[30]; (4) elevated activation of autophagy^[16-18,31] [Figure 1]. As such, dormant BCSCs are inherently resistant to traditional chemotherapeutic agents and radiation that target rapidly dividing tumor cells. In the succeeding sections, we highlight the role of autophagy in regulating mammary tumorigenesis and dormancy-associated phenotypes during metastatic progression and relapse.

CONTEXT-DEPENDENT ROLE OF AUTOPHAGY IN TUMOR PROGRESSION

Macroautophagy (hereafter referred to as autophagy) is a highly conserved process that maintains cellular homeostasis through the lysosomal degradation of proteins and organelles, a phenomenon that is tightly controlled by autophagy-related genes (ATGs)^[32]. The autophagosome cargo protein, p62/sequestosome 1 (SQSTM1), binds to degradation targets and facilitates selective autophagy^[33]. Indeed, during the activation of autophagy, ATGs mediate the recycling of p62/SQSTM1-tagged cargo through the formation of double-membrane vesicles, termed autophagosomes, which fuse with lysosomes to form autophagolysosomes. Lysosomal fusion facilitates the degradation of nonfunctional cellular components and also functions

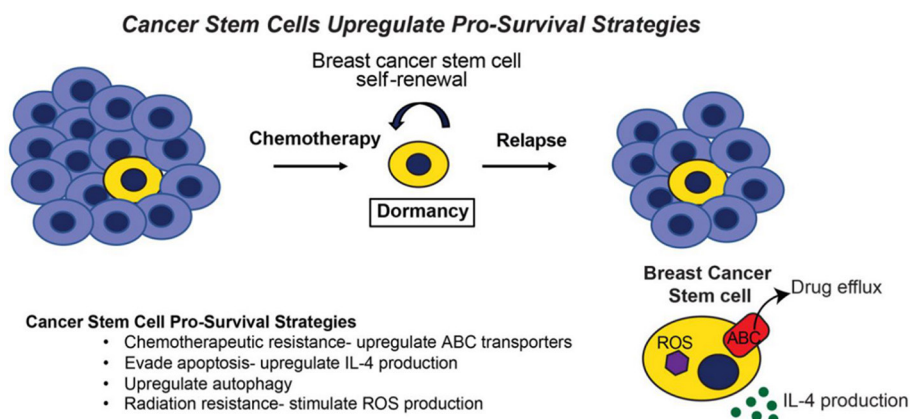


Figure 1. Cancer stem cells upregulate pro-survival strategies. Early in mammary tumor development, breast cancer cells are shed and disseminated from the growing lesion, ultimately colonizing distant metastatic sites before clinical detection of a primary breast tumor. Upon breast cancer diagnosis, neoadjuvant chemotherapy in conjunction with surgical resection, or more traditionally, surgery followed by adjuvant chemotherapy are both effective in eliminating the bulk the primary tumor cells. In contrast to bulk tumor cells, breast cancer stem cells manage to survive chemotherapeutic treatment by upregulating a number of pro-survival strategies, thereby contributing to metastatic relapse following a period of remission and dormancy. In doing so, cancer stem cells can (1) upregulate ABC transporter expression, which evades the cytotoxic activities of chemotherapies; (2) enhance IL-4 production, which inhibits apoptosis; (3) activate autophagy; (4) induce ROS production, which confers resistance to radiation. In addition, breast cancer stem cells also evade apoptosis by lying dormant for years or even decades, a pathophysiological state that further protects these cells from the cytotoxic activities of chemotherapy and radiation, and from the apoptotic activities engendered by metabolic, hypoxic, and environmental stressors

to meet the energy demands of a cell in periods of environmental stress^[32,34] [Figure 2]. Recent basic and clinical research findings have highlighted the context-dependent role of autophagy in regulating tumorigenesis. Indeed, in the earliest stages of tumor growth and development, autophagy functions as a tumor suppressor, thereby limiting tumor growth. However, once primary tumors or their metastases are established, autophagy can promote tumorigenesis by subverting stress responses, and consequently, facilitating tumor cell survival and disease progression^[35] [Figure 3]. At present, a thorough understanding of the molecular mechanisms that enable autophagy to both suppresses or promote mammary tumorigenesis is lacking, as are cell- and context-specific signals that underlie the paradoxical functions of autophagy in breast cancers. Future studies need to address these important questions as a means to uncover novel therapeutic strategies aimed at modulating autophagy in patients with metastatic breast cancer.

AUTOPHAGY AND TUMOR SUPPRESSION

Anecdotal evidence indicates that autophagy can act as a barrier to prevent tumor initiation in a number of solid tumors, including those of the breast. For instance, autophagy is readily induced by the tumor suppressors PTEN and p53, while their inactivation in developing neoplasms inhibits autophagy, as does oncogenic activation of PI3K/AKT and BCL2^[36]. Likewise, monoallelic deletion of the autophagy regulator, beclin-1, is observed in 40%-75% of breast and ovarian human tumors^[37], suggesting that autophagy functions to suppress tumor initiation. Accordingly, genetic inactivation of beclin-1 in mice predisposes their development of a variety of tumors, findings consistent with the notion that autophagy regulates cellular homeostasis and prevents tumor initiation^[37-39]. Furthermore, activation of the transcription factor NRF2 elicits deregulation of autophagy due in part to aberrant accumulation of p62/SQSTM1 that can promote tumor formation^[40-42]. Indeed, under tonic conditions, NRF2 interacts with Keap1, which targets NRF2 for ubiquitin-mediated degradation. The interaction between Keap1 and NRF2 can be prevented by the accumulation of p62/SQSTM1, thereby: (1) inhibiting the activation of autophagy; (2) stabilizing NRF2 expression, leading to its transcriptional activation^[40-43]. Finally, emerging evidence suggests a role for autophagy in maintaining genomic integrity, as metabolic stress induced by loss of autophagy can promote DNA damage and chromosomal instability^[44]. Indeed, when confronted with DNA damage, autophagy-

Autophagy: A Pro-Survival Strategy Employed by Breast Cancer Stem Cells

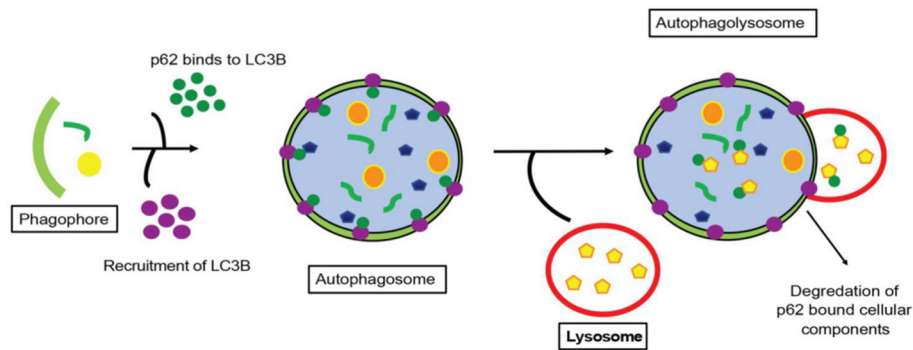


Figure 2. Autophagy: a pro-survival strategy employed by breast cancer stem cells. One of the pro-survival strategies employed by breast cancer stem cells during their acquisition of dormant states is autophagy, which facilitates the recycling of damaged or unnecessary organelles and/or proteins as a means to provide energy during periods of metabolic stress. Upon initiation of autophagy, the phagophore encircles those cellular contents targeted for autophagic degradation. LC3 is recruited to the phagophore and subsequently binds to the cargo adaptor protein, p62/SQSTM1. Upon doing so, a double membrane structure called the autophagosome forms and encircles cellular candidates for autophagic degradation. Subsequently, the autophagosome binds to the highly acidic lysosome to form the autophagolysosome, wherein p62/SQSTM1 bound cellular contents are degraded

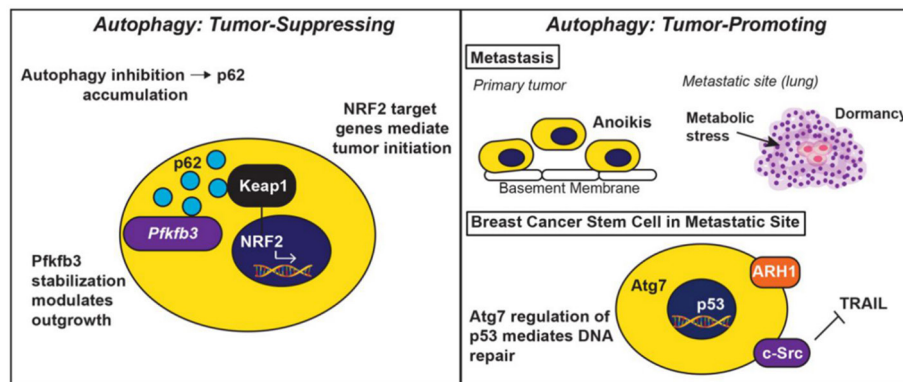


Figure 3. The tumor-suppressing and tumor-promoting activities elicited by autophagy. Autophagy functions to suppress tumor initiation (left panel), as well as to promote tumor development and progression (right panel). In early stages of tumor formation or during periods of metastatic dormancy, autophagy is tumor suppressive. Upon autophagy inhibition, p62/SQSTM1 accumulates and stabilizes Pfkfb3, leading dormant breast cancer stem cells to initiate metastatic relapse. Additionally, p62/SQSTM1 also inhibits the interaction between Keap1 and NRF2, thereby preventing NRF2-mediated expression of genes operant in tumor initiation (left panel). In stark contrast, autophagy provides established tumors with pro-survival phenotypes, including protection from anoikis and intrinsic cellular stressors encountered during metastatic dormancy. Likewise, autophagy protects breast cancer stem cells by ensuring for their resistance to the apoptotic stimuli housed within the metastatic microenvironment (e.g., Src-mediated TRAIL resistance), and to chemotherapeutic insults (e.g., Atg7-mediated p53 regulation of DNA repair). Finally, dormant cells can upregulate ARH1 to induce autophagy and promote the activation of pro-survival signaling systems that ensure for their survival

deficient cells exhibit diminished homologous recombination (HR) repair of damaged DNA that arises due to proteasomal degradation of checkpoint kinase 1 (Chk1)^[45,46]. While non-homologous end joining (NHEJ) appears to be largely unaffected by autophagy inhibition, the diminished HR proficiency in these cells can render them more sensitive to DNA damage, especially if NHEJ is subsequently impaired^[45,46]. Collectively, these findings identify important mechanisms whereby autophagy functions to suppress malignant transformation and tumor development.

AUTOPHAGY AND TUMOR PROMOTION

In contrast to its tumor suppressing functions, autophagy can also serve as a tumor promoting process, particularly by: (1) enhancing the ability of DTCs to traverse the metastatic cascade; (2) inhibiting

immunosurveillance by tumor infiltrating immune cells. During metastasis, cells shed from the primary tumor must invade through the extracellular matrix, intravasate into blood vessels, survive the turbid flow of the vasculature, extravasate, and finally colonize a distant metastatic site^[47]. Not surprisingly, cells traversing the metastatic cascade experience a variety of cellular stressors and vastly different tissue microenvironments, including changes in the (1) composition of the extracellular matrix composition; (2) availability of nutrients due to alterations in vascular and lymphatic networks; (3) biomechanical properties of metastatic sites; (4) tumor immunosurveillance programs^[48-50]. Importantly, autophagy activation protects DTCs during periods of metabolic stress encountered by anoikis and entry into foreign microenvironments^[51-53], and by bouts of dormancy at distant metastatic sites^[54,55].

AUTOPHAGY INHIBITS TUMOR IMMUNOSURVEILLANCE

Tumor immunosurveillance is a critical physiological process that inhibits the development and progression of mammary tumors. Accumulating data in the literature indicate that tumor immunosurveillance programs are an all-encompassing system that involves not only the adaptive immune system and cytotoxic effector pathways, but also the release of a complex set of cytokines and chemokines that coalesce to prevent tumor development^[50]. Although the molecular mechanisms used by tumor cells to escape immunosurveillance are varied, recent evidence has implicated a role for autophagy in mediating this phenomenon. Indeed, autophagy activation has been shown to inhibit immune cell killing of tumor cells as a means to promote escape from immunosurveillance and DTC outgrowth^[50]. In doing so, autophagy activation can target the activation of Signal Transducer and Activator of Transcription 3 (STAT3), a gene commonly dysregulated in breast cancer that also plays a prominent role in regulating the immune system^[56]. For instance, the activation of autophagy can induce the phosphorylation and stimulation of STAT3 in tumors, an event that initiates cellular cross-talk between tumor and immune cells that ultimately suppresses Cytotoxic T Lymphocyte (CTL)-mediated lysis of tumor cells^[56,57]. Likewise, autophagy can inhibit natural killer cell (NK)-mediated tumor cell killing by degrading granzyme B, a serum protease that is released by NK cells during NK-mediated cell killing^[58,59]. Finally, breast cancer development and metastatic progression is critically dependent upon Epithelial-Mesenchymal Transition (EMT) programs^[60]. Interestingly, autophagy is activated as carcinoma cells traverse the EMT program, with the resulting post-EMT mesenchymal-like cells exhibiting elevated levels of autophagy relative to their pre-EMT epithelial-like counterparts. Importantly, EMT-mediated activation of autophagy inhibits CTL-mediated antitumor immunosurveillance in a beclin-1-dependent manner^[50,61-63]. Collectively, these studies highlight the tumor intrinsic and extrinsic functions of autophagy, with the latter function, fulfilling an essential role in governing the fidelity of tumor immunosurveillance mechanisms.

DORMANT BCSCS AND AUTOPHAGY ACTIVATION

BCSCs can lie dormant for decades before recurring as metastatic lesions in breast cancer patients. During this time, disseminated BCSCs must survive nonpermissive tumor environments, while simultaneously maintaining their viability and the capacity for tumor initiation^[5,64]. Emerging evidence implicates autophagy as an essential feature in maintaining the phenotypes associated with BCSCs, particularly their resistance to chemotherapies and hypoxic microenvironments^[16,18,54,55,65-67]. The mechanisms whereby autophagy promotes BCSC survival at metastatic sites are varied and include the ability to confer resistance to apoptotic stimuli (e.g., Src-mediated TRAIL resistance in bone metastases^[68]), to chemotherapeutic insults (e.g., DNA repair via Atg7 and p53 by Atg7^[69]), and to cellular stressors^[70]. Similarly, aberrant expression of ARHI (aplasia Ras homolog member 1) can elicit autophagy activation and modulate the survival of dormant cells in preclinical models of ovarian cancer, further implicating autophagy as an essential mediator of dormant cell survival^[71]. Finally, tumor cells that possess defects in autophagy readily accumulate p62/SQSTM1, an event that alters p62/SQSTM1 function and contributes to tumorigenesis^[17]. The aforementioned studies highlight the oncogenic and pro-survival activities of autophagy that contribute to tumor progression, as well as the

acquisition and eventual emergence from metastatic dormancy. In the succeeding sections, we discuss the implications of targeting the dichotomous roles of autophagy in clinical settings.

CLINICAL TARGETING AND MODULATION OF AUTOPHAGY

Numerous clinical trials have aimed with varying degrees of success to inhibit or stimulate autophagy as a potential cancer therapeutic^[35]. Despite significant investments in preclinical and clinical investigations, no FDA-approved drugs designed to modulate autophagy have been approved for the treatment of primary or metastatic breast cancers. This clinical deficit reflects the challenges associated with the dichotomous roles played by autophagy during mammary tumor development and metastatic progression, and with the inability of science and medicine to fully appreciate the downstream consequences of autophagy modulation in metastatic disease settings.

INHIBITORS OF AUTOPHAGY

At present, nearly 32 human clinical trials have been undertaken to assess the efficacy of autophagy modulating agents [Table 1], either administered alone or in combination with standard-of-care chemotherapeutics (www.clinicaltrials.gov). Pharmacological inhibition of autophagy in clinical settings is primarily accomplished using chloroquine, or a closely related molecule, hydroxychloroquine. Chloroquine functions to block autophagosome-lysosome fusion by preventing the acidification of the lysosome, thus inhibiting autophagy^[72,73]. While the vast majority of studies include either chloroquine or hydroxychloroquine in combination with standard-of-care regimens, one recent study utilized a novel proteasome inhibitor, MLN9708, as a means to assess the impact of autophagy in conferring breast cancer resistance to the cytotoxic activities of doxorubicin^[74]. Interestingly, administration of MLN9708 to breast cancer cells resulted in autophagy activation in a manner paralleling previous connections between proteasomal inhibitor and autophagy^[75,76]. Moreover, MLN9708 enhanced the sensitivity of breast cancer cells to doxorubicin in a manner that was inversely correlated with the extent of autophagy activation^[74]. As such, future studies need to assess the effectiveness of combining proteasomal and autophagy inhibitors with cytotoxic chemotherapies (e.g., doxorubicin).

Additional translational insights into how autophagy inhibition impacts cancer cell survival has been accomplished using a combination of pharmacologic (e.g., chloroquine and/or hydroxychloroquine) and genetic (e.g., knockdown of autophagy associated genes) approaches. In general, these studies support the concept that inactivation of autophagy limits the development and spread of human cancers. Interestingly, recent evidence indicates that the molecular mechanisms underlying the cytotoxic activities of chloroquine and hydroxychloroquine are distinct from those employed to inhibit autophagy. Indeed, induction of lysosomal membrane permeabilization was insufficient to elicit apoptosis in cells treated with chloroquine. Rather, the cytotoxic activities of chloroquine were found to manifest subsequent to mitochondrial membrane permeabilization^[77], and to reduced expression and activity of JAK3 and DNMT1^[78]. Precisely how these alternative targets and activities attributed to chloroquine contribute to its clinical successes and failures remains an important line of research in the field of autophagy modulation.

STIMULATORS OF AUTOPHAGY

In light of the dichotomous activities autophagy plays during tumorigenesis, clinical investigation has also evaluated the impact of stimulating autophagy as a means to limit the growth and spread of cancers. Indeed, mTOR (mammalian target of rapamycin) is the primary pathway targeted pharmacologically as a means to induce autophagy in human breast cancers. For instance, several studies have investigated the importance of rapamycin^[79], Everolimus^[80,81], and Temsirolimus^[80,82] as potential inducers of autophagy in clinical settings. In general, autophagy activation elicited in response to mTORC1 inactivation is a byproduct of the intended

Table 1. Cancer clinical trials evaluating autophagy modulation

Clinical trial title	Status	Conditions	Interventions
Autophagy Bladder Cancer	Not yet recruiting	Bladder Cancer	
Sorafenib Induced Autophagy Using Hydroxychloroquine in Hepatocellular Cancer	Recruiting	Hepatocellular Cancer	Drug: Sorafenib (SOR) Drug: Hydroxychloroquine (HCQ)
A Phase I/II/Pharmacodynamic Study of Hydroxychloroquine in Combination With Gemcitabine/Abraxane to Inhibit Autophagy in Pancreatic Cancer	Active, not recruiting	Advanced Adenocarcinoma Metastatic Adenocarcinoma	Drug: Hydroxychloroquine (HCQ) Drug: Gemcitabine
Hydroxychloroquine, Palbociclib, and Letrozole Before Surgery in Treating Participants With Estrogen Receptor Positive, HER2 Negative Breast Cancer	Recruiting	Anatomic Stage I Breast Cancer AJCC v8 Anatomic Stage IA Breast Cancer AJCC v8 Anatomic Stage IB Breast Cancer AJCC v8 Anatomic Stage II Breast Cancer AJCC v8 Anatomic Stage IIA Breast Cancer AJCC v8 Anatomic Stage IIB Breast Cancer AJCC v8 Anatomic Stage III Breast Cancer AJCC v8 Anatomic Stage IIIA Breast Cancer AJCC v8 Anatomic Stage IIIB Breast Cancer AJCC v8 Anatomic Stage IIIC Breast Cancer AJCC v8 Anatomic Stage IV Breast Cancer AJCC v8 Estrogen Receptor Positive HER2/Neu Negative MKI67 Positive Postmenopausal Prognostic Stage I Breast Cancer AJCC v8 Prognostic Stage IA Breast Cancer AJCC v8 Prognostic Stage IB Breast Cancer AJCC v8 Prognostic Stage IIA Breast Cancer AJCC v8 Prognostic Stage IIB Breast Cancer AJCC v8 Prognostic Stage III Breast Cancer AJCC v8 Prognostic Stage IIIA Breast Cancer AJCC v8 Prognostic Stage IIIB Breast Cancer AJCC v8 Prognostic Stage IIIC Breast Cancer AJCC v8 Prognostic Stage IV Breast Cancer AJCC v8	Drug: Hydroxychloroquine Drug: Letrozole Drug: Palbociclib
Imaging Tumor Hypoxia With 18F-EF5 PET in Recurrent or Metastatic Clear Cell Ovarian Cancer	Active, not recruiting	Ovarian Cancer Ovarian Neoplasms	Drug: 18F-EF5 PET/CT scan Procedure: Optional biopsy
Enzalutamide and Metformin Hydrochloride in Treating Patients With Hormone-Resistant Prostate Cancer	Active, not recruiting	Prostate Cancer	Drug: Enzalutamide Drug: Metformin Hydrochloride
Vorinostat Plus Hydroxychloroquine Versus Regorafenib in Colorectal Cancer	Recruiting	Colorectal Cancer	Drug: Vorinostat Drug: Hydroxychloroquine Drug: Regorafenib
Sirolimus or Vorinostat and Hydroxychloroquine in Advanced Cancer	Active, not recruiting	Advanced Cancers	Drug: Hydroxychloroquine Drug: Sirolimus Drug: Vorinostat
MLN9708 and Vorinostat in Patients With Advanced p53 Mutant Malignancies	Active, not recruiting	Advanced Cancers	Drug: MLN9708 Drug: Vorinostat
Akt Inhibitor MK2206 and Hydroxychloroquine in Treating Patients With Advanced Solid Tumors, Melanoma, Prostate or Kidney Cancer	Active, not recruiting	Adult Solid Neoplasm Hormone-Resistant Prostate Carcinoma Recurrent Melanoma Recurrent Prostate Carcinoma Recurrent Renal Cell Carcinoma Stage IIIA Cutaneous Melanoma AJCC v7 Stage IIIB Cutaneous Melanoma AJCC v7 Stage IIIC Cutaneous Melanoma AJCC v7 Stage IV Cutaneous Melanoma AJCC v6 and v7 Stage IV Prostate Cancer AJCC v7 Stage IV Renal Cell Cancer AJCC v7	Drug: Akt Inhibitor MK2206 Drug: Hydroxychloroquine Other: Laboratory Biomarker Analysis Other: Pharmacological Study
Hydroxychloroquine + Vorinostat in Advanced Solid Tumors	Active, not recruiting	Malignant Solid Tumour	Drug: Hydroxychloroquine Drug: Vorinostat
Novel Molecular Targets for Ductal Carcinoma In Situ (DCIS)	Active, not recruiting	Breast Cancer	Other: Biomarkers
Observational Study of Biomarker During Liver Surgery	Recruiting	Liver Cancer Hepatobiliary Tract Adenomas and Carcinomas	Procedure: Hepatocellular carcinoma
Pantoprazole and Docetaxel for Men With Metastatic Castration-Resistant Prostate Cancer	Active, not recruiting	Prostate Cancer	Drug: Pantoprazole

A Randomized, 2x2 Factorial Design Biomarker Prevention Trial of Low-dose Aspirin and Metformin in Stage I-III Colorectal Cancer Patients.	Recruiting	Tertiary Prevention in Colon Cancer	Drug: Aspirin (ASA) + Metformin (MET) Drug: ASA Drug: MET Drug: Placebos
Randomized Phase II Trial of Pre-Operative Gemcitabine and Nab Paclitaxel With or Without Hydroxychloroquine	Active, not recruiting	Pancreatic Cancer	Drug: gemcitabine Drug: abraxane Drug: hydroxychloroquine
The Treatment of Advanced Lung Cancer With Dribbles Antigen by Targeting Activation of Tcells	Not yet recruiting	Carcinoma, Non-Small-Cell Lung	Biological: Dribble vaccine
Pre-Operative Trial (PGHA vs. PGH) for Resectable Pancreatic Cancer	Recruiting	Pancreatic Cancer Resectable	Drug: Gemcitabine, Nab-Paclitaxel, hydroxychloroquine and Avelumab Drug: Gemcitabine, Nab-Paclitaxel, and hydroxychloroquine
Sunitinib Malate and Hydroxychloroquine in Treating Patients With Advanced Solid Tumors That Have Not Responded to Chemotherapy	Active, not recruiting	Adult Solid Neoplasm	Drug: Hydroxychloroquine Other: Laboratory Biomarker Analysis Other: Pharmacological Study Drug: Sunitinib Malate
The Role of Fibroblast Activation in Uterine Fibroid	Not yet recruiting	Uterine Fibroid	Genetic: Measurement of protein expression in tissue and/or blood samples.
International Cooperative Phase III Trial of the HIT-HGG Study Group (HIT-HGG-2013)	Recruiting	Glioblastoma WHO Grade IV Diffuse Midline Glioma Histone 3 K27M WHO Grade IV Anaplastic Astrocytoma WHO Grade III Diffuse Intrinsic Pontine Glioma Gliomatosis Cerebri	Drug: Temozolomide + Valproic Acid Drug: Temozolomide + Chloroquine
Short Course Radiation Therapy With Proton or Photon Beam Capecitabine and Hydroxychloroquine for Resectable Pancreatic Cancer	Active, not recruiting	Pancreatic Cancer	Drug: Capecitabine Drug: Hydroxychloroquine Radiation: Proton or Photon Radiation Therapy
Androgen Deprivation Therapy Muscle Protein Metabolism and Blood Glucose	Recruiting	Prostate Cancer Resistance	Drug: Zoladex
Autophagy Inhibition to Augment mTOR Inhibition: A Phase I/II Trial of RAD001 and Hydroxychloroquine in Patients With Previously Treated Renal Cell Carcinoma	Active, not recruiting	Metastatic Clear Cell Renal Cell Carcinoma	Drug: Hydroxychloroquine Drug: RAD001
Dabrafenib/Trametinib/Hydroxychloroquine for Advanced Pretreated BRAF V600 Mutant Melanoma	Recruiting	Melanoma	Drug: Dabrafenib Drug: Trametinib Drug: Hydroxychloroquine
The Addition of Chloroquine to Chemoradiation for Glioblastoma,	Not yet recruiting	Glioblastoma Astrocytoma, Grade IV	Drug: Chloroquine
The Addition of Chloroquine to Chemoradiation for Glioblastoma	Recruiting	Glioblastoma Multiforme	Drug: Chloroquine Radiation: Radiotherapy Drug: Temozolomide
Gemcitabine, Docetaxel, and Hydroxychloroquine in Treating Participants With Recurrent or Refractory Osteosarcoma	Recruiting	Recurrent Osteosarcoma Refractory Osteosarcoma	Drug: Docetaxel Drug: Gemcitabine Drug: Hydroxychloroquine
The BAMM Trial: BRAF, Autophagy and MEK Inhibition in Metastatic Melanoma: A Phase I/2 Trial of Dabrafenib, Trametinib and Hydroxychloroquine in Patients With Advanced BRAF Mutant Melanoma	Recruiting	Advanced BRAF Mutant Melanoma	Drug: Trametinib 2 mg daily Drug: hydroxychloroquine (HCQ)
Characterization of the Mechanisms of Resistance to Azacitidine	Recruiting	Myelodysplastic Syndromes or Acute Myeloid Leukemia With Multilineage Dysplasia	
TN-TC11G (THC+CBD) Combination With Temozolomide and Radiotherapy in Patients With Newly-diagnosed Glioblastoma	Not yet recruiting	Glioblastoma	Drug: TN-TC11G Drug: Temozolomide Oral Product Radiation: Radiotherapy
Hepatocellular Carcinoma in Patients With a Cirrhosis Due to an Alcoholic or a Non Alcoholic Fatty Liver Disease	Recruiting	Hepatocellular Carcinoma	Other: blood collection

List of clinical trials (www.clinicaltrials.gov) that are currently active, recruiting, or not yet recruiting patients for clinical trials to study how autophagy modulation, primarily through chloroquine or hydroxychloroquine treatment, influences tumor growth and progression

drug target, thereby producing synergistic cell killing in the form of autosis (i.e., autophagic cell death^[83]). Along these lines, several clinical trials associated with mTOR modulation have sought to overcome endocrine resistance associated with hormone receptor positive breast cancer treatments. Unfortunately, single agent modification of autophagy by administration of mTOR pathway inhibitors has proven to be

highly ineffective at restoring endocrine sensitivity to estrogen receptor-positive breast cancers. Likewise, combining autophagy modulators with anti-estrogens has also failed to significantly improve the clinical course of these patients, with severe toxicities being associated with Everolimus^[80]. Thus, similar to the strategy of autophagy inhibition, the clinical utility of autophagy activation to eliminate metastatic breast cancers awaits additional mechanistic and translational investigation.

CONCLUSION

Metastatic dormancy is mediated by BCSCs and responsible for the majority of breast cancer-associated deaths. An inherent property of BCSCs reflects their ability to activate a variety of pro-survival strategies to circumvent metabolic stress within the metastatic niche, and to overcome therapeutic insults mediated by chemotherapies and radiation. The activation of autophagy has proven to be a critical component of the pro-survival strategies employed by BCSCs, especially when confronted with nutrient deprivation, with inhospitable tissue microenvironments, with cytotoxic agents, and with dormancy-associated phenotypes. Indeed, preclinical evidence implicates important roles for autophagy modulation in the treatment of breast cancer. However, the paradoxical functions of autophagy to both suppress and promote tumorigenesis has clearly hampered the development and implementation of effective autophagy modulators for the treatment of metastatic breast cancer. Accordingly, several important avenues of basic and clinical investigation need to be achieved in order to generate effective autophagic agents. First, studies need to determine the extent to which chemotherapeutic drugs rely upon autophagy modulation when inducing their cytotoxic activities in target cells. Indeed, these so-called “off-target” effects on autophagy may underscore either directly or indirectly the extent to which a therapeutic regimen is effective, or alternatively, is rendered insensitive. Second, additional efforts need to be directed at identifying improved autophagy modulating drugs, particularly those that are effective against metastatic disease. Third, enhancing our understanding of how the tumor microenvironment impacts the targeting of autophagy-directed drugs is also warranted^[84-86]. Finally, efforts directed at developing biomarkers capable of identifying patients most likely to benefit from autophagy modulation needs to be undertaken to minimize potential untoward side effects (e.g., disease progression, emergence from dormancy, and metastatic relapse) of this course of treatment. Ultimately, addressing these research avenues will provide new inroads for strategies aimed at targeting autophagy vulnerability in BCSCs, and consequently, at eliminating metastatic relapse.

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Author's contributions

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Drafted and revised the manuscript: Flynn ALB, Schiemann WP

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Conflicts of interest

All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Chemotherapy-induced immunological breast cancer dormancy: a new function for old drugs?

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Abstract

Breast cancer remains the main cause of cancer-related mortality for women world-wide. Main cause of death is the development of therapy-resistant metastases. Relapses occur with a bimodal temporal distribution, with a first peak at 1-2 years after initial therapy and a second peak 2-3 years later. This discontinuous growth kinetics is consistent with the notion that disseminated cancer cells can remain dormant over a prolonged period of time before resuming growth. How cancer cells enter, sustain and exit dormancy, are unanswered questions with relevance to cancer biology, monitoring and therapy. Investigating mechanisms of breast cancer dormancy remains challenging, as in patients the condition is elusive and experimentally there are only a few models that recapitulate the clinical condition. Thus, developing new models to identify clinically relevant mechanisms and candidate therapeutic targets may open new avenues for novel therapies to induce and prolong dormancy. We have observed that cells surviving chemotherapy can enter a state of immunological dormancy. Using this model, we identified IRF-7/Interferon type I/IFNRA as signaling axis essential for this effect. Here we will review concepts and recent developments in cancer metastasis and dormancy with emphasis on breast cancer, and elaborate strategies to exploit them therapeutically.

Keywords: Breast cancer, chemotherapy, resistance, dormancy, T lymphocytes, interferon

INTRODUCTION

With a few exceptions, as in the case of brain or liver cancer, the main cause of cancer-related death is not the primary tumor itself but rather the formation of secondary tumors, so called metastases, in vital organs,



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in particular lung, liver, bone or brain, leading to organ destruction and failure, resistance to therapy and cachexia^[1,2]. Metastasis formation is believed to be a highly inefficient process^[3]. The main rate limiting step in the metastatic process appears to be the ability of tumor cells to adapt to the new environment^[4,5]. During this adaptation step at the metastatic site, cells have to establish bidirectional paracrine communication with a new tissue different from the primary site, acquire novel survival capacities and escape immune destruction. While cell autonomous processes, such as genetic evolution and epigenetic modifications, altered gene expression and metabolic adaptation, are essential to the metastatic process, the microenvironments of the primary tumor and of the metastatic site are equally critical determinants of metastasis formation^[5-8]. Tumor angiogenesis, the remodeling of the extracellular matrix, the activation of local resident cells and the recruitment of inflammatory cells provide essential contributions to the metastatic process, including in breast cancer^[9-11]. At diagnosis, only a minority of cancers have already formed clinically overt metastases (i.e., stage IV)^[12]. Those that progress to metastatic disease can do with strikingly different kinetic. For example, lung and colorectal cancers mostly relapse within 1-3 years after diagnosis and the 5-year survival rates for these cancers are about 20% and 60%, respectively^[13,14]. Conversely, in prostate cancer relapses occur late, with over 90% of the patients still alive 15 years after initial diagnosis^[15]. In breast cancer, relapses occur with a peculiar bimodal distribution: a first peak appears generally 1-2 years and a second peak 4-5 years after surgery, followed by a tailed extension up to 15 years^[16,17].

BREAST CANCER SUBTYPES AND ADJUVANT THERAPIES

Breast cancer is the most common cancer diagnosed among women. In spite of improved management over the past 30 years, it remains the leading cause of cancer-related mortality for women world-wide^[18]. Therapy and prognosis are largely determined by the biological and molecular characteristics of the primary tumor and its size and spreading at time of diagnosis^[19,20]. There are three main clinically relevant biological subtypes: Oestrogen/Progesterone receptor positive breast cancer (ER⁺/PR⁺), HER2 amplified (HER2⁺) breast cancer and Triple Negative Breast Cancer (TNBC; i.e., ER⁻, PR⁻, HER2⁻)^[21-23]. Based on gene expression signatures four main molecular subtypes have been reported: Luminal A and B, HER2⁺, and basal-like, which overlap largely, but not fully with the ER⁺/PR⁺, HER2⁺ and TNBC biological subtypes, respectively^[24,25]. Both biological and molecular classifications have prognostic and predictive (therapeutic) relevance^[19,20,24,25]. ER⁺ tumors are treated with adjuvant anti-estrogen therapies (e.g., tamoxifen) while HER2⁺ tumors are treated with HER2 inhibitors (e.g., trastuzumab), in addition to radiotherapy and chemotherapy, if necessary^[19,26]. TNBC, has no molecular target useful for targeted therapy yet and adjuvant radio- and chemotherapies are still the standards of care^[22,27]. The rationale for administering adjuvant therapy after surgery is to eradicate disseminated tumor cells (DTC) or micro-metastases to decrease the risk of relapse. A large body of evidence from adjuvant studies suggest that ER⁺ breast cancer benefits less from chemotherapy compared to ER⁻ breast cancer^[28]. This is particularly true for the luminal A molecular subtype of ER⁺ breast cancer, which has a low rate of proliferation. Luminal A tumors have lower rates of pathologic complete response to chemotherapy compared to the highly proliferative ER⁺ luminal B breast cancer subset, as demonstrated with neo-adjuvant anthracycline/taxane-based chemotherapy^[29,30]. For HER2⁺ breast cancer the introduction of HER2 inhibitors as adjuvant therapy, in combination with taxane-based chemotherapy, has vastly decreased the risk of metastatic progression and greatly improved survival in this cancer subtype^[23,31]. TNBC is highly proliferative and respond better to chemotherapy compared to ER⁺ cancers^[22,32]. Today no established targeted therapy exists for TNBC^[33]. Interestingly, a fraction of TNBC are rich in tumor infiltrating lymphocytes and this infiltration has been associated with improved disease-free survival and overall survival OS, suggesting that immune cells may contribute to therapy success and implying the possibility of applying check-point inhibitors-based immunotherapies for these patients^[10,22].

In spite of an approx 30%-35% decrease in mortality over the past 35 years due to combined systematic early detection and improved adjuvant therapies, there are still about 20%-25% of breast cancer patients, all stages combined, that will eventually succumb to their disease due to formation of therapy resistant metastases.

This corresponds to about 95,000 and 40,000 women in Europe^[1] (EU 28) and the United States^[2], dying every year, respectively^[18,19,34]. As of today, there are no effective, curative therapies for metastatic disease. Therapy-resistance and therapy-related toxicity limit therapeutic options^[35].

METASTATIC DISSEMINATION

Cancer metastases is a multistage process. Cancer cells have to first escape from the primary tumor, survive in the circulation as circulating tumor cells (CTC), seed at distant sites as DTC and grow to colonize the new tissue and form secondary tumors^[36-38]. Growing evidence indicates that metastases are formed by a subset of tumor cells with “stem cell-like” features^[39-41] that also associated with resistance to treatments and dormancy^[42-44]. Accordingly, molecules controlling stem cell maintenance and differentiation^[10,22,45] have been implicated in metastasis, including Wnts, BMPs, TGF β family members, Notch, CD44 and integrins^[46,47]. Cancer stem cells (CSCs), in contrast to normal adult stem cells, seem able to revert the hierarchy so that a differentiated cancer cell can revert and recover the stem-like features, while normal, differentiated somatic cells are not able to do so. Thus, CSCs may be rather defined by function than lineage and may represent a form of adaptation of cancer cells to cellular or microenvironmental stress^[48-50]. This plasticity may be one reason why by eliminating CSCs as proposed as a new therapeutic approach to eradicate cancer, may actually not be as effective as anticipated^[51-54]. Acquisition of CSCs traits has been associated with Epithelial-to-Mesenchymal Transition (EMT), a condition endowing cancer cells with increased migratory, invasive, metastatic and therapy resistance capacities^[53,55,56]. For example, breast cancer cells undergoing EMT acquire a cell surface phenotype (i.e., CD44^{high}/CD24^{low}) associated with CSCs properties^[57]. Accordingly, EMT is a reversible process, as cells that disseminated through EMT and lost epithelial features, can revert back to their epithelial phenotype through an opposite process called Mesenchymal-to-Epithelial Transition (MET)^[8]. Both CSCs and EMT are features that are heavily influenced by the microenvironment such as the vascular niches or inflammation (See below).

The genetic and epigenetic basis of metastasis is still not fully elucidated^[58]. A current paradigm relies on the notion that the accumulation and selection of genetic mutations and epigenetic alterations is the basis of clonal evolution at the primary site and metastatic dissemination is its ultimate expression^[59]. This notion is supported by the clinical observation that primary tumor size is a main risk factor for metastasis. This suggests that metastasis formation occurs rather in late disease stage as the end product of an evolutionary processes in the primary tumor (linear model of metastasis)^[8,37,60,61]. According to this model many driver mutations found in the primary tumor are present at the metastatic site, and only a few additional mutations accumulate between primary tumor and metastases^[62-64], including in breast cancer^[65-68]. In the other hand, comparative genomic hybridization analysis in breast and other cancers revealed that DTCs display significantly more genetic aberration than in the primary tumors^[69-72]. These observations imply that metastatic cells disseminate early during tumor development and then progress independently from the primary tumor through multiple steps of genetic mutations. Therefore, the parallel progression model of metastasis has been proposed^[60]. Importantly, the two models are not mutually exclusive: a first vague of cancer cells may disseminate early during tumor formation, for example at the time of oncogene activation or EMT induction^[73-75], followed by the late dissemination of cells that acquired metastatic properties through local evolution^[64,76]. Recently, evidence for the parallel progression model in breast cancer was reported by using experimental models. By studying metastasis in a HER2-driven murine model of breast cancer, Harper *et al.*^[77] showed that cancer cells migrate away from early lesions shortly after HER2 activation. In this model over 80% of the metastases were derived from early disseminated cancer cells. Using the MMTV-HER2 breast tumor model, Harper *et al.*^[77] identified a subpopulation of ERBB2⁺/p-p38^{low}/p-ATF^{low}/TWIST1^{high}/E-CAD^{low} early cancer cells that are invasive and can spread to distant organs (early disseminated cancer cells - eDCC). By using intra-vital imaging they showed that ErbB2⁺ eDCC precursors invaded locally, intravasated and lodged in target organs through a WNT-dependent EMT-like dissemination program. Strikingly, although the majority of eDCCs were TWIST1^{high}/E-CAD^{low} and

dormant, they eventually formed metastasis. This experimental observation supports the notion that DTC/DCC can remain dormant for prolonged periods before resuming growth to form macroscopic metastases. As current adjuvant treatment in breast cancer seems to have reached a plateau in term of survival benefits, understanding how DTC and micro-metastases adapt to the distant environment, survive and eventually resume growth to form macroscopic metastases may identify new therapeutic opportunities^[1,8,78].

In both the linear and parallel tumor progression models, the genomic instability of tumor cells is the basis of the evolutive process. The variability emerging among tumor cells within the same tumor tissue is referred to as intratumoral heterogeneity and is also found within metastases^[79]. This heterogeneity may also be responsible to the presence of tumor cells with low and high tumorigenic potentials, the latter being CSCs or cancer initiating cells (CICs)^[80]. Moreover, it is believed that heterogeneity also exists inside the CSC population itself. In such a scenario, metastasis progression and resistance to anti-tumor treatment are thought to be due to clonal evolution and selection much alike Darwinian evolution^[59]. Intratumoral heterogeneity may also contribute to tumor dormancy or escape from it. Marusyk *et al.*^[81] have used the MDA-MB-468 tumor cell model *in vivo* to show that a IL-11 expressing tumor population is able to drive non-cell-autonomous tumor growth from dormant tumor cells. This indicates that re-establishment of certain heterogeneity is necessary for tumor growth after seeding or treatment. In line with this observation, Aceto *et al.*^[82] have reported that, CTC clusters have higher ability to seed metastasis compared to single CTC, involving at least in part altered DNA methylation^[83]. Furthermore, Kmiecik *et al.*^[84] showed that the heterogeneity of breast cancer cells in the levels of IFN- γ receptor α expression could determine a selective dormancy. Tumor cells expressing high levels of IFN- γ receptor α are eliminated by CD8⁺ T cells, while tumor cells with low expression levels do not die and remain dormant in the presence of IFN- γ producing CD8⁺ T cells. Thus, tumor heterogeneity contributes to tumor dormancy by providing cells with different genetic and biological features.

METASTATIC BREAST CANCER DORMANCY

As mentioned before, breast cancer metastasis occurs with a bimodal distribution with two peaks: a first one 1-2 years and a second one at 4-5 years after surgery, followed by a tailed extension up to 15 years^[17,85,86]. These observations are inconsistent with a model of continuous growth kinetics and rather suggestive of discontinuous growth, thereby implying a period of dormancy^[16,17,85-87]. Clinical observations also revealed that timing of appearance of metastasis has a similar profile for the different breast cancer subtypes, suggesting that after primary tumor removal, DTC from distinct subsets evolve following similar patterns but with different dominances (i.e., TNBC and HER2⁺ cancers tend to relapse at the early peak, compared to ER⁺/PR⁺ cancer which tend to relapse at the second peak or later^[85,86,88,89]). These observations also suggest that relapses occurring at peaks may follow inducible and reproducible patterns based on defined mechanisms, for example tumor surgery^[90], while relapses occurring in between or in the tailed extension may be due to unpredictable or random events, such as genetic mutations, epigenetic modifications or unrelated inflammatory events^[65,67,68,91,92]. Accordingly, these clinical observations were modelled mathematically by considering known basic hazard rates and unknown variables^[93,94]. Dormant disseminated cancer cells and micrometastases have been reported in breast cancer patients^[95] and in experimental animal models^[77,96-98]. Although dormancy is a common phenomenon in breast cancer, the underlying biological mechanisms remain ill characterized. Dormancy might be functionally considered as a transitional, metastable step of cell adaptation to a novel external stress, in particular a “foreign” soil. DTC lacking survival capabilities would rapidly die, while those that have acquired the latter would immediately grow to form macroscopic metastases without latency [Figure 1]. Mechanistically, three forms of cancer dormancy have been described and many genes and molecules involved identified^[99-102]: cellular dormancy, microenvironmental (angiogenic) dormancy and immunological dormancy. These three forms of dormancy are not mutually exclusive and it is likely that clinical dormancy is owed by their combination and interrelation.

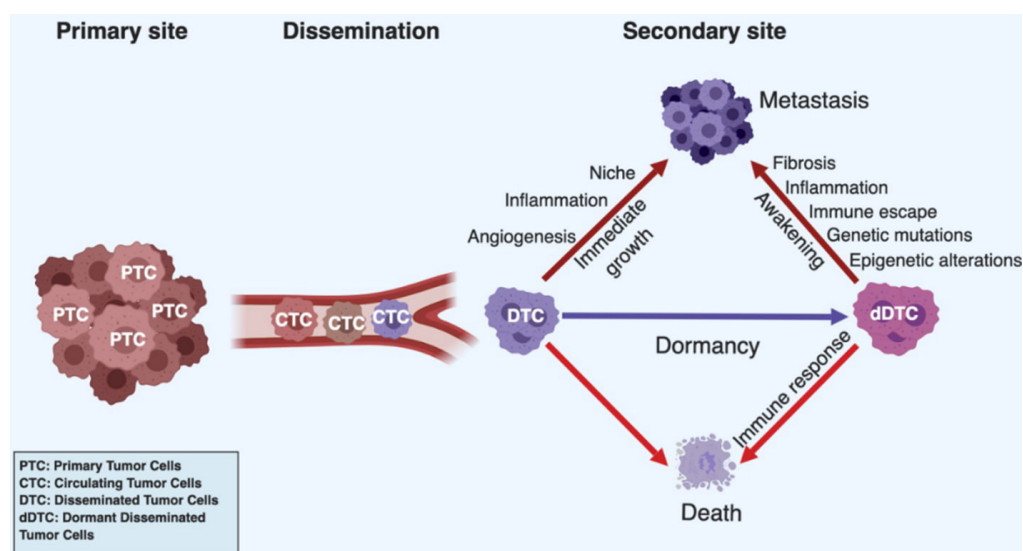


Figure 1. Dormancy in cancer progression. Tumor cells can leave the primary tumor site and enter the systemic circulation as circulating tumor cells (CTCs). Once surviving CTCs have reached a target organ, they seed into a new tissue as disseminated tumor cells (DTCs). Their fate is diverse depending on their cell autonomous capacities and complementary cues provided by the local environment. DTCs can rapidly die if they fail to adapt to the new condition or are killed by the immune system. They can immediately resume proliferation if they have acquired full autonomy for cell survival and proliferation or the local microenvironment provide missing complementary cues. In addition, proliferating cells have to evade the immune system. Alternatively, DTC or small tumor cell clusters, can enter a state of dormancy if cell autonomous or microenvironmental signals are sufficient to maintain survival but do not effectively support growth or the immune system keeps them in check by preventing their expansion. Dormant tumor cells can eventually die by exhaustion, be killed by the immune system or resume proliferation and generate clinically relevant metastases at later time points

CELLULAR DORMANCY: SURVIVAL OF NON-PROLIFERATING SOLITARY CELLS

Dormant DTC cells have developed mechanisms of survival in a foreign environment, but not yet those allowing unrestricted growth. They enter a state of cell cycle arrest (i.e., G₀-G₁) and survive as non-proliferating solitary cells or as small cell clusters. Accordingly, solitary dormant cancer cells should be negative for the proliferation marker Ki67 as well as for apoptosis markers such as the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Entry into dormancy and subsequent reactivation seem to be regulated by intrinsic programs and by contextual cues, similar to those involved in the physiological regulation of adult tissue stem cells^[103]. Lack of signaling from the matrix seems to play a role in this form of dormancy, as loss of $\alpha 5 \beta 1$ integrin expression or function and inhibition of uPAR induce dormancy through the inhibition of the RAS-RAF-ERK signaling pathway, activation of p38/JNK signaling, and induction of p53/Rb-dependent cell-cycle arrest^[103-105]. By studying DTC in an experimental model of head and neck squamous cell carcinoma, Sosa *et al.*^[106] have shown that epigenetic upregulation of orphan nuclear receptor NR2F1 (COUP-TF1) plays a critical role in maintaining DTCs in a dormant state. This finding has been further extended by a study in breast cancer patients, in which NR2F1 was tested as a dormancy marker. Breast cancer patients with < 1% NR2F1^{high} DTC in bone marrow aspirate had all systemic relapse within 12 months, while only half of the patients with > 50% NR2F1^{high} remained metastasis-free^[107].

Alterations in cell signaling have been found associated with tumor dormancy. High levels of ERK1/2 activity lean toward a higher proliferation, so the ratio of ERK1/2 to p38 MAPK regulates the cell cycle suggesting that the cross talk between mitogenic and stress signals may be relevant to induce cellular dormancy^[108]. Impinging on the PI3K signaling cascade was shown to lead to quiescence and the activation of autophagy^[109]. Dormant tumor cells express high levels of ARHI, an inhibitor of the PI3K-AKT cascade, and ARHI silencing awakens dormant cells of several tumor types, and promotes their proliferation^[110,111]. Consistently, very low or absent AKT signaling in DTC from breast cancer patient has been shown to correlate with the state of dormancy^[112]. Strikingly, however, mTOR, a known target of AKT, is found to be

activated in quiescent cancer cells. This activation is independent of AKT and is maintained by the small GTPase RHEB with anti-apoptotic activity and itself under control of the stress-regulated transcription factor ATF6 α and high p38 kinase activity. Thus, mTOR seems to be a critical node integrating diverse signaling pathways regulating dormancy^[111,113,114]. Recently Malladi *et al.*^[115] have shown that human breast and lung carcinoma cells express stem-cell like SOX transcription factors, which induces autocrine expression of DKK1, a WNT inhibitor, resulting in a state of metastatic latency and immune evasion consistent with dormancy.

Interaction with the extracellular matrix is also implicated in controlling dormancy^[116], paralleling the role of cell-matrix interaction in physiological CSC niches^[117]. For example, the uPAR interaction with $\alpha 5 \beta 1$ integrin increases ERK activity and integrin binding to fibronectin fibrils suppresses p38 activity, increases ERK activity and promotes cell proliferation. Accordingly, low uPAR-expressing cells that are growth arrested (dormant) *in vivo*, have a high p38/ERK activity ratio and fail to assemble fibronectin fibrils and ligate $\alpha 5 \beta 1$ integrin^[104]. Integrin $\alpha 5 \beta 1$ was shown to promote survival of growth-arrested breast cancer cells reminiscent of breast cancer dormancy in bone marrow^[118]. Collagen-rich matrix (fibrosis) at the metastatic site is also a critical determinant of DTC transition from dormancy to metastatic growth^[116]. For instance, hepatocellular carcinoma cells that colonize rigid matrix resume growth and proliferation through TGF- $\beta 1$ signaling, while cells colonizing a softer matrix remain dormant^[116]. The proliferative switch of dormant DTCs in response to fibrosis in a mouse model of breast cancer was shown to be also mediated by $\beta 1$ integrin^[116]. 3D-in vitro models and further *in vivo* studies demonstrated the critical role of type I collagen in the proliferative fate of DTCs^[119-121]. Another matrix protein, periostin, produced by TGF- $\beta 1$ -stimulated stromal fibroblasts and angiogenic blood vessels^[47,122], can drive DTCs escape from dormancy through WNT signaling in breast carcinoma^[123]. Interfering with extracellular matrix-integrin interaction, in particular $\beta 1$ integrins, has been proposed as therapeutic approach to promote dormancy, including in breast cancer^[124,125].

Autophagy appears to promote DTCs survival and dormancy by maintaining DTCs viable under conditions of microenvironmental stress^[126]. Autophagy also promotes survival of DTC against chemotherapy-induced cell stress^[126]. On the other side, in breast cancer the lack of autophagy is associated with early tumor recurrence and escape from dormancy^[127]. It is important to note, that cellular dormancy is not just a feature of cancer cells but it also occurs in normal cells. For example, hair follicle (bulge) stem cells, muscle stem cells (satellite cells), hematopoietic and liver stem cells are rather quiescent under homeostatic conditions and can be rapidly activated during tissue regeneration and repair^[128-130]. These observations raised the idea that cancer cells may hijack the signaling cascade used physiologically by these cells to enter, maintain and exit dormancy during homeostatic and regenerative conditions^[103,131].

SENESCENCE, A SPECIAL FORM OF CELLULAR DORMANCY?

The term senescence was originally introduced to describe primary human fibroblasts in culture, that after reaching a maximal number of passages (cell divisions) entered a state of permanent growth arrest^[132,133]. Senescent cells remain metabolically and synthetically active but show significant alterations in morphology^[132-134]. Following these original observations, scientists discovered that replicative senescence is driven by telomere shortening^[135]. Telomerase, an enzyme reconstituting telomers, is expressed in germ line cells, early embryonic cells, but not in normal cells, and is re-expressed in most cancer cells^[134]. Besides telomere shortening, other stimuli have been found to induce the senescence^[136] including DNA damage^[137], chemotherapy^[138] and oncogene activation^[139]. Despite the nature of the stimulus initiating the senescence cascade, the signals ultimately converge to the p53/p21 and/or p16^{INK4a}/pRB pathways^[136]. The tumor suppressor proteins DEC1 (Deleted in Esophageal Cancer) and Decoy Receptor 2 are also used as senescence markers in some cell types^[140]. Importantly, when oncogenic HRAS (HRAS^{G12V}) was used to transform immortalized embryo fibroblasts into tumorigenic cells^[141], unexpectedly, it induced senescence in normal cells associated with the accumulation of p53 and p16^{INK4a}^[142]. Thus, senescence may act as a

tumor-suppressor mechanism in response to oncogene activation^[133]. Generally, overexpression or sustained activation of one of the tumor suppressors p53, p21, p16^{INK4a}, or pRB is sufficient to induce senescence^[136,143-145]. Importantly, growth arrest caused by senescence is considered as irreversible as senescent cell could not be stimulated to resume proliferation by exposure to growth factors^[136,141,143]. However, the genetic or epigenetic alternations which cause the shift of the senescence maintaining mechanisms, such as the inactivation of tumor suppressor genes p53 and/or p16^{INK4a}, could potentially push the cells to re-enter the cell cycle^[136,143].

It is not clear whether senescence is one of the mechanisms that drives tumor dormancy and late relapse, but there are some potential links suggesting so. Senescence-associated secretory phenotype (SASP) defines the spectrum of factors secreted by senescent cells. It consists of a mixture of chemokines, cytokines, growth factors and proteases, many of which are pro-inflammatory^[136]. The cytokine GM-CSF (granulocyte-macrophage colony-stimulating factor, also known as CSF2), one of the components from SASP, induces differentiation of myeloid dendritic cells, which present tumor-associated antigens (TAAs), resulting in the activation of the immune system, enhanced immunosurveillance and improved tumor control^[146,147]. Furthermore, Braumüller *et al.*^[148] showed that adaptive TH1 cell are capable, via the combined secretion of IFN- γ and TNFR to induce tumor cells senescence. In conclusion, while senescence is a mechanism capable of negatively controlling tumor growth, its relevance in dormancy is not fully demonstrated.

MICROENVIRONMENTAL DORMANCY: DEFICIENT SUPPORT FROM THE MICROENVIRONMENT

In this form of dormancy, the fate of the DTCs is mainly controlled by the immediate, and possibly distant, host environment^[126,149]: cancer cells proliferate but fail to grow as a tumor mass because proliferation is balanced by cell death^[99]. This situation has been described first when disseminated cancer cells fail to induce blood vessels^[150]. During the early stage of tumor spreading, DTCs associate to preexisting (coopted) blood vessels where oxygen and nutrient levels are highest^[151]. Importantly, quiescent mature blood vessels keep DTCs in a state of dormancy through angiocrine communication^[122]. With the growing tumor mass, however, the increasing metabolic demand call for the formation of novel blood vessels through an angiogenic switch^[152]. Several molecules, including hypoxia-inducible factor 1, vascular endothelial growth factor (VEGF), placental growth factor and angiopoietin-1, are induced upon metabolic stress or hypoxia and initiate endothelial cell sprouting from preexisting vessels^[153]. Angiogenic endothelial sprouts secrete periostin and TGF- β 1 to create a microenvironment that promotes DTCs exit from dormancy and accelerates proliferation^[122]. Thus, the failure of initiating the angiogenic switch will keep the tumor mass small, a condition called angiogenic dormancy^[154]. Conversely, a short-term angiogenic burst may awaken dormant tumor cells^[155]. For example, in the mouse model of Lewis lung carcinoma, dormant micrometastatic cells can be induced to grow by the expression of VEGF and the recruitment of bone-marrow-derived endothelial progenitor cells^[156].

Importantly, inflammatory cells recruited to the tumor microenvironment are critical inducer of the angiogenic switch and lack of recruitment may therefore contribute to angiogenic dormancy^[11,157]. In particular the polarization of tumor associated macrophages toward the M2 phenotype results in the formation of a metastatic niche favoring tumor cell outgrowth^[158]. In addition, the angiogenic endothelium triggers a T helper 2-mediated inflammatory response, which can accelerate metastatic outgrowth in tumor models^[159]. The hemopoietic stem cell niche also supports quiescence and survival through the CXCL12/CXCR4 pathway and Src pathway^[47,160] as well as the TGF- β 2-rich bone marrow microenvironment^[161]. On the other hand, expression of VCAM1 on DTCs promotes escape from dormancy. This is due to the recruitment of osteoclast progenitors via α 4 β 1 integrin binding to VECAM1 causing the breakdown of the bone matrix and stimulation of DTC to grow and form metastases^[162,163]. Likewise, metastatic outgrowth following skeletal traumas was associated with bone remodeling in the reactivation of DTCs via TNF α , IL1 β , IL6 and prostaglandin E2 production^[163]. Thus, the cellular tumor microenvironment, in particular

inflammatory cells, endothelial cells and osteoclasts should be considered as critical regulators of tumor dormancy.

IMMUNOLOGICAL DORMANCY: ACTIVE CONTROL BY THE IMMUNE SYSTEM

The immune system can specifically recognize tumor cells through TAAs and initiate an anti-tumor immune response through a multi-step process called immunoediting^[164,165]: initially, the immune response can effectively eliminate cancer cells and no metastases are formed (elimination phase). If a balance between the tumor suppressive immune response and tumor evasion is achieved (equilibrium phase), the tumor mass remains constant resulting in a state of immunological dormancy^[166]. Tumor cells may eventually succeed in evading the immune system through a combination of genetic evolution and exhaustion of the immune system, and resuming growth to form macroscopic metastases (escape phase). Evasion involves multiple mechanisms, including the down-regulation of TAAs or MHC molecules, secretion of immunosuppressive cytokines, (e.g., IL-10 or TGF- β), recruitment of regulatory T cells (Treg), myeloid derived suppressor cells (MDSC) or alternatively (M2) - polarized macrophages or by expressing immunosuppressive molecules on the cell surface, such as PDL1 or B7^[167-169], in multiple cancer types, including breast cancer^[170]. The occurrence and relevance of immunological dormancy in human cancer is difficult to grasp, as the absence of biomarkers of dormancy makes it hard to investigate in patients. Also, there is paucity of clinically relevant *in vivo* model for experimental studies^[100]. Pommier *et al.*^[171] have recently reported that patients and mice with pancreatic ductal adenocarcinoma contained single quiescent DTCs lacking MHC-I expression, which enabled them to escape immunity and establish latent metastases. Four cases of breast cancer transmission to immunosuppressed transplant recipients from a single, clinically healthy donor have been recently described^[172]. The latency time to metastasis formation ranged from 16 months to 6 years, and transmissions did not occur in a patient that discontinued immunosuppression. This unintentional situation suggested that donor tissues harbored DTCs, which persisted in a latent state because of immune control, while the immunosuppression of the recipient allowed the DTCs to resume growth^[172]. Once cells have escaped immunosurveillance it is unlikely that they can re-enter a second state of immunological dormancy, because the mechanisms that eluded immunosurveillance are generally part of the genetic or epigenetic tumor clonal evolution and therefore irreversible^[59,91].

The recent observation that immunosuppressive immune cells promote angiogenesis^[157], and that some angiogenic factors, including VEGF, are immunosuppressive, established an important cross-talk between these two systems^[173-175]. They implicate that suppression of angiogenesis may stimulate the immune system and that reversal of immunosuppression may inhibit angiogenesis in a bi-directional cross-talk. In support of this notion, several trials have been performed to improve the anti-tumor immune response with anti-angiogenic drugs^[176]. Combination of anti-angiogenic agents with immunotherapy is currently being considered as strategy to improve the response rates and duration of immunotherapy^[177]. For example, VEGF has been shown to suppress the immune-response by impinging on maturation of dendritic cells^[178,179]. Thus, inhibition of VEGF abrogates its immunosuppressive effect and improves the antitumor effect of adoptive cellular immunotherapy^[180]. Combining anti-VEGF therapies with check-point inhibitors (e.g., anti-PD-L1) has shown synergy and positive outcomes in phases I to III studies, particularly in patients with high VEGF levels^[177]. Using the 4T1 experimental model of TNBC we have demonstrated that inhibition of tumor angiogenesis with the anti-VEGFR-2 antibody DC101, attenuated the inhibitory effect of MDSC on T cell proliferation and decreased the frequency of Tregs in primary tumors and lung metastases^[181]. Combined angiopoietin-2 and VEGF inhibition was shown to promote superior vascular regression, tumor necrosis, and enhanced the perivascular recruitment of cytotoxic T lymphocytes, as compared to the single agents in multiple cancer models. Addition of a PD-1 blocker unleashed the cytotoxic activity resulting in improved tumor control^[182,183]. These findings support the rationale for combining anti-angiogenic therapy with immune checkpoints inhibitors in cancer therapy, including in breast cancer. In addition to immunosuppressive angiogenic molecules, endothelial cells themselves also play a direct role in modulating

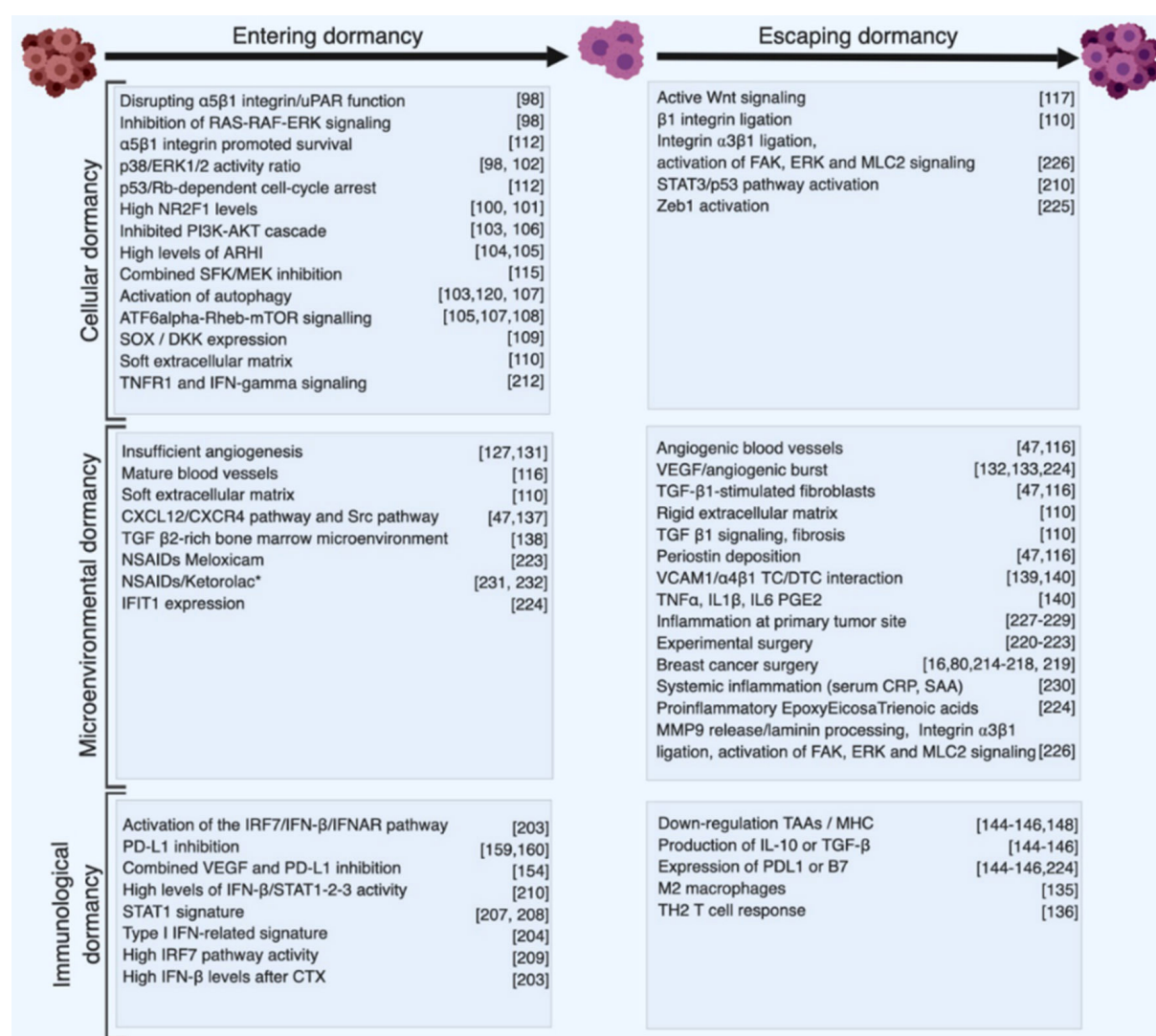


Figure 2. Synthetic summary of cellular and molecular events and interventions associated with dormancy. Cellular and molecular events promoting entering into dormancy or escape from dormancy are listed based on their implication in the three forms of dormancy. Classification is based on the main mechanisms and does not consider potential cross talk between different types of dormancy

cellular dormancy. Quiescent endothelial-derived thrombospondin-1 induces sustained quiescence of DTC in breast cancer, while sprouting vessels wake dormant DTC and promote their outgrowth^[122,184,185] [Figure 2].

DORMANT CANCER CELLS ARE RESISTANT TO THE CYTOTOXIC EFFECTS OF CHEMOTHERAPY

Adjuvant chemotherapy significantly improves breast cancer patient survival, in particular for TNBC, by decreasing the hazard of relapse after surgical removal of the primary tumor^[26,32,186-188]. Relapses and metastases however still occur in a fraction of patients and are likely due to tumor cells that had already invaded the surrounding tissue, lymphatic vessels and lymph nodes, or disseminated through the blood stream to the bone marrow and distant sites prior to surgery and resume grow at a later time point^[60,77,189]. As it is generally assumed that adjuvant chemotherapy acts by killing tumor cells, a corollary of this assumption is that relapses are due to DTC resuming growth at a later time point after surviving chemotherapy. Dormant, poorly proliferative DTC may not be affected by chemotherapy and may persist upon treatment since chemotherapeutic drugs mainly target highly proliferating cells. This has been shown for instance in

acute lymphoblastic leukemia, colorectal, lung, liver and breast cancers^[190-196]. In addition, chemotherapy pre-exposed DTC may develop mechanisms of chemoresistance and become less responsive to subsequent chemotherapies, as is often observed at relapse in patients. CSCs have been shown to be chemo-resistant and to be responsible for post-therapy relapses^[197]. Chemotherapy causes enrichment of CSCs thereby facilitating recurrences and resistance to further chemotherapies in multiple cancers including glioma, ovarian, liver, colon, breast cancers^[197]. Resistance involves multiple mechanisms, such as activation of signaling pathways prominent in stem cells (e.g., WNT, NOTCH, HEDGEHOG)^[198-200], but also pathways that are frequently mutated and activated in cancer, in particular the EGFR-HER2/PI3K/PTEN/Akt/mTORC pathway^[198,200,201]. CSC are often enriched at sites of chronic hypoxia leading to the activation of the HIF pathway^[200,202]. Interestingly, HIF activation leads to the initiation of protective pathways, including WNT and NOTCH, and genes of the ATP-binding cassette (ABC) drug transporter family members, such as MDR1, MRP1 and ABCG2 which are responsible for the efflux of cytotoxic drugs from the cells^[203-208]. Additional, HIF-independent mechanisms have been reported^[209]. In short, the mechanisms behind the long-term beneficial effects of adjuvant chemotherapy remain in part elusive and cannot be fully explained by the direct cytotoxic activity of chemotherapy as dormant/CSC that are mostly responsible for late relapses that are highly resistant to chemotherapy.

IMMUNE RESPONSE AND CHEMOTHERAPY IN BREAST CANCER

Cumulating evidence indicates that tumor infiltrating lymphocytes (TILs) play an active role in controlling progression and clinical outcome in breast cancer, particularly in highly proliferative TNBC and HER2⁺ cancers^[210-212], and in conjunction with chemotherapy^[213,214]. This is particularly relevant to TNBC, as these cancers present the richest presence of TILs, most notably CD8⁺ T lymphocytes, and tertiary lymphoid structures^[211,215,216]. Increased numbers of infiltrating TIL in TNBC, are associated with an improved pathological complete response to chemotherapy^[217], decreased rates of recurrences and improved survival^[210,216,218]. Evaluation of TILs in breast cancer has been recommended as an immunological biomarker with prognostic and potentially predictive values, mainly in TNBC and HER2-amplified breast cancers^[219]. In TNBC, expression of antigen presenting MHC class II pathway molecules is associated with a better outcome, consistent with the hypothesis that a functional antigen presentation pathway may trigger a protective antitumor immune response in response to chemotherapy^[220]. Ladoire *et al.*^[221] demonstrated that pathological complete response to neoadjuvant chemotherapy of breast carcinoma resulted in the disappearance of tumor-infiltrating FOXP3⁺ regulatory T cells and the increase in tumor infiltrating cytotoxic T_H1⁺ and granzyme B⁺ T cells, consistent with the induction of an antitumor immune response by chemotherapy. While the association between lymphocytic infiltrates, and improved outcome after chemotherapy appears to be strongest in TNBC and HER2⁺ breast cancer subtypes, the association with luminal tumors is less clear, and may be limited by the reduced immune infiltration or by the greater tumor heterogeneity of these tumors^[222]. Several recent experimental studies have shown that chemotherapy can induce a therapeutic anti-tumor immune response. For example, Ma *et al.*^[223,224], reported that anthracycline-based chemotherapy induces the release of ATP by dying breast cancer cells, which promotes the recruitment and differentiation of CD11c⁺CD11b⁺Ly6C^{high} antigen presenting cells. Depletion or preventing tumor infiltration by these cells abolished the anti-tumor immune responses elicited by anthracyclines^[223,224].

Besides these desired immunological effects, chemotherapy can also induce unwanted, immune-mediated tumor-promoting effects. For example, increased expression of TNF α in the breast tumor microenvironment due to chemotherapy, induces CXCL1/2 expression via NF- κ B activation in breast cancer cells and initiates a paracrine loop involving myeloid cell-derived S100A8/9 to enhance cancer cell survival and chemo-resistance. Inhibition of CXCR2 blunt this TNF α -induced response and augments the efficacy of chemotherapy, particularly against breast cancer metastasis^[225].

Taken together, there is growing evidence to support the notion that chemotherapy, in particular those based on anthracyclines, can elicit an effective anti-tumor immune response in breast cancer, mainly in the TNBC and HER2⁺ subtypes.

CHEMOTHERAPY-INDUCED IMMUNOLOGICAL BREAST CANCER DORMANCY

One important question emerging from these studies is whether the cytotoxic activity of chemotherapy and the elicited immune response, dominated by CD8⁺ T lymphocytes, may be sufficient to effectively kill cancer cells or whether additional mechanisms may be involved. This is particularly relevant to dormant DTC as these cells are naturally less responsive to chemotherapy due to the fact that they are not or low proliferative. We were interested in the possibility of whether a short chemotherapy treatment, induce a long-lasting immune response leading to immunological dormancy. We recently addressed this question experimentally^[226]. To this end, we treated the TNBC-like 4T1 cells with high dose Methotrexate and Doxorubicin *in vitro* and characterized the *in vitro* and *in vivo* behavior of the surviving cells (MR20 and DR500 derived from Methotrexate and Doxorubicin treatment, respectively). The hallmark of the surviving cell lines was the dormant phenotype at the primary (MR20 in the mammary gland) or at the metastatic (MR20 and DR500 in the lung) site. MR20 cells grew significantly slower *in vitro* compared to parental 4T1 cells and this was due to increased cell death, while there was no significant alteration in the cell cycle. When injected orthotopically in the mammary fat pad of immunocompetent BALB/c mice, only about half of the MR20-injected mice developed tumors with a longer latency (between 6 week and 4 months) compared to parental 4T1 cells (between 2 and 4 weeks). These tumor cells grew with nearly the same rate as parental 4T1 tumors and were highly metastatic. DR500 cells formed primary tumors but no lung metastases. Both conditions were consistent with dormancy. In BALB/c mice MR20 and DR500 cells twisted the immune response from CD11b⁺ Gr1⁺ MDSC-dominated to CD4/8 T cell, B cell and DC dominated response. When injected *in vivo* in immunodeficient NSG mice, however, treated cells formed tumors without latency. We then performed a genome wide gene expression analysis of parental 4T1, MR20 and DR500 cells and the dominant trait observed was a type I IFN gene expression signature and the sustained activation of the IRF7/IFN-β/IFNAR pathway. Upregulated IRF7 expression in treated cancer cells was responsible for suppressed mobilization of CD11b⁺ Gr1⁺ MDSCs, increased expansion of DCs, T and B lymphocytes and chemo resistance. Silencing IRF7 or blocking IFNAR1 resulted in escape from dormancy *in vivo*. Elevated levels of IFN-β were present in the blood of mice injected with dormant cells, while MR20 cells escaping dormancy and forming late tumors no longer expressed IFN-β. Interestingly, the dormant D2.0R murine breast cancer cells that are generally considered as a model of cellular dormancy, also induced a T-cell twisted immune response and had a constitutive active IRF7/IFN-β/IFNAR pathway [Figure 3]. To collect evidence that the activation of the type I IFN pathway was associated with a better response to chemotherapy in patients, we monitored IFN-β levels in serum samples of ER⁻ breast cancer patients treated with neoadjuvant chemotherapy (TOP trial NCT00162812). Patients with detectable IFN-β during neoadjuvant therapy had significantly longer distant metastasis free survival (DMFS) compared to patients with undetectable levels^[226].

TYPE I IFN RESPONSE TO CHEMOTHERAPY MEDIATES IMMUNOLOGICAL DORMANCY IN BREAST CANCER

Taken together our results demonstrate that sustained activation of the IFN-β/IFNAR/IRF7 signaling axis in chemotherapy-treated TNBC-like murine breast cancer cells instigates immunological dormancy. Elevated levels of IFN-β in the serum of TNBC patients during adjuvant therapy correlates with a shorter DMFS. Acute exposure of breast cancer cells to chemotherapy induced IFN-β expression^[226]. Thus IFN-β may be considered as a potential therapeutic tool to improve chemotherapy efficacy and clinical outcome as well as a potential predictive biomarker to identify responding vs. non-responding patients. While the implication of type I IFN in dormancy is novel, previous reports have implicated it in acute anti-tumor

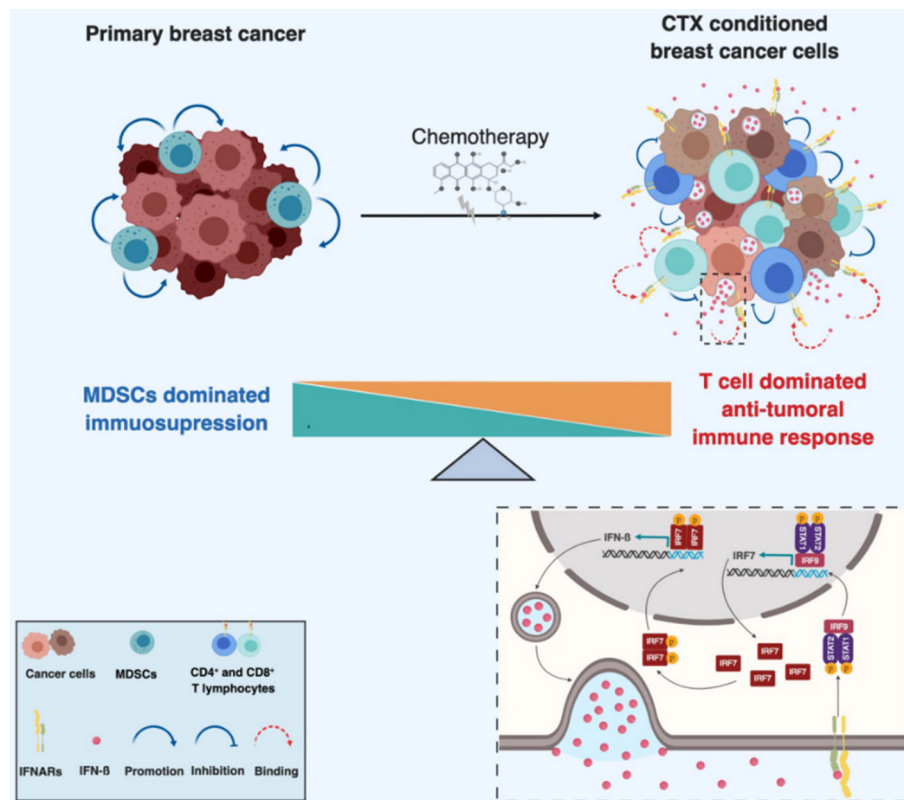


Figure 3. Schematic model of chemotherapy-induced immunological dormancy. Primary breast cancer cells escape immune elimination by inducing the expansion of myeloid derived suppressive cells (MDSC) which also promote tumor growth. Chemotherapy induces a type I IFN response in treated tumor cells, resulting in an autocrine and self-sustained increase of IRF7 expression and activation, which in turn induces expression and secretion of IFN- β . Secreted IFN- β binds to IFNAR and induces signaling in both immune cells and tumor cells. IFNARs signaling in tumor cells activates STAT1/STAT2/IRF9 complex which further induces the expression of IFN- β responsive genes including IRF7 resulting in a sustained autocrine IFN- β expression and secretion. Paracrine activation of IFNARs on immune cells stimulates the expansion of tumor suppressive lymphocytes (e.g., CD4 $^{+}$ and CD8 $^{+}$ T cells) and prevents the mobilization of MDSCs, resulting in the switch of the immune response from immunosuppressive to anti-tumoral

response to chemotherapy. Sistigu *et al.*^[227] reported that anthracycline-based chemotherapy rapidly induced the production of type I IFN in cancer cells via TLR3 activation resulting in CXCL10 secretion promoting an anti-tumor immune response mimicking those induced by viruses. Moreover, a type I IFN-related signature predicted clinical responses to anthracycline-based chemotherapy in breast cancer patients. Using a panel of ER $^{+}$ breast cancer PDXs before and after chemotherapy, Legrier *et al.*^[228] demonstrated activation of the IFN/STAT1 pathway and expression of IFN-inducible genes, early after chemotherapy treatment. IFN- α deficient DC were shown to accumulate in aggressive breast cancers favoring the expansion of Tregs implicating that IFN- α deficiency may contribute to tumor immune tolerance and poor clinical outcome^[229]. Previous studies based on tumor-derived IFN signatures have shown that IFN-regulated genes may correlate with favorable outcomes. A STAT1 signature before therapy was associated with a better response to neoadjuvant chemotherapy^[230] and better prognosis in TNBC and HER2 $^{+}$ breast cancers^[231]. High IRF7 pathway activity in human breast cancer predicted bone relapse-free survival and, and protected mice against bone metastasis^[232]. In the same study, treatment with IFN- α improved bone metastasis-free survival^[232]. High level of IFN- β activates STAT1, STAT2 and STAT3 to facilitate cell autonomous cellular dormancy of melanoma repopulating cells^[233]. Taken together, our recent data extend the role of type I IFN in immunoediting in cancer^[234] to a putative role in inducing immunological dormancy after chemotherapy in TNBC. An important outstanding question raised by our study, concerns the effector mechanism of T cell mediated dormancy. While it is likely that direct CD8 $^{+}$ T cell mediated killing and immune control is involved, particularly since CD4 $^{+}$ Th1 T cells produce IFN- γ which upregulates MHC expression on

tumor cells thereby facilitating killing by CD8⁺ T cells^[235], it is plausible that additional mechanisms may be involved. One possibility is suppression of tumor angiogenesis by the secretion of anti-angiogenic factors, such as CXCL9 and CXCL10 by CD4⁺ T cells^[235]. Another putative mechanism is induction of senescence. CD4⁺ Th1 T cells also produce TNF- α , which in combination with IFN- γ induces an irreversible state of cellular senescence keeping DTC dormant^[235,236]. Ongoing projects in our lab are aimed at unraveling the effectors steps.

BREAKING DORMANCY: IMPACT OF INFLAMMATION

Clinical and experimental observations indicate that dormant tumor cells can resume proliferation and generate macroscopic metastases at various times after primary tumor treatment^[17,85]. Escape from dormancy could be initiated by cell autonomous events, such as oncogenic mutation or inactivation of tumor suppressor genes. Alternatively, alterations in the host micro- or macro-environment may promote escape from dormancy. Metastatic growth following surgical removal of the primary tumor itself is often observed in clinical settings with predictable patterns, including in breast cancer^[16,86,237-241]. Reconstructive surgery after mastectomy for breast cancer significantly accelerates relapse rates proportionally to the extent of surgery, when compared to primary surgery. However, no worsening in long-term survival was reported, consistent with an effect on breaking dormancy and accelerating progression but not altering the overall outcome^[242]. Enhancement of metastatic growth induced by experimental surgery has been long observed in animal models, suggesting that surgical wounding itself may be directly involved in breaking dormancy^[243-245]. Recently Krall *et al.*^[246] described an experimental model that links the wound-healing response after surgery to the outgrowth of DTC at distant sites. The link is mediated by the systemic inflammatory response induced after surgery suppressing a tumor-specific T cell response, thereby resulting in tumor growth. Consistent with these findings, perioperative anti-inflammatory treatment significantly reduced tumor outgrowth. Epoxyeicosatrienoic acids, a family of pro-inflammatory molecules, stimulated escape from dormancy in several tumor models independently of the primary tumor and was associated with increased production of VEGF by endothelial cells^[247]. Recently, it was reported that lung inflammation promotes escape from tumor latency by inducing ZEB1 expression, a regulator of the EMT^[248]. Another recent study reported that neutrophil extracellular traps (NET) produced by neutrophils during repeated acute inflammation awaken dormant cancer cells in a mouse model of breast cancer^[249]. The releasing of neutrophil elastase and MMP9 from NET then remodel laminin in the extracellular matrix rendering it accessible to $\alpha 3 \beta 1$ integrin. Ligated $\alpha 3 \beta 1$ integrin leads to the activation of FAK, ERK and MLC2 signaling resulting in the awakening of the dormant cancer cells^[249].

Clinical and experimental evidence suggests that sustained inflammation may also promote relapses^[91]. Correlations between inflammation at primary tumor site and risk or recurrences were reported for several cancers including oral^[250], endometrial^[251] and breast cancers^[250,252]. Elevated levels of circulating C-reactive protein and serum amyloid A, two proteins of the inflammatory response, are associated with reduced overall survival in breast cancer, independently of body mass index, age and tumor type and stage, consistent with inflammation being involved in breaking dormancy^[253]. Interestingly, perioperative administration of the analgesic nonsteroidal anti-inflammatory drug (NSAID) ketorolac was reported to suppress early breast cancer relapse particularly in TNBC patients^[254] and to reduce distant recurrences in patients with increased BMI^[255]. Taken together, there is compelling clinical and experimental evidence indicating that inflammation promotes breast (and other) cancer relapses by breaking dormancy.

OUTLOOK AND PERSPECTIVES

Tumor dormancy is widely accepted as one discrete step during multistage tumor progression and bears considerable therapeutic potential^[256]. The rapid translation of this innovative concept to the patient is limited by the paucity of therapeutic tools currently available in the clinic. Treatments directly aimed at DTC

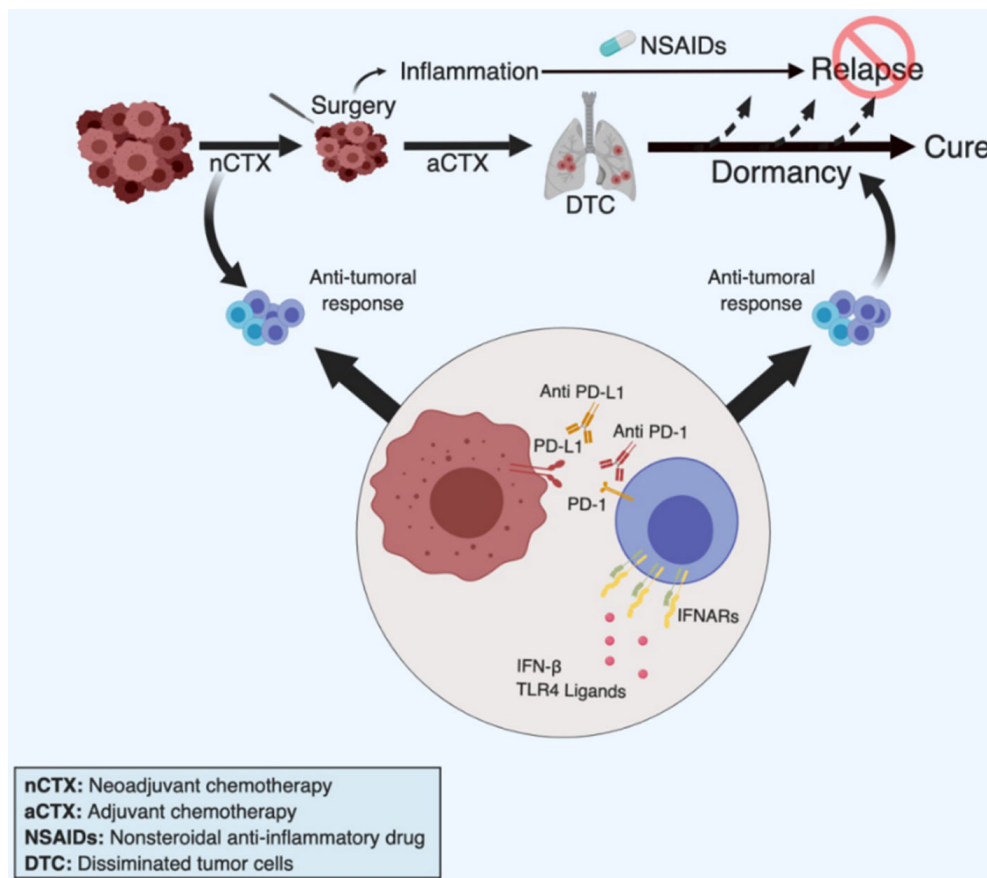


Figure 4. Strategies to improve chemotherapy-induced immunological dormancy. Based on work by others and us, we propose four clinically feasible approaches to induce or maintain breast cancer dormancy, primarily in TNBC. Firstly, we propose neo-adjuvant chemotherapy (nCTX) to promote chemotherapy-induced immune response. Chemotherapy may be pursued as adjuvant therapy (aCTX) if necessary. Secondly, during and following surgery we propose the administration of NSAIDs (in particular Ketorolac) to prevent surgery-associated inflammation that may potentially promote relapses. Thirdly, the cytotoxic immune response could be stimulated by providing type I IFN, or inducers of an interferon response such as TLR4 ligands, in particularly in low IFN-producing patients. Fourthly, addition of checkpoint inhibitors, such as anti-PD1/PDL-1 antibodies, may be applied to maintain the immune response active

by critical cell survival and proliferation pathways (e.g., PI3K-AKT or MAPK pathways), stem cell pathways (e.g., WNT, NOTCH) or cell adhesion molecules (e.g., β_1 integrin) would be virtually excluded for such an approach given their expected long-term systemic toxicities. We are proposing here selected strategies based on limiting the host (unwanted) inflammatory response and stimulating the (wanted) anti-tumoral-immune response that may be rapidly tested in clinical-translation studies in breast cancer [Figure 4].

NSAIDs

There is growing clinical and experimental evidence that inflammation can trigger cancer relapse, in particular in breast cancer, and that NSAIDs treatment can prolong dormancy and delay or reduce relapses^[246,248,249,252,254,255,257]. The overall positive safety profile of aspirin and other NSAIDs would make them realistic candidate drugs for such long-term therapies^[258]. Even more interesting, as a short perioperative treatment with ketorolac has been shown to significantly decrease the risk of breast cancer relapses particularly in obese patients^[255], a short term NSAID treatment at time of surgery may have long-lasting effects through suppression of surgery-associated inflammation.

Neoadjuvant chemotherapy

A second approach to consider, is to exploit the ability of chemotherapy to elicit an effective immune response in breast cancer, particularly in lymphocyte-infiltrated TNBC or HER2⁺ tumors^[211,212,222,224,226,227,259].

Our and other's results indicate that chemotherapy induces an effective anti-tumor immune response upon tumor cell treatment^[214,226,227]. Clinically this implies that neo-adjuvant/pre-operative chemotherapies may be more effective in inducing a long-lasting, protective immune response compared to classical adjuvant/post-operative therapies, due to the larger targeted tumor mass^[224,260]. Indeed, neoadjuvant chemotherapy is already used in highly proliferative breast cancers (i.e., Luminal B, HER2⁺ and TNBC) with high frequencies of pathological complete responses (pCR). Interestingly, paclitaxel, in combination with trastuzumab, induced a high rate of pCR in HER2⁺ patients, likely due to the synergy between the immunomodulating properties of these drugs^[260]. Ladoire *et al.*^[221] reported that pCR to breast cancer neoadjuvant chemotherapy was associated with the disappearance of tumor-infiltrating FOXP3⁺ Tregs and recruitment of CD8⁺ T cells, consistent with the induction of an antitumor immune response by chemotherapy.

Type I IFN

In our experimental model we have shown that Type I IFN response is essential to elicit a state of immunological breast cancer dormancy^[226]. Others have shown that exogenous addition of type I IFN boosted an insufficient response to chemotherapy in an experimental model of breast cancer and that a type I IFN-related signature predicted clinical responses to anthracycline-based chemotherapy in breast cancer patients^[227]. We demonstrated that patients with high levels of serum IFN- β during neoadjuvant therapy have a better outcome compared to patients with low levels^[226]. This suggests that administration of type I IFN during neo-adjuvant therapy may be effective in mounting a long-lasting immune response in particular in those patients with low endogenous type I IFN levels. Type I IFN, in particular IFN- α has been already tested as immunostimulatory anti-cancer agent, especially in melanoma and kidney cancers, albeit with mild results, in part also due to the need of repeated administrations and its intrinsic toxicity^[261-263]. Alternatively, less toxic inducers of Type I IFN response, such as TLR-ligands of STING stimulators may be considered^[227,264].

Check point inhibitors

A complementary strategy to IFN administration could be the use of check point inhibitors, in particular anti-PD-1/PD-L1 antibodies to relieve tumor-induced immunosuppression^[265]. In breast cancer, immunotherapy is being explored, in particular in patients with tumors expressing PD-L1 and infiltrated with lymphocytes^[266]. Potential response to PD-1 or PD-L1 inhibitors was demonstrated in metastatic TNBC^[267] and HER2⁺ breast cancers^[268]. However, because the number of potential neoantigens available for immune response in most breast cancers, responses are modest compared with other cancers such as lung and melanoma, the use of check-point inhibitors may require combination with other therapies^[269]. Therefore, combination with neo-adjuvant chemotherapy (causing the release of tumor antigens), anti-angiogenesis therapies (suppressing inhibitory cues)^[182] or Type I IFN (acting immunostimulating)^[270] may be more effective and should be explored.

Biomarkers of dormancy

There are currently no specific non-invasive biomarkers to monitor breast cancer dormancy of clinical utility^[271]. Such markers would allow personalized follow up and accelerate therapeutic decisions in case of evidence of disease progression. CD44⁺/CD24⁻ CTC subsets along with combinatorial expression of uPAR and b1 integrin, as well as proliferation and apoptosis markers in CTC of early breast cancer patients, have been explored for potential use as biomarker of dormancy or aggressiveness^[272,273]. Genomic analysis (i.e., SNP/CNV) of circulating ctDNA was shown to potentially identify breast cancer patients with dormant/minimal residual disease^[274]. Also, serum inflammatory markers might serve as biomarkers of relapse in disease-free patients, as inflammation is associated with escape from dormancy but will likely be unspecific and of limited sensitivity^[275]. Serum IFN- β levels were associated with longer DMFS (as a surrogate of dormancy) in our model of chemotherapy-induced dormancy and during neoadjuvant therapy in patients with favorable outcome^[226]. Also, we observed a shift in peripheral blood leucocyte populations in our

experimental model, from a Gr1⁺CD11b⁺ cell dominated response in mice with progressive tumors toward a CD4⁺/CD8⁺-, B cell and CD11c⁺ cell-dominated response in mice with dormant tumors^[226]. Thus, Serum IFN- β levels and immunophenotyping should be explored for their potential use as biomarkers of breast cancer dormancy.

CONCLUSION

Advances in the understanding of the mechanisms of breast cancer dormancy have raised the hope to therapeutically exploit dormancy to prevent relapses and overt metastatic disease. To date many potential therapeutic targets and strategies have been considered and proposed for clinical testing^[100,185]. However, many of these approaches would be difficult to apply to patients due to lack of suitable drugs, potential long-term toxicity and over all complexity in the in their clinical translation. Recent reports implicating a T cell based immune response in the therapeutic effects of chemotherapy including dormancy, have opened novel perspectives for a feasible clinical translation. Administration of chemotherapy before tumor removal (i.e., neoadjuvant chemotherapy) may be explored for improved effects on immunological dormancy compared to conventional, post-surgery, adjuvant chemotherapy. Drugs with potential beneficial effects on promoting or prolonging dormancy, such as NSAIDs to suppress inflammation, type I interferons, check-point inhibitors or anti-angiogenic drugs to stimulate the immune response, are available for clinical use and could be tested in combination with chemotherapy. Thus, the observation that chemotherapy can induce a state of immunological dormancy adds a new therapeutic effect to the older class of anti-cancer drugs and opens unanticipated therapeutic opportunities for clinical translation in breast cancer (and possibly other cancers).

DECLARATIONS

Acknowledgments

We apologize for not being able to cite all published work relevant to this topic due to space limitation and selective focus of the article.

Authors' contributions

Conceiving the paper: Peyvandi S, Lan Q, Lorusso G, Rüegg C

Writing the paper: Peyvandi S, Lan Q, Lorusso G, Rüegg C

Performed the work of immunological dormancy in the laboratory: Peyvandi S, Lan Q, Lorusso G, Rüegg C

Availability of data and materials

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Conflicts of interest

All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Operative treatment of metastatic breast cancer in the spine with regard to molecular phenotypes

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Abstract

With more than one million new diseases per year breast cancer is the most common malignancy in women. Metastatic breast cancer remains an incurable disease and the spinal column is most likely affected by metastases. A significantly prolonged patient survival is the consequence of modern oncologic treatment options in the last decade. Surgical treatment of vertebral metastases has become an increasing focus for spine surgeons. With the turn of the millennium it was possible to classify breast cancer into four intrinsic phenotypes with various survival rates. Well known scoring systems help surgeons to evaluate the patient's prognosis and to choose adequate treatment options. However, tumor entities are differentiated without regard to the molecular subtypes. In this article we describe surgical treatment options in metastatic lesions to the spine with regard to molecular phenotypes of breast cancer malignancy. It is crucial to correctly estimate the expected survival time to plan invasiveness of therapy regarding metastatic spine surgery.

Keywords: Spine, metastases, breast cancer, phenotypes, subtypes, molecular level, prognosis

INTRODUCTION

Breast cancer is one of the most common cancer diseases metastasizing to the spine and the second leading cause of deaths in woman related to cancer^[1]. Modern treatment and diagnostic concepts including bisphosphonates go along with considerable longer survival times and a reduced rate of skeletal



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complications^[2-4]. Until 2015 mean survival times of 27 months were described, actual studies report mean survival times of 55 months with a 85% incidence of spinal metastases^[2-15]. An increased need for surgical treatment of complications related to spinal metastases is the consequence. Due to mortality and morbidity rates related to metastatic breast cancer every surgical treatment should be considered carefully. Well evaluated scoring systems (revised Tokuhashi-or Tomita score, revised Bauer score, Van-der-Linden-score, Karnofsky index) help surgeons to individually chose optimal treatment methods and to predict life expectancies in cancer patients. But these scores only differentiate between tumor entities whereas respective tumor phenotypes are not taken into account^[16-21]. With the turn of the millennium five different intrinsic subtypes of breast cancer were reported by means of gene expression profiles^[14-22]. Four phenotypes are decisive in daily clinical routine: luminal A, luminal B, HER2-enriched and basal like/triple-negative. With an accordance of 70%-80%^[23] these phenotypes are assessed with immunohistochemical pathologic markers (e.g., ER, PgR, HER2, KI-67/grading) as standardized gene expression profiling is available with partial coverage^[24]. In this article we describe the molecular parameters of metastatic breast cancer to predict survival time more precisely and to adequately calculate surgical therapies.

ASSESSMENT OF PROGNOSIS

The development of oncologic treatment options in primary breast cancer (in particular medical therapy) led to considerable improved survival rates in the last decade^[2-9,12-15,25-27]. Immunohistochemical diagnostic tools provide a sufficient molecular phenotyping in breast cancer [Table 1].

Visceral, skeletal or cervical spine metastases, surgical complications and advanced patient's age are reported with different data in literature and therefore are not recommended as negative predictors^[13]. In contrast, unimodal postoperative therapies, a disease-free interval less than 24 months, a high number of axillary lymph node metastases and progress after first-line therapy mark assured predictors of shorter survival^[4,10,13]. Furthermore, patients' wishes and preferences, symptoms, biological age, intrinsic breast cancer subtype, tumor burden (number of metastasized organs) and prior therapies have to be taken into account^[4].

MOLECULAR PREDICTORS

Around the turn of the millennium, Sørlie und Perou^[14,22] published fundamental studies to classify breast cancer at the molecular level with relevance to clinical course of disease. As tumor-biological classifications increasingly become more complex, modern oncologic diagnostic and therapy options interact with highly specialized regulatory mechanisms of cells and cell cycles^[8,28]. The actual breast cancer classification [Table 1] is based on histopathological examinations of a biopsy from the breast. Classifying breast cancer^[1-3,5-9,12-15] by estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor 2 (HER-2) and a proliferation marker (KI-67) is global standard^[23,24,28-33]. With these molecular genetic parameters breast cancer is differentiated into the four intrinsic phenotypes, which are clinically relevant [Table 1]. Hormone receptor status comprises the combination of ER and PgR status^[3,8,12]. Furthermore, endocrine responsive subtypes (Luminal A and-B), triple negative and HER-2 over expressing immunophenotypes are differentiated^[2,3,8,13,14,23]. Luminal-A and Luminal-B phenotypes are clinically distinguished by proliferation rate (e.g., KI-67), grading or a multigen signature^[2,3,5-9,15,24,30].

The common classification is based on the ubiquitous availability of these diagnostic methods, which enable comparable studies and therapy concepts. With regard to metastatic breast cancer mean survival times of 60 months are reported in the current literature^[4,10,34] in case of endocrine responsive phenotypes (Luminal-A and-B) compared to 26 months until the year 2015^[2,3,35]. Compared to PGR positive (PgR+) status, patients with PgR negative (PgR-) status have a 59% higher mortality risk^[3]. In patients with hormone receptor negative (HR-) status the mean survival time is reduced by 11 months and mortality risk is 52%

Table 1. Molecular phenotypes of metastatic breast cancer with related survival times^[26]

Molecular Phenotype	Luminal A	Luminal B	HER2-enriched	Triple-negative-status
	ER and PR positive Low Proliferation Rate (Ki-67)	ER and/or PR positive High Proliferation Rate (Ki-67)	HER2-positive	ER and PR and HER2-negative (Basal-like)
Mean survival time	60 months	60 months	56.5 months	15-19 months

Table 2. Karnowsky Performance Scale from 1949^[34]

Score	Parameter
100	Normal; no complaints; no evidence of disease
90	Able to carry on normal activity; minor signs or symptoms of disease
80	Normal activity with effort; some signs or symptoms of disease
70	Cares for self; unable to carry on normal activity or to do active work
60	Requires occasional assistance, but is able to care for most of their personal needs
50	Requires considerable assistance and frequent medical care
40	Disabled; requires special care and assistance
30	Severely disabled; hospital admission is indicated although death not imminent
20	Very sick; hospital admission necessary; active supportive treatment necessary
10	Moribund; fatal processes progressing rapidly
0	Dead

higher compared to hormone receptor positive (HR+) status^[6]. Biggest progress regarding survival times and therapy options was achieved in HER-2 enriched phenotypes in the last decade. Until some years a HER-2 positive status was associated as a negative predictor with a mean survival time of 5-9 months. After the introduction of new Anti-Her-2 therapeutics (Trastuzumab, Pertuzumab, T-DMI) the mean survival time is reported with 56.5 months^[2-4,10,34,36].

Whereas triple negative phenotypes (ER-, PgR-, HER-2-) were reported with a mean survival time of 6-9 months until the year 2015, actual data show a prolonged survival time of 15-19 months^[2-6,9,10,13-15,26-28,34,35,37,38].

SCORE SYSTEMS

Life expectancy in metastasized tumor diseases is estimated with well known and evaluated score systems. The Karnofsky-Index [Table 2] dating from 1948 describes patients' general condition and physical resilience^[17,21,39]. Furthermore, the Karnofsky-Index is part of different score systems (Tokuhashi and revised Tokuhashi score, Oswestry Disability Index, Van-der-Linden-Score)^[18,20,21,39,40]. Patients describe pain with the numeric or visual analogue scale (NAS/VAS)^[41]. Vertebral stability is estimated by the Spinal Instability Neoplastic Score (SINS) [Tables 3 and 4]^[21,42], Harrington Score^[21,43] or Taneichi score^[44]. The revised Tokuhashi score [Tables 5 and 6]^[18,34] and Tomita score [Tables 7 and 8]^[19] are worldwide accepted to determine individual life expectancy in malignancy and to plan optimal treatment options. The modified Bauer score^[16], Van-der-Linden score^[20] and Oswestry-risk index^[39] are named for the sake of completeness.

The revised Tokuhashi^[18] and Tomita score^[19] merely discern tumor entities (breast, prostate, lung, thyroid) and in both scores breast cancer is assessed with a favorable prognosis. The four different phenotypes of breast cancer are not included. A life expectancy limited to 5 to 9 months was published until 2015 concerning HER-2 enriched and triple negative phenotypes^[2-6,9,10,13-15,26-28,34,35,37,38,45]. Therefore, modifications in the revised Tokuhashi^[18] and Tomita score^[19] were suggested^[2,3] and highly invasive anterior-posterior spine surgery should be evaluated critically. Based on present-day knowledge we can no longer emphasize these recommendations. Due to better oncologic treatment options actual studies estimate mean survival times of 15-19 months with regard to the triple negative and 56.5 months concerning HER-2 enriched phenotypes^[4,6,7,10,15,26]. A mean survival time of 15-19 months implies that several patients live longer and

Table 3. Spinal Instability Neoplastic Score for the determination of vertebral body stability from 2010^[23]

SINS parameter	Description	Score
Location	Junctional (C0-2, C7-T2, T11-L1, L5-S1)	3
	Mobile spine (C3-6, L2-4)	2
	Semirigid segments (T3-10)	1
	Rigid segments (S2-5)	0
Mechanical pain	Yes	3
	Sometimes	1
	No	0
Bone lesion	Lytic	2
	Lytic/blastic (mixed)	1
	Blastic	0
Spinal alignment	Subluxation/translation present	4
	<i>De-novo</i> -deformity (Kyphosis/Scoliosis)	2
	Normal alignment	0
Vertebral body collapse	> 50% collapse	3
	< 50% collapse	2
	No collapse with > 50% body involved	1
	None of the above	0
Posterolateral involvement	Bilateral	3
	Unilateral	1
	None of the above	0

Table 4. Interpretation of Spinal Instability Neoplastic Score^[23]

Score	Stability
0-6	Stable
7-12	Potentially unstable
13-18	Unstable

Table 5. Revised Tokuhashi-Score parameter^[48]

Score	0	1	2	3	4	5
Karnofsky-score (%)	10-40	50-70	80-100			
Extraspinal bone metastases	3 or more	1-2	0			
Spinal metastases	3 or more	2	1			
Frankel	Frankel A/B	Frankel C/D	Frankel E			
Visceral metastases	Unremovable	Removable	None			
Primary site of cancer	Lung Stomach Bladder Esophagus Pancreas Osteosarcoma	Liver, Gall Bladder Unidentified	Others	Kidney Uterus	Rectum	Breast Thyroid Prostata Carzinoid

Table 6. Interpretation of the revised Tokuhashi-Score^[48]

Score	Prognosis of survival	Recommendation
0-8	85% of patients survive up to 6 months	Conservative therapy or palliative surgery
9-11	73% of patients survive > 6 months; 30% > 12 Monate	Palliative surgery or in exceptions tumor excision
12-15	95% of patients survive > 12 Monate	Tumor excision

these patients have to be filtered out. According to Tomita *et al.*^[19] and Tokuhashi *et al.*^[18] anterior spine surgery is indicated with a life expectancy of 12 months or more. If the patient is in good general condition and asks for surgery, we therefore recommend anterior-posterior techniques after critical evaluation. Furthermore, in 2012 Majeed^[45] evaluated 55 patients according to the revised Tokuhashi^[18] and Tomita score^[19] and reported continuously longer survival times than initially estimated. Majeed^[45] concluded, that prognosis of survival times is not reliable with the current scoring systems.

Table 7. Tomita-Score^[50]

Prognostic factor	Score
Tumor growth	
Slow (Breast, Prostate, Thyroid)	1
Moderate (Kidney, Uterus)	2
Rapid (Lung, Liver, Stomach, Colon, CUP)	3
Visceral metastases	
None	0
Treatable	2
Not treatable	4
Bone metastases	
Solitary	1
Multiple	2

Table 8. Interpretation of the Tomita-Score^[50]

Score	Mean survival time (months)	Treatment goal	Recommendation
2-3	50	Local long-term monitoring	Wide or marginal excision
4-5	23.5	Local mid-term monitoring	Tumor bordering or intralesional excision
6-7	15	Local short-term monitoring	Palliative surgery
8-10	6	Terminal supply phase	Limited palliative surgery or no operative intervention

THERAPEUTIC OPTIONS REGARDING SPINAL METASTASES

With an incidence of 85% breast cancer metastases to the spine may lead to instability with pain, pathologic fractures and neurologic deficits in up to 10%^[21,29,46]. Furthermore, there are severe socioeconomic aspects with 50% of woman have to change the working environment and 37% of woman involved can temporarily or permanently work no longer^[4]. The primary therapy of painful metastases to the spine without relevant loss of stability is radiation and spine surgeons are not necessarily involved. Potentially unstable and painful lesions with a SIN Score > 7^[42] (+/-neurological deficits) are demonstrated to the consulting spine surgeons. Mandatory in every patient is a critical individual evaluation of prognosis to choose correct therapy options^[4,8,11,14,21,29,35,38,45-48]. Improving or maintaining the quality of life is the decisive therapeutic target in an incurable palliative situation. Wishes and priorities of these patients beside the status of metastases, previous therapy lines and general condition mainly influence the appropriate treatment^[4].

Beside local radiation orthotic devices are the main conservative treatment tools to stabilize the spinal column and reduce pain. Concomitantly as systemic osteoprotective therapy a bisphosphonate in combination with calcium and Vitamin D or Denosumab (monoclonal antibody) mark the standard additive medication in advanced breast cancer with bone metastases. With the best response in diverse tumor entities up to 62% of recalcification post radiatio is described in breast cancer spinal metastases^[4,8,21,47,49,50].

Various surgical treatment options can reduce pain and stabilize the spine. Bilateral, percutaneous balloon kyphoplasty as a minimal invasive treatment tool^[13,36,46,47,49,51-54] may not restore vertebral height but correlates with pain reduction. Thermal ablation of vertebral metastases with radio frequency ablation (RFA)^[50,53,55-57] may be combined with kyphoplasty to reduce the likelihood of tumor recurrence. Posterior instrumentation with a screw and rod system is the gold standard in spine surgery to stabilize unstable tumor lesions. In case of spinal stenosis due to tumor the decompression of neural structures is reached via laminectomy and tumor debulking^[13,29,43,46,47,50,52-55]. According to Tomita *et al*^[19] palliative anterior surgery with vertebral body replacement (VBR) [Figures 1-3] can be recommended in patients with a life expectancy > 12 months^[46,47,58,59]. Highly invasive surgical options like *en-bloc* spondylectomy in Tomita technique or vertebral column resection with a mandatory 360 reconstruction [Figure 4] mark curative treatment options in case of solitary spinal metastases^[46,47,58,59].

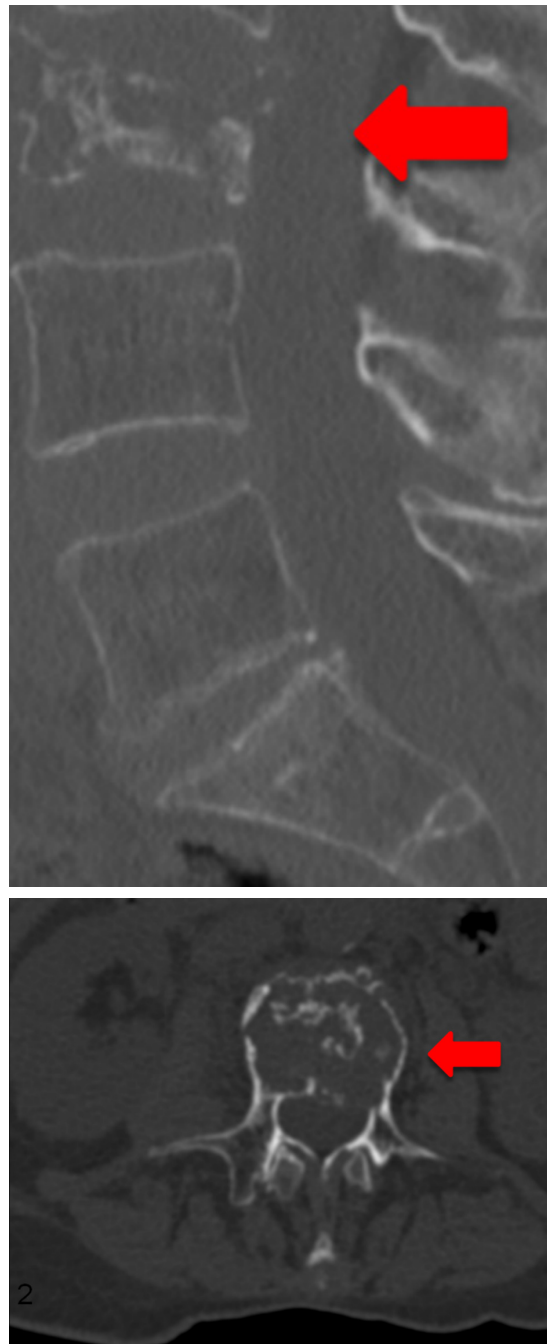


Figure 1. 73-year-old female patient with multiple metastatic breast cancer ER: 95%; PgR: 0%; HER2-Score 1+; Ki-67: 20%. Sagittal and axial section of a total spine CT scan: unstable metastatic destruction of L3

The various techniques mentioned above can be combined as required. Additional measures like (partial-) resection of soft tissue, thoracic wall or pelvis with potential correction or reconstruction of deformity complete the portfolio^[47]. Modern adjuvant oncologic concepts comprise radio-, chemo- or hormone therapy^[8,47,50].

Due to the wide range of therapy options mentioned above it is impossible for a sole attending physician to determine the individual therapeutical regimes. Such complex decision making demands an interdisciplinary setting like a tumor conference or board^[4]. The spine surgeon determines the individual therapy of



Figure 2. Sagittal section (T2-weighted) of a total spine MRI with contrast agent: metastatic destruction of L3 and multiple metastases

spinal metastases in breast cancer patients. An improved quality of life results of preserved mobility and autonomy with less pain. If possible, pathologic fractures and neurologic deficits up to paraplegia should be avoided^[2,3,8,18,19,29,42,43,47,50].

SPECIAL CASE: SOLITARY METASTASIS

Prognostic statements are especially important in case of a solitary, locally curable metastasis (principle of limited metastasis^[4]) and a treatable primary tumor in a curative way. For us the question was, if solitary metastasis with any phenotype is always to be treated the same way [Figure 5].

Either a Ct-controlled or an open biopsy finally ensures the histopathological phenotype. Until the year 2015 the curative therapeutic approach with *en-bloc* spondylectomy according to Tokuhashi *et al*^[18] and Tomita *et al*^[19] was only recommended in hormone positive receptor status (luminal A and B) with a median survival time of 26 months^[2,13]. In triple-negative or Her-2 enriched phenotypes with an estimated survival time of 5-9 months, a limited posterior instrumentation (screw and rod system) and decompression of neural structures was indicated^[2,13]. Modern oncologic treatment concepts provide clearly longer survival times and therefore, according to Tokuhashi *et al*^[18], curative treatment options (mean survival time > 12 months) can principally be applied to all phenotypes in breast cancer with solitary metastasis. In the worst case (triple-negative phenotype) the mean survival time is actually reported with 15-19 months^[2-4,10,34,35].

Indicating *en-bloc* spondylectomy will never be an automatism and controversial discussions in tumor boards can be expected. Critically evaluation of the individual wish, priorities, general condition,



Figure 3. Postoperative sagittal total spine radiograph: posterior instrumentation L1-L5 with a screw and rod system, decompression via laminectomy, anterior stabilization with an insitu distractable vertebral body replacement of L3

comorbidities, previous lines of treatment and the biological age must influence the decision^[4]. In tumor conferences a (neo-)adjuvant systemic therapy (endocrine therapy, Anti-HER-2 therapy, chemotherapy, osteoprotective therapy) is determined according to tumor load (TNM system) and biology. An adjuvant loco regional radiation therapy is mandatory in case of short edges of the resected areas in preparation slides^[4].

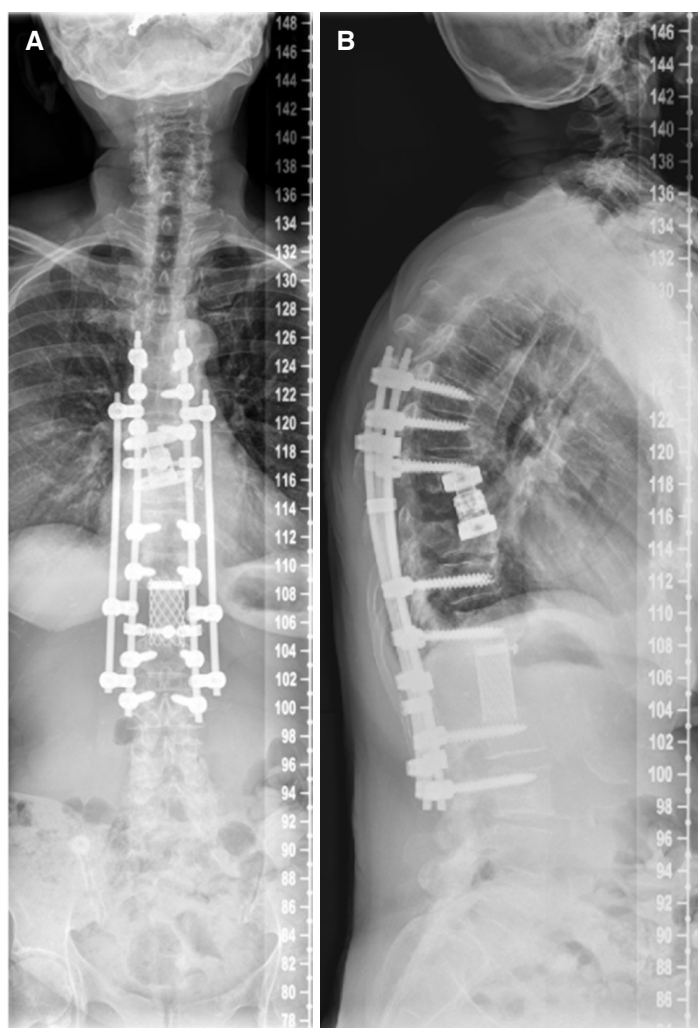


Figure 4. Two plane full spine radiographs of a 65 year old female patient with metastatic breast cancer(A, B). En-bloc spondylectomy in Tomita technique at the Th9 and L1 level with a time interval of 10 years. Anterior in situ distractible cage at the Th9 level and Harms cage at the L1 level, posterior 4-rod instrumentation Th6-L3

CONCLUSION

Evaluating prognosis by means of molecular tumor typing, previous course of disease and actual status of metastases is mandatory prior to elective surgery of breast cancer spine metastases.

Mean survival times of 60 months in endocrine responsive (Luminal A and B) phenotypes, 50 months in HER-2 enriched phenotypes and 15-19 months in triple negative phenotypes are described in actual literature.

The patients's general condition, biological age, intrinsic breast cancer subtype, comorbidities, tumor burden (number of metastases to organs) and previous therapies influence decision making.

Regardless of molecular phenotype solitary spinal metastases may be treated with a curative therapeutic approach.

Close cooperation of all experts participating interdisciplinary tumor boards are essential to determine adequate therapy strategies.

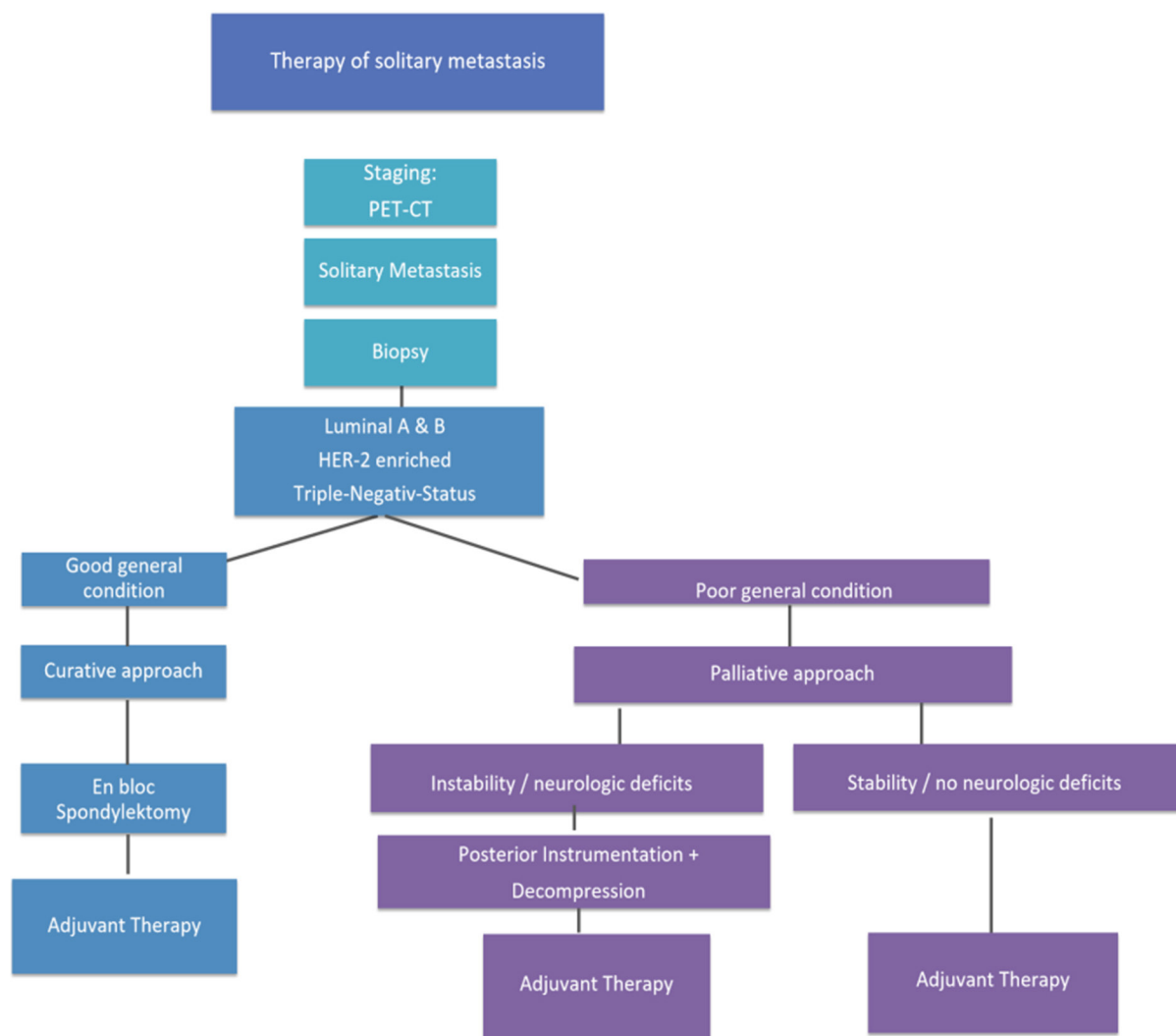


Figure 5. Heidelberg Spine Research Group Therapeutic Algorithm of Solitary Spine Metastasis in Breast Cancer

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: Adler D, Akbar M

Performed data acquisition, as well as provided administrative, technical, and material support: Pepke W

Availability of data and materials

Data is available for readers.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Original Article

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Differential expression and function of the endogenous lactate receptor, GPR81, in ER α -positive/HER2-positive epithelial vs. post-EMT triple-negative mesenchymal breast cancer cells

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Abstract

Aim: Lactate can signal through the endogenous lactate receptor, GPR81, which is expressed in some cancers. Lactate metabolism is altered by the metastasis-promoting process of epithelial-mesenchymal transition (EMT). This study examined the expression and function of GPR81 in breast cancer samples, and in receptor-positive epithelial vs. triple-negative post-EMT mesenchymal breast cancer cells.

Methods: GPR81 mRNA expression was examined by breast cancer microarray, and by a Kaplan-Meier survival curve. Using 3-dimensional culture conditions, GPR81 mRNA expression in epithelial and mesenchymal breast cancer cell lines was measured by qRT-PCR. GPR81 siRNA was used to assess the role of GPR81, alone or in conjunction with tamoxifen, in the regulation of MCT1 and MCT4 lactate transporters, intracellular lactate, and cell proliferation and survival.

Results: GPR81 mRNA levels were elevated in receptor-positive breast cancer, relative to non-tumor and triple-negative samples, and correlated with increased survival. GPR81 expression was elevated in the two epithelial breast cancer cell lines vs. the corresponding post-EMT mesenchymal cell lines. GPR81 knock-down in epithelial MCF7 cells caused: 1, selectively lower mRNA and protein expression of the MCT1 transporter, but not MCT2 or MCT4



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transporters; 2, lower levels of intracellular lactate; and 3, decreased proliferation and survival in lactate only-containing conditions. GPR81 siRNA plus tamoxifen displayed additive suppressive effects on MCT1 expression and cell viability.

Conclusion: GPR81 promotes the ability of epithelial breast cancer cells to import lactate for energy use. As such, GPR81 represents a potential target for treatment of hormone-positive breast cancer cells, and may be prognostic for higher grade breast cancer.

Keywords: GPR81, lactate metabolism, MCT transporters, MCT1, tamoxifen

INTRODUCTION

Invasive breast cancer (IBC) is the most predominant form of cancer among women worldwide and the second leading cause of cancer-related deaths in women in the United States^[1,2]. Moreover, breast cancer is a complex and heterogeneous disease, which includes a range of pathological subtypes associated with different clinical behavior, treatment options and outcomes. The most common subtype of IBC, termed Luminal A, is characterized by an adhesive epithelial phenotype and defined by the overexpression of the estrogen receptor α (ER α) that underlies the endocrine-based treatment of this subtype^[3]. Although Luminal A IBC is a relatively non-aggressive type with a good response to adjuvant endocrine therapy and a high survival rate, failure of early detection is associated with metastatic disease, which in turn is responsible for the majority of deaths in breast cancer patients.

The metastatic dissemination of epithelial IBC cells is initiated by the reprogramming of cellular regulatory pathways that induce a coordinated downregulation of cell-cell adhesion and the acquisition of motility and invasiveness in a subpopulation of cancer cells. Epithelial-mesenchymal transition (EMT) has been proposed as a key regulatory program that drives these early metastasis-related changes^[4]. The process of EMT can be induced by exposure of cancer cells to an altered microenvironment (e.g., hypoxia, pro-inflammatory cytokines), often in conjunction with somatic mutations^[5,6].

We previously reported that prolonged mammosphere culture induced EMT in two distinct epithelial breast cancer cell lines, MCF-7 and BT-474 cells, and generated stable populations of mesenchymal cancer cells, MCF-7M and BT-474M^[7,8]. EMT promoted a more glycolytic phenotype compared to the parental epithelial breast cancer cells that primarily use oxidative phosphorylation to produce ATP (submitted manuscript). The altered metabolic phenotype induced by EMT involved enhanced aerobic glycolysis (i.e., the Warburg effect) along with higher rates of lactate production and extracellular acidification.

As a result of the glycolytic shift induced by EMT, mesenchymal cells co-export higher amounts of lactate and protons to the extracellular microenvironment, thereby creating an acidic microenvironment that has been associated with tumor progression^[9]. Elevated levels of lactate within the tumor microenvironment have been associated with metastasis and poor prognosis of cancer patients^[10-12]. Once exported, lactate can act both as a carbon source for other cells and as a signaling molecule. For example, lactate produced by highly glycolytic cancer cells can be utilized by neighboring oxidative cancer cells for ATP production, a phenomenon known as “metabolic symbiosis”^[13,14]. As a “signaling molecule”, lactate can regulate migration, angiogenesis, immune escape and metastasis^[15-17].

Intracellular and extracellular lactate levels are determined, in part, by different isoforms of the lactate/H⁺ symporter, termed monocarboxylate transporters (MCTs)^[18,19]. MCT isoforms are lactate/proton co-transporters, and thus contribute to the acidification and lactate accumulation and signaling in the tumor microenvironment^[20].

However, different isoforms have different affinities for lactate and pyruvate, which influences whether they act predominantly as cellular exporters or importers of lactate and H⁺. MCT1 is a ubiquitous lactate/H⁺ symporter that is involved in both import and export of lactate, whereas MCT4 is primarily involved in lactate/H⁺ export. Both MCT1 and MCT4 are differentially expressed in several cancers, including breast cancer^[21]. Additional important players in lactate shuttling between cancer cells are the lactate dehydrogenases A and B (LDHA and LDHB) that catalyze the reversible conversion of pyruvate to lactate, alter the NAD⁺/NADH ratio and thus glycolytic flux, and have also been implicated in EMT and metastasis^[22].

Previous studies have identified the cell-surface G-protein-coupled receptor, GPR81, as an endogenous lactate receptor. GPR81 family of receptors consists of three highly homologous members, GPR109a, GPR109b and GPR81, which are regulated by the specific agonists, 3-hydroxybutyrate, 3-hydroxyoctanoate, and lactate, respectively^[23]. GPR81 has been mainly studied in adipocytes, in which the receptor is coupled to Gi/q. GPR81 activation by lactate decreases cAMP production, which ultimately reduces lipolysis^[24]. Recently GPR81 has been shown to be upregulated in several cancers (pancreas, colon, liver, breast, cervix) and its expression levels associated with tumor growth and metastasis^[25-29]. In this current study, we examined GPR81 expression in the context of EMT in breast cancer. We provide evidence that GPR81 promotes lactate transport by MCT1 in hormone-positive breast cancer cells and its expression is crucial for their cell proliferation and survival.

METHODS

Cell culture

BT-474 and MCF-7 cell lines were obtained from the American Type Culture Collection (Manassas, VA). EMT in MCF-7 and BT-474 cells was induced using prolonged mammosphere culture method as reported previously^[7,8]. BT-474 and MCF-7 and their corresponding EMT-derived BT-474-M and MCF-7-M cells were grown in DMEM/F-12 containing 10% heat inactivated FBS (Gibco, Grand Island, NY) and 1 × MycoZapTM Plus-CL antibiotic (Lonza, Walkersville, MD). BT-474 and MCF-7 cells were additionally supplemented with 1 × Insulin-Transferrin-Selenium solution (Gibco, Grand Island, NY). All cells were incubated at 5% CO₂ and at 37 °C.

3-dimensional culture

CorningTM MatrigelTM GFR (Fisher Scientific) or MatrigelTM (Corning) was used as culture matrix following a modified version of the previously described protocol for 3-dimensional on-top assay^[30]. Twenty-four-well plates were coated with 200 μL of the mix of 1:1 MatrigelTM/Physiological modified medium supplemented with 17 μmol/L Insulin-Transferrin-Selenium solution (Gibco, Grand Island, NY) and kept at 37 °C for 30 min to allow the MatrigelTM to solidify. All cell lines were suspended in physiological modified medium supplemented with 1× insulin and seeded into the coated twenty-four-plates. Epithelial and mesenchymal cells were seeded in 250 μL at 100,000 cells/well and 20,000 cells/well, respectively, and incubated at 37 °C for 30 min to allow cell attachment to the Matrigel. All wells were overlaid with 250 μL of physiological modified medium containing 4% MatrigelTM. Culture medium was replaced with fresh medium daily and cells were used for each assay after seven days, unless otherwise indicated.

Physiological modified medium and drugs

RPMI 1640 Medium Modified without L-glutamine, amino acids and glucose (powder, R9010-02; US Biological, Swampscott, MA) was supplemented with the following: from Sigma (St. Louis, MO) - 1X MEM amino acid mixture (minus glutamine) (M5550), 1× Lipid Mixture 1 (L0288), 14.3 mmol/L sodium bicarbonate powder (S5761); from ThermoFisher/Gibco, (Waltham, MA) -1× non-essential amino acid mixture (11140), 10% heat-inactivated FBS (16000069) and 15 mmol/L HEPES (15630-106); from (Lonza, Walkersville, MD) - 1× MycoZapTM Plus-CL antibiotic.

Unless otherwise noted, this medium was also supplemented with physiological levels of: glucose (5 mmol/L; Gibco A24940), sodium pyruvate (0.1 mmol/L; Gibco 11360-070), Na-lactate (1 mmol/L; Sigma L7022) and L-glutamine (0.5 mmol/L; Sigma G7513. This same medium, but without phenol red (powder, R9010-01; US Biological, Swampscott, MA, was used in assays where cells were treated with β -Estradiol or Tamoxifen.

MCT1 inhibitor SR 13800, β -Estradiol and Tamoxifen (Tocris Bioscience, Bristol, UK) solutions were used at concentrations of 100 nmol/L, 10 nmol/L and 1 μ mol/L, respectively.

Imaging of 3-dimensional structures

Cells were cultured in 3-dimensional Matrigel and glass bottom 24-well plate (MatTek Corporation, MA) for seven days. Phase contrast images of the 3-dimensional structures of epithelial and mesenchymal cells were imaged using a Zeiss Axio Observer inverted microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY) equipped a QImaging Retiga EXi CCD digital camera (QImaging, Surrey, BC). For immunofluorescence images cell lines grown in 3-dimensional Matrigel at the seventh day were fixed in 2% formaldehyde in 1 \times PBS for 20 min. Cells were washed with 1 \times PBS and then treated with 0.1% Triton-X 100 for 10 min. Cell were washed with 1 \times PBS and blocked with 1% bovine serum albumin (BSA) for 20 min. After blocking, cells were incubated with F-actin-staining solution for 20 min at room temperature. F-actin-staining solution was prepared with 5 μ L Alexa-488 Phallotoxin (Invitrogen, Carlsbad, CA) stock in 200 μ L 1 \times PBS for each well to be stained. SYTOXTM orange dye (Invitrogen, Carlsbad, CA) was used to stain nuclei. Images were captured using a Zeiss Pascal confocal system with a 40 \times 1.2 NA objective (Carl Zeiss Microscopy, Narashige, MN).

Transient transfection of siRNA

MCF-7 cells were seeded in 250 μ L at a density of 150,000 cells/well in twenty-four-well plates coated with 200 μ L of the mix of 1:1 MatrigelTM/Physiological modified medium supplemented with 17 μ mol/L Insulin-Transferrin-Selenium solution (Gibco, Grand Island, NY) and transiently transfected with Trilencer-27 human siRNA (siNT and siGATA3 "A") at a final concentration of 10 nmol/L (Origene, Rockville, MD) with LipofectamineTM RNAiMAX transfection reagent (Thermo Scientific, Rockford, IL). After 8 h of transfection cells were overlaid with 250 μ L of physiological modified medium containing 4% MatrigelTM. Culture medium was replaced with fresh medium every day and cells were used for each assay after 72 or 96 h after transfection.

Growth and viability assays

MCF-7 cells were seeded at a density of 150,000 cells/well and recovered from Matrigel by incubating them with 5 mM EDTA (Gibco, Grand Island, NY) in ice cold 1 \times PBS for 30 minutes at 4 $^{\circ}$ C and centrifuged for 5 min at 1000 rpm. Recovered cells were digested with 0.25% trypsin for 4 min and resuspended in DMEM with 10% FBS. The cell number was calculated on hemocytometer at specific time point. Viability assays were performed three times for each cell group.

Real-time qPCR and primers

Total RNA was isolated from cell lines using TRIzol Reagent (Ambion RNA, Carlsbad, CA), following the manufacturer's instruction. cDNA was synthesized 1 μ g of total RNA using iSCRIPT cDNA synthesis kit (Bio-Rad, Hercules, CA). SYBR green based SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA) was utilized to perform real-time PCR from 50 ng of cDNA. Gene expression was normalized to TATA-box binding protein using $2^{-\Delta C_t}$ method. Specificity of the primer sets was confirmed by melting curve analysis.

cDNA arrays

Real-time qPCR was performed in two TissueScanTM breast cancer and normal tissue cDNA arrays I and II (Origene, Rockville, MD). Gene expression was normalized to normal tissue using $2^{-\Delta C_t}$ method.

Glucose uptake and lactate production

Cells were cultured and transfected in 3-dimensional MatrigelTM with physiological modified medium for 72 or 96 h and medium was changed daily. Glucose and lactate concentrations in the culture medium were determined by fluorometric based Glucose Assay Kit and Lactate Assay Kit (BioVision, Inc., Milpitas, CA) according to the vendor's instructions using Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT). The amount consumed or produced by cells was determined by comparing the concentration in the medium incubated without cells and then normalized to cell number. These assays were performed three times with two experimental replicates for each cell type.

Western blot and antibodies

Cells were collected from 3-dimensional MatrigelTM culture (as described previously) and lysed with RIPA buffer (TEKnova, Hollister, CA) containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Rockford, IL). The lysates were clarified by centrifugation at 4 °C for 10 min at 12,000 ×g. The concentration of protein in the supernatants was measured using BCA assay (Thermo Scientific Pierce, Rockford, IL). Total proteins (20 µg/lane) were separated on a 10% SDS-PAGE and were transferred to nitrocellulose membrane. The membranes were blocked with 5% BSA in 1× TBST (β-actin) or with 5% non-fat milk (MCT1, MCT4 and Cleaved PARP) and subsequently incubated with primary and secondary antibodies according to the vendor's instructions. Blots were visualized by using Amersham ECL prime (GE Healthcare Life Sciences, Buckinghamshire, UK) and imaged using G:box imaging system (SynGene, Cambridge, UK). Primary antibodies to Cleaved PARP (1:1000) was given by Dr. Claffey from Cell Signaling Technology (Danvers, MA); β-actin (1:2500) and MCT1 (1:1000) were purchased from Abcam (Cambridge, MA). MCT4 (1:1000) was purchased from Millipore (Billerica, MA). All images were imported to Image J for pixel grayscale intensity analysis. Western blot analysis was performed three times using two independent protein samples for each cell group.

Nuclear magnetic resonance spectroscopy and data analysis

Cells were collected from 3-dimensional MatrigelTM culture (as described previously) and lysed with 1:1:1 methanol/chloroform/water in sequence with 5 s of gyration between additions. In order to optimize chemical extraction, non-trypsin-based methods were employed as follows^[31]. A 10 µL sample was then removed for cell counting. Resulting lysed solution was then allowed to separate overnight at 4 °C to form resultant aqueous and organic layers. Aqueous layer containing metabolites of concern were then manually extracted and resulting solution was subjected to addition of Chelex (approximate 3 mg/mL) for removal of divalent ions, which was then removed through vacuum filtration using Buchner filter. Solution was then lyophilized overnight to form solid components. 800 µL of D₂O with 10 mmol/L EDTA and 1 mmol/L 4, 4-dimethyl-4-silapentane-1-sulfonic acid (DSS) was added and solution was centrifuged at 3000 rpm for 5 min prior to placement in standard 5 mm nuclear magnetic resonance (NMR) tube for ¹H NMR.

¹H NMR experiments were performed on 500 MHz Agilent spectrometer with HCN cold probe and a VNMRs console. Default pre-saturation pulse sequences were utilized with the following parameters: pulse-width = 7.2 µs, sweep-width = 8389.26 Hz, 2460 complex points, D₁ = 1.5 s, SATPWR = 6 dB on H₂O signal, and 64 transients. Resulting ¹H NMR underwent Fourier Transformation and data was further analyzed in MestReNova NMR software, version 9.0.0. Processing template for all spectra had the following time domain corrections: drift correction to tail points of 5% and apodization through an exponential of 1.5 Hz. The frequency domain had global phase correction and a baseline correction using Bernstein polynomial of $n = 3$. Reference signal was set as the singlet produced off of DSS at 0 ppm. Lactate and Pyruvate were identified on the spectra through prior titration analyses and the absolute area under the curve was used for their methyl groups with respect to methyl groups found off of DSS with the usage of Line-Shape analysis. These areas were used to calculate concentrations by a ratio of the absolute area to the number of protons

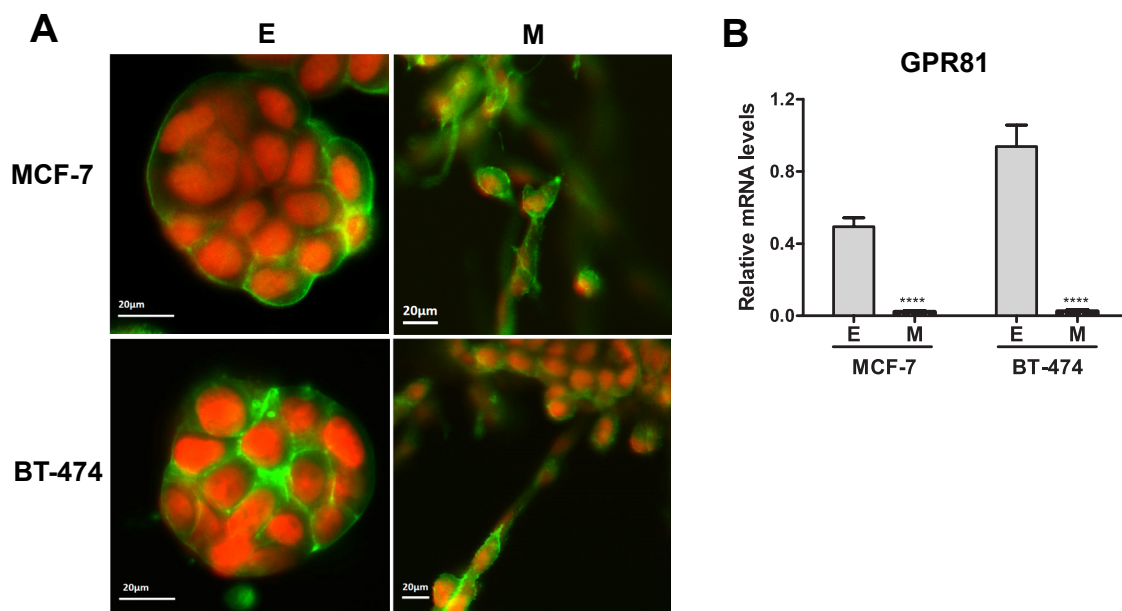


Figure 1. GPR81 expression of epithelial MCF-7 and BT-474 cells and mesenchymal MCF-7M and BT-474M in 3-dimensional Matrigel culture. A: fluorescence microscopy images of 3-dimensional structures of proliferating epithelial (E) and mesenchymal (M) cells in 3-dimensional Matrigel with physiological modified medium for 7 days. Green, Phalloidin staining of F-actin; red/orange, nuclei counterstained with SYTOXTM orange nucleic acid (red); B: real-time qPCR analysis of GPR81 in epithelial (E) and mesenchymal (M) cells. Results are presented as mean \pm S.E.M. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ (ordinary one-way ANOVA)

in the signal used. Resulting concentrations in millimolar were then exported into an Excel spread sheet containing cellular counts to determine a normalized concentration per number of cells.

Statistical analysis

Ordinary one-way ANOVA followed by Sidak's multiple comparison post-test or two-way ANOVA was performed to determine statistical significance. $P < 0.05$ was considered to be significant. Statistical analysis was performed using GraphPad Prism software (La Jolla, CA).

RESULTS

Endogenous lactate receptor GPR81 is upregulated in epithelial breast cancer cells

Epithelial (E) MCF-7 and BT-474 cell lines and the corresponding post-EMT mesenchymal (M) MCF-7M and BT-474M were cultured in a more physiologically relevant system using 3-dimensional Matrigel and physiological modified medium (referred as MPM culture). Under these conditions epithelial breast cancer cells grew as tightly adherent spheroids for 1 week [Figure 1A; "E" boxes]. The two mesenchymal cell lines also formed tight spheroids initially, but after 2 days began invading the Matrigel [Figure 1A; "M" boxes]. After 1 week of MPM culture, cells were examined for the expression of the endogenous lactate receptor GPR81. Epithelial spheroids expressed relatively high levels of GPR81, whereas mesenchymal cells showed no or very low expression [Figure 1B].

GPR81 is highly expressed in human hormone-receptor-positive breast cancer tissues

To determine whether GPR81 was present in human breast cancer tumors, we analyzed mRNA gene expression profiles from 74 human breast cancer samples and 12 non-tumorigenic (NT) breast tissues [Figure 2A]. We grouped these samples in non-tumorigenic (NT), estrogen receptor positive (ER⁺), amplification of the human epidermal receptor growth factor 2 (HER2⁺) and triple negative breast cancer (TNBC) according to their clinical information provided by the supplier. Higher levels of GPR81 mRNA were noted in hormone-

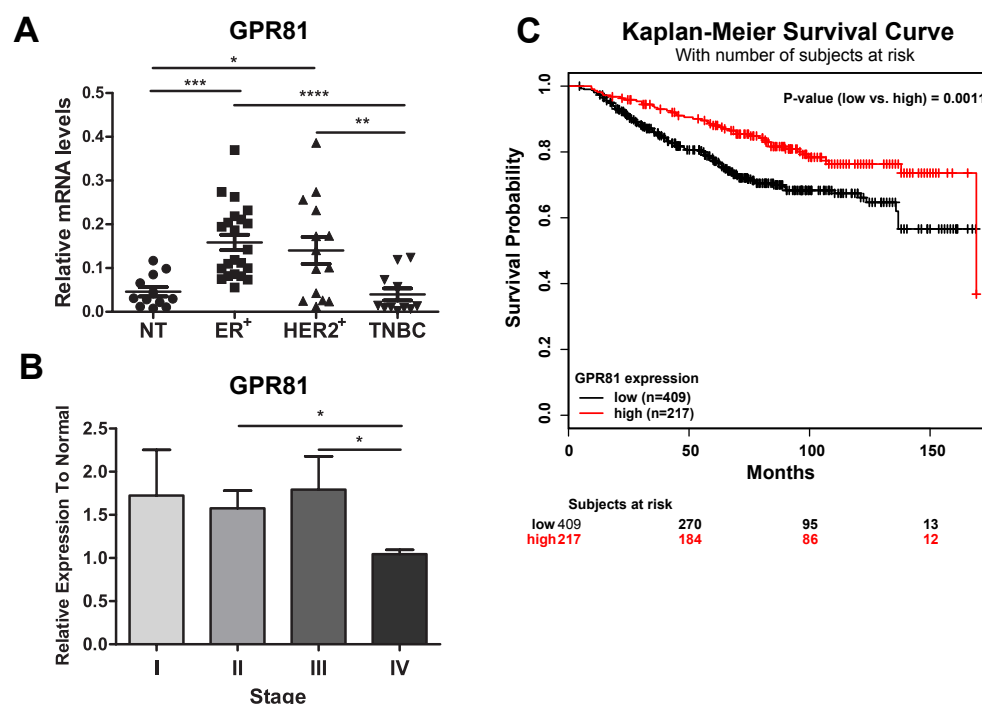


Figure 2. GPR81 is highly expressed in human hormone-receptor-positive breast cancer tissues. A: relative mRNA expression of GPR81 in ER⁺ ($n = 32$), HER2⁺ ($n = 17$) and TNBC ($n = 12$) breast cancer tissues to normal tissue ($n = 12$); B: relative mRNA expression of GPR81 in stage I ($n = 21$), II ($n = 32$), III ($n = 25$) and IV ($n = 4$) of breast cancer; C: Kaplan-Meier plot of estimated overall survival in breast cancer patients ($n = 626$) with low expression of GPR81, below the median (black line) or high expression of GPR81, above the median (red line). Log-rank test P -value = 0.0011. Results are presented as mean \pm S.E.M. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (two-way ANOVA)

positive breast cancer samples (ER⁺ and HER2⁺) and significantly lower levels were found in the NT and TNBC samples [Figure 2A]. We also analyzed the relative gene expression of GPR81 by the stage of human breast cancer. Breast cancer stage II-III had significantly higher GPR81 expression than stage IV [Figure 2B]. Additionally, the Kaplan-Meier survival plot showed that high expression levels GPR81 was strongly associated with better prognosis and overall survival in breast cancer patients [Figure 2C] ($P = 0.0011$) ($n = 626$)^[32]. We also tested GPR81 expression in other breast cancer cell lines. Hormone-receptor positive HC1500 cells had higher expression of the lactate receptor and lactate importer compared to the TNBC, MDA-MB231 cells [Supplementary Figure 1A-C]. These results demonstrate that GPR81 is mainly expressed in hormone positive and less aggressive types of human breast cancer.

GPR81 regulates expression of the gene involved in lactate import in MCF-7 epithelial breast cancer cell line

A previous study reported that GPR81 regulates the expression of lactate transport related genes MCT1 and MCT4 in pancreatic cancer cells through an as yet unknown mechanism^[26]. To determine the role of the endogenous lactate receptor GPR81 in the lactate metabolism of estrogen positive breast cancer cells, we silenced GPR81 (~60%) in MCF-7 cancer cells using siRNA (siGPR81) up to four days in MPM culture [Figure 3A]. GPR81 knockdown in MCF-7 cells led to a 2-fold reduction mRNA and protein expression of the lactate importer, MCT1, but had no effect on the lactate exporter, MCT4 [Figure 3B and C]. In addition, GPR81 did not significantly alter the expression of other lactate metabolism-related genes (LDHA, LDHB, MCT2) [Supplementary Figure 2].

GPR81 regulates lactate uptake in MCF-7 epithelial breast cancer cell line

We found that the two epithelial breast cancer cell lines had higher steady state levels of intracellular lactate and pyruvate than the corresponding post-EMT mesenchymal cell lines^[33]. Given that MCT1 is primarily a

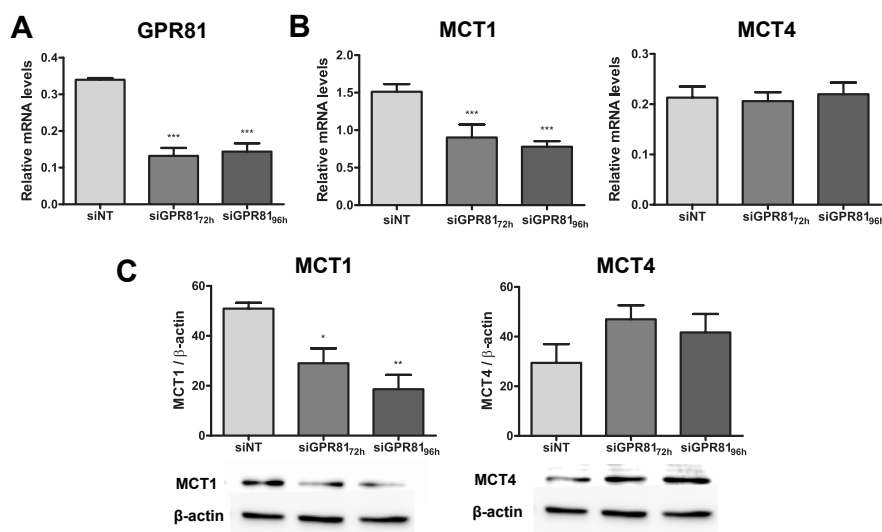


Figure 3. GPR81 regulates expression of the gene involved in lactate import in MCF-7 epithelial breast cancer cell line. A: real-time qPCR analysis of GPR81; B: MCT1 and MCT4 mRNA levels in MCF-7-siNT (control) and MCF-7-siGPR81 cultured in 3-dimensional Matrigel with physiological modified medium at 72 and 96 h after transfection; C: Western blot analysis of cell lysates from MCF-7-siNT and MCF-7-siGPR81 cells used to detect protein expression levels of MCT1 and MCT4. β -actin was used as a loading control. The bars represent the mean \pm S.E.M of three independent experiments; * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001 (ordinary one-way ANOVA)

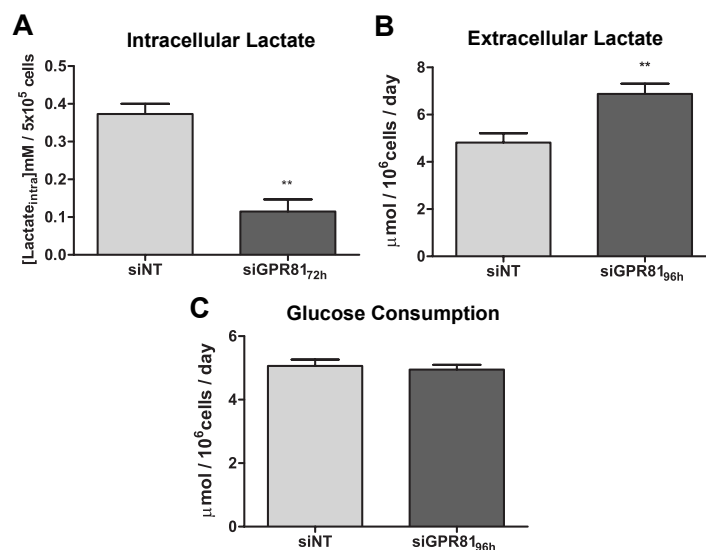


Figure 4. GPR81 regulates lactate uptake in MCF-7 epithelial breast cancer cell lines. A: intracellular lactate of MCF-7-siNT (control) and MCF-7-siGPR81 measured using NMR analysis after 72 h of transfection; B: lactate concentration in the medium of MCF-7-siNT 10 nmol/L and MCF-7-siGPR81 10 nmol/L were measured after 96 h of transfection; C: glucose consumption rate of MCF-7-siNT and MCF-7-siGPR81 were measured after 96 h of transfection. MCF-7-siNT and MCF-7-siGPR81 cells were cultured and transfected in 3-dimensional Matrigel with physiological modified medium and measurements were taken after 24 h of culture in fresh medium. The bars represent the mean \pm S.E.M of three independent experiments; * P < 0.05; ** P < 0.01; (ordinary one-way ANOVA)

lactate importer, we hypothesized that the reduction of MCT1 expression may affect the lactate uptake of the epithelial MCF-7 breast cancer cells. To test this hypothesis, we measured the intracellular lactate of non-targeted control MCF-7-siNT and MCF-7-siGPR81 in MPM culture using NMR analysis. MCF-7-siGPR81 cells had significant lower intracellular lactate compared to MCF-7-siNT cells [Figure 4A], indicating reduced lactate uptake of MCF-7-siGPR81 cells. Additionally, GPR81 knockdown resulted in higher levels of lactate

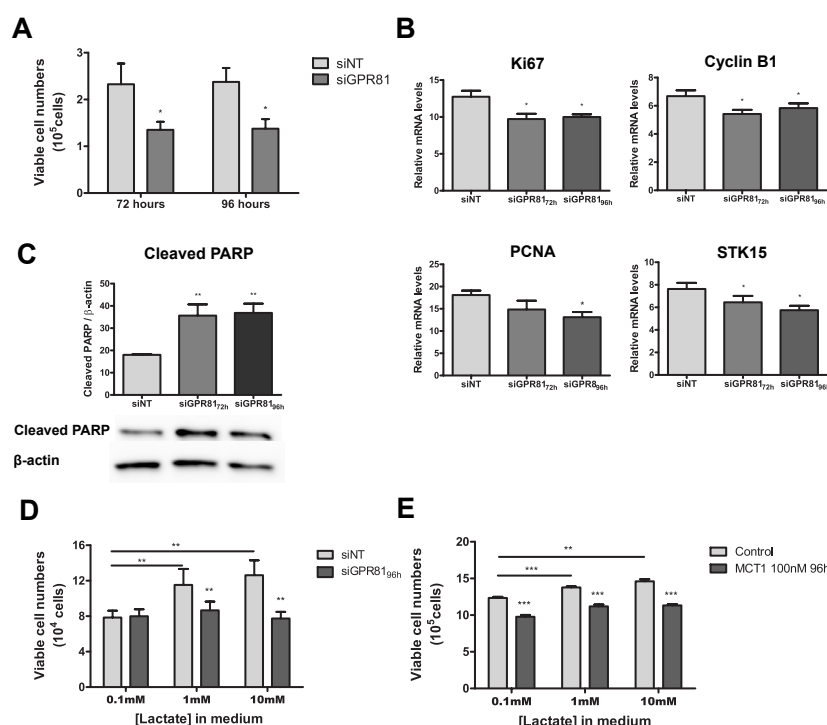


Figure 5. GPR81 is required for cancer cell proliferation and cancer cell survival when lactate is the primary fuel source. A: viable cell counts of MCF-7-siNT (control) and MCF-7-siGPR81 after 72 and 96 h of transfection and subtracting the initial cell number; B: real-time qPCR analysis of proliferation markers: Ki67, Cyclin B1, PCNA and STK15; C: Western blot analysis of cell lysates from MCF-7-siNT and MCF-7-siGPR81 cells after 72 and 96 h of transfection used to detect protein expression levels of the apoptotic marker cleaved-PARP. β -actin was used as a loading control; D: MCF-7-siNT and MCF-7-siGPR81 breast cancer cells were cultured in 3-dimensional Matrigel with physiological modified medium containing only lactate at 0.1 mmol/L, 1 mmol/L and 10 mmol/L concentration for 96 h; E: MCF-7 breast cancer cells were cultured in 3-dimensional Matrigel with physiological modified medium containing only lactate at 0.1 mmol/L, 1 mmol/L and 10 mmol/L concentration and treated with or without MCT1 inhibitor SR 13800 for 96 h. Viable cell counts after treatment. Results are presented as mean \pm S.E.M. of three independent experiments; * P < 0.05; ** P < 0.01; *** P < 0.001 (ordinary one-way ANOVA)

in the culture medium but not control MCF-7-siNT cells [Figure 4B]. We did not detect significant change in glucose consumption [Figure 4C]. Taken together, these data suggest the role for GPR81 in the regulation of the lactate uptake of the epithelial MCF-7 breast cancer cells.

GPR81 is required for cancer cell proliferation and cancer cell survival when lactate is the primary fuel source

Lactate has been previously suggested as an alternative energy source for aerobic breast cancer cells^[13,14,34]. To determine whether GPR81 plays a role in breast cancer cell proliferation, we studied the increase in viable cell numbers and the expression of cell proliferation makers (taken from the OncotypeDx recurrence score assay) in control MCF-7-siNT and MCF-7-siGPR81 cells. Silencing of GPR81 led to about 40% reduction in cell proliferation of MCF-7-siGPR81 [Figure 5A] and decreased relative gene expression of proliferation makers; Ki67, Cyclin B1, PCNA and STK15 [Figure 5B]. Furthermore, the expression of the cell apoptosis marker, cleaved PARP, was significantly increased by 2-fold in MCF-7-siGPR81 cells when compared to control MCF-7-siNT cells [Figure 5C].

To determine whether GPR81 expression is required for cell growth and survival, we cultured control MCF-siNT and MCF-7-siGPR81 cells in 3-dimensional Matrigel with physiological medium lacking glucose, glutamine and pyruvate and with 0.1 mmol/L, 1 mmol/L or 10 mmol/L lactate as the main available nutrient source. Despite of the significant increase in cell number of the MCF-7-siNT cells when cultured in medium containing only 1 mmol/L and 10 mmol/L lactate, GPR81 silencing prevented cell growth under these nutrient-limited

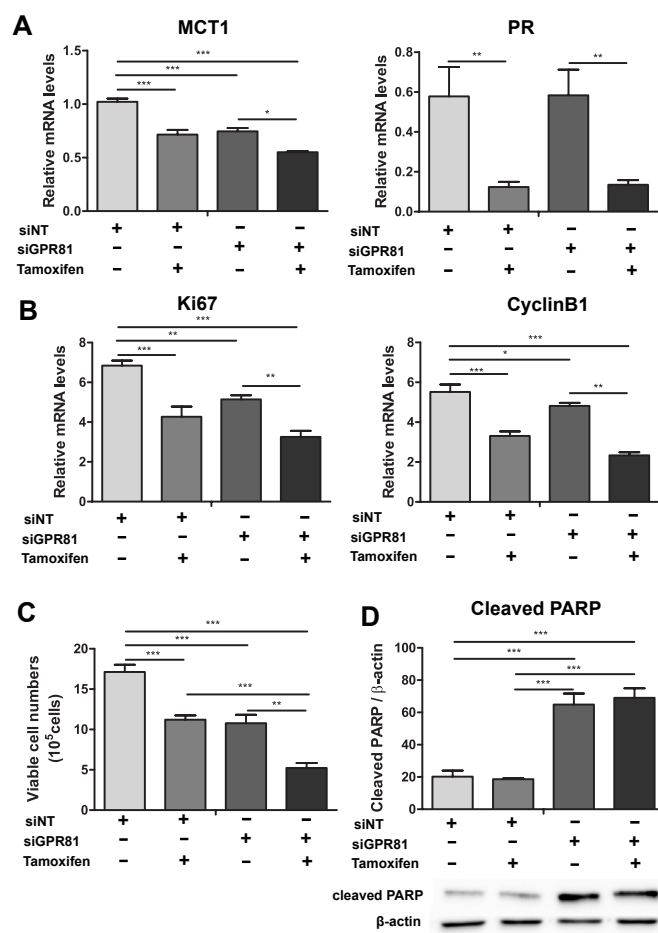


Figure 6. Additive effect of GPR81 knockdown and Tamoxifen treatment in reducing the cell proliferation and increasing cell apoptosis in epithelial MCF-7 breast cancer cells. MCF-7-siNT (control) and MCF-7-siGPR81 cancer cells were treated with or without 1 μ mol/L Tamoxifen for 96 h in 3-dimensional Matrigel with physiological modified medium. A: real-time qPCR analysis of lactate importer MCT1; and progesterone receptor PR; B: relative mRNA expression of proliferation markers: Ki67 and Cyclin B1; C: viable cell counts of epithelial MCF-7-siNT and MCF-7-siGPR81; D: Western blot analysis of cell lysates from MCF-7-siNT and MCF-7-siGPR81 used to detect protein expression levels of the apoptotic marker cleaved-PARP. β -actin was used as a loading control. Results are presented as mean \pm S.E.M. of three independent experiments * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001 (ordinary one-way ANOVA)

conditions [Figure 5D]. Similarly, we found that treatment with the MCT1 inhibitor also prevented cell growth when in MCF-7 cells were culture in nutrient-limited conditions [Figure 5E]. Taken together, these data support the physiological role for GPR81 in the regulation of lactate uptake, cell proliferation and survival of the epithelial MCF-7 breast cancer cells under nutrient-limited conditions.

Tamoxifen treatment in reducing cell proliferation and increasing cell apoptosis has additive effect on GPR81 knockdown

We observed here that 1 μ mol/L Tamoxifen further reduced MCT1 gene expression in GPR81-silenced MCF-7 cells treated for 4 days [Figure 6A]. The effect of Tamoxifen was confirmed by the reduced expression of the progesterone receptor^[35]. We next evaluated the effects of Tamoxifen and GPR81 silencing in cell proliferation and apoptosis in ER+ MCF-7 breast cancer cells. GPR81 knockdown and Tamoxifen treatment had an additive effect on the reduction of cell proliferation markers; Ki67 and Cyclin B1 [Figure 6B], as well as viable cell numbers of MCF-7 cells in MPM culture [Figure 6C]. Additionally, cell apoptosis was significantly increased only in MCF-7-siGPR81 cells independently of Tamoxifen treatment, showed by the increased expression of the apoptotic marker cleaved PARP [Figure 6E]. These data suggest GPR81 regulates cell proliferation and apoptosis.

DISCUSSION

The metabolic changes that cancer cells undergo in order to support macromolecule biosynthesis, growth and survival, are collectively referred to as “metabolic reprogramming”, now considered a hallmark of cancer cell biology^[36,37]. Increasing evidence has linked metabolic reprogramming to metastatic transformation. For example, it has been shown that the EMT-transcription factor SNAIL mediates the suppression of mitochondrial respiration and enhances glycolysis in breast cancer^[38,39]. In the accompanying report in this issue, we demonstrated that EMT induced a metabolic shift to a less oxidative and more glycolytic metabolism in two epithelial cell lines MCF-7 and BT-474, as evidenced by increased glucose uptake and lactate production along with increased expression of enzymes and transporters. These studies suggest that metastatic transformation or EMT facilitates the metabolic shift toward glycolysis in some forms of breast cancer.

We also observed that metabolic reprogramming induced by EMT was associated with a striking switch in the expression of the lactate transporters MCT1 and MCT4. The two epithelial breast cancer cell lines expressed high levels of MCT1 and imported lactate as an alternative energy source. In contrast, the mesenchymal cells showed a marked decreased expression of MCT1 and upregulation of the lactate exporter MCT4. Our findings are consistent with those of Hussein and Brooks^[40], who reported MCT1 was expressed in MCF7 cells, but not in mesenchymal, triple-negative MDA-MB-231 cells, that were maintained in 2-dimensional culture containing high glucose medium. Similarly, MCT4 expression was lower in MCF7 cells as compared to the MDA-MB-231 cells. Using 2-dimensional culture and DMEM/F12 media with high glucose levels, Baenke *et al.*^[41] examined MCT4 expression and function in 17 breast cancer cell lines. Although their findings differed from ours in that MCT4 expression was highest in HER2-positive cell lines, these authors reported that MCT4 suppression increased mitochondrial oxidation, linking MCT4 to a more glycolytic phenotype. This is consistent with MCT4 promoting a glycolytic phenotype, and also raises the prospect that breast cancer cells can adapt to targeted therapies in part through metabolic reprogramming. In contrast to our finding of reciprocal expression between MCT1 and MCT4, Pinheiro *et al.*^[42] used immunohistochemistry to analyze primary breast carcinomas for MCT1 and MCT4, and found MCT1 expression occurring most frequently in the Basal-like subtype and in higher grades of breast cancer. It is also important to note that expression of MCTs does not always correlate to lactate/H⁺ transport. For example, hypoxia-induced carbonic anhydrase IX enhanced the activity of MCT1 to export lactate and H⁺ in MCF7 cells in 2-dimensional culture with physiological (5 mmol/L) glucose levels. MCT4 upregulation was previously reported in triple negative breast cancer and high levels of this lactate exporter were associated with poor prognosis and survival^[41,43].

Lactate has been identified as the ligand for the endogenous cell-surface G-protein-coupled receptor 81 (GPR81). GPR81 was first discovered and mostly studied in adipocytes, in which lactate activates GPR81 to decrease the cAMP production to ultimately reduce lipolysis^[24]. GPR81 is highly expressed in neurons^[44], and when activated, it is able to modify electrical activity of primary neuronal cells^[45]. Also, the activation of GPR81 by lactate has been identified as the initial step for an anti-inflammatory response in pancreas, liver and uterus^[46-48].

More recently, GPR81 expression has been found to be upregulated in several types of cancer, including pancreatic, colon, liver, breast, lung and cervical cancers, and in several cases high GPR81 expression was associated with tumor growth, chemoresistance and metastasis^[25-28]. It should also be noted that previous studies indicate that lactate activates GPR81 in the millimolar range (1-5 mmol/L), and in human breast cancer tissues lactate was found to be at concentrations as high as 8 mmol/L^[14], suggesting that GPR81 and downstream signaling pathways could be constantly activated by lactate in the breast tumor microenvironment. Our study provides the first examination of GPR81 expression in the context of EMT, and indicates that GPR81 expression is dependent on the type of breast cancer cell. We showed that hormone-positive epithelial breast cancer cells highly expressed GPR81. In contrast, EMT induction and

transformation of epithelial breast cancer cells into triple negative mesenchymal cancer cells was associated with significantly reduced expression of GPR81. Lee *et al.*^[25] reported that GPR81 expression is significantly increased in breast cancer patients compared to normal mammary tissues. We also observed a significant association between GPR81 overexpression with ER α -positive and human epidermal growth HER2 positive human breast cancer tissues as opposed to triple negative breast cancer. This is consistent with previous observations that ER α -positive breast cancer tissues overexpressed GPR81^[25]. In addition, GPR81 was expressed at higher levels in the first three stages of breast cancer (Stage I, II and III) as compared to Stage IV, and GPR81 expression correlated with better overall survival of breast cancer patients. These findings suggest that GPR81 may be an important regulator in hormone-positive breast cancer and could be used as a prognostic marker in the progression of breast cancer.

GPR81 was reported to regulate the expression of genes involved in lactate metabolism, including lactate transporters, MCT1 and MCT4, in pancreatic cancer cells^[26]. In partial agreement, our study revealed that GPR81 specifically regulates the lactate importer MCT1, but not lactate exporter MCT4, in epithelial breast cancer cells. Oxidative breast tumor cells with high expression of the lactate importer, MCT1, have been reported to import and oxidize extracellular lactate, a mechanism that is essential for cell viability under glucose deprivation^[14,34,49]. In fact, Lee *et al.*^[25] showed that epithelial breast cancer cells imported and utilized ¹⁴C-lactate for mitochondrial respiration. We previously showed that inhibition of MCT1 reduced cell proliferation in epithelial but not mesenchymal cancer cells when lactate was used as the primary extracellular fuel source. Here we report that GPR81 silencing caused downregulation of MCT1 and reduced cell growth in epithelial breast cancer cells grown in complete MPM medium. We reported that the two epithelial breast cancer cell lines contained significantly higher intracellular lactate and pyruvate than the two corresponding post-EMT mesenchymal cell lines (submitted manuscript). In the present study, we observed that silencing of GPR81 in MCF7 cells led to lower intracellular lactate and higher levels of lactate in the culture media, indicating reduction in lactate uptake. Furthermore, we found a protective effect of GPR81 against apoptosis, which was previously described in epithelial MCF-7 breast cancer cells, in which GPR81 activation triggered the PI3K/Akt signaling pathway to inhibit apoptosis^[25]. Together these results suggest a specific regulation of GPR81 on lactate importer MCT1, which affects cell proliferation, apoptosis and survival of hormone-positive epithelial breast cancer cells.

In adipocytes, GPR81 is coupled to Gi/q which inhibits adenylate cyclase activity and decreases the production of cAMP. However, a previous study did not find a significant change in cAMP levels when GPR81 was silenced in epithelial MCF-7 breast cancer cells^[25], suggesting that GPR81 regulates MCT1 expression by another signaling pathway in this cells. Alternate GPR81-associated signaling that is independent of the PKA/cAMP pathway, but involves the GPR81-binding protein β -arrestin, has been reported in monocyte/macrophage^[48]. Further studies are needed to examine whether the β -arrestin pathway or other pathways are linked to GPR81 signaling in epithelial breast cancer, as these pathways may provide targets for future development of adjuvant therapies for Luminal A breast cancer.

DECLARATIONS

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Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: Tafur D, White B

Performed NMR studies, data acquisition, analysis and interpretation: Svrcek P

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All authors declared that there are no conflicts of interest.

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Review

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Cellular plasticity and metastasis in breast cancer: a pre- and post-malignant problem

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Abstract

As a field we have made tremendous strides in treating breast cancer, with a decline in the past 30 years of overall breast cancer mortality. However, this progress is met with little affect once the disease spreads beyond the primary site. With a 5-year survival rate of 22%, 10-year of 13%, for those patients with metastatic breast cancer (mBC), our ability to effectively treat wide spread disease is minimal. A major contributing factor to this ineffectiveness is the complex make-up, or heterogeneity, of the primary site. Within a primary tumor, secreted factors, malignant and pre-malignant epithelial cells, immune cells, stromal fibroblasts and many others all reside alongside each other creating a dynamic environment contributing to metastasis. Furthermore, heterogeneity contributes to our lack of understanding regarding the cells' remarkable ability to undergo epithelial/non-cancer stem cell (CSC) to mesenchymal/CSC (E-M/CSC) plasticity. The enhanced invasion & motility, tumor-initiating potential, and acquired therapeutic resistance which accompanies E-M/CSC plasticity implicates a significant role in metastasis. While most work trying to understand E-M/CSC plasticity has been done on malignant cells, recent evidence is emerging concerning the ability for pre-malignant cells to undergo E-M/CSC plasticity and contribute to the metastatic process. Here we will discuss the importance of E-M/CSC plasticity within malignant and pre-malignant populations of the tumor. Moreover, we will discuss how one may potentially target these populations, ultimately disrupting the metastatic cascade and increasing patient survival for those with mBC.



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Keywords: Cell plasticity, pre-malignant plasticity, breast cancer, epithelial-mesenchymal transition/cancer stem cell, metastasis

INTRODUCTION: THE MORTALITY OF METASTATIC BREAST CANCER

Breast cancer (BC) is the most commonly diagnosed cancer among women, and the second leading cause of cancer related deaths in women^[1]. The statistics highlight the importance of metastasis in BC mortality: in patients with distant metastasis, 5-year survival rates are only 22% (13% at 10 years), compared to 90% for patients with local disease^[2]. Furthermore, for those patients with metastatic BC (mBC), there are currently no effective treatment options. Understanding how BC cells escape the primary tumor, spread to distant organs, initiate outgrowth at a distant site, and then developing therapies to target those metastatic processes remains a significant clinical challenge. Our understanding of the metastatic cascade has increased in recent years: cells must degrade the extracellular matrix (ECM) surrounding them, extravasate into the circulatory or lymphatic system and circulate throughout the body, intravasate into the new organ tissue, and regain their proliferative capacity^[3-5]. Yet, the molecular mechanisms driving each of these processes, all of which are important for a successful metastatic event, remain unresolved.

A complicating factor to our understanding of BC metastasis is the heterogeneous milieu of the primary tumor site, or tumor micro-environment (TME), which is comprised of epithelial, endothelial, immune, and stromal cells. It is important to note that the epithelial populations can be subdivided into two distinct groups, malignant and pre-malignant. The malignant population has completed the transformation process through loss of tumor suppressive mechanisms and a gain of oncogenic signaling, via genetic mutation or sustained growth factor or cytokine signaling. Conversely, pre-malignant refers to a spectrum of points on the path towards transformation. Pre-malignant cells drift further from normalcy as they acquire mutations and engage aberrant signaling. If intact, a tumor suppressive response may be engaged to halt the transformation process, however if lost, the cell may progress to a fully transformed endpoint. The complex cellular composition within the TME results in a network of secreted factors and ECM proteins, which also profoundly influence metastatic potential^[6-9]. Numerous TME factors can drive epithelial cells to undergo epithelial-mesenchymal transition (EMT) and acquire cancer stem cell (CSC) properties, which we refer to as epithelial-mesenchymal (E-M)/CSC plasticity^[10-14]. Seminal work has defined E-M/CSC plasticity as an important step in metastasis and is often investigated from the perspective of a malignant population. However, malignant cells are not the only populations capable of undergoing E-M/CSC reprogramming. Recent evidence has demonstrated a remarkable ability of pre-malignant epithelial cells to take on a more invasive phenotype able to intravasate and disseminate to secondary sites following signaling cues from the TME^[15,16]. Here, we discuss the challenges of targeting various cell populations and the signaling pathways that contribute to the cellular plasticity driving mBC. Importantly, we will explore the impact of pre-malignant cells escaping senescence by undergoing E-M/CSC reprogramming to gain invasive, metastatic, and tumor-initiating properties. Identifying determinants of metastasis, such as E-M/CSC plasticity, and advancing our ability to target the drivers of plasticity will have a significant impact on survival for those with mBC.

SETTING THE STAGE: HETEROGENEITY WITHIN THE PRIMARY SITE

A major challenge in dealing with BC is the heterogeneity that accompanies it. With three distinct clinical subtypes, estrogen and progesterone receptor positive (ER+/PR+), human epidermal growth factor receptor 2 positive (HER2+), and triple negative breast cancer (TNBC; negative for ER, PR, HER2 expression), finding the proper treatment options can be difficult^[17-19]. Therapies targeting ER/PR hormone signaling via selective estrogen receptor modulators (SERMs) or aromatase inhibitors (i.e., Tamoxifen and Arimidex) have significantly improved patient survival and have made this subtype more manageable. Likewise, HER2+

tumors can be treated with antibodies or kinase inhibitors targeting HER2 signaling (i.e., Trastuzumab and Lapatinib) and are often met with success provided the disease is caught early; the efficacy of these therapies drastically decreases with late stage, metastatic HER2+ BC^[20-23]. Conversely, TNBC currently lacks a targeted therapy tailored to a specific driver oncogene and is most often treated with cytotoxic chemotherapies. Patients with TNBC exhibit an increased risk of metastatic dissemination resulting in higher clinical stage at diagnosis and lower disease-free survival compared to patients with non-TNBC cancers^[24]. Much like metastatic HER2+ BC, metastatic TNBC is not effectively treated, highlighting the need for better therapies targeting those cells which progress beyond the primary site and are ultimately responsible for patient mortality and morbidity. Further challenges in treating BC involve the heterogeneous nature of the tumor cells themselves, a phenomenon often referred to as intra-tumoral heterogeneity (ITH)^[25]. Evidence suggests that across a panel of human cancers, including breast, increased ITH correlates with decreased overall survival, and therapy resistance^[26,27]. Furthermore, high ITH inversely correlates with low tumor infiltrating lymphocytes, which are often associated with increased patient survival^[28-36].

The path to ITH is complex and involves a series of genetic and epigenetic events throughout the transformation process which permit normal human mammary epithelial cells (HMEC) to develop into fully malignant cancer cells^[37-44]. Progress in RNA and DNA sequencing technologies have helped shape the evolutionary picture of HMEC; losing tumor suppressor function (TP53 mutations or RB loss) and acquiring oncogenic drivers (MYC, HER2, or CCND1 amplification or PIK3CA mutations)^[45-49]. Genetic alterations lead to the expansion of a pre-malignant population which progressively acquires additional genetic and epigenetic changes until one or more cells become fully transformed^[50,51]. These additional mutations are numerous, and genomic profiling has found a wide variety of changes in copy number, chromatin alterations, chromosomal rearrangements, and point mutations throughout the genome from single cell sequencing of bulk tumor tissue in TNBC^[36,52,53]. Not only does this dynamic process of transformation alter the cancer cell itself, but transforming cells have a substantial impact on the surrounding environment. Evidence suggests that the accumulation of mutations within epithelial cells can lead to a dysregulated secretory network, including a number of inflammatory cytokines linked to poor prognosis, therapy failure, and disease recurrence (IL-6, IL-8, TGF- β , CCL2, TNF- α , IL-17 and others)^[54-59]. This dysregulated secretory network in turn, changes the cellular composition of the TME, leading to a reciprocal cross-talk between non-cancerous stromal cells and the transforming epithelial cells. Overall, this demonstrates the immense complexity of the tumor, as the heterogeneity described above culminates in a highly diverse TME, with an array of cell types, secreted factors, and structural make up.

Importantly, not all epithelial cells that begin the transformation process reach full malignancy. As a cell senses aberrant activation of signaling pathways/gene induction, it may enact intact tumor suppressive mechanisms, resulting in senescence^[60-66]. Senescence is a major growth-inhibiting and tumor-suppressive barrier that must be bypassed *in vivo* during transformation en-route to tumor development^[63,67-73]. Large senescent cell populations can be found at various stages of tumor development, further contributing to tumor heterogeneity. Remarkably, an investigation by Cotarelo *et al.*^[74] was able to identify the presence of senescent cells in approximately 83.7% of the human invasive breast carcinomas examined, suggesting their involvement throughout the transformation process and as tumors evolve and progress. Since the 129 tumors surveyed in this study were from untreated patients, the origin of senescence within these invasive BC is an *in vivo* physiological response.

Long thought inert, bystanders within the tumor, senescent cells have gained considerable interest for their potential impact on the tumor as a whole. Despite being growth-arrested, senescent cells remain viable, metabolically active, and play an important role in the developing TME^[75-77]. A hallmark of senescent cells is the secretion of a wide variety of growth factors, pro-inflammatory cytokines, chemokines, and proteinases, a characteristic termed the senescence-associated secretory phenotype (SASP) [Figure 1]^[78,79]. Under normal

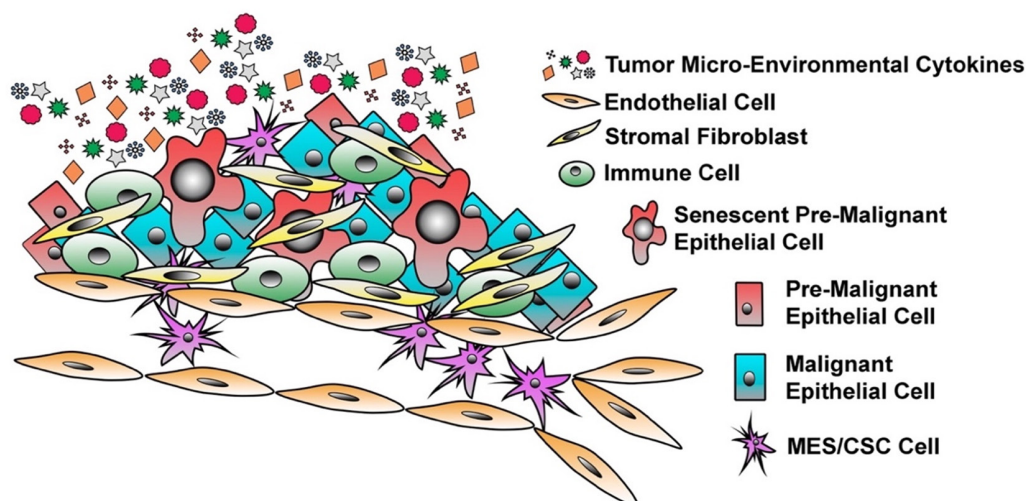


Figure 1. The Heterogeneity of Breast Cancer. Breast cancer is a heterogeneous disease with a highly dynamic tumor micro-environment (TME). Within the primary site, one can observe the presence of epithelial cells that have undergone E-M/CSC plasticity, malignant epithelial cells, pre-malignant cells, senescent epithelial cells, stromal fibroblasts, infiltrating immune cells, and endothelial cells. The presence of all of these diverse cell types results in a distinct and complex milieu of secreted factors within the TME that influence, tumor progression, disease recurrence, and cell plasticity

conditions, the SASP-factors act in an autocrine manner to maintain the senescence program and recruit immune cells into the local environment^[80-83]. However, paracrine signaling by SASP components can also influence the behavior of adjacent cells, engaging signaling programs that contribute to tumor progression and therapy failure^[64,84-89]. A collection of recent studies has demonstrated the ability of senescent cells and SASP components in the TME to drive cellular E-M plasticity and the expansion of a CSC-like cell population^[90,91]. In fact, the SASP program can promote stemness within both senescent cells and neighboring cells, both *in vitro* and *in vivo*, through secretion of potent inflammatory cytokines associated with disease recurrence, and overall poor prognosis^[92,93]. More specifically, less aggressive luminal MCF-7 cells were treated with conditioned medium harvested from senescent populations experiencing SASP. Exposure to conditioned media led to a more CD24LO/CD44HI invasive/stem like population similar to already aggressive MDA-MB-231 cells, which was dependent upon IL6 and IL8, two well defined SASP-factors, secretion^[94]. Furthermore, sustained hyper-activation of signal transducer and activator of transcription 3 (STAT3) by SASP components plays a critical role in induction of an invasive and stem-like program^[95]. Taken together, the presence of malignant, pre-malignant, and senescent epithelial cells creates a diverse TME suitable to drive E-M/CSC plasticity within the tumor and contribute to metastatic, therapy-resistant, and tumor-initiating phenotypes. Below we will discuss how E-M/CSC plasticity contributes to these deadly phenotypes responsible for patient mortality.

AN IDENTITY CRISIS: MESENCHYMAL VS. EPITHELIAL

Each step along the metastatic cascade presents a new environmental context and challenge that a potentially metastatic cell must adapt to in order to thrive. This adaptation involves changes in a cell's state. Cellular plasticity is defined as the ability of a cell to acquire new biological properties due to intrinsic and extrinsic cues. It is important to recognize that plasticity is most often a highly dynamic and reversible process that can be used to describe multiple cellular changes (i.e., differentiation, metabolism, response to immune cells, motility, and cell fate). Throughout this review, we will refer to cellular plasticity as the ability for cells to undergo E-M/CSC plasticity, that is, cells shifting between epithelial/non-CSC and mesenchymal/CSC states. E-M/CSC plasticity is important in imparting invasive and motile phenotypes as well as cells acquiring tumor-initiating potential and reduced sensitivities to therapy.

The switch from epithelial to mesenchymal state facilitates dissolution of cell-cell junctions (due to the repression of cell adhesion proteins E-Cadherin, EPCAM, and CD24) and increased ability to remodel the surrounding ECM [due to matrix-metalloprotease proteins (MMP), adamalysins (ADAMs), and differential integrin expression]^[3,96-101]. The corresponding cytoskeletal and ECM remodeling imparts a migratory and invasive phenotype important for metastasis. Lineage tracing experiments confirm that epithelial-to-mesenchymal transition (EMT) occurs *in vivo*, and that it precedes metastasis in murine BC models^[102-104]. Through intravital imaging, Beerling and colleagues showed that mesenchymal tumor cells have a unique and specific migratory behavior that results in greater circulating tumor cells (CTC), increased tumor cells within the lungs, and metastasis; in contrast, the more abundant epithelial cell population remained non-motile and less metastatic^[104]. It should be noted that others, Zheng *et al.*^[105] and Fischer *et al.*^[106] have employed *in vivo* lineage tracing models and reported that EMT is not required for metastasis. As Beerling and colleagues discuss, many of these reports rely on fixed gene manipulation (for example, gene silencing or protein overexpression) to experimentally test an EMT-underlies-metastasis hypothesis. It is possible that such artificial manipulation is not able to recapitulate physiologic events and, in this way, contributes to discrepancies in findings. Other small, but crucial, details could play a further role in some discrepancies: (1) EMT may be indispensable to metastasis for select cancer subtypes, but dispensable for others; (2) reliance on activation of a single gene reporter (e.g., Fsp1) to capture and "tag" an EMT event restricts the sensitivity of the model system; (3) criteria for how the EMT program is identified, such as the panoply of specific "epithelial" or "mesenchymal" proteins that are induced or suppressed, may also lead to false-negatives if these identifying protein sets are incongruent across cancers and cancer subtypes. Regarding the latter point, Zheng *et al.*^[105] and Fischer *et al.*^[106] reported on Vimentin and E-cadherin status, but each represents just one exemplar for mesenchymal or epithelial cell state and ultimately, these may not be the most relevant. Finally, evidence that metastases occur in the absence of EMT does not preclude a potential for EMT to enhance cancer cell metastasis.

The gain of migratory and invasive properties which accompany E-M plasticity occurs concomitantly with changes in global signaling programs and gene expression. Several key transcription factors (TF) have been identified as master regulators of EMT: SNAI1 (Snail), SNAI2 (Slug), ZEB1, ZEB2, TWIST1, and TWIST2^[107-112]. These TF are typically kept silenced in adult cells when plasticity is unnecessary but become aberrantly activated by TME factors to induce EMT^[113,114]. EMT-TF expression strongly correlates to regions of the tumor with mesenchymal marker positivity, notably, the invasive front of the tumor where mesenchymal cells act as 'trailblazers' that initiate and guide local metastasis^[108,115-118].

Tissue-invasive cells that encounter vessels may intravasate into vascular or lymph networks and be disseminated throughout the body as CTC. CTC are not only present in measurable quantities in patients with BC, but their abundance is predictive of overall survival and directly correlates with the likelihood of relapse following treatment^[119-123]. Moreover, CTC display a wide range of markers associated with cells that have or are undergoing E-M/CSC reprogramming. CTC often exhibit a decrease in epithelial markers CD24, E-Cadherin (CDH1), EPCAM and an increase in well-known mesenchymal and CSC markers (ZEB1, Snail, CD44, Vimentin)^[116,124-127]. Critically, CTC are capable of tumor initiation at a secondary site, and more importantly demonstrate remarkable plasticity by engaging specific molecular programs that dictate the organ-specificity of metastases^[128-133]. Overall, mesenchymal cells are simply more well-suited to the task of escaping the primary site and reaching distant organs.

However, escaping the primary site and circulating throughout the lymph or cardiovascular system is not sufficient for metastases to develop. Once a mesenchymal cell has reached a secondary organ, it must embed itself in the new tissue and flourish in order to establish a metastatic outgrowth; not all cells have this capability^[3,134]. CSC have been deemed the "roots" of primary and secondary site outgrowth due to their tumor-initiating capacity and their ability to differentiate into multiple lineages, recreating the heterogeneity

seen in the primary site^[135-137]. Since Al-Hajj *et al.*^[138]'s initial isolation of CSC from BC models, marked by CD44HI/CD24LO cell surface expression profile, the field has made significant insight into the CSC paradigm^[138]. Shortly after, Ginestier *et al.*^[139] demonstrated an increase in aldehyde dehydrogenase 1 (ALDH1) activity strongly correlated with both normal and malignant stem/progenitor cells within BC. CSC have been isolated from nearly every human cancer through identification of surface marker expression of nearly 40 different markers which vary from cancer to cancer (CD133, CD44, CXCR4, CD90, *etc.*)^[140]. Further complicating matters, our understanding of the dynamics of breast cancer stem cell (BCSC) populations is poorly understood. For instance, Liu *et al.*^[141] identified ALDH1 activity to inversely correlate with CD44 expression in BC models, suggesting a complex make up of phenotypes contributing to tumor initiation, which further work is required to better understand. Regardless of differences in marker expression, isolated CSC populations all exhibit similar characteristics in terms of their ability to initiate tumor formation in limiting dilution, produce multiple cell lineages, maintain tumor-initiating potential through periods of metastatic latency, and survive cytotoxic therapies due to a wide range of resistance mechanisms^[142,143]. Seminal papers from the Weinberg and Puisieux groups provided the first evidence of a link between shifting from epithelial to mesenchymal identity and the acquisition of CSC properties in BC^[144,145]. Prior to this, the existence of CSC and the occurrence of EMT in malignant neoplasms had each been garnering significant attention, but an association between the two phenotypes had yet to be demonstrated. Specifically, induction of EMT led to a shift in surface marker profiles from CD24HI/CD44LO to CD24LO/CD44HI, resulting in (1) the ability to generate tumorspheres and tumors (self-renewal potential); and (2) the ability to give rise to differentiated daughter cells^[144-148].

One implication of this finding is that breast CSC may not solely arise through malignant transformation of a pre-existing 'normal' mammary stem cell. More recent work, including ours, has described the ability for transformed epithelial/non-CSC populations to acquire mesenchymal/CSC properties in response to autocrine or paracrine signaling initiated from secreted factors within the TME. Importantly, the ability to fluidly move between epithelial/non-CSC and mesenchymal/CSC states has been shown to facilitate metastatic outgrowth [Figure 2A], as cells are believed to reactivate a proliferative program and reacquire epithelial cell phenotypes at the site of metastasis^[12-14,137,141,149-163].

THE QUIET NEIGHBOR: PRE-MALIGNANT CELLS AND PLASTICITY

E-M/CSC plasticity is a major contributor to tumor heterogeneity, progression towards metastasis, and therapy failure. Yet, cellular plasticity also plays a crucial role in normal physiology, such as embryonic development, wound healing, and tissue remodeling^[164,165]. For instance, differentiated epithelial cells can de-differentiate or trans-differentiate, wherein a differentiated cell may change state into an alternate lineage. These forms of plasticity are seen routinely in skin cells, hepatocytes, colon epithelium, pancreatic acinar cells, and others as they undergo a phenotypic transition in order to repair and sustain the homeostatic nature of the tissue^[166-170]. In fact, cellular plasticity of mammary epithelial cells has been suggested to be a driver of the heterogeneity seen in BC^[171-173].

Similarly, normal mammary gland organogenesis and homeostasis requires the presence of mammary stem cells (MaSCs) in the mammary gland epithelium that differentiate into both progenitor cells and differentiated cells^[174]. Remarkably, many of the markers used to distinguish normal MaSCs overlap with those used to identify breast CSC including mRNA expression profiles^[174]. Interestingly E-M plasticity is also essential for normal mammary gland development and maintenance. For example, E-M plasticity is crucial for MaSC migration as well as the expansion and invasion of terminal end buds (TEB) during mammary gland development^[174]. Much like the mechanisms that guide stemness in MaSC, the upregulation of multiple EMT-TFs, such as Snail and Twist, was uncovered in the microenvironment of the TEB by genomic transcriptional analysis^[175]. E-M plasticity in non-MaSC cells that comprise the normal mammary gland guides cell polarity and the organization of mammary cells into their well-defined glandular structures^[176].

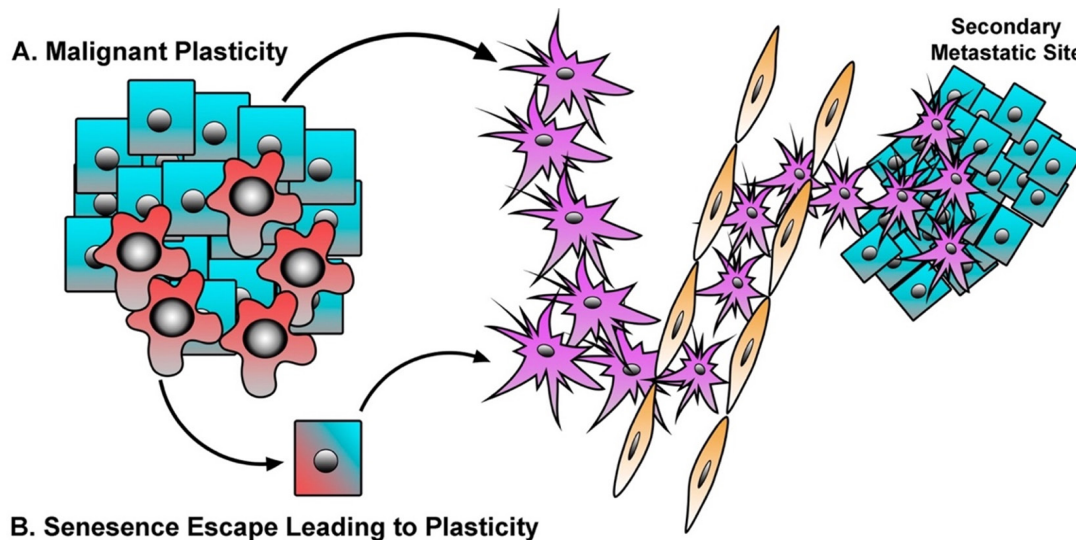


Figure 2. Malignant and Senescent Epithelial Cells Contribute to Metastasis. A: malignant cells respond to TME cytokines or intrinsic genetic alterations in order to drive E-M/CSC plasticity, resulting in greater metastasis, enhanced disease recurrence, and therapeutic resistance; B: pre-malignant cells undergo senescence in response to aberrant oncogene or cytokine signaling. However, in some instances a small population of senescent cells may undergo E-M/CSC plasticity, and thus escape the tumor suppressive barrier of senescence and further contribute to the metastatic phenotype

Given its importance in tissue remodeling and homeostasis, the normal processes governing cellular plasticity are strictly regulated^[177-179].

As discussed above, senescence serves as an important tumor suppressive barrier to prevent transformation and the outgrowth of a dysregulated and uncontrolled population. Interestingly, the induction of senescence in normal cells often results in the simultaneous emergence of mesenchymal/stem-cell markers in conjunction with senescence markers, and loss of proliferative capacity. For example, senescence leads to the induction of CD44 expression, a cell surface marker regularly used to distinguish breast CSC from non-CSC, and often expressed as malignant cells acquire MES/CSC properties and undergo plasticity^[138,180-183]. As small subsets of senescent cells dismantle the senescence program, the emergent population of proliferating cells can harbor a mesenchymal/stem-cell phenotype that can persist throughout the remainder of the transformation process, ultimately yielding a more invasive and aggressive cancer cell^[184-187]. Li *et al.*^[15] demonstrated that many of the transcriptional changes observed in BC are also initiated in normal HMEC as they escape senescence. In their study, HMEC were shown to have a “pre-transformation” transcriptome and exhibited a partial EMT following their senescence escape^[15]. Similarly, normal HMEC that have spontaneously escaped replicative senescence exhibit a greater mesenchymal and CD24LO/CD44HI CSC-phenotype^[16,188]. Further studies have suggested cell cycle regulator Cyclin A1 and tumor suppressors p53 and p16 can act as “gatekeepers” to maintain cells in an epithelial state. Dysregulation of these proteins in epithelial cells can result in the initiation of the mesenchymal/stem-cell program, which interestingly corresponds with the escape from senescence^[189,190]. Moreover, induction of EMT (via Snail, Twist, and ZEB1 expression) prior to a cell’s engagement of oncogene-induced senescence, prevents senescence altogether and results in the induction of a CSC program and tumor initiation^[191-196].

In addition to pre-malignant cells, cytotoxic therapies can drive cancer cells into a therapy-induced senescence (TIS). Again, cells that escape TIS have acquired a senescence-associated stemness^[197-199]. Milanovic *et al.*^[200] have shown that cells undergoing TIS express a variety of stem-cell associated markers, and that TIS in B-cell lymphomas exhibit a gene signature which mirrors that of adult tissue stem cells, conferring a highly aggressive phenotype responsible for tumor relapse. The emergence of a stem-like population following the

engagement of senescence may be an inherent phenomenon that also occurs during other stress-induced senescence responses (i.e., oncogenes, replication stress, γ -irradiation) among different cell types^[200-202]. Altogether, these findings suggest that, the mesenchymal/stem-cell program engaged during senescence may have significant negative consequences if those cells can overcome the signals maintaining senescence, resulting in cells with greater ability metastasize and survive therapy [Figure 2B]. Thus, we suggest that therapies that target senescent cells would limit the reservoir of aggressive cells harboring a senescence-associated stemness responsible for therapy failure and relapse. In the following section we will discuss the potential of targeting E-M/CSC plasticity within malignant, pre-malignant, and senescent populations.

HALTING METASTASIS: TARGETING PRE- AND POST-MALIGNANT CELL PLASTICITY

Liquid biopsies from patients with early-stage BC receiving neoadjuvant therapy can be used to identify subjects at high risk of recurrence by quantifying the number of CTC. Furthermore, expression of mesenchymal markers in the CTC correlates with poor prognosis and response to therapy^[138,203-206]. With the advent of single cell analysis techniques, our understanding of the evolution and diversity of tumor cells that are responsible for invasion, metastasis, and therapy failure is expanding. For example, single-cell qPCR has identified mesenchymal/CSC gene expression patterns in early-stage breast cancer micro-metastases^[137]. In contrast, later-stage metastases (from the same PDX tissue) are more heterogeneous, more proliferative, express differentiation markers, and display greater similarity to the primary tumors. The findings are consistent with the idea that mesenchymal/CSC initiate metastatic outgrowth at a secondary site, followed later by increased proliferation and differentiation. More recent single cell RNA-sequencing (scRNA-seq) studies have confirmed that EMT in primary tumors proceeds through distinct, hybrid states, ranging from completely epithelial to completely mesenchymal^[207]. These E-M hybrids, which harbor the greatest level of plasticity, are more efficient at intravasating, extravasating to the lungs, and forming metastases^[208]. Underlying this biology, E-M hybrids have distinct chromatin landscapes and transcriptional profiles. *In situ* analysis identified increasing vascularization and immune cell infiltration (particularly macrophages) nearest the E-M hybrids and fully mesenchymal cells^[208]. A separate scRNA-seq study determined that, in response to chemotherapy, emerging chemo-resistant cells undergo transcriptional changes consistent with EMT. In most patients, this chemo-resistant transcriptional program was not evident before treatment but acquired via transcriptional reprogramming following treatment^[209]. These studies and others make a strong case that epithelial tumor cells can be induced into a drug-tolerant, E-M hybrid cell state by chemotherapy^[141,209-214].

Identifying and targeting the pathways responsible for this chemo-resistant reprogramming would help improve the efficacy of chemotherapy. In a recent example, SRC kinase inhibition prevented the *de novo* generation of chemo-resistant cells^[209]. Importantly, this chemo-sensitization was temporally dependent, and only effective if SRC inhibition occurred after chemotherapy, when the signaling responsible for generating the chemo-resistance phenotype had become activated. More recently, Cazet *et al.*^[215] identified cross-talk between TNBC models and cancer-associated fibroblasts (CAF), which promoted stemness and drug resistance in the cancer cells via paracrine Hedgehog (Hh) signaling. A key developmental pathway, Hh signaling requires receptor mediated binding of Hh to Patched (PTCH), resulting in Smoothened (SMO)-mediated nuclear translocation of Gli1. Gli1 then acts as a transcription factor, mediating Hh pathway transcriptional changes^[216]. Hh signaling is often reactivated in a subset of BC, and specifically in the context of TNBC, a paracrine manner^[217]. After targeting paracrine Hh signaling *in vivo*, via two clinically available smoothened inhibitors (SMOi) Vismodegib and Sonidegib, Cazet *et al.*^[215] observed a suppression of cancer cell plasticity and increased sensitivity to docetaxel. Most importantly, in a phase I clinical trial, 3 of 12 patients with metastatic TNBC observed clinical benefit from combinatorial therapy of SMOi and docetaxel (one with complete response), similar to treatment paradigms we suggest above. SMOi have been beneficial for basal cell carcinomas and medulloblastomas where tumors rely on cell-autonomous hedgehog signaling, however the work here suggests the ability to target the TME in order to dampen cancer cell plasticity, and

achieve a greater therapeutic response^[216,218,219]. Below we will address additional potential therapeutic avenues one may use to dismantle E-M/CSC plasticity in order to prevent metastatic dissemination, secondary site outgrowth, or re-sensitize cancer cells to standard of care therapies.

TARGETING MALIGNANT POPULATIONS

STAT3

STAT3 is persistently activated in cancer cells, as it is a downstream effector of several receptor tyrosine kinases (RTKs) commonly activated by growth factors and cytokines^[220-222]. We, as well as others have demonstrated that, persistent STAT3 activation in cancer cells induces mesenchymal and CSC properties, inhibits apoptosis, and maintains a more un-differentiated phenotype^[12,14]. Therefore, STAT3 is a promising therapeutic target. A number of small-molecule inhibitors of STAT3 (KI16; BP-5-087; WP1066) are currently in development and combination therapies, with BCR-ABL1 or BRAF inhibitors, have shown positive results in the treatment of several cancer types^[223-225]. In the context of TNBC, a recent phase Ib/II study combining the cancer stemness inhibitor Napubacasin (BB608), which prevents STAT3 activation, with weekly administrations of paclitaxel showed improvement in metastatic patients whose cancer had progressed while on a taxane-based regimen (NCT01325441).

PI3K, Akt and mTOR

The PI3K/Akt/mTOR pathway is involved in several cell processes, including proliferation, metabolism and motility, therefore it is not surprising that its dysregulation corresponds to uncontrolled proliferation and propagation in a wide spectrum of cancers. The role of the PI3K/Akt/mTOR pathway in maintaining cell plasticity in cancer has been documented in several publications^[226-228]. In the context of breast cancer, *PIK3CA* mutations have been observed in each of the different subtypes, but mostly in hormone receptor-positive tumors where it's associated with disease progression and resistance to endocrine therapy. Each *PIK3CA* mutation results in an abnormal activation of the alpha subunit of PI3K, that with the beta subunit is the most common in breast tissue^[229]. *PIK3CA* mutations appear to hold prognostic and predictive value in hormone receptor-positive, HER2-negative advanced or metastatic breast cancer. Several studies show how targeting tumors carrying a *PIK3CA* mutation with *PIK3CA* inhibitors increased the PFS of patients^[230]. In January 2019, results from the phase III SANDPIPER clinical trial (NCT02340221) were posted. This international, multicenter, randomized, double-blinded, placebo-controlled study was designed to test the efficacy of a combo of the *PIK3CA* SMI taselisib and the synthetic estrogen receptor antagonist fulvestran versus placebo and fulvestran in the treatment of ER-positive, HER-2-negative locally advanced or metastatic breast cancer harboring a *PIK3CA* mutation in patients with disease recurrence after or during treatment with aromatase inhibitor (AI). SANDPIPER is the first placebo-controlled trial testing the efficacy of a mutant-specific PI3K inhibitor^[231]. Taselisib is specifically directed against the alpha isoform of *PIK3CA*, however it can inhibit the gamma and delta isoforms as well, thus causing an increase in toxicity mostly involving the gastro-intestinal tract. Another phase III clinical trial, BELLE-2 (NCT01610284) is analyzing the effects of the pan-PI3K inhibitor buparlisib in combination with fulvestran compared to fulvestran and placebo combo. Unfortunately, also buparlisib showed important side effects, particularly hyperglycemia^[229]. Several other PI3K inhibitors are currently under investigation in clinical trials, including apelisib (NCT02437318 - SOLAR-1), which showed encouraging clinical benefits in the majority of patients enrolled^[232]. In the future, research efforts should be more focused at inhibiting exclusively the alpha subunit of *PIK3CA*, thus reducing the risk of toxicity and side effects. Interestingly, GDC-0077 from Genentech appears to be extremely more specific towards the alpha subunit of PIK3A over other subunits, thus representing a potentially less toxic alternative to other inhibitors and is currently investigated in a phase I clinical trial alone or in combination with other agents, such as palbociclib, letrozole and fulvestran (NCT03006172).

While the role of mTOR signaling in promoting a CSC phenotype is still controversial, its activation in BC appears to be essential for colony formation *in vitro* and tumorigenicity^[233]. Furthermore, mTOR signaling

increases aldehyde dehydrogenase 1 (ALDH1) activity^[234,235]. IGF-1R activation signaling through PI3K/Akt/mTOR represents a promising target in BC as an abnormal activation of PI3K can also lead to an increased activation of STAT3 through enhanced expression of the chemokine (C-X-C motif) receptor 4 (CXCR4)^[236,237]. IGF-1R is activated in the 50% of breast cancer patients. Several phase III clinical trials targeting IGF-1R with small molecule inhibitors have so far failed due to side effects such as hyperglycemia and metabolic syndrome caused by the homology of IGF-1R and insulin receptor (IR) and IGF elevation in response to disruption of glucose homeostasis. On the other hand, monoclonal antibodies specifically targeting IGF-1R or its ligand have shown a higher specificity by sparing the insulin metabolism from any inhibitory effect in preclinical models^[238]. Early phase trials have been setup to determine the efficacy of an antibody-based inhibition of IGF-1R signaling by targeting either the receptor or the ligand in combination with aromatase inhibitors or mTOR inhibitors but with small success^[239]. A more complex therapeutic protocol combining inhibitors of PI3K or other downstream effectors of IGF-1R would probably be more beneficial.

Several drugs targeting the PI3K/mTOR pathway have shown a selective effect on CSCs, inhibiting their growth and sensitizing them to traditional chemotherapy. Since 2007, when the Food and Drug Administration (FDA) approved the mTOR inhibitor temsirolimus for the treatment of advanced renal carcinoma, new generations of compounds have been developed. Everolimus, is a dual PI3K/mTOR inhibitor, which blocks all PI3K class I isoforms as well as mTORC1 and 2, thus preventing the development of CSCs when combined with letrozole^[240,241]. Interestingly, mTOR is also inhibited by metformin (1,1-dimethylbiguanide hydrochloride), usually prescribed for the treatment of type 2 diabetes. Metformin preferentially kills CSCs over non-CSCs and reduces tumorsphere formation and CSC marker expression (CD133, CD44 and ALDH1)^[242]. Similar effects have been achieved with the antibiotic salinomycin, which selectively kills breast CSC^[243]. The plant-derived chemotherapeutic molecule rottlerin induces apoptosis in breast CSC by inhibiting PI3K/Akt/mTOR pathway^[244]. More recently, the PI3K/mTOR dual inhibitor VS5584 has shown promising results by delaying tumor recurrence through selective killing of CSC after chemotherapy^[245].

Notch

Notch signaling is known to be involved in different cellular processes, including differentiation and proliferation. Perturbation in these processes can be caused by mutations in Notch or one of its effectors. Mutations of the Notch pathway are the hallmark of many subtypes of cancer, including BC, where cells overexpressing Notch have increased CSC markers (SOX2 and OCT4) and phenotypes (tumorsphere formation)^[246-248]. Moreover, Notch promotes EMT and metastasis in TNBC cells and Notch inhibition can prevent EMT both *in vitro* and *in vivo*^[249]. For example, 3,6-dihydroxyflavone (3,6-DHF) causes a significant reduction of CSCs *in vitro* and blocks lung metastasis by specifically down regulating Notch, Hes-1 and c-Myc^[250]. 3,6 DHF shows potent chemo-preventive properties against breast carcinogenesis both *in vitro* and *in vivo*, although a mechanism has not been identified yet, besides an epigenetic increase in the synthesis of miR-34a, a potent down-regulator of Notch and thus of EMT in breast cancer^[251]. Furthermore, 3,6-DHF down-regulates the expression of Notch's target genes *Hes1*, *c-Myc* and the EMT mediators SNAIL, Twist and Slug by compromising the formation of the transcriptional complex NICD-CSL-MAML^[252,253]. More recently, it has been shown that Notch3 inhibition by siRNA silencing increases TNBC sensitivity to gefitinib in EGFR-Tyrosine kinase inhibitors (TKI)-resistant cells by blocking the nuclear translocation of activated EGFR^[254].

Wnt and β -catenin

The Wnt pathway is involved in the maintenance of breast CSCs by promoting self-renewal and plasticity through PAF (proliferating cell nuclear antigen-associated factor)^[255]. The Wnt ligand, Frizzled, is upregulated in high-grade tumors, including more aggressive forms of BC, and can cause EMT and metastasis through non-canonical STAT3 activation^[256]. Inhibitors targeting the Wnt pathway have been developed and are showing promise in BC models. In particular, the Wnt/beta-catenin inhibitor CWP232228, which blocks

beta-catenin binding to T-cell factor (TCF) in the nucleus, prevents the proliferation of breast CSC, selectively depleting CD133-positive and ALDH1-high cells both *in vitro* and *in vivo*^[257,258].

TARGETING SENESCENT POPULATIONS

As described in the previous section, cells escaping senescence exhibit increased invasive and tumor-initiating properties. On top of that, senescent cells secrete a variety of cytokines and growth factors as part of the SASP. The production of these factors into the TME has been shown to drive E-M/CSC plasticity in neighboring cells, as well as the senescent cells themselves, expanding the population that can facilitate metastasis and drug resistance. Moreover, the chronic presence of senescent cells can impair local tissue function, create a highly inflammatory environment, and in some instances exacerbate the side effects of chemotherapies^[259,260]. Considering conventional treatments such as chemo- and radio-therapy often induce senescence in both cancer and normal cells, it seems pertinent to target senescent cells and clear them from the local tissue. The concept for targeting senescent cells was brought to light by Lee *et al.*^[261] by exploiting the high lysosomal β -galactosidase activity in senescent cells, cytotoxic drugs encapsulated in galacto-oligosaccharides particles (galNP beads) can target chemotherapy-induced senescent cells in mice^[262]. Pre-clinical results showed a significant regression of tumor xenografts after treatment with galNP beads loaded with doxorubicin in combination with palbociclib^[263]. Moreover, senescent-cell accumulation in mice can be reduced by treatment with potential senolytic agent, Navitoclax (ABT-263), a small molecule inhibitor of the anti-apoptotic proteins BCL-2 and BCLxL^[264,265]. However, targeting senescent cells is a relatively new concept, and further insights into the signaling mechanisms which senescent cells rely on is needed.

CONCLUSION

To date our ability to target BC metastases have been largely unsuccessful. With patient survival falling to 22% for those that reach distant and wide-spread disease, the ability to target cells at various stages of the metastatic cascade is greatly needed. Here, we have focused on an epithelial cancer cell's ability to out-manuever cytotoxic agents by changing cell state; E-M/CSC plasticity. This induced reprogramming often reduces sensitivity to therapy by a number of mechanisms, creating an immense problem with effectively removing the disease. We propose to remove the molecular "escape hatch" which provides cells that have undergone E-M/CSC reprogramming a sustained advantage in survival and resurgence. An approach that combines readily available small molecule inhibitors of plasticity-inducing pathways in conjunction with commonly used front-line therapies should increase therapeutic sensitivities. Often, pathway-selective small molecule inhibitors that make it to the clinic are used with the objective of inducing growth inhibition or cell death. However, many of these inhibitors may present a wide range of side effects by acting on non-tumor cells or show little efficacy as a single agent^[266-268]. Instead, a low dosage may prevent toxicity or off-target effects while reducing a tumor cells ability to undergo reprogramming to a more MES/CSC-like state. In doing so, these combination therapies may overcome the MES/CSC-like programs which promote therapy failure and metastatic disease progression, ultimately rendering populations sensitive to currently used chemotherapies and increasing overall patient survival.

Cell plasticity is garnering significant interest in the field of cancer biology, as we attempt to better understand the metastatic process, and those drivers behind it. However, we do not yet fully understand what allows an epithelial cell to undergo MES/CSC reprogramming or what pathways maintain this newly attained phenotype. While most of the work understanding plasticity has been done on malignant populations, recent studies have hinted at the ability of pre-malignant populations to undergo MES/stem-cell reprogramming, albeit often halted by intact tumor suppressive mechanisms, leading to senescence. Here, we discussed the ability of pre-malignant epithelial cells to undergo a MES/stem-cell reprogramming that is comparable to that observed with malignant cells. However, much remains to be discovered about the importance of these pre-malignant populations. We still do not yet know which cells within a pre-malignant population have escaped the

senescence “barrier”, which in turn generate more aggressive sub-populations capable of driving metastasis. Identifying, or molecularly defining, which cells possess the capability to escape senescence may allow us to predict the aggressiveness of a patient’s metastatic burden and open potential avenues for targeting cells with the potential to escape senescence. Ultimately, targeting the signaling responsible for plasticity, both in pre-malignant and fully transformed cells, will enhance the efficacy of chemotherapies and suppress a key driving force responsible for patient death.

DECLARATIONS

Authors’ contributions

Figure concept/design and writing of the manuscript: Smigiel JM

Writing of the manuscript: Taylor SE, Bryson BL, Tamagno I, Polak K, Jackson MW

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All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

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Consent for publication

Not applicable.

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Original Article

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Significance of trace element quantities in the prostatic secretion of patients with benign prostatic hyperplasia and prostate cancer

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Abstract

Aim: Benign prostatic hyperplasia and prostate cancer is an internationally important health problem of the man, particularly in developed countries. The aim of this exploratory study was to evaluate whether significant difference in the levels of Zn and some other trace elements of prostatic fluid exist between the inflamed and malignantly transformed prostate.

Methods: Prostatic fluid levels of Br, Fe, Rb, Sr, and Zn were prospectively evaluated in 52 patients with benign prostatic hyperplasia and 24 patients with prostate cancer. Measurements were performed using ¹⁰⁹Cd radionuclide-induced energy dispersive X-ray fluorescent microanalysis. Prostatic fluid samples were divided into two portions. One was used for cytological study to exclude prostatitis, while the other was intended for trace element analysis.

Results: Mean values \pm standard error of means for concentration (mg/L) of trace element in the prostatic fluid of hyperplastic prostate were: Br 2.32 ± 0.30 , Fe 11.5 ± 1.8 , Rb 1.70 ± 0.23 , Sr 1.41 ± 0.26 , and Zn 488 ± 42 . The contents of Rb and Zn were significantly lower (approximately 3.2 and 7.7 times, respectively) in fluid of cancerous prostate compared with those in fluid of hyperplastic prostate.

Conclusion: There are significant differences in trace element contents in the fluid of hyperplastic and malignantly transformed prostate. The great decrease in levels of Rb and Zn in the fluid of cancerous prostate might demonstrate



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an involvement of these trace elements in etiology and pathogenesis of malignant prostate tumors. It was supposed that the differences in Rb and Zn levels in prostatic fluid can be used as tumor markers.

Keywords: Benign prostatic hyperplasia, prostate cancer, prostatic fluid, trace elements, energy-dispersive X-ray fluorescent analysis

INTRODUCTION

Prostate cancer (PCa) is the most prevalent nonskin male cancer in many populations, including USA, Scandinavia, West European states, Australia, New Zealand, and others^[1,2]. Globally, PCa ranks second in incidence and the fifth in mortality in men, and represents a substantial public health burden^[1,2]. Although the etiology of PCa is unknown, several risk factors including age and diet have been well identified. Thus, the risk of having PCa drastically increase with age, being three orders of magnitude higher for the age group 40-79 years than for those younger than 39 years^[3,4]. The odds of PCa diagnosis by age 79 years are one in six among countries with a high sociodemographic index.

Benign prostatic hyperplasia (BPH) is an internationally important health problem of the man, particularly in developed countries, and represents the most common urologic disease among of men after the age of fifty^[4-7]. Incidence of histological BPH could be over 70% at 60 years old and over 90% at 70 years old^[4,8]. To date, we still have no precise knowledge of the biochemical, cellular and molecular processes underlying the pathogenesis of BPH. Although the influence of androgens and estrogens has been demonstrated, hormonal factors alone may not fully explain BPH development^[9,10].

Thus, the both PCa and BPH is the very common urologic disease in adult males. Moreover, use systematic review methods provide the statistical evidence that the association between PCa and BPH is significant^[11,12]. BPH can be a cause of an elevated prostate specific antigen level in blood^[13]. In these cases it is difficult to differentiate BPH from PCa because the findings of imaging modalities like TRUS and conventional MR Imaging can mimic those of PCa. Even biopsy doesn't play promising role in the diagnosis of BPH. As a result of this, BPH may be misdiagnosed as a malignant condition and end up in aggressive surgical management resulting in increased morbidity. This warrants the need of reliable diagnostic tool which has ability not only to diagnose BPH reliably but also to differentiate it from the PCa.

It was reported that the risk of having PCa and BPH depends on lifestyle and diet, including the intake of zinc (Zn) and some other trace elements (TE)^[14-18]. TE have essential physiological functions such as maintenance and regulation of cell function, gene regulation, activation or inhibition of enzymatic reactions, and regulation of membrane function. Essential or toxic (mutagenic, carcinogenic) properties of TE depend on tissue-specific need or tolerance, respectively^[19]. Excessive accumulation or an imbalance of the TE may disturb the cell functions and may result in cellular degeneration or death^[19-24].

In our previous studies a significant involvement of Zn and some other TE in the function of prostate was observed^[25-39]. Moreover, it was found that intracellular Zn and calcium (Ca) excess is one of the main factors in the etiology of prostate cancer^[16-18,24,25]. One of the main functions of prostate gland is a production of prostatic fluid^[40] with extremely high concentration of Zn and some other chemical elements. The first finding of remarkable high level of Zn concentration in human expressed prostatic fluid (EPF) was reported in the beginning of 1960s^[41]. Analyzing EPF expressed from prostate of 8 apparently healthy men aged 25-55 years it was found that Zn concentration varied in range from 300 to 730 mg/L. After this finding several investigators have suggested that the measurement of Zn level in EPF may be useful as a marker of prostate secretory function^[42,43]. It promoted a more detailed study of Zn concentration in EPF of healthy subjects and in those with different

prostate diseases, including PCa^[43,44]. A detailed review of these studies, reflecting the contradictions within accumulated data, was given in our earlier publication^[44].

In present study it was supposed by us that apart from Zn the levels of some other TE in EPF have to reflect a difference between possible functional suppression of hyperplastic prostate and functional disintegration of cancerous prostate. Thus, this work had four aims. The first one was to present the design of the method and apparatus for micro analysis of bromine (Br), iron (Fe), rubidium (Rb), strontium (Sr), and Zn in the EPF samples using energy dispersive X-ray fluorescence (EDXRF) with radionuclide source ¹⁰⁹Cd. The second aim was to assess the Br, Fe, Rb, Sr, and Zn concentration in the EPF samples received from patients with BPH and PCa. The third aim was to evaluate the quality of obtained results and to compare obtained results with published data. The last aim was to compare the concentration of Br, Fe, Rb, Sr, and Zn in EPF samples of hyperplastic and cancerous prostate gland.

METHODS

Specimens of EPF were obtained from 52 patients with BPH (mean age 63 ± 6 years, range 52-75 years) and from 24 patients with PCa (mean age 65 ± 10 years, range 47-77 years) by qualified urologists in the Urological Department of the Medical Radiological Research Centre using standard rectal massage procedure. In all cases the diagnosis of BPH or PCa has been confirmed by clinical examination, including morphological results obtained during studies of biopsy and resected materials. Patients with BPH combined with chronic prostatitis or prostatic stones were excluded from the study. Subjects were asked to abstain from sexual intercourse for 3 days preceding the procedure. Specimens of EPF were obtained in sterile containers which were appropriately labeled. Twice 20 μ L of fluid were taken by micropipette from every specimen for trace element analysis, while the rest of the fluid was used for cytological and bacteriological investigations to exclude prostatitis. The chosen 20 μ L of the EPF was dropped on 11.3 mm diameter disk made of thin, ash-free filter papers fixed on the Scotch tape pieces and dried in an exsiccator at room temperature. Then the dried sample was covered with 4 μ m Dacron film and centrally pulled onto a Plexiglas cylindrical frame.

To determine concentration of the elements by comparison with a known standard, aliquots of solutions of commercial, chemically pure compounds were used for a device calibration^[45]. The standard samples for calibration were prepared in the same way as the samples of prostate fluid. Because there were no available liquid Certified Reference Material (CRM) ten sub-samples of the powdery CRM IAEA H-4 (animal muscle) were analyzed to estimate the precision and accuracy of results. Every CRM sub-sample weighing about 3 mg was applied to the piece of Scotch tape serving as an adhesive fixing backing. An acrylic stencil made in the form of a thin-walled cylinder with 11.3 mm inner diameter was used to apply the sub-sample to the Scotch tape. The polished-end acrylic pestle which is a constituent of the stencil set was used for uniform distribution of the sub-sample within the Scotch surface restricted by stencil inner diameter. When the sub-sample was slightly pressed to the Scotch adhesive sample, the stencil was removed. Then the sub-sample was covered with 4 μ m Dacron film. Before the sample was applied, pieces of Scotch tape and Dacron film were weighed using analytical balance. Those were again weighed together with the sample inside to determine the sub-sample mass precisely.

The facility for radionuclide-induced energy dispersive X-ray fluorescence included an annular ¹⁰⁹Cd source with an activity of 2.56 GBq, Si(Li) detector with electric cooler and portable multi-channel analyzer combined with a PC. Its resolution was 270 eV at the 6.4 keV line. The facility functioned as follows. Photons with the 22.1 keV energy from ¹⁰⁹Cd source are sent to the surface of a specimen analyzed, where they excite the characteristic fluorescence radiation, inducing the K_{α} X-rays of trace elements. The fluorescence radiation got to the detector through a 10 mm diameter collimator to be recorded.

Table 1. Energy dispersive X-ray fluorescence data of Br, Fe, Rb, Sr, and Zn contents in the IAEA H-4 (animal muscle) reference material compared to certified values (mg/kg, dry mass basis)

Element	Certified values			This work results
	Mean	95%CI	Type	Mean \pm SD
Fe	49	47-51	C	48 \pm 9
Zn	86	83-90	C	90 \pm 5
Br	4.1	3.5-4.7	C	5.0 \pm 1.2
Rb	18	17-20	C	22 \pm 4
Sr	0.1	-	N	< 1

Mean: arithmetical mean; SD: standard deviation; C: certified values; N: non-certified values

Table 2. Some basic statistical parameters of Br, Fe, Rb, Sr, and Zn concentration (mg/L) in prostate fluid of patients with BPH and PCa

Disease	Element	Mean	SD	SEM	Min	Max	Median	Per. 0.025	Per. 0.975
BPH 52-75 years <i>n</i> = 52	Br	2.32	1.84	0.30	0.230	8.70	1.62	0.268	5.84
	Fe	11.5	10.8	1.8	1.06	54.1	9.31	1.09	38.9
	Rb	1.70	1.41	0.23	0.210	5.04	1.46	0.254	5.04
	Sr	1.41	1.09	0.26	0.230	4.79	1.12	0.300	4.02
	Zn	488	302	42	45.0	977	427	81.4	962
PCa 47-77 years <i>n</i> = 24	Br	4.51	7.19	2.27	0.697	24.3	2.08	0.704	20.4
	Fe	21.7	28.8	8.7	7.70	107	13.9	7.70	86.8
	Rb	0.53	0.38	0.11	0.013	1.39	0.422	0.024	1.26
	Sr	1.70	2.15	0.76	0.230	6.83	0.872	0.275	5.95
	Zn	62.0	98.3	20.1	2.82	371	21.6	3.43	358

M: arithmetic mean; SD: standard deviation; SEM: standard error of mean; Min: minimum value; Max: maximum value; Per. 0.025: percentile with 0.025 level; Per. 0.975: percentile with 0.975 level; DL: detection limit; BPH: Benign prostatic hyperplasia

The duration of the Zn concentration measurement was 10 min. The duration of the Zn concentration measurement together with Br, Fe, Rb, and Sr was 60 min. The intensity of K_{α} -line of Br, Fe, Rb, Sr, and Zn for EPF samples and standards was estimated on calculation basis of the total area of the corresponding photopeak in the spectra.

All EPF samples for EDXRF were prepared in duplicate and mean values of TE contents were used in final calculation. Using the Microsoft Office Excel programs, the summary of statistics, arithmetic mean, standard deviation, standard error of mean, minimum and maximum values, median, percentiles with 0.025 and 0.975 levels was calculated for TE concentrations in EPF of hyperplastic and cancerous prostate. The difference in the results between two groups of samples (BPH and PCa) was evaluated by the parametric Student's *t*-test and non-parametric Wilcoxon-Mann-Whitney *U*-test.

RESULTS

Table 1 depicts our data for Br, Fe, Rb, Sr, and Zn mass fractions in ten sub-samples of CRM IAEA H-4 (animal muscle) and the certified values of this reference material. Of 4 (Br, Fe, Rb, and Zn) TE with certified values for the CRM IAEA H-4 (animal muscle) we determined contents of all certified elements [**Table 1**]. Mean values ($M \pm SD$) for Br, Fe, Rb, and Zn were in the range of 95% confidence interval. Good agreement of the TE contents analyzed by ^{109}Cd radionuclide-induced EDXRF with the certified data of CRM IAEA H-4 [**Table 1**] indicate an acceptable accuracy of the results obtained in the study of the prostatic fluid presented in **Tables 2-4**.

Table 2 presents certain statistical parameters (arithmetic mean, standard deviation, standard error of mean, minimal and maximal values, median, percentiles with 0.025 and 0.975 levels) of the Br, Fe, Rb, Sr, and Zn concentrations in EPF of patients with BPH and PCa.

Table 3. Median, minimum and maximum value of means of Br, Fe, Rb, Sr, and Zn concentration (mg/L) in prostate fluid of patients with BPH and PCa according to data from the literature

Disease	Element	Published data [Ref.]			This work results
		Median of means (n)*	Minimum of means M or M ± SD, (n)**	Maximum of means M ± SD, (n)**	M ± SD
BPH	Br	-	-	-	2.32 ± 1.84
	Fe	-	-	-	11.5 ± 10.8
	Rb	2.35 (1)	2.35 ± 1.85 (11) ^[43]	2.35 ± 1.85 (11) ^[43]	1.70 ± 1.41
	Sr	-	-	-	1.41 ± 1.09
	Zn	459 (7)	268 (7) ^[46]	9870 ± 10130 (11) ^[47]	488 ± 302
PCa	Br	-	-	-	4.51 ± 7.19
	Fe	-	-	-	21.7 ± 28.8
	Rb	1.11 (1)	1.11 ± 0.57 (15) ^[43]	1.11 ± 0.57 (15) ^[43]	0.53 ± 0.38
	Sr	-	-	-	1.70 ± 2.15
	Zn	65.4 (6)	34.7 ± 34.6 (13) ^[44]	722 (3) ^[49]	62.0 ± 98.3

M: arithmetic mean; SD: standard deviation; (n)*: number of all references; (n)**: number of samples; BPH: Benign prostatic hyperplasia

Table 4. Comparison of mean values (M ± SEM) of Br, Fe, Rb, Sr, and Zn concentration (mg/L) in prostate fluid of patients with BPH and PCa

Element	Age groups				Ratios
	BPH	PCa	Student's <i>t</i> -test <i>P</i> value	<i>U</i> -test* <i>P</i> value	PCa to BPH
Br	2.32 ± 0.30	4.51 ± 2.27	≤ 0.364	> 0.05	1.94
Fe	11.5 ± 1.8	21.7 ± 8.7	≤ 0.272	> 0.05	1.89
Rb	1.70 ± 0.23	0.53 ± 0.11	≤ 0.000024 [#]	< 0.01 [#]	0.31
Sr	1.41 ± 0.26	1.70 ± 0.76	≤ 0.729	> 0.05	1.21
Zn	488 ± 42	62.0 ± 20.1	≤ 0.00000001 [#]	< 0.01 [#]	0.13

#significant difference (*P* ≤ 0.05). M: arithmetic mean; SEM: standard error of mean; *Wilcoxon: Mann Whitney *U*-test; BPH: Benign prostatic hyperplasia

The comparison of our results with published data for Br, Fe, Rb, Sr, and Zn concentrations in EPF of hyperplastic and cancerous prostate^[41-44,46-50] is shown in Table 3. A number of values for Zn concentrations in EPF were not expressed on a wet mass basis in the cited literature. Therefore, we calculated these values using the published data for water - 93.2%^[50].

The ratios of means and the differences between mean values of Br, Fe, Rb, Sr, and Zn concentrations in EPF of patients with BPH and PCa are presented in Table 4.

DISCUSSION

The mean values and all selected statistical parameters were calculated for 5 (Br, Fe, Rb, Sr, and Zn) TE concentrations [Table 2]. The concentrations of Br, Fe, Rb, Sr, and Zn were measured in all, or a major portion of EPF samples of hyperplastic and cancerous prostate.

The mean of Zn concentration obtained for BPH group of prostate fluid, as shown in Table 3, agrees well with median of means cited by other researches^[41-44,46-50]. The mean of Rb concentration obtained for EPF samples of CP group agrees well with our data reported 38 years ago^[43]. No published data referring to Br, Fe, Rb, and Sr concentrations in EPF samples of patients with CP were found.

In the EPF samples of cancerous prostate our results were comparable with published data for Zn concentrations [Table 3]. The mean of Rb concentration obtained for EPF samples of PCa group was some lower than our data reported 38 years ago^[43]. No published data referring to Br, Fe, and Sr concentrations in EPF samples obtained from patients with PCa were found.

From Table 4, it is observed that in EPF samples of PCa group the concentrations of Rb and Zn are 3.2 and 7.7 times, respectively, lower than levels of these trace elements in EPS of patients with BPH.

The range of means of Zn concentration reported in the literature for EPF of untreated hyperplastic prostate (from 268 mg/L to 9,870 mg/L) and cancerous prostate (from 34.7 to 722 mg/L) varies widely [Table 3]. This can be explained by a dependence of Zn content on many factors, including age, ethnicity, mass of the gland, presence of benign prostatic hyperplasia, and others. Not all these factors were strictly controlled in cited studies. Another and, in our opinion, leading cause of interobserver variability was insufficient quality control of results in these studies. In many reported papers EPF samples were dried at high temperature or acid digestion. Sample digestion is a critical step in elemental analysis and due to the risk of contamination and analytes loss contributes to the systematic uncontrolled analysis errors^[51-53]. Thus, when using destructive analytical methods it is necessary to control for the losses of TE, for complete acid digestion of the sample, and for the contaminations by TE during sample decomposition, which needs adding some chemicals. It is possible to avoid these not easy procedures using non-destructive methods. Therefore, sample-nondestructive technique like ¹⁰⁹Cd radionuclide-induced EDXRF, which was developed and used by us^[54,55] is good alternatives for TE determination in EPF samples.

The ¹⁰⁹Cd radionuclide-induced EDXRF developed to determine TE concentrations in prostate fluid is micro method because sample volume 20 µL (one drop) is quite enough for analysis. It is another advantage of the method. Amount of human prostatic fluid collected by massage of the normal prostate is usually in range 100-500 µL^[56] but in a pathological state of gland, particularly after malignant transformation, this amount may be significantly lower. Therefore, the micro method of ¹⁰⁹Cd radionuclide-induced EDXRF developed to determine TE concentrations in prostate fluid is available for using in clinical studies.

Characteristically, elevated or deficient levels of TE and electrolytes observed in EPF of cancerous prostate are discussed in terms of their potential role in the initiation, promotion, or inhibition of prostate cancer. In our opinion, abnormal levels of TE in EPF of cancerous prostate could be the consequence of malignant transformation. Compared to other fluids of human body, the prostate secretion has higher levels of Rb and Zn and some other TE. These data suggests that these elements could be involved in functional features of prostate. The suppressed prostatic function can be both a cause and a consequence of BPH. However, malignant transformation is accompanied by a loss of tissue-specific functional features, which leads to a significant reduction in the contents of elements associated with functional characteristics of the human EPF (Rb and Zn).

Our findings show that concentration of Rb and Zn are significantly lower in EPF of cancerous prostate as compared to their concentrations in EPF of hyperplastic prostate [Table 4]. Thus, it is plausible to assume that levels of these trace elements in EPF can be used as tumor markers. However, this subjects needs in additional studies.

This study has several limitations. Firstly, analytical techniques employed in this study measure only five TE (Br, Fe, Rb, Sr and Zn) concentrations in EPF. Future studies should be directed toward using other non-destructive analytical methods which will extend the list of TE investigated in EPF of hyperplastic and cancerous prostate. Secondly, the sample size of PCa group was relatively small. It was not allow us to carry out the investigations of TE contents in PCa group using differentials like histological types of tumors, stage of disease, and dietary habits of healthy persons and patients with PCa. Despite these limitations, this study provides evidence on cancer-specific Rb and Zn level alteration in EPF and shows the necessity the need to continue TE research of EPF in prostatic diseases.

In conclusion, in this work, TE measurements were carried out in the EPF samples of hyperplastic and malignant prostate using non-destructive instrumental EDXRF micro method developed by us. It was shown

that this method is an adequate analytical tool for the non-destructive determination of Br, Fe, Rb, Sr, and Zn concentration in the EPF samples of human prostate. It was observed that in the EPF of cancerous prostate contents of Rb and Zn significantly lower in a comparison with those in the EPF of hyperplastic prostate. In our opinion, the decrease in levels of Rb and Zn in the EPF of cancerous prostate might demonstrate an involvement of these TE in etiology and pathogenesis of malignant prostate tumors. It was supposed that the changes of Rb and Zn levels in the EPF samples can be used as tumor markers.

DECLARATIONS

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Authors' contributions

Collected EPF samples, designed the EDXRF of samples, and carried out the statistical analysis of results: Zaichick V

Managed the literature searches, wrote the first draft of the manuscript, and translated the manuscript into English: Zaichick S

Read and approved the final manuscript: Zaichick V, Zaichick S

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

All studies were approved by the Ethical Committees of the Medical Radiological Research Centre, Obninsk. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent for publication

Not applicable.

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Review

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The role of hypoxia-induced factor 1 α in breast cancer

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Abstract

Breast cancer usually grows very quickly, becoming insensitive to blood flow in nearby veins; because of that, inside solid tumors it's possible to find a hypoxic environment, in other words, an environment where oxygen is less available. Another feature of cancer is its angiogenesis rate, because of the high energy demand, new blood vessels must be produced to take nutrients inside the solid tumor mass. Even with normal blood flow bringing the cancer oxygen and nutrients, its cells favor hypoxia, in an event known as Warburg Effect. According to the Warburg Effect, cells, even with normal oxygen rates, prefer to use fermentation instead of the citric acid cycle to produce ATP. For the cancer to operate normally in hypoxia, a transcription factor family is activated, known as hypoxia-induced factors (HIF), composed of a HIF-1 β and a HIF-1 α subunits. As HIF-1 α is expressed during hypoxia, it is a great target for treatments and a breast cancer biomarker. Because of the role of HIF-1 α in cancer and the high incidence of breast cancer worldwide, this review was performed in order to bring the most recent results concerning the role HIF-1 α can exert in breast cancer development and progression.

Keywords: Breast neoplasms, hypoxia-induced factor 1, HIF-1 α

INTRODUCTION

Cancer, an extremely aggressive heterogeneous disease, is the second leading cause of death in the world^[1]. The hallmarks of cancer are evasion of apoptosis, self-sufficiency in growth signals, insensitivity to anti-



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growth factors, sustained angiogenesis, limitless replication potential, and tissue invasion/metastasis^[2]. To proliferate, tumors must form their own vascular network and blood supply, which is due to the influence of tumor angiogenesis factors. However, the new vascular network that is formed differs from that found in healthy tissues because it presents a series of structural and functional alterations. These alterations may interfere with the adequate supply of blood and oxygen, leading to hypoxia, a state in which the organism is functioning with reduced and/or no oxygen supply^[3]. In order for the cancer to prevent its metabolism from being diminished or ceased in this situation, a family of transcription factors called hypoxia-induced factors (HIF) is activated.

HIF is known to act on hematopoiesis, angiogenesis, apoptosis, cell reproduction, cancer cell metabolism among other functions^[2-5]. HIF-1 α action is complemented by the transcriptional co-activator with PDZ-binding motif (TAZ), activating different genes that are involved in the same cascade. Both HIF-1 α and TAZ play an important role in remodeling the extracellular matrix, one of the important steps for tissue invasion to occur; these genes act as co-activators of each other, potentiating the expression of HIF-1 α target genes^[6,7].

HIF-1 α is especially important in the metabolism of cancers, being part of several metabolic pathways that cancers use, such as metabolism in hypoxia, angiogenesis, cell reproduction, *etc.* Thus, cancers with a high rate of metastasis and recurrence - as is the case with breast cancer - have their physiology strongly linked to HIF-1 α . Based on that, this review sought to group the most recent experimental studies on the role that HIF-1 α plays in the development and progression of breast cancer - one of the most incident and deadly cancers worldwide - resistance to conventional treatments, as well as proposals for new treatments based on its expression and/or inhibition.

HIF-1 α MODULATION UNDER HYPOXIA

The genes in HIF family are composed of two subunits: HIF-1 α (or one of its isoforms, HIF-2 α or HIF-3 α) and HIF-1 β (which is expressed constitutively, acting on a range of transcriptional systems)^[8]. The HIF-1 α subunit, when in normoxia, is rapidly degraded via the 26S ubiquitin-proteasome pathway by the von Hippel-Lindau tumor suppressor protein; in situations of hypoxia, however, it has increased activity^[2-4].

Under periods of normoxia, proline residues of HIF-1 α are hydrolyzed by prolyl-4-hydroxylases. Hydroxylated HIF-1 α is then recognized by VHL and ubiquitinated by the E3 ubiquitin-protein ligase complex. After, its proteasomal degradation occurs^[9]. Breast cancer grows very rapidly and usually the tumor microenvironment has reduced oxygen distribution^[10]. In situations of hypoxia, hydroxylation of HIF-1 α does not occur, after which it is translocated and accumulates in the nucleus, where it binds to HIF-1 β ; the HIF-1 α /HIF-1 β complex binds to hypoxia-response element, adhering to hypoxia target genes such as vascular endothelial growth factor receptor (VEGF) and insulin-like growth factor 2 (IGF-2), stimulating angiogenesis and proliferation^[9]. Inhibition of HIF-1 α generated inhibition of signaling by VEGF/VEGFR2 factors, which in turn prevented angiogenesis^[10].

A model of HIF-1 α modulation in hypoxia involves receptor of activated protein C kinase-1 (RACK1) and heat shock protein-90 (HSP90)^[11]. In it, human rhomboid family-1 (RHBDF1) controls the competition between RACK1 and HSP90 for binding to HIF-1 α . The interaction between RACK1 and HIF-1 α results in its degradation, however prior to that RHBDF1 binds to RACK1, giving way for HIF-1 α to bind to HSP90, resulting in its stabilization. This model would explain the high levels of HIF-1 α , Rack1, HSP90 and RHBDF1 in various cancer cells, including breast cancer. This facilitation of HIF-1 α activity as a result of the action of RHBDF1 would also explain the relationship between the high protein level of RHBDF1 in distant metastases and lymph nodes^[11].

The RAS protein activator 1 (RASAL1) protein is known to be involved in the progression of hypoxia resistance in breast cancer cells. The increase of intracellular Ca²⁺ ions stimulates RASAL1, which leads to

attenuation of RAS activation and mitogen-activated protein kinase (MAPK) activity and contributes to tumor progression through weakened anti-RAS activity^[12]. In periods of hypoxia, reactive oxygen species accumulation activates PI3k/Akt (Protein-kinase B) and MAPK/ERK (extracellular signal-regulated kinase) pathways, essential for the maintenance of carcinogenesis, tumor angiogenesis and activation of HIF-1 α .

PROGNOSIS VALUE OF HIF-1 α EXPRESSION

Several genes are influenced by HIF-1 α expression. In addition to the high expression of HIF-1 α being directly associated with tumor growth rate, metastatic potential, and consequently to the poor prognosis of patients, it is also associated with tumor progression, since as the cancer stage advances, HIF-1 α expression increases^[13]. Thus, the possibility of using HIF-1 α as a prognostic marker has already been discussed by several authors^[14-16].

HIF-1 α up-regulates Snail expression - a gene involved in induction of the epithelial-mesenchymal transition (EMT) - promoting increased migration and aggressiveness of breast cancer cells. HIF-1 α together with Snail are responsible for the down-regulation of E-Cadherin expression, promoting EMT^[17]. After inhibiting HIF-1 α and Snail expression in triple negative breast cancer (TNBC) cells by adding small doses of farnesyltransferase inhibitors, the following mRNA levels of HIF-1 α expression pathway genes were also decreased: Snail, glucose transporter 1 (GLUT1), pyruvate dehydrogenase kinase 1 and lactate dehydrogenase A, all involved in the metabolism of hypoxia.

There is also a correlation between HIF-1 α protein expression and the erythropoietin receptor protein in aggressive breast cancer samples, which leads to the worsening of the cases, since both play an important role in angiogenesis^[18]. γ -secretase is an intramembranous protease that cleaves transmembrane proteins^[19]. HIF-1 α containing the γ -secretase complex also contributes to the migration and invasiveness phenotype of breast cancer cells. Another gene that is influenced by HIF-1 α is heregulin, a growth factor that binds to Erb-B2 receptor tyrosine kinase 3 (ErbB3) and ErbB4 receptors with a known role in cell proliferation, survival, motility and tumorigenesis^[20]. According to the authors, stimulation of the ErbB3 receptor and the activation of the P-Rex1/Rac1 pathway via induction of C-X-C Motif Chemokine Receptor 4 are transcriptionally mediated by HIF-1 α .

In breast neoplasms, SETD8, a methyltransferase that methylates H4K20, along with a transcription factor related to cancer progression, has the function of regulating the increase and of N-Cadherin expression and the decrease of E-Cadherin^[21]. This modulation would be responsible for the epithelial-mesenchymal transition (EMT) phenotype, which increases the invasive potential of breast cancer cells. An interaction between SETD8 and HIF-1 α was identified, as well as co-localization in the human embryonic kidney 293T and MDA-MB-231 cell nuclei, indicating that they interact with each other. SETD8 was seen to slightly regulate HIF-1 α expression, and SETD8 silencing increased the acetylation and hydroxylation of HIF-1 α , demonstrating that SETD8 plays a role in the stabilization of HIF-1 α . It was also found that SETD8 and HIF-1 α expression in patients with TNBC and HER2+ breast cancer were higher than in the Luminal A and Luminal B molecular profile^[21].

Interleukin-17 (IL-17) is a cytokine produced by T Helper lymphocytes associated with inflammatory diseases that has also been related to onset, development and metastasis of tumors^[22]. IL-17 can modulate the expression of some genes in breast cancer cells by altering their adhesion properties through pathways of expression including that of HIF-1 α . Tumor necrosis factor α (TNF- α), in turn, is a cytokine associated with cell survival, apoptosis, inflammation and immunity^[22]. In addition, TNF- α regulates the expression of cell adhesion proteins, which aid in the determination of a metastatic phenotype in tumor cells. Increased levels of HIF-1 α were also found in groups of cells treated with IL-17 and TNF- α in a dose-dependent manner^[22].

HIF-1 α expression in breast cancer cells influences AKT and ERK pathways, which promote angiogenesis, further worsening the patient's prognosis^[10]. Another transcription pathway that was also seen to be more expressed along with the high expression of HIF-1 α was the PI3K/AKT pathway, which is important in signaling proliferation, invasion, angiogenesis, etc.^[23].

HIF-1 α expression is directly related to the expression of VEGF^[16,24] and Axl (Tyrosine-kinase receptor), which is described as being more expressed in a hypoxic environment and acts in much the same way as HIF-1 α (angiogenesis, metastasis, invasion, etc.). It was also seen that both aided in the regulation of EMT, which in turn is an important step in the progression and metastasis of cancers^[24].

When the increase of HIF-1 α expression is regulated by lysophosphatidic acid receptor 2, it has been associated with increased proliferation, migration and cell invasion parameters^[15,24]. An increase in the expression of the *EZH2* gene (enhancer of zeste homologue 2, known to act on the aggressive profile of tumors) is completely linked to that of HIF-1 α ^[25]. Patients expressing high levels of HIF-1 α and *EZH2* had worse prognoses, data showing that both can potentially lead the individual to death^[25]. A direct relationship between the high HIF-1 α expression and a greater p53 expression has also been described, which is an aggravating factor for the progression of breast cancer, since the loss of p53 function implies an increase in vascular endothelial growth factor (VEGF) expression mediated by HIF-1 α , and consequently, increased angiogenesis^[13].

An assessment of HIF-1 α and nuclear factor-kappa B (NF- κ B) levels as prognostic factors in breast cancer, related to the sub-type of cancer and overall survival (OS) was done by Rajkovic-Molek *et al.*^[26]. Activation of HIF-1 α and NF- κ B (detected by nuclear staining) was found in 41% and 31% of tumors, respectively. In addition, HIF-1 α was related to worse OS, making its expression an unfavorable prognostic factor^[26].

HIF-1 α IN DRUG RESISTENCE

The high expression of HIF-1 α has been related to worse prognosis not only by the pathways affected that lead to more aggressive tumors, but also by its association with resistance to hormone, chemo - and radiotherapies in breast cancer^[27,28].

Tamoxifen is a drug which acts as an estrogen receptor antagonist, widely used in the treatment of breast cancer, as well as in prevention in women who are prone to develop breast cancer^[29]. Lactate generated through cell fermentation in breast cancer (mediated by HIF-1 α and the Warburg effect) is related to high rates of resistance to Tamoxifen, and can therefore be a marker of resistance to treatment^[29,30]. HIF-1 α expression inhibits estrogen receptor α (ER α), which contributes to Tamoxifen resistance and a worse prognosis. Tumors expressing HIF-1 α along with ER α were observed to have a much more aggressive profile^[29]. Letrozole, in turn, is an aromatase inhibitor, a class of drugs used to treat ER positive breast neoplasms^[31]. That the non-hypoxic expression of HIF-1 α mediated the resistance to Letrozole effected by HER2 was also seen. HIF-1 α protein synthesis increases when activation of phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (mTOR) pathway by HER2 occurs in long term letrozole-treated cells. As a result, HIF-1 α up-regulates the expression of breast cancer resistant protein, which contributes to resistance to Letrozole^[31]. HIF-1 α is also involved in AI resistance in acquired cases and/or in *de novo* cases, showing a potential therapeutic target for resistance to Letrozole and other AIs.

Trastuzumab is a monoclonal antibody against the HER2 domain, one of the most commonly used drugs for the treatment of HER2+ breast cancers. The induction of resistance to Trastuzumab in breast cancer cells through the activation of the signal transducer and activator of transcription protein (STAT3) pathway/HIF-1 α / hairy and enhancer of split-1 (Hes-1) leading to a down-regulation of phosphatase and tensin homolog (PTEN)

was evaluated^[32]. Knockdown of HIF-1 α in Trastuzumab-resistant cells was observed to cause a decrease in the proliferation of HIF-1 α , just as the up-expression of HIF-1 α in cells caused resistance to the anti-proliferative effect of Trastuzumab, showing that there is a direct link between the levels of HIF-1 α expression and resistance to Trastuzumab. HIF-1 α up-regulates HES-1 expression, a negative regulator of the PTEN promoter, implying that HIF-1 α can modulate PTEN expression in Trastuzumab-resistant cells with the constitutive activation of the STAT3 protein^[32].

4-Hydroxynonenal (4-HNE) is produced in the lipid peroxidation of cells and is present at high levels in invasive breast cancer cells. The role of 4-HNE in the stabilization of HIF-1 α in breast cancer cells was assessed by Li *et al.*^[32]. Sirtuin-1 (SIRT1) and Sirtuin-3 (SIRT3) are covalently modified by 4-HNE, leading to SIRT3 deacetylase activity. SIRT3 was also observed to destabilize the HIF-1 α protein and inhibit its activity. Regulation of cell growth, invasion and expression of VEGF may be the result of the destabilization of the HIF-1 α protein by the inhibition of SIRT3, induced by 4-HNE. These results led researchers to conclude that 4-HNE is involved in resistance to chemotherapeutic drugs via up-regulation of HIF-1 α ^[33].

Zhong *et al.*^[34] studied the role of HIF-1 α in the regulation of radiation-induced autophagy in mammary neoplasia cells. Michigan Cancer Foundation-7 (MCF-7) in cultures mimicking hypoxia had greater survival of cells when exposed to ionizing radiation. Isogenic cells constitutively expressing HIF-1 α small hairpin RNA decreased the expression of HIF-1 α and also the formation of colonies. This fact suggested that the HIF-1 α knockdown increased radiosensitivity.

Long non-coding RNAs (LncRNAs) are non-coding transcripts involved in the modulation of various signaling pathways, acting as oncogenes or tumor suppressors during tumorigenesis^[35]. The urothelial carcinoma-associated 1 (UCA1) lncRNA, which induces adriamycin resistance in MCF-7 cells, was used to test for resistance to tamoxifen in breast cancer cells. Cells expressing UCA1 lncRNAs were transfected with an up-expressing HIF-1 α vector, and UCA1 lncRNA was found to be regulated in ER+ cells in a HIF-1 α -dependent manner^[35]. UCA1 expression was also observed to be increased by an miR-18 - HIF-1 α feedback loop.

HIF-1 α INHIBITION AS THERAPEUTIC AGENT

As seen previously, the high expression of HIF-1 α is associated with a worse prognosis and resistance to antineoplastic treatments. The low expression of HIF-1 α , by its turn, is linked to several metabolic alterations, since it influences the regulation of other genes that are important in the process of metastasis of breast cancer^[36,37]. The low expression of HIF-1 α leads to decreased expression of the *Snail* and twist-related protein 1 (*TWIST1*) genes, signaled by transforming growth factor β 1 (TGF- β 1), a protein that stimulates cell differentiation and proliferation that is known to regulate the EMT process in tumor cells^[36]. Both *Snail* and *TWIST1* are genes most commonly expressed in hypoxia. The silencing of HIF-1 α led to the termination of TGF- β -regulated breast cancer cell invasion^[36]. Reduced expression of HIF-1 α increased apoptosis when on serum starvation. At the same time, Caspase 3 fragments were identified with enhanced activity, indicating that HIF-1 α maintains cell growth and survival by inhibiting apoptosis^[38]. It was also observed that reduced expression of HIF-1 α inhibited TNBC migration and invasion ability^[38]. Thus, given the importance of the role of HIF-1 α in the development and progression of neoplasias, this gene has become the target of several studies that have been carried out to identify or create HIF-1 α inhibitors that could be used as therapeutical agent, as can be seen in the studies below.

Brachyury is a transcription factor of the T-Box family characterized by a highly conserved DNA binding domain called T-Domain, which is directly related with the progression of tumor cells^[39]. High Brachyury expression was related to a significant increase in HIF-1 α expression in MCF-7 and T47D cells. Akt/PTEN

signaling is known to be a regulator of HIF-1 α expression, and on that account the specific inhibitor LY290004, which inhibited Akt phosphorylation and consequently prevented Brachyury-induced HIF-1 α expression, revealing that Brachyury is a transcription factor that can be targeted in breast cancer treatments along with HIF-1 α ^[39].

A HIF-1 α putative inhibitor is T2A or TSN^[36,40-42]. Breast cancer cells treated with this drug showed a decrease in expression levels of HIF-1 α and VEGF proportional to the concentration of T2A administered^[36]. T2A was also used to inhibit EMT in breast cancer cells via HIF-1 α ^[40]. Two cell lines with hypoxia-induced doxorubicin (DOX) resistance in relation to T2A, MCF-1 and HCC1937, were analyzed. T2A decreased the DOX resistance of both lines in hypoxia partially via HIF-1 α ^[40,41]. It was also found that T2A reduced the viability and proliferation rate in both cell lines, indicating that T2A can target the cell cycle^[40].

Dutasteride, a double-blocker of type 1 and type 2 isoforms of the steroid-5 α -reductase (*SRD541*) gene is used in the treatment of benign prostatic hyperplasia. As *SRD541* is highly expressed in breast tumor cells, Dutasteride was tested in MDA-MB-231 and MDA-MB-468 cells *in vitro*^[43]. MDA-MB-231 cells showed a decrease in VEGF expression, while the detection of HIF-1 α was at the limit of detection of the technique employed. MDA-MB-468 cells had decreased VEGF and HIF-1 α , showing the potential effect of Dutasteride treatment in response to chemotherapy of TNBCs via altered protein expression of HIF-1 α and VEGF.

In order to verify the effects of pigallocatechin-3-gallate on the expression of HIF-1 α and VEGF in breast cancer cells, MCF-7 cells were treated with different concentrations of this compound^[44]. These cells had a dramatic decrease in growth and decreased expression of VEGF and HIF-1 α in a dose-dependent manner. Also working with cells, Dewangan *et al.*^[23] evaluated the inhibitory effects of centchroman, a non-steroidal oral contraceptive on HIF-1 α . As was expected, an elevated expression of HIF-1 α in cells prior to treatment was observed, which was inhibited with drug administration. After treatment, a decrease in VEGF/VEGFR2 expression was observed, with a consequent decrease in angiogenesis^[23]. Sarkar *et al.*^[10], on the other hand, demonstrated that the administration of phenethyl isothiocyanate, an organic sulfur of the Isotiosianate family found in cruciferous plants decreased HIF-1 α expression in normoxia and hypoxia. In addition, administration of N-acetyl cysteine, a known antioxidant, which had been used as a positive control was also observed to inhibit HIF-1 α expression. A correlation analysis was then performed to try to determine the association between the parameters involved in the low regulation of HIF-1 α by phenethyl isothiocyanate. A positive correlation was found between HIF-1 α and HSP90, and these two parameters were correlated with reactive oxygen species (ROS). According to the authors, ROS can be considered the main regulator of both HIF-1 α and HSP90^[10].

The drug M410 [(Z)-3,4,5-trimethoxystilbene-3'-O-phosphate disodium, a CA4 analog], which is a potent tubulin polymerization inhibitor in bovine brain *in vitro*, had its inhibitory potential for HIF-1 α evaluated by Yang *et al.*^[45] Upon treatment, the expression of HIF-1 β was found to be unaltered; however, HIF-1 α expression was decreased. The amount of VEGF and glucose transporter 1 messenger RNA in the cells was also analyzed, which was very low in relative to normality, showing that M410 was directly involved in the transcriptional activity of HIF-1 α ^[45].

DCQ (2-benzoyl-3-phenyl-6,7-dichloroquinoxaline 1,4-dioxide) contains an N-oxide portion (present in hypoxia-activated drugs used in cancer treatments) similar to tirapazamine (TPZ) used in treatments against other types of cancer, but inefficient in breast cancer^[46]. Similarly to the results with TPZ treatments found in the literature, DCQ induced an increase in p-Akt levels and a reduction in p-mTOR levels suggesting possible anti-translational activity in MCF-7 cells, which would decrease the rates of HIF-1 α . DCQ was identified as a promising drug in the treatment of breast cancer because it exhibits pro-apoptotic and anti-metastatic features by reducing HIF-1 α and the post-HIF-1 α gene signaling cascade^[46].

Extracellular matrix metalloproteinase inducer (EMMPRIN) glycoprotein, also known as cluster of differentiation 147 (CD147), is a densely glycosylated type I transmembrane glycoprotein, highly expressed in tumors^[47]. This glycoprotein is known to induce a number of malignancy characteristics in breast cancer cells, such as invasiveness, angiogenesis, anchorage independent growth and chemioresistance. SUM102 and BT474 cells were treated with human recombinant EMMPRIN, in which the levels of STAT3 and HIF-1 α were observed to be increased. To investigate the role of HIF-1 α , the cells were infected with lentivirus carrying HIF-1 α shRNA^[47]. HIF-1 α was observed to increase the percentage of CD24 surface markers (stem-like cells) and decreased the percentage of CD44 via STAT3. In addition, miR-106a/b targeted STAT3 and HIF-1 α , and mammary neoplasms treated with miR-106a/b had decreased STAT3 and HIF-1 α , and consequently a significant attenuation of stem-like characteristics^[47]. Also working with miRNA, Pakravan *et al.*^[48] evaluated whether the transfer of exosomes from mesenchymal stem cells (MSCs) containing miR-100 - a tumor suppressor - could have an effect on angiogenesis in breast cancer cells. The miR-100 present in MSCs exosomes was found to inhibit the expression of HIF-1 α and mTOR in MDA-MB-231 and MCF-7 cells, which consequently inhibited VEGF expression, contributing to the control of angiogenesis^[48].

To understand the functioning of pathways of acquisition of malignancy characteristics, notorious in breast cancer, Kuo *et al.*^[49] treated several breast cancer cell lines with pharmacological doses of dimethyl-2-ketoglutarate (DKG). DKG induced HIF-1 α in 6 cell lines, rapidly but transiently, and DKG treatment was shown to create a “pseudohypoxic” state in normoxia. An increase in succinate and fumarate rates was then seen, along with a decline in SDH and FH levels. This imbalance of metabolites may then impair PHD2 activity, stabilizing HIF-1 α and reprogramming the surface of breast cancer cells^[49]. The phenotype was maintained for up to 3 cell passages, and an increase of Carbonic anhydrase 9 (CAIX) (which is one of the genes downstream of the HIF-1 α signaling pathway) was seen in DKG-treated breast cancer cells. Taking into account that CAIX is seen in high-grade cancers, this result suggests mitochondrial dysregulation may induce tumorigenicity^[49]. Considering that, Tosatto *et al.*^[50] evaluated mitochondrial calcium uniporter (MCU) in the progression of breast cancer cells. The MCU is a selective channel that absorbs Ca²⁺ ions in the mitochondria, which is correlated with infiltration in lymph nodes and tumor size. Silencing of the MCU drastically decreased mitochondrial ROS levels and down-regulated HIF-1 α . When treated with Paraquat (a substance that generates superoxide production), there was an increase in the transcription of HIF-1 α , which led the researchers to relate HIF-1 α to an effector of MCU depletion^[50].

A novel class of an antigenic strategy known as the decoy approach was used by Zhu *et al.*^[51], who showed that cells treated with a HIF-1 α decoy had the transcriptional activity of HIF-1 α inhibited and entered apoptosis.

HIF-1 α INHIBITION BY NATURAL COMPOUNDS

Still considering the inhibition of HIF-1 α as antineoplastic therapy, we have listed here the most recent works dealing with natural compounds, be they extracted from animal plants or microbes.

Manassantin A (MA), a compound isolated from the organism *Saururus cernuus*, which was identified as a potent inhibitor of HIF-1 α was used in an attempt to inhibit the function of HIF-1 α in breast cancer cells^[9]. Several MA derivatives were synthesized in the search for one presenting low toxicity and a good inhibitory effect on HIF-1 α . LXY6090 was developed which initially inhibited HIF-1 α activity well. The compound was used in T47D, MCF-7 and MX-1 lines, and a decrease in the protein levels of HIF-1 α in the cells was observed in a dose-dependent manner. LXY6090 also inhibited the accumulation of HIF-1 α mRNA by promoting proteasome degradation in a VHL-dependent way. In addition to these effects, LXY6090 was also observed to inhibit the hypoxia-induced protein expression of HIF-1 α target genes such as VEGF and IGF-2, demonstrating a great potential as the target of further research for the treatment of breast cancer associated with an up-expression of HIF-1 α .

Another compound that is known to modulate glycolysis in tumor cells is oroxylin A (OA), an active flavonoid found in the plant *Scutellaria baicalensis* Georgi, widely used in Chinese medicine. In breast cancer cells, OA was observed to decrease HIF-1 α expression at the protein level. SIRT3 acts as a tumor suppressor by regulating the mitochondrial protein manganese superoxide dismutase, reducing ROS levels. In hypoxia, HIF-1 α is stabilized by the inhibition of prolyl hydroxylases by ROS generation. The regulation of O²⁻ (Superoxide anion) by SIRT3 plays an important role in the activation of PHD induced by OA, destabilization of HIF-1 α and suppression of glycolysis^[52].

Ascofuranone is an antibiotic with antifungal properties that has been isolated from cultures of the phytopathogenic fungus *Ascochyta viciae*. In addition to its antibiotic and antiviral effects, it also has some physiological effects such as hypolipidemic activity, suppression of hypertension, immuno-modulation, and amelioration of type II diabetes^[53]. Therefore, the effect of Ascofuranone on the expression of VEGF and HIF-1 α was examined, in order to determine if the antibiotic would also act on angiogenesis. Ascofuranone decreased HIF-1 α expression with a consequent decrease of VEGF expression in MDA-MB-231 cells in a dose-dependent manner. Ascofuranone was subsequently shown to inhibit the phosphorylation of Akt, mTOR and p70S6K, inhibiting epidermal growth factor-induced protein synthesis^[46]. Another compound that suppress HIF-1 α by the Akt/mTOR/p70S6K and MAPK pathways in a dose-dependent manner is Ft1 (notoginsenoside Ft1) a saponin isolated from *Panax notoginseng*^[54].

Andrographolide (Andro), a substance extracted from *Andrographis paniculata*, an Asian plant used for fever, diarrhea and infectious diseases, also has anti-tumor characteristics such as attenuation of cell invasion, inhibition of cell cycle progression, among others^[55]. Thus, the authors evaluated the relationship between Andro and HIF-1 α and its possible antitumor effect in breast cancer cells. In cultures of MDA-MB-231 and T47D cells with Andro, a decrease in HIF-1 α levels was observed in a dose-dependent manner^[55]. It was also found that Andro down-regulated HIF-1 α -mediated VEGF expression, which would be an indicator of improvement in angiogenesis.

That some lactobacilli have a cytotoxic effect on neoplastic cells has already been described in the literature. Based on that, Esfandiary et al.^[56] decided to use the culture supernatant of *Lactobacillus crispatus* (*L. crispatus*) (LCS - *L. crispatus* supernatant) and *Lactobacillus rhamnosus* (*L. rhamnosus*) (LRS - *L. rhamnosus* supernatant) in cultures of TNBC MDA-MB-231. It was found that LRS had a greater cytotoxic effect on MDA-MB-231 cells than LCS. Treatment with LRS led to a decrease in mRNA levels of HIF-1 α , HSP90 and SCL2A1 (GLUT1), whereas LCS treatment down-regulated the expression of HSP90, SHARP1 (gene encoding proteins that act on the degradation of HIF-1 α) and VHL.

Diallyl trisulfides is an organic sulfur compound present in garlic that modulates various biological processes and its effects were studied on the process of metastasis in breast cancer cells^[57]. According to the authors, the compound at different concentrations inhibited not only the transcription but also the translation of HIF-1 α ^[57].

Berberine is an alkaloid purified from a plant of the genus *Berberis*, widely used in Eastern medicine, and exhibits many pharmacological effects such as antihypertensive, antibacterial and antitumor effects. Berberine was observed to inhibit the expression of P-glycoprotein (P-gp), which when up-expressed is a marker of drug resistance. AMP-activated protein kinase (AMPK) is also seen in processes of resistance to antitumor treatments, since it is a P-gp modulator in human cancer cells in hypoxia^[58]. Hypoxia was observed to induce AMPK activation, elevating the protein levels of AMPK, P-gp and HIF-1 α in MCF-7 cells. HIF-1 α was subsequently found to be a protein of the AMPK pathway and that regulates P-gp expression in MCF-7 cells, and that small doses of Berberine increased DOX chemosensitivity by inhibiting AMPK, subsequently downregulating the expression of HIF-1 α and of P-gp. Yet high doses of Berberine down-regulated HIF-1 α , which induced p53 activation, leading the mammary tumor cells to apoptosis *in vitro*^[58].

CONCLUSION

In this review, several experimental studies the role of HIF-1 α in breast cancer, whether in *in vitro* or *in vivo* models, have been included. HIF-1 α has an immense potential both as a target for various breast cancer treatments and for the prevention and diagnosis of breast cancer. The importance of research with HIF-1 α is undeniable. Because of its wide range and several signaling pathways, it has a huge potential for use in the treatment, follow-up and diagnosis of breast cancer. Many innovative treatments using chemicals, drugs and substances exogenous to the body have been employed to better understand the way HIF-1 α works in the organism. Analysis of the different points in the HIF-1 α expression cascade is of upmost importance in order to gain greater knowledge about how to use HIF-1 α in favor of the treatment and prevention of breast cancer and other diseases in which this factor plays an important role.

However, that there is a dearth of studies using HIF-1 α directly has been observed, since most of the articles found used HIF-1 α as a complement to confirm the data obtained with other genes/proteins/signaling pathways, or only as one of the components of the work. It is hoped that in the future more research will be done using HIF-1 α to better understand its function, and new ways of using the knowledge of the functioning of this transcription factor to our advantage.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: Carlos HFP, Glaucia LV, Fernando F, Beatriz CAA

Performed data acquisition, as well as provided administrative, technical, and material support: Carlos HFP, Jessica AE, Matheus MP, Glauco SAA, Beatriz CAA

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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A new view of the mammary epithelial hierarchy and its implications for breast cancer initiation and metastasis

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Abstract

The existence of mammary epithelial stem cell (MaSC) populations capable of mediating mammary gland development and homeostasis has been established for over a decade. A combination of lineage tracing and mammary gland transplantation studies has affirmed that MaSCs and their downstream progenitors are organized in a hierarchical manner; however, these techniques have failed to illuminate the complete spectrum of epithelial intermediate populations or their spatial and temporal relationships. The advent of single cell sequencing technology has allowed for characterization of highly heterogeneous tissues at high resolution. In the last two years, the remarkable advances in single cell RNA sequencing technologies have been leveraged to address the heterogeneity of the mammary epithelium. These studies have afforded fresh insights into the transcriptional differentiation hierarchy and its chronology. Importantly, these data have led to a major conceptual shift in which the rigid boundaries separating stem, progenitor, and differentiated epithelial populations have been deconstructed, resulting in a new more fluid and flexible model of epithelial differentiation. The emerging view of the mammary epithelial hierarchy has important implications for mammary development, carcinogenesis, and metastasis, providing novel insights into the underlying cellular states that may promote malignant phenotypes.

Keywords: Mammary gland, mammary epithelial cells, breast cancer, stem cell



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INTRODUCTION

Many of the processes that occur during mammary gland morphogenesis are appropriated during the formation and malignant progression of breast cancer. These include the regeneration and hormonally-driven proliferative expansion of the mammary epithelium, remodeling of the extracellular matrix and invasion into surrounding stroma, aversion of apoptosis, and recruitment of vasculature^[1,2]. Thus, understanding the endocrine, cellular, and molecular mechanisms that control mammary gland development should provide critical insights into the processes that also underlie breast cancer growth and metastases and reveal novel approaches for preventing and treating aggressive disease. This review focuses on recent advances in our understanding of the development of the mammary epithelial cell hierarchy, revealing the abundant progression of cell states, many of which could undergo malignant transformation and give rise to the heterogeneous disease known as “breast cancer”.

The mammary gland is a uniquely dynamic organ that undergoes extensive remodeling throughout life in response to cytokine signaling and hormonal cues^[3,4]. The primitive mammary epithelium is first detected around embryonic day 11.5 (E11.5) in the mouse. Between embryonic day 16 and embryonic day 18 (E18), this rudimentary structure invades the mammary fat pad, forming a primitive ductal structure. During puberty, the mammary epithelium expands to form a branched, ductal network that invades the stroma until it reaches the edge of the mammary fat pad. This ductal structure is maintained into adulthood where it remains highly flexible in response to oscillating levels of ovarian hormones during the estrous cycle, undergoing extensive proliferation and apoptosis^[5]. In pregnancy, a unique hormonal milieu triggers the formation of secretory alveolar structures to form at the terminal end of each duct. Alveolar units function to produce milk proteins during lactation. Once lactation has ceased, the gland undergoes a process of involution in which massive architectural remodeling occurs, resulting in an epithelial structure that resembles the pre-pregnant state. Although morphologically similar, genetically engineered mouse models permitted tracing of “terminally” differentiated lactating cells and revealed that these cells contribute to the remodeled post-involuting gland and are molecularly distinct from their virgin counterparts^[6,7]. The events of expansion, differentiation, and repopulation of the mammary tree reoccur with each subsequent pregnancy indicating extensive plasticity of the cells that comprise the gland. Closer examination reveals that the mammary gland consists of two main epithelial lineages, the inner luminal lineage and the outer myoepithelial lineage. The luminal lineage can be further subdivided into mature ductal and milk-producing alveolar cells. Myoepithelial cells are commonly referred to as “basal” since they are located on the basal side of the luminal cells^[8]. These cells are highly contractile, and function to facilitate the movement of milk through the ducts.

The ability of the gland to undergo successive rounds of reconstruction in response to multiple pregnancies implicates the existence of a mammary stem cell (MaSC) population capable of generating all mature cell types within the gland. Studies utilizing transplantation and lineage tracing techniques further established that mammary epithelial differentiation is organized in a hierarchical, branched pathway wherein multipotent MaSCs give rise to a series of downstream intermediates^[8-10]. With each differentiation step, cells become increasingly lineage-restricted moving from multi- to bi- to uni-potent progenitor states until terminally differentiating into mature epithelial populations.

Discoveries over the last 50 years have been integral to our understanding of the mammary epithelial cell hierarchy; however, the advent of single-cell (sc) sequencing technologies coupled with microfluidic cell sorting has recently uncovered intricacies in lineage relationships and heterogeneity of the mammary epithelium. Recent evidence has demonstrated that each cell is unique in its transcriptomic signature^[11-14]. This revelation has redefined the differentiation cascade as a continuous trajectory of heterogeneous populations passing through gradual transcriptional states. These studies suggest that historical, rigid, models of the epithelial hierarchy should be replaced by a model that incorporates the diverse transcriptional profiles and

paths represented throughout development, underscoring the immense diversity and flexibility that likely underlies mammary gland development and gives rise to breast cancer heterogeneity. Here we discuss the recent studies utilizing scRNA-sequencing of mammary epithelium and how these new findings have shifted our current perspectives of mammary gland development and breast cancer.

HISTORICAL PERSPECTIVE OF THE MAMMARY EPITHELIAL HIERARCHY

In 1959, DeOme and colleagues developed the mammary transplantation assay^[15]. Using this technique, transplantation of murine ductal fragments^[16,17] or dissociated suspensions of normal mammary epithelium^[18] into the cleared fat pad of a recipient mouse resulted in the regeneration of a complete ductal structure. Smith and Medina then reported that morphologically distinct cells with regenerating capacity could be found throughout the entire mammary tree and persisted throughout pregnancy, lactation, and involution^[19]. Both the frequency and regenerative ability of these cells was not affected by age or reproductive history^[19,20]. Later studies demonstrated that mammary gland reconstitution was achievable upon transplantation of a single cell. This was first reported by Kordon and Smith, using serial transplantation and limiting dilution assays of mammary epithelium labeled with unique viral insertions generated by mouse mammary tumor virus (MMTV)^[21]. Similarly, by labeling cells with a LacZ transgene, Shackleton *et al.*^[22] confirmed this finding and demonstrated that stem cell populations were enriched in the basal epithelial fraction (defined by Lin⁻CD29^{hi}CD24⁺ cell surface markers). Additionally, cells from reconstituted glands harbored the same repopulating activity of the original and were capable of fully differentiating into milk-producing alveolar cells during pregnancy. These studies implicated the existence of a long lived multipotent mammary stem cell population (MaSC), capable of giving rise to all downstream lineages within the gland.

Following the disclosure of MaSCs, the search for molecular markers specific to the stem cell population became a major focus within the field. Transplantation of luminal vs. basal epithelial populations revealed that cells exhibiting stem-like properties were located within the basal fraction and could be enriched by FACS sorting using CD49f^{hi}CD29^{hi}CD24^{+/mod}EpCAM⁺Sca1^{low} expression as markers^[22-25]. Additionally, expression of s-SHIP^[26], LGR5^[27-29], Lrp5/6^[30], and Axin2^[31] enriched for populations with repopulating activity upon transplantation; however, stem cell activity was not exclusive to these subsets and the degree to which these markers may enrich for overlapping stem cell populations was not clear^[10]. Difficulty surrounding the identification of MaSC markers was due, in part, to the rarity of MaSCs in the adult gland. The frequency of repopulating cells was found to be higher during embryogenesis (~7% of total cells by E18 compared to ~2%-5% frequency in the adult gland)^[22,23,32,33]; however, enriched populations of fetal (f)MaSCs displayed altered gene expression profiles from their adult counterparts further complicating the isolation and characterization of a multipotent population^[32].

Transplantation studies demonstrated the existence of a stem cell entity that was capable of multi-lineage differentiation within the adult gland. Yet, various studies employing lineage tracing analyses led to confusion surrounding these findings. While some groups provided evidence to support multipotent cells, others reported that only unipotent cells remain after birth. Early lineage tracing experiments by Van Keymeulen *et al.*^[34], used the promoters of the keratin 14, 5, and 8 (K14, K5, K8) genes, to drive tracer expression and track both luminal (K8) and myoepithelial (K14, K5) lineages. This analysis demonstrated that prior to birth, K14⁺ cells were multipotent; however, after birth, these cells became unipotent, only giving rise to myoepithelial cells. Cells labeled by the K8 promoter revealed a second unipotent population responsible for maintaining the luminal lineage after birth. These unipotent populations supported epithelial expansion during puberty and pregnancy underscoring their long lived capacity within the gland. Additionally, lineage tracing at the single cell level revealed that unipotent populations were dispersed throughout the ductal tree, estimating that within each major duct, ~20 luminal and ~15 basal unipotent cells contribute to ductal expansion during puberty^[35]. Furthermore, by employing clonal analyses at saturation using DOX-inducible systems to trace luminal (K8rtTA-CreTetO/

Rosa-TdTomato) and myoepithelial (K14rtTA-CreTetO/Rosa-TdTomato) lineages, Wuidart *et al.*^[36] also reported unipotent progenitors as solely responsible for maintenance of adult lineages. Evidence of luminal unipotent progenitors that are restricted to the alveolar fate has also been reported. In this case, the “parity-induced mammary epithelial cell” (PI-MEC) population consists of alveolar-like cells that originate during a female’s first pregnancy^[6]. Unlike the majority of alveolar cells, PI-MECs do not undergo apoptosis during involution. Rather, these cells remain as a residual population within the parous gland and serve as alveolar precursors during subsequent pregnancies^[6,37].

Contrary to these results, some genetic fate-mapping studies have supported the existence of multi- and bi-potent mammary epithelial cell populations within the adult. For example, tracing of the Wnt target protein C receptor (Procr) illustrated the presence of a multipotent cell within the basal layer of the adult gland that was capable of giving rise to all mature lineages^[33]. Furthermore, studies have also reported bipotent progenitors in the adult gland. By combining a multi-color Cre reporter with three dimensional imaging techniques, Rios and colleagues traced luminal (ELF5+) and basal (Keratin 5+) cells during puberty and adulthood^[28]. This analysis resulted in the tracing of discreet clonal patches of luminal or basal cells as well as patches containing both lineages, indicating a common cellular origin^[28]. Similarly, an inducible Cre reporter mouse line, driven by the Axin2 promoter, labeled both bipotent and unipotent cells within the adult gland^[38]. Further complicating these studies, Axin2+ cells can undergo cell fate switching^[38]. This phenomenon has also been reported in mammary epithelial cells that are positive for Lgr5^[29]. Interestingly, Axin2+ unipotent cells are basally-restricted during adulthood but become bipotent during pregnancy, suggesting that cell fate switching may occur in response to hormonal cues^[10,38]. Pregnancy-induced bipotent mammary epithelial cells were also recently reported in a study by Song *et al.*^[39]. Lineage tracing of K8+ luminal cells revealed bipotent luminal cells that gave rise to basal progeny during pregnancy or upon stimulation with estrogen or progesterone. Additionally, transplantation of luminal-derived basal cells into cleared fat pads of recipient mice, demonstrated their capacity to generate new mammary gland structures with normal morphology.

The conflicting results generated from transplantation studies with those from lineage tracing has been a source of controversy in the field. The primary question still remains: does a multipotent stem cell exist after birth as indicated by transplantation assays; or are progenitor populations solely responsible for postnatal development? Inherent flaws within transplantation and lineage tracing methods likely contribute to the conflicting evidence within the field.

Transplantation assays have been highly scrutinized since this method includes the dissociation of mammary epithelial cells from their normal tissue architecture followed by their reintroduction into a new mammary tissue environment that has undergone invasive surgical resection. The drastic micro-environmental changes that epithelial cells must endure for this procedure has raised concerns that this assay may impart multipotent potential onto cells that would otherwise be restricted to specific differentiation outcomes^[10]. For example, Wnt/-catenin-responsive cells only give rise to myoepithelial cells during puberty and pregnancy, however; upon transplantation these cells can regenerate both luminal and myoepithelial lineages^[38]. Additionally, in lineage tracing experiments, K14+ cells are restricted to the myoepithelial lineage after birth; however, transplantation of K14-labeled cells from 4-week old mice results in the formation of new ductal structures containing both myoepithelial and luminal cells^[34]. The ability of transplantation to alter stem cell fate has also been demonstrated in other systems, including hair follicle development^[40] and hematopoiesis^[41]. It is plausible that differences in the microenvironment such as stromal, hormonal, and inflammatory cues, may impart phenotypic changes in mammary epithelial cell populations, activating primitive precursor pathways in their lineage. This may be especially relevant in the case of mammary transplants as the surgical resection needed to clear the innate epithelia likely elicits a “wound healing” response in the local environment of the transplant. Thus, transplantation assays may not accurately represent stem cell biology as it would intrinsically occur *in vivo*.

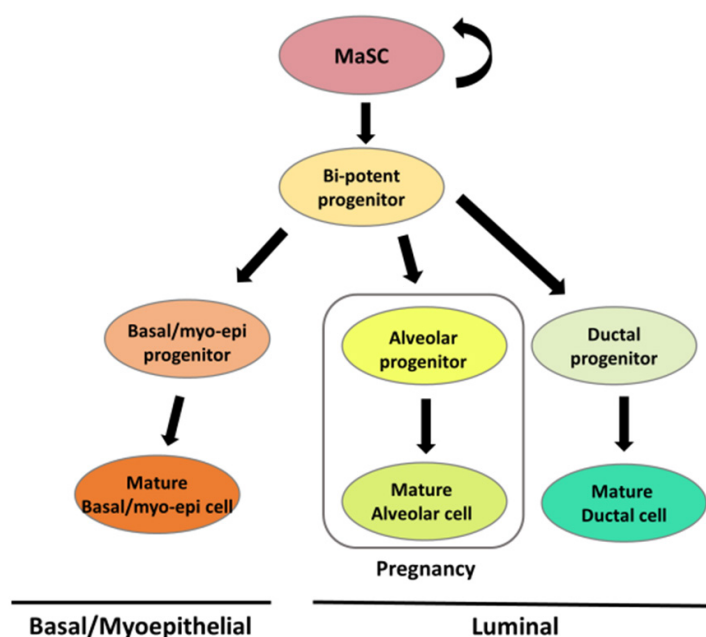


Figure 1. Historical model of the mammary epithelial cell differentiation hierarchy. Multipotent mammary stem cells (MaSCs) have the ability to self-renew and give rise to all downstream lineages. Bipotent stem cells give rise to committed unipotent progenitor populations that maintain basal/myoepithelial and luminal lineages. These unipotent cells are responsible for maintaining mature basal/myoepithelial and luminal lineages in the postnatal gland. Two luminal progenitor populations have been reported, one giving rise to alveolar cells during pregnancy and the other responsible for maintaining the mature ductal cells

Although lineage tracing allows for the visualization of cellular differentiation within the context of the normal tissue environment, it is not without its own set of limitations. The ability to detect rare heterogeneous populations of cells using genetic lineage tracing is highly dependent on recombination efficiency. High variability in the extent of recombination not only exists between mouse models (i.e., K5-Cre versus K14-Cre) but also between individual mice from the same strain^[42]. In addition, labeling efficiency varies significantly throughout the ductal tree of a single mammary gland^[42]. Several explanations for this variability have been postulated^[8,10,42]. Inadequate strength and specificity of the promoter driving recombination could result in inconsistent labeling. This may be particularly relevant to quiescent stem cells that are transcriptionally uncommitted to a certain lineage. Additionally, inducible creERT2 models are reliant on accurate dosing of Tamoxifen, a selective estrogen receptor (ER) modulator, to induce sufficient recombination. Since mammary gland development is highly dependent on ER signaling, tamoxifen administration, even at low doses, can alter normal mammary gland biology and lead to false readouts^[43].

Taken together, the results from both transplantation and lineage tracing studies have led to a highly compartmentalized view of the mammary epithelial hierarchy in which fetal MaSCs give rise to a common bipotent progenitor which differentiates into lineage-restricted and unipotent luminal and myoepithelial progenitors. Unipotent progenitors then give rise to mature myoepithelial and luminal lineages (for detailed reviews see Visvader and Stingl^[10], 2014, and Yang *et al.*^[44], 2017) [Figure 1]. Although transplantation and lineage tracing methods have been fundamental to our understanding of mammary epithelial biology, it has become increasingly evident that these methods are insufficient to fully characterize rare or highly heterogeneous cell populations. Gene expression signatures on bulk isolates of the major epithelial compartments including luminal progenitors, mature luminal, and MaSC-enriched basal epithelial cells, have been generated^[45]. However, the utility of these signatures to identify critical molecular regulators and markers of epithelial subsets has been limited due to the heterogeneity of each epithelial population in which only a small fraction of cells expresses the same set of transcripts (as in the case of stem populations within the basal fraction)^[22,32,46]. As a single cell can give rise to an entire mammary gland, it is imperative that the

molecular states and instructive signals occurring at the single cell level be examined to fully grasp how individual cells traverse the differentiation cascade as well as discern how specific cell states can re-emerge in breast cancer and contribute to metastasis.

A NEW VIEW OF THE MAMMARY HIERARCHY

Single-cell sequencing has emerged as a powerful tool capable of uncovering the transcriptional heterogeneity of diverse cell types within a tissue^[47]. Although transcriptome profiling of single cells was reported over 20 years ago^[48,49], only recently has it become a widely utilized technique. Technological advances in areas such as microfluidics and in situ barcoding have allowed for high throughput, cost effective sequencing of hundreds to thousands of cells within a given sample^[50]. Furthermore, the ability to analyze large numbers of cells at high resolution has mandated the development of innovative approaches for downstream data analysis. Software tools have recently become available to infer evolutionary history and lineage relationships within heterogeneous cell populations^[51].

Recently, several independent laboratories have utilized scRNA-seq to identify epithelial subpopulations and their differentiation trajectory within the mammary gland^[11-14]. Together these studies have examined the complete mammary developmental cycle encompassing embryonic through adult stages and pregnancy through post-weaning. The transcriptional landscape generated from these studies has provided critical insight into epithelial differentiation, transforming the way in which we view the mammary epithelial hierarchy. The new model emerging from these studies emphasizes the fluid and gradual progression of epithelial differentiation and reveals a greater degree of cellular heterogeneity than had previously been acknowledged.

SINGLE-CELL SEQUENCING OF THE MAMMARY EPITHELIUM

Prior to birth, sc-RNA sequencing has shown that the epithelium consists of a continuum of primitive cellular states in which unique subpopulations could not be detected^[12]. This supports previous findings generated from gene expression analysis of bulk fMaSCs, demonstrating that fMaSCs are comprised of a single CD24+CD49f+ population^[32]. During embryogenesis, the majority of mammary epithelial cells exhibit dual expression of both luminal and myoepithelial signature genes^[12,32,52]. This hybrid gene expression signature is associated with multipotent potential and a loss of dual lineage expression as cells become restricted to unipotent potential^[52].

At birth, many mammary epithelial cells maintain a hybrid gene expression profile^[12]. This dual lineage signature may include rare bipotent progenitor populations previously observed by lineage tracing studies^[28,38]. However, the majority of this population likely represents cells spanning various transitional stages of lineage restriction. Cells become increasingly more lineage restricted throughout puberty; however, the precise timing of the major bifurcation event between luminal and basal cells remains unclear. In work by Pal *et al.*^[53], scRNA-sequencing of mammary epithelium from 2 (pre-puberty), 5 (puberty), and 10 (adult) week-old mice demonstrated that before puberty, the mammary epithelial cell populations are largely homogeneous, expressing a basal (myoepithelial)-like gene signature. Upon puberty, a fraction of cells upregulate expression of luminal genes such as Epcam, and Keratins 8, 18, and 19, suggesting that bifurcation initiates at this time^[53]. However, Giraddi *et al.*^[12], specifically assessed changes in the early postnatal epithelium by examining glands from mice at day zero (PN0) and day 4 (PN4) after birth. In contrast to the report by Pal and colleagues, they found that two distinct cellular populations, one containing myoepithelial cells and the other containing luminal precursors, begin to emerge as early as PN4. While these studies confirm that the mammary epithelium remains in a primitive state until postnatal stages during which lineage restriction commences, the specific timing of those commitments remains unclear.

Sc-RNA sequencing of adult stages clearly demonstrate that mature epithelial cells are transcriptionally segregated into basal (myoepithelial) and luminal lineages^[11,12,53]. Within these overarching phenotypes, up to 15 transcriptionally distinct clusters (11 luminal and 4 basal) have been reported across nulliparous and reproductive stages^[11]. However, the majority of these subpopulations cannot be characterized by expression of a single gene, indicating that cells remain highly heterogeneous and many of these clusters may represent transient states of differentiation. The luminal lineage within the nulliparous gland undergoes multiple differentiation events. Luminal progenitors give rise to a series of intermediate states that bifurcate to generate a hormone responsive lineage (ER+) and a secretory alveolar lineage (ER-)^[11,12,14]. Upon pregnancy, ER- cells undergo massive expansion to produce the alveolar cells needed for lactation. The expanded population expresses genes associated with proliferation, fatty-acid transport, and lipid biosynthetic and homeostatic processes^[11]. Recent lineage tracing studies have proposed that ER+ and ER- luminal lineages arise from independent progenitor populations^[54,55]. In work by Wang *et al.*^[54], PROM1+ progenitors were found to give rise to ER+ luminal cells whereas SOX9+ progenitors give rise to ER- cells. Thus, as heterogeneous luminal populations undergo gradual differentiation, the acquisition of SOX9 or PROM1 expression may be a major determinant of further luminal cell fate specification.

STEM AND PROGENITOR POPULATIONS DETECTED BY SINGLE-CELL RNA SEQUENCING

The sc-RNA-sequencing studies reported to date have not detected a single transcriptionally distinct multipotent stem cell population within the adult murine gland^[11,12,53]. However, dormant populations that possess stem potential under specific environmental cues may exist. In a study by Sun *et al.*^[13] mammary epithelial stem cell markers including Bcl11b^[56], Tspan8^[57], and Procr^[33] were detected in the adult gland; however, expression on these genes was mutually exclusive, indicating each labels an independent population. Further analysis of a novel population marked by Cdh5+, revealed increased stem activity within this group of cells as demonstrated by both mammosphere and *in vivo* transplantation assays^[13].

In human breast, bipotent progenitor populations within the adult gland have been identified in cells isolated from reduction mamoplasties^[14]. Using Monocle software analysis of sc-RNA sequencing data collected from seven individuals, Nguyen *et al.*^[14], mapped both luminal and myoepithelial lineages to a common cellular origin. These cells exhibited a basal gene expression signature as well as expression of ZEB1. This contrasts with the inability to identify subpopulations of bipotent progenitors using sc-RNA sequencing of mouse mammary glands. Discrepancies between mouse and human studies may be due to differences in the methodologies employed to generate scRNA-seq profiles [Table 1]. These include epithelial cell isolation methods, read depth, and data analysis and interpretation. For example, in work by Giraddi *et al.*^[12] (2018) and Bach *et al.*^[11] (2017), sc-RNA sequencing clustering results were based on epithelial cells that were positive for EPCAM expression. Thus, there remains the possibility that an EPCAM-stem cell population was overlooked in the murine studies. Additionally, cells double positive for the major luminal and basal cell markers, cytokeratin 8 and 14 respectively, are detected within the basal epithelial layer of human adult breast tissue^[14]. This is in contrast with mouse basal epithelium where double positive cells are not detected after birth^[22]. This difference in dual cytokeratin expression coupled with the interpretation that cells expressing both luminal and basal markers represent bipotent progenitors may contribute to the differences between mouse and human scRNA-seq findings. Furthermore, evidence from transplantation assays suggests that stem populations are widely distributed within the gland^[16,17,19]; however, it is unknown if specific subpopulations are spatially restricted. In this regard, it has been reported that a group of highly quiescent MaSCs, marked by Tspan8 expression, are located within proximal regions of the adult murine gland, closest to the nipple^[57]. Thus, discrepancies between data sets may also arise depending on area of the mammary gland from which the sequenced cells were collected.

Luminal progenitors that are first identified as mixed lineage cells gradually gain luminal orientation during postnatal development^[53]. By puberty, a distinct luminal progenitor population can be detected. However,

Table 1. Comparison of scRNA-seq methodologies used to examine the mammary epithelial hierarchy

Author	Species and developmental time points examined	Cell isolation method	scRNA-seq Platform	Number of cells sequenced	Sequencing system	Read coverage (x) or Number of reads	Genes detected per cell
Bach <i>et al.</i> ^[11] 2017	Mouse: Nulliparous Day 14.5 Gestation Day 6 Lactation 11 days post-involution	Lin ⁻ (Ter119 ⁺ CD31 ⁻ CD45 ⁻) EpCAM ⁺	10 × Genomics Chromium	25,010	Illumina HiSeq 2,500	~ 162 million	2,118
Pal <i>et al.</i> ^[53] 2017	Mouse: Pre-puberty (2 weeks) Puberty (5 weeks) Adult (10 weeks)	Lin ⁻ (Ter119 ⁺ CD31 ⁻ CD45 ⁻) CD24 ⁺	10 × Genomics Chromium	3,308	NextSeq 500	Not reported	> 1,500
			Fluidigm C1	460	Illumina HiSeq 2000	2 million	8000
Giraddi <i>et al.</i> ^[12] 2018	Mouse: Embryonic day 16 Embryonic day 18 Post-natal day 4 Adult	EpCAM ^{low-high} Cd49f ^{medium-high}	10 × Genomics Chromium	6,424	Illumina HiSeq 2,500	5000-50,000	500-2000
			Fluidigm C1	262	Illumina HiSeq 2,500	> 1.5 million	4000-9000
Sun <i>et al.</i> ^[13] 2018	Mouse: Adult Day 12 Gestation	CD24 ^{Mid} CD29 ^{Hi} (Basal) CD24 ^{Hi} CD29 ^{Lo} (Luminal)	Fluidigm C1	318	Illumina HiSeq 2,500	> 1 million	12,688
Nguyen <i>et al.</i> ^[14] 2018	Human: Mamoplasties from age matched post-pubertal and pre-pubertal females	CD49f ^{hi} EpCAM ⁺ (Basal) CD49f ⁺ EpCAM ^{hi} (luminal) EpCAM ⁺ CD49f ^{hi/lo}	Fluidigm C1	868	Illumina HiSeq 2,500	~ 1.6 million	~ 4,500
			10 × Genomics Chromium	24,646	Illumina HiSeq 4000	~ 60,000	> 500 < 6000

scRNA-sequencing has further uncovered novel subcategories within the greater luminal progenitor population. For example, prior to puberty, CD55 is a marker of rare basal cells that give rise to a subset of luminal progenitors during adulthood^[53]. CD55+ cells within the luminal fraction exhibit increased colony forming ability compared to CD55- cells^[53]. In addition, populations positive for both CD55 and the progenitor marker, CD14 possess an even greater colony-forming ability. Luminal progenitors acquire additional complexity during pregnancy. Single-cell analysis of luminal progenitors in the nulliparous adult compared to post-involution glands revealed permanent transcriptomic alterations within a subset of post-involution cells^[11]. Following involution, luminal progenitors retain expression of genes associated with lactation and milk production. It is likely that this population overlaps with the previously described PI-MEC population^[6].

CELLULAR PLASTICITY OF THE MAMMARY EPITHELIUM

The ability of mammary epithelial cells to express both luminal and myoepithelial signature genes at various stages of maturation and reproduction suggests that a fraction of cells may maintain lineage plasticity throughout life^[12,14,52,53]. Studies investigating chromatin accessibility within the mammary epithelium at fetal and adult stages have demonstrated that bi-lineage gene expression is achievable due to epigenetic regulation of genes associated with multiple lineages^[12,58]. In a recent study by Dravis *et al.*^[58], gene expression profiles and open chromatin regions were compared between fetal (f)MECs, luminal progenitors, and mature lineages using RNA-seq and ATAC-seq, respectively. fMaSCs expressed both canonical luminal and basal genes and gene loci were associated with open chromatin, reflecting their immature, multi-lineage state (consistent with Giraddi *et al.*^[12]). While, the chromatin architecture of adult myoepithelial cells resembled that of fMaSCs in which luminal and myoepithelial gene loci were associated with open chromatin; only genes associated with the myoepithelial lineage were expressed. These results indicate that although myoepithelial cells express lineage-restricted genes, they retain the flexibility to adopt a luminal identity similar to multipotent fMaSCs^[58]. This may explain the multipotent capacity of basal cells observed

upon transplantation^[22,23]. Several epigenetic factors have been shown to play a role in defining mammary epithelial cell fate. These include the histone methylation reader POGZ^[59], the polycomb-repressive complex-1 member, BMI1^[60,61], and the polycomb-repressive complex 3 member, EZH2^[62]. BMI1 and EZH2 also play key roles in metastasis of breast cancers, likely due to their activation of more primitive cell fates^[63-66]. Yet how these players collaborate to orchestrate the epigenetic continuum that drives fate determination and heterogeneity is not fully understood.

Differential chromatin accessibility of both myoepithelial and luminal genes in basal cells may explain the ability of transcriptional drivers to induce cell fate switching in committed cells. In a recent example, overexpression of Notch1 in Smooth muscle actin (SMA)+ or K5+ myoepithelial cells, was sufficient to commit cells to a luminal fate^[67]. Complementary to this, overexpression of p63 in luminal cells was sufficient to reprogram them into a myoepithelial state^[52]. Although the mechanism of p63-mediated cell fate switching is unknown, chromatin remodeling is likely required as mature luminal cells exhibit repressed chromatin at basal gene loci^[58]. SOX10 has also emerged as a major transcriptional regulator of epithelial cell fate^[58]. Within fMaSCs, SOX10 motifs are enriched at accessible chromatin regions flanking highly expressed genes. Furthermore, tumors expressing high SOX10 levels exhibit neural crest-like features^[58] and high SOX10 expression is correlated with the aggressive, basal-like breast cancer subtype. As embryonic neural crest cells are multipotent and highly mobile^[68], SOX10 reprogramming may lead to primitive cellular states that could contribute to aggressive tumor phenotypes.

A NEW MODEL FOR THE MAMMARY EPITHELIAL HIERARCHY

Cumulative evidence from the recent papers utilizing sc-RNA-sequencing has revealed that the historical models by which the mammary epithelial hierarchy has been traditionally represented do not accurately portray the complexity of the system^[11-14,53]. Previous models in which cellular states are depicted as discrete populations that differentiate along restricted paths is an oversimplification [Figure 1]. Instead, recent results indicate that heterogeneous cell populations gradually advance, and likely also retreat, through a differentiation trajectory^[11,12,53]. This is supported by the work from several groups, reporting a vast array of epithelial transcriptional profiles throughout the stages of mammary gland development^[11-14,53]. Similar findings have redefined the hierarchical visualization of hematopoiesis^[69]. Using the new visual representation of hematopoietic differentiation suggested by Laurenti and Gottgens as an example, we propose a similar paradigm to represent the mammary epithelial hierarchy [Figure 2]. As depicted in this model, groups of heterogeneous epithelial cells traverse through the differentiation landscape, passing through cellular states that have been previously defined including fMaSCs, luminal and myoepithelial progenitors, and mature luminal and myoepithelial lineages. The newly reported heterogeneity of these populations implies that each cell may take a slightly different transcriptional path from the next as it undergoes differentiation. Additionally, the array of cellular states detected within the gland at any one time suggests that variation in temporal dynamics of differentiation may exist between individual cells. Moreover, this model implies significant forward and reverse plasticity of cell states that could be impacted by the microenvironment. This could partially explain historical difficulty in attempting to isolate and characterize specific subpopulations; however, further studies are needed to test these predictions.

IMPLICATIONS FOR TUMOR INITIATION AND METASTASIS

Breast cancer is an amalgam of diseases that exhibit both inter- and intra-tumoral heterogeneity^[70]. Gene expression profiling has led to the classification of five overarching subtypes, including luminal A, luminal B, HER2+, basal-like, and claudin-low^[71-74]. However, within each subtype, tumors can exhibit further variability in gene expression, molecular function, and drug susceptibility conveying distinct patient outcomes. The heterogenic nature of breast cancer is thought to arise from the combination of cellular origin, genetic and epigenetic changes, and environmental context^[1,10,70,75].

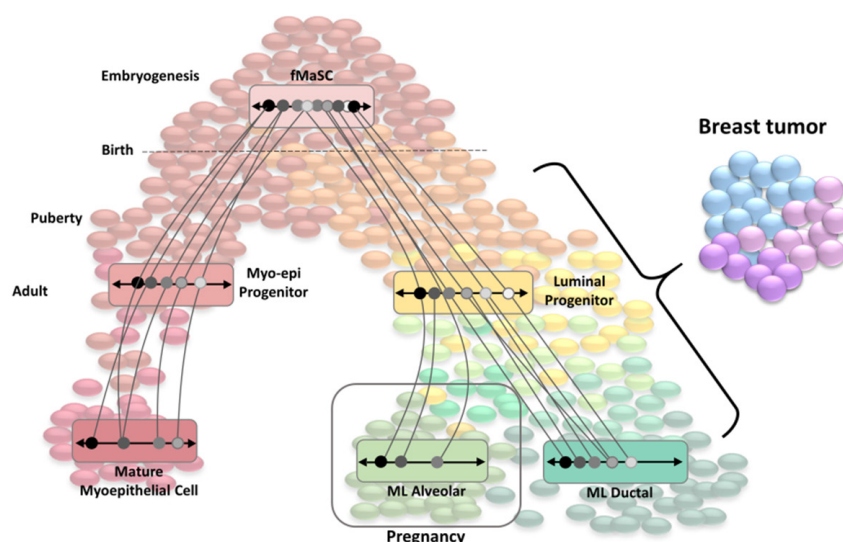


Figure 2. A progression model of mammary epithelial cell fate specification. Mammary epithelial differentiation occurs as cells pass through a continuous cascade of transcriptional states, with the majority of cells becoming increasingly more lineage-restricted throughout life and in response to pregnancy. Progressive lineage restriction is represented by gradual changes of the colors of cells (red, orange, yellow, green and blue). As cells differentiate, they pass through previously defined phenotypic compartments [fetal mammary stem cells (fMaSCs), Luminal and myoepithelial (myo-epi) progenitors, mature luminal (ML) ductal and alveolar cells and mature myoepithelial cells (rectangles)]. Grayscale circles (ranging from white to black) on the horizontal lines represent the various epigenetic and transcriptomic states found within each phenotypic compartment with the number and different shading patterns representing the degree of heterogeneity within each major phenotypic group. Arching and angled lines connecting those circles reflect the various transcriptional and epigenetic trajectories that cells pass through before eventually terminating into mature, differentiated lineages (myoepithelial, luminal, and alveolar). Cells at various points along the differentiation cascade may serve as tumor initiators, leading to inter- and intra-tumoral heterogeneity. Figure adapted from Girardi *et al.*^[12]

Molecular profiling studies have revealed that the gene expression patterns of cancer subtypes align with those of normal mammary epithelial cell lineages^[76,77]. This suggests that tumor subtypes may originate from distinct mammary epithelial subpopulations. It is widely speculated that epithelial stem/progenitor populations may serve as tumor initiating cells since their longevity and ability to self-renew affords the accumulation of genetic mutations. This has been supported by gene expression profiling studies. For example, the gene expression profile of claudin-low tumors most closely resembles that of MaSC/basal epithelial cells, whereas basal-like tumors more closely resemble luminal progenitors^[45,72,76,78]. Further evidence supporting luminal progenitors as the cell of origin for the basal-like subtype has been shown in tumors harboring BRCA-1 mutations^[76,77,79]. More recently, sc-RNA-sequencing of human breast epithelial cells demonstrated that cells expressing luminal progenitor genes correlated most highly with basal-like breast cancers from the METABRIC dataset^[14]. Despite these findings, not all cancer subtypes have been correlated with a cell of origin and the inability to isolate pure populations of mammary stem/progenitor cells has impeded functional testing. Moreover, it is also feasible that cancers acquire the transcriptional profiles of specific cell states due to the mutations that drive tumor development or progression and that the cells of origin may not be consistent with final tumor deposition. In this case, identifying the contextual cues that promote normal epithelial hierarchical progression would likely reveal pathways that are also activated or suppressed in tumors that promote their phenotypes.

The new view of the mammary epithelial hierarchy suggests that the continued search for a tumor cell of origin based on molecular profiles that have historically been used to characterize the major epithelial populations (mature luminal, myoepithelial, luminal progenitor ext.) is unlikely to reveal discreet cells from which different breast cancer subtypes emanate. The complexity of the normal epithelium suggests a similar heterogeneity is present in the primary and secondary tumors that arise from it. Indeed, a study comparing sc-RNA-seq profiles generated from triple-negative patient tumors revealed that a subset of cells within

each tumor expressed profiles resembling a spectrum of epithelial differentiation statuses^[80]. It now can be presumed that cells residing anywhere along the continuous differentiation trajectory may acquire tumor-initiating mutations [Figure 2] and that the location of the cell within the differentiation hierarchy will determine its transcriptional status and influence whether acquired mutations will be oncogenic. This is most consistent with the inability to identify a single lineage that contributes to HER2/Neu-induced tumors because amplification or overexpression of the ERBB2 gene may occur in an array of cell states rather than a single cell of origin for all tumors^[81,82]. Similar findings have been reported in leukemia, in which progenitor cells rely on the activation of the β -catenin pathway for oncogenic transformation^[83]. Although it is noted that some populations may be more susceptible to mutagenic events than others^[84,85], the possibility that phenotypically similar tumors arise from cells residing in the exact same cellular state is slim. This notion is supported by the transcriptional heterogeneity observed across breast cancer patient tumors within the same subtype^[86]. Several genetic mutations have been associated with the stratification of breast cancer subtypes. These include mutations in GATA3, RUNX1, or NCOR1 which are associated with the Luminal A subtype; whereas mutations in the tumor suppressor retinoblastoma, RB1, or CDH1 have been associated with luminal B tumors^[10,87,88]. Although these mutations are associated with the acquisition of a specific breast cancer subtype, it is likely the combination of the preexisting epigenetic and transcriptional status of the cell along with the transforming mutations that explain tumor heterogeneity and give rise to the variable therapeutic responses observed between patients.

Accumulating evidence suggests that a rare population of stem-like progenitor cells are responsible for tumor initiation, progression, metastasis, and therapeutic resistance^[89]. These cells are referred to as cancer stem cells (CSCs) or “tumor initiating cells” and share many characteristics with normal mammary epithelial stem cells including the ability to self-renew and differentiate. Additionally, CSCs share gene expression profiles that closely resemble those of primitive mammary epithelial cells, including the expression of stem and EMT associated genes^[90]. This has led to many to posit that CSCs arise from mammary stem/progenitor cells, however definitive evidence for the origin of CSCs in breast cancer is lacking^[91]. The extensive heterogeneity within the mammary epithelium that has been revealed by scRNA-seq makes it reasonable to presume that many cellular states have the potential to give rise to CSCs. Furthermore, the unique transcriptional and epigenetic context of an epithelial cell prior to transformation likely determines the aggressive nature of the arising tumor, including its ability to metastasize. Indeed, many studies have identified transcriptional regulators of normal mammary development that also encourage aggressive behaviors during cancer. This is the case for transcriptional drivers of stem cell and EMT programs including SOX9^[92], SLUG^[92], and SOX10^[58]. In a study by Guo *et al.*^[92], expression of both SOX9 and SLUG was found to be necessary for maintenance of the MaSC phenotype. Moreover, coexpression of SOX9 and SLUG in breast cancer cell lines was sufficient to induce stem cell-like properties, including activation of an EMT program and metastasis-seeding abilities. Similar results were observed for SOX10^[58]. Binding of SOX10 within regions of open chromatin that flank genes associated with EMT or with embryonic neural crest cells (NCC) was found to promote migratory and invasive behavior within mammary tumor cells. Thus, it is possible that epithelial cells expressing high levels of transcriptional drivers of EMT and stem-like states at the time of transformation may be predisposed to malignant phenotypes. Likewise, the chromatin accessibility of genes associated with processes such as EMT and NCC may ‘prime’ cells for aggressive behaviors associated with CSC phenotypes.

The formation of CSCs and their ability to seed new tumors at distant sites is also highly dependent on the surrounding microenvironment^[93-95]. Notably, contextual stimuli such as exposure to TGF β , can initiate stem cell and EMT programs in normal mammary epithelial cells that then promote their transition to a CSC state^[94]. The resulting CSCs remain highly susceptible to surrounding cues. This is especially relevant in the context of metastasis, in which Paget’s “seed and soil” hypothesis proposes that a tumor-permissive environment is required for disseminating tumor cells to engraft and metastasize into new tissue sites^[93,96]. As breast cancer arises from the normal epithelium, the contextual cues regulating normal epithelial

behavior are likely mirrored in breast cancer cells. Therefore, understanding the reciprocal interactions between epithelial cells and their niche should also inform the mechanisms underlying tumor initiation and metastasis.

In this regard, the recent studies using sc-RNA-sequencing outside the context of disruptive transplantation assays, have revealed that epithelial populations may be more highly influenced by the surrounding environmental cues than previously appreciated. As discussed earlier, previous studies using transplantation assays demonstrated the multipotent potential of rare mammary epithelial cells within the adult gland^[22,23]. However, lineage tracing analyses were unable to replicate these results and recent sc-RNA-sequencing has further supported the lack of multipotent cells during adulthood. Thus, it has become increasingly apparent that introduction of isolated cells into wounded stromal environment may elicit cellular phenotypes that are not supported under normal physiological conditions. A similar phenomenon is likely to occur with breast cancer initiation and progression. While acquisition of an “oncogenic” mutation may occur in varied cells along the differentiation hierarchy, its impact on tumorigenesis and progression may rely upon the stromal microenvironment, particularly an environment that is reflective of wound repair such as that which occurs with high breast density^[97], obesity^[98], and in post-lactational remodeling^[99]. The same is also likely to be true with metastasizing cancer cells. Instructional interactions between the epithelium and surrounding stroma are well established. This was most exquisitely demonstrated in extensive studies by Smith and colleagues revealing that the local microenvironment of the mammary gland is sufficient to reprogram progenitor cells from completely different tissues (including testicular and embryonic stem cells) into mammary progenitors that were capable of forming normal mammary gland structures^[100-104]. Furthermore, alterations in the microenvironment may influence cellular transformation. In work by Barcellos-Hoff and Ravani^[105], irradiating the cleared fat pads of recipient mice, lead to alterations in growth factor activity and extracellular matrix composition. Following irradiation, implantation of non-tumorigenic mammary epithelial cells resulted in tumor formation more readily in irradiated mice. Conversely, epithelial tumor cells injected into non-irradiated mammary fat pads reverted to form normal mammary gland ducts rather than tumors^[106].

The effects of contextual cues on the epigenetic and transcriptional status of major transcriptional regulators has also been recently highlighted. Although SOX10 is highly expressed in cells isolated from MMTV-PyMT tumors, this expression was lost when cells were grown in 2D culture conditions^[58]. The loss of SOX10 expression was associated with a switch from open to closed chromatin at the SOX10 locus, emphasizing the role of environmental cues on epigenetic status. This is not a new concept, as changes in the epigenetic landscape are known to occur in response to hormonal exposure^[62,107].

CONCLUSION

The scRNA-sequencing studies reviewed here have provided a detailed transcriptional map of mammary epithelial differentiation. However, these data indicate that previously underappreciated complexity undoubtedly underlies the regulation of the heterogeneous transcriptional networks observed. The unique transcriptional status within each epithelial cell is likely correlated with a unique epigenetic profile and it is anticipated that these profiles have a considerable level of plasticity that is dictated by the microenvironment and, in the case of tumors, mutational status. Studies examining the chromatin accessibility within bulk isolations of epithelial populations (MaSCs, basal, luminal and luminal progenitors) have demonstrated that each population is associated with distinctive active and repressed regions, many of which are associated with functional consequences and specific tumor subtypes^[12,58,107]. The emerging ability to simultaneously assess both the transcriptional and epigenetic status at a single cell level should allow for clearer understanding of the lineage relationships in the normal gland and in tumors as well as reveal whether tumor cells can readily transition through states as has been observed in the normal breast. It is important to note, however, that

while single cell sequencing technologies can provide high resolution maps of cellular states, they cannot make definitive predictive connections to the resulting cellular fates. The use of newly developed technologies, such as “CellTagging” that allow for early transcriptional statuses to be linked to cellular fates will allow for a clearer understanding of epithelial lineage relationships within the mammary gland^[108]. Furthermore, studies layering single cell technologies that assess transcriptional, epigenetic, and proteomic data within the normal gland, primary tumor, and associated metastatic lesions should provide important information regarding the extent of plasticity of primary and metastatic tumor cells that can be therapeutically leveraged to promote differentiation of breast cancers into less malignant states.

DECLARATIONS

Authors' contributions

Conceptualized and drafted the manuscript: Anstine LJ

Revised the manuscript for important intellectual content: Keri R

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Both authors declared that there are no conflicts of interest.

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Original Article

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Paxillin serine 178 phosphorylation in control of cell migration and metastasis formation through regulation of EGFR expression in breast cancer

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Abstract

Aim: Paxillin is a well-known multidomain scaffold protein that is involved in the regulation of cell-matrix adhesion dynamics, a process required for the tumor cell migration and invasion. Phosphorylation of the serine residue 178 requires c-Jun NH2-terminal kinase (JNK) activation, which occurs downstream of epidermal growth factor receptor (EGFR)-mediated signaling and drives cell migration. In this study, we investigated the significance of paxillin Ser178 phosphorylation in breast cancer progression.

Methods: We employed the rat mammary carcinoma MTLn3 cell line with which we established stabile variants of both wild type and mutant GFP-paxillin constructs. With those, we next performed several in vitro assays including cell proliferation, migration and focal adhesion dynamics. Finally, we monitored the metastatic spread of both cell line variants in an orthotopic mouse model for breast cancer.

Results: Here we show that expression of the phospho-defective mutant paxillinS178A in the metastatic mammary adenocarcinoma MTLn3 cell-line significantly decreased EGF-induced cell migration, which was correlated with impaired focal adhesion dynamics. Moreover, paxillinS178A attenuated lung metastasis formation in an orthotopic *in vivo* mammary gland tumor/metastasis model, demonstrating the importance of JNK-mediated paxillin phosphorylation in breast cancer progression. Expression of paxillinS178A caused a decrease in EGFR expression,



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while re-expression of EGFR in MTLn3-paxillinS178A cells fully restored EGF-driven cell motility and focal adhesion dynamics. Furthermore, re-expression of EGFR in MTLn3-paxillinS178A rescued spontaneous metastasis from breast to lung.

Conclusion: Overall our data show an important role for JNK-mediated paxillin Ser178 phosphorylation in the regulation of EGFR expression and thereby, in EGF-driven cell migration and metastasis formation.

Keywords: Paxillin, c-Jun NH2-terminal kinase, focal adhesion, epidermal growth factor receptor, cell migration, metastasis, breast cancer

INTRODUCTION

Breast cancer represents the most common type of cancer among women. The formation of metastases, which is a determinant of the prognosis of cancer patients, involves distinct cellular processes including cell migration, invasion, intra- and extravasation and proliferation. These processes are regulated by growth factors, cytokines and cellular matrix molecules^[1-6]. An important regulator of cell proliferation and migration is the receptor tyrosine kinase epidermal growth factor receptor (EGFR) whose stimulation by epidermal growth factor (EGF) results in the activation of downstream signaling pathways including different mitogen-activated protein kinases cascades: extracellular signal-regulated kinase (ERK), p38 and c-Jun NH2-terminal kinase (JNK)^[7-11].

The JNK group of kinases has essential roles in cancer development including regulation of the survival/proliferation balance as well as cell migration^[7,9,12,13]. Dual Thr and Tyr phosphorylation of JNK by upstream MAP kinases results in JNK activation and nuclear translocation. In the nucleus, JNKs phosphorylate and activate transcription factors including members of the AP-1 family such as c-Jun. The JNK-AP-1 pathway regulates the expression of genes involved in the cell cycle, survival and apoptosis and extracellular matrix homeostasis^[14-22]. Besides phosphorylation of transcription factors, several cytoskeleton-associated adaptor and signaling proteins have recently been identified as direct JNK substrates including β -catenin^[23] and paxillin^[24,25]. Since both proteins are well known to be involved in cell-matrix and cell-cell contacts, the direct JNK-mediated phosphorylation of these targets may also influence cell migration.

Paxillin is a 68 kD multidomain adaptor protein associated with focal adhesions^[26-31]. It is long known to function as a scaffold to integrate multiple signaling pathways involved in matrix adhesion dynamics and cell migration^[27,31-36], and recently, it was shown to play a role in the nucleus in relation to mRNA transcription and subsequent translation^[37,38]. Few studies have identified paxillin as a JNK substrate^[8,24,25]. EGF-driven JNK activation results in JNK-mediated phosphorylation of paxillin at Ser178 and is required for cell migration^[9,13,19,25,39]. In addition, expression of a phospho-defective Ser178 to Ala mutant of paxillin inhibited cell migration^[9,19,24]. How JNK-mediated phosphorylation of paxillin regulates cell migration is still under investigation. It is still unclear how this phosphorylation event is relevant for breast cancer metastasis formation^[40,41].

Here we explored the role and mechanism of paxillin Ser178 phosphorylation in breast tumor progression using the highly metastatic breast tumor cell line MTLn3 as a model. We show that ectopic expression of paxillinS178A significantly decreased EGF-dependent signaling and cell migration. Using an orthotopic mammary gland tumor/metastasis model, we demonstrate that the JNK-mediated phosphorylation of paxillin at Ser178 is essential for efficient metastasis of MTLn3 cells to the lung. Expression of mutant paxillinS178A was found to reduce EGFR expression, whereas re-expression of EGFR rescued the defected tumor cell migration and metastasis formation. Our data indicate that the phosphorylation of Ser178 of

paxillin by JNK can regulate cell migration and metastasis formation via modulation of the EGFR-signaling pathway.

METHODS

Chemicals and antibodies

Alpha modified minimal essential medium without ribonucleosides and deoxyribonucleosides (α -MEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), trypsin and geneticin (G418 sulphate) were from Life Technologies. Rat tail collagen type I was from Upstate Biotechnology. LipofectAMINE Plus transfection reagents were from Invitrogen. Primary antibodies were anti-paxillin (BD), anti-tubulin, anti-GFP (Sigma, St. Louis, MO), anti-pT183/pY185-JNK (Promega), anti-Phospho-Thr202/Tyr204 ERK1/2, anti-pSer473-AKT (Cell Signaling), anti-pSer178-paxillin (Abcam, Cambridge, UK), anti-EGFR for Western blot (rabbit polyclonal, Cell Signaling Technology) and FACS/immunostaining (mouse monoclonal, Calbiochem). All secondary antibodies were from Jackson. The Western-Star immunodetection system (Tropix kit) was from Applied Biosystems and ECL Plus reagent was from Amersham. Hoechst 33258 and rhodamine-phalloidin were from Molecular Probes and Aqua Poly/Mount was from Polysciences.

Cell culture

MTLn3 cells were cultured as before^[42]. To generate stable cell lines, MTLn3 cells were transfected with GFP-paxillin or GFP-paxillinS178A kindly provided by Huang et al.^[24] were transfected using LipofectAMINE plus reagents according to manufacturer's procedures. Stable transfectants were selected using G418 and individual clones were picked and kept at 100 μ g/mL G418. For EGF experiments, 70%-80% confluent cells were starved for 4 h followed by exposure to EGF (10 nmol/L). Human EGFR was expressed in GFP-paxillin S178A using retroviral transfection followed by FACS sorting as described previously^[43]. Retroviral transduction of GFP-paxillin cells with a pMSCV-blast-hEGFR retroviral vector, followed by blasticidin selection (12.5 μ g/mL) was used to generate GFP-paxillin S178A EGFR cells.

Luciferase reporter assay

MTLn3 cells were transiently transfected with the reporter constructs pGL3-Tata-5xE3AP1^[44], pGL3-Tata-5xCol1TRE, pGL3-Tata-5xJun2 kindly provided by Dr. Hans van Dam (LUMC) using Lipofectamine Plus reagent^[42]. 1 μ g of expression reporter plasmids together with 1 μ g of a renilla-luciferase construct (as an internal control for transfection efficiency) were transfected in both clones WT and S178A. After 48 h, cells were serum starved for 4 h then lysed and analyzed for luciferase activity using a luminescence plate reader.

Proliferation, attachment and wound healing assay

For proliferation assay, cells were plated in complete medium in 6 wells-plates and cultured for 24, 48, 72 or 96 h at which time point the amount of cells was determined by counting. For cell attachment assay, 1 h serum-starved cells were seeded in complete medium on collagen-coated 6 wells-plates for 30, 60, 90 and 120 min, and following a PBS wash, attached cells were trypsinized and counted. For wound healing assays, monolayer cells were scratched using a pipette tip to generate a wound followed by a wash with medium and incubation in α -MEM supplemented with 1% (v/v) FBS for 20 hrs. Wounds were photographed using phase contrast microscopy with a Nikon Coolpix digital camera directly after scratching and after 20 h. Wound closure was determined using Image J software.

Live cell imaging

Random cell migration

Cells were cultured in glass-bottom plates overnight and starved for 4 h followed by imaging for 1-3 h on a Nikon TE 2000-E microscope in a humid climate of 37 °C and 5% CO₂ with either DIC (Differential Interference Contrast) or fluorescence microscopy. Subsequently, cells were treated with EGF and time-

lapses were captured with 20× objective. Per biological replicate, there were 3 wells treated similarly and 2 positions per well were imaged. About 30–40 cells were followed over time in each field of view, which means that we analyzed the behavior of more than 120 cells per biological replicate. When used, the JNK inhibitor SP600125 (20 μmol/L) was added 30 min prior to stimulation. Cell speed was determined with a homemade macro written in Image-Pro Plus (Media Cybernetics Inc., Silver Spring, MD).

Total internal reflection fluorescence and fluorescence recovery after photobleaching

Total internal reflection fluorescence (TIRF) microscopy was performed with a Nikon TE 2000-E microscope in a climate control chamber. To determine the turnover of GFP-tagged paxillin proteins in individual focal adhesions, fluorescence recovery after photobleaching (FRAP) was performed as follows: photobleaching was applied to a small area covering a single focal adhesion for 1 s with laser intensity of 50 μW. Redistribution of fluorescence was monitored with 100 ms time intervals at 7.5 μW starting directly after the bleach pulse. Approximately 20 focal adhesions (each in distinct cells) were averaged to generate one FRAP curve for a single experiment. All measurements were performed at 37 °C and the experiment was performed on three different days. The relative fluorescence intensity of individual focal adhesion was calculated at each time interval as follows: $I_{rel}(t) = (F_{at}/F_{A0})$, where F_{at} is the intensity of the focal adhesion at time point t after bleaching and F_{A0} is the average intensity of the focal adhesion before bleaching.

Gel electrophoresis and immunoblotting

Equal protein amounts (25 μg; Bradford protein assay) were separated on 7.5% polyacrylamide gels and transferred to PVDF membranes (Millipore). Membranes were blocked in 5% (w/v) BSA in TBS-T and probed with primary antibody overnight followed by sufficient washes and incubation with secondary antibodies. Alkaline phosphatase-conjugated secondary antibodies for phospho-proteins were detected with the Western-Star immunodetection system. For detection of horseradish peroxidase-conjugated antibodies, ECL Plus reagent was used, followed by visualization on a Typhoon Imager 9400.

Immunofluorescence

Cells were plated on collagen-coated glass coverslips. Cells were briefly washed in PBS, followed by fixation in 3.7% formaldehyde for 10 min at room temperature. After washing, coverslips were blocked in TBP (0.1% (w/v) Triton X-100, 0.5% (w/v) BSA in PBS, pH 7.4). Incubation with primary antibodies diluted in TBP containing 0.05% (w/v) NaN₃ was carried out overnight at 4 °C. Coverslips were mounted on glass slides using Aqua Poly/Mount.

RNA isolation and DNA array analysis

Total RNA was isolated from all MTLn3 clones using TRIzol reagent (Invitrogen Corp.). Five microgram of RNA was used for cDNA synthesis. A custom cDNA kit (Invitrogen Corp.) with T7-(dT)₂₄ primer was used for this reaction. Biotinylated cRNA was generated from the cDNA reaction using the BioArray high yield RNA transcript kit (Affymetrix Inc., Santa Clara, CA). cRNA was then fragmented (5X fragmentation buffer: 200 mmol/L Tris acetate, pH 8.1, 500 mmol/L potassium acetate, 150 mmol/L magnesium acetate) at 94 °C for 35 min before chip hybridization. Following the manufacturer's protocol, fragmented cRNA was added to the hybridization mixture. For DNA array, HG-U133A from Affymetrix were hybridized for 16 h in a GeneChip Fluidics Station 400 and scanned with a GeneArray Scanner. The Human Genome U133A set of microarray represents ~14,500 human genes. Affymetrix GeneChip Microarray software was used for basic analysis. Samples were normalized to the average hybridization intensity on each chip. The study was performed for all 6 clones in duplicate. Gene Spring 6.0 (Silicon Genetics, Redwood City, CA) software was used for data analysis. Data mining of the list of genes was done using Enrichr (<http://amp.pharm.mssm.edu/Enrichr/>), an online gene set enrichment analysis web tool from the Ma'ayan Lab^[45,46].

Stable shRNA-mediated gene knockdown

MC7 cells were transduced with lentiviral shRNA constructs coding for a non-targeting control sequences shCtrl (SHC002) and a sequence targeting the coding region of PXN (TRCN0000123138) (Mission/

Sigma-Aldrich, Zwijndrecht, The Netherlands). The cells were selected by puromycin (sc-108071, Santa Cruz Biotechnology, Heidelberg, Germany). Knockdown efficiency was verified by Western Blot and immunofluorescent staining.

***In vivo* tumor growth and metastasis formation**

Six-week-old Rag2^{-/-}γC^{-/-} mice were obtained from in-house breeding. Animals were housed in individually ventilated cages under sterile conditions containing 3 mice per cage. Sterilised food and water were provided *ad libitum*. To measure spontaneous metastasis, tumor cells (5×10^5) were injected into the right thoracic mammary fat pads as described previously^[10]. After 4 weeks, the lungs were excised as well as the primary tumor which was also weighed. For quantification of all GFP-paxillin positive macro- and micro-metastases, the flat side of the right lung was analysed with the immunofluorescence microscope using a 10× objective lens (NA 0.25). Next the right lung and primary tumor were fixated in 4% paraformaldehyde. Paraffin sections of the lungs (5 μm) were stained with hematoxylin and eosin followed by histological analysis.

Statistical analysis

When not indicated, all experiments were performed in biological triplicates. As all data sets in this study follow a normal distribution, therefore were compared with Student's *t*-test (two-tailed, equal variances) using GraphPad Prism 6.0. Results were considered to be significant if *P* value < 0.05.

RESULTS

EGF-induced cell migration of MTLn3 cells is dependent on JNK activation and associated with paxillin Ser178 phosphorylation

First we determined the role of JNK-paxillin signaling in EGF-induced migration of the highly metastatic MTLn3 rat mammary adenocarcinoma cell line. EGF exposure caused membrane ruffling [Supplementary Figure 1], random cell migration and scattering of MTLn3 cells [Figure 1A and Video 1]. An inhibitor of JNK, SP600125, blocked the migration almost completely, which was associated with increased cell clustering of the MTLn3 cells. This clustered phenotype was associated with increased localization of β-catenin at cell-cell contacts [Figure 1B and Supplementary Figure 2]. Visual inspection of the movies indicates that inhibition of JNK reduces persistent movement [Figure 1C] and quantitative analysis of the cellular tracks reveal that the velocity of both individual and clustered cells was significantly impaired [Figure 1D]. By immunofluorescence, we observed that active phosphorylated JNK co-localizes with paxillin at focal adhesions in MTLn3 cells [Supplementary Figure 3B] as was previously observed in renal epithelial cells^[47,48]. EGF treatment induced a transient phosphorylation of paxillin at Ser178 in association with JNK activation, while SP600125 prevented phosphorylation of JNK, c-Jun as well as paxillin at Ser178 indicating the importance of EGF-induced JNK activation for paxillin phosphorylation [Figure 1E and Supplementary Figure 3C]. Together, these data indicate that in MTLn3 cells EGF-induced JNK activation mediates cell migration possibly via the phosphorylation of paxillin at Ser178.

PaxillinS178A mutant inhibits MTLn3 cell motility *in vitro*

To further investigate the role of paxillin Ser178 in cell migration, we generated MTLn3 cell-lines stably expressing either GFP-tagged paxillin-wt or phospho-defective mutant GFP-paxillin in which the serine residue 178 was replaced by alanine (further referred to as paxillinS178A). Three independently obtained paxillin-wt clones and paxillinS178A clones were selected for further experiments. MTLn3 clones stably expressing GFP-wt-paxillin or GFP-paxillinS178A were evaluated by flow cytometry, Western blotting [Supplementary Figure 4A] and immunofluorescence [Figure 2A]. Expression levels were approximately equal in all clones. Although GFP-paxillinS178A still localized at focal adhesions, cells were smaller and clustered with enhanced β-catenin-containing cell-cell contacts while cells expressing the GFP-wt-paxillin were stretched with large lamellipodia and almost no cell-cell contacts [Figure 2A and Supplementary Figure 4B]. The paxillinS178A-induced cell clusters [Figure 2B and Supplementary Figure 4C] resembled

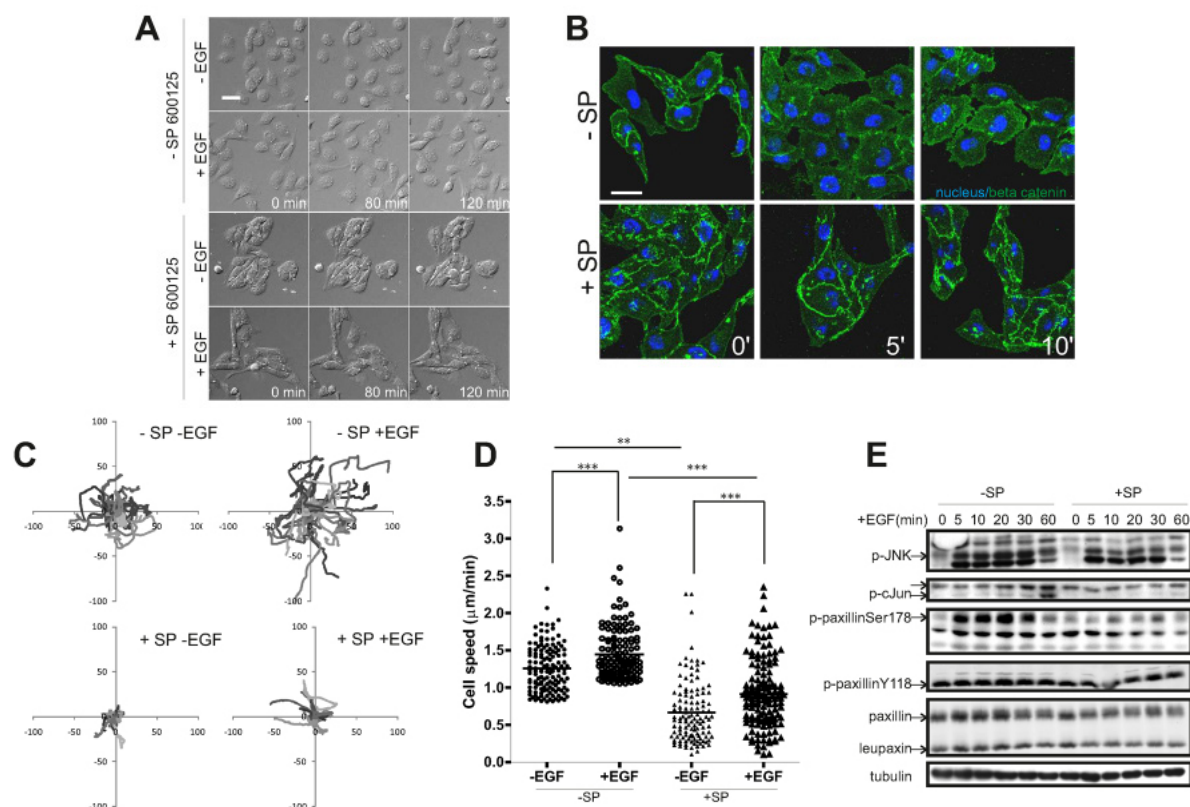


Figure 1. c-Jun NH2-terminal kinase (JNK)-mediated phosphorylation of paxillin Ser178 plays a role in tumor cell migration. MTLn3 cells were either untreated or treated with EGF (10 nmol/L) in the absence or presence of the JNK inhibitor SP600125 (20 μ mol/L). A: migration of these cells was observed by live DIC microscopy. Snapshots of the time-lapse made for 2 h are shown, scale bar is 50 μ m. See movie M1; B: at 0, 5 and 10 min after treatment cells were fixed and stained for the nucleus (blue) and β -catenin (green). Scale bar is 20 μ m; C: overall migration trajectories of individual cells of one representative experiment (only one position from the 6 technical replicates of one biological replicate); D: average cell speeds of about 100 cells per treatment imaged in one biological replicate were plotted. This graph shows the data for one representative biological replicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; E: the JNK signaling pathway was analyzed by Western Blotting using the indicated antibodies. The arrows indicate the phospho specific bands of the different antibodies. The paxillin antibody detects also a paxillin family member leupaxin encoded by LPXN, which has a much lower molecular weight than paxillin encoded by PXN

those formed in SP600125 treated cells [Figure 1B]. Given the prominent role of paxillin in focal adhesion formation and dynamics, a process required for cell spreading, we next examined the effect of paxillinS178A expression on cell attachment and spreading. Significantly less paxillinS178A cells adhered shortly after plating compared to paxillin-wt cells [Figure 2Ca]. Furthermore, while most of paxillin-wt cells had already spread most of the paxillinS178A cells remained rounded and presented a smaller surface area even after three hours of spreading [Figure 2Cb]. We also determined the effect of paxillinS178A on directed cell migration in an artificial wound healing assay [Figure 2D and Supplementary Figure 4D]. While wt-paxillin cells closed the wound by 83%, paxillin-S178A cells had only closed 25% of the wound after 20 h.

PaxillinS178A affects EGF-induced cell migration and focal adhesion dynamics

In a random cell migration assay paxillin-wt cells rapidly formed lamellipodia and became highly motile while paxillinS178A cells showed decreased cell motility and responded less to EGF stimulation [Figure 3A and Video 2]. Since paxillinS178A most likely acts as a dominant negative construct in these cells, it may compete for the localization of endogenous paxillin at focal adhesions and prevent the phosphorylation of endogenous paxillin at Ser178 by JNK. Indeed, EGF stimulation of paxillinS178A cells induced negligible Ser178 phosphorylation of endogenous paxillin whereas in paxillin-wt cells, both endogenous and GFP-paxillin-wt were phosphorylated at Ser178 after EGF treatment [Supplementary Figure 5]. Importantly, an

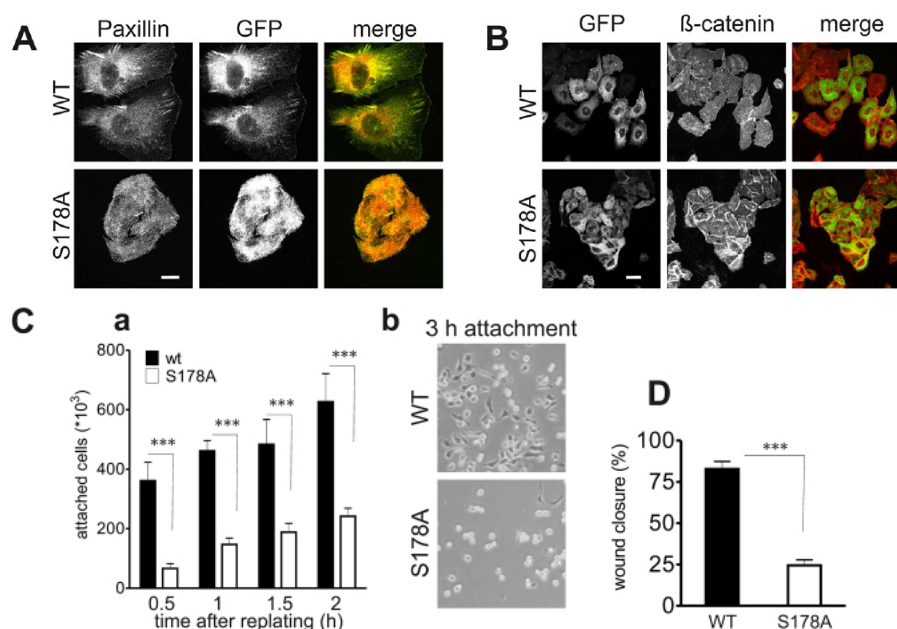


Figure 2. Expression of paxillinS178A decreases cell spreading and directed cell migration. GFP-paxillin-wt and GFP-paxillinS178A MTLn3 cells were generated and three independent clones were used for further research. A: endogenous paxillin (red) colocalized with ectopically expressed GFP-paxillin-wt and GFP-paxillinS178A (green). Scale bar is 10 μ m; B: cell clusters were detected using β -catenin (red) and GFP-paxillin (green) staining. Scale bar is 20 μ m; C: cells were analyzed for cell adhesion (a). Cells were replated on collagen-coated plastic culture dishes. The number of attached cells was counted at different time points after replating. Columns show the mean of three independent experiments; bars show SE, *** $P < 0.001$. The spreading after 3 hrs of both wildtype and mutant cells was assessed using phase-contrast pictures (b); D: directed cell migration capacity was assessed using a woundhealing assay. The wound closure was measured at three different location in the wound after 24 h. The assay was repeated three times. Columns show the mean of three independent experiments; bars show SE, *** $P < 0.001$. All three adhesion related assays were demonstrating a defect in the GFP-paxillinS178A cells

EGF-induced mobility shift of endogenous paxillin was observed in both WT and S178A cell-lines, indicating that most of the other paxillin modifications were unaffected [Supplementary Figure 5].

To understand the mechanism of the inhibitory effect of paxillinS178A on cell migration, we determined the dynamics of focal adhesions in WT and S178A cells using TIRF microscopy. MTLn3 cells expressing paxillin-wt showed a high focal adhesion turnover which was enhanced upon EGF stimulation. In contrast, paxillinS178A cells showed a much slower rate of FA disassembly either in the presence or absence of EGF [Figure 3B and Video 3]. The decreased focal adhesion dynamics could not be explained by a changed in mobility of GFP-paxillinS178A as determined by FRAP experiments. Indeed, both under serum-free conditions and upon EGF stimulation, the rates and percentages of fluorescence recovery of GFP-paxillin-wt and GFP-paxillinS178A were similar [Figure 3C].

GFP-paxillinS178A expression impairs metastasis formation of MTLn3 cells in an orthotopic breast tumor model

We next determined whether paxillin Ser178 was important for spontaneous lung metastasis formation. The MTLn3 cell line has been established as a suitable cell model to study metastasis formation from mammary gland tumors to the lung^[10]. We injected GFP-paxillin-wt (clone #2) and GFP-paxillinS178A (clone #2) cells into the mammary fat pads of immunodeficient Rag2^{-/-} γ ^{-/-} mice. After three weeks mice were sacrificed for the analysis of the primary mammary gland tumors as well as lung metastases. All primary tumors remained GFP-positive, indicating expression of wt or paxillinS178A GFP-paxillin continuously during the experiment. The edges of the GFP-paxillin-wt tumors were more invasive-like compared to those of GFP-paxillinS178A tumors [Figure 4A]. Yet, the weight of the primary tumor was not significantly altered

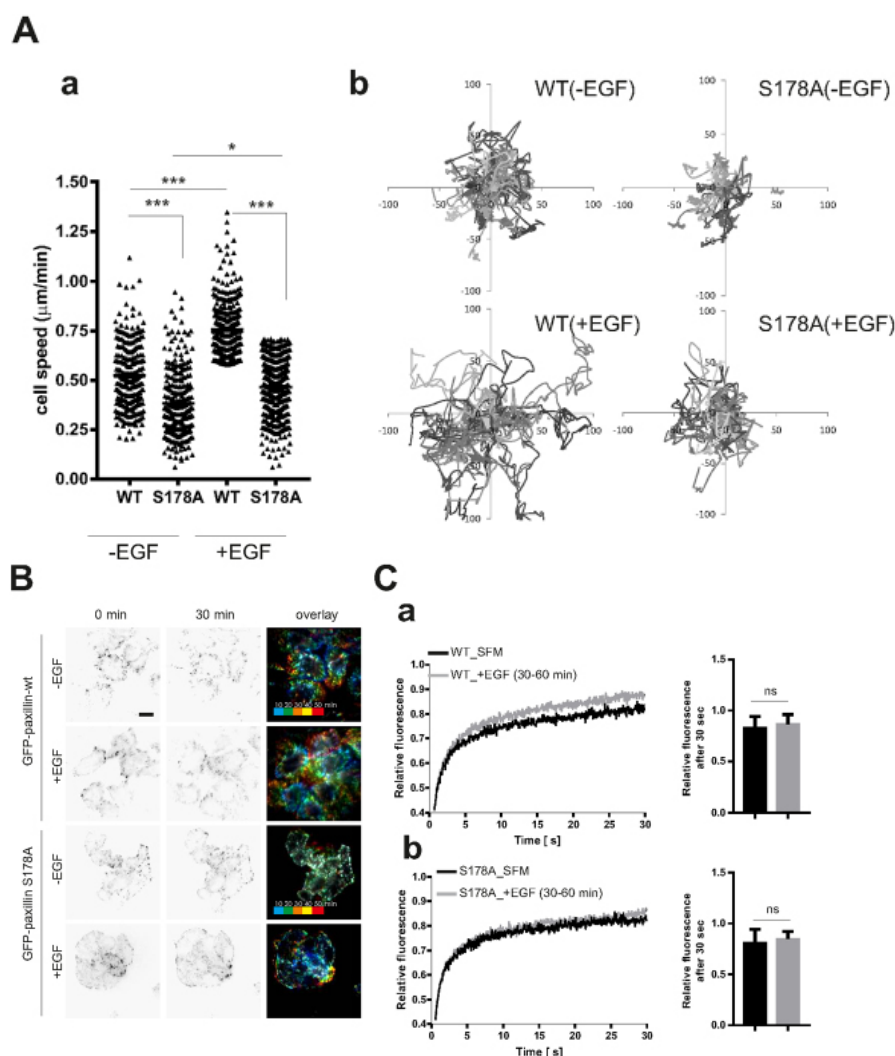


Figure 3. EGF-driven cell migration is inhibited in paxillinS178A cells because of impaired focal adhesion turnover. MTLn3 cells were either untreated or treated with EGF (10 nmol/L). A: migration was observed for 10 h by epi-fluorescence microscopy PaxillinS178A reduced cell speed average (about 100 cells per condition were imaged in one biological replicate). This graph shows the data for one representative biological replicate. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (a) and directional cell movement (b). See also Supplementary movie M2; B: matrix adhesions dynamics in MTLn3 cells was visualized with TIRF microscopy. See also Supplementary movie M3. Overlay of different timeframes were generated in red, green and blue. Focal adhesions in white, as observed for PaxillinSer178, represent unchanged (less dynamic) focal adhesions. Scale bar is 20 μm ; C: protein dynamics was measured with the spot bleaching technique and showed similar dynamics for both GFP-paxillinS178A (b) and GFP-paxillin-wt (a). Approximately 20 focal adhesions (each in distinct cells) were averaged to generate one FRAP curve for a single experiment. The mean relative fluorescence of both GFP-paxillin-wt and GFP-paxillinS178A both in SFM and upon EGF stimulation shows no significant difference after 30 s of recovery

by GFP-paxillinS178A [Figure 4B]. GFP-paxillinS178A MTLn3 cells formed significantly less spontaneous lung metastases than GFP-paxillin-wt MTLn3 cells [Figure 4C and D], and these metastases also had a less invasive phenotype [Figure 4C]. Our data show for the first time an important role for phosphorylation of paxillin on Ser178 in breast cancer progression.

Ectopic paxillinS178A expression results in EGFR down regulation at mRNA and protein levels

Next we sought to determine the possible mechanism by which paxillinS178A affects tumor cell migration and metastasis formation. Using Affimetrix microarrays, we analyzed the differentially expressed genes (DEG) between GFP-paxillinWT and GFP-paxillinS178A MTLn3 clones. Using a very low p-value, a comparison between WT and mutant clones delineated 134 genes that were differentially expressed of which 84 were down-regulated and 50 up-regulated [Supplementary Table 1]. In Figure 5Aa, we plotted

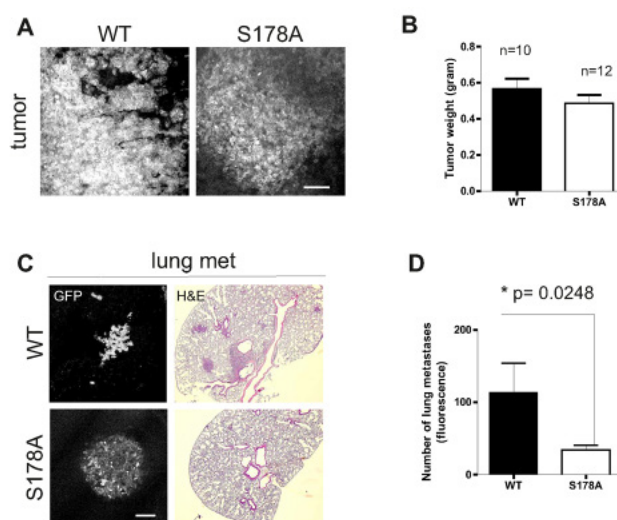
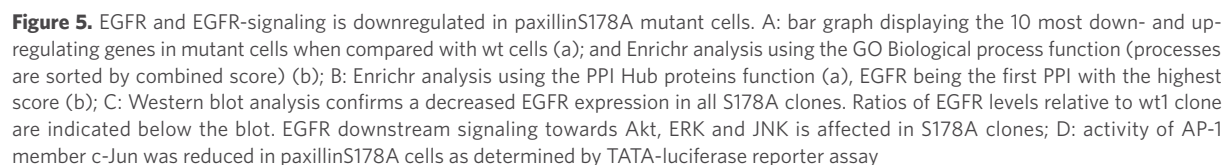


Figure 4. Expression of paxillinS178A impairs lung metastasis formation *in vivo*. One representative clone of either GFP-paxillin-wt or GFP-paxillinS178A MTLn3 cell-lines were used for *in vivo* experiments. A: Primary tumors were imaged using two-photon intravital imaging. Scale bar is 100 μ m; B: no significant difference in tumor weight was measured between the wt mice ($n = 10$) and S178A mice ($n = 12$); C: two-photon intravital microscopy, H&E staining of lung slices; D: quantification of the number of GFP-positive lung metastases demonstrated that paxillinS178A reduced lung metastasis formation. Scale bar is 100 μ m

the fold changes of the 10 most down- and 10 most up-regulated genes. Strikingly, EGFR was significantly down-regulated in the mutant clones. A pathway analysis of the complete set of genes using Gene Ontology revealed that pathways involved in intermediate filament assembly, cell proliferation and regulation of β -catenin transport were significantly altered when compared to the wt clones [Figure 5Ab]. Furthermore, a protein-protein interaction analysis of those same DEGs highlighted the EGFR network as most significantly altered [Figure 5B]. Paxillin localizes at focal adhesions and EGFR signaling is regulated and trans-activated at focal adhesions by both integrins^[49,50] and FAK^[51,52]. Therefore we reasoned that paxillinS178A may disturb the EGFR signaling pathway. In agreement with our microarray analysis, all three paxillinS178A clones had much lower levels of EGFR protein than wt clones. As a consequence, downstream EGFR signaling towards Akt and ERK were also reduced in S178A clones [Figure 5C]. Since EGF also caused JNK activation in control MTLn3 cells [Figure 1], we also determined the activation of JNK signaling pathway in these cells. Indeed, paxillinS178A expression also inhibited EGF-induced JNK activation [Figure 5C], which was associated with a reduction in c-Jun transcriptional activity as determined by luciferase reporter assays [Figure 5D]. These data indicate that paxillinS178A affects the expression of EGFR possibly through the regulation of c-Jun-mediated EGFR transcription^[53,54], thereby disturbing downstream signaling pathways that are essential in the cell migration process.

Ectopic expression of human wt-EGFR in paxillinS178A cells restores EGF-driven cell motility and lung metastasis formation

To determine whether paxillinS178A reduced tumor cell migration and metastases formation via EGFR downregulation, we re-expressed EGFR in the mutant cells [Supplementary Figure 6A]. The EGFR re-expression induced a more spread phenotype in paxillinS178A cells [Figure 6A and Supplementary Figure 6B]. The EGF-driven cell migration was rescued and the protein turnover of paxillinS178A at focal adhesions was slightly faster only upon EGF stimulation [Figure 6B and Supplementary Figure 6C]. This was associated with a sustained activation of both JNK and ERK after EGF exposure [Supplementary Figure 6D]. Next we determined whether EGFR re-expression also restored the capacity of MTLn3 paxillinS178A cells to metastasize to the lungs. For this purpose, we injected GFP-paxillinS178A cells and EGFR-GFP-paxillinS178A cells into the mammary fat pads of immunodeficient Rag2^{-/-} mice, although we were aware that EGFR expression would decrease during the course of the experiment. The tumor growth of paxillinS178A and EGFR-paxillinS178A



cells was similar in the both groups [Figure 6Cb] and all tumor cells in the primary tumors were GFP-positive [Figure 6Ca]. Mice injected with GFP-paxillinS178A cells that expressed EGFR showed an approximately threefold increase in the lung tumor burden in [Figure 6D]. These data indicate that the reduced metastasis formation of GFP-paxillinS178A cells is directly related to the expression levels of EGFR.

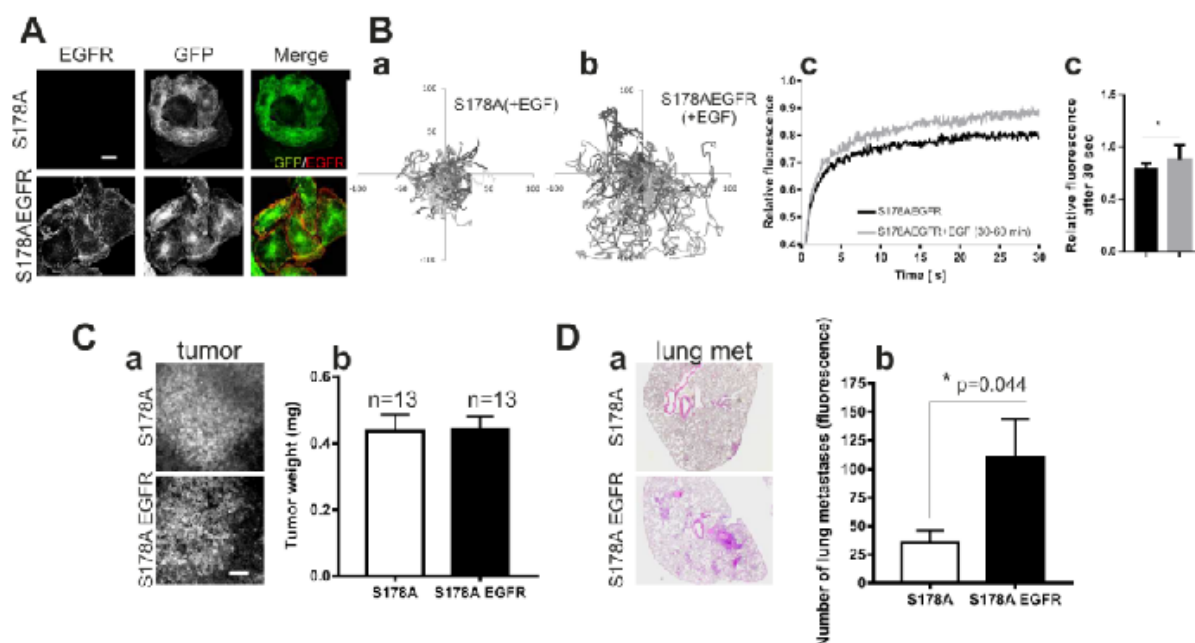


Figure 6. Re-expression of wt-EGFR fully restores EGF-driven cell motility and lung metastasis formation. A: immunostaining with a specific mouse monoclonal antibody against human EGFR confirmed the ectopic expression of EGFR in S178A cells; paxillinS178A (green) and human EGFR (red). Scale bar is 10 μm ; B: trajectories of the movements of S178A and S178AEGFR cells are plotted in (a) and (b) respectively (X and Y are in μm). Protein dynamics was quantified with spot bleaching technique and the fluorescence recovery after 30 sec was slightly faster upon EGF stimulation, * $P < 0.05$. (c); C: two-photon imaging of GFP-positive lung metastases, scale bar is 100 μm . (a). There was no significant difference in tumor weight of S178A ($n = 13$) and S178AEGFR ($n = 13$) groups (b); D: H&E staining of lung slices of S178A and S178AEGFR groups (a). Number of GFP-positive metastases counted in the fresh lungs at time of sacrifice is shown (b)

DISCUSSION

Our data indicate that EGF-induced JNK activation is essential for MTLn3 cell migration. Phosphorylation of paxillin on Ser178 in MTLn3 cells was induced upon EGF stimulation in a manner sensitive to the JNK inhibitor, and expression of paxillinS178A inhibited cell motility. Such a role for paxillin Ser178 in cell migration is in agreement with observations made in different tumor cell lines^[7,9,13,24,25,40,55-60]. Here we further demonstrate that the JNK-paxillin axis regulates both the dynamics of focal adhesions through modulation of paxillin protein dynamics at focal adhesions as well as the stability of β -catenin-based adherence junction formation. We propose that Ser178 phosphorylation may induce a conformational change, thereby affecting paxillin interactions with other focal adhesion components. Alternatively, the decreased EGFR expression in paxillinS178A cells affects downstream signaling pathways that indirectly modifies focal adhesion dynamics as well as adherence junction stability. Indeed, re-expression of EGFR in paxillinS178A cells reversed the epithelial-like phenotype and rendered paxillinS178A again less immobile at focal adhesions, suggesting that this is not an intrinsic characteristic of paxillinS178A, but is rather due to altered signaling in cells mediated by downstream EGFR signaling pathways. This needs further investigation.

So far no *in vivo* data on the specific role of paxillin Ser178 in metastasis formation have been presented. Here, we demonstrate that the Ser178 of paxillin is essential for spontaneous metastasis formation in an orthotopic breast tumor/metastasis model. Our *in vivo* data on the specific role of paxillin Ser178 in metastasis formation is in line with previously published data using a knock-down of MLK3 in MDA-MB-231. Indeed in the later study, they found that Ser178 phosphorylation of paxillin was associated with the metastatic phenotype^[40,59]. Importantly in our study, we demonstrate that specifically the Ser178 of paxillin is essential for spontaneous metastasis formation in an orthotopic breast tumor/metastasis model. Our findings are indicative for a role for the JNK-paxillin pathway in the regulation of the expression of EGFR, thereby severely affecting the capacity of tumor cells to migrate and metastasize. Indeed, reduced levels of EGFR were observed in

three independent paxillinS178A MTLn3 clones, resulting in decreased EGF-induced activation of signaling pathways downstream of EGFR. Given the fact that EGFR is often highly expressed in advanced breast cancer, and that EGFR antagonists inhibit spontaneous metastasis formation of MTLn3 cells, we anticipated that the reduced EGFR expression was an essential component of the inhibited cell migration and metastasis formation. Indeed, re-expression of the wt-EGFR in the paxillinS178A cells did rescue the migratory phenotype of MTLn3 paxillinS178A cells as well as their metastatic capacity under *in vivo* conditions. This regulation of the EGFR by paxillin phosphorylation has not been described before. Curiously, a recent study demonstrated that knockdown of MCLK in mammary MCF10A cells induces increase in cell migration through enhanced phosphorylation of paxillin at Ser178 which was linked to an increase in JNK activity and very interestingly to a significant up-regulation of EGFR at protein level^[60]. This is striking and yet would suggest that our findings might not be specific to our cell model. In our study, paxillinS178A MTLn3 cells showed reduced c-Jun transcriptional activity. In MCF7 cells stable overexpression of c-Jun induces an increase in EGFR expression suggesting that AP-1 transcription factors can regulate EGFR transcription levels^[53]; such a regulation is also observed in keratinocytes^[54]. Therefore, we propose a role of the JNK-c-Jun signaling pathway in the regulation of EGFR expression at transcriptional level. Our own data in MCF7 cells that have increased ectopic expression of EGFR show that depletion of paxillin results in significant downregulation of EGFR expression as well as downstream signaling such as AKT and ERK [Supplementary Figure 7]. Thus, an alternative explanation may be that JNK-mediated phosphorylation of paxillin Ser178 is required for efficient EGFR endocytosis and recycling, an essential component in its activation^[61]. Hence this will affect proper JNK activation and AP-1 transcription factor activation thereby providing a positive feedback for EGFR expression^[62]. Alternatively, paxillin may affect the expression of EGFR by its known role in the regulation of gene expression through its interaction with ERK^[63,64], poly-A-binding protein^[65], Abl^[66,67] and steroid receptors, or through its own ability to undergo nucleocytoplasmic shuttling^[37,38,68-71].

Our findings indicate that the JNK-paxillin axis modulates the scattered phenotype of MTLn3 cells. Both inhibition of JNK as well as ectopic expression of paxillinS178A reversed the scattered phenotype towards a more epithelial-like morphology with the formation of E-cadherin/ β -catenin cell-cell junctions. This morphological switch was observed under serum-starved and EGF-treated conditions. The (in)direct tyrosine phosphorylation of β -catenin by EGFR and other receptor tyrosine kinases such as c-Met is known to destabilize its binding to E-cadherin. Because of the low EGFR expression in the paxillin mutant cells, we anticipate that this results in de-phosphorylation of β -catenin leading to its localization to the cell-cell contacts. These data suggest that paxillinS178A does not affect the intrinsic molecular components and machinery required for the scattered phenotype and support the notion that the effect of paxillinS178A is rather related to the defects in the EGFR signaling pathways. Consequently, the defects in *in vivo* metastasis formation of MTLn3 paxillinS178A cells are most likely largely due to defects EGF signaling. This fits with our observations that ectopic EGFR expression itself is sufficient to again allow metastasis formation of paxillinS178A cells.

In summary, in MTLn3, engagement of EGF receptors by EGF triggers rapid activation of JNK, leading to the phosphorylation of paxillin on Ser 178 which facilitates adhesion turnover thus promoting rapid migration. In the MTLn3 cell lines that ectopically express GFP-paxillin-S178A mutants, we found that the EGF receptor is downregulated at both the protein and mRNA levels. Paxillin S178A mutant protein might associate with JNK preventing its activation and consequently affecting AP1 activity (c-Jun phosphorylation) and consequently EGFR expression. In our model [Figure 7], still some links need to be further determined such as how exactly paxillin regulates via JNK EGFR expression. Given the essential role of Ser178 phosphorylation in the migration and metastasis formation, monitoring this paxillin phosphorylation in tumor samples from patients may be indicative of the activation of this pro-metastatic pathway and possibly predictive for the disease prognosis. Moreover, with the further development of specific JNK inhibitors^[72,73] it is anticipated that novel targeted therapies that antagonize the migratory/invasive behavior of tumor cells may be used in the clinic in the future. Further work in this area is required.

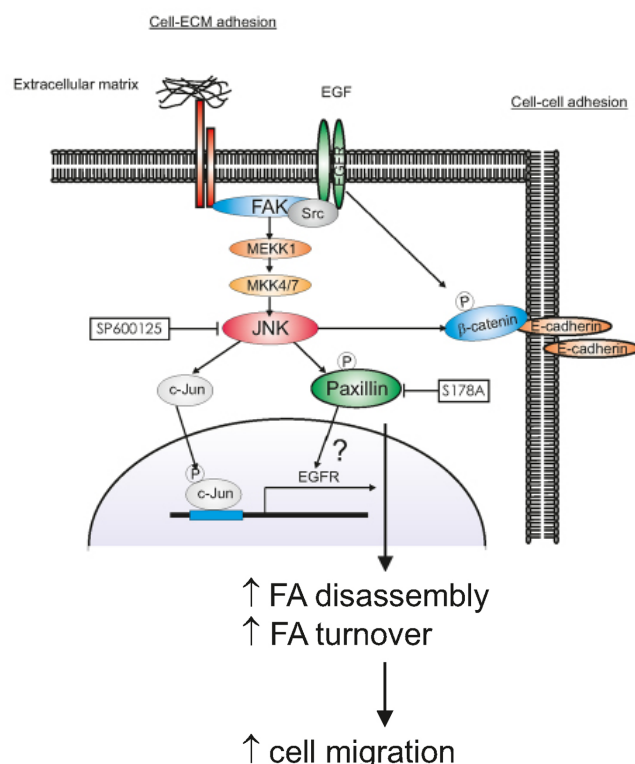


Figure 7. Diagram depicts the hypothesized signaling pathways for JNK-paxillin dependent cell migration. Engagement of EGF receptors by EGF triggers rapid activation of JNK, leading to the phosphorylation of paxillin on Ser 178 which might facilitate adhesion turnover thus promoting rapid migration. Upon EGF stimulation, JNK phosphorylates also c-Jun which regulates EGFR transcription and β-catenin which delocalizes from the cell-cell contact breaking down the adherens junctions. Paxillin S178A mutant protein might associate with JNK preventing its activation and consequently affecting AP1 activity and EGFR expression. Inactive JNK as well as down-regulation of EGFR result in de-phosphorylation of β-catenin and formation of stable cell-cell contact in MTLn3 cells

DECLARATIONS

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Authors' contributions

Carried out the experiments: Verkoeijen S, Ma YF, van Roosmalen W, Lalai R, van Miltenburg MHAM, Le Dévédec SE

Wrote the manuscript with support from van de Water B: Verkoeijen S, Ma YF, Le Dévédec SE

Conceived the original idea and helped supervise the project: van de Water B

Supervised and finalized the project: Le Dévédec SE

Availability of data and materials

All data are made available through supplemental data. Materials are available on request.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Circulating microRNAs and liquid biopsy: murine xenograft models for technical validation of clinical protocols

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Abstract

In oncology, liquid biopsy is applied to detect with high efficiency clinically relevant analytes, such as tumor cells, cell-free nucleic acids, and exosomes in peripheral blood and other body fluids of cancer patients. Liquid biopsy is considered one of the most advanced non-invasive diagnostic systems useful, in the next future, for enabling personalized treatments in precision medicine. Medical actions include, but are not limited to, early diagnosis, staging, prognosis, anticipation (lead time) and prediction of therapy responses, as well as follow up. Experimental system for validation of the proposed liquid biopsy approaches is highly needed. In this review article we will discuss the establishment of xenotransplanted mouse model systems for the validation of liquid biopsy protocols aimed to identify changes in the miRNA plasma content. Human colon cancer HT-29 and LoVo cells have been xenotransplanted and miR-221-3p and miR-222-3p have been comparatively analyzed in cultured HT-29 and LoVo cells, xenotransplants and plasma samples.

Keywords: Liquid biopsy, circulating tumor RNA, microRNA, xenograft



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LIQUID BIOPSY: A NEW FRONTIER FOR CANCER DIAGNOSTICS

In the field of cancer diagnosis and treatment, liquid biopsy is a new diagnostic tool that investigates circulating tumor cells (CTC) and/or cell-free nucleic acids in the peripheral blood [Figure 1A]. Liquid biopsy is considered one of the most advanced non-invasive diagnostic systems. It provides key molecular information relevant to important clinical decisions and, being “longitudinal” (it can be repeated as many times as needed), it fits the idea of precision medicine possibly more than other “static” techniques based on the analysis of tissue nucleic acids^[1-5]. Diagnostic actions made possible by liquid biopsy include, but are not limited to, early diagnosis, staging, prognosis, prediction of therapy response and follow up during therapeutic intervention^[6-11].

In addition to the use of CTCs^[12-15] and circulating tumor DNA (ctDNA)^[16-18], other important targets for liquid biopsy are circulating microRNAs (miRNAs)^[19-24], a family of small (19 to 25 nucleotides in length) noncoding RNAs playing important roles in controlling post-transcriptional gene expression. Regulatory miRNAs reduce protein synthesis through selective interactions with complementary sequences of target messenger RNAs (mRNAs)^[25-27]. Single or multiple mRNAs can be targeted at their 3'-UTR, CDS, 5'-UTR sequences, and it is calculated that more than 60% of human mRNAs are microRNA targets^[26]. Dysregulation of microRNAs has been associated with a variety of human pathologies, including cancer^[28-31]. In this case miRNAs behave both as tumor promoters (oncomiRNAs and metastamiRNAs) and tumor suppressor molecules^[29], depending on their mRNA targets (oncosuppressor mRNAs or mRNA coding oncoproteins, respectively) with opposing activity on cancer cells. Based on this, it is not surprising that circulating cell-free miRNAs have been actively investigated as liquid biopsy analytes. OncomiRNAs are abundant in several extracellular body fluids, where they are protected and stabilized by exosome-like structures and small intraluminal vesicles produced by a variety of cells (including cancer cells)^[32-36]. Hence, elevated levels of several miRNAs (including miR-221, miR-222, miR-141, miR-92a, miR-21, miR-155, miR-506, miR-4316, miR-4772-3p, and miR-29a) are present in the blood from patients with colorectal carcinomas (CRC) and may contribute to diagnosis and prognosis^[21,37-42]. Furthermore, it is well established that miRNAs may help in monitoring therapeutic approaches. For instance, Ogata-Kawata *et al.*^[22] reported that serum exosomal miRNA levels (let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, and miR-23a) were higher in CRC patients than controls, were already detectable at early disease stages, and that they were significantly down-regulated after surgical resection.

TECHNOLOGIES FOR MICRORNA ANALYSIS

In order to quantify miRNAs in the plasma and other body fluids isolated from cancer patients, several types of technologies for RNA analysis have been proposed^[43-51]. Quantitative real-time PCR (RT-qPCR)^[52], NGS RNA sequencing^[53], miRNA microarray analysis^[54], and digital PCR^[55] are the most used [Table 1] and can be employed not only for tissue or cells but also for highly diluted samples, such as body fluids. One of the major limits of RT-qPCR and ddPCR is the limited number of miRNAs that can be quantified for single run. This problem was partially solved by introduction of TaqMan low density arrays, that allows to quantify the content of a significant number of miRNAs (about 700 miRNAs) using PCR-based methods^[56]. In addition to these methodologies, other technologies have been described for direct miRNA detection from serum samples. For example, Chapin *et al.*^[57] proposed rolling circle amplification (RCA) based on the use of a universal adapter ligated to the targets captured on encoded gel microparticles. The system allows the multiplexed profiling of miRNA at sub-femtomolar concentration. Interestingly, Williams *et al.*^[58] proposed a miRNA detection technique able to amplify miRNAs directly in body fluids, avoiding upstream sample preparation. The technique, based on isothermal target amplification, has a sensitivity positioned in the femtomolar range. Other conventional technologies normally proposed for miRNA detection in cells or tissues, such as northern blotting^[59], are not suitable for miRNA detection in body fluids, due to the low sensitivity of the technology, requiring therefore large amounts of RNA. Other unconventional miRNA

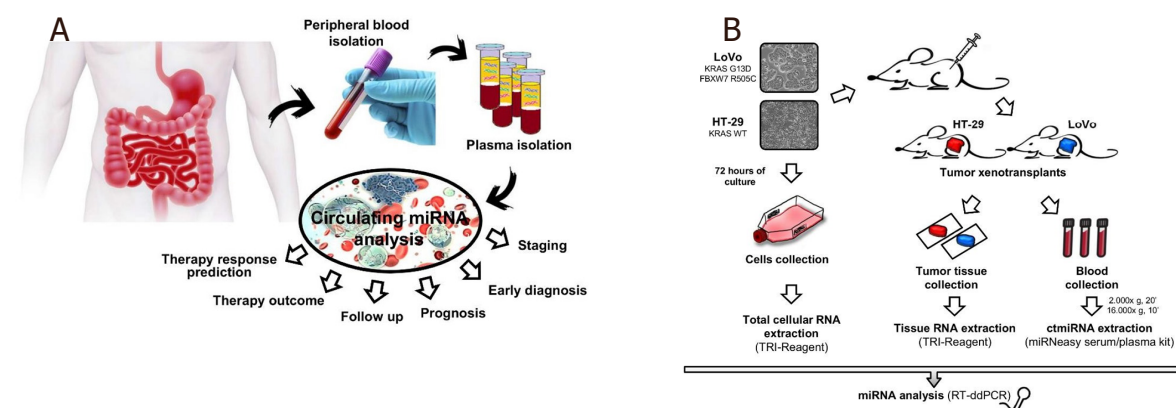


Figure 1. Flow chart describing the experimental *in vivo* model system. All the procedures have been performed according to Directive 2010/63/EU and Italian Decree Law 26/2014. They were approved by the EU Research Executive Agency, the Intramural Regina Elena Board for Animal Welfare, and the Italian Ministry of Health (700-2015-PR, dated July 17, 2015). Tumor xenotransplants were established by inoculating 3×10^6 cells from the HT-29 and LoVo cell lines in the flank of 4-month old Nu/CD1 mice (Charles River Laboratories, Italy). Xenotransplants were allowed to grow to two different sizes (300 and 1000 mm^3 , 6 mice per group for each of the two cell lines). Tumors were taken at sacrifice along with blood. Frozen tissues were used as the source of miRNAs. Blood was collected in 6 mL BD Vacutainer K2E tubes (BD, 368857) and centrifuged within 1 h at $2000 \times g$ for 20 min at 4°C . Plasma was recovered and further centrifuged at $16,000 \times g$ for 10 min at 4°C to remove cell debris, and stored at -80°C until extraction

Table 1. Technologies applied to miRNA detection

Technologies	Biological fluid	Major results obtained	Ref.
Real-time quantitative PCR	Plasma	RNA was isolated from human plasma samples of healthy donor and the content of three miRNAs chosen to represent moderate- to low-abundance plasma miRNAs (miR-15b, miR-16, and miR-24) was evaluated using TaqMan RT-qPCR assays	[52]
NGS RNA sequencing	Serum	A NGS RNA sequencing (Solexa sequencing) approach, followed by RT-qPCR data validation was employed to identify miRNAs able to predict prognosis of non-small-cell lung cancer. Four miRNAs (miR-486, miR-30d, miR-1 and miR-499) were significantly associated with overall survival	[53]
MicroRNA microarray analysis	Serum	A global miRNA profiling was performed, using GeneChip miRNA Arrays (Affymetrix) in prostate adenocarcinoma transgenic mouse models identifying 46 miRNAs significantly altered compared to healthy controls	[54]
Droplet digital PCR	Plasma	Droplet digital PCR platform was used to assess levels of miRNAs (miR-221, miR-222 and miR-141) released into the culture supernatants of colorectal carcinoma cell lines and mouse blood plasma obtained from xenograft models	[55]
MicroRNA low Density Arrays	Plasma	Circulating miRNAs profile was examined in mice bearing human small cell lung cancer tumor xenografts using human TaqMan Low Density Array. The analysis allows to identify a panel of differentially expressed miRNAs, in a stage dependent manner	[56]
Rolling circle amplification	Serum	The rolling circle amplification of a universal adapter sequence selectively ligated to targets captured on encoded gel microparticles is employed for multiplexed profiling of miRNAs at sub-femtomolar concentration. The protocol was optimized to cover a dynamic range of magnitude (300 aM to 40 pM). Moreover, miRNAs can be directly detected in small quantities of unprocessed serum samples avoiding RNA extraction or target-amplification steps	[57]
Isothermal ramification amplification (RAM)	Plasma	Isothermal RAM allows the direct amplification of miRNAs without upstream sample preparation. The presence of microRNA promotes base-stacking hybridization, and subsequent amplification between two universal strands, under isothermal conditions. A sensitivity in the range of femtomolar is provided	[58]
Northern blotting	Tissues	An highly efficient microRNAs detection method based on northern blot analysis was set-up using LNA (locked nucleic acid) probes able to increase the sensitivity of at least 10-fold compared to conventional DNA probes	[59]
Bead-based flow Cytometry	Tissue/cell lines	Oligonucleotide-capture probes complementary to miRNAs of interest were coupled to carboxylated 5- micron polystyrene Beads impregnated with variable mixtures of two fluorescent dyes each representing a single miRNA. After the reverse transcription miRNAs are amplified by polymerase chain reaction and hybridized to the capture beads, beads were then analysed using a flow cytometer capable of measuring bead colour	[60]

detection techniques have been proposed in recent years such as bead-based flow cytometry^[60] but at the moment they are employed only for miRNA detection in tissues or cellular samples.

EXPERIMENTAL MODEL SYSTEMS FOR LIQUID BIOPSY

Given the fast pace of technological evolution and the clinical complexity of human cancers, there is an increasing need for experimental *in vivo* systems and associated validation technologies. A robust analysis of bio-fluids must acknowledge the fact that liquid biopsy is a complex strategy requiring the achievements of several key points, including pre-analytical steps, post-analytical optimization, and careful selection of optimal analytes for specific biological queries. *In vivo* model systems might be very useful to address and isolate these many individual variables (that are both technical and biological), and validate complex multi-step approaches. It is surprising, in this respect, that only few reports are available focusing on the use of animal models. *In vivo* model systems for the detection of circulating tumor cells and DNA have been proposed^[61-63]. These include injection of cancer cells into their orthotopic site of origin (e.g., a “recap” of natural tumor onset), injection of tumor cells directly into the bloodstream of the animal (to recap distant seeding), genetically engineered mouse and mice xenografted with patient-derived tumors^[64-69] (to mimic “true” tumors). As to the analysis of circulating miRNAs in these experimental settings, several reports are available^[52,54-56,70,71] [Table 2].

Mice xenografted with human tumor cell lines or patient-derived tumor

The pattern of circulating miRNAs has been extensively studied in mice xenografted with tumor cells and patient-derived tumors. Different independent studies firmly demonstrated that miRNAs released in the circulation by the tumor xenograft are distinct from the “background” mouse miRNAs pattern. This is a key point, since pre-existing miRNAs present in the mouse body fluids are identical in sequence to most human miRNAs and hence they might be a powerful confounding parameter, possibly altering conclusions and implications of any circulating miRNA signature. In this respect, the use of laboratory mouse strains has the advantage that its “background” mouse miRNA pattern is stable and easily quantifiable. Mitchell *et al.*^[52] demonstrated that several miRNAs originating from xenografted human prostate cancer cells are present in the mouse circulation (one of the most interesting being miR-141), and are readily measured in plasma, allowing a clear distinction between tumor xenografted and control tumor-free mice. Waters *et al.*^[70] observed a complex miRNAs dysregulation in the circulation of athymic nude mice subcutaneously injected with MDA-MB-231 cells. Some miRNAs (such as miR-10b) were undetectable in the circulation, others (miR-195 and miR-497) were significantly decreased, miR-221 content did not change, and a positive correlation was observed between miR-497 and miR-195. This study highlighted distinct roles of miRNA subsets in the circulation and in disease dissemination and progression, all of which may be candidates as molecular targets for diagnosis as well as design of systemic therapy. Gasparello *et al.*^[55] studied liquid biopsy in mice bearing CRC xenografts, demonstrating gateways regulating the levels of circulating tumor-derived miRNAs (ctmiRNAs), e.g., cell-specific roadblocks that determine whether a given cell xenotransplants releases or retains a specific miRNA. These roadblocks are often not present in cultured cells, and build “barriers” to detection in a liquid biopsy format.

Genetically engineered mouse model systems

Genetically engineered mouse models (GEMMs) manipulate target oncogene or tumor suppressor expression in mice in order to promote tumor development. Transgenic and knockout GEMMs have provided important models for identifying tumor-associated and metastasis-associated genes that can lead to tumor formation and disease progression. In addition, GEMMs have been applied to the development of liquid biopsy methods based on the analysis of circulating microRNAs. Selth *et al.*^[54] performed a global miRNA profiling and identified a set of miRNAs exhibiting significantly altered serum levels in transgenic mice models of prostate cancer (i.e., Transgenic Adenocarcinoma of Mouse Prostate mice). Global miRNA profiling identified 46

Table 2. Examples of experimental systems to study miRNA content in liquid biopsy

Experimental mouse system	miRNA studied	Major results obtained	Ref.
Mouse models of prostate cancer xenografted with 22Rv1 human prostate cancer- derived cells	miR-141-3p	MicroRNAs derived from human prostate cancer xenografts are readily measured in plasma of mouse models allowing a clear distinction between tumor-xenografted mice and controls	[52]
Transgenic mouse model of prostate cancer	miR-141-3p miR-375 miR-298 miR-346	Global miRNA profiling allows to identified a set of ten miRNAs significantly altered in serum of transgenic mice compared to healthy controls	[54]
Mice bearing colorectal carcinoma cell lines xenografts	miR-141-3p miR-221-3p miR-222-3p	Evidences of gateways regulating the levels of circulating miRNA	[55]
Mouse models bearing human small cell lung cancer (SCLC) tumor xenografts	miR-95 miR-141-3p miR-200a-3p miR-200b-3p miR-200c-3p miR-210-3p miR-335-3p miR-375 miR-429	A panel of 10 miRNAs are dysregulated in tumor bearing mouse models. The same miRNAs were also confirmed to be altered in stage dependent manner in plasma isolated from SCLC patients	[56]
Mouse models of breast cancer xenografted with MDA-MB-231 cells	miR-10b-5p miR-195-5p miR-497-5p miR-221-3p	A complex miRNA dysregulation in the circulation athymic nude xenografted mice was detected compared to tumor-free controls	[70]
Foxp3 heterozygous Scurfy mutant (Foxp3 ^{sf/+}) female mice. The loss of Foxp3 expression, due to the frameshift mutation leads to the spontaneous development of breast cancer and lung metastases.	miR-200c-3p miR-141-3p	Despite levels of miR-200c and miR-141 were found to be lower in Foxp3 ^{sf/+} tumor cells than in normal breast epithelial cells, plasma levels of miR- 200c and miR-141 in the Foxp3 ^{sf/+} mice increased during tumor progression and metastasis	[71]

miRNAs at significantly altered levels in the serum of mice with advanced prostate cancer compared to healthy mice used as controls. Interestingly, four miRNAs altered in mice (mmu-miR-141, mmu-miR-298, mmu-miR-346 and mmu-miR-375) were also found to be expressed at higher levels in the serum of patients with metastatic prostate cancer compared with control subjects. Moreover, three of these (hsa-miR-141, hsa-miR-298 and hsa-miR-375) were upregulated in prostate tumors compared with normal prostate tissue, suggesting that they are directly released from the tumor into the blood as disease progresses. This study was the first to demonstrate that specific serum miRNAs (miR-141, miR-298 and miR-375) are common between human prostate cancer and a mouse model of the disease, highlighting the potential of such models for the discovery of novel biomarkers.

Zhang *et al.*^[71] investigated FOXP3-inducible breast cancer cells, Foxp3 heterozygous Scurfy mutant (Foxp3^{sf/+}) female mice, and patients with breast cancer for characterization of the formation and regulation of the miR-200 family in breast cancer cells and circulation. While levels of miR-200c and miR-141 were lower in Foxp3^{sf/+} tumor cells than in normal breast epithelial cells, plasma levels of miR-200c and miR-141 in the Foxp3^{sf/+} mice increased during tumor progression and metastasis. Interestingly, the levels of miR-200c and miR-141 were higher in plasma from patients with metastatic breast cancer than in plasma from those with localized breast cancer, with benign breast tumors, with a family history of breast cancer, or from healthy controls. The conclusion of the work reported by Zhang *et al.*^[71] supports the concept that miR-200c and miR-141 are regulated by a FOXP3-KAT2B axis in breast cancer cells, and circulating levels of miR-200c and miR-141 are potential biomarkers for early detection of breast cancer metastasis. Moreover, they highlight the idea that roadblocks evolve during the natural history of tumors.

LIQUID BIOPSY IN MICE BEARING COLORECTAL CARCINOMA XENOGRAFTS OBTAINED AFTER IMPLANTATION OF HT-29 AND LOVO CARCINOMA CELLS

Analysis of miRNA content has been recently performed in mice xenografted with colon cancer cell lines^[55]. Among the different xenografted models the one based on the implantation of the HT-29 and LoVo CRC

cells was found the most efficient for miRNA detection. The HT-29 cells were derived from a KRAS-WT, differentiated colorectal adenocarcinoma^[72,73], while LoVo cells (originally described as Dukes' type C, grade IV) harbor a heterozygous KRAS c.38G>A mutation (G13D)^[74].

TUMOR XENOGRAPHS AND PLASMA PREPARATION

Figure 1B shows the study workflow based on the tumor xenografts as models for liquid biopsy to assess plasma levels of circulating miRNAs. In this study workflow, miRNAs are compared considering (1) *in vitro* cultured tumor cells; (2) tumor xenografts; and (3) blood plasma samples. The HT-29 and LoVo cell lines were selected as proxies of clinically evident cancers and sources of soluble analytes. MicroRNAs were extracted from both cell lines, matched tumor tissue and blood plasma samples and were then subjected to ddPCR and RT-qPCR analysis. Tumor xenotransplants were established by inoculating HT-29 and LoVo cells in the flank of 4-month old Nu/CD1 mice. Tumors were taken at sacrifice along with blood. Frozen tissues were used as the source of miRNAs. For the analysis of ctmiRNA, blood plasma was treated to disrupt exosomes and denature miRNA-binding proteins with QIAzol Lysis Reagent. After the addition of 400 amoles of cel-miR-39-3p (an equalizer), total RNA was purified and reverse transcribed. Finally, droplet digital PCR (RT-ddPCR) assays for microRNA content analysis were performed to quantify the levels of miR-221-3p^[37,75] and miR-222-3p^[76]. Droplets were analyzed using the QX200 Droplet Reader, and data analysis was performed with QuantaSoft version 1.7.4 (Bio-Rad, Hercules, CA, USA).

TUMOR XENOGRAPHS AND PLASMA PREPARATION: MAJOR RESULTS

The main point of this study is focused on determining whether the pattern of plasma miRNA content recapitulates HT-29 and LoVo cells and xenografted tumors. A representative example of miR-222-3p content is shown in Figure 2A and all the quantitative data for miR-221-3p and miR-222-3p are presented in Figure 2B. The miRNA levels were independently assessed by RT-qPCR and ddPCR results, obtaining similar results, as reported elsewhere^[55]. Of course, in the quantitative analysis shown in Figure 2B and concerning the plasma miRNA quantitation, we have taken into account the fact that cross-species miRNA homology might influence our *in vivo* results. Accordingly, we quantitated baseline, endogenous miR-221-3p and miR-222-3p levels in tumor-free, healthy nude mice. As expected (the sequences of mouse miR-221-3p and miR-222-3p are identical to those found in human cells) both RT-ddPCR and RT-qPCR demonstrated that circulating miRNAs were detectable even in the absence of tumor growth. However, the differences between tumor-bearing and tumor-free mice were clearly appreciable for both miRNAs. Figure 2B shows that the miR-222-3p content is higher than miR-221-3p content in HT-29 and LoVo cells, and in tumor and plasma samples isolated from HT-29 and LoVo xenografted mice, despite the miR-222/miR-221 ratio is much higher in plasma in comparison with cell and tumors. This is consistent with the “gateway” effect mentioned above and discussed in deep in Gasparello *et al*^[55]. This issue is particularly of interest, since detailed knowledge of the molecular mechanisms underlying the release of circulating analytes is still lacking. Alternatively, the *in vivo* response of xenotransplanted mice to tumor cell injection might contribute to the reported unbalanced content of miR-222/miR-221. The proposed system is expected to help in verifying the underlying cellular mechanisms.

CONCLUSION

Circulating miRNAs have been recently used as biological markers for early diagnosis, prognosis, prediction of response to therapy and clinical outcome, particularly in a liquid biopsy setting^[1-11,77-79]. Liquid biopsy is a powerful tool applicable to all or most human cancers, including colorectal, lung, melanoma, and breast neoplasms^[80,81]. From a more general viewpoint, tumor-xenotransplanted mice and other *in vivo* models may have an important role because they resolve biological variables from technical variables (such as handling and storage of biological fluids, pre-analytical processing, as well as DNA and RNA isolation protocols) that

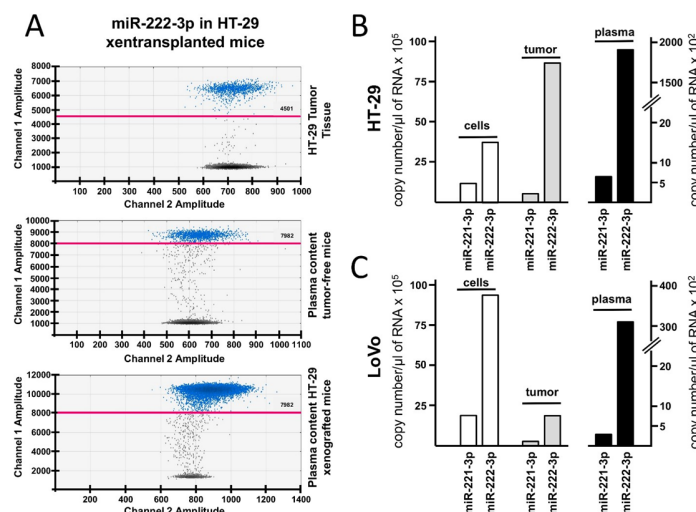


Figure 2. Content of microRNA miR-221-3p and miR-222-3p in HT-29 and LoVo cells, HT-29 and LoVo tumors and plasma isolated from HT-29 and LoVo xenotransplanted mice. A: representative Reverse Transcription droplet digital PCR plots performed using RNA isolated from tumor cells, plasma from tumor-free mice, and plasma from xenografted mice; B, C: content of miR-221-3p and miR-222-3p in cells, xenotransplanted tumors and plasma. RNA was extracted from frozen cell (5×10^5) pellets by the TRI-Reagent (Sigma-Aldrich, St.Louis, MO, USA), the procedure is described in Gasparello *et al.*^[55]. All RNAs were stored at -80°C until the use. Reverse Transcription droplet digital PCR assays for microRNA expression analysis were performed to quantify the levels of miR-221-3p and miR-222-3p. 300 ng of total RNA (from cells and tissues) and the RNA isolated from 150 μL of plasma were reverse transcribed and analyzed for miR-221-3p and miR-222-3p as described by Gasparello *et al.*^[55]

might affect efficient marker detection by liquid biopsy^[82,83]. Liquid biopsy of cancer is mainly based on the analysis of circulating tumor cells and/or cell-free nucleic acids in the peripheral blood of cancer patients, as well as in other body fluids suitable for diagnostic assessment. Among these, cerebrospinal fluid for tumors of the central nervous system, saliva for tumors affecting the head and neck, pleural effusion in the case of respiratory tract cancers and urine for urinary tract cancers. We propose that *in vivo* xenotransplant models monitoring miRNAs may find application in all the body fluids, contributing to assess the relevance of clinical liquid biopsy. The importance of *in vivo* model systems adds to the established role of liquid biopsy in complementing key limitations of surgical tissue biopsy. These include, but are not limited to: (1) invasiveness and inherent patient compliance; (2) a static representation of the tumor pathology strictly limited to the tumor tissue sampling; (3) ethical and practical issues preventing repeated tissue biopsy, particularly at unaccessible (or difficult to access) body sites; (4) tumor heterogeneity, especially during progression and metastatic dissemination (making multiple sampling necessary); (5) easier and real-time patient monitoring by non-invasive liquid biopsy. Therefore, although liquid biopsy approach still suffers from important drawbacks (fragmentation of cfDNA, instability of RNA, low yield of isolated samples to be analyzed and variable presence of normal DNA and RNA), this approach is generally deemed of great potential interest for future applications, patent development, and clinical trials, and mouse xenotransplants may be an important “shortcut” to application and technical streamlining.

Among possible application of mouse models we suggest: (1) studies on the relationship between the tumor size and the plasma miRNAs content (e.g., miR-222/miR-221 ratios); (2) analysis of the “gateway” hypothesis involved in the selection of released microRNAs (e.g., miR-221 and miR-222); (3) studies concerning the possible local and systemic responses of normal cells and tissues to xenotransplant procedure (tumor seeding); (4) analysis of the effects on miRNA plasma content on the susceptibility to experimental treatment of xenografted mice with physical and/or chemotherapeutic agents; (5) verification of the selectivity of the effects on plasma miRNA content of miRNA targeting and relative delivery approaches; (6) usage as key tools for the comparison of different analytical strategies including, among others, different PCR/RT-qPCR and NGS platforms, instruments and protocols, as well as PCR-free methods^[84-86]. Among possible limits of

the mouse xenograft model systems here presented are the differences between man and mouse with respect to ctDNA and microRNA dynamics in respect to their vasculature. Therefore we should carefully consider the sharply different ratios between the dimension of implanted tumors, the mouse body weight and the blood volume on one hand and those related the same parameters (i.e., tumor weight, body weight and blood total volume) in CRC patients. In this respect the analysis of the miRNome in liquid biopsy obtained when tumors of different dimensions are employed in mouse xenograft model systems might clarify whether the ratio between tumor size and mouse body weight or blood volume might affect the results. This might also be of interest for developing algorithms in human clinical settings.

DECLARATIONS

Authors' contributions

Revised and approved the final manuscript: Gasparello J, Allegretti M, Papi C, Giordani E, Giacomini P, Gambari R, Finotti A

Wrote the manuscript: Allegretti M, Giacomini P, Gambari R, Finotti A

Performed the literature search: Allegretti M, Giacomini P, Gambari R, Finotti A

Critically analyzed the existing literature: Allegretti M, Giacomini P, Gambari R, Finotti A

Designed the figures and created the tables: Gasparello J, Papi C, Giordani E, Gambari R, Finotti A

Availability of data and materials

Not applicable.

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Conflicts of interest

The author declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Radiotherapy of brain metastases from small-cell lung cancer: standards and controversies

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Abstract

Small-cell lung cancer (SCLC) has a high propensity to metastasize into the brain. Radiotherapy plays a major role in the treatment of brain metastases (BM) from SCLC. Whole-brain radiotherapy (WBRT) is the standard treatment of BM from SCLC. However, the neurocognitive toxicity and modest efficacy of this approach have led to the increased use of stereotactic radiosurgery. We have no strong evidence for the use of different forms of radiation (WBRT vs. radiosurgery) in SCLC, because BM from this primary tumor were excluded from clinical trials. In this review, the use of radiation in form of WBRT or radiosurgery is discussed in distinct clinical indications: as a primary treatment and at relapse; without prior use of prophylactic cranial irradiation (PCI); and after PCI. Combinations of radiotherapy with chemotherapy are discussed as BM in SCLC occur rarely as a sole event.

Keywords: Small-cell lung cancer, brain metastases, whole-brain radiotherapy, radiosurgery

INTRODUCTION

Small-cell lung cancer (SCLC) has a higher propensity than other solid tumors to spread to the brain. As many as 40%-50% of SCLC patients will develop brain metastases (BM) during the course of their disease^[1]. Apart from a higher risk of occurrence than in other cancers, BM from SCLC have distinct clinical characteristics that differ from BM from all other solid tumors. These differences are reflected in radio- and chemo-sensitivity and high aggressiveness with rapid propagation in the brain. The latter property makes BM from SCLC hardly suitable for local treatment such as surgery or radiosurgery. Unlike BM from



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other cancers whole brain radiotherapy (WBRT) remains a standard treatment even if BM are suitable for radiosurgery. The well recognized radio- and chemo-sensitivity of SCLC are also present in the case of BM. Despite their radio- and chemo-responsiveness, the prognosis of BM from SCLC remains very poor with median survival after WBRT of 3.0-4.7 months in both retrospective and prospective studies^[2-5]. The high risk of developing BM with a dismal disease course once BM have occurred and their high radiosensitivity have led to many trials assessing the value of prophylactic cranial irradiation (PCI). To date, SCLC is the only solid tumor in which the prolongation of survival with the use of PCI has been demonstrated for localized stage (LS) SCLC in a meta-analysis^[6]. A total dose of 25 Gy in 10 fractions was established in a randomized trial as a standard dose of PCI for responders to initial therapy of LS SCLC^[7]. In addition, for extensive stage (ES) SCLC, there were some indications that the use of PCI is of value for prolongation of survival^[6] and this was demonstrated in one randomized trial^[8].

The present review summarizes the problems related to radiotherapy of BM from SCLC with an emphasis on the distinctiveness of this approach in relation to management of BM from solid tumors. Different approaches in specific indications for treatment of BM from SCLC are discussed.

PROGNOSTIC FACTORS

Patients with BM from SCLC are usually excluded from trials on management of BM due to the different biological behavior of the primary tumor. The question arises if existing prognostic scores for overall survival in patients with BM are also relevant for BM from SCLC. The pivotal RTOG prognostic score was published after carrying out a recursive partitioning analysis (RPA) of pretreatment characteristics of patients in the randomized trials on BM. The prognosis of BM patients was related to the presence of three prognostic factors: performance status, presence of extracranial disease and age. Class 1 with the best prognosis included patients younger than 65 years, with control of extracranial disease, and with performance status ≥ 60 in Karnofsky performance status (KPS) score. Class 3 with the poorest prognosis included patients with KPS < 70 regardless of the presence of all other factors. Class 2 with intermediate prognosis included patients who did not meet the criteria of classes 1 or 3^[9]. These three prognostic factors are still the basis of all contemporary, more refined prognostic scores, even if new factors are included. However, in the original RTOG database, only 4% of patients had SCLC histology^[9]. Retrospective studies have confirmed the validity of the prognostic stratification of BM from SCLC according to the RTOG RPA class criteria. In 132 SCLC patients who received WBRT, the median survival for classes 1, 2, and 3 was 2, 4.5, and 2 months, respectively ($P = 0.003$). However, there were only eight (4%) patients in class 1^[2]. In 154 patients with BM of whom 98% received WBRT as part of their treatment, the median survival for classes 1, 2, and 3 was 8.6, 4.2, and 2.3 months, respectively ($P = 0.002$). Only 5% of patients from this group met the criteria of RPA class 1^[2]. The rarity of RPA class 1 among patients treated for BM from SCLC is related to the aggressiveness of the primary tumor. Brain-only metastases from SCLC with cured primary are an extremely rare event, as demonstrated by EORTC phase II trial, in which the accrual of patients with brain-only metastases was so slow that the study was stopped before the required number of patients was reached^[4].

In the more contemporary disease-specific graded prognostic assessment (DS-GPA), the prognostic factors for patients with BM were attributed separately for respective primary tumors. For lung cancer, based on the results of 1833 NSCLC and 281 SCLC patients, apart from the prognostic factors from RTOG RPA class (extracranial disease, age and KPS) the number of BM (1 vs. 2-3 vs. > 3) reached prognostic significance. When patients with SCLC were analyzed separately, the number of BM was also significantly prognostic for survival^[10]. The retrospective study demonstrated that median survival for 30 patients with single BM was 7 months compared with 2.9 months for 98 patients with multiple BM from SCLC ($P = 0.005$)^[2]. In another small retrospective study, the number of BM also had an impact on survival^[11]. The prognostic significance of the number of BM for SCLC may be counterintuitive, because we still treat this disease with WBRT based

on the rationale that due to rapid progression, the subclinical disease foci exist in the whole brain at the onset of macroscopic BM. On the other hand, published data indicate that also in the case of SCLC, biological behavior may differ in a single BM compared with multiple BM. Thus, a disease that occurs with single or oligo- brain metastases may have different, more favorable prognosis than poly-metastatic brain disease at its onset. The latter would have greater inherent aggressiveness. However, we cannot exclude that in the case of less advanced brain involvement, more aggressive treatment strategies are employed. In the retrospective analysis of 52 patients who received WBRT for single BM, the use of surgery in combination with WBRT was related to improved survival compared with WBRT alone, with median overall survival of 19 and 5 months, respectively ($P = 0.03$)^[12]. In the largest retrospective study on patients with BM from SCLC that included 229 patients treated with WBRT, the number of BM did not retain prognostic significance. Apart from the factors included in the RTOG RPA score, the time of occurrence of BM in relation to the diagnosis of the primary (synchronous *vs.* metachronous BM) and initial response to chemotherapy had a significant impact on survival. Patients presenting with synchronous BM and initial good response to chemotherapy had improved overall survival compared with patients presenting with metachronous BM and non-/poor responders to initial chemotherapy^[13]. The authors proposed a new BM from SCLC score (BMS-score), which included RTOG RPA class and synchronous *vs.* metachronous BM presentation. Initial response to chemotherapy was not included in the score, because the evaluation of the response to chemotherapy may not be practical and as a result may prevent the use of the score on a large scale^[14].

Concluding the discussion of the issue of prognostic factors in BM from SCLC, we should emphasize the unquestioned value of the RTOG RPA classes. The role of the number of BM should be reconfirmed, especially in the era of rapid expansion of local treatment of BM, also in SCLC. The timing of occurrence of BM, that is, synchronous *vs.* metachronous presentation, and the initial response to chemotherapy require further evaluation.

TREATMENT OF BM WITH SYNCHRONOUS PRESENTATION

BM that occur synchronously with primary diagnosis of SCLC represent different clinical scenarios and require different management compared with BM that are diagnosed metachronously, that is, at the relapse of SCLC. We may distinguish four categories of this synchronous presentation that require different management.

BM diagnosed during an initial staging of SCLC

When asymptomatic BM are part of the dissemination of SCLC, either as a sole distant site or as one of many distant locations, treatment usually starts with chemotherapy. The efficacy of chemotherapy for BM was questioned in the past, because the brain was considered a pharmacological sanctuary due to the brain-blood barrier (BBB) that prevented drugs from penetration into the brain. However, tumor invasion likely disrupts the BBB, because there are many clinical observations of the efficacy of chemotherapy in BM from SCLC. Pooled data from five studies reported a 66% response rate (RR) in 64 patients with synchronous BM^[15]. However, there are also contrary data indicating that the RR in the brain is lower than the systemic RR; with 27% *vs.* 73% RR for brain and extracranial sites, respectively, in 24 asymptomatic patients with BM from SCLC who received cyclophosphamide, doxorubicine, and vincristine^[16]. However, an aggressive and rapid disease course prompts the initiation of systemic therapy so as not to miss the opportunity to administer chemotherapy that may stop extracranial systemic and local disease progression that causes bothersome and/or life threatening symptoms.

The question remains, whether chemotherapy should be followed by WBRT in such patients, even if the use of chemotherapy results in a complete response in the brain. We have no prospective data on this point. However, one randomized trial compared the use of teniposide alone *vs.* teniposide + WBRT 30 Gy in 120

patients with progression in the brain after or during first-line treatment. The data from this study showed that omission of WBRT in the treatment of BM led to a significantly shorter time to progression in the brain in patients managed without WBRT, with 8% vs. 35% of patients free of progression in the brain at 6 months in those treated with and without WBRT, respectively. Overall survival was short (~ 3.5 months) in both groups^[5]. The demonstrated risk of rapid progression in the brain without the use of WBRT suggests the benefit of combining chemotherapy with WBRT also in these patients, regardless of the chemotherapy response-with the awareness that no strong evidence for such an approach exists.

Histology of SCLC found during a craniotomy performed for brain tumor without prior diagnosis of lung primary with this histology

This is a special case when the unexpected SCLC histology of a removed brain tumor leads to performing diagnostic procedures to find a lung primary and evaluate its extension. When the lung primary is found, further management does not differ from the scenario when BM are found during initial staging of SCLC, namely chemotherapy followed by WBRT. Patients with BM from SCLC were not included in the trials on the use of tumor bed radiosurgery with the omission of WBRT, because of the increased risk of dissemination outside the tumor bed^[17,18]. Thus, also in these cases, WBRT remains the standard of care. A special presentation of BM SCLC represents a situation in which after removal of the brain tumor neither primary, nor signs of extracranial extension are found despite meticulous diagnostic procedures including PET-CT and bronchoscopy. In such a scenario, the justification for the use of chemotherapy may be questioned - again, we have very limited evidence. The omission of chemotherapy as first-line treatment in such patients with careful monitoring may be an option in more fragile patients.

BM developed during first-line treatment for ES-SCLC when no baseline brain imaging was performed

Brain imaging in asymptomatic patients with ES-SCLC is not systematically performed. In the EORTC trial which demonstrated a survival advantage with the use of PCI, brain imaging was not part of the standard staging procedures and only 19% of randomized patients had baseline brain CT or MRI^[8,19]. Obviously, if in such patients during first-line chemotherapy symptomatic BM occur, WBRT is given and chemotherapy is continued or discontinued after completion of brain irradiation depending on the systemic chemotherapy response and performance status of the patient.

BM diagnosed before PCI for LS SCLC or ES SCLC in the case of pre-PCI MRI

Brain imaging has not always been a standard procedure before PCI qualification in either LS SCLC or ES SCLC. Some prospective studies reported using CT scans, some did not require any imaging, and some did not mention any requirements for imaging use^[20]. NCCN guidelines recommend pre-PCI MRI for patients with response to initial therapy^[21]. These guidelines are strictly followed in the USA; as highlighted in a recent survey, up to 96% out of 309 US radiation oncologists performed pre-PCI MRI^[22]. In contrast, some European IASLC and ESTRO experts indicated in a survey on the practice of PCI use for ES SCLC that the restrictions in reimbursement for MRI and problems with its availability were the main reasons of performing PCI in such patients. With MRI surveillance, patients would avoid brain irradiation, unnecessary in some cases^[23]. In one study, patients with initial diagnosis of LS SCLC had baseline MRI performed. Complete responders who qualified for PCI after treatment completion had a second, pre-PCI MRI; 13 out of 40 (32.5%) patients had BM in pre-PCI MRI. Patients with pre-PCI detected BM had worse prognosis than those without BM in pre-PCI MRI (17% vs. 74% for 1-year survival, respectively, $P = 0.0001$). This difference was seen despite the higher and more intense WBRT doses in patients with BM. However, one major limitation of this study was that PCI was applied late, that is, 4-10 months after diagnosis. Moreover, this finding does not support the routine use of pre-PCI MRI, because even giving higher WBRT doses did not reverse the poor prognosis in these patients^[24]. Similar observations from a larger group of patients were presented at the IASLC World Conference on Lung Cancer in 2018. From 119 LS SCLC patients referred for PCI after

definitive chemoradiotherapy with baseline brain MRI, 25 (21%) harbored BM on pre-PCI MRI, and 23 were asymptomatic. The duration of chemo-radiotherapy was the only prognostic factor for occurrence of pre-PCI BM. Patients with BM in pre-PCI MRI and duration of chemo-radiotherapy that exceeded 4.5 months had significantly shorter survival^[25]. These findings are in line with modeling radiobiological studies that sought to elucidate dose-response relationship for PCI in SCLC as a function of time interval between time of treatment of primary tumor and initiation of PCI. The nearly linear dose-response relationship for reduction in BM was demonstrated for “early PCI” (up to 60 days from the start of treatment of the primary tumor) in doses of 0-35 Gy given in 2-Gy fractions. When PCI was delayed for over 60 days, a significant threshold in dose-response was observed, which is consistent with a fast growth rate of untreated subclinical BM from SCLC^[26]. Thus, it is important to initiate PCI early from the start of treatment. However, the guidelines do not recommend the concomitant use of PCI with chemotherapy because of apprehension concerning the toxicity of such an approach. What we learn from these studies is that every effort should be made to avoid unnecessarily prolonging chemo-radiotherapy for SCLC and to start PCI as quickly as possible after the end of treatment of the primary tumor. Pre-PCI MRI and higher WBRT dose not change a prognosis of asymptomatic patients in which progression in the brain occurs during initial therapy. However, in some patients with ES SCLC without BM on pre-PCI MRI, MRI surveillance without baseline PCI may be recommended as in a Japanese trial, in which patients with MRI surveillance did not have a survival benefit with the addition of PCI^[27]. Pre-PCI MRI is also a pre-requisite for PCI with hippocampal avoidance.

TREATMENT OF BM WITH METACHRONOUS PRESENTATION

Despite a high initial response rate on chemotherapy, the majority of patients with LS SCLC and practically all with ES SCLC will relapse within the first year after treatment. After first-line chemotherapy, only extremely limited therapeutic options exist. Median survival after second-line chemotherapy is 3-6 months in clinical trials^[28]. In addition, BM occur rarely as a sole event. The majority of patients with BM simultaneously have extracranial disease progression^[4]. The limited therapeutic options and poor survival after relapse determine the poor outcome of treatment of metachronous BM. Thus, a metachronous presentation, that is, occurrence after first-line treatment, appears as an adverse prognostic factor^[13,14]. Treatment strategies for BM that occur at the relapse differ with regard to the earlier use or not of PCI.

Radiotherapy for BM that occur without prior PCI

When the use of chemotherapy for asymptomatic BM that occur simultaneously with the diagnosis of primary is not debatable, the use of chemotherapy for metachronous BM is conditioned by a number of factors, such as the site of progression, brain only vs. brain and extracranial site, previous response to chemotherapy, the time interval from the last line of chemotherapy, the extent of extracranial disease, and performance status. We have no strong evidence for the use of chemotherapy for BM from SCLC. In the Cochrane Database of Systematic Reviews, only three small randomized trials involving 192 participants that dealt with chemotherapy for BM from SCLC were identified^[29]. In one study, 120 patients with BM and concurrent systemic failure were randomized to receive teniposide with and without WBRT. Patients in the combined modality arm had higher RR in the brain (57%) than patients treated with teniposide alone (22%), ($P < 0.001$). Patients who received WBRT also had longer time to progression in the brain than patients treated with chemotherapy alone, ($P = 0.005$). Overall survival did not differ greatly between the groups (median survival: 3.5 months in the combined modality arm and 3.2 months for chemotherapy alone arm; $P = 0.087$)^[5]. Only one trial compared chemotherapy with no chemotherapy; 33 patients received WBRT for BM from SCLC (first line, $n = 5$; recurrence, $n = 28$) and were randomized to WBRT alone vs. WBRT plus topotecan. No significant difference in survival was found between these two groups^[30]. The Cochrane Database of Systematic Reviews identified one other chemotherapy trial that compared two schedules - sequential and concomitant-of combination chemotherapy (teniposide plus cisplatin) with WBRT. This trial included only 39 patients and no difference in overall survival and response rate for either combination was demonstrated, although patients

in the concomitant arm had a higher rate of myelosuppression^[31]. Thus, the available evidence is insufficient to determine the effectiveness of chemotherapy for the treatment of BM from SCLC. We have no evidence that chemotherapy improves brain tumor control and overall survival in those patients. However, when BM are accompanied by systemic progression and the option of giving chemotherapy for a specific patient exists, chemotherapy is given based on the recognized chemo-responsiveness of this SCLC.

Although WBRT remains the standard therapeutic strategy for these patients, this approach is not based on the results of randomized trials. In addition, the effectiveness of WBRT in patients with widespread metastatic disease is debatable. We cannot exclude that some of these patients may be treated with chemotherapy alone. Another dubious indication for WBRT in these patients involves BM in RTOG RPA class 3, that is, patients with poor performance status (KPS < 70). In one retrospective analysis of 132 patients with BM from SCLC who received WBRT, the median survival for 27 (20%) patients from RPA class 3 was 2 months^[2]; similarly, in another trial that included 154 such patients, the median survival for 51 (33%) patients from RPA class 3 was 2.3 months^[3]. With such a short survival, the benefit of WBRT is doubtful. One prospective trial that also included patients with SCLC histology aimed to determine whether WBRT had any benefit in terms of symptoms palliation in 91 RTOG RPA class 3 patients. All patients received WBRT and were asked to complete a questionnaire about their symptoms before and 1 month after WBRT. Only 43 (47%) patients completed the questionnaire after WBRT, the other patients died or were not able to respond to the survey questions because of further deterioration of performance and/or neurological status. In the group of patients who completed both questionnaires, the intensity of symptoms of the disease significantly increased after WBRT. This result challenges the value of WBRT for patients with poor performance status^[32]. Recently, the limited value of WBRT in patients with BM from NSCLC who were unsuitable for resection or radiosurgery in terms of survival, quality of life, and reduction of the dose of steroids was demonstrated in a large randomized trial^[33]. Taking into account, the short survival of patients with BM from SCLC, the value of considering WBRT in these patients as a standard of care should be subject to further research.

Radiotherapy for BM that occur after PCI

PCI reduces the risk of BM in both LS SCLC and ES SCLC, as shown in a meta-analysis and prospective randomized trials^[6,8,27]. Nevertheless, a substantial proportion of patients still develop brain failure after PCI. The 3-year BM rate after PCI was 33% vs. 59% without PCI in a meta-analysis of 987 patients from seven randomized trials that compared treatment of SCLC with and without PCI^[6]. In ES SCLC, the risk of symptomatic BM after PCI was 15% vs. 40% without PCI^[8]. In a Japanese prospective trial that compared treatment with and without PCI with staging and strict surveillance with brain MRI, the 1-year BM rate was as high as 33% with PCI vs. 59% in patients without PCI^[27]. These results indicate that 15%-30% of patients will still develop BM after PCI. Such failure represents a special therapeutic challenge, because reirradiation of the whole brain that has already received a biological dose of ~ 30 Gy risks meaningful neurotoxicity. On the other hand, the short survival of such patients may prevent them from the occurrence of late toxic effects, and in the short term, reirradiation at moderate doses may be beneficial, especially in symptomatic patients, taking into account the radio-sensitivity of SCLC. Even if localized treatment options like radiosurgery or less often resection appear as appropriate strategies in the irradiated region, most of these patients recur as multifocal BM that are unsuitable for radiosurgery. At the 2018 IASLC World Conference on Lung Cancer, one study reported that 60% of 32 patients who recurred in the brain after PCI were unsuitable for radiosurgery and WBRT may be considered for such patients^[34]. There are very few reports about whole brain reirradiation after PCI. The largest series of patients treated with brain radiation after PCI with a median time of 14 months (range, 4-42 months) demonstrated that in 76 patients receiving a PCI dose of 30 Gy in 15 fractions, repeat WBRT with doses of 20-30 Gy in fractional doses of 2 Gy was given to 66 (88%) patients and radiosurgery of 18-24 Gy in a single fraction to 13 (18%) patients. Median survival after repeat WBRT and radiosurgery was 3 months and 5 months, respectively, with a range of 0-12 months for both treatment types; 40% of 44 symptomatic patients improved after reirradiation. No serious, grade > 2 toxicity was observed in these patients^[35]. These results indicate that WBRT with moderate doses may be beneficial in

Table 1. Characteristics and outcome of patients undergoing radiosurgery (RS) for brain metastases (BM) from small-cell lung cancer (SCLC)

Author, year	Number of included patients	Number of BM treated/doses of SRS	Number of patients undergoing prior WBRT (details on previous treatment)	Median overall survival from RS (months)	Local control	Distant control in the brain
Bernhardt <i>et al.</i> ^[35] , 2016	13	Maximum: 4 / 18-24 Gy	13 (PCI: 30 Gy in 15 fractions)	5 (range: 0-12)	Not provided	Not provided
Rava <i>et al.</i> ^[37] , 2015	40	Single: 15 2-3: 15 ≥ 4: 10/ No details	37 (27: WBRT, 10: PCI)	6.5 (range: 4.1-8.9)	Actuarial 1-year: 69%	Actuarial 1-year: 22%
Harris <i>et al.</i> ^[36] , 2012	51	Single: 22 2-3: 18 ≥ 4: 11/10-24 Gy (median: 18 Gy)	51 (35: WBRT, 16: PCI)	5.9	Actuarial 1-year: 57%	Actuarial 1-year: 42%
Wegner <i>et al.</i> ^[40] , 2011	44	Median: 1 (range: 1-14)/14-20 Gy (median: 18 Gy)	36 (18: WBRT at median dose: 30 Gy in 10 fractions; 6 WBRT combined with SRS; 9: PCI, 3: PCI + WBRT for relapse)	9	Actuarial 1-year: 86%	Crude rate of failure: 61%
Olson <i>et al.</i> ^[39] , 2012	27	Median: 2 (range:1-6)/15-24 Gy (median: 20.5 Gy)	27 (19: WBRT, 8: PCI)	3	Actuarial 1-year: 75%	Actuarial 1-year: 31%
Yomo <i>et al.</i> ^[41] , 2015	70	Median: 2 (range:1-21)/12-22 Gy (median: 20 Gy)	23 (16: WBRT, 7: PCI) + 1 Hypofractionated partial brain irradiation	7.8 (range: 0.6-56)	Actuarial 1-year: 77%	Actuarial 1-year: 53%
Nakazaki <i>et al.</i> ^[38] , 2012	44	Median: 5 (range:1-98)/10-21 Gy (median: 20 Gy)	44 (34: WBRT with median dose of 30 Gy, 10: PCI)	5.8 (range: 0.5-24)	33 out of 44 evaluated: in 10 out of 33 failure: 70% crude local control	33 out of 44 evaluated: in 24 out of 33 failure: 28% crude distant control

Abbreviations: PCI: Prophylactic Cranial Irradiation; WBRT: Whole-Brain Radiotherapy

patients who are not candidates for radiosurgery and the short survival of such patients prevents them from the development of serious late neurotoxicity.

In the case of a limited number of BM < 3 cm in diameter, the minimal invasiveness and ease of use of radiosurgery make it the preferred salvage method after prior PCI for patients with life expectancy > 3 months. Retrospective data indicate that for patients with good performance status, radiosurgery for BM in SCLC gave results that were at least comparable with WBRT in terms of survival, with the median range of 3-9 months^[30-36]. However, some reports reported lower local control after radiosurgery for BM from SCLC than for BM from other solid tumors. One-year local control rates were < 70% in evaluated patients^[36-41], whilst in prospective trials on radiosurgery with the exclusion of SCLC histology, these rates were 70%-90%^[42,43]. Distant brain control was also at the lower limit or < 60% as reported in prospective trials on radiosurgery alone^[36-41]. This may be related to the known aggressiveness of SCLC, but also to the inclusion of patients with multiple (> 4) BM, which may affect these results. Recently, it was demonstrated that radiosurgery without WBRT in patients with 5-10 BM was not inferior to that in patients with 2-4 BM in terms of survival^[44]. Table 1 summarizes the results of radiosurgery for BM from SCLC used as a salvage method after WBRT, as well as a first-line irradiation. To conclude, radiosurgery is an attractive treatment option after prior PCI and should be used if the technical possibilities for its use exist; however, WBRT at moderate doses is also feasible. WBRT is the treatment of first choice in patients who are unsuitable for radiosurgery or symptomatic patients with limited life expectancy (< 3 months).

REDUCTION OF NEUROTOXICITY IN BRAIN RADIOTHERAPY FOR SCLC

WBRT in SCLC plays a role in prevention in the form of PCI and remains the standard in the treatment of BM. However, we have evidence that WBRT has a detrimental effect on neurocognitive functioning. In a

phase III trial of standard-dose *vs.* high-dose PCI for LS SCLC (RTOG0212), patients underwent evaluation for cognitive toxicity and quality of life effects. Patients receiving the higher-dose PCI were found to have a 25% increase in the rate of chronic cognitive toxicity compared with the standard-dose arm. However, 62% of patients receiving the standard-dose PCI also developed cognitive toxicity, as assessed by the Hopkins Verbal Learning Test (HVLT) Delayed Recall score^[45]. These data indicate that even the standard-dose of PCI is associated with neurocognitive toxicity. It is also argued that with the improvement of survival, neurotoxicity has greater chance to occur and negatively impact quality of life^[46]. Thus, the strategies to reduce neurotoxicity in PCI and WBRT warrant further investigation. One of these strategies is hippocampal avoidance (HA) during WBRT, based on the principle that proliferating neuronal progenitor cells in the subgranular zone of the hippocampus play an essential role in memory function. Thanks to technological advances and the availability of IMRT techniques, PCI and WBRT for BM with HA have been extensively explored and even used in routine practice. The RTOG 0933 trial demonstrated that HA during WBRT for BM was associated with a mean relative decline in the HVLT-Revised Delayed Recall score from baseline to 4 months of 7.0% (95% confidence interval, 4.7%-18.7%), which was a significant improvement compared with the historical control ($P = 0.0003$)^[47]. Recently, for the first time, the benefit of HA in WBRT for BM in terms of preservation of neurocognitive function without compromising intracranial control was confirmed in 518 patients included in a phase III trial (NRG Oncology CC001). The 6-month neurocognitive function failure rate was 69% after WBRT without HA compared with 58% when using HA^[48].

However, the safety of this approach for SCLC remains to be demonstrated, because the risk of impaired intracranial control *via* underdosing some parts of the brain in the HA area persists in this very aggressive malignancy. Some reports support the safety of such an approach, indicating the risk of failure in the HA zone to be $< 5\%$ ^[25,49]. In contrast, other reports warn of the rapid implementation of HA in SCLC, with an increased risk of failure of $> 10\%$ in the HA zone^[50,51]. These contradictory data suggest that a potential small benefit of hippocampal sparing in limiting the neuropsychological sequelae of brain radiation may be obtained at the risk of failure in the spared region. In modeling studies, HA PCI in SCLC was more cost-effective than conventional PCI provided that the risk of developing BM was not increased by more than 14%, or if neurocognitive dysfunction rates were reduced by at least 40%^[52]. These findings strongly support the continued enrollment in ongoing cooperative group randomized trials on the value of HA PCI in SCLC: NRG CC003 (USA), PREMER (Spain)^[53], NCT01780675 (Holland).

Other strategy to reduce neurocognitive deterioration in patients after brain irradiation is the use of neuroprotective medication. The only agent that seems to be effective in this indication is memantine. It was shown in the prospective study, that it delayed cognitive deterioration after WBRT, although at 24 weeks this did not reach significance ($P = 0.059$)^[54]. Recently performed systematic review concluded that studies on other medications, like methylphenidate and donepezil remain inconclusive in this regard^[55].

CONCLUSION

To conclude, a standard radiation method for BM from SCLC remains WBRT. Stereotactic radiosurgery is reserved for relapses after PCI or prior WBRT for overt BM. However, the advancement in technologies allow for treatment of multiple metastases with omission of WBRT. Safety and benefit of such an approach remain to be confirmed in a prospective trial. All such efforts are welcomed. The ENCEPHALON trial launched at Heidelberg University Hospital randomizes patients with up to 10 metastases from SCLC to radiosurgery of all lesions *vs.* WBRT. Primary endpoint of the study is neurocognitive function. Secondary endpoints are intracranial control, overall survival and toxicity^[56]. Only such direct comparisons will give evidence for safety of omission of WBRT in BM from SCLC. Mature data from the trials evaluating safety of HA in SCLC are also eagerly awaited. Short survival of such patients with both WBRT and stereotactic methods shows also that radiotherapy is not a sufficient treatment method in this indication. Combinations

of radiotherapy with chemotherapy should be further evaluated. It was demonstrated in a randomized trial that Atezolizumab added to standard chemotherapy with Carboplatin and Etoposide in ES SCLC improved overall survival, however patients with BM represented only 8.5% of the included cohort^[57]. Thus, further research is needed to investigate the role of immunotherapy in patients with BM from SCLC.

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The author solely contributed to the manuscript.

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Review

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Diagnosis and management of brain metastases: an updated review from a radiation oncology perspective

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Abstract

Brain metastasis are the most common intracranial malignancy in the adult population. Their incidence has increased dramatically over the last 20 years, as a result of the increasing number of cases stemming from lung and breast cancer together with the higher cancer survival rates due to diagnostic and therapeutic advances. More than 40% of cancer patients develop brain metastases during the course of their disease: specifically, they appear in 50% of patients with lung cancer, more than 25% of patients with breast cancer, and 20% of patients with melanoma. Diagnosis is made using different imaging approaches, such as computed tomography and magnetic resonance imaging, accompanied by clinical manifestations and a history of malignancy supporting the diagnosis of a brain metastasis. Current treatment options should be oriented to the patient's current performance, the number of intracranial and extracranial lesions, and related factors. Although surgical resection and whole-brain radiotherapy have been standard treatments for many years, numerous treatment modalities have become more easily available and accepted worldwide, producing more favorable and reliable results. Among these is stereotactic radiosurgery, and the latest clinical trials support this treatment.



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Keywords: Brain metastases, whole-brain radiation therapy, stereotactic radiosurgery, graded prognostic assessment

INTRODUCTION

Brain metastases are the most common intracranial malign neoplasms in adult patients, with 170,000 new cases per year reported in the USA alone^[1]. The incidence of brain metastasis has been on the rise the last 20 years, resulting from an increase in the number of cases of lung and breast cancer^[2] and the fact that cancer survival rates have been increasing thanks to new therapeutic advances and the availability of central nervous system imaging technologies for diagnosis. More than 40% of patients with cancer develop brain metastases: specifically, they appear in 50% of patients with lung cancer, more than 25% of patients with breast cancer, and 20% of patients with melanoma^[3,4].

Multiple epidemiological studies have been conducted regarding brain metastases. These have had different methods and limitations; however, a comparison between them can bring an understanding of the incidence of brain metastasis. Such studies include the initial work done by Goumudsson in Iceland, which reported an incidence of 2.8 cases per 100,000 people^[5], and Percy *et al.*^[6], where the incidence found was as high as 11.1 cases per 100,000. The Barnholtz-Sloan *et al.*^[7]'s study derived greater validity from its use of the register of the Metropolitan Detroit Cancer Surveillance System from 1973 to 2001, carrying information on an approximate population of 4.5 million patients, in which the observed incidence of brain metastasis in patients with any kind of neoplasm was 9.6%.

Considerable variability in the incidence of brain metastasis has been found, which may be attributable to limited available data, such as autopsy reports or general hospital records; nevertheless, beginning with the first records of cancer, a similar incidence of brain metastasis has been observed, which may be attributable to the fact that it is an exclusively oncological population limited to a particular state or region and follow-up of the same patients^[8,9].

Generally, lung cancer is the foremost cause of brain metastasis, with studies reporting incidences of 12% to 65%^[10] of all patients with primary lung cancer. Among the most commonly associated histologies for brain metastasis are small-cell lung cancer and adenocarcinoma. In a high percentage of patients with lung cancer, diagnosis is performed after the onset of neurological symptoms.

Breast cancer is the main cause of brain metastases in women, with reported incidences between 5% and 30% of all breast cancer cases. Unlike the case of lung cancer, in breast cancer the diagnosis of brain metastasis usually follows well after the initial diagnosis of cancer. Diagnoses of melanoma have increased over the last several years, and this malignant neoplasm has the greatest capacity to develop into brain metastasis, with incidences from 12% to 90% [Figures 1-3]. Incidences of 7% to 10% of all patients with renal cancer and 1% to 4% of all gastrointestinal tumors have been reported^[11].

The therapeutic management of patients with brain metastases depends on the localization and number of brain lesions, primary tumor biology, and disease extension. The overall survival from the moment of diagnosis for untreated patients is 1-2 months, which can be extended to 6 months in patients who receive conventional radiotherapy and chemotherapy^[4].

PATHOPHYSIOLOGY

The genesis of metastasis requires several complex and sophisticated steps to occur first. These include genetic, epigenetic, and biological changes known as the “metastatic cascade”^[12,13]. This process begins with the detachment of a tumor cell from its primary lesion and the invasion of the surrounding tissue,



Figure 1. Left parietal lesion

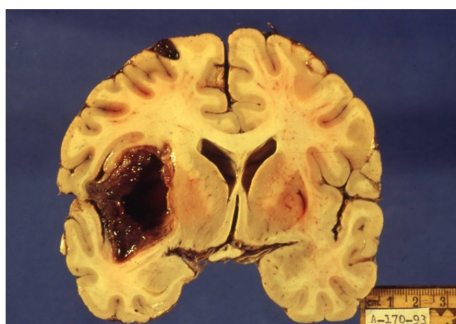


Figure 2. Macroscopic view of a lesion located in the left temporal lobe, with dimensions of 5 cm× 4 cm× 3 cm, from the cystic-necrotic center. A subepidural lesion appears in the parietal region on the same side

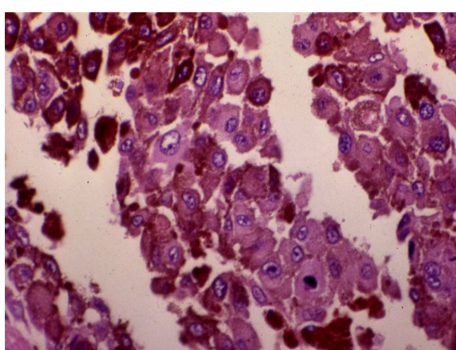


Figure 3. Histologically, this lesion is composed of epithelioid-like cells with moderate eosinophilic cytoplasm and well-defined cytoplasmic junctions between some cells. The nuclei are vesicles with an apparent nucleolus. A brown pigment characteristic of melanocytic cells is seen

including the basement membrane, which is followed by intravasation in the blood vessels, hematogenous and lymphatic dissemination, the production of circulating tumor cells in brain capillaries, and then extravasation. Finally, the cancer cells must colonize the surrounding tissue and induce angiogenesis and cell proliferation, forming secondary lesions^[14].

The blood-brain barrier is a functional and anatomical barrier that plays an important role in the interaction between the cerebral microenvironment and metastatic colonization^[15]. In this process, tumor cells survive

an inflamed cerebral microenvironment that is appropriate for their development and growth, which is known as their niche^[16].

Tumor cells can evade growth-suppressing factors and inhibitors of cell proliferation through mechanisms that include resistance to apoptosis, overexpression of Bcl-2 and Bcl-xL, and the inhibition of proapoptotic pathways Bax and Bim^[12]. Tumor cells can be dedifferentiated, migrate to a distant site, survive apoptosis, disseminate, and redifferentiate. The activation of cells in the adjacent stroma by paracrine signaling with pro-tumor factors maintains the growth of the tumor, intensifying genomic instability and epigenetic dysregulation^[11,13].

Tumor cells adhere to the endothelium of recipient tissue and act as macrophages, creating pseudopods and penetrating cell-to-cell junctions, subsequently gaining access to normal tissue parenchyma to activate angiogenesis and develop new vessels for its nutrition, in this way promoting the growth of secondary injuries^[17]. Circulating cells attract platelets due to the proteins they express on their surface, which protects them from the immune system. Likewise, metastatic cells activate mechanisms to escape immunity by reducing the expression of TAP1, which decreases the effects of T-cell-mediated death^[18].

DIAGNOSIS

Although clinical manifestations of a history of malignant disease with central nervous system metastasis potential may obviously occur, it will not always be possible to obtain histological confirmation of these lesions [Figure 3]. In such cases, complementary image studies may play an important role in determining the best management for these patients.

Image studies

Magnetic resonance imaging (MRI) is the tool of choice when brain metastasis is suspected, due to its high sensitivity and specificity, which support its high capacity to detect smaller lesions than those that appear in computed tomography (CT) with or without contrast; it is also associated with fewer bone artifacts in posterior fossa. However, if MRI cannot be used, CT is still a valid option^[19].

CT will show isodense lesions in contrast with brain parenchyma, which will intensify with the application of intravenous contrast medium. When lesions appear hyperdense, one may suspect secondary bleeding, especially in histologies associated with high spontaneous bleeding risk (including choriocarcinoma, melanoma, and renal carcinoma); there are also other findings that are secondary to lesions and that can be easily visualized, such as hydrocephaly, ring-enhancing cerebral lesions, and brain herniation^[19,20].

The sensitivity and specificity of CT scans are 92% and 99%, respectively, and they are considerably higher in tumors that have a high incidence of central nervous system metastasis, such as non-small-cell lung cancer^[21]. MRI exhibits an ability to detect lesions smaller than 1 cm, up to 70% more sensitive than CT, and this increases in cases of multiple metastases^[19]. MRI has other beneficial characteristics, for example, in the use of distinct sequences such as T1, T2, FLAIR, diffusion, and perfusion, which can be used along with spectroscopy to increase sensibility and specificity^[22].

The majority of metastatic lesions are hypointense in T1 sequence images, which can be an indication of hemorrhage and necrosis. In T2 weighted images, the majority of lesions appear hyperintense [Figure 4]. Bleeding appears as acutely hypointense and becomes hyperintense as chronicity develops, while vasogenic edema appears hyperintense. The use of contrast significantly increases its sensitivity and specificity for the detection of brain metastases relative to simple MRI^[23].

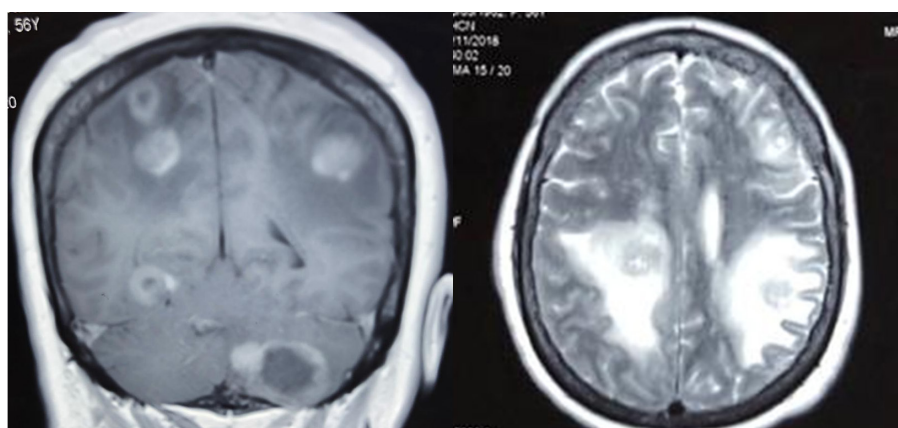


Figure 4. Magnetic resonance imaging of a patient with multiple brain metastases. Axial T2 slice sequence shows perilesional edema in the junction of white and gray matter

Table 1. Spectroscopy

Metabolite/marker	Function	As found in brain metastases	Range (parts per million)
Creatine	Metabolism	Internal standard	3.0
Choline	Cellular membrane turnover	Increased	3.2
Lipids	Necrosis	Increased	0.9-1.4
Lactate	Anaerobic metabolism/necrosis	Increased	1.3
N-acetylaspartate	Neuronal viability	Decreased	2.0

SPECTROSCOPY

Spectroscopy is an MRI technique that allows the metabolic characteristics of lesions to be evaluated, which may help distinguish between benign lesions and malignant lesions which would be difficult to differentiate using MRI alone. Spectroscopy can be performed for single or multiple tumor regions (unique voxel or multivoxel) to detect certain ranges of specific metabolites in brain tissue, such as choline, creatinine, lipids, lactate, and N-acetyl-aspartate (NAA)^[22,23].

The analysis of these metabolites is helpful for distinguishing metastasis from necrosis, gliosis, and vasogenic edema [Table 1].

Creatine is the most stable metabolite in brain tissue, although it can be diminished in malignant primary tumors such as high-grade gliomas. It is present in both white and gray matter, which enables it to be used as an internal reference for the remainder of metabolites, which can change in the context of metastatic brain disease^[24,25].

Choline is a marker of cell change: it is a structural part of cell membrane phospholipids, with a greater presence in white matter than in gray matter. It is elevated where there are high-grade cell changes, and it has a relationship with creatine, such that both appear elevated, which helps orient a diagnosis of brain metastasis^[26].

Because lipids are a structural component of cell membranes, they appear elevated in the case of severe cell damage, even with necrosis. Lactate is a main metabolite associated with anaerobic metabolism, and necrosis is common in brain metastases^[26]. NAA is found at high concentrations in normal brain tissue, making it a marker for cell integrity and normal tissue structure; this marker appears at low concentrations in brain metastases^[25,26].

Spectroscopy may increase the accuracy of MRI in differentiating tumors from necrosis in patients with metastases who have been irradiated, using choline/NAA and choline/creatine ratios^[26]. According to a meta-analysis of 261 patients, it can also guide a clinician's therapeutic approach by adding information that could contribute to a differential diagnosis of primary malignant tumors such as high-grade gliomas; the study concluded that the most useful spectroscopy parameter is the choline-NAA ratio, preferably using a multi-voxel technique in the peritumoral region^[27]. However, in contrast, a meta-analysis of advanced MRI metrics that included 24 spectroscopy studies found no reliability of this technology in distinguishing high-grade gliomas from metastases using the choline-creatine ratio; the authors had excluded cases in which the choline-NAA ratio was used due to insufficient data^[28].

Hence, there is a lack of robust data supporting the routine use of MRI spectroscopy for differentiating metastases from high-grade gliomas, but it remains an important tool supporting the differential diagnosis of brain metastases from benign and low-grade gliomas and necrosis in irradiated patients^[29].

TREATMENT

The therapeutic approach to patients with brain metastasis is intended to relieve symptoms, such as headache, vomiting, and neurological focalization; the success of this largely depends on the presence of cerebral hypertension syndrome secondary to perilesional cerebral edema. Surgical management continues to be the standard of treatment, accompanied by radiotherapy using two different techniques: stereotactic radiosurgery (SRS) or whole-brain radiation therapy (WBRT) (or a combination of these)^[30].

Management of brain edema

The treatment of cerebral edema with systemic steroids such as dexamethasone has been practiced for more than five decades^[31]. Its efficacy is well known in terms of the clinical improvement of symptoms associated with vasogenic edema through its reduction of capillary permeability and regulation of the elevation of tight endothelial junctions involved in the physiology of the blood-brain barrier by binding to glucocorticoid receptors^[32-34].

In actuality, the pharmacological measures previously adopted for the management of edema, including osmotic diuretics such as mannitol and glycerol and loop diuretics such as furosemide, are no longer considered standard for the treatment of cerebral edema secondary to metastasis. Because osmotic diuretics cause a redistribution of fluid from the intracellular space to the extracellular space, they reach a plateau within a few hours, reducing their effectiveness^[35].

Even though there is not a defined dexamethasone scheme, the most utilized dose in patients with clinical cerebral edema is 4 to 8 mg/day^[33].

According to a recent joint update of the American Society of Clinical Oncology and the Society for Neuro-Oncology, they endorse a practice guideline regarding the role of steroids in brain tumors. The recommended dose depends on symptom severity with an initial dose of 8 mg in mildly symptomatic patients and 16 mg in severely symptomatic patients^[36].

Management of seizures

In patients who have not presented with seizures, the use of an antiepileptic agent in a prophylactic manner is currently not recommended, following a systematic review by Perry *et al*^[37]. In that study, no significant differences were found in reduction of risk of convulsive episodes in patients who were recently diagnosed with brain metastases and had never presented with seizures, between administration of the drug and only observation.

Table 2. RPA classes

Prognostic factors	Class I	Class II*	Class III
Age (years)	< 65	Any	Any
Controlled primary tumor	Yes	Any	Any
KPS	> 70	> 70	<7 0
Extracranial metastasis	No	Any	Any
Estimated survival (months)	7.1	4.2	2.3

Abbreviations: RPA: recursive partition analysis; KPS: Karnofsky performance score. *All patients not in class I or III.

By contrast, the use of anticonvulsants in patients with seizures, although it does not have an impact on overall survival, is useful for the relief and decrease of the number of convulsive episodes. The drugs used most often that have been proven to be effective and safe include levetiracetam, oxcarbazepine, and topiramate^[38,39].

INITIAL ASSESSMENT

A therapeutic approach must be preceded by an adequate assessment of the patient's condition, evaluating parameters such as functional status and extracranial disease so that the best available treatment may be offered, without compromising the oncological outcome or overtreating patients in whom poor survival may be anticipated and for whom support measures are more adequate.

PROGNOSTIC SCALES IN BRAIN METASTASES

At present, several useful prognostic scales are available for the clinical decision-making process, the first of which is recursive partition analysis (RPA by Gaspar *et al.*^[40]), developed in 1997. This scale was formed by the Radiation Therapy Oncology Group (RTOG) clinical trials that defined three prognostic classes according to four prognostic factors: performance according to the Karnofsky performance scale (KPS), control of the primary disease, presence or absence of extracerebral disease, and age greater or less than 65 years, dividing patients with brain metastases and their respective median survivals into three prognostic classes [Table 2].

Patients with a class III RPA are usually candidates for only supportive care, with local management performed either through surgery or radiotherapy for patients with classes I and II^[40,41].

The histological type of the primary tumor should also be considered an important prognostic factor; however, the previous scale does not take this into account. There is a more specific scale for primary disease that takes melanoma, gastrointestinal tumors, breast cell carcinoma, renal cell carcinoma and non-small-cell lung carcinoma into account. This scale is known as DS-GPA^[42,43].

The prognosis derived from these scales depends on various prognostic factors in relation to each histology, namely, age, KPS score, presence or absence of extracranial metastasis in the case of lung carcinoma, number of cerebral metastases in the case of lung carcinoma, melanoma, and renal cell carcinoma. Likewise, breast cancer, with its several molecular patterns that determine prognosis, is integrated into this scale [Table 3].

For more information on the estimation of global survival according to tumor type and characteristics, we recommend visiting a website made for this purpose: <http://brainmetgpa.com/>.

SURGICAL MANAGEMENT

Surgery plays an important role in the management of brain metastases, enabling a definitive histologic diagnosis in patients with no previously known history of cancer, allowing clinicians to alleviate the symptoms of intracranial hypertension (thus providing immediate relief to patients), and serving as a

Table 3. Disease-specific GPA

Disease-specific GPA						
Histology	Prognostic factors	Score				
		0	0.5	1	1.5	2
NSCLC/SCLC	AGE	> 60	50-60			< 50
	KPS	< 70	70-80			90-100
	ECM	YES				NO
	#BM	> 3	2-3			1
Score						
MELANOMA/RCC		0	1			2
	KPS	< 70	70-80			90-100
	#BM	> 3	2-3			1
Score						
BREAST		0	0.5	1	1.5	2
	KPS	< 60	60	70-80	90-100	
	ER/PR/HER2	Triple negative		ER/PR (+), HER2 (-)	ER/PR (-), HER2 (+)	Triple positive
	AGE	> 70	< 70			
Score						
GASTROINTESTINAL		0	1	2	3	4
	KPS	< 70	70	80	90	100

Abbreviations: GPA: graded prognostic index; NSCLC: non-small-cell lung carcinoma; SCLC: small-cell lung carcinoma; RCC: renal cell carcinoma; KPS: Karnofsky performance score; ECM: extracranial metastasis; #BM: number of brain metastases; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2

primary therapeutic approach. However, in recent years it has been displaced by advanced radiotherapy techniques such as SRS.

The American Society for Radiation Oncology recommends surgical resection in patients with an expected survival of at least 3 months, lesions larger than 3-4 cm, and who are amenable to safe, complete resection followed by WBRT or SRS to the cavity^[44].

Whole-brain radiotherapy

WBRT has been considered a mainstay treatment for brain metastases since the publication of Chao *et al.*^[45], who proposed irradiating the whole brain through two opposed lateral fields with the inferior margin of each field lying along the line running from the supraorbital ridge through the external auditory meatus to the foramen magnum; the other margins of the rectangular field project 2 cm beyond the forehead, vertex, and occiput onto bolus bags that surround the head. This was the first WBRT technique described using 250 kv X-rays in 38 patients with brain metastasis. The authors reported that 63% of the enrolled patients demonstrated reduced symptoms associated with brain metastasis, with a relief duration of 3-4 months^[45].

Dose and fractionation for WBRT

Dose and fractionation schemes are based, not on the radiation sensitivity of the primary tumor, but rather on the tolerance of healthy brain tissue as described in the QUANTEC report from 2010 (maximum dose [Dmax] of 60 Gy with an estimated rate of symptomatic brain necrosis of 3%)^[46]. Taking this into account and with a biologically equivalent dose (BED) with an α/β ratio of 3 for a normal brain, we cite the most used radiation schemes with their BED in Table 4.

Because the primary objective of this type of treatment is the palliation of symptoms, the most common prescription is 30 Gy in 10 fractions. This is based on the results of the first two randomized trials conducted by the RTOG, in which they compared four different radiation schemes including 3000 rad delivered in 2 weeks and 2000 rad in 1 week, and reported no differences in survival, time to progression, and symptom relief [Table 5]^[47].

Table 4. Most used radiation therapy schemes for WBRT

Dose and fractionation	BED (Gy)
30 Gy/2 weeks	60
20 Gy/1 week	46.67
37.5 Gy/3 weeks	68.75
40 Gy/4 weeks	66.67

Abbreviations: BED: Biologically equivalent dose. Gy: Gray

Table 5. Former RTOG dose and fractionation protocols for brain metastases

RTOG brain metastasis protocols			
First study		Second Study	
Scheme	# patients	Scheme	# patients
3000 rad/2 weeks	233	2000 rad/2 weeks	447
3000 rad/3 weeks	217		
4000 rad/3 weeks	233	3000 rad/2 weeks	228
4000 rad/4 weeks	227	4000 rad/3 weeks	227

Considerations that can be taken into account as the physician decides on one fractionation scheme over another are the patient's performance status, estimated survival, and histology of the primary tumor because choriocarcinoma, melanoma, and renal cell carcinoma, among other types, present a higher risk of bleeding^[43,48,49].

The shorter-course fractionation of 20 Gy in 1 week is preferable for most patients with poor performance status, to avoid unnecessary treatment time, as it has demonstrated similar survival benefits as longer treatment schemes^[50]. However, other fractionation schemes such as 37.5 Gy in 3 weeks is recommended in patients who have received a stereotactic radiosurgery boost with one metastatic lesion and should be considered in patients with one to three lesions^[51].

WBRT, unlike SRS, is associated with lower intracranial relapse, but when the whole brain is irradiated with this technique, it may also lead to greater cognitive deterioration (reflected as short-term memory loss), especially in patients with a longer life expectancy (> 6 months). In Aoyama *et al.*^[52], global survival did not significantly differ between treatment techniques (8.0 vs. 7.5 months) but there was a difference in the presentation of new metastases (63.7% vs. 41.5%) WBRT as shown [Figure 5](#).

Role of Radiosurgery in the treatment of brain metastases

SRS-based treatment began in 1951, with its implementation by Lars Leksell. It uses multiple rays of radiation, which converge three-dimensionally on a localized objective, either static or mobile, giving a high dose to a unique fraction with a high fall-off. This minimizes the damage to the adjacent tissue^[53].

Luckily, more than half of brain metastasis patients present with three or fewer lesions at diagnosis. It has been demonstrated that both surgical treatment and SRS lead to longer overall survival in these patients, especially for lone lesions smaller than 30 mm, where SRS has an overall survival comparable to microsurgery. However, it is important to take into account that although brain metastases tend not to invade more than a few millimeters of adjacent tissue, local recurrences are common after resection, meaning that adjuvant treatment with radiotherapy after surgery is imperative^[54]. To reduce cognitive impairment in such patients, the use of SRS has grown in use as an alternative to WBRT in the first 6 weeks following surgery, with the goal being to maintain local control in surgery and preserve neurocognitive functions without lowering quality of life^[52].

Clinical presentation with a single metastasis appears in only 10% to 20% of patients, where treatment with SRS following surgery improves both local recurrence rates and death due to neurological causes^[55,56].

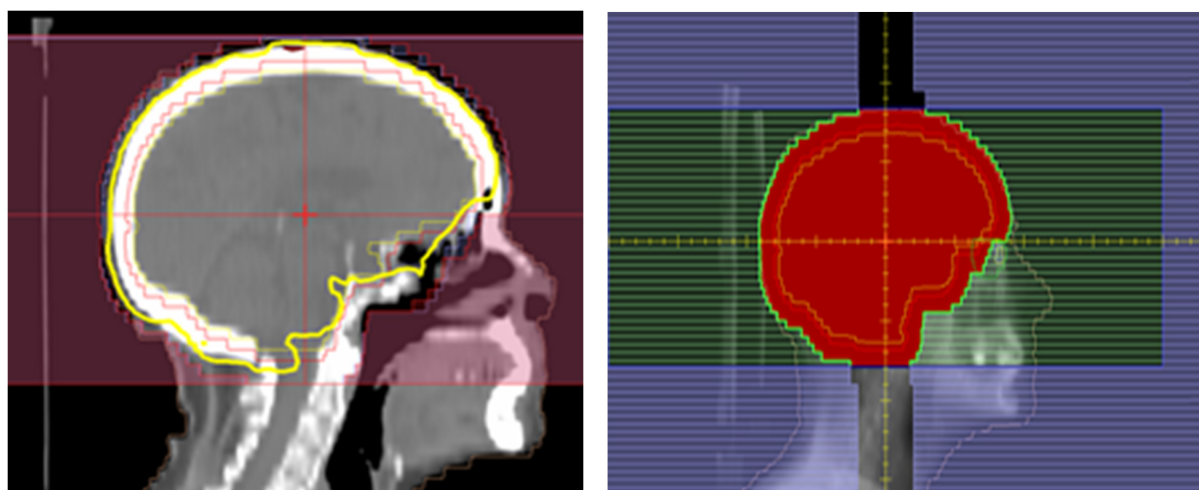


Figure 5. Whole-brain radiation therapy treatment plan using a 3D conformal technique with two opposite lateral fields

One of the first aleatorized studies employed SRS as a boost in patients with one to three brain metastases that had previously received treatment with WBRT, showing an improvement in functional status in all patients and an improvement in overall survival in patients with a single metastasis^[52,57].

The retrospective series published by Wang *et al.*^[58] in 2015 analyzed patients with brain metastases, comparing GammaKnife SRS alone, GammaKnife SRS with WBRT, surgery and SRS (as an adjuvant treatment to the surgical cavity), and a triple modality (surgery, SRS, and WBRT). For patients with a single metastasis and those with multiple lesions, the triple modality treatment was found to have greater positive effects on median survival than GammaKnife SRS alone. That study was not a prospective trial, and it also found better results for bimodal treatment than for GammaKnife surgery alone (as opposed to previous clinical trials). The authors concluded that WBRT is a good alternative as a rescue treatment for patients who had previously received SRS^[58,59].

SRS has broadened the terrain of the primary treatment of brain metastasis, especially in patients with good functional status and in those who have one to three metastases at diagnosis with limited extracranial disease^[58] [As shown in Figures 6 and 7]. Therefore, it is important to note that better global control of metastasis can be obtained with WBRT and SRS, which have an impact on local control and overall survival^[51]. Patients whose disease is more limited in extent (up to four metastases for SRS protocols) may be treated with WBRT and SRS or SRS alone without improvement in their overall quality of life. A worsening of cognitive performance is found in patients treated with WBRT plus SRS, which affects their quality of life and ultimately their overall survival. These results were found by the trials conducted by Brown *et al.*^[60] and Chang *et al.*^[61]. ASTRO and NCCN guidelines agree in recommending management with SRS in patients with a limited number of brain metastases.

The utility of SRS for patients with five or more brain metastases is unclear. The only prospective study that has evaluated patients with these characteristics was conducted by Yamamoto *et al.*^[62], who assessed 208 patients with 5 to 10 metastases, 531 patients with 2 to 4 metastases, and 455 patients with a single metastasis, with a maximum lesion diameter of 3 cm. The most important result was that the number of brain metastases did not affect overall survival, while the volume of intracranial tumors ranged from 0.02 to 13.9 cc, and the average survival for patients with 5 to 10 metastases was 10.8 months. Deaths from neurological causes did not exceed 10% and there were no significant differences among groups. Finally, it was concluded that the progression of systemic disease was the main cause of death, the initial number of

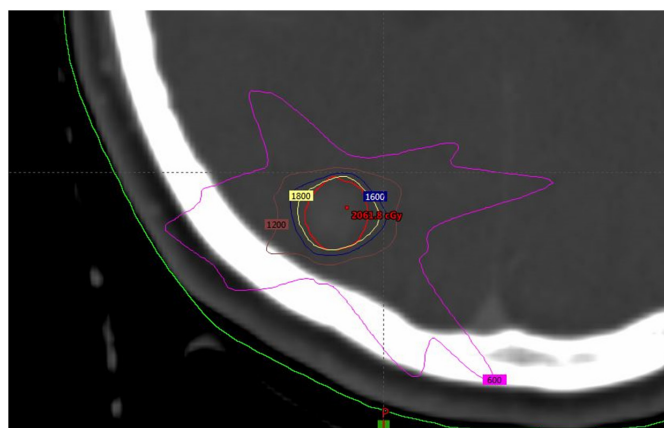


Figure 6. Treatment-planning dose for SRS of a single lesion, with the dose distribution for one target prescribing 20 Gy 95% with the following specification: isodose lines: red 20 Gy, yellow 18 Gy, blue 16 Gy, brown 12 Gy, pink 6 Gy. Software: Eclipse External Beam Planning system version 15.5 HD MLC

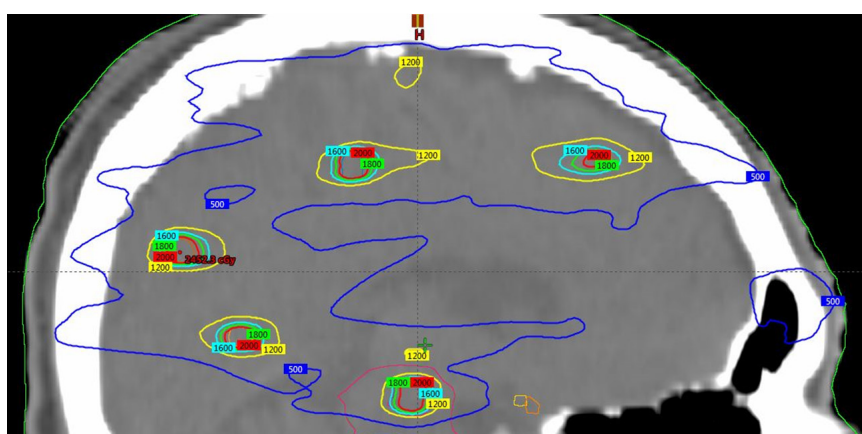


Figure 7. Multiple-target planning showing the dose distribution for multiple targets, prescribing 20 Gy 95% to each of them with the following specification: isodose lines: red 20 Gy, green 18 Gy, light blue 16 Gy, yellow 12 Gy, dark blue 6 Gy. Software: Eclipse External Beam Planning system version 15.5 HD MLC

metastases did not impact local control, and the rate of distant metastasis failure was lower in patients with a single metastasis, although this advantage seemed to be lost for those with two or more metastases^[62].

Because 50% of patients with brain metastases will present with new lesions, it is necessary to continue surveillance using serial MRI when only SRS is used, with the intention of identifying the progression of the disease and enabling early management to limit neurological deficits. Success in the control of new distant metastases varies from 22% to 90%, with different results reported by different studies. Many such studies have reported a correlation between the initial number of metastases and the progression of disease (according to Chang *et al.*^[61], the presence of 15 or more metastases is a risk factor). In addition to age, KPS, histology, and RPA, the total irradiated volume is also likely to impact overall survival, however, neither the number of metastases nor the total volume of treatment is yet considered a criterion for the selection of patients for treatment with SRS^[51].

Brown's and Chang's trial^[60,61] indicated that treatment with WBRT affects cognitive function in a significant way in all analyzed aspects, and opinions on the management of brain metastases converge on the use of SRS, even for multiple brain metastases, to avoid cognitive deficit^[55].

SRS TREATMENT DOSE

As described in the protocol RTOG 9005, treatment dose is inversely proportional to metastatic lesion size. The suggested dose is 24 Gy for tumors smaller than 20 mm, 18 Gy for tumors from 21 to 30 mm, and 15 Gy for tumors from 31 to 40 mm^[63]. It is not known whether the dose used for lesions smaller than 20 mm can be safely incremented above 27 Gy; hence, the general consensus still recommends a 24 Gy dose. However, because the organs at risk are so near, a single-dose treatment modality is associated with higher rates of toxicity, meaning that hypofractionated treatment plans are more appropriate for local control with acceptable toxicity^[64].

In 2014, Minniti published results of a study of hypofractionated SRS in which lesions under 20 mm received 36 Gy in three fractions, and lesions larger than 20 mm received 27 Gy in three fractions, resulting in 2 years of local control and an overall survival rate of 72% and 25%, respectively^[65]. In 2016, Navarra published the results of a similar study, administering a dose of 27 Gy in three fractions to lesions of 21 to 30 mm and a dose of 32 Gy in four fractions to lesions of 31 to 50 mm. The technique resulted in local control and an overall survival at 2 years of 96% and 33%, respectively^[66].

CONTOURING OF BRAIN METASTASES

Before a treatment plan is complete, an MRI with gadolinium contrast should be performed for fusion with axial CT. GTV is defined as gadolinium enhancement, identified in MRI with a 1 mm margin. A 1-3 mm margin is used for GTV in a geometrical form to obtain the total treatment volume (planning target volume)^[63].

SRS boost after WBRT

Several randomized trials that employed SRS as a boost after WBRT in newly detected patients with one to three surgically unresectable brain metastases have reported no added toxicity and improved performance in all patients and also an important benefit in the overall survival of patients with a single metastasis^[51,52,57]. In addition, a recent review of five studies involving 2,728 patients reported a survival benefit in all patients who received a combined modality based on prognostic criteria (RPA or DS-GPA) regardless of the number of brain metastases^[67].

Cavity radiation therapy

A panel of international experts has recommended the following to contour CTV in SRS for patients with completely resected brain metastases. CTV must include the entire surgical cavity showing enhancement of contrast, using axial images in the T1 sequence of brain MRI with gadolinium contrast, excluding edema as determined by MRI. CTV must include the surgical path shown in the MRI or CT. If the presurgical tumor is in contact with the dura mater, CTV must include a 5-10 mm margin, until overlap with bone structures is achieved. If the presurgical tumor is not in contact with the dura mater, the CTV must include a 1-5 mm margin, until contact with bone structures is achieved. If a presurgical tumor is in contact with the venous sinus, CTV must include a 1-5 mm margin along the sinus^[68].

Late toxicity implications: radiation cognitive syndrome

Radiation cognitive syndrome is a poorly understood entity, and currently there is no validated long-term treatment or prevention; 50%-90% of patients who receive WBRT exhibit disabling cognitive function including declines in learning capability, processing speed, memory, and attention. This undermines the patient's overall quality of life. Radiation is associated with inflammation, gliosis, demyelination, vascular abnormalities, and necrosis, which in turn may lead to such cognitive issues^[69].

Cognitive deterioration is usually reported 6 months to 1 year after WBRT, but may be seen as early as 1 month after treatment. In addition, cognitive decline tends to be progressive and irreversible. Many

radiation and pharmacologic approaches to address this syndrome are being studied, and while further data are needed for any one medical solution to become the standard of care, the results generated to date suggest a positive outlook for future treatment options^[70].

As new radiotherapy techniques have appeared, treatment plans have been developed that, in addition to tumor control, aim to reduce cognitive deterioration through the preservation of the hippocampus. Indeed, the RTOG 0933 trial^[71] demonstrated the preservation of cognitive function by reducing the dose to the hippocampus to not more than 9 Gy at 100% of the volume and a maximal hippocampal dose of 16 Gy. This was achieved by manually contouring the hippocampus on a fused MRI-CT image set and expanding by 5 mm to generate “hippocampal avoidance regions.” The mean relative cognitive decline from baseline to 4 months was 7.0%, significantly lower than in controls. The study demonstrated that conformal avoidance of the hippocampus during WBRT was associated with preservation of memory and quality of life. Nonetheless, this procedure poses a risk of generating new brain metastases within the avoidance regions. However, this risk is not fully quantified and further data are needed to validate this technique within a phase III setting.

CONCLUSION

Although brain metastasis is the most common malignant intracranial tumor, it is closely linked to unfavorable outcomes. Its incidence has increased dramatically, due to a greater number of newly diagnosed cancer patients and the broader therapeutic options available today, which have led to better disease control and longer overall survival. The majority of patients are not candidates for surgical resection, so radiotherapy remains the standard of care. The possibility of a cure for an oligometastatic disease has been gaining increasing attention in recent years. The management of these patients has changed immeasurably over the past few decades: not many years ago, the prognosis and survival of such patients was for a short life expectancy, with poor disease control. At present, there are several treatment options available. The choice among these modalities depends on several factors, such as the functional state of the patient and the availability of equipment and treatment techniques at the given medical center. Before the 1990s there was no GPA prognostic scale, much less an RPA, which are quite useful for decision making.

To date, no prospective studies have evaluated the use of SRS relative to WBRT for patients with more than four brain metastases. However, the current tendency in several hospitals around the world is to avoid WBRT, due to the toxicity and neurological deterioration attendant on that treatment, especially in developed countries. Consequently, there has been a shift to highly sophisticated techniques, such as SRS. A randomized phase III study is currently running at The Odette Cancer Center and the Princess Margaret Cancer Center (University of Toronto) in patients with 5 to 20 cerebral metastases who are receiving treatment with SRS without WBRT versus SRS plus WBRT, with the primary outcome being to compare neurocognitive decline between the approaches, as this is a common late side effect in patients receiving radiotherapy.

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Authors' contributions

All the authors contributed in an equitable way in the conception, bibliographic search and writing of this review article.

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Review

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Peptide nucleic acid-based targeting of microRNAs: possible therapeutic applications for glioblastoma

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Abstract

A large and incremental number of non-coding RNAs, including microRNAs (miRNAs) have been recently demonstrated to play a very important role in human pathologies, including cancer. Therefore, microRNAs have been proposed as therapeutic targets and molecules exhibiting anti-miRNA activity or mimicking functional miRNAs have been developed. Among biomolecules proposed in anti-miRNA therapy, peptide nucleic acids (PNAs) are appealing, in consideration of their stability and efficacy in recognizing RNA targets. PNAs against tumor associated miRNAs have proven to be efficient in inducing anti-tumor effects both *in vitro* and *in vivo*. For instance, PNAs targeting miR-155-5p are able to induce apoptosis in glioma cell lines and to enhance the sensitivity to temozolomide (TMZ) in TMZ resistant glioma cells. *In vivo*, PNAs anti-miR-21 were found to exhibit anti-tumor effects associated with improved survival when administered to animals with intracranial gliomas.

Keywords: Peptide nucleic acids, glioma, microRNAs, miRNA targeting, delivery, apoptosis, temozolomide

MICRORNAS IN ONCOLOGY

MicroRNAs (miRNAs) are a family of evolutionary conserved small (19 to 25 nucleotides in length) non-coding RNAs playing important roles in the post-transcriptional control of gene expression. This control is operated at the level of mRNA translation and is based on the miRNA-dependent recognition of 3'UTR, CDS and 5'UTR mRNA sequences. This molecular recognition leads to a reduction of protein synthesis^[1-4]. Single or multiple mRNAs can be targeted by a single miRNA, while a single mRNA can be recognized and



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functionally controlled by several miRNAs (eventually differentially expressed in cells of different histotype); following these considerations, it is calculated that more than 60% of human mRNAs can be considered molecular targets of miRNAs^[5,6]. It has been reported in different studies and reviews that the miRNA/mRNA interaction occurs at the level of the RNA-induced silencing complex (RISC), a ribonucleoprotein which incorporates one strand of a single-stranded RNA (in our case a microRNA), acting as a template to recognize complementary mRNA transcripts^[1-4]. This molecular interaction is associated to (1) repression of translation or (2) mRNA degradation, depending on the level of miRNA complementarity with nucleotide sequences of the target mRNA^[3,4]. Since their discovery and first characterization, the number of human microRNAs identified and deposited in the miRBase database (miRBase v.22, www.mirbase.org) is much more than 2600^[7-9] and the research studies on microRNAs have confirmed the very high complexity of the networks constituted by miRNAs and RNA targets^[7].

Changes of microRNA expression have been demonstrated to be associated with different human pathologies, and guided alterations of specific miRNAs have been suggested as novel approaches to develop innovative therapeutic protocols^[10-13]. Several reports conclusively demonstrated that microRNAs are deeply involved in tumor onset and progression, behaving as tumor promoting miRNAs (oncomiRNA and metastamiRNAs) as well as tumor suppressor miRNAs^[14-19]. In general, a miRNA able to promote cancer targets mRNA coding for tumor-suppressor proteins, while microRNAs exhibiting tumor-suppression properties usually target mRNAs coding oncoproteins^[15].

Targeting oncomiRNAs and mimicking tumor-suppressor miRNAs: overcoming drug resistance

With respect to targeting oncogenic RNAs and mimicking tumor-suppressor miRNAs in translational medicine, it should be underlined that these non-coding RNAs are suitable targets for therapeutic interventions, as summarized in Figure 1^[10-13]. The use of modified miRNA mimetics, either synthetic or produced by plasmid or lentiviral vectors, might lead to potentiation of miRNA functions (miRNA replacement therapy)^[20-24]. In this case the miRNA replacement molecules (mimicking the miRNA functions to be up-regulated) are transfected to target cells [Figure 1A, step “a”] where interact with the mRNA to be modulated [Figure 1A, step “b”]. This interaction leads to down-regulation of this mRNA and associated suppression of protein production [Figure 1A, step “c”, dotted arrows]. According to this procedure, it is possible to mimic the activity of tumor-suppressor miRNAs (down-regulated in tumors) to achieve down-regulation of miRNA-regulated oncogenes^[23,24].

On the contrary, forced down-regulation of miRNA biological functions can be obtained using a large variety of well-characterized miRNA-inhibitor oligomers (such as in the case of direct miRNA antisense therapy based on RNA, DNA, LNA and other DNA analogues)^[25-31], miRNA sponges^[32-38], mowers^[39] or through miRNA masking strategy that interferes with miRNA function by masking the miRNA binding site of target mRNA through hybridization with complementary molecules^[40-42] [see Table 1]. In this case the miRNA inhibitors [“a”, “b” and “c” of Figure 1B, suppressing the miRNA functions to be down-regulated] are transfected to target cells where they interact with the microRNA target [Figure 1B, step “d”]. This interaction prevents the target miRNA (for instance an oncomiRNA in protocols designed to develop anti-tumor therapy) to bind the specific 3'UTR sequence of the regulated mRNA, causing up-regulation of this mRNA and the associated increase of protein production [Figure 1B, step “e”]. According to this procedure, it is possible to inhibit the activity of oncomiRNAs (up-regulated in tumors) to achieve up-regulation of miRNA-regulated tumor suppressor genes.

Interestingly, in the case of the development of anti-cancer protocols, targeting miRNAs in drug-resistant tumor cell lines has been associated with partial or total reversion of the drug-resistance phenotype, as reported in different studies^[43-48].

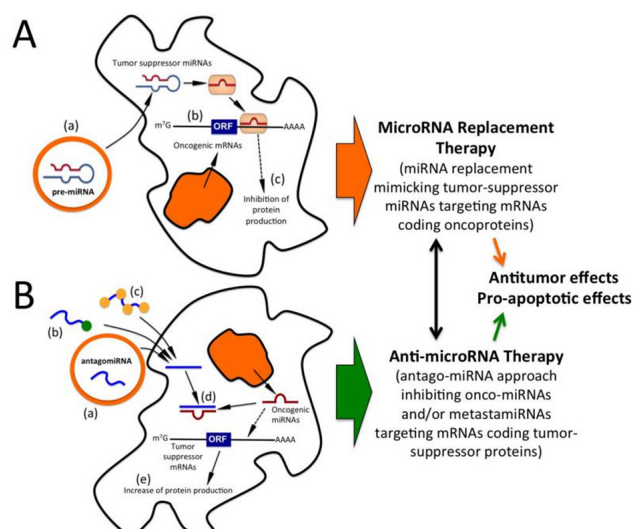


Figure 1. Scheme summarizing the miRNA replacement (A) and anti-miRNA (B) approaches to modify miRNA-regulated gene expression. In panel A the miRNA replacement molecule is transfected to target cells (a) where it interacts with the mRNA to be modulated (b) causing inhibition of protein production (c, dotted arrow). In panel B the miRNA inhibitors (a-c) are transfected to target cells (d) where they interact with the microRNA target preventing its binding to the specific 3'UTR sequence (dotted arrow) of the regulated mRNA (d). This causes up-regulation of this mRNA with increased protein production (e). Three examples of antagomiRNA molecules are shown: microparticle delivered antagomiRNAs (a), peptide-delivered molecules (b, peptide in green), or chemically-modified molecules (chemical modifications in yellow) to increase biological functions (for instance resistance to enzymatic degradation or delivery efficiency to target cells)

Table 1. Experimental strategies for inhibition of microRNA functions

Strategy	Bioactive molecules	Mechanism(s) of action	Biological effects	References
Use of microRNA inhibiting molecules	RNA, DNA, LNA and other DNA analogues, PNAs and PNA analogues	Sequence-specific hybridization to miRNA targets	Up-regulation of the expression of miRNA-regulated mRNAs	Weiler <i>et al.</i> ^[25] , 2006; Lu <i>et al.</i> ^[26] , 2009; Lennox <i>et al.</i> ^[27] , 2011; Obad <i>et al.</i> ^[28] , 2011; Elmen <i>et al.</i> ^[29] , 2008; Stenvang <i>et al.</i> ^[30] , 2008; Staedel <i>et al.</i> ^[31] , 2015
Use of miRNA sponges	Circular RNAs (circRNAs) and long-non-coding RNAs (lncRNAs)	Inhibition of miRNAs by circRNA-miRNA or lncRNA-miRNA interactions	Up-regulation of the expression of mRNAs regulated by sponged miRNAs	Ebert <i>et al.</i> ^[32] , 2007; Ebert <i>et al.</i> ^[33] , 2010; Kluiver <i>et al.</i> ^[34] , 2012; Kluiver <i>et al.</i> ^[35] , 2012; de Melo <i>et al.</i> ^[37] , 2015; Tay <i>et al.</i> ^[38] , 2015
Use of mowers	Synthetic devices containing multiple bulged miRNA binding sites and named them "miRNA-mowers"	"Mowing down" miRNA expression (just like a lawn mower)	Up-regulation of the expression of mRNAs regulated by the "mowed down" miRNAs	Liu <i>et al.</i> ^[39] , 2012
MirNA masking	DNA, LNA, PNAs and analogues	Binding to mRNA and interference with the binding of miRNA to its target site	Up-regulation of the expression of "masked" mRNAs by inhibition of miRNA binding	Wang <i>et al.</i> ^[40] , 2011; Bak <i>et al.</i> ^[41] , 2013; Murakami <i>et al.</i> ^[42] , 2014

PEPTIDE-NUCLEIC ACIDS

Peptide Nucleic Acids (PNAs) are DNA analogues described for the first time by Nielsen *et al.*^[49], in which the sugar-phosphate backbone has been replaced by N-(2-aminoethyl)glycine units^[50-53] [see Figure 2 for the chemical general structure]. PNAs are very interesting molecules for sequence-specific alteration of gene expression, since are capable of forming Watson-Crick double helices after efficient sequence-specific hybridization with complementary DNA and RNA^[54]. Furthermore, they are able to generate triple helix with double-stranded DNA and to perform strand invasion^[55]. In virtue of these biological activities, PNAs have

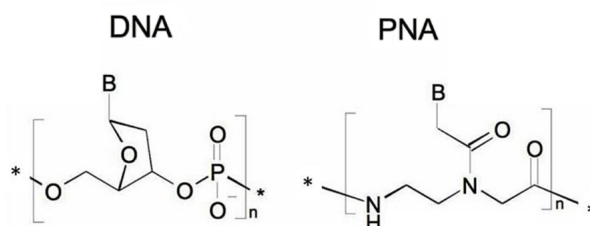


Figure 2. Scheme outlining the differences between DNA (left) and PNA (right) monomers

been demonstrated to be very efficient tools for pharmacologically-mediated alteration of gene expression, both *in vitro* and *in vivo*^[56,57].

Summarizing, PNAs and PNA-based analogues were employed as antisense molecules targeting mRNAs, triple-helix forming molecules targeting eukaryotic gene promoters, artificial promoters, decoy molecules targeting transcription factors^[54-56]. Relevant in the context of the present review article, PNAs have been demonstrated to be able of altering miRNA functions, both *in vitro* and *in vivo*^[58-62]. This has been recently reviewed by Manicardi *et al*^[63].

While well-controlled studies are needed to compare the *in vivo* activity of PNAs to those of LNA, RNA and other molecules exhibiting anti-sense potential, several *in vivo* studies on PNAs have been already reported to sustain the usefulness of these molecules and their derivatives.

Gupta *et al.*^[64] tried to target miR-210 with an antisense γ -peptide nucleic acids (γ PNAs), exhibiting superior RNA-binding affinity, improved solubility, and favorable biocompatibility. The rationale for this approach is that miR-210 is overexpressed in hypoxic cancer cells and is a key player for the adaptation of tumor cells to hypoxia. For cellular delivery, they encapsulated the γ PNAs in poly(lactic-co-glycolic acid) (PLGA) nanoparticles. The results obtained show that γ PNAs targeting miR-210 cause significant delay in growth of a human tumor xenograft in mice compared to conventional PNAs. Furthermore, histopathological analyses show considerable necrosis, fibrosis, and reduced cell proliferation in γ PNA-treated tumors compared to controls^[64]. In another paper, Cheng *et al.*^[11] efficiently inhibited the function of oncomiRNA miR-155 in a tumor mouse model after the design and realization of a peptide-(anti-miRNA)PNA construct able to target the tumor microenvironment and to transport the anti-miRNA PNA across the cellular plasma membranes under the acidic conditions which characterize solid tumors. A final example is that published by Yan *et al.*^[65], demonstrating that PNA-mediated targeting of miR-21 causes inhibition of growth and migration of breast cancer MCF-7 and MDA-MB-231 cells *in vitro*, and tumor growth *in vivo* when nude mice were employed.

GLIOBLASTOMA

Glioblastoma multiforme (GBM), a grade IV glioma, is a lethal malignant tumor accounting for 42% of the central nervous system tumors, the median survival being 12-15 months^[66-71]. The current standard therapeutic management of GBM is based on neurosurgery followed by chemoradiotherapy by fractionated external-beam radiotherapy and systemic chemotherapeutic treatment with temozolomide (TMZ) and other agents, including repurposed drugs (such as metformin, disulfiram, rapamycin and derivatives, chloroquine, ionidamine)^[71]. There are only very limited possibilities for the treatment of subsequent recurrences, generally with minimal clinical efficacy^[69]. Among novel therapeutic strategies for GBM, of interest are inhibitors of aberrantly activated cell signaling pathways, including those regulated by growth factors and their receptors, such as epidermal growth factor family and their receptors^[72], platelet-derived growth factors and their receptors^[73]. In addition, innovative targets for GBM experimental therapy might

be insulin-like growth factors, fibroblast growth factor and their receptors^[74,75]. Excellent review articles describing current therapeutic approaches and novel trends in GBM management are available^[69,76-82].

In conclusion, since no curative treatment is available and the most used first-line drug, temozolomide (TMZ), is only able to cause an increase of the life expectancy^[69], new drugs are urgently needed for the implementation of therapeutic protocols for anti-glioma treatments. Moreover, a high proportion of gliomas become with time TMZ-resistant. Therefore, a deep interest does exist for combined treatments on TMZ-resistant glioma cells in order to induce therapeutic relevant response, including, but not limited to, apoptosis^[81,82].

MICRORNAS IN GLIOMAS: VARIABILITY AMONG THE PATIENTS HAMPERS THE IDENTIFICATION OF POSSIBLE THERAPEUTIC TARGETS FOR PERSONALIZED TREATMENTS

Several studies available from the recent literature clearly support the involvement of microRNAs in gliomas^[83-93], outlining a large number of miRNAs demonstrated to be dysregulated in these tumors. Of course, these studies might indicate miRNA targets to be proposed for the development of protocols for therapeutic intervention in glioma, including strategies useful to tackle the issue of drug resistance^[94-96]. Comprehensive analysis of microRNA expression profile in malignant glioma tissues has been reported by Piwecka *et al.*^[97], Banelli *et al.*^[98], Ciafrè *et al.*^[99]. The analysis of microRNAs is also associated with tumor onset and progression. For instance a miRNA signature was found associated with glioblastoma tumor tissues. Up-regulated miRNAs were miR-221, miR-222, miR-22, miR-296-3p, miR-195, miR-155, miR-152, miR-132, miR-146b-5p, miR-149, miR-129-3p, miR-34a, miR-671-5p, miR-10a. Down-regulated miRNAs were let-7b, miR-767-5p, miR-505, miR-301b, miR-181a, miR-20a, miR-19a, miR-19b, miR-106a^[97-99]. In any case, a large consensus does exist on the fact that tumor tissues are highly heterogeneous with respect to molecular and genetic features, supporting the concept that GBM represents a heterogeneous type of neoplasm when the microRNA patterns are considered^[100,101].

However, due to global high-throughput profiling it is possible to select miRNAs that are at high risk of being deregulated in the majority of patients. Thus, there is a considerable hope for utilizing miRNAs as targets in prospects of glioma therapy. A partial list of validated miRNAs dysregulated in gliomas (with their proposed mRNA targets) is reported in [Supplementary Table S1](#).

Among the possible microRNA targets involved in glioma, miR-155-5p appears to be of relevant interest for the following reasons: (1) miR-155-5p may play an important role in the transformation of normal neural stem cell toward glioma stem cells^[102]; (2) the elevated expression level of miR-155-5p promotes the proliferation and invasion of glioblastoma cells through suppressing GABA receptors^[103], FOXO3a^[104] and MXI1^[105]; (3) an oligonucleotide targeting miR-155-5p was shown to sensitize glioma cells to taxol-induced apoptotic death^[106]; and (4) miR-155-5p overexpression is considered as a major molecular feature of glioblastoma, which can discriminate this malignancy from a similar intracranial tumor, oligodendroglioma^[107]. Therefore, miR-155-5p alone or together with other miRNAs is a predictive biomarker for glioma prognosis^[107,108]. On the other hand, glioma-targeting therapy based on miR-155-5p anti-oligonucleotides is believed to be efficient to inhibit the progression of glioma^[109].

However, several recent studies have pointed out that the translation of the analysis of the expression and content of miR-155-5p and other miRNAs in gliomas into therapeutic strategy is hampered by the high variability of microRNAs among patients^[92,100,101]. For instance, in analyzing 31 tumor specimens against paired normal tissue, Yan *et al.*^[92] found a significant upregulation of miR-155 in tumor tissues. This study was based on the analysis of surgically dissected glioma specimens and their surrounding brain tissues. In spite of the increased miR-155 expression, a large proportion of tumor samples exhibited a nearly normal

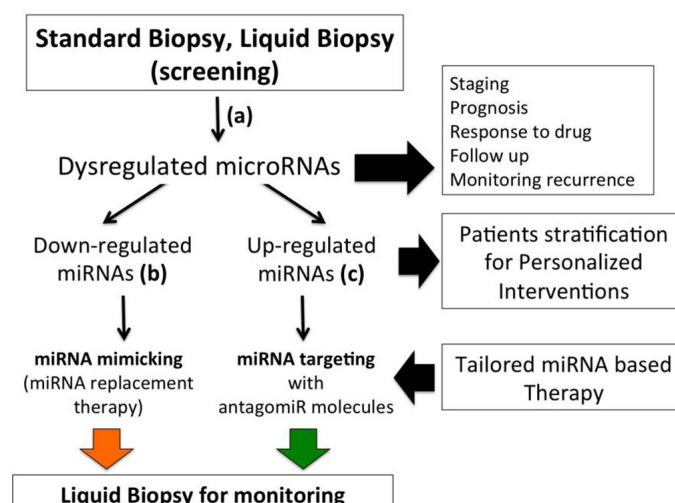


Figure 3. Scheme outlining the strategy to identify dysregulated microRNAs (a) in tumor patients and design possible therapeutic approaches based on anti-miRNA therapy and miRNA-replacement. The activity of down-regulated miRNA might be replaced with the use of miRNA mimicking molecules (b); the activity of up-regulated miRNAs might be counteracted with antagomiRNA molecules (c). A partial list of dysregulated miRNAs in gliomas is depicted in Supplementary Table S1

level of miR-155, supporting the concept that characterization of the GBM patients is required before the activation of a miRNA-155 targeting approach. Therefore, the analysis of the miRNA pattern appears to be a required step in the road of personalized therapy on precision medicine based on miRNA therapeutics.

Figure 3 indicates an overall strategy for determining miRNA targets in cancer patients, on the road of personalized therapy in precision oncology. The first step (step “a”) is the characterization of the miRNA profile in tumor tissues, performed with either surgery-based biopsy or liquid biopsy. The characterization of dysregulated miRNAs (down-regulated, “b” and up-regulated, “c”) will be helpful for staging, prognosis of the neoplasia as well as for determining the response of the patients to drugs. In addition, the analysis of dysregulated miRNAs might allow stratifying patients with respect to expression of specific miRNAs, with the final objective to propose a therapeutic intervention (either based on anti-miRNA or miRNA-replacement approach, as outlined in Figure 1). Liquid biopsy will be the best choice to monitor the outcome of this tailored therapeutic approach^[110].

PEPTIDE NUCLEIC ACID-MEDIATED TARGETING OF MICRORNAS IN GBM CELL LINES

The studies focusing on the possible use of PNAs targeting microRNAs are few. The first report was published by Brognara *et al.*^[62], who found that a PNA targeting miR-221 (R8-PNA-a221), bearing an oligoarginine peptide (R8) enabling efficient uptake by glioma cells^[58,59,62], strongly inhibited miR-221-3p in U251, U373 and T98G glioma cells. This inhibition of miR-221-3p activity was associated with increased expression of the miR-221 target p27Kip1, analyzed by RT-qPCR and by Western blotting^[62,66]. In a second study, Bertucci *et al.*^[111] reported the efficient combined delivery of temozolomide and the same anti-miR221 PNA using mesoporous silica nanoparticles^[111]. More recently, high levels of apoptosis on glioma cell lines were obtained following co-treatment with two PNAs, one targeting miR-221-3p, the other targeting miR-222-3p. In addition, Seo *et al.*^[113] showed the use of a PNA targeting miR-21 as *in vivo* inhibitor of glioma U87 cells.

A further example is a PNA against miR-155-5p. The possibility of obtaining clinically-relevant effects following targeting miR-155-5p with PNA-based molecules is shown in Figure 4 and elsewhere reported in the study by Milani *et al.*^[109], describing the synthesis and validation of a PNA targeting miR-155-5p on the temozolomide-resistant T98G glioma cell line^[66].

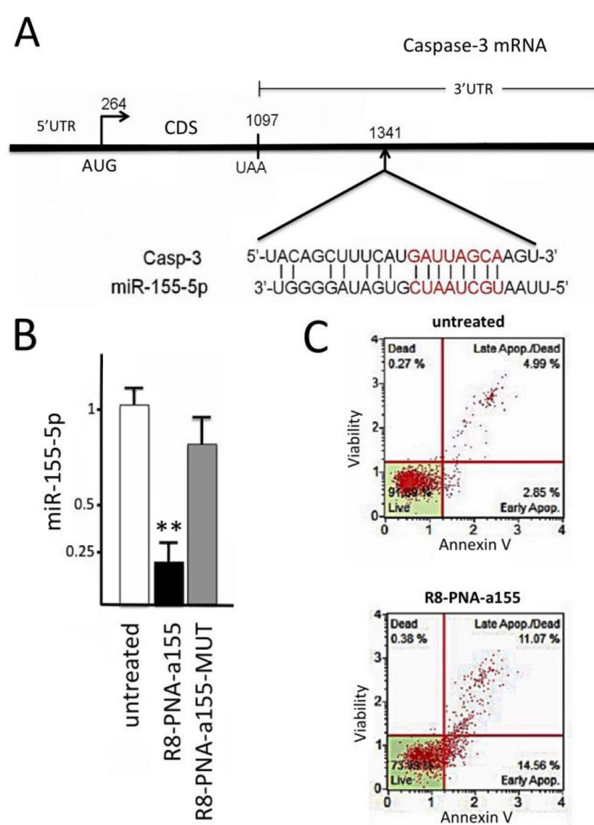


Figure 4. A. Location of a miR-155-5p binding site within the 3'UTR sequence of Caspase-3 mRNA. B. Effects of cell treatment with 4 μ M R8-PNA-a155 and R8-PNA-a155-MUT for 48 h on the miR-155-5p hybridization signal. C. Increase of T98G apoptotic cells after treatment for 48 h with 4 μ M R8-PNA-a155. Modified from Milani *et al.*^[109]

For efficient delivery, the PNA was conjugated with an octoarginine tail (R8-PNA-a155). Apoptosis was analyzed, and the effect of this PNA was associated with a reversion of drug-resistance phenotype. The specificity of the PNA effects at the cellular level was analyzed by RT-qPCR [see Figure 4B], suggesting that the effects of R8-PNA-a155 are specific. The studies on apoptosis [Figure 4C] confirmed that the R8-PNA-a155 demonstrated the pro-apoptotic effects, inducing apoptosis of TMZ-treated T98G cells.

FINAL REMARKS ON MIRNA THERAPEUTICS BASED ON PNA MOLECULES: FROM THE PAST TO FUTURE THERAPEUTIC APPLICATIONS

The data available on the recent literature support the concept that the anti-miRNA strategy [see Figure 1] could lead to therapeutic relevant inhibition of miRNA dependent effects and that PNA-based anti-miRNA molecules are very promising reagents to regulate tumor cell growth. Further research on PNA analogues to increase efficiency of delivery, stability and control of intracellular distribution for specific targets, i.e., mature miRNA, pre-miRNA or pri-miRNA, are further steps for the selection of best candidate drugs. Finally, the studies on miRNA targeting strongly indicate that multiple miRNA targeting, might lead to significant improvement in the efficacy of the treatment. This last conclusion supports also the concept of designing multifunctional PNA-containing systems enabling to perform targeting of different mRNA sequences.

Considering PNAs as anti-miRNA reagents, it should be underlined that one of the most important challenges in PNAs technology is their delivery to cells^[114,115], in particular their low uptake by eukaryotic cells. In order to solve this drawback, several approaches have been considered. One of the several approaches

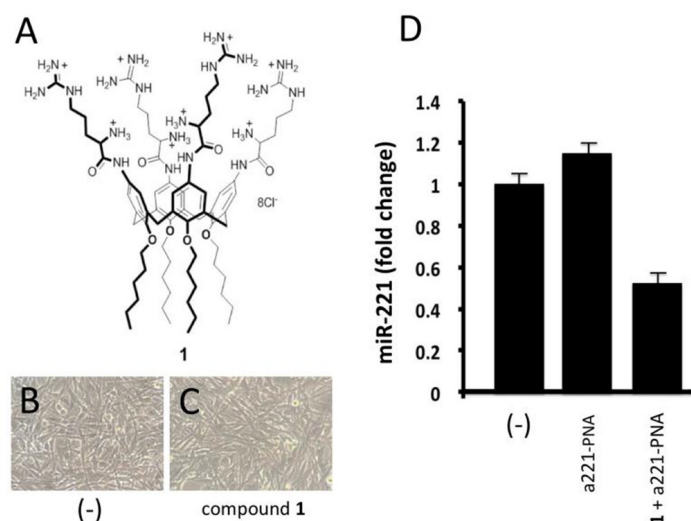


Figure 5. A: Structure of the macrocyclic multivalent tetraargininocalix[4]arene **1** used as non-covalent vector for anti-miRNA PNAs; B,C: Effects of compound **1** on morphology of U251 glioma cells: lack of cytotoxic effects. D. Effects of free PNA (a221-PNA) and a221-PNA delivered with compound **1** on miR-221-3p in treated U251 cells. Inhibition of miR-221-3p expression is obtained only when a221-PNA is vehiculated by compound **1**. Modified from Gasparello *et al.*^[125]

undertaken to solve this issue is the conjugation with carrier peptides^[116-118], in particular those sensitive to microenvironment changes^[11]; anti-miRNA activity was indeed observed for instance by conjugation of PNAs to polyarginine (poly-R) tails^[58,59,62] or by modification of the PNA backbone with cationic amino acid side chains^[58,119]. An alternative strategy to chemical modification of PNAs is the use of carriers able to perform non-covalent and reversible interactions with the PNA structure.

In this context, it was actually already explored the delivery of PNAs and PNA derivatives or analogues with liposomes^[120], polymer nanoparticles^[121] and pseudovirions^[122], and by co-transfection with partially complementary DNA^[123]. Inorganic nanocarriers, such as nanozeolites^[124] or mesoporous silica nanoparticles^[111] have been also used for cellular delivery of PNAs, maintain their biological functions. Recently, we have reported results relative to the delivery ability of a macrocyclic multivalent tetraargininocalix^[4]arene “**1**” [Figure 5].

In conclusion, several studies demonstrate that efficiently delivered PNAs might be of great interest in the inhibition of miRNA activity. This opens new and still unexplored avenues to non-viral gene therapy, especially when PNA-based strategies to target multiple miRNA sequences will be available^[126]. In consideration of the high patient-to-patient variability of the miRNome, multiple miRNA targeting should be considered a key feature in the road of personalized therapy in precision medicine.

As far as delivered PNAs *in vivo* to experimental models of GBM, local delivery of nanoparticles should be considered a promising therapeutic strategy that bypasses the blood-brain barrier, minimizes systemic toxicity, and enhances intracranial drug distribution and retention. In this respect, Seo *et al.*^[113] developed nanoparticles loaded with PNAs inhibiting miR-21, a microRNA overexpressed in GBM and retaining oncogenic features. These authors employed a block copolymer of poly(lactic acid) and hyperbranched polyglycerol to deliver an anti-miR-21 PNA, showing that efficient intracellular delivery was facilitated, leading to miR-21 suppression and PTEN upregulation and apoptosis of human GBM cells. This anti-miR-21 PNA was also administered by convection-enhanced delivery to animals with intracranial gliomas, inducing significant miR-21 knockdown and chemosensitization, resulting in improved survival when combined with chemotherapy^[113]. This study demonstrates the feasibility and promise of local administration

of nanoparticles carrying an anti-miR-21 PNAs for the development of protocols for adjuvant therapy for GBM. Interestingly, the delivered PNAs were found to cause long-term survival in treated mice at a level much higher with respect to antisense RNAs.

DECLARATIONS

Authors' contributions

Revised and approved the final manuscript: Gambari R, Gasparello J, Finotti A

Wrote the manuscript, performed the literature search, and critically analyzed the existing literature: Gambari R, Gasparello J, Finotti A

Designed the figures and created the tables: Gambari R, Finotti A

Availability of data and materials

Not Applicable.

Financial support and sponsorship

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Original article

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Determination of cytokine regulated glycan expression by using molecularly imprinted polymers targeting sialic acid

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Abstract

Cancer cells often have an increased amount of glycans, such as sialic acid (SA), on the cell surface, which normally play an important role in cell growth, proliferation and differentiation. In this study, SA expression is determined by fluorescent nanoprobe, molecularly imprinted polymers, SA-MIPs. The nanoprobe is synthesized with an imprinting approach to produce tailor-made fluorescent core-shell particles with high affinity for cell surface SA. Inflammation and cytokine production are well known tumor promoters, modulating the cellular microenvironment, including an aberrant cell surface glycan pattern. The recombinant cytokines IL-4, IL-6, IL-8 and a cocktail of cytokines collected from stimulated T leukemia Jurkat cells were used to induce *in vitro* inflammation in two cell lines, and thereafter analyzed with the use of SA-MIPs and flow cytometry. One of the cell lines showed a different binding pattern of SA-MIPs after treatment with recombinant cytokines and the cytokine cocktail. This study shows that SA-MIPs can be an important tool in the investigation of overexpressed glycans in the tumor microenvironment.

Keywords: Cancer, cytokine, glycans, molecularly imprinted polymers, sialic acid

INTRODUCTION

Abnormal cell growth can be initiated through inflammation^[1]. Cancer cells are able to reshape the microenvironment by expression of tumor-promoting chemokines and cytokines^[2,3]. Fast growing cancer



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cells outpace their blood supply and become nutrient and oxygen deprived. This results in necrotic cell death at the tumor's core and this releases pro- inflammatory cytokines, such as IL-1.

Cytokines act as cell regulators of many different biological processes including cell growth, differentiation, metabolism, immunity and inflammation. Cytokines can enhance cancer cell growth by modulating the cellular microenvironment or by affecting the cells directly. Pro- inflammatory cytokines can be oncogenic and thereby inducing elevated levels of pro- invasive factors such as metalloproteinase-2 (MMP-2) and epithelial growth factor.

Pro-inflammatory cytokines including TNF- α , IL-1 β , IL-6 and IL-8 are regulated by the transcription factor NF- κ B, which is involved in activating genes in neoplastic transformation^[1,4]. Highly glycosylated intestinal mucins, such as MUC2 and MUC4, are regulated through NF- κ B and by the gp130/STAT3 pathways, respectively^[5,6].

Sialic acid (SA), or neuraminic acid, is the outermost sugar molecule of glycans, thus forming the outer surface of cells by being attached to proteins or lipids bound to the plasma membrane. SA is very important for the function of living organisms since it is involved in cell processes like proliferation, differentiation, angiogenesis, invasiveness and metastasis^[7,8]. The over-expression of SA creates a negative charge on the cell surface, which is important in cell-cell and cell-matrix communication. An increased level of SA on the cell membrane, which have been reported on malignant and metastatic cancer cells, makes cells repel each other leading to an increased motility^[9]. In addition, it has been proven that aberrant expression of in particular the α 2,3-SA variants are related to tumor adhesion and invasion^[10,11].

Today, there is a lack of tools for specific targeting to glycans with high affinity. Lectins and glycan-specific antibodies have been used by many research groups to detect altered glycosylation, but the current tools do not perform with high specificity or affinity^[12]. We have developed fluorescent nanoprobes to make convenient targeting and imaging of cell surface SA possible^[13]. Based on other glycan specific receptors, we have developed SA-imprinted molecularly polymers (SA-MIPs) by using silica core particles and implemented NBD-fluorophores, which have favorable spectroscopic properties^[13,14].

In this study, two different cancer cell lines, MCF-7 and RAW 264.7, were stimulated with recombinant IL-4, IL-6, IL-8 and a cocktail of cytokines obtained by stimulating Jurkat T leukemia cells with phytohemagglutinine (PHA). The resulting expression of SA on the membrane of the stimulated cancer cells was analyzed with flow cytometry using both lectins and the SA-MIPs. One of the cell lines showed an increased binding of the SA-MIPs after treatment with recombinant cytokines and with the cytokine cocktail from PHA-stimulated Jurkat cells.

MATERIAL AND METHODS

Cell culture

The cell lines MCF-7 (ATCC HTB-22), RAW 264.7 (ATCCTIB-71) and Jurkat (ATCCTIB-152) were obtained from the American Type Culture Collection (ATCC/LGC Standards, Teddington, UK). MCF-7 and Jurkat cells were cultured in RPMI-1640 medium (Invitrogen, San Diego, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 50 μ g/mL gentamycin (Invitrogen) (complete medium). RAW 264.7 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and penicillin-streptomycin (Invitrogen). The cell lines were incubated in 37 °C with 5% CO₂ in 100 % humidity.

Stimulation of Jurkat cells

5 \times 10⁶ Jurkat cells in 5 mL of were stimulated in complete medium with the addition of 10 μ g/mL of PHA (Sigma Aldrich, St Louis, USA) for 72 h. The cells were harvested and centrifuged at 300 xg for 10 min. The supernatant was kept at 4 °C until used for cell experiments.

Addition of recombinant cytokines

2×10^6 of each of MCF-7 and RAW 264.7 cells were seeded in T25 flasks. Next day, the medium was removed and the different recombinant cytokines IL-4, IL-6, IL-8 (Peprotech, Rocky Hill, NJ, USA), all added at a final concentration of 40 ng/mL, or undiluted PHA- stimulated Jurkat supernatant was added to the cell culture flasks. One flask served as control with cells and medium alone. The cells were harvested after 3 days of incubation by trypsinization, two washes with phosphate buffered saline (PBS, Invitrogen) and thereafter stained with SA-MIPs according to below.

Preparation of polymers

The polymers (SA-MIPs) were prepared as described previously by Shinde *et al*^[13]. Before the SA-MIPs were used for cell-based experiments, the dried SA-MIPs were resuspended in PBS and sonicated for 8 min with a VWR sonicator. The stock solution of 1 mg/mL was further sonicated and diluted in PBS prior to use.

Flow cytometry analysis

SA-MIPs

1×10^6 cells/sample were stained with SA-MIPs or left unstained as a control. The cells were washed twice with 2 mL PBS and thereafter 100 μ L of SA-MIPs with indicated concentration was added to the cells. 100 μ L of PBS was used as a negative control. The cells were incubated with SA-MIP for 30 min at 4 °C in the dark, and were thereafter washed three times with 2 mL PBS and analyzed using flow cytometry (BD Biosciences, Accuri C6 Flow Cytometry, NJ).

Lectins conjugated with biotin

1×10^6 cells/sample were stained with biotin-conjugated lectins (Vector Labs, Burlingame, CA, USA) or left unstained as a control. The cells were washed twice with 2 mL PBS. 100 μ L of biotin-conjugated lectins, MAL I (from *Maackia amurensis*) or SNA (from *Sambucus nigra*) at concentrations of 10 μ g/mL was added to the cells and 100 μ L of PBS was used as a negative control. The cells were incubated with biotin- conjugated lectins for 30 min on 4 °C and were thereafter washed three times with 2 mL PBS. Thereafter 100 μ L of a 1:100 dilution of streptavidin-FITC (Agilent Technologies, Santa Clara, CA, USA) was added to the cells and incubated for 20 min at 4 °C in the dark. After the incubation, the cells were washed three times with 2 mL PBS and analyzed using flow cytometry (Accuri C6 Flow Cytometry).

ELISAs for IL-2, IL-6, IL-8 and TNF- α

96-well ELISA plates were coated overnight at room temperature with 100 μ L/well of diluted capture antibody against IL-2, IL-6, IL-8 or TNF- α , respectively, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The plates were washed three times with PBS-Tween (PBS-T), blocked by adding 300 μ L/well of blocking buffer (1% BSA in PBS) and thereafter incubated at room temperature (RT) for 1 hr. After three washes with PBS-T, the plates were incubated with 100 μ L/well IL-2, IL-6, IL-8 or TNF-standards, respectively, diluted in reagent diluent, and 100 μ L/well of samples collected from the Jurkat cell supernatant. After an incubation of 2 h at RT, the plates were washed three times and thereafter 100 μ L/well of 50 ng/mL biotin-linked detection antibody against IL-2, IL-6, IL-8 or TNF- α , respectively, were added and incubated at RT for 2 h. Then washed the plates three times and incubated with streptavidin-HRP at room temperature for 20 min. After three washes with PBS-T, the plates were incubated with 100 μ L/well with substrate solution (1:1 mixture of H₂O₂ and tetramethylbenzidine, TMB) at RT for 20 min. After that, 100 μ L/well of stop solution was added and the plates were analyzed with a spectrophotometer at 450 nm.

Statistical methods

Mean and standard deviation were used for statistical analysis of all calculations.

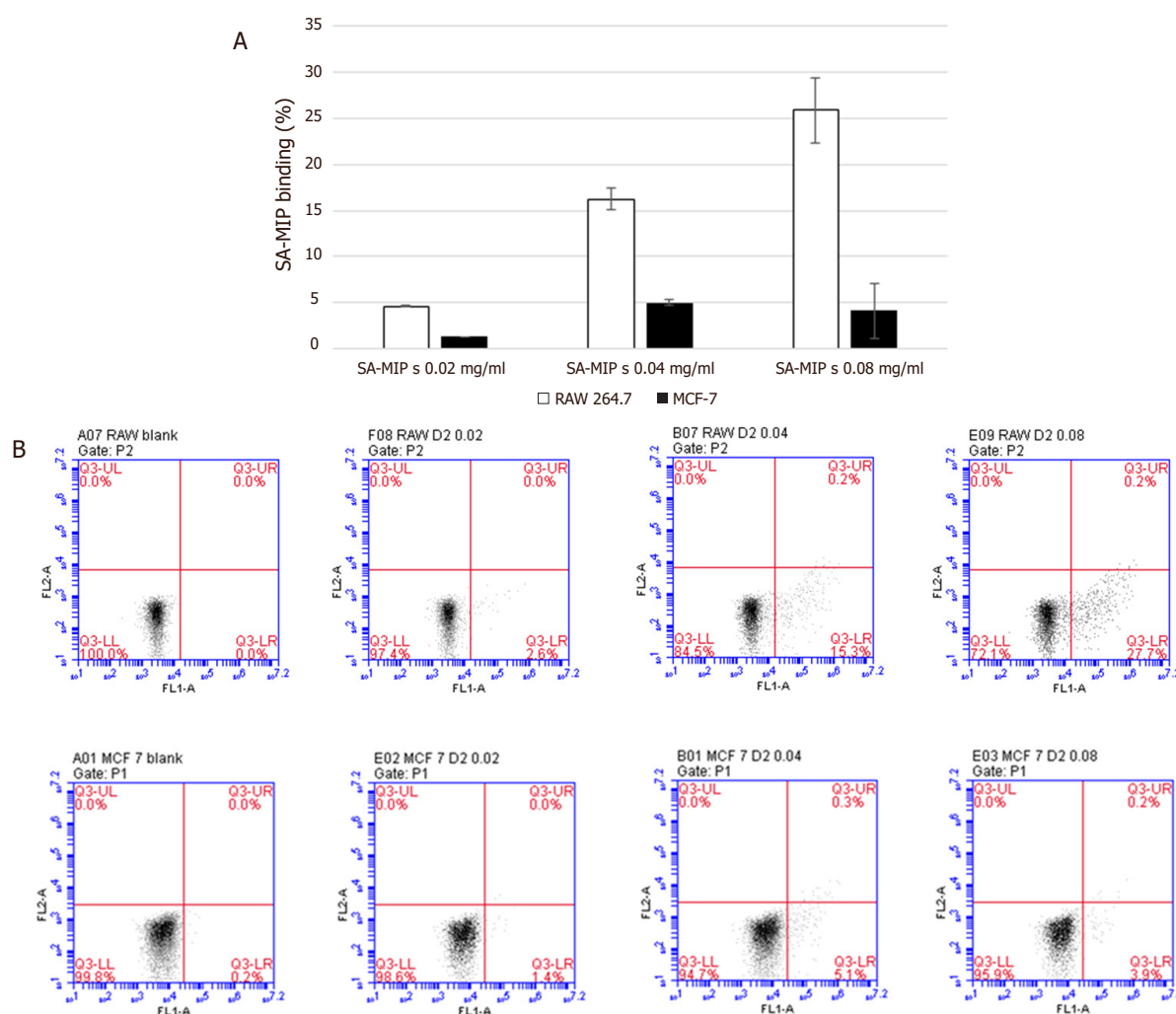


Figure 1. SA-MIP binding to RAW 264.7 cells and MCF-7 cells. A: Results of cells RAW 264.7 cells and MCF-7 cells stained with different concentrations of SA-MIP. B: Flow cytometry diagrams present the positive cells for SA-MIP concentrations 0.02, 0.04 and 0.08 mg/mL, as dot-plot diagrams, respectively. The experiment shown in A represents the average of least three performed

RESULTS

Evaluation of SA-MIP binding to MCF-7 and RAW 264.7 cells

The breast cancer cell line MCF-7 and the macrophage cell line RAW 264.7 were tested for SA-MIP binding at different concentrations. Addition of increased concentration of SA-MIP resulted in higher SA-MIP binding [Figure 1A]. At the highest concentration of SA-MIPs used, 0.08 mg/mL, the staining result was around 5% cell binding for MCF-7 cells and 65% cell binding for RAW 264.7 cells. The dot-plot diagrams for RAW 264.7 cells (Upper figures) and MCF-7 cells (Lower figures) are shown in Figure 1B.

SA-specific lectins show an increased binding to MCF-7 and RAW 264.7 cells after treatment with a cytokine cocktail

First, the expression of SA on the cell lines were confirmed by using the lectins MAL I and SNA [Figure 2]. Then, the cells were treated with the cytokine cocktail from PHA-stimulated Jurkat cells, and a significant increased binding of the lectins could be detected in both cell lines [Figure 2].

Cytokine treated MCF-7 and RAW 264.7 cells show a variation in SA-MIP binding

Both cell lines were treated with the recombinant cytokines IL-4, IL-6 or IL-8. Alternatively, the cytokine cocktail from PHA-stimulated Jurkat cells was used. For the RAW 264.7 cells, treatment with IL-4 showed

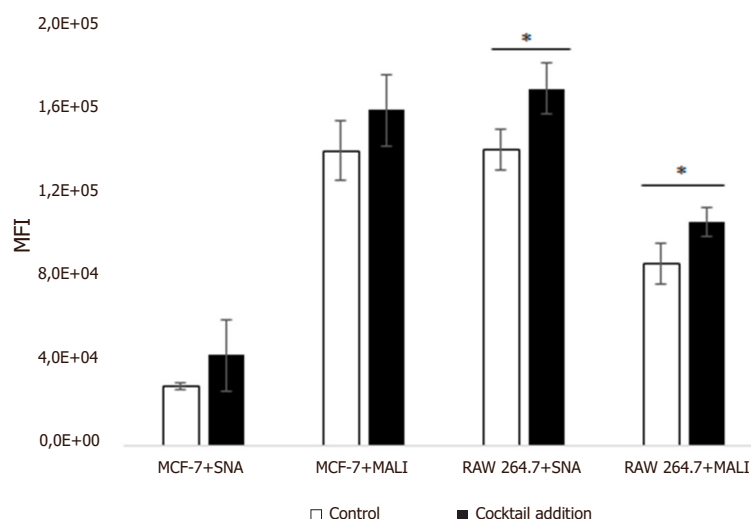


Figure 2. Lectin binding to RAW 264.7 cells and MCF-7 cells. Results of cells RAW 264.7 cells and MCF-7 cells stained with the MAL I and SNA lectins. Flow cytometry results present the mean fluorescence intensity for the lectins and a small increase in binding of the lectins after treatment with the cytokine cocktail. The experiment shown represents the average of least three performed

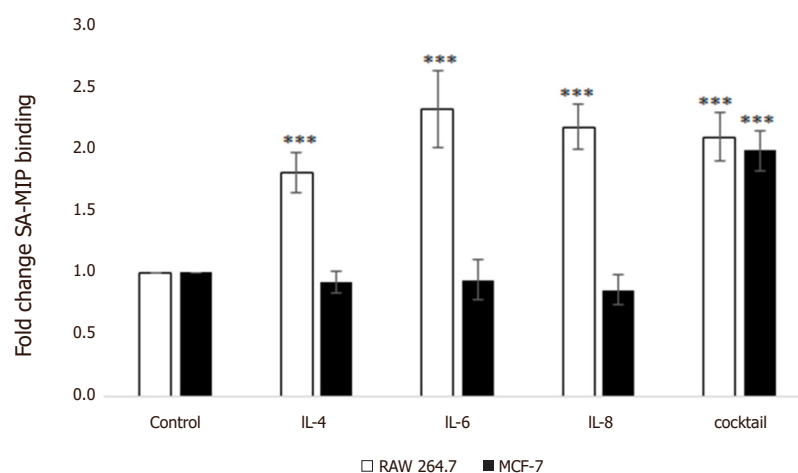


Figure 3. Flow cytometry of cytokine-treated RAW264.7 cells showing increase in SA-MIP binding after IL-4, IL-6, IL-8 or cocktail treatment. Results of cells RAW 264.7 cells and MCF-7 cells stained with SA-MIP. Flow cytometry results were re-calculated to fold change of response compared to control. The experiment shown represents the average of least three performed

an increase in SA-MIP binding in both cell lines, whereas IL-6 and IL-8 showed an even larger decrease [Figure 3]. The cytokine cocktail with unknown content influenced the SA-MIP binding in a similar manner as IL-6 and IL-8. All additions of cytokines to RAW 264.7 cells were found to be significantly different from the control, as determined by statistical calculations.

For the MCF-7 cell line, addition of the recombinant cytokines IL-4, IL-6 or IL-8 did not influence any increase of SA-MIP binding to the cells [Figure 3]. No statistical significant difference could be determined for cytokine treatment of MCF-7 cells. However, addition of the cytokine cocktail induced a 2-fold significant increase of SA-MIP binding in MCF-7 cells. To elucidate the cytokine content in the cocktail, ELISA was performed.

Table 1. Cytokine content in PHA-stimulated Jurkat cells was analyzed with sandwich ELISAs detecting IL-2, IL-6, IL-8 and TNF-, respectively

Cytokines analyzed with ELISA in PHA-stimulated Jurkat cell sample	Cytokines detected (pg/mL)
IL-2 negative control	0
IL-2 stimulated sample	17
IL-4 negative control	n.d.
IL-4 stimulated sample	n.d.
IL-6 negative control	0
IL-6 stimulated sample	0
IL-8 negative control	0
IL-8 stimulated sample	427
TNF-negative control	0
TNF-stimulated sample	12

PHA-stimulated Jurkat cells produce high amounts of IL-8

The supernatants from stimulated and unstimulated Jurkat cells were investigated with sandwich ELISA specific for IL-2, IL-6, IL-8 and TNF- respectively [Table 1]. The levels of IL-2 and TNF- were low, but showed an increase compared to control. In contrast, more than 400 pg/mL of IL-8 was detected in the PHA-stimulated Jurkat cell supernatant. No IL-8 could be detected in the unstimulated control. IL-6 could not be detected in either stimulated or unstimulated samples.

DISCUSSION

In the tumor microenvironment, the cells of the immune system communicate with each other by releasing chemokines and cytokines to either favor antitumor immunity or enhance tumor progression. Thereby the cells control the immune and inflammatory milieu in a sophisticated fashion.

In this study we demonstrate that the SA-expression of RAW 264.7 and MCF-7 cells treated with recombinant cytokines *in vitro* could be analyzed with the use of SA-MIPs and flow cytometry. Interestingly, the cytokine-treated cells showed a modulated SA-MIPs binding pattern compared to the unstimulated controls. Stimulating macrophage RAW264.7 cells with IL-4, IL-6 or IL-8 cause an up-regulation of SA-MIP binding, while for the MCF-7 cells no increase could be determined. The effect of inflammatory cytokines on pancreatic cancer cells has been reviewed by Roshani *et al.*^[1]. In other studies, IL-4 did not have any effect, but IL-6 and IL-8 were associated with poor prognosis and increased aggressiveness, respectively. IL-4 is an anti-inflammatory cytokine expected to down-regulate SA, while IL-6 and IL-8 both up-regulate the SA expression. Indeed, IL-6 and IL-8^[15-17], as well as anti-inflammatory cytokines IL-10 and tumor growth factor-beta (TGF- β)^[15,18,19] have been commonly shown to be elevated in pancreatic patients. Also, TNF- and IL- were upregulated compared to controls, but all studies in pancreatic cancer patients did not come to the same conclusions^[1]. Other studies have proven that the levels of IL-4, IL-6 and IL-8 in pancreatic cancer patients is higher than normal^[15]. An important remark is that the pattern of cytokine expression varies between studies and even contradicting results have been obtained. Bassaganas *et al.*^[20] studied pancreatic ductal adenocarcinoma cell lines treated with different cytokines. They found that treatment with either IL-1 or IL-6 resulted in an increased carbohydrate antigen and SA level. Also, the levels of mRNA of precursor of carbohydrate antigens was enhanced. Moreover, Dima *et al.*^[19] found an association between higher levels of circulating TNF- α with poorer prognosis. These findings indicate that inflammatory cytokines can be pursued as potential prognostic biomarkers as well as therapeutic targets.

Escaping immune recognition is now a recognized hallmark of cancer^[21]. Chemokines and cytokines can play a critical role in the immune evasion. Tumor cells can escape host immunity by producing immunosuppressive cytokines as well as by recruiting regulatory immune cells with immunosuppressive functions.

The cellular expression pattern of glycosyltransferases and Lewis antigens detected in the normal mucosa can change during neoplastic transformation. The stomach mucosa was investigated for Lewis antigens in the presence of pro-inflammatory cytokines^[4]. The level of one type of Lewis antigen decreased after treatment with IL-1 or IL-6, whereas the levels of other carbohydrate antigens were unaffected^[4].

Lectins are plant-derived macromolecules specific for carbohydrate moieties. The MAL lectin specifically bind the sialyl α 2,3 Gal β 1,4GlcNAc/Glc trisaccharide sequence^[22]. Expression of α 2,3-SA residues of N-cadherin was shown to be altered in metastatic melanoma cell lines^[23]. The expression of α 2,3-SA residues in breast cancer using MAL-staining were analyzed by Cui *et al.*^[24] The results suggested that high levels of α 2,3-SA residues was closely associated with lymph node metastasis and invasive depth in breast cancer patients. The highly metastatic breast cancer cell line MDA-MB-231 had higher expression of α 2,3-sialic acid residues compared to T-47D and MCF-7 depending on the mRNA levels of α 2,3-ST genes. Indeed, the tumor microenvironment can direct the level of inflammation. Also, the metastatic potential and malignancy of cancer cells is closely associated with aberrant sialylation pattern^[24].

The enzyme that adds α 2-6linked sialic acids, β -galactoside α 2-6 sialyltransferase (ST6Gal-I), is known to be upregulated in many tumor types including colon adenocarcinomas^[25], and high expression levels have been associated with poor prognosis and metastasis. ST6Gal-I sialylation of membrane glycoproteins contributes to metastasis by enhancing cell motility and invasion through the extra cellular matrix (ECM). Increased negative charged properties of sialic acids was correlated with reduced adhesiveness of tumor cells and may be suitable for conformational change of integrin and enhances its function in cell-ECM interactions^[26].

Interestingly, in our study we show a high expression of α 2,3-SA on both cell lines, whereas the α 2,6-SA, as analyzed with the lectin SNA, displayed a lower expression on the MCF-7 cells. It has been shown that high levels of sialylation of cell surface glycoconjugates can significantly increase metastasis of colon carcinoma cells and human melanoma cells.

In addition to directly modulating cell motility, α 2,3-SA residues are involved in the synthesis of sialyl Lewis X determinants, which are the major ligands for endothelial E-selectin^[27]. The sialyl Lewis X structure on malignant cells is suggested to facilitate tumor cell dissemination by mediating the tumor-endothelial cell interactions^[28,29]. Overall, many studies support the hypothesis that an increase of sialylation of cancer cells play an important role in tumor metastasis.

In the present study, the MCF-7 cell line showed a small increase of the SA-MIP binding after treatment with the cytokine cocktail derived from PHA-stimulated Jurkat cells. On the other hand, the SA-MIP binding when treating the RAW264.7 cell line with the same cocktail was not different compared to the effect of recombinant IL-6 or IL-8. Interestingly, an increase in binding of the lectins MAL and SNA was also detected after cytokine cocktail treatment. By analyzing with ELISA, we show that the cocktail contains increased amounts of IL-8, as well as low levels of IL-2 and TNF- α . IL-6 could not be detected in the PHA-stimulated samples. IL-8 is a chemokine, functioning by attracting cells to a site of infection, and it is therefore tempting to speculate that the increased SA-MIP binding seen by the RAW264.7 cells, could be due to a regulated SA-expression in response to IL-8. The natural ligands for SA are selectins and siglecs^[30]. Indeed, the rolling of cancer cells ectopically expressing the selectin ligands on endothelial cells is potentially a crucial step favoring the metastatic process *in vivo*^[31]. The combined analysis of SA and the targeting of SA to the ligands selectins and siglecs will be attractive for further investigations.

In conclusion, cancer cell migration and invasion is controlled by protein glycosylation and the ECM. SA is one of several important players in this crucial process. Inflammation and cytokine production will modulate the cellular microenvironment. We have studied cell lines *in vitro* that showed that one of the cell

lines showed a significantly increased binding of the SA-MIPs after treatment with recombinant cytokines and with the cytokine cocktail from PHA-stimulated Jurkat cells. Here we demonstrate that SA-MIPs can be an important tool in the investigation of overexpressed glycans in the tumor microenvironment.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: Zhang Y, Llapashtica K, Wingren AG

Performed data acquisition, as well as provided administrative, technical, and material support: El-Schich Z

Provided administrative, technical, and material support: Shinde S, Sellergren B

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Original Article

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Monoclonal antibodies to the exon 18 encoded moiety of NCAM

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Abstract

Aim: Exon 18 expression of NCAM has been recognized as a biomarker for small cell lung cancer (SCLC). To use this finding for an improved diagnosis of SCLC and personalized treatment of patients, techniques to identify and quantitate E18, the exon 18 encoded protein moiety of NCAM, are needed. We developed three monoclonal antibodies for this purpose.

Methods: The his-tagged E18 antigen was expressed in *E. coli* and, after purification, used to immunize mice. Hybridoma's were isolated by standard procedures and tested for their reaction with E18.

Results: Three monoclonal antibodies, MUM-1, MUM-4 and MUM-6 were obtained. They reacted with E18 in western blots, with SCLC cell line NCI-H82, but not with unrelated his-tagged proteins. Only permeabilized NCI-H82 cells stained with the antibodies, confirming the intracellular position of E18. Next an enzyme-linked immunosorbent assay was developed using the earlier isolated monoclonal antibody MUMi-21B2, coated on the surface of microtiter wells as capture antibody and biotinylated MUM-6 as second antibody. Using streptavidin conjugated to horse radish peroxidase a linear dose response curve to his-tagged E18 antigen was obtained between 0 and 5 µg/mL with a sensitivity of at least 0.5 µg/mL or 50 ng/well.

Conclusion: Four monoclonal antibodies are available to be used in assays for the identification and quantification of SCLC biomarker E18. This will enable the development of liquid biopsies to follow the tumor load in patients.



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Keywords: Small cell lung cancer, tumor biomarker, monoclonal antibodies, NCAM exon 18

INTRODUCTION

Small cell lung cancer (SCLC) is strongly associated with smoking. It is a very aggressive form of cancer due to resistance to chemotherapy and rapidly dividing metastatic disseminations. SCLC represent about 15% of all lung cancer cases and annually kills about 250,000 people^[1]. At the initial diagnosis metastatic lesions are already present in about two thirds of the patients^[2]. Patients die as a result of the disease at a median of 10 to 12 months after diagnosis^[3]. Over many decades survival rates have not improved^[4]. SCLC is a neuroendocrine tumor. There are three other types of neuroendocrine lung tumors [Table 1]. A hallmark of these tumors is the presence of neuroendocrine peptides such as NCAM. NCAM is expressed as 120, 140 and 180 kDa isoforms, all derived through alternative splicing from a single gene. NCAM has been described as a biomarker for SCLC, but is also present on other cell types^[5].

Biomarkers for SCLC could lead to an important improvement in the treatment of SCLC. These markers could be used to monitor the tumor load in patients, allowing early diagnosis and monitoring the effect of therapeutic interventions. We recently described the expression of NCAM exon 18 as a potential biomarker for SCLC^[5]. More recently we have further validated this biomarker. More SCLC biopsies were tested. These were all positive for NCAM exon 18 expression. Expression was also found in large cell neuroendocrine carcinoma (LCNEC) (unpublished data). In this paper we describe the generation and characterization of monoclonal antibodies directed to E18, the exon 18 encoded moiety of NCAM. We intend to use these antibodies for the quantification of E18 in extracellular vesicles present in the blood of SCLC patients.

METHODS

Production of monoclonal antibodies

Preparation of antigen

Recombinant his-tagged E18 was prepared in *E. coli*, using a plasmid with a codon usage optimized for *E. coli* (GeneArt/Thermo Fisher Scientific), and purified on a Ni²⁺-NTA agarose column and E18-encoding DNA was cloned into the pCI mammalian expression vector as described^[5].

Immunization of mice and preparation of hybridoma's

Three Balb/c mice were immunized 4 times. At zero time intramuscularly with 2 times 50 µg in 50 µL of the pCI vector containing E18-encoding DNA and at 3 and 8 weeks subcutaneously with 10 µg his-tagged E18 and 12 µg (in 25 µL) AbISCO-100 (Isconova, Sweden). The final immunization was at 10 weeks with 10 µg his-tagged E18 without adjuvant subcutaneously and the same amount intraperitoneally. Three days after the last immunization spleen cells were isolated and fused with SP2/0 myeloma cells according standard procedures^[9]. Cells were plated in nine 96 well microtiter plates.

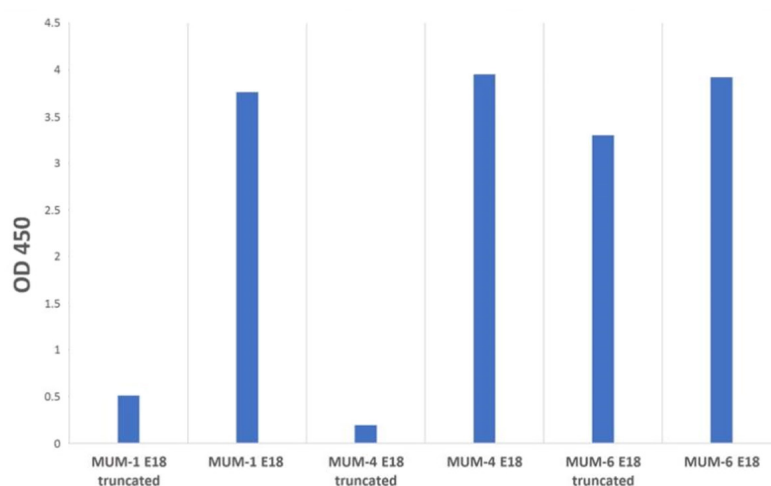
Screening and characterization

The supernatants of the microtiter wells were tested in an enzyme-linked immunosorbent assay (ELISA) as described^[5]. Wells were coated with his-tagged E18 or an unrelated his-tagged protein. This resulted in 6 candidates positive for E18 and negative for unrelated his-tagged proteins. These hybridoma's were subcloned and characterized by four methods. First, an ELISA was carried out on full length E18 and a shortened version of E18 (Supplementary Figure 1 by Vander Borghet *et al.*^[5]). Second, immunohistochemistry on SCLC cell line NCI-H82 and control cell line HCT116. Third flow cytometry of intact and permeabilized NCI-H82 cells and last western blotting. Experimental details can be found in the study by Vander Borghet *et al.*^[5]. Isotyping was carried out with the MMT1 kit of AdD Serotec (presently Biorad).

Table 1. Neuroendocrine tumors of the lung

Name	Smoking-associated	% of all cases of lung cancer	Overall Survival 5 y	Mitotic index*	Ki-67 index [#]
SCLC	Yes	15-20	< 5%	Median = 80	50%-100%
LCNEC	Yes	3	15%-57%	Median = 70	40%-80%
TC	No	1-2	92%-100%	< 2	< 5%
AC	No	0.1-0.2	61%-68%	2-10	< 20%

Data from Ref.[6,8]. *Measure for rate of cell division; [#]Ki-67 is a marker protein for cell division. SCLC: small cell lung cancer; LCNEC: large cell neuroendocrine carcinoma; TC: typical carcinoid; AC: atypical carcinoid.

**Figure 1.** Reaction of monoclonal antibodies MUM-1, MUM-4 and MUM-6 with (truncated) E18 measured by an ELISA

Setting up of an ELISA

An ELISA was set up, using one antibody coated to the microtiter wells as a capture antibody and a second biotinylated antibody for detection. The best combination of antibodies was MUMi-21B2 as a capture antibody and MUM-6 as second antibody. Full experimental details can be found in [Supplementary Figure 1](#).

RESULTS

Three monoclonal antibodies, MUM-1 (IgG1 kappa), MUM-4 (IgG1 kappa) and MUM-6 (IgG1 lambda), were fully characterized and produced. The ELISA confirmed that they all react with E18. MUM-6 also reacted with the truncated form of E18 that was used to generate MUMi-21B2 [\[Figure 1\]](#).

The monoclonal antibodies were also tested in immunohistochemistry assays on SCLC cell line NCI-H82 and control colon carcinoma cell line HCT116. In comparison antibody RNL-1, directed to an extracellular part of NCAM, was used. [Figure 2](#) shows the results for MUM-4 and MUM-6. A strong reaction of RNL-1 to NCI-H82 cells and no reaction to HCT116 cells that lack NCAM is seen. The signal with MUM-4 and MUM-6 is weaker but clearly present. A stronger signal was obtained when the cells were permeabilized with 4% paraformaldehyde/0.5% triton X-100 (data not shown). Intact cells did not stain with the MUM-1, MUM-4 and MUM-6. This could be demonstrated most convincingly by flow cytometry. The results for MUM-1 are shown in [Figure 3](#).

DISCUSSION

The further validation of NCAM exon 18 expression in cancer cells is ongoing. More SCLC and LCNEC biopsies will be analyzed. These are the two most aggressive neuroendocrine lung cancers, but it will be also of interest to analyze the milder [\[Table 1\]](#) “typical carcinoid” and “atypical carcinoid”. However, our focus

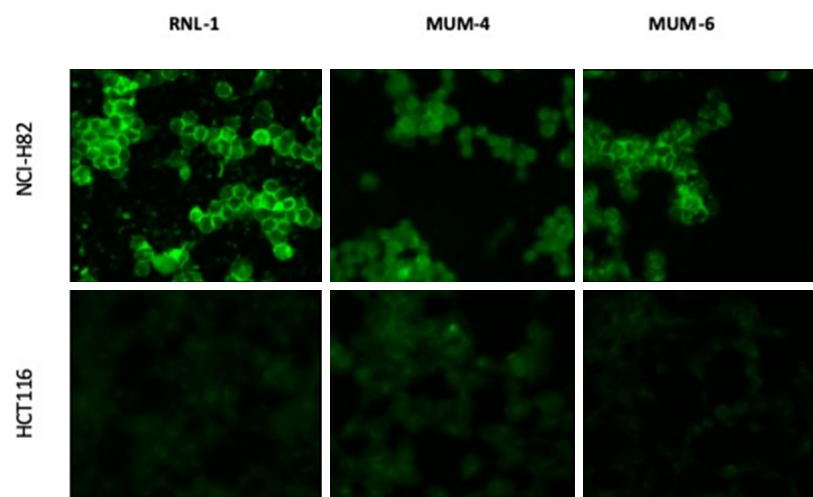


Figure 2. Immunohistochemistry of SCLC cell line NCI-H82 and colon carcinoma cell line HCT116. The results for MUM-4 and MUM-6 are shown, together with the staining with RNL-1, a monoclonal antibody directed to an extracellular part of NCAM. Cells were permeabilized with methanol/acetone as described^[5] or, for the MUM-6 panel, with 4% paraformaldehyde (15 min) and 0.5% Triton-X100 (10 min)

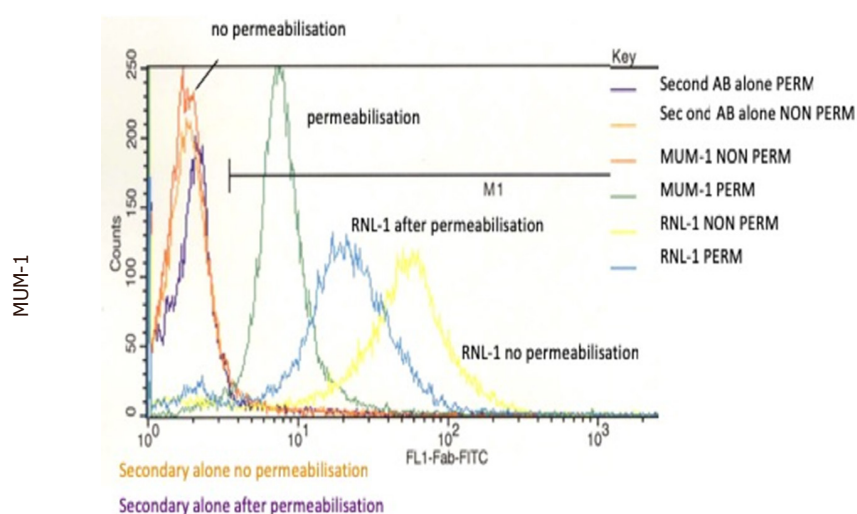


Figure 3. Reaction of MUM-1 with SCLC cell line NCI-H82 measured with flow cytometry. Methanol/acetone permeabilized cells were compared with intact cells. A signal was only found in permeabilized cells. This difference was not found with antibody RNL-1 directed to an external region of NCAM

will be on clinical applications of E18 as a biomarker for SCLC. A main problem in the treatment of SCLC is that patients initially respond well to first line chemotherapy, even in advanced disease, but most patients relapse within 6 months. Promising alternative targeted and immune therapies have been identified^[10-12], but due to the rapid progression of the disease the time window to study these alternatives is limited. Ideally clinical treatment should be guided by closely monitoring the evolution of the disease^[12,13]. Our assumption is that the presence of E18 will reflect the tumor load. We intend to develop methods to analyze the presence of E18 in liquid biopsies. Serial sampling would give information on disease burden, responses to treatment and on disease relapse. Promising results have been obtained with circulating tumor cells (CTCs)^[14], but there is a large variation in CTC numbers and heterogeneity in the expression of NCAM^[14]. Our present strategy is to use tumor derived extracellular vesicles (EVs) as a correlate of tumor load. EVs are constantly formed by cells, either from endosomal membrane compartments (exosomes) or from the

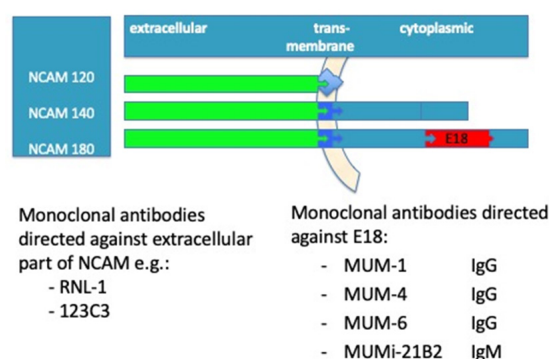


Figure 4. Strategy for the isolation of extracellular vesicles (EVs) derived from SCLC cells. SCLC-specific EVs are enriched and purified by RNL-1 and/or 123C antibodies, specific for the extracellular moiety of NCAM^[19-21], coupled to magnetic beads. In the next step the presence of E18 in the EVs will be measured with one of the specific antibodies. We will choose the antibody with the best signal to noise ratio

plasma membrane. The membranes of EVs contain in principle all cellular surface proteins and, within the membrane, all cytoplasmic components^[15]. Virtually all SCLC cells express NCAM^[16,17]. We will enrich for NCAM-containing EVs, using immunoaffinity capture methods^[18] [Figure 4].

DECLARATIONS

Authors' contributions

Design and execution of experiments: Vander Borghet A, Duysinx M, Ummelen M
Manuscript drafting, literature analysis and research strategy: van der Zeijst BAM

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

The animal experiments were carried out at the University of Maastricht, and were approved by the Animal Experiments Committee of the University of Maastricht (project number 2005-043).

Consent for publication

Not applicable.

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Review

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The potential for estrogen disrupting chemicals to contribute to migration, invasion and metastasis of human breast cancer cells

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Abstract

Estrogen disrupting chemicals are environmental compounds which mimic, antagonize or interfere in the action of physiological estrogens. They occur naturally (plant phytoestrogens) but the majority are man-made compounds, which, through their use in agricultural, industrial and consumer products, have become widely present in human tissues including breast tissue. Since exposure to estrogen is a risk factor for breast cancer, estrogen disrupting chemicals may also contribute to breast cancer development. This review discusses evidence implicating estrogen disrupting chemicals in increasing migratory and invasive activity of breast epithelial cells, in epithelial-to-mesenchymal transition, and in growth of breast tumours at metastatic sites as well as the primary site. Mechanisms may be through the ability of such chemicals to bind to estrogen receptors, but unlike for proliferation, effects on cell migration and invasion are not limited to estrogen receptor-mediated mechanisms. Furthermore, whilst effects on proliferation can be measured within hours/days of adding an estrogen disrupting chemical to estrogen-responsive breast cancer cells, effects on cell migration occur after longer times (weeks). Most studies have focused on individual chemicals, but there is now a need to consider the environmentally relevant effects of long-term, low-dose exposure to complex mixtures of estrogen disrupting chemicals on mechanisms of metastasis.

Keywords: Aluminium, breast cancer, estrogen disrupting chemicals, invasion, metastasis, migration, paraben, personal care products, UV filter.



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INTRODUCTION

Estrogen disrupting chemicals are exogenous environmental compounds that can interfere in the action of endogenous estrogens. Through their ability to bind to estrogen receptors, they may be able to mimic or antagonize the cellular actions of physiological estrogens^[1]. They may also influence synthesis, transport, metabolism and clearance of physiological estrogens^[1]. Some are found naturally in plants (phytoestrogens) or fungi (mycoestrogens), but the majority are man-made compounds to which the human population is widely exposed through their use in agricultural, industrial and consumer products^[1]. Such compounds are used as components of pesticides and herbicides in agricultural, urban and domestic environments. They are widely used in industrial applications and occur as byproducts of combustion from vehicles, aircraft and ships. These compounds are found as components in plastics, with applications from building materials to food/water containers, in detergents used for cleaning in industrial and domestic settings, and as flame retardants in soft furnishings. A range of components added to personal care products are also now known to possess estrogen disrupting activity^[1]. Some more specific examples are shown in Table 1. Intake to the human body may be by the oral route (food/water) or inhalation (air pollution) or dermal absorption (products applied to skin or present in the air). So ubiquitous are the many estrogen disrupting chemicals in modern life that exposure is almost never from a single source but rather from multiple sources reflecting not only occupational exposures but also personal lifestyle choices.

Since exposure to estrogens is an established risk factor for breast cancer^[2], it is thought that estrogen disrupting chemicals may also contribute to breast cancer development if they are present in human breast tissue at sufficient concentrations^[1]. Development of cancer is a lengthy and complex process, that originates from a loss of growth control, and culminates in metastatic tumour spread. Both genetic and environmental influences interact to enable the associated hallmarks of cancer to develop^[3]. The hallmark of sustained cell proliferation in breast cancer is well established as involving estrogens^[4] and probably also estrogen disrupting chemicals^[5]. Since metabolism of some endogenous estrogens gives rise to genotoxic compounds^[6] and since some estrogen disrupting chemicals are also genotoxic^[1], a role has been recognized in generating genomic instability which is an underlying enabling characteristic of cancer^[3]. However, the role of endogenous and exogenous estrogens in contributing to the processes of metastatic tumour spread are less well established. This is especially important in the context of breast cancer where the main cause of mortality arises from growth of metastatic tumours^[2,7]. This review will discuss current evidence suggesting that endogenous and exogenous estrogens can contribute to increasing migratory and invasive activity of breast epithelial cells which are hallmarks of cancer required for the metastatic process^[3].

ENDOGENOUS ESTROGENS AND METASTASIS

Cellular actions of estrogens are mediated through binding to specific receptor proteins^[8]. There are two types of nuclear estrogen receptors, estrogen receptor α (ER α) and estrogen receptor β (ER β) which exist as multiple isoforms and which function as ligand-activated transcription factors to influence patterns of gene expression^[8]. Final outcomes are dependent on the affinity of ligand binding to receptor, the concentrations of receptors in the target cells and the presence of co-acting factors/transcription factors^[8]. In addition, estrogenic ligands can also act through binding to membrane-associated molecules such as the G-protein-coupled estrogen receptors^[8,9].

Metastasis involves a complex series of events whereby the malignant cells break away from the primary tumour, invade through local tissue, infiltrate circulatory vessels (blood and/or lymph) and at distant sites exit the vessels to form new foci of cancer cell growths (colonization)^[7]. Endogenous estrogens can influence all these processes including immune evasion and the angiogenesis needed for effective metastatic colonization^[10]. In their capacity to regulate growth of estrogen-responsive human breast cancer cells^[2,4], estrogens can influence metastatic tumour growth at both the primary and secondary sites^[2].

Table 1. Estrogen disrupting chemicals: example compounds, uses in the environment, and sources of human exposure

Use in the environment	Example compounds	Source of human exposure
Plant phytoestrogens	Genistein, daidzein	Edible plant material (diet, dietary supplements)
Pharmaceuticals	Ethinylestradiol	Contraceptive pill, hormone replacement therapy, cosmetics
Pesticides	DDT, lindane, pyrethroids	Pesticide products, animal fat (diet)
Herbicides	Glyphosate	Garden weedkillers
Industrial	Polychlorinated biphenyls	Animal fat (diet)
By-product of incineration	Polychlorinated dioxins	Inhaled; animal fat in diet
Flame retardant	Polybrominated organics	Domestic environment from use on soft furnishings
Plastics, epoxy resins	Bisphenol A	Storage of food and beverages (diet); domestic environment
Plastics	Phthalate esters	Domestic consumer products
Detergent	Alkyl phenols	Domestic environment
Preservative	Parabens	Personal care products, food, pharmaceuticals
Antiperspirant	Aluminium chlorohydrate	Underarm antiperspirants
Antimicrobial	Triclosan	Personal care products, domestic consumer products
Absorb ultraviolet light	Benzophenones	Suncare products, other cosmetics, clothing
Fragrance	Butylphenylmethylpropional, benzyl salicylate, musks	Personal care products, domestic consumer products
Conditioning/spreading agent	Cyclic volatile methylsiloxanes	Personal care products
Cigarettes	Cadmium	Cigarette smoking

DDT: dichlorodiphenyltrichloroethane

However, endogenous estrogens are also now known to be able to influence cell motility, cell migration and invasive behavior of human breast cancer cells through altering expression of proteins and transcription factors key to the process of epithelial to mesenchymal transition (EMT). EMT is a process in which the epithelial cells lose their polarity and strong cell-cell adhesion properties in order to assume a more mesenchymal phenotype lacking the polarization and the strong cell-cell interactions. The cell adhesion junctions of epithelial cells are reliant on high levels of E-cadherin which is a transmembrane protein linked to the cytoskeleton by α - and β -catenin, and EMT is typically associated with reduction in levels of these adhesion proteins. It is also associated with altered levels of transcription factors (such as slug and snail) which control expression of the adhesion proteins^[7]. This allows the cells to break away from neighbouring cells and to become generally more motile. Human breast cancer cell lines which possess ER α (ER α +) tend to be less invasive, less metastatic and possess higher levels of E-cadherin than those which lack ER α (ER α -)^[11]. Knockdown of ER α in the ER α + cell lines has been shown to result in decreased E-cadherin and increased slug expression^[12]. Conversely, transfected overexpression of ER α in the ER α - cell lines has been shown to enable estrogen-mediated increase in E-cadherin and decrease in slug^[12]. In the ER α + MCF-7 human breast cancer cell line, estradiol can increase EMT whilst the antiestrogen tamoxifen can reduce EMT^[13] suggesting that the mechanism is ER-dependent. However, there appear to be multiple molecular mechanisms involving not only genomic alterations to levels of E-cadherin and associated transcription factors such as slug^[12] but also non-genomic alterations via c-src and phosphorylation of focal adhesion kinase^[14,15].

In order to facilitate invasion through local tissue, the cells need also to secrete greater levels of extracellular proteases which aid in the digestion of the surrounding extracellular matrix (ECM). Some of the extracellular proteases identified specifically in human breast cancer cells are matrix metalloproteinases (MMPs)^[16,17] and cathepsin D^[18]. It is notable that one of the first estrogen-regulated genes to be identified was cathepsin D^[19] and studies now also show effects of estrogen on expression of MMPs^[16]. These studies attest to the role of estrogen in altering proteases which can degrade the ECM.

Estrogens are also known to play a role in the processes of angiogenesis^[10,20]. Vascular endothelial growth factor (VEGF) is a key angiogenesis promoting factor and the VEGF gene has been shown to possess functional estrogen response elements indicating it is an estrogen-responsive gene^[21]. Estrogen treatment

has also been reported to increase intra-tumoural angiogenesis^[22] and VEGF levels^[23] in mouse models of breast cancer.

ESTROGEN DISRUPTING CHEMICALS AND METASTASIS

On the basis that endogenous estrogens can influence processes necessary for metastasis, it would seem plausible that estrogen disrupting chemicals will also contribute if they can enter breast tissue and target metastatic tissues at sufficient concentrations. Whilst endogenous estrogens are subject to physiological regulation in terms of both concentration and timing, it is noteworthy that the estrogen disrupting chemicals are not regulated in this manner. The presence of estrogen disrupting chemicals in the body will follow rather intake from environmental exposures balanced by rates of elimination and ability of individual chemicals to bioaccumulate. Many estrogen disrupting chemicals tend to be lipophilic accumulating in body fat over the years such that body burdens increase with age^[1]. In this context it may be significant that 80% of breast cancers occur in women over the age of 50^[24] at a time when endogenous estrogens are reduced following menopause but exogenous estrogen disrupting chemical body burdens would be continuing to increase.

Estrogen disrupting chemicals and metastatic colonisation

Metastasis of breast cancer cells begins with local spread within the breast and to the underarm lymph nodes. The pattern of more distant dissemination is less predictable with preferred sites of colonization being brain, bone, lungs and liver^[7]. Stephen Paget's original "seed and soil" hypothesis proposed that preferred sites of metastasis would reflect a favourable organ microenvironment in which the metastatic breast cancer cells could grow^[25]. His hypothesis was based on the tenet that "when a plant goes to seed, its seeds are carried in all directions, but they can only live and grow if they fall on congenial soil". For breast cancer cells, one important favourable element for cell growth would be an "estrogenic" environment and it is in this context that estrogen disrupting chemicals may influence sites of metastatic spread. Estrogen disrupting chemicals have been widely measured in human populations across the globe in body fluids such as blood and urine and in various body tissues^[1]. With such widespread contamination of the human body, it is becoming accepted that there are ramifications for human health including effects on male and female reproduction and on cancers in reproductive tissues^[1]. The presence of estrogen disrupting chemicals across many body tissues could act to change a previously non-favourable location into a favourable environment for colonization and growth of human breast cancer cells into metastatic tumours.

Evidence for effects of estrogen disrupting chemicals on the metastatic process

There is now mounting evidence for effects of the parabens, ultraviolet (UV) filters, aluminium salts, triclosan, phthalates, bisphenol A (BPA), dioxins and phytoestrogens in the processes of EMT, motility, migration and invasion, and relevant studies of breast cells are discussed below. Most studies to date have been based *in vitro* but there are some animal models which demonstrate measurable effects also *in vivo*. Many of the mechanisms may involve mimicking the action of endogenous estrogens through the ability of these estrogen disrupting chemicals to bind to estrogen receptors, but unlike for proliferation, the effects on migration and invasion do not seem to be limited to ER-mediated mechanisms. Furthermore, whilst effects on proliferation can be measured within hours/days of adding an estrogen disrupting chemical to estrogen-responsive breast cells, effects on migration tend to occur after a longer time frame of weeks. The reasons why the time taken is longer remain unknown but would be important to determine in future research.

Parabens

The alkyl esters of *p*-hydroxybenzoic acid (parabens) are added as preservatives to personal care products, pharmaceuticals and foods^[1]. Of the five commonly used esters (methylparaben, ethylparaben,

n-propylparaben, *n*-butylparaben and isobutylparaben) 99% of the human breast tissues were found to contain at least one of the esters, and 60% contained all five esters^[26]. Although the parabens bind more weakly to estrogen receptors than do the endogenous estrogens, their efficacy is not weak provided sufficient concentration is present^[27]. It is therefore noteworthy that the concentrations of parabens measurable to human breast tissues are considerably higher (in the micromolar range)^[26] than the levels of endogenous 17 β -estradiol (in the nanomolar range)^[2,28]. Previous work has demonstrated that parabens can increase proliferation of estrogen-responsive human breast cancer cells in cell culture at concentrations measurable in some human breast tissue samples^[29] through estrogen receptor-mediated mechanisms^[27]. However, parabens have now been found to also increase migratory and invasive activity of human breast cancer cells in culture^[30]. Migratory activity was measured using scratch assays, time-lapse microscopy and xCELLigence technology^[30]. Invasive activity was measured using matrix degradation assays and invasion through matrigel on the xCELLigence system^[30]. Unlike the proliferative effects which were measurable within days of addition of the parabens^[27,29], the increased migratory and invasive properties only developed in the cells after long-term exposure (20 weeks)^[30]. Western immunoblotting showed an associated downregulation of E-cadherin and β -catenin in the long-term paraben-exposed cells, which could be consistent with a mechanism involving EMT^[30].

One technology which has been particularly useful in determining adhesion, migration and invasion of breast cells following exposure to estrogen disrupting chemicals has been the ACEA BioSciences xCELLigence technology, and the increased cell migration following long-term exposure of MCF-7 human breast cancer cells to paraben which was measured using this technology is shown in Figure 1A. This technology works on the basis of a modified Boyden chamber in which two chambers are separated by a membrane. Migration of cells through the membrane on the base of the upper chamber can be monitored in real time at any pre-determined time interval as the cells move onto the undersurface of this membrane located at the top of the lower chamber. The CIM-plate-16 contains 16 wells which can be monitored independently but simultaneously to measure cell migration/invasion in real time through 8 μ m pores in the membrane onto gold electrodes on the underside of the membrane using the ACEA BioSciences xCELLigence analyser system. Cell movement onto the gold electrodes is measured as electrical impedance (cell index). Collated traces of cell index are shown in Figure 1A for cell migration through uncoated membranes following 20 weeks of prior treatment with or without 5×10^{-4} M methylparaben or 10^{-5} M *n*-butylparaben (conditions as published in reference 30).

UV filters

Chemicals which can absorb UV light (UVA and/or UVB) are added to consumer products either to protect the skin of the user from UV damage (sunscreen products) or to protect the product itself from UV damage during storage^[31]. They are also used in textiles marketed as UV protective clothing^[32]. Widespread use of benzophenone-3 (BP-3), octylmethoxycinnamate (OMC) and 4-methylbenzilidenecamphor (4-MBC) has led to their detection in environmental water and soil samples^[33] and they have been recently also measured as present in human breast tissues^[34]. One or more of these UV filters were quantifiable in 84% of the breast tissue samples and in at least one breast region of 95% of the women^[34]. BP-3, OMC and 4-MBC all possess estrogenic activity in reporter gene assays in estrogen-responsive MCF-7 human breast cancer cells^[31,35]. Long-term exposure (23 weeks) to any one of these three compounds has now also been found to increase migratory activity in MCF-7 cells using scratch assays, time-lapse microscopy and xCELLigence technology, and to increase invasion through matrigel as measured using xCELLigence technology^[35]. The increased cell migration following long-term treatment (23 weeks) of the MCF-7 cells with or without 10^{-5} M of BP-3 or OMC is shown in Figure 1B using xCELLigence technology (conditions as published in reference 35). However, increased motility of estrogen-unresponsive MDA-MB-231 human breast cancer cells was also observed after long-term exposure to each of the three compounds, implying the increased migratory activity was not confined to estrogen responsive cells^[35]. Furthermore, molecular mechanisms

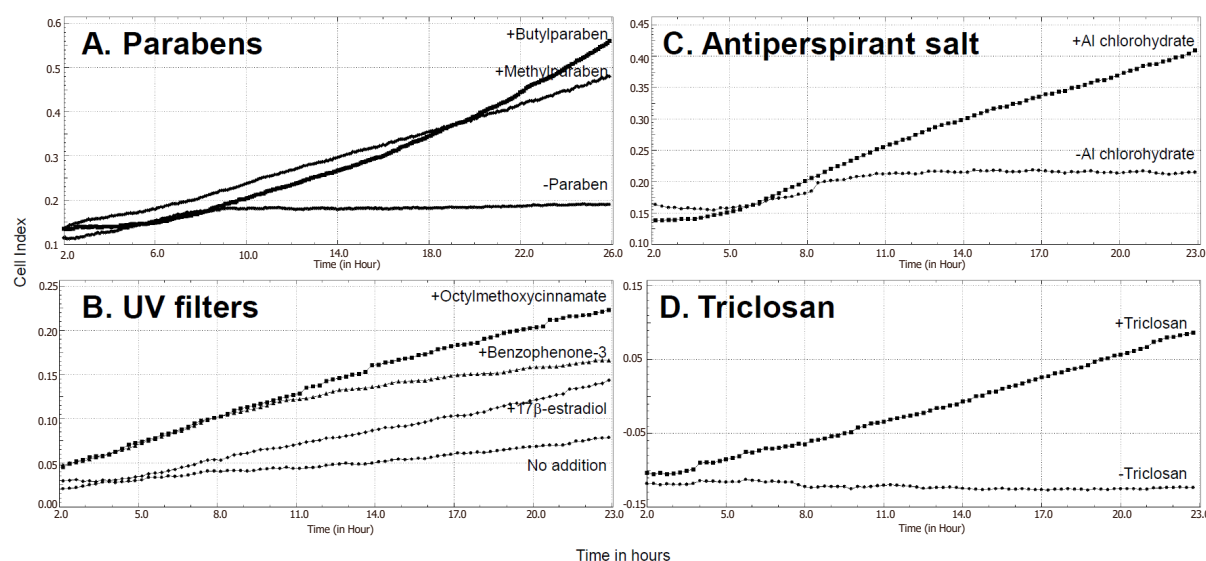


Figure 1. Increases in migration of MCF-7 human breast cancer cells following prior long-term exposure to several estrogen disrupting chemicals contained in personal care products. Cell migration was measured in real-time as electrical impedance (cell index) using xCELLigence technology with uncoated CIM-16 plates. Prior to the assay, cells had been grown: A: for 20 weeks with no addition, 5×10^{-4} M methylparaben or 10^{-5} M n-butylparaben; B: for 23 weeks with no addition, 10^{-8} M 17β -estradiol, 10^{-5} M octylmethoxycinnamate or 10^{-5} M benzophenone-3; C: for 32 weeks with or without 10^{-4} M aluminium chlorohydrate; D: for 9 weeks with or without 10^{-7} M triclosan. Conditions were as published for the parabens^[30], UV filters^[35], and aluminium chlorohydrate^[42]. Data for triclosan are unpublished personal results. UV: ultraviolet

differed between compounds and cell lines, with a noted loss of β -catenin only after long-term exposure to OMC in the MCF-7 cells and an increase in MMP-2 after long-term exposure to OMC and 4-MBC in the MDA-MB-231 cells^[35].

Aluminium-based antiperspirant salts

Aluminium-based salts are used as antiperspirant in underarm cosmetics and dermal absorption of aluminium from this use has been implicated in the development of breast cancer^[36]. Aluminium has been measured in human breast tissue^[37] and breast cyst fluid^[38] at higher levels than in blood, and in nipple aspirate fluid at higher levels in samples taken from women with than without breast cancer^[39]. Aluminium, as well as several other metal ions, is a metalloestrogen^[40] and in the form of the antiperspirant salts aluminium chloride or aluminium chlorohydrate it can displace radiolabeled 17β -estradiol from estrogen receptors and regulate estrogen-responsive gene expression^[41]. However, aluminium has also been shown to increase migratory and invasive activity of human breast cancer cells, although since effects were found not only in estrogen-responsive MCF-7^[42] but also estrogen-unresponsive MDA-MB-231^[43] cells, estrogen-independent mechanisms of action must also exist. The increased cell migration following long-term (32 weeks) treatment of MCF-7 cells with or without 10^{-4} M aluminium chlorohydrate are illustrated in Figure 1C using xCELLigence technology (conditions as published in reference 42). More recent animal model research has shown that non-transformed murine mammary gland (NMuMG) epithelial cells exposed to aluminium chloride *in vitro* were transformed as judged by soft agar assay, and then when injected into three mouse strains with increasing immunodeficiency formed tumours and metastasised *in vivo*^[44]. Untreated cells formed tumours and metastasized to a limited extent in the highly immunodeficient and natural killer (NK) cell deficient NSG mouse strain but not in the less permissive and NK cell competent NOD SKID strain or nude strains. In contrast, NMuMG cells transformed *in vitro* by the aluminium chloride formed large tumours and metastasized in all three mouse models^[44].

Triclosan

Triclosan [5-chloro-2-(2,4-dichlorophenoxy)phenol] is a chlorinated aromatic compound which is added as a broadspectrum antimicrobial agent to consumer goods. It was first used as a hospital scrub but is now added widely to personal care products and homeware products^[45]. It possesses estrogenic activity^[46] and has been detected in human milk^[45] implying its presence in the human breast. It can increase proliferation of estrogen-responsive human breast cancer cells^[45]. More recently, exposure of MCF-7 human breast cancer cells to triclosan has been shown to increase migration and development of EMT associated with downregulation of E-cadherin and upregulation of N-cadherin, snail and slug^[47]. The increased cell migration observed in our laboratory following long-term (9 weeks) exposure of MCF-7 cells to 10^{-7} M triclosan is shown in Figure 1D using xCELLigence technology (personal unpublished data).

Phthalates

Esters of phthalic acid (phthalates) are used as plasticisers in the manufacture of plastics. However, in addition to plastic goods, they are also found in many personal care products, adhesives, paints, air fresheners, food products, pharmaceuticals and textiles^[1]. Many of the esters are listed by the Organisation for Economic Cooperation and Development (OECD) as high production volume chemicals^[48], with some of the most used being diethylphthalate (DEP), dibutylphthalate (DBP), benzylbutylphthalate (BBP) and di-(2-ethylhexyl)phthalate (DEHP). Metabolites of these esters are widely detected in human urine samples of the US population^[49] and can be measured in human milk^[50] implying their presence in the human breast. Many of these widely used esters and their metabolites possess estrogenic activity and can increase growth of estrogen-responsive human breast cancer cells^[51,52]. Using a stem cell-derived human breast epithelial cell line R2d, DBP and BBP were found to induce EMT through an ER-mediated increase in mesenchymal markers and decrease in epithelial markers^[53]. Furthermore, BBP has been reported to increase growth of tumours and lung-derived metastases in an *in vivo* mouse xenograft model using breast cancer stem cells. The mechanism was linked to an arylhydrocarbon receptor-mediated increase in sphingosine-1-phosphate receptor 3 (S1PR3) signaling since downregulation of this pathway reduced both the tumour growth and the lung metastasis but the role of estrogen in this remains unknown^[54].

Bisphenol A

BPA is used in the manufacture of polycarbonate plastics and epoxy resins which are found ubiquitously in building materials and consumer products^[55]. It is used to line food storage containers and water bottles from which it can leach out when heated^[56]. It is listed by the OECD as a high production volume chemical^[48]. A large literature links BPA to adverse health effects in animals and humans^[55], and it is measurable in human body tissues including breast milk^[57] which implies its presence in the human breast. Through its estrogenic activity, it is known to stimulate the proliferation of estrogen-responsive human breast cancer cells^[56]. Animal models have revealed that prenatal exposure can enhance breast carcinogenesis in the chemically induced DMBA-mouse model^[58] and alone can also cause disruption to the mouse mammary tissue increasing susceptibility to breast cancer^[59]. However, chronic exposure to BPA in drinking water during adulthood was also shown to increase tumour burden and incidence of metastasis in a transgenic mouse model that spontaneously develops tumors through overexpression of erbB2^[60]. More recent *in vitro* experiments with human breast cancer cells are beginning to suggest that BPA may increase cell migration and invasion. Exposure of SkBr3 human breast cancer cells to BPA (10^{-8} M) increased cell motility and downregulated the transcription factor FOXA1^[61]. FOXA1 repression is thought to be characteristic of EMT because it is an activator of the E-cadherin gene^[62]. Exposure of the human breast cancer cell lines MDA-MB-231 and BT-549 led to increased invasiveness with an associated increased expression of MMP-2 and MMP-9^[63].

Dioxins

Polychlorinated dibenzodioxins (PCDDs) (dioxins) are highly toxic, bioaccumulative environmental pollutants generated as byproducts of combustion^[64]. They are carried by air, washed off the land by

rainwater into rivers and lakes and then pass up the food chain dissolved in animal fat. They are measured ubiquitously in human tissues^[64]. There are 75 congeners of which the most toxic is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)^[64] and has been shown to exert molecular actions both through ER-mediated and AhR-mediated mechanisms^[65]. Gene targets of the AhR include activation of slug and some MMPs^[66]. In the ER+, AhR+ MCF-7 human breast cancer cells, TCDD has been found to downregulate E-cadherin and reduce cell-cell contacts in a JNK-dependent mechanism^[67]. However, antiestrogenic actions of TCDD have been repeatedly reported over the years^[65], and so it is noteworthy that TCDD has been reported to inhibit mammary tumour metastasis *in vivo*^[68,69].

Phytoestrogens

Phytoestrogens are found in over 300 plant species and can be ingested by humans through consumption of plant material either in diet or as dietary supplements^[70]. Flavonoids include genistein and daidzein found in soybeans and other legumes, coumestans in clover and young sprouting legumes, prenylflavonoids in hops. The most prevalent non flavonoids are lignans in cereals, fruits and vegetables. Although many purified phytoestrogens display estrogenic activity *in vitro*, the estrogenic actions on cell proliferation are concentration-dependent with only the lower doses increasing proliferation of estrogen-responsive human breast cancer cells, whilst higher doses inhibit^[71], and many phytoestrogen-containing plant products are considered to have anti-tumour activity^[70]. It is interesting therefore that emerging data seem to suggest that several phytoestrogens can act to reduce breast cancer cell migration and invasion including lignans^[72], soy-derived daidzein^[73], and formononetin^[74]. Triclosan-induced EMT can be reversed by kaempferol^[47]. However, one mouse model of breast cancer has shown that consumption of soy isoflavones increased growth of metastatic tumours in bone and lung^[75].

THE ISSUE OF LOW-DOSE MIXTURES

The studies discussed above provide evidence that some individual estrogen disrupting chemicals can influence components of EMT and processes of migration and invasion in human breast cancer cells. However, since many hundreds of estrogen disrupting chemicals have now been measured in the human breast^[1], the environmental reality is that, in today's world, human breast cells *in vivo* are not exposed to single chemicals but to complex mixtures of pollutant chemicals. This is especially poignant in view of many estrogen disrupting chemicals being lipophilic and the human breast being a fatty organ. It is also highly relevant in the context of the many non-monotonic responses which have been reported for environmental endocrine disrupting chemicals in general, often with stronger responses at the relatively lower doses tested^[76]. There are therefore outstanding questions as to the effects of exposure to complex mixtures of estrogen disrupting chemicals where each is present at low dose. For proliferation, it has been shown that mixtures of such chemicals can increase cell proliferation where the same concentrations of the estrogen disrupting chemicals individually do not, in what has now been nicknamed "the something from nothing" effect^[77]. Even mixtures of the different esters of parabens can add together to give increased cell proliferation^[29]. This is relevant environmentally where human breast tissue samples were found to contain differing levels of the individual paraben esters^[26] and implies that the same outcomes can arise from different mixtures of estrogen disrupting chemicals^[29]. Clearly in ER-mediated mechanisms, such as in increasing cell proliferation, each chemical will act according to its relative estrogen receptor binding affinity but low doses of different chemicals can add together until a maximum proliferative signal is achieved. As yet, there seems to be no published information as to whether the same effect of mixtures can be achieved on development of EMT or on increasing cell migration/invasion. Furthermore, since these studies are technically long and labour-intensive, the range of concentrations are often lacking in order to determine the extent to which non-monotonic responses may be occurring.

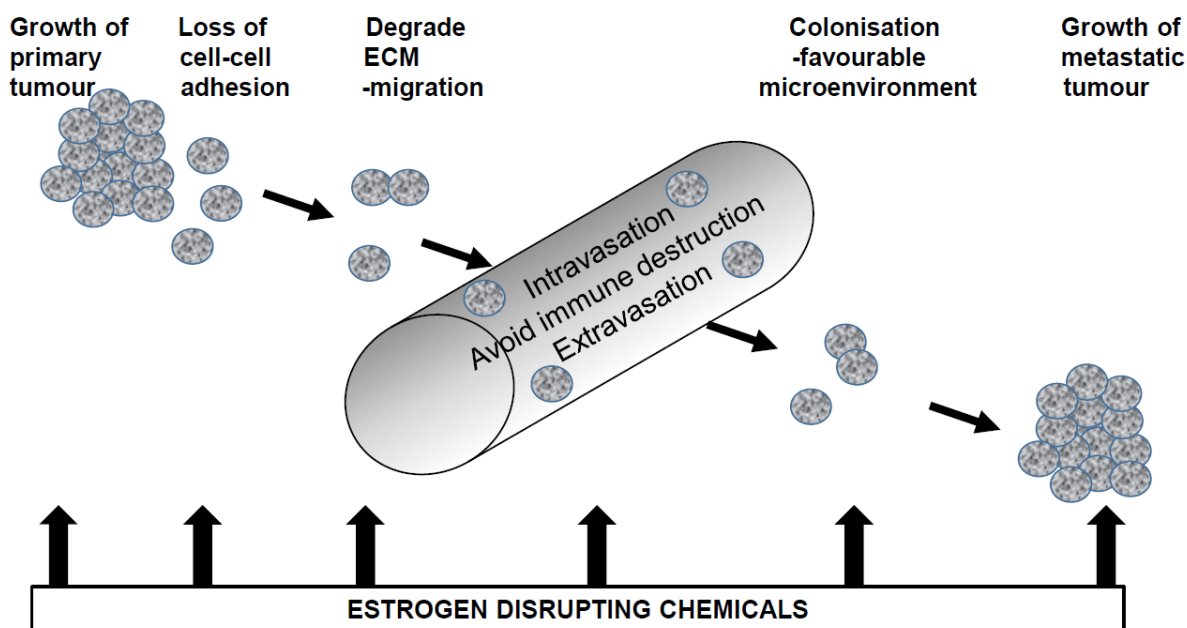


Figure 2. Diagrammatic representation of how estrogen disrupting chemicals may influence metastasis of breast cancer cells. Through their estrogenic activity, they may increase growth of cells at the primary site in the breast or at metastatic sites. They may enable EMT, increase cell migration and cell invasion. Through their widespread presence in the human body, they may provide a favourable microenvironment for colonization and metastatic tumour growth

CONCLUSIONS

It can be concluded that there is mounting evidence for a role of estrogen disrupting chemicals in contributing to the processes of metastatic tumour spread and this is summarized in Figure 2. Estrogen disrupting chemicals may contribute to loss of cell-cell adhesion, development of EMT and increased secretion of ECM-degrading proteases leading to increased cell motility, migration and invasion. However, estrogen disrupting chemicals are also likely to play a role in creating a favourable microenvironment for colonization and growth of metastatic tumours at distant sites. Unpredictable awakening from dormancy^[7] could result from altered environmental exposures or indeed sudden release of estrogen disrupting chemicals from fat stores such as at times of weight loss. It is well established that estrogen disrupting chemicals are passed from mother to child in breast milk as they are mobilized with the fat in the milk^[78]. Such detoxification of the the mother's breast may provide an alternative explanation for the protective effect of breast feeding on incidence of breast cancer^[79]. Likewise, release of estrogen disrupting chemicals from storage could change the microenvironment for "dormant niches"^[7] causing renewed proliferation.

Research is now needed to answer outstanding questions concerning the effects of long-term exposure to low doses of chemicals and the effects of complex mixtures of chemicals^[80]. Since estrogen disrupting chemicals are widely measurable in human tissues and some can bioaccumulate with age, then it follows that breast cells are exposed long term which requires long term cell culture modelling for further understanding. This is especially poignant in the acquisition of increased migratory and invasive properties described above which generally took weeks rather than days to develop. The effects of mixtures of chemicals each at low dose will be more difficult to resolve because personal lifestyle choices will inevitably result in differing chemical contents between individuals. Furthermore, due to the additive and complementary mechanisms of estrogen disrupting chemicals^[81], different mixtures may have the same outcomes which makes tracing individual culprit chemicals impossible. However, if specific environmental exposures can be uncovered, then avoidance would be a good strategy for prevention. In 2001, I proposed a hypothesis that regular application of constituents of underarm cosmetics might play a role in the rising

incidence of breast cancer if sufficient of the constituent chemicals were absorbed either from long-term exposure on the skin (these products are not washed off) or from damaging the skin by shaving prior to cosmetic application^[82,83]. This was proposed partly due to the disproportionately high incidence of breast cancer in the upper outer quadrant of the breast which is coincidentally also the site of application of these chemicals. Many years on, it is now known that many of the constituent chemicals can be absorbed through even intact skin after a single application^[84,85] and that they are measurable in human breast tissue or human milk (see above for references). Published studies have linked the levels of some of these estrogen disrupting chemicals in the body tissues with personal care product usage^[86,87]. However, it is also clear that these chemicals are getting into many body tissues other than breast and therefore could be expected to create distant estrogenic microenvironments which could influence not only the growth of the primary tumour but also growth of metastatic tumours. If excessive use of personal care products is involved in the development of metastatic breast cancer, then reduction or cessation in use could provide a prevention strategy.

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This review was written solely by the author and reflects the author's views.

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The author declares that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Case Report

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Syndrome of inappropriate antidiuresis in prostate adenocarcinoma with neuroendocrine differentiation: a case report and literature review

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Abstract

Syndrome of inappropriate antidiuresis (SIAD) is a common paraneoplastic syndrome commonly associated with thoracic malignancies, gastrointestinal cancers and kidney tumors. It is defined as hyponatremia in euvolemic patients, often due to abnormal secretion of antidiuretic hormone by tumor cells. Tolvaptan, a vasopressin-2-receptor antagonist, is currently recommended for patients affected by SIAD with mild or moderate symptoms. Among patients with prostatic cancer, SIAD represents a rare condition but it is frequently associated with poorly differentiated adenocarcinoma or pure small-cell carcinoma histotype. We report a case of SIAD appeared at disease progression in a 60-year-old male patient with acinar adenocarcinoma with neuroendocrine differentiation together with a literature review.

Keywords: Hyponatremia, prostate cancer, syndrome of inappropriate antidiuresis, neuroendocrine differentiation

INTRODUCTION

Prostate cancer is the most common non-cutaneous cancer in Western men, with estimated 1,600,000 cases annually^[1]. The recent World Health Organization (WHO) 2016 classification



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of neuroendocrine (NE) tumors of prostatic adenocarcinoma (PAC) includes the following four categories: Adenocarcinoma with neuroendocrine differentiation, Well-differentiated NE tumor (carcinoid tumor), Small cell NE carcinoma and Large cell NE carcinoma. Gleason score and grade group are the standard approach to histologic grading of PAC, but not for the NE components, and it is an important independent prognostic factor^[2,3]. Clinical presentation and prognosis usually depend on local infiltration and metastatic spread. Paraneoplastic syndromes including syndrome of inappropriate antidiuresis (SIAD) are rare, mostly in poorly differentiated PAC, frequently with NE features^[4]. SIAD is commonly associated with small cell lung cancer (SCLC), non-small cell lung cancer (NSCLC), gastrointestinal cancers, kidney cancers and mesothelioma^[5]. In the last few years, several international and national guidelines have been published in order to improve clinical managing of this electrolyte disorder^[6]. Oral tolvaptan, a vasopressin-2-receptor antagonist, has been approved by FDA and EMA for treatment of euvolemic hyponatremia due to SIAD with mild or moderate symptoms. Vaptans demonstrated to improve serum sodium concentration in patients with SIAD but there are still few data about their possible effect on survival of cancer patients^[7].

CASE REPORT

A 60-year-old male with PAC and abdominal lymph nodes and axial bone metastasis came to our Department. An informed consent to procedures and therapy was obtained from the patient. At first observation, the patient was in good clinical condition (Performance status according to Eastern Cooperative Oncology Group-ECOG-PS = 0) and he did not present significant comorbidities other than well-controlled arterial hypertension.

PAC diagnosis occurred in June 2014 when, after onset of urinary frequency and dysuria, patient underwent prostate biopsies. Histological examination reported prostatic acinar adenocarcinoma Gleason score 5 + 4 = 9 (grade group 5, with NE differentiation = 5%) [Figure 1]. PSA was 22 ng/mL.

After diagnosis, a CT scan showed left iliac chain, para-aortic and inguinal bilateral lymphadenomegalies and osteoblastic metastasis to lumbar spin, multiple ribs and hip with no fracture risk. A bone scintigraphy confirmed bone involvement [Figure 2]. Laboratory values did not show clinically significant abnormalities.

In August 2014 the patient started complete androgen deprivation therapy with oral Bicalutamide 50 mg daily along with intramuscular Leuporelin 11.25 mg/mL quarterly. Denosumab 120 mg monthly was started to prevent skeletal events. Serum PSA showed initial complete biochemical response (0.3 ng/mL). In November 2014 PSA raised to 10 ng/mL. The patient presented with clinical worsening including increased urinary frequency and dysuria. Considering biochemical and clinical progression, Bicalutamide was stopped and first-line chemotherapy with Docetaxel 75 mg/m² intravenously every three weeks was started. The patient underwent five cycles of chemotherapy until May 2015, continuing Denosumab and Leuporelin. PSA decreased to 8.2 ng/mL, with limited clinical benefit. After multidisciplinary evaluation, the patient was considered eligible for radiotherapy on the prostatic region. This treatment was performed between May 2015 and July 2015 (total dose: 5400 cGy).

After radiation therapy, PSA raised to 17.4 ng/mL and the patient was referred to our department for dorsal and abdominal pain. A total-body CT scan performed in August 2015 showed disease progression, including rectal thickening, multiple liver metastasis, peritoneal carcinosis and increase of abdominal and pelvic lymphadenomegalies. CT also showed disseminate osteoblastic metastasis [Figure 3].

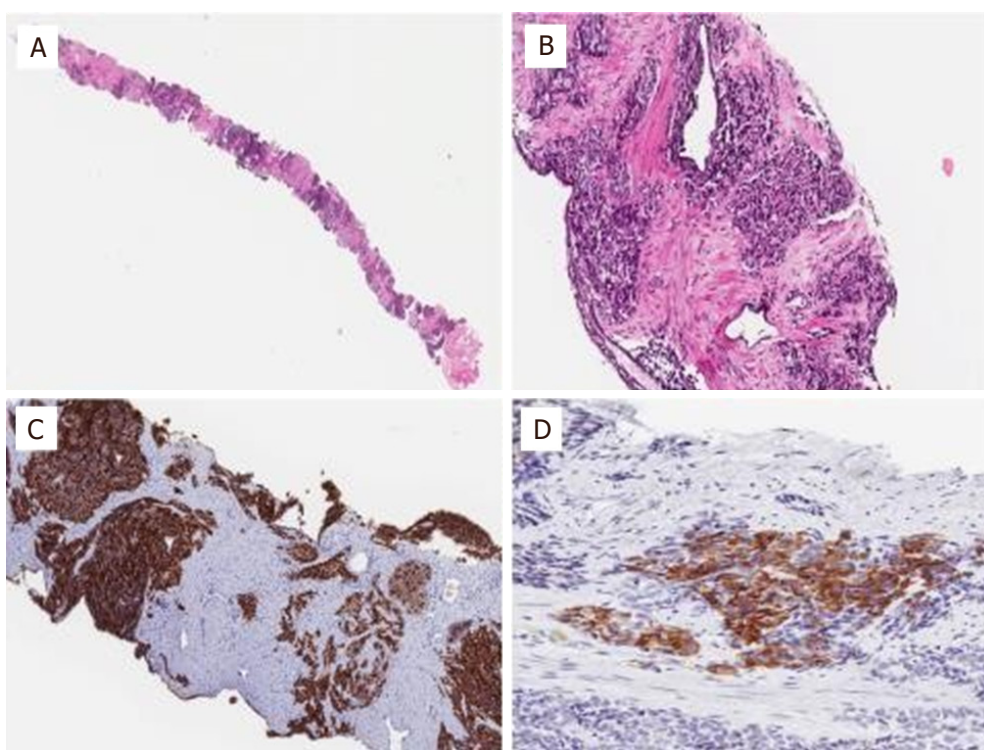


Figure 1. A: Prostate biopsy extensively involved by acinar adenocarcinoma with NE differentiation (5%) at low magnification (hematoxylin and eosin-H&E); B: prostate biopsy with solid area of adenocarcinoma at high magnification (H&E); C: prostate biopsy with diffuse positivity for prostate specific membrane antigen (PSMA, immunohistochemistry); D: prostate biopsy with focal positivity for synaptophysin (immunohistochemistry)

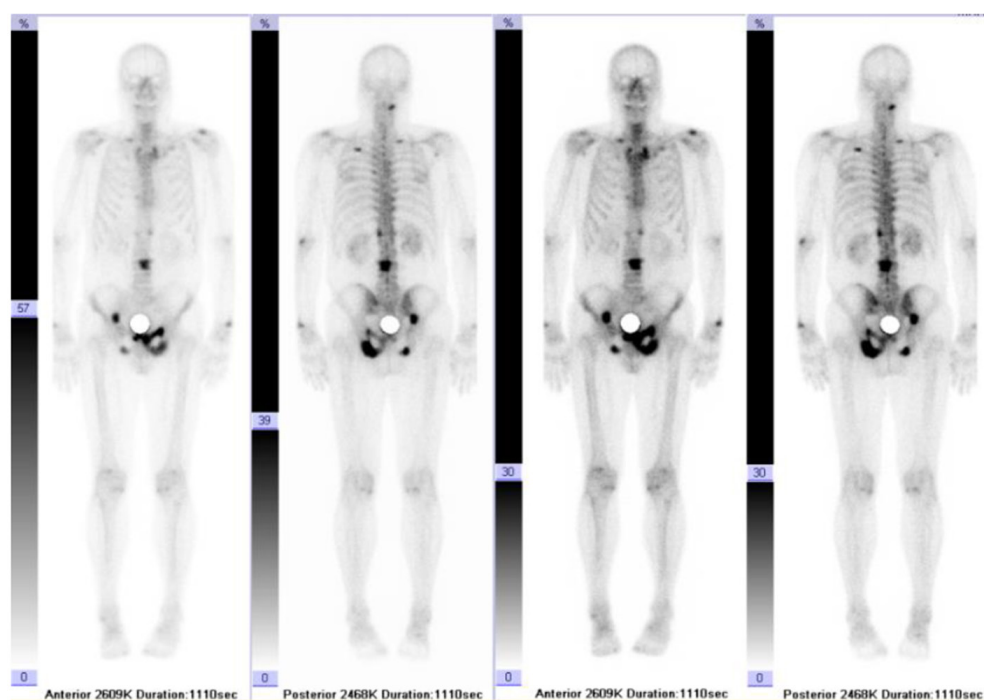


Figure 2. Bone scintigraphy confirming skeletal involvement

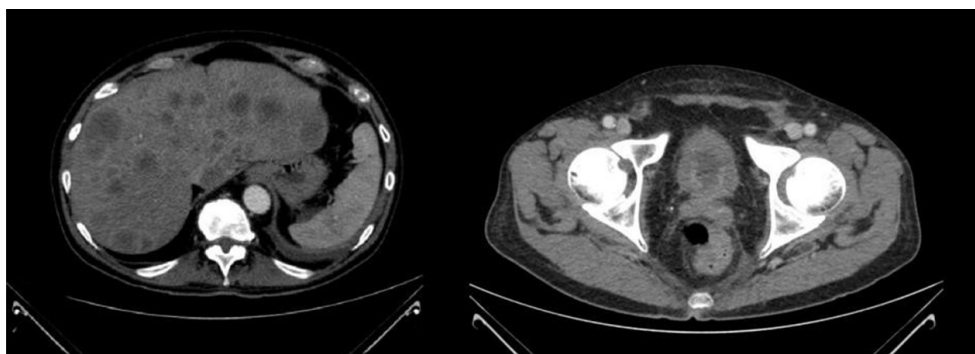


Figure 3. CT scan showing liver metastatization and rectal thickening

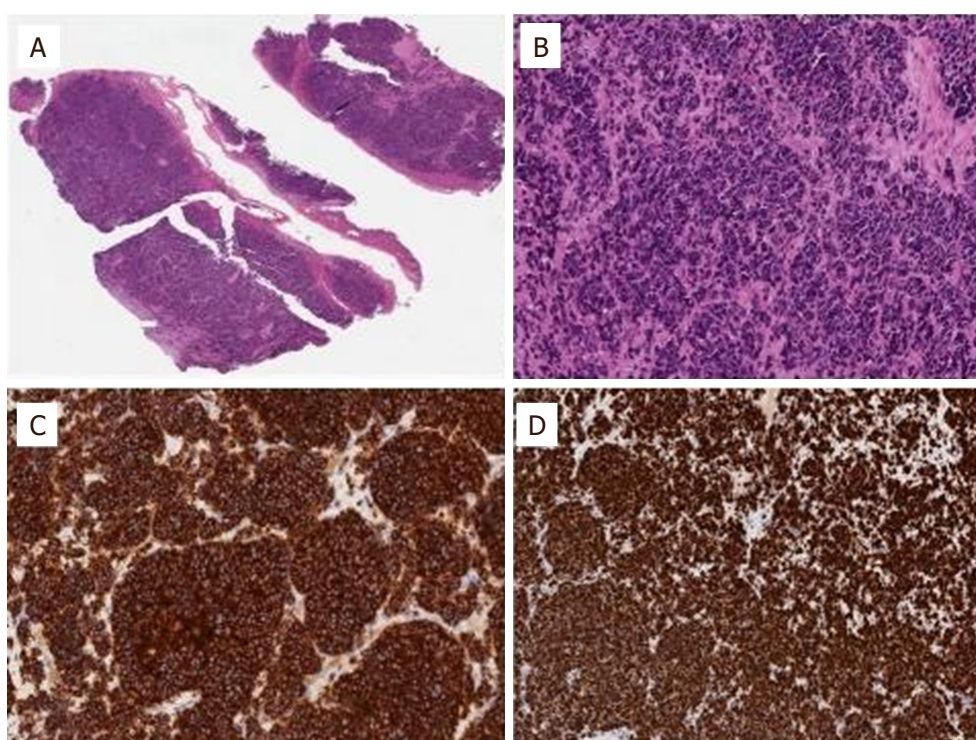


Figure 4. A: Liver metastasis at low magnification (H&E); B: Liver metastasis at high magnification showing small cell carcinoma (H&E); C: Liver metastasis showing diffuse positivity for synaptophysin (immunohistochemistry); D: Liver metastasis showing high proliferation rate (Mib1, immunohistochemistry)

Patient was hospitalized at the Surgery Department in our hospital where he underwent colostomy to palliate bowel obstruction and third-segment liver lesion biopsy. The histological examination of the liver showed metastasis of small cell NE carcinoma (Mib1 = 90%) without prostatic features at immunohistochemistry [Figure 4 and Table 1].

After that, the patient came back to our observation in September 2015 in fair conditions (ECOG-PS = 1), with partial pain control, constipation and mild mental confusion. At physical examination, no neurological impairment was reported.

Laboratory evaluation showed PSA = 26.7 ng/mL, NSE = 1238 ng/mL, chromogranin A = 454 ng/mL, rise of cholestasis values grade 1 according to Common Terminology Criteria for Adverse Events (CTCAE) v4.0 and first occurrence of moderate hyponatremia (125 mEq/L).

Table 1. Comparison between prostate and liver histology

	Prostate	Liver
Morphology	Acinar adenocarcinoma with neuroendocrine differentiation (5%), Gleason score 5 + 4 = 9 (grade group 5)	Small cell neuroendocrine carcinoma
PSMA	Diffuse positivity	Negative
Synaptophysin	Focal	Diffuse
Mib1	50%	90%

PSMA: prostate specific membrane antigen

After Endocrinological Consultant, we performed the following evaluations: glucose = 96 mg/dL, serum creatinine = 0.35 mg/dL, blood urea nitrogen = 9.8 mg/dL, plasma osmolality = 259 mOsm/kg, urinary osmolality = 322 mOsm/kg, urinary calcium = 2.4 mg/dL, urinary sodium = 115 mEq/L, urinary potassium = 5.3 mEq/L, TSH = 1.1 mcU/mL, ACTH = 14 pg/mL, plasmatic cortisol = 9.9 mcg/dL, venous bicarbonates = 25 mmol/L. Therefore, considering also euvolemic status and no concomitant use of diuretics, we diagnosed SIAD and started treatment with Tolvaptan 15 mg/die in October 2nd 2015 carefully monitoring plasmatic sodium every six hours. Sodium increase was < 10 mmol/die up to Na^+ = 133 mEq/L and the patient continued progressively to reduce Tolvaptan dosage till a maintenance dose of 3.75 mg/die, monitoring plasmatic sodium daily.

Considering last histological examination and the clinical benefit with vaptan, after evaluation, the patient started a second-line chemotherapy with Cisplatin 80 mg/m² i.v. day 1 and Etoposide 100 mg/m² i.v. days 1-3 every three weeks. After three cycles of chemotherapy, the patient experienced grade 3 anemia, grade 2 thrombocytopenia, grade 4 leucopenia and grade 4 neutropenia, requiring blood transfusion and administration of granulocyte growth factor and antibiotics. No other toxicities were reported. Patient underwent a fourth cycle with reduced doses. PSA value was stable, while NE tumor markers increased.

In December 2015, patient experienced bone disease progression at CT scan. A month later, he died due to liver failure.

DISCUSSION

Hyponatremia is defined as serum sodium concentration (Na^+) lower than 135 mmol/L^[8,9] and it is one of the most common electrolyte disorders occurring in cancer patients with an estimated incidence between 5% and 20%^[10,11]. The most frequent causes include SIAD, due in most cases to ectopic production of antidiuretic hormone, extracellular fluid depletion or renal toxicity of chemotherapy, especially platinum-based^[5].

The main diagnostic criteria for SIAD are: euvolemic status, reduced plasma osmolality < 275 mOsm/kg, increased urine osmolality > 100 mOsm/kg, increased urinary sodium > 30 mEq/L, normal kidney, thyroid and adrenal function. Additional diagnostic criteria are: no use of diuretics, reduced blood uric acid < 4 mg/dL, reduced blood urea nitrogen (BUN) < 10 mg/dL, increased sodium renal excretion fraction > 1% and increased urea excretion fraction > 55%^[6].

Correct management of hyponatremia represents an important issue for cancer patients considering its well-demonstrated role in increasing mortality, costs and length of hospitalization^[12-14]. Treatment depends on underlying causes of hyponatremia but also on severity and time of onset. In the context of SIAD, vaptans represent a relevant therapeutic option for mild-moderate reduction^[15].

Literature review revealed that most PAC-associated SIAD cases are high grade and high stage adenocarcinoma. Actually, SIAD can be linked to neuroendocrine evolution at disease progression. Pure small-cell carcinoma of the prostate is extremely rare, accounting for < 0.5% of all cases^[16]. About 50% of small cell carcinomas (SCC) have a history of usual PAC. Frequently, at initial diagnosis SCC are seen admixed with adenocarcinoma of the prostate^[17]. In these cases, Gleason score is assigned only to the conventional adenocarcinoma component. According to the SEER database, the presence of concomitant high-grade PAC is an independent predictor of poorer cancer-specific mortality^[18].

Recent data on *in vitro* and *in vivo* studies related to the molecular mechanism of NE transdifferentiation of PCa cells suggest that PCa cells undergo a transdifferentiation process to become NE-like cells, acquiring NE phenotype and NE markers. Common molecular alterations between PAC and SCC components of mixed prostate tumors under conditions of androgen deprivation sustain the hypothesis that small cell carcinoma represents a transdifferentiation from PAC^[19]. The model of divergent-differentiation consider instead the hypothesis of a common stem cell progenitor that can differentiate into both adenocarcinoma and SCC. According to this model, hormonal therapy determinates a selective pressure resulting in development of SCC from a subpopulation of hormone-independent cells and consequently inducing hormonal resistance^[20]. The more aggressive behavior compared with conventional PAC is due to expression of genes involved in cellular proliferation, mitosis, neuroendocrine differentiation, along with downregulation of genes encoding cell adhesion molecules^[21]. This biological molecular phenotype is the reason behind the high proliferation rate, the rapid spreading and the great tendency to metastasize. Further preclinical results, deriving mostly from SCLC cell lines, seem to confirm the relation between hyponatremia, directly or through vasopressin receptors and cell proliferation, resistance to programmed cell death, angiogenesis and metastatization. Mitogen-activated protein kinase (MAPK) pathway is the main intracellular signalling which leads to tumoral growth and spreading^[22,23]. SIAD onset may guide physicians to perform a tumor biopsy in order to obtain a new morphological and immunohistochemical characterization^[24].

Neuroendocrine prostatic cancer is generally managed by chemotherapy regimens similar to those for SCLC. Prognosis is poor with a median survival of 7 months.

It is still unclear how SIAD arises from prostatic carcinoma with neuroendocrine differentiation but it may involve an ectopic production of ADH from carcinoma cells, as detected immunohistochemically in the tumor tissue^[25].

In our case-report, SIAD appeared at progression of disease, with metastatic spread to the liver. In particular a dedifferentiation of cancer cells was reported, which lost prostatic markers to acquire fully neuroendocrine characteristics. Only focal neuroendocrine prostatic markers were reported in the primary prostatic cancer [Figure 1], while liver metastasis presented neuroendocrine carcinoma [Figure 4]. Our case supports the hypothesis that histological evolution to neuroendocrine features accompanies to metastatic spread and often with paraneoplastic syndrome as SIAD.

DECLARATIONS

Authors' contributions

Conception and design of the study and performed data analysis and interpretation: Fiordoliva I, Marcantognini G, Rinaldi S

Performed data acquisition, as well as provided administrative, technical and material support: Cimadamore A, Montironi R, Berardi R

Availability of data and materials

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Written informed consent for diagnostic and therapeutic procedures was obtained.

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Editorial

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Introduction to this special issue - “Autophagy and Cancer: current biology and drug development”

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It is my pleasure, as one of the editorial board members, to introduce the readers of *JCMT* to this special issue entitled “Autophagy and Cancer: current biology and drug development”.

Autophagy is a fundamental process for cells to degrade unwanted proteins/damaged organelles and also to recycle cellular components. Since its discovery on 1960's, a vast amount of effort has been made in understanding the physiological role/s of this process. For example, it is now known that mitosis, apoptosis, and autophagy are inter-connected and inter-regulated in cells^[1]. It is also known that upregulation of autophagy is a double-edged sword that promotes both cell survival and cell death, depending on the circumstances. However, the pathological role/s of autophagy in normal-to-cancer cell transformation, tumor development, and tumor drug resistance was not clear until the arrival of various breakthrough discoveries in the past 15 years. Noticeably, it has been demonstrated that dysregulation of autophagy (and probably downregulation) induces genomic instability in non-cancerous cells and subsequently promotes tumorigenesis^[2]. In contrast, upregulation of autophagy has been shown to enhance the survival ability of cancer cells in response to various micro-environmental stresses and different chemotherapeutic agents^[3]. Therefore, autophagy is currently a “hot” cellular pathway target for the development of cancer therapeutics^[4-7].

This special issue contains reviews focusing on recent understandings on the regulation of autophagy in non-cancerous cells and dysregulation of this process in cancer cells. Reviews on recent advances in the development of autophagy modulators for cancer treatment are also included in this special issue.



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Review

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Muscle mitochondria and oxidative metabolism as targets against cancer cachexia

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Abstract

Cancer cachexia is a debilitating syndrome mainly characterized by muscle and fat wasting, leading to the progressive loss of body weight and complicating the management of cancer patient. In particular, the loss of muscle weight is a negative prognostic factor, being associated with chemotherapy toxicity and reduced survival. Increased inflammation and protein dysmetabolism are some of the impairments that lead to muscle wasting in cancer patients. Together with these alterations, tumor growth and chemotherapy administration may affect mitochondrial function, impinging on the muscle energy metabolism. Indeed, therapeutic approaches poised to correct both hypercatabolism and mitochondrial alterations could be effective in preventing cancer-induced muscle wasting. Among the non-pharmacological approaches, exercise training is one of the best modulator of muscle physiology able to impinge on both protein and energy metabolism. However, the wasting phenotype that characterizes cancer patients could be not compatible with physical training, prompting the development of different strategies to improve muscle metabolism. The aim of this mini-review is to discuss both the beneficial effects and the limitations of exercise training in cancer cachexia and the adoption of drugs able to modulate exercise-induced pathways.

Keywords: Muscle wasting, exercise, PGC-1 α , trimetazidine, erythropoietin



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INTRODUCTION

Cachexia is a metabolic disorder that occurs in different chronic diseases, including cancer, heart failure, kidney disease, chronic obstructive pulmonary disease, sepsis and rheumatoid arthritis^[1]. The prevalence of cachexia is particularly high in cancer patients, ranging from 50% to 80%, depending on tumor type^[2]. Cancer cachexia is characterized by body weight loss, systemic inflammation and metabolic alterations. The occurrence of this syndrome affects patient's quality of life and increases both morbidity and mortality due to cardiovascular complications, immune disorders and nutritional deficiency^[3,4]. Body weight loss is mainly due to the consistent depletion of muscle and fat mass, that is frequently worsened by chemotherapy toxicity^[5]. Humoral mediators and altered energy balance activate proteolysis in the skeletal muscle of cancer hosts, ultimately leading to protein and organelle disposal^[6]. However, the specific blockade of intracellular muscle proteolytic systems does not prove effective in counteracting cancer-induced wasting^[7-9], focusing the investigation to upstream pathways that regulate muscle metabolism and energetics. In particular, several lines of evidence show that alterations of the energy metabolism and mitochondrial impairments may contribute to the onset and progression of muscle wasting in cancer^[10]. In this regard, cancer hosts are characterized by low nutrient intake and increased energy expenditure^[1]. Whereas the former is induced by anorexia, dysphagia and/or altered absorption, due to chemotherapy or to tumors with esophageal/gastrointestinal localization^[11], the latter is enhanced by tumor metabolism, inflammation and mitochondrial alterations^[1]. Notably, one of the first evidences of energy wasting in cancer cachexia derive from studies on uncoupling proteins (UCPs), whose expression is increased in the skeletal muscle and adipose tissue of tumor-bearing animals and cancer patients^[12-15]. Along this line, a time-course analysis in lewis lung carcinoma (LLC)-bearing mice has shown that mitochondrial ROS emission, mitochondrial degradation and respiratory function are impaired prior to the loss of muscle mass in the host mice and, interestingly, some of these alterations appear early after tumor implantation^[16]. A proteomic profiling of both skeletal and cardiac muscles of C26-bearing mice, another syngeneic model of cancer cachexia, has revealed an impaired expression of proteins involved in energy homeostasis and mitochondrial function^[17]. Consistently, cachectic cancer patients show altered levels of proteins accepted as markers of mitophagy, such as Parkin, PTEN-induced putative kinase 1 (PINK1) and BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), and mitochondrial dynamics, such as mitofusin 2 (Mfn2) and mitochondrial fission 1 protein (Fis1)^[18,19]. In addition, it is well established that anti-cancer treatments alter mitochondrial homeostasis. In this regard, healthy mice treated with FOLFOX or FOLFIRI (the combination of 5-fluorouracil and leucovorin with either oxaliplatin or irinotecan, respectively) present with reduced mitochondrial mass and oxidative capacity in the skeletal muscle^[20]. Similar results have been reported in C26-bearing mice treated with chemotherapy (oxaliplatin and 5-fluorouracil)^[21], showing that anti-cancer treatment significantly increased the lifespan of C26-bearing mice but exacerbate muscle wasting as compared to untreated C26 hosts^[21]. Such wasting effect has been associated with reduced peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and cytochrome c expression and with increased markers of mitophagy, together with impaired oxidative capacity and ATP levels^[21]. Using a proteomic approach, alterations of the tricarboxylic acid (TCA) cycle and impaired expression of markers of mitochondrial fusion, fission and biogenesis have been reported in both untreated and chemotherapy-treated C26-bearing mice^[22]. A metabolic profiling performed in the same C26 hosts exposed to chemotherapy shows that glycolysis and β-oxidation are also impaired^[23].

Overall, since muscle metabolic phenotype in cancer hosts is severely impaired by both tumor growth and chemotherapy, strategies targeting mitochondria and/or improving the oxidative capacity could usefully integrate a multimodal therapy for cancer cachexia.

EXERCISE AS A MODULATOR OF MUSCLE METABOLISM

Physical activity is associated with several metabolic adaptations that particularly affect the skeletal muscle^[24]. Depending on the type and frequency of exercise, different targets are preferentially regulated.

Acute exercise leads to transient muscle molecular changes (e.g., temporary increase in specific mRNA expression), whereas chronic exercise induces physiological adaptations that could modify muscle phenotype^[25]. Regarding the differences in terms of exercise type, resistance training imposes a low-repetition and high-intensity demand on the skeletal muscle, whereas endurance exercise does the opposite^[26], resulting in specific skeletal muscle adaptations. Thus, resistance training leads to the induction of myofibrillar protein synthesis and increased anaerobic capacity, whereas endurance exercise sessions mainly lead to improved mitochondrial density and function, oxygen delivery and uptake, anti-oxidant defenses and resistance to fatigue^[25]. The beneficial effects of exercise have been tested in different chronic conditions, including coronary heart disease, type 2 diabetes mellitus, obesity and aging^[24,27]. Notably, both exercise types exert beneficial effects in cachectic animals. Resistance training by ladder climbing has been reported to relieve body weight loss, muscle wasting and inflammation in tumor-bearing rats^[28,29] and to increase the expression of factors associated with myogenesis in cachectic mice^[30]. In *Apc^{+/-Min}* mice overexpressing IL-6, endurance exercise prevents body weight loss, improves insulin sensitivity, increases mitochondrial biogenesis and muscle oxidative capacity and restores normal signaling through Akt and 5' AMP-activated protein kinase (AMPK)^[31,32]. Endurance exercise by voluntary wheel running is effective also in C26-bearing mice, reducing body weight loss, muscle wasting and the expression of markers of autophagy and proteasome-dependent degradation^[33]. However, the combination of the two types of exercise could result in better outcomes in cancer hosts. This was suggested by the results of an exercise protocol in which the slope was increased in a mild intensity run protocol using a motorized wheel. Indeed, this protocol mixed endurance exercise, due to the moderate and continuous training, to the resistance exercise, resulting by the increased hill as compared to the flat treadmill run. Notably, C26 hosts performing this exercise protocol have shown improved muscle wasting and strength, together with reduced autophagy and oxidative stress markers^[34]. On the contrary, endurance exercise with treadmill in a flat mode does not succeed in improving cachexia in C26 hosts^[35]. The effect of exercise through a motorized wheel has also been tested in a model of cancer (C26 tumor) and chemotherapy-induced muscle wasting, partially preventing the loss of muscle mass and strength^[21]. These improvements are associated with a partial restoration of mitochondrial homeostasis^[21]. Notably, exercise increases mitochondrial mass, normalizes the levels of PINK1, BNIP3 and Mfn2, improves the succinate dehydrogenase (SDH) activity and increases energy stores^[21]. In a recent study, the resistance exercise component has been further increased by combining the motorized wheel to the ladder climbing. This combined protocol relieves muscle wasting in C26-bearing mice, reducing autophagy markers and increasing muscle oxidative metabolism^[36].

The effects of physical training in cancer patients may vary depending on tumor type, stage and exercise modalities. A systematic review analyzing the effectiveness of aerobic exercise during chemotherapy treatment in cancer patients, has shown beneficial effects in terms of both quality of life and muscle function^[37]. In chemotherapy-treated breast cancer patients, exercise increases muscle fiber cross-sectional area, citrate synthase activity, mitochondrial complex protein levels and muscle capillarization^[38]. Supervised progressive resistance training increases muscle strength in pancreatic cancer hosts^[39]; similarly, it decreases fatigue and improves quality of life in head and neck cancer patients^[40]. Although these encouraging data, exercise has some limitations in terms of feasibility and patient compliance. In this regard, a study in a cohort of 196 cancer patients with established cachexia has shown a scarce adherence to exercise programs, with low ability to perform either aerobic or resistance training^[41]. Along this line, cancer patients with severe or refractory cachexia have a lack of interest in, or cannot cope with, exercise or do not complete the initially planned program^[42]. In addition, exercise is not free of risks and may harm cancer patients presenting with co-morbidities (anemia or cardiac dysfunctions)^[43] or with excessive loss of muscle mass and low energy availability. Depending on exercise type and duration, physical activity may worsen body and muscle wasting also in cachectic animals. Notably, in C26-bearing mice 2-week-long treadmill run exacerbates body and muscle wasting as compared to sedentary tumor bearers^[35]. In

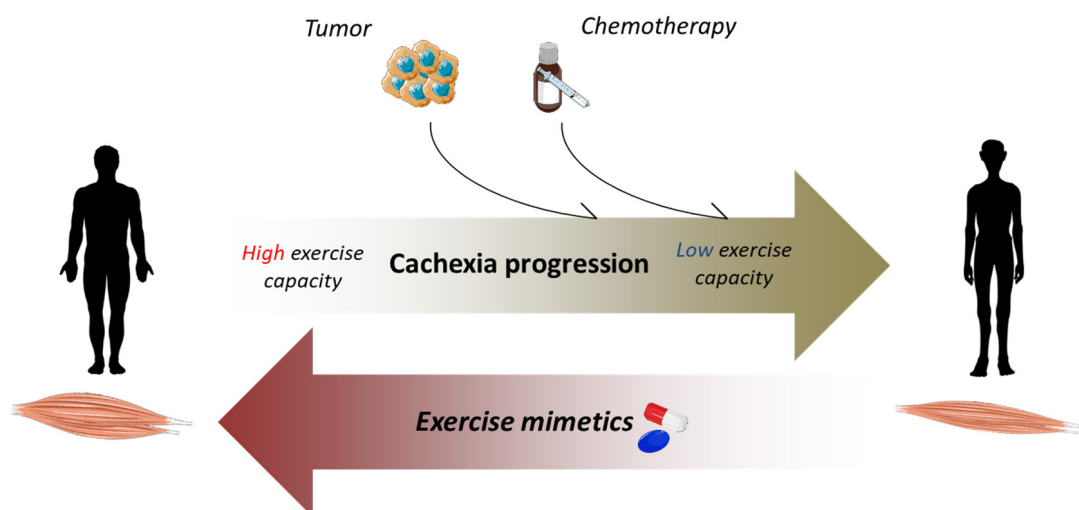


Figure 1. Exercise mimetics may positively affect cachexia progression and low exercise performance. In cancer patients, tumor growth and chemotherapy may induce anemia, cardiac dysfunction and mitochondrial alterations, leading to loss of muscle mass and reduced exercise performance. Exercise-like drugs may relieve muscle wasting and/or support cancer patients practicing exercise

addition, exercise performed in late-stage cachexia reduces the survival of C26-bearing mice exposed to chemotherapy^[21]. Given these premises, strategies that circumvent the limitations of exercise or improve exercise capacity could be useful for cancer hosts who are intolerant to physical training^[44] [Figure 1].

TARGETING EXERCISE-INDUCED PATHWAYS AND MUSCLE METABOLISM AS THERAPEUTIC STRATEGY

One of the most relevant player in the exercise-induced adaptations in the skeletal muscle is the co-transcription factor PGC-1 α , that responds to energy demand by enhancing the transcription of genes involved in mitochondrial biogenesis and turnover, leading to increased muscle oxidative capacity^[44,45]. In this regard, the ablation of PGC-1 α in mice has been shown to blunt the increase of autophagy and mitophagy induced by exercise, to decrease mitochondrial mass and to impair exercise performance^[45,46]. Conversely, PGC-1 α overexpression increases mitochondrial content, fast-to-slow muscle fiber switch and induces a set of genes associated with energy metabolism^[47,48]. Moreover, PGC-1 α transgenic animals show an improvement of satellite cell activation and proliferative potential^[49]. Indeed, PGC-1 α plays an important role in modulating muscle homeostasis and its downregulation is correlated with different wasting conditions, such as aging, muscle disuse and denervation^[50-52]. In aged mice, PGC-1 α overexpression has been shown to counteract the loss of muscle mitochondrial enzymes, to increase markers of autophagy and to preserve both neuromuscular junctions^[47] and exercise capacity^[53]. In muscle disuse and denervation, increased PGC-1 α expression prevents muscle atrophy and the activation of catabolic pathways^[50,52]. In cancer cachexia, muscle PGC-1 α protein expression is reduced in different animal models and such impairment does not always reflect a reduction of the transcript level^[54]. Otherwise, some studies also reports an increase of PGC-1 α gene expression^[54]. Independently from PGC-1 α , muscle mitochondrial mass and metabolism are severely affected in tumor-bearing animals^[10], suggesting that PGC-1 α is not the only determining factor in impaired mitochondrial function. In cachectic animals, conflicting results are also reported regarding PGC-1 α overexpression. Whereas Wang *et al.*^[55] demonstrate that the muscle-specific upregulation of PGC-1 α does not protect LLC-bearing mice from muscle atrophy in the same animal model Pin *et al.*^[35] show increased muscle mass and reduced atrogin-1 and muscle RING-finger protein-1 (*MuRF-1*) gene expression as compared to wild-type tumor bearers. However, both the studies suggest that forcing the expression of PGC-1 α in the skeletal muscle could have some limitations, such as the increase in tumor mass^[35,55].

The effects of boosting mitochondrial biogenesis have been tested also in cancer and chemotherapy-induced muscle wasting. In this condition, PGC-1 α overexpression does not improve muscle mass, whereas this effect is achieved by moderate exercise^[21]. This could suggest that enhancing mitochondrial biogenesis is not sufficient to mimic the effects of exercise and that it does not necessarily reflect an increase of mitochondrial function, both being affected in chemotherapy-treated tumor hosts^[21].

The modulations triggered by exercise may be mimicked by pharmacological and dietary compounds^[44,56]. Some of them have been extensively studied, such as 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), glitazones, metformin and sirtuin 1 (SIRT1) activators, also reporting their effectiveness in preventing cancer-induced muscle wasting^[10,44]. Also erythropoietin (EPO), an endogenous cytokine essential for the growth and differentiation of red blood progenitor cells, has shown some exercise-like effects that go beyond the increase in oxygen delivery. Indeed, the EPO receptor (EpoR) is expressed in different tissues other than hematopoietic cells, such as heart, skeletal muscle, adipose tissue, brain and pancreas^[57]. In this regard, EPO stimulates SIRT1 signaling in human cardiomyocytes treated with doxorubicin, increasing the levels of PGC-1 α , nuclear respiratory factor 1 (NRF1), citrate synthase and cytochrome *c* oxidase IV^[58]. EPO exposure of C2C12 myocyte cultures and primary skeletal myoblasts *in vitro* results in increased mitochondrial mass, PGC-1 α levels, citrate synthase activity and oxygen consumption rate^[59]. Similarly, transgenic mice expressing high levels of EPO show increased proportion of oxidative fibers and improved mitochondrial activity in the skeletal muscle, together with increased PGC-1 α and AMPK activation^[59]. Recombinant EPO administration to humans also results in increased mitochondrial oxidative phosphorylation and electron transport capacity^[60]. Consistently, LLC-bearing mice treated with EPO show increased SDH activity, ATP content and PGC-1 α expression in the skeletal muscle^[35]. These effects are enhanced by combining exercise training and EPO, protecting the mitochondrial compartment and resulting in increased muscle strength as compared to untreated sedentary cancer hosts^[35].

Another modulator of muscle metabolism is trimetazidine (TMZ). The effects of TMZ are primarily related to the ability to block β -oxidation, by inhibiting the 3-ketoacyl-CoA thiolase (3-KAT) activity in the mitochondrial matrix^[61]. This inhibition results in a metabolic shift towards glucose oxidation, optimizing oxygen utilization and decreasing lactate levels^[62]. TMZ is used to treat chronic stable angina and ischemic cardiomyopathy^[63], reducing symptoms and improving exercise performance^[63,64]. The effects on patients with cardiovascular diseases could be also related to the protection of the mitochondrial compartment. In this regard, TMZ is able to correct the toxicity induced by palmitate in cultured cardiomyocytes, increasing mitochondria mass, volume and function^[65]. Positive effects have been achieved by TMZ also on C2C12 myotubes exposed to TNF- α or to growth factor deprivation. Notably, TMZ induces the activation of anabolic pathways and reduces the expression of atrogin-1 and MuRF1, protecting C2C12 myotubes from atrophy^[66]. Of relevance, TMZ is also able to induce autophagy *in vitro*^[66]. Moving to *in vivo* models, TMZ improves muscle strength of aged mice, coupled with a muscle metabolic shift towards oxidative metabolism, suggested by increased levels of the slow myosin heavy chain (MyHC) isoform^[67]. Similarly to aged muscle, TMZ induces exercise-like effects also in the skeletal muscle of tumor-bearing animals. In this regard, TMZ administration partially protects C26-bearing mice from muscle atrophy (myofiber cross sectional area) and dysfunction, increasing slow MyHC content, SDH-positive myofibers and markers associated with mitochondrial mass and biogenesis^[68]. An increase in vascular endothelial cadherin (Ve-cadherin) and vascular endothelial growth factor (VEGF) expression, two known markers of angiogenesis, has also been detected in tumor-bearing mice after TMZ administration^[68]. Of relevance, TMZ seems to induce its effects quickly in the skeletal muscle. Indeed, 15 min *ex-vivo* TMZ treatment of EDL muscle (mostly composed by glycolytic fibers) is enough to reduce both contraction and relaxation rates^[68], favoring the occurrence of a phenotype typical of slow-twitch muscle^[69].

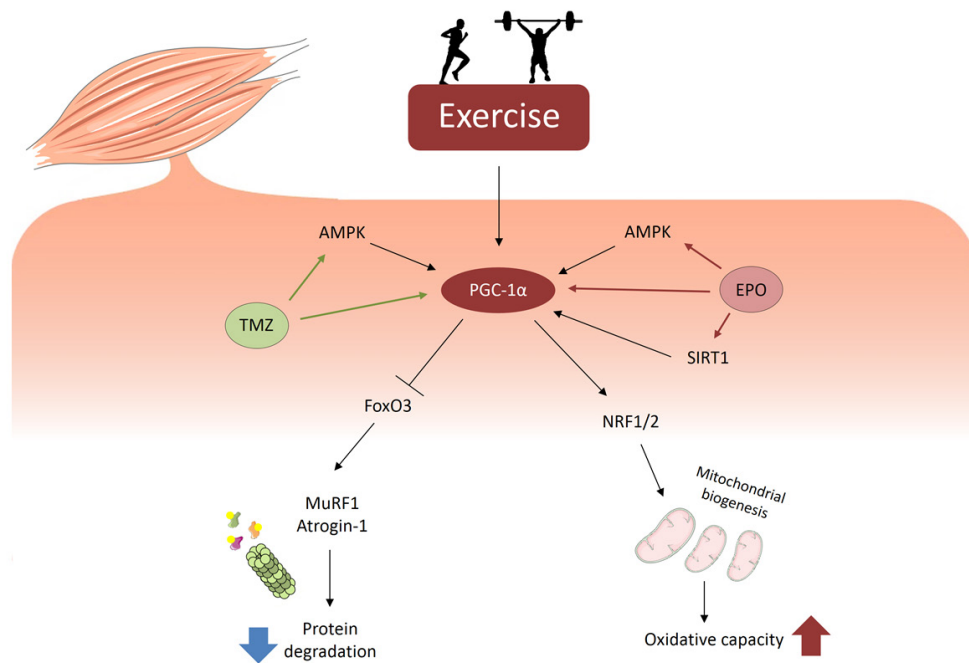


Figure 2. Exercise-like responses in the skeletal muscle may be induced by drugs acting on PGC-1 α expression and activity. Exercise modulates different pathways involved in the regulation of protein homeostasis and energy metabolism. During exercise, PGC-1 α activation increased mitochondrial biogenesis, favoring oxidative capacity, and inhibits FoxO3, reducing proteasome-dependent degradation. Similarly to exercise, EPO and TMZ are able to activate energy sensors, such as AMPK and SIRT1, that induce PGC-1 α and its downstream targets

Exercise is also able to activate myogenesis, impinging on satellite cells (SCs) proliferation and differentiation^[70]. Accordingly, TMZ treatment modulates the behavior of differentiating murine myoblast, increasing fusion capacity, markers of myogenesis and MyHC levels^[71]. These effects are associated with increased expression of metabolic regulators such as PGC-1 α and activated AMPK, suggesting an increase in oxidative metabolism^[71]. Similar results have been obtained in isolated murine SCs, in which TMZ increases some markers of differentiation as compared to control^[71]. Myogenesis is impaired in cachectic animals and cancer patients, likely contributing to muscle wasting^[72,73]. In this regard, the effect of TMZ on myogenesis markers has been tested on C26-bearing mice, showing increased levels of MyoD and Myogenin^[71]. Such results suggest that TMZ could potentially protect against myogenesis impairments.

CONCLUSION

The research on the etiopathogenesis of cancer cachexia has unraveled different metabolic alterations that lead to the wasting phenotype of cancer patients. Exercise training is one of the best approaches able to correct the metabolic impairments of tumor hosts, such as inflammation, hypercatabolism and energy deficit. However, being the skeletal muscle severely affected in cancer cachexia, often the physical (and psychological) state of cancer patients is not permissive for practicing exercise. Also due to these reasons the search for pharmacological strategies able to modulate exercise-induced pathways is rapidly growing. In this regard, exercise beneficial effects in cancer cachexia could depend, partially at least, on the expression of PGC-1 α . Consistently, drugs able to enhance the expression of this co-transcription factor, together with strategies that increase mitochondrial function, could be effective in improving muscle wasting [Figure 2]. However, beyond the alterations of the mitochondrial homeostasis, muscle depletion relies also on inflammation, activation of catabolic pathways and, frequently, depression of protein synthesis. Along this line, a combinatorial therapy targeting each of these alterations could be the best choice to counteract cancer-induced muscle wasting.

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Original Article

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Cancer stem cell subpopulations in metastatic melanoma to the brain express components of the renin-angiotensin system

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Abstract

Aim: There is increasing appreciation of the role of the renin-angiotensin system (RAS) in carcinogenesis with recent evidence showing expression of the RAS by cancer stem cells (CSCs) in different types of cancer. We have recently demonstrated the presence of three CSC subpopulations within metastatic melanoma (MM) to the brain: a Melan-A⁺ subpopulation and a Melan-A⁻ subpopulation within the tumor that express OCT4, SALL4, SOX2 and NANOG; and a pSTAT3⁺ subpopulation localized to the CD34⁺ endothelium of microvessels within the tumor. In this study we investigated the expression and localization of components of the RAS in relation to these CSCs in MM to the brain.

Methods: 3, 3-diaminobenzidine immunohistochemical (IHC) staining of components of the RAS: pro-renin receptor (PRR), angiotensin converting enzyme (ACE), angiotensin II receptor 1 (AT1R1) and angiotensin II receptor 2 (AT1R2) was performed on the same ten samples of MM to the brain included in our previous study. Immunofluorescence IHC staining of these components of the RAS was performed with embryonic stem cell markers OCT4 and NANOG, and endothelial marker CD34, on two of the samples of MM to the brain from the original cohort of ten patients. Western blotting ($n = 5$) and NanoString mRNA analysis ($n = 4$) were performed on samples of MM to the brain to confirm protein and mRNA expression of these components of the RAS, respectively.



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Results: DAB IHC staining showed the presence of PRR, ACE, ATIIR1 and ATIIR2 in all ten samples of MM to the brain. IF IHC staining showed that the CSC subpopulations in MM to the brain expressed PRR, ATIIR1 and ATIIR2; and a CSC subpopulation on the endothelium of the microvessels expressed ACE. Western blotting and NanoString mRNA analysis confirmed protein and mRNA expression of these components of the RAS, respectively.

Conclusion: CSCs in MM to the brain expressed components of the RAS. Targeting the CSCs using modulators of the RAS may be a novel therapeutic approach for treating this aggressive cancer.

Keywords: Malignant melanoma, metastatic, cancer stem cells, renin-angiotensin system, cancer, brain

INTRODUCTION

Metastatic melanoma (MM) to the brain occurs in 30% of melanoma patients and accounts for 5%-8% of all brain tumors^[1]. For many years, the median survival of MM to the brain is 6-9 months following mainstay treatments including surgical excision, stereotactic radiosurgery and external beam radiotherapy^[2-5]. Investigations into MM to the brain have identified real time preferential hematogenous dissemination of tumor cells^[6], with survival of these cells within brain capillaries, migration to breach the blood brain barrier, angiogenesis and intraparenchymal proliferation^[7].

Although immunotherapies that enhance T-lymphocyte activity such as Ipilimumab, anti-cytotoxic T-lymphocyte associated antigen-4 and anti-programmed cell death-1 inhibitors can improve the median survival, some cause significant toxicities^[8,9]. Up to 50%-60% of MM patients harbor the BRAF V600 somatic mutation^[10]. Vemurafenib, a BRAF inhibitor, increases the median survival to 16 months^[11] but despite an initially good response, progression-free survival remains poor, at seven months^[5]. Used together, kinase inhibitors, BRAF-targeted and MEK-targeted small molecule drugs relieve intracranial symptoms and induces remission but cessation due to toxicity causes rapid intracranial and/or extracranial disease progression^[12]. The short period of remission after neuro-mimicry and individualized tumor targets^[13] has been attributed to the initiation, propagation and differentiation of cancer stem cells (CSCs)^[14].

The CSC concept proposes that cancer is caused by CSCs that possess the ability for uncontrolled growth and propagation^[15]. CSCs have been demonstrated in many types of cancer including breast carcinoma^[16], glioblastoma^[17] and oral cavity squamous cell carcinoma (OCSCC)^[18-20]. We have recently identified and characterized three CSC subpopulations within MM to the brain: a Melan-A⁺ subpopulation and a Melan-A⁻ subpopulations that express embryonic stem cell (ESC) markers OCT4, SALL4, SOX2 and NANOG within the tumor, and a pSTAT3⁺ subpopulation localized to the CD34⁺ endothelium of microvessels within the tumor^[21].

The RAS has been linked to carcinogenesis for some time^[22]. In the classical RAS, angiotensinogen (ANG) is converted to angiotensin I (ATI) by renin. ATI is then converted to angiotensin II (ATII) by angiotensin converting enzyme (ACE). The RAS is implicated in central nervous system disorders such as Parkinson's disease and dementia with neuroprotective and neurotoxic features although its role in carcinogenesis is not discussed in a recent review^[23]. GB cells in culture express ANG, pro-renin, ACE, ATII, angiotensin II receptor 1 (ATIIR1) and angiotensin II receptor 2 (ATIIR2); and renin inhibitors decrease DNA synthesis, induce apoptosis and reduce viable cell numbers^[24]. Renin and its precursor pro-renin bind to pro-renin receptor (PRR) to activate the MAPK signaling cascades. PRR is associated with increased cell proliferation, decreased apoptosis and highly expressed in pancreatic ductal adenocarcinoma^[25]. CSC subpopulations in GB^[26] and OCSCC of different subsites^[27-29] express components of RAS.

We here hypothesized expression of the RAS by CSCs in MM to the brain and investigated the expression and localization of components of the RAS: PRR, ACE, ATIR1 and ATIR2, in relation to the putative CSC subpopulations we have recently identified^[21], using immunohistochemical (IHC) staining, Western blotting (WB) and NanoString mRNA analysis.

METHODS

Tissue samples

Samples of MM to the brain from ten patients were sourced from the Gillies McIndoe Research Institute Tissue Bank and used in this study that was approved by the Central Health and Disabilities Ethics Committee (Ref. 15CEN28) with written informed consent from all participants.

Histology and IHC staining

Hematoxylin and eosin (H&E) staining was performed on 4 µm thick formalin-fixed paraffin-embedded sections of MM to the brain from ten patients included in our previous study^[21], to confirm the presence of the tumor by an anatomical pathologist (HDB). 3,3-diaminobenzidine (DAB) IHC staining for CD34 (ready-to-use, cat# PA0212, Leica), Melan-A (ready-to-use, cat# PA0233, Leica), ACE (1:100; cat# MCA2054, AbD Serotec, Kidlington, UK), PRR (1:2000; cat# ab40790, Abcam), ATIR1 (1:30; cat# ab9391, Abcam), ATIR2 (1:2000; cat# NBP1-77368, Novus Biologicals, LLC, Littleton, CO, USA) as well as NANOG (1:100; cat# ab80892, Abcam, Cambridge, UK), OCT4 (1:1000; cat# ab109183, Abcam) and ERG (1:200; EPII, Cell Marque, Rocklin, CA, USA), diluted with BondTM primary antibody diluent (cat# AR9352, Leica), was performed as previously described^[30].

To localize the components of the RAS in relation to the putative Melan-A⁺ and Melan-A⁻ OCT4⁺/SALL4⁺/SOX2⁺/NANOG⁺ CSC subpopulations within the tumor, and the pSTAT3⁺ subpopulation on the endothelium of microvessels within MM to the brain^[21], immunofluorescence (IF) IHC staining was performed on two representative samples of MM to the brain from the original cohort of ten patients included for DAB IHC staining. Components of the RAS were co-stained with ESC markers OCT4 or NANOG as the surrogate markers for the Melan-A⁺ and the Melan-A⁻ OCT4⁺/SALL4⁺/SOX2⁺/NANOG⁺ CSC subpopulations, or endothelial markers ERG or CD34 for the pSTAT3⁺ subpopulation on the endothelium of microvessels within the tumor. Antibodies used for PRR and ATIR2 detection were VectaFluor Excel anti-rabbit 594 (ready-to-use; cat# VEDK-1594, Vector Laboratories) and Alexa Fluor anti-mouse 488 (1:500; cat#A21202, Life Technologies). Antibodies for ACE and ATIR1 detection were VectaFluor Excel anti-mouse (ready-to-use; cat# VEDK2488, Vector Laboratories) and Alexa Fluor anti-rabbit 594 (1:500; cat# A21207, Life Technologies).

Human tissues used for positive controls were placenta for PRR, liver for ATIR1, and kidney for ATIR2 and ACE. Negative controls were used in secondary and tertiary antibody staining by omitting the primary antibodies on a randomly selected sample of MM to the brain.

Image analysis

DAB IHC stained-slides were viewed and images were captured with an Olympus BX53 light microscope fitted with an Olympus SC100 digital camera (Olympus, Tokyo, Japan), and processed with the CellSens 2.0 software (Olympus). IF IHC-stained images were captured using an Olympus FV1200 biological confocal laser-scanning microscope and processed with CellSens Dimension 1.11 software using 2D deconvolution algorithms (Olympus).

WB

Five snap-frozen samples of MM to the brain from the original cohort of ten patients underwent WB as described previously^[28], using the primary antibodies: anti-PRR (ATP6IP2, 1:500; cat# ab40790, Abcam),

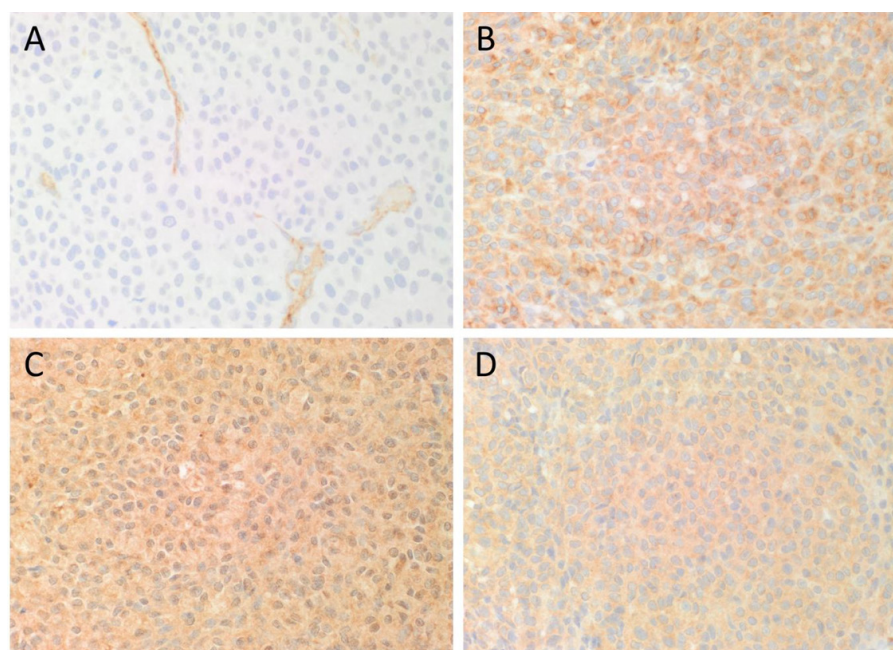


Figure 1. Representative DAB IHC-stained sections of metastatic melanoma to the brain showing endothelial staining of ACE (A, brown), and mostly cytoplasmic staining of PRR (B, brown), ATIIR1 (C, brown) and ATIIR2 (D, brown). Nuclei were counter-stained with hematoxylin (A-D, blue). Original magnification: 200 \times . DAB: 3, 3-diaminobenzidine; IHC: immunohistochemical; ATIIR1: angiotensin II receptor 1; ATIIR2: angiotensin II receptor 2; PRR: pro-renin receptor; ACE: angiotensin converting enzyme

anti-ATIIR1 (AT2R1, 1:500; cat# sc-1173, Santa Cruz, CA, USA), anti-ATIIR2 (1:5000; cat# ab92445, Abcam), anti-ACE (1:200; cat# sc-12184, Santa Cruz) and anti- β -actin (1:2000 cat# ab8226, Abcam). Secondary antibodies used were Alexa Fluor 647 rabbit anti-mouse for β -actin (1:2000 cat# A21202, Thermo Fisher Scientific), donkey anti-goat HRP (1:10000; cat# ab97120; Abcam) and a rabbit anti-goat SuperclonalTM biotin conjugated secondary antibody (1:20000; cat# A27013, Thermo Fisher Scientific) followed by a PierceTM Streptavidin Poly-HRP (1:5000, cat# 21140, Thermo Fisher Scientific) at 4 °C for 10 min for the ACE tertiary cascade. β -actin antibody probing was performed with the iBindTM Flex device (cat# SLF2000, Life Technologies) using primary mouse monoclonal anti- β -actin (1:2000; cat# ab8226, Abcam) and secondary donkey anti-mouse AlexaFluor 488 (1:2000; cat# A21202, Thermo Fisher Scientific). Clarity Western ECL (cat# 1705061, Bio-Rad) was used to visualize HRP detected bands and the Chemi Doc MP Imaging System (Bio-Rad) and Image Lab 5.0 software (Bio-Rad) were used for detection and analysis. Positive controls were mouse brain extract for PRR and ATIIR1, mouse lung protein extract for ACE and PC3 cell lysate for ATIIR2. Negative controls were mouse kidney for PRR, human tonsil for ACE, mouse kidney for ATIIR1 and NTERA2 for ATIIR2.

NanoString mRNA analysis

RNA extraction, as previously described^[27], was performed on four snap-frozen samples of MM to the brain from the same cohort of ten patients included in DAB IHC staining underwent. RNA was subjected to NanoString nCounterTM Gene Expression Assay (NanoString Technologies, Seattle, WA, USA) as completed by New Zealand Genomics Ltd (Dunedin, NZ). Probes for the genes encoding PRR (NM_005765.2), ATIIR1 (NM_000685.3), ATIIR2 (NM_000686.3), ACE (NM_000789.2) and the housekeeping gene GAPDH (NM_002046.3) were designed and synthesized by NanoString Technologies.

RESULTS

Histology and DAB IHC staining

H&E staining (data not shown) demonstrated the presence of MM in all ten samples that stained positively for the melanoma marker Melan-A (data not shown). The endothelium of the microvessels expressed

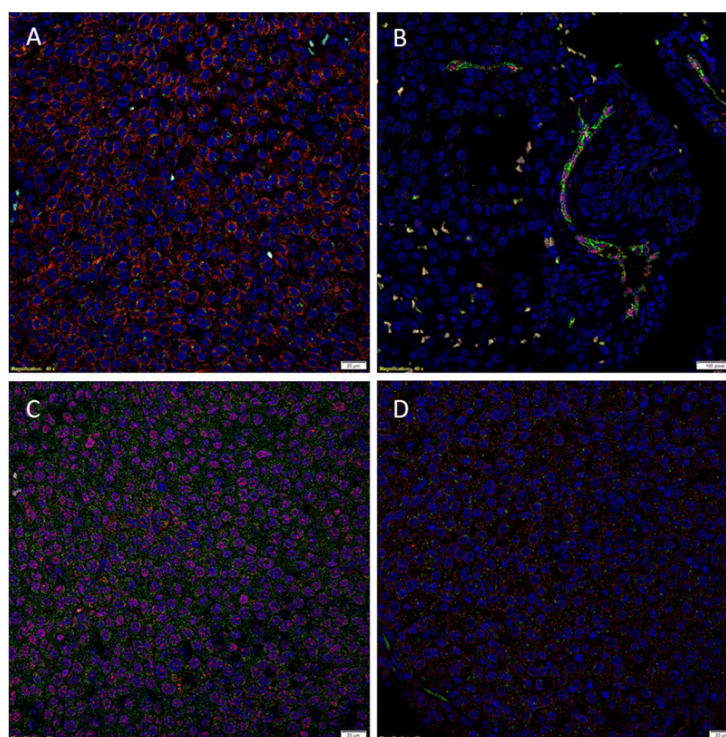


Figure 2. Representative IF IHC-stained sections of metastatic melanoma to the brain demonstrating cytoplasmic expression and some nuclear expression of PRR (A, red) on the OCT4⁺ (A, green) cells. ACE (B, green) was expressed by the ERG⁺ (B, red) endothelium of the microvessels. Some NANOG⁺ (C, red) cells expressed ATIIR1 (C, green). ATIIR2 (D, red) was expressed in the cytoplasm of the OCT4⁺ (D, green) cells. Cell nuclei were counterstained with 4', 6'-diamidino-2-phenylindole (A-D, blue). Scale bars: 20 μ m. IF: immunofluorescence; IHC: immunohistochemical; PRR: pro-renin receptor; ATIIR1: angiotensin II receptor 1; ATIIR2: angiotensin II receptor 2; ACE: angiotensin converting enzyme

ACE [Figure 1A]. The tumor tissue widely expressed PRR [Figure 1B], ATIIR1 [Figure 1C] and ATIIR2 [Figure 1D], mostly in the cytoplasm.

Human tissues used for positive controls: placenta for PRR, liver for ATIIR1, and kidney for ATIIR2 and ACE demonstrated the expected staining patterns [Supplementary Figure 1]. The omission of the primary antibody provided a control for the secondary antibody (data not shown).

IF IHC staining

IF IHC staining showed cytoplasmic expression of PRR [Figure 2A, red] on the OCT4⁺ [Figure 2A, green] CSCs within MM to the brain. ACE [Figure 2B, green] was expressed only on the ERG⁺ [Figure 2B, red] endothelium of the microvessels. ATIIR1 [Figure 2C, green] was expressed by the NANOG⁺ [Figure 2C, red] CSCs, while ATIIR2 [Figure 2D, red] was expressed on the OCT4⁺ [Figure 2D, green] CSCs in MM to the brain.

Supplementary Figure 2A-H displays individual IF IHC stains shown in Figure 2. Supplemental Figure 2I shows the appropriate negative control on a section of MM to the brain by the omission of the primary antibody.

WB

PRR, at a molecular weight of 40 kDa, was present in all five samples of MM to the brain [Figure 3A]. The negative control of mouse kidney also showed a comparatively faint band. ACE, at 190 kDa, was detected in all five samples, with multiple smaller molecular weight bands detected in all the samples [Figure 3B], possibly due to degraded protein. ATIIR1 was seen in two of the five samples at 45 kDa, with a 28-kDa

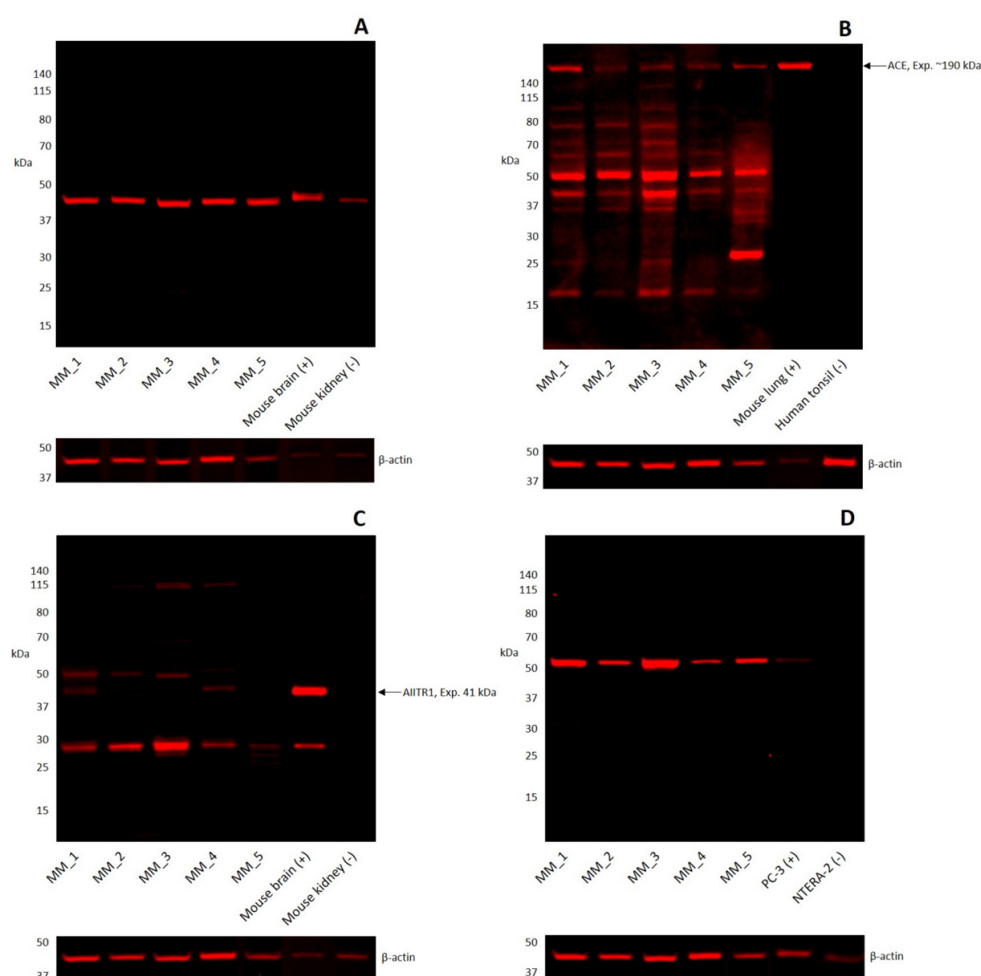


Figure 3. Representative images of Western blots performed on five samples of metastatic melanoma to the brain. PRR was detected in all five samples at 40 kDa (A). ACE was detected in all five samples at 190 kDa, with multiple lower bands consistent with protein degradation (B). ATIIR1 was detected at 45 kDa in two samples and at 28 kDa in all five samples (C). ATIIR2 was present at 50 kDa in all five samples (H). Positive controls were mouse brain extract for PRR (A), mouse lung protein extract for ACE (B), mouse brain for ATIIR1 (C), PC3 cell lysate for ATIIR2 (D). Negative controls were mouse kidney for PRR (A), human tonsil for ACE (B), mouse kidney for ATIIR1 (C), and NTERA2 for ATIIR2 (D). PRR: pro-renin receptor; ATIIR1: angiotensin II receptor 1; ATIIR2: angiotensin II receptor 2; ACE: angiotensin converting enzyme

band was also present in all five samples and the positive control [Figure 3C], possibly due to degradation. ATIIR2 was seen in all five samples at 50 kDa [Figure 3D].

NanoString mRNA analysis

NanoString mRNA analysis revealed very high levels of mRNA expression for PRR, moderate levels of ACE and low levels of ATIIR1 mRNA expression, while ATIIR2 was below detectable levels [Figure 4].

DISCUSSION

We have recently identified three CSC subpopulations within MM to the brain: a Melan-A⁺ subpopulation and a Melan-A⁻ subpopulations that express the ESC markers OCT4, SALL4, SOX2 and NANOG, and a pSTAT3⁺ subpopulation localized to the CD34⁺ endothelium of the microvessels within the tumor^[21]. This study demonstrated the expression of PRR, ATIIR1 and ATIIR2 by these CSCs subpopulations we have identified. The demonstration of widespread expression of these cell surface receptors by DAB IHC staining, may be due to their relatively abundant expression within the samples we have examined. Using

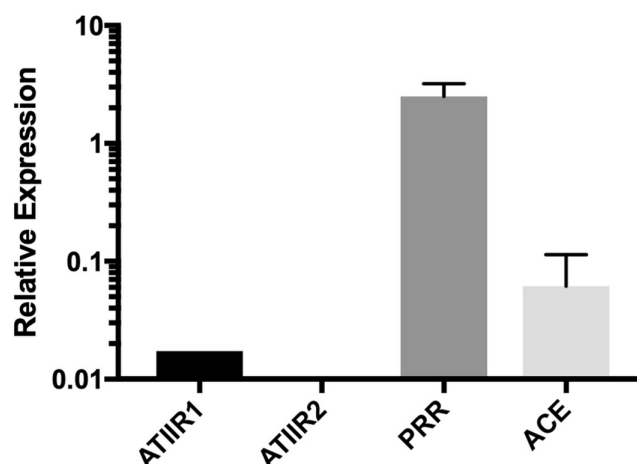


Figure 4. Relative expression of mRNA transcripts of PRR, ACE, ATIIR1 and ATIIR2 by NanoString mRNA analysis of four samples of metastatic melanoma to the brain showing high levels of expression of PRR, moderate levels for ACE, low levels for ATIIR1 and undetectable levels for ATIIR2, relative to the housekeeping gene GAPDH. PRR: pro-renin receptor; ATIIR1: angiotensin II receptor 1; ATIIR2: angiotensin II receptor 2; ACE: angiotensin converting enzyme

OCT4 and NANOG as surrogate markers, IF IHC staining revealed localization of PRR, ATIIR1 and ATIIR2 to the OCT4⁺/OCT4⁺/SALL4⁺/SOX2⁺/NANOG⁺ CSC subpopulations while ACE was localized the CSC subpopulation on ERG⁺ endothelium of the microvessels which we have shown to express pSTAT3 previously. The expression of PRR, ACE, ATIIR1 and ATIIR2 was confirmed by WB. NanoString mRNA analysis showed high levels of expression of PRR, lower expression levels of ACE and ATIIR1. ATIIR2 was below detectable levels which may be due to mRNA degradation or its transient presence during transcription within the samples examined.

We have previously reported expression of PRR, ATIIR1 and ATIIR2 by the SOX2⁺ CSC subpopulation, and exclusive expression of ACE by the subpopulation on the endothelium of the microvessels, in human isocitrate dehydrogenase-wildtype GB^[26] and OCSCE affecting different subsites^[27-29]. mRNA expression of renin, ACE, ATIIR1 and ATIIR2 has been demonstrated on primary cultured human keratinocytes, melanocytes, dermal fibroblasts and dermal capillary endothelial cells, but ATIIR2 is not detected in melanocytes^[31].

Cellular proliferation associated with ATI-treated and ATII-treated infantile hemangioma cell culture has shown to be diminished by ACE inhibitor ramipril and the ATIIR2 antagonist PD123319, and is enhanced by ATIIR2 agonist CGP42112^[32], implying a role for the RAS peptides in stem cell proliferation.

Blockade of the RAS leads to inhibited growth of colorectal cancer liver metastases in the regenerating liver^[33] and recent reports of increased overall survival of GB patients treated with angiotensin receptor blockers^[34], support the role of the RAS in cancer. It is exciting to speculate that CSCs in MM to the brain maybe a novel therapeutic target by modulating the RAS, although more work including a larger sample size, control tissue samples and *in vitro* and *in vivo* functional work is needed to determine the precise role of the RAS in this aggressive cancer.

In conclusion, in this study we have demonstrated the expression and localization of components of the RAS: PRR, ACE, ATIIR1 and ATIIR2 in MM to the brain. PRR, ATIIR1 and ATIIR2 are localized to the CSC subpopulations within the tumor while ACE is expressed by the CSC subpopulation on the endothelium of the microvessels. Targeting the CSCs using modulators of the RAS may be a novel therapeutic approach for MM to the brain.

DECLARATIONS

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Authors' contributions

Formulated the study hypothesis: Itinteang T, Tan ST
Designed the study: Itinteang T, Wickremesekera AC, Tan ST
Recruited patients and obtained study samples: Wickremesekera AC, Parker A, Koeck H
Interpreted the DAB IHC data: Itinteang T, Brasch HD, Wickremesekera AC, Tan ST
Interpreted the IF IHC data: Itinteang T, Wickremesekera AC, Tan ST
Interpreted the WB and NanoString mRNA analysis data: Itinteang T, Tan ST
Drafted the manuscript: Wickremesekera AC, Itinteang T, Tan ST
All authors commented on and approved the manuscript.

Availability of data and materials

Data supporting the findings of this study can be obtained by contacting the corresponding author.

Financial support and sponsorship

None.

Conflict of interest

All authors declared that there are no conflicts of interest. TI, PFD and STT are inventors of the provisional patents Cancer Diagnosis and Therapy (No.PCT/NZ2015/050108) and Cancer Therapeutic (PCT/NZ2018/050006), and provisional patent application Novel Pharmaceutical Compositions for Cancer Therapy (US/62/711709).

Ethics approval and consent to participate

This study was approved by the Central Health and Disabilities Ethics Committee (Ref. 15CEN28) with written informed consent from all subjects in accordance with the Declaration of Helsinki.

Consent for publication

Not applicable.

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Original Article

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A logarithmic rapid desensitization protocol: initial experience in carboplatin hypersensitivity reactions

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Abstract

Aim: Hypersensitivity reactions to carboplatin are not an infrequent adverse event in ovarian cancer patients. However, reintroduction of platinum-containing schedules is the standard of care in platinum-sensitive recurrent ovarian cancer. Rapid desensitization is a procedure for gradual reintroduction of drug. It allows a safe administration of medications that are beneficial for the management of patients after certain types of hypersensitivity reactions. It is indicated in cases in which there are no reasonable therapeutic alternatives.

Methods: We performed a descriptive retrospective study of high-grade ovarian cancer patients with known carboplatin hypersensitivity reactions that were treated with a 13-steps rapid desensitization protocol with 3 different solutions and infusion rates. The procedure followed a mathematic model (gradual increases with a relationship between doses following a geometric series) which is called logarithmic rapid desensitization protocol (LRDP). The aim was to describe the safety of the LRDP in terms of number and severity of infusion reactions and the effectiveness in the rate of cycles completely administered.

Results: Four different patients diagnosed with recurrent platinum-sensitive ovarian cancer with a previous infusion reaction were included. LRDP was administered in 19 different cycles. LRDP was administered safely in all 19 cycles, only 2 patients had a mild cutaneous reaction in 4 different cycles during LRDP (21.05%). The foreseen dose of carboplatin was fully administered in all cycles.



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Conclusion: LRDP with carboplatin is a feasible and safe protocol in patients with previous infusion reaction to carboplatin. The protocol might allow a safe administration of drugs, that are beneficial for the management of patients, after certain types of hypersensitivity reactions, and it is indicated in cases in which there are no reasonable therapeutic alternatives.

Keywords: Drug allergy, hypersensitivity, desensitization, rapid protocol, logarithmic model, carboplatin

INTRODUCTION

Platinum-based chemotherapeutic agents have been used for the treatment of numerous solid tumors affecting several locations (such as breast, ovarian, endometrial, lung, and gastrointestinal tract). As a result of its extended use, platinum compounds have produced an increased number of reactions^[1,2]. Hypersensitivity reactions are much more frequent with platins than with other drugs. Reactions to carboplatin are a frequent adverse event with an overall incidence of 1%-44% supposing a challenge in the management of ovarian cancer.

In ovarian cancer, platinum-responsiveness have been a classical prognostic factor. Reintroduction of platinum-containing schedules in patients with platinum-responsiveness relapse (> 6 months of interval) is widely recommended^[3-4]. Carboplatin has shown in a randomized trial similar survival than cisplatin but with better safety profile and quality of life^[5]. Since then carboplatin-containing chemotherapy has been considered standard and, as a consequence, relapsed ovarian cancer patients are frequently exposed to an important number of carboplatin infusions.

Positive carboplatin skin tests and the presentation pattern of these reactions suggest that type I pathway immunological mechanisms are involved. Moreover, platinum-specific IgE can be also found in serum among exposed refinery workers and it supports the theory of type IgE-mediated reaction. Furthermore, clinical symptoms related to a reaction to carboplatin range from a mild rash to severe anaphylaxis; thus, different types of immunologic hypersensitivity seem to be implicated^[1].

The fact that the reintroduction of platinum is a major issue in platinum-sensitive patients has prompted the development of desensitization protocols. Rapid desensitization is a procedure for gradual reintroduction of drug at a low dose by dissolving it at a low and intermediate concentration, until reaching the target dose. It allows a safe administration of medications that are beneficial for the management of patients after certain types of hypersensitivity reactions, and it is indicated in cases in which there are no reasonable therapeutic alternatives.

We have performed a retrospective study with the aim of assessing the effectiveness and safety of a logarithm (geometric series) rapid desensitization protocol (LRDP) of carboplatin in patients with platinum-sensitive ovarian relapse that had a previous allergic reaction during carboplatin infusion.

METHODS

Design

This is a pivotal study with the objective of assessing effectiveness and safety of LRDP, in terms of number and severity of infusion reactions, in patients with previous reactions to carboplatin exposure and proportion of completely administered carboplatin-containing schedules. A retrospective review of the clinical chart of patients diagnosed with ovarian cancer in our institution since January 2007 was performed. Data extracted from the clinical chart included: (1) diagnosis, pathological variables and demographic data; (2) data from initial infusion reaction; (3) skin tests; and (4) data from the outcome during LPRD and type of reactions occurring during this protocol.

Classification of the initial reaction bore three aspects: characteristics, severity and timing (time interval between drug administration and the reaction).

Characteristics of clinical symptoms associated with the initial hypersensitivity reactions were categorized by organ system involvement. Cutaneous symptoms (oedema of the face and hands, flushing, pruritus, urticaria or angioedema), respiratory symptoms (dyspnea or wheezing or oxygen desaturation, coughing, nasal congestion), abdominal symptoms (nausea, emesis/vomiting, diarrhea, or abdominal pain), laryngeal angioedema/throat tightness, or cardiovascular symptoms (chest pain, hypotension, hypertension, or tachycardia), neurological/muscular (vision disturbances, weakness, unusual taste, hallucinations, or neurological compromise) according to previous publications^[1,2].

Hypersensitivity severity: A mild rash may be the first manifestation of reactions. Hypersensitivity reactions were classified as mild (reactions limited to the skin), moderate (features suggesting respiratory - dyspnea, wheeze -, cardiovascular - dizziness, diaphoresis, chest tightness - or gastrointestinal involvement - nausea, vomiting, abdominal pain -) or severe (hypoxia, hypotension and neurological compromise - confusion, collapse or incontinence -) according to the scale proposed by Brown^[6].

As for the timing of reaction, hypersensitivity reactions were categorized as immediate or delayed. Immediate reactions were turned up usually during drug administration while delaying reactions were presented after drug administration.

This retrospective study was approved by the local Ethics Committee of Hospital Clinico Universitario of Valencia (resolution number 320) in November 2016.

Patients

Patients included had a histological diagnosis of high-grade ovarian carcinoma and a platinum-sensitive relapsed defined as recurrence after at least 6 months of platinum-free interval. Being considered candidates for the LRDP, patients must have presented a hypersensitivity reaction during carboplatin infusion in a previous cycle. Carboplatin hypersensitivity is a late event^[7]. Consistent with these studies, all reactions in our population occurred after re-exposure to at least seven previous cycles of carboplatin.

In all cases, the initial infusion reaction had been evaluated and managed by emergency staff of the outpatient Oncology department.

Exclusion criteria were an impossibility to understand or to sign informed consent or lack of expected benefit with the reintroduction of carboplatin according to the clinician's opinion.

All patients with a previous type I reaction underwent a skin test with carboplatin before LRDP. Skin testing was performed at least 4 weeks after the initial reaction to minimize the likelihood of false negative results. Drug was diluted further in water with 5% dextrose for testing. For prick test, a drop of carboplatin (10 mg/mL) was applied to the volar surface of the forearm followed by pricking. For intradermal injections 0.03 mL (of a 1:100 dilution followed, if the result was negative, by an 1:10 dilution). A positive reaction was defined as a wheal with a diameter at least 3 mm large that produced by a negative control. Histamine prick (10 mg/mL) was used as a positive control^[1,2].

Informed consent was obtained from each patient before skin testing and desensitization procedures.

Treatment

Before (24-72 h previous) administration of LRDP, a blood test including creatinine and hemogram was performed. Patients were then seen in the outpatient unit of Medical Oncology. Treatment was only

administered if neutrophils count was ≥ 1500 and platelet count $\geq 100,000$. A total dose of carboplatin was always calculated according to the most recent creatinine levels.

According to the severity of the previous reaction and the results of the skin test, the first cycle of the LRDP was administered under an intensive monitoring in an Intensive Care Unit or under a lighter monitoring surveillance in beds of the Oncology Department inpatient area.

A standard premedication with corticosteroids and antagonists of histamine receptors was administered before desensitization to all patients. Metoclopramide hydrochloride 10 mg and dexamethasone 8 mg were given intravenously before initiation of LRDP as standard emesis prophylaxis. Histamine blockade (H1/H2) was performed with 5 mg of parenteral dexchlorpheniramine (5 mg/mL amp) and ranitidine (50 mg i.v.).

The management of reaction during desensitization was intended to block the effects of mast cell mediators, including histamine, prostaglandins and leukotrienes. If symptoms of a hypersensitivity reaction was developed during the desensitization procedure, the infusion was stopped.

In case of a mild reaction, 50 mg of parenteral dexchlorpheniramine (5 mg/mL) was administered. For severe or recurrent reactions, 40 mg of parenteral methylprednisolone (sodium succinate 0.5 mg/kg intravenously) and epinephrine 0.3 mL (1 mg/mL) were also added. Bronchoespasm and throat tightness was treated with inhaled B-agonists. Flushing was treated with aspirin and montelukast. Once symptoms have resolved, the protocol was resumed and the infusion was restarted at the point where the reaction occurred. All desensitization procedures were prescribed and supervised by the allergy and oncology departments and were conducted under physician supervision.

This 13-step LRDP combined gradual increases in the rate of infusion and concentration of carboplatin, administering the total dose over 5 h [Table 1].

The total target dose of carboplatin was calculated using the Cockcroft-Gault's formulation based on the area under the curve with a creatinine level obtained in no more than 24 h previous to the LRDP. Three different solutions A, B and C were employed with a total volume of 50 mL, 100 mL and 500 mL of water with 5% dextrose respectively and delivered in 13 consecutive steps. The concentrations of the solutions were 0.1 mg/mL for infusion A, 1 mg/mL for infusion B and 2 mg/mL for infusion C. Solution A was used for steps 1 to 7, solution B for steps 8 to 11 and solution C for steps 12 and 13. The total dose of carboplatin administered in the last step was calculated by subtracting the cumulative dose given in the steps 1-12 from the total target dose.

The initial dose was approximately in the order of a 10^{-4} times lower (in a rank of $0.2-3 \times 10^{-4}$) that the target dose and each step deliver twice the dose of the previous step. All step concentrations are arranged in a geometric series with a factor two. The first term of the series is 1/32 and the common ratio is 2 (1/32, 1/16, 1/8, 1/4, 1/2, 1, 2,). The rate of the infusion was adjusted every 15 min. The final step 13 maintained a constant rate of infusion in order to deliver the remainder of the total carboplatin dose.

RESULTS

From February 2011 to November 2014, 4 patients with platinum-sensitive recurrence of ovarian cancer that had presented a documented hypersensitivity reaction to the latest carboplatin infusion were treated with LRDP.

Patients characteristics and type of infusion reaction are shown in Table 2. Cutaneous reaction was the most frequent type of reaction to standard carboplatin administration in our series. All patients presented

Table 1. Logarithmic rapid desensitization protocol

Step	mg	mL	Flow rate (mL/h)	Time (min)	Cumulated time
Solution A					
1	0.03125	0.325	1,25	15	15 min
2	0.0625	0.625	2.5	15	30 min
3	0.125	1.25	5	15	45 min
4	0.25	2.5	10	15	1 h
5	0.5	5	20	15	1 h 15 min
6	1	10	40	15	1 h 30 min
7	2	20	80	15	1 h 45 min
Cumulated dose 4 mg					
Solution B					
8	4	4	16	15	2 h
9	8	8	32	15	2 h 15 min
10	16	16	64	15	2 h 30 min
11	32	32	128	15	2 h 45 min
Cumulated dose 60 mg					
Solution C					
12	64	32	128	15	3 h
cumulated dose 128 mg					
13	Subtraction (final target dose - total cumulated dose in steps 1-12)		200	2-3 h	5-6 h

Solution A: Concentration 0.1 mg/mL 5 mg in 50 mL; solution B: Concentration 1 mg/mL 100 mg in 100 mL; solution C: Concentration 2 mg/mL 1000 mg in 500 mL

any type of cutaneous reaction. According to severity, the half of patients, 2 out of 4, experienced a mild reaction (either pruritus, urticaria or angioedema).

One patient (1/5) developed a moderate reaction to standard infusion of carboplatin with cutaneous symptoms (palmar rash, pruritus, urticaria), respiratory symptoms (dyspnea) and cardiovascular symptoms (dizziness and diaphoresis).

Another patient (1/5) presented a severe reaction, including cutaneous (edema of the face and hands/flushing with palmar erythema, pruritus, urticaria), respiratory (dyspnea and oxygen desaturation) and cardiovascular symptoms (hypotension).

All 4 patients included had developed a reaction to standard carboplatin after at least 7 previous cycles of carboplatin (range 8th-14th cycle of carboplatin).

Regarding skin test results, only one patient had positive skin tests to carboplatin cutaneous exposure at their initial evaluation and the remaining 3 patients were negative. The patient who had specific-IgE (in skin) showed a moderate reaction in relation to the initial reaction and only a cutaneous reaction during the desensitization.

In total, 19 cycles of LRDP carboplatin were administered (in all cases successfully) under the LRDP. The rate of reactions during desensitization procedure was 21.05% (4 reactions out of 19 cycles), of whom all 4 reactions were considered as mild (palmar and/or facial erythema with pruritus). Despite these reactions, once symptoms have resolved, the foreseen carboplatin dose was fully administered after the protocol was resumed.

DISCUSSION

This report describes our experience with 19 rapid desensitizations with carboplatin in 4 patients that had a previous hypersensitivity reaction during standard carboplatin infusion. The procedure is successful, and we have been able to complete all the desensitizations undertaken.

Table 2. Characteristics of patients: type and severity of initial infusion reactions and during LRDP

Patient	Type of reaction	Timing	Severity	Skin test	Number of cycles of carboplatin inducing reaction	Schedule that induced reaction	Number of cycles with LRDP	Schedule during LRDP	Reaction during LRDP
Patient 1	Cutaneous	Immediate	Mild	-	8th (2nd line)	Carboplatin Paclitaxel	6	Carboplatin DLP	None
Patient 2	Cutaneous	Immediate	Mild	-	9th (2nd line)	Carboplatin Gemcitabine	2	Carboplatin Gemcitabine	None
Patient 2 (II)							4	Carboplatin DLP	None
Patient 3	Cutaneous Respiratory Cardiovascular	Immediate	Moderate	+	14th (3th line)	Carboplatin Gemcitabine	2	Carboplatin Gemcitabine	Cutaneous (2/2)
Patient 4	Cutaneous Respiratory Cardiovascular	Immediate	Severe	-	11th (3th line)	Carboplatin Gemcitabine Bevacizumab	5	Carboplatin monotherapy	Cutaneous (2/5)

LRDP: logarithmic rapid desensitization protocol

Carboplatin has had an increasing use and there has been an increase incidence of reactions. When a reaction occurs, options are desensitizing or substituting with a different agent. Successful replacement of carboplatin by cisplatin has been shown in patients with gynecological malignancies^[8,9]. However, the possibility of developing a reaction to the substituting platinum agent may be as high as 25%^[10] and cases of fatal cisplatin reactions have been reported^[11]. In this context, substitution of carboplatin must be considered with caution. Because of, desensitization protocols have been successfully used to manage hypersensitivity reactions^[1,2,12,13].

Goldberg *et al.*^[14] reported a desensitization regimen in two patients based in serial dilutions, with administration of increasing concentrations of carboplatin. Patients subsequently received infusions of 10^{-3} , 10^{-2} , and 10^{-1} of the total of the carboplatin dose. The final infusion contained 90% of the total drug dose.

Castells has developed a successful protocol of desensitization which would be supported by a basic biochemical mechanism: hypo-responsiveness in mast cells associated to suboptimal and increasing doses delivered at fixed time intervals (15 min). Therefore, specific mast cell and basophils tolerance could be due to the molecular stabilization of membrane, that would permit surpass a threshold antigen concentration required to activate these cells^[15]. Based on these dates, a 12-step protocol with a standardized three-resolution was generated which allows, for gradual increases in the infusion rate, to administer the target dose over 5-8 h. Doses of antigen must be delivered at fixed time intervals. Steps 1-11 last 15 min, and step 12 was prolonged to complete the target dose. The rate of the infusion was changed every 15 min, which each step delivering approximately twice (2 or 2.5) the dose of the previous step. The final step 12 maintained a constant role of infusion to deliver the remainder of the total dose. According to the description of the own authors, bag A contains a 100-fold lower amount of final target dose diluted in 250 mL (water with dextrose 5%); bag B contains a 10-fold lower amount of final target dose diluted in 250 mL and bag C contains final target minus a cumulative dose of previous steps, diluted in 250 mL^[1,2,12,13].

We have chosen a different path to calculate drug concentration of each bag, not giving importance to the concentration of each solution in relation to the target dose as other models. In order to facilitate the calculations, we selected a concentration based on mathematic concepts on the unit. The solution A had a concentration of 10^{-1} mg/mL (0.1 mg/mL) of the drug, the solution B had a concentration of 1 mg/mL and solution C (one only step) was calculated with a concentration of 2 mg/mL and including the total remaining dose of the drug in order to be administered at a faster speed. The initial dose of protocol had not a direct relationship with the target dose even though was approximately in order of 10^{-4} (approximately in a rank of $0.2-3 \times 10^{-4}$) lower than the target dose (if this dose is in the rank of 1-1000 mg) and this allowed us to apply the protocol in a simpler way. The procedure followed a mathematic model (gradual

increases with a relationship between doses following a geometric series) which is called LRDP. We have implemented this protocol consisting of 13 doses delivery stages, underlining the accurately relationship between doses in a geometric series. Progression of the doses occurred logarithmically. Each step delivered exactly twice the dose of previous step, in such a way that all step concentrations arranged in a geometric series with a factor two. The first term of the series is 1/32 and the common ratio is 2 (1/32, 1/16, 1/8, 1/4, 1/2, 1, 2,). The total quantity of volume in each of the three bags with each of the solutions is not very different from the volume to administer. Therefore, in case of a potential mistake in the schedule, the risk for the patient is minimized.

To enhance patient safety, administration of LRDP was performed by a multidisciplinary team including physicians and nursing staff from the Departments of Allergy, Medical Oncology and Pharmacy. LRDP with carboplatin is a feasible and safe protocol in patients with previous infusion reaction to carboplatin.

CONCLUSION

We have performed a retrospective study with the aim of assessing the effectiveness and safety of a LRDP with carboplatin in patients with a previous reaction. It is a promised protocol for administration of carboplatin after an infusion reaction.

The protocol might allow a safe administration of drugs (it can be applied to other drugs because the therapeutic dose of the majority of drugs is in a range between 1-1000 mg), that are beneficial for the management of patients, after certain types of hypersensitivity reactions, and it is indicated in cases in which there are no reasonable therapeutic alternatives.

DECLARATIONS

Authors' contributions

Designed the study and wrote the manuscript: Burches E, Pérez-Fidalgo JA
Contributed to data collection: Ferriols F, González-Barrallo I, Cervantes A

Availability of data and materials

Data are available in data base/Clinical Data Repository of Hospital Clinico de Valencia.

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Conflict of interest

All authors declared that there are no conflicts of interest.

Ethics approval and consent to participate

This retrospective study was approved by the local Ethics Committee of Hospital Clinico Universitario of Valencia (resolution number 320) in November 2016.

Consent for publication

Not applicable.

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Review

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Dietary fatty acids and adipose tissue inflammation at the crossroad between obesity and colorectal cancer

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Abstract

Excess adiposity, a worldwide-growing pathological condition, is now recognized as a main risk factor for most chronic diseases including colorectal cancer (CRC). Obese subjects show an increased cancer incidence with obesity representing an important indicator of survival, prognosis, recurrence and response to therapy. A low-grade chronic inflammation of metabolically active tissues including the adipose tissue (AT), defined as meta-inflammation, is a main feature of obesity. Fatty acids (FA), the main AT components, are important modulators of inflammation, and the type of FA stored in AT critically affects tissue functions. Their profile within AT mirrors FA dietary intake but also depends on a metabolic control. Obesity, changes in the habitual diet, weight loss or pathological conditions like CRC influence FA profile of AT pointing to these molecules as important actors in AT dysfunction and meta-inflammation, that characterize metabolic diseases and may favor cancer development. Worth of note, diet is receiving growing attention as a main determinant in cancer prevention due to its capacity to modulate immune response and inflammation. Alterations in the balance between different families of FA may contribute to generate a pro-inflammatory profile with deleterious effects on metabolic and immune homeostasis at both local and systemic levels. This review focuses on FA as regulators of human AT inflammation discussing the role of obesity-, diet-, and weight loss-associated changes in FA profile in this process. The relevance of FA composition of AT in linking diet, obesity and CRC will be also reviewed.



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Keywords: Obesity, adipose tissue, inflammation, diet, weight loss, fatty acids, colorectal cancer

INTRODUCTION

The overweight and obesity epidemic represents a rapidly growing health threat in several countries. Excess adiposity is associated with increased incidence of several cancers and represents an important indicator of survival, prognosis, recurrence and response to therapy in many tumors, including colorectal cancer (CRC). The risk of developing CRC is significantly increased in obese subjects, with abdominal obesity being more predictive than overall obesity, and is highly modifiable by diet^[1]. Dietary habits and excessive adiposity can not only influence cancer growth but also shape host immune response^[2]. White adipose tissue (AT), now recognized as the largest endocrine organ, plays a key role in metabolic and immune homeostasis^[3]. This tissue influences many local and systemic physiological and pathological processes by virtue of its capacity to secrete a large number of hormones, cytokines/chemokines/adipokines, extracellular matrix proteins, lipid metabolites and growth factors^[4]. In condition of chronic positive energy balance, AT undergoes profound modifications including adipocyte expansion, induction of hypoxia, and mitochondrial function alterations that lead to tissue remodeling, inflammation and metabolic dysfunction^[5]. These events tightly couple with dramatic changes in the immune cell repertoire and functions^[6,7] shifting the balance of cell subsets and soluble mediators toward a pro-inflammatory profile. Indeed, growing evidence indicates that meta-inflammation - a chronic low-grade inflammatory state occurring in metabolically active tissues including the AT - characterizes obesity and contributes to the impairment of immune functions, thus representing a key determinant in the development of obesity-related morbidities, including cancer^[8].

Evidence suggests that lipids, especially fatty acids (FA), the main components of AT, play an important role not only in obesity development but also in the interplay between excessive adiposity and development of associated diseases^[9]. Dietary lipids derived from plants and animals encompass FA (saturated, SFA, monounsaturated, MUFA, and polyunsaturated, PUFA), their derivatives including mono-, di-, and triglycerides and phospholipids, as well as sterols such as cholesterol. Among FA, SFA are mainly found in animal food, but a few plant food is also high in saturated fats, such as coconut, coconut oil, palm oil, and palm kernel oil. MUFA can come from both various plant-based (e.g., vegetable oils and nuts) and animal-based sources (e.g., red meats and high-fat dairy products). PUFA are essential FA as they cannot be synthesized from precursors in the diet, and derive primarily from plant-based sources. However, ω 3 PUFA can also be found in fish oils^[10,11].

The type of FA stored in AT, besides adipocyte fat overload, critically affects tissue functions. FA can directly or indirectly modify immune and inflammatory responses by several mechanisms, acting on cell surface and intracellular receptors that control cell signaling and gene expression^[12,13]. Recent studies have indicated that dietary FA quality rather than quantity has major implications in meta-inflammation development. In fact, FA exhibit either pro- or anti-inflammatory activity depending on their chemical structure^[11,13]. In general, long-chain SFA have been associated with inflammation while short-chain FA show anti-inflammatory effects^[14]. On the other hand ω 3 PUFA favor anti-inflammatory profiles, while ω 6 PUFA, with a few exceptions, are endowed with pro-inflammatory activity^[10]. Lastly, the effect of MUFA is more debated, with evidence for either anti-inflammatory or weak pro-inflammatory responses^[11,13].

The FA composition of AT is widely considered a marker of medium- and long-term dietary fat intake, with a general agreement that FA content in AT mirrors their relative abundance in the diet^[15]. However, FA profile in AT also depends on, at a certain extent, a metabolic control. Indeed, different AT sites exhibit different FA compositions^[16,17] as well as different rates of FA turnover^[18] and active remodeling^[19]. Furthermore, while some ω 3 PUFA such as docosahexaenoic acid are preferentially stored, others like

eicosapentaenoic acid are preferentially released or turned over during lipolysis^[19]. More recently, evidence points to a relevant role of enzymes involved in FA metabolism in defining plasma and tissue FA profiles. Human studies indicate that inter-individual variation in desaturase genes is due to both genetic and lifestyle factors, highlighting that the function of these enzymes may influence disease risk^[20].

In the following sections we will overview obesity-, diet-, and weight loss-associated changes of FA profiles by focusing on human AT. The role of FA as potential regulators of inflammation in metabolically active tissues, in particular AT, and as a link between obesity and CRC development will be discussed.

OBESITY-ASSOCIATED FATTY ACID PROFILES OF ADIPOSE TISSUE AND THEIR RELATIONSHIP WITH DIETARY INTAKE

Subcutaneous (SAT) and visceral (VAT) white AT depots constitute AT bulk. Their body distribution shows person-to-person variations and depends on several factors such as age, nutrition, sex, and energy homeostasis of the individual AT^[21]. VAT and SAT show significant variations in anatomical, cellular, molecular, and physiological characteristics^[22] and play different roles in metabolic syndrome development^[21]. In fact, excessive VAT accumulation is commonly associated with insulin resistance, markers of oxidative stress and inflammation, high risk of type 2 diabetes (T2D), dyslipidemia, and high mortality^[23-25]. Conversely, SAT accumulation is associated with improved insulin sensitivity and lower risk of T2D. Furthermore, metabolically beneficial adipokines (i.e., leptin and adiponectin) are secreted in higher amounts by SAT, whereas pro-inflammatory mediators are more abundantly secreted by VAT, regardless the body weight^[22]. Interestingly, in obese subjects, SAT exhibits a higher content of SFA with respect to VAT that instead shows a higher accumulation of MUFA as well as a higher activity of stearoyl-coenzyme A desaturase 16 (SCD16) and SCD18. Conversely, a similar PUFA composition has been reported for both fat depots^[16,17].

Some studies investigated obesity-associated FA profile of AT that can be modified by specific diets, weight loss or in pathological conditions like CRC. However, the patterns of FA accumulation distinguishing lean with respect to obese subjects are poorly known. A summary of the main differences in FA profiles of AT related to fat depot, obesity, weight loss and cancer, are shown in Table 1.

Despite any difference in SFA, MUFA and PUFA ($\omega 3$ and $\omega 6$) total content in the VAT of lean with respect to obese subjects, a clear-cut decrease in the $\omega 3/\omega 6$ ratio was detected in the latter^[26]. Furthermore, analysis of individual members of the $\omega 6$ PUFA family unraveled that arachidonic acid content increases in obese subjects with respect to lean while γ linolenic acid, the only member of the $\omega 6$ PUFA family endowed with anti-inflammatory activity, decreases in the same subjects^[27]. Moreover, a higher palmitoleic acid content and SCD1 index have been reported in SAT of obese *vs.* lean subjects^[28]. Likewise, our study demonstrated that higher levels of palmitoleic and stearic acids together with a higher desaturation index (i.e., SCD1 $\Delta 9$ -18) are found in the VAT of obese subjects^[29].

The FA composition of AT reflects not only the dietary intake but also endogenous fat processing. Thus, changing the nature of the fat consumed has a profound influence on the type of FA available to the body and may alter AT composition^[30]. In this regard, our most recent data show a higher arachidonic acid dietary intake in obese subjects as well as a higher arachidonic acid content of VAT^[27,29]. The relative proportion of some PUFA (i.e., linoleic, α linolenic, eicosapentaenoic and docosahexaenoic acids) as well as of SFA (i.e., palmitic acid) and MUFA (i.e., palmitoleic acid) in SAT was also found to mainly reflect their dietary intake in a large Swedish cohort study of adult men^[31]. Likewise higher levels of oleic acid in SAT were observed when overweight subjects received a MUFA- rather than a SFA-rich diet^[32]. The association between dietary FA and their composition in specific AT was studied in a cohort of obese subjects from the

Table 1. Fat depot-, obesity-, weight loss- and cancer-related differences in fatty acid profile of visceral and subcutaneous adipose tissue

Comparison	AT localization	FA profile and lipid metabolism enzymes	Ref.
SAT vs. VAT ^a		SFA > total MUFA < total PUFA Equal total $\omega 3$, $\omega 6$ and $\omega 3/\omega 6$ Enzymes < SCD16/SCD18	[16,17]
Obese vs. lean	VAT	SFA Equal total; > SA MUFA Equal total; > POA PUFA Equal total $\omega 3$ and $\omega 6$; < GLA; > AA; < $\omega 3/\omega 6$ Enzymes > SCD1 $\Delta 9$ -18	[26,27,29]
	SAT	MUFA > POA Enzymes > SCD1	[28]
Weight loss vs. baseline ^b (Diet)	SAT	SFA < total and PA; > SA MUFA < POA; > OA PUFA < ALA and trans-LA; > EPA, DHA, LA, DGLA, AA and DTA	[34-37]
Weight loss vs. baseline ^b (Surgery)	SAT	SFA < total and PA MUFA Equal total; > OA; < POA PUFA > total $\omega 3$ and $\omega 6$, EPA, DPA, LA, GLA and AA Enzymes > Elongase 3, 5, 6 ^c	[38]
CC/CRC vs. healthy	VAT	SFA > or < total PUFA > total $\omega 6$, DGLA, AA and DTA; < ALA and SDA; < $\omega 3/\omega 6$ Enzymes > SCD1 $\Delta 9$ -18; < FASN	[26,27,29,45,74,80]
	SAT	SFA Equal total MUFA Equal or > total PUFA Equal or < ALA; > DPA, AA, GLA and DGLA; equal LA; < $\omega 3/\omega 6$	[72-75]

^aFA content of SAT versus VAT. ^bstudies analyzing FA profiles in VAT upon weight loss are not present in the literature. ^cmeasured as mRNA expression. ALA: α linolenic acid; AA: arachidonic acid; DHA: docosahexaenoic acid; DGLA: dihomo- γ -linolenic acid; DPA: docosapentaenoic acid; DTA: docosatetraenoic acid; EPA: eicosapentaenoic acid; GLA: γ linolenic acid; LA: linoleic acid; OA: oleic acid; PA: palmitic acid; POA: palmitoleic acid; SA: stearic acid; SDA: stearidonic acid; VAT: visceral adipose tissue; SAT: subcutaneous adipose tissue

Mediterranean area. Significant correlations were found for oleic, linoleic and α linolenic acids and for total $\omega 6$ PUFA between the subjects' habitual diet and AT composition. Interestingly, VAT accumulation was positively associated with $\omega 6$ PUFA and inversely associated with MUFA and $\omega 3$ PUFA^[17]. The subsequent assessment of the dietary pattern, AT composition and degree of obesity showed that despite a similar dietary pattern among the groups, overweight and morbidly obese individuals ($\text{BMI} \geq 40 \text{ kg/m}^2$) have a higher MUFA content than obese subjects ($\text{BMI} \leq 40 \text{ kg/m}^2$), probably due to differences in SCD1 activity, slightly higher in morbidly obese than in obese subjects^[33].

WEIGHT LOSS AND DIET-INDUCED MODIFICATIONS OF ADIPOSE TISSUE FATTY ACID PROFILES

The association between individual FA content and body weight is rather complex due to the fact that distinct FA share dietary sources and metabolic pathways. Furthermore, a complex interplay among lifestyle, bulk intake of nutrients together with the genetic background influences FA composition of AT in addition to their dietary intake. Likely due to this complexity, only few studies investigated the association between specific FA or FA profile of AT with dietary intake, and assessed the effects of changes in FA composition of AT on the health status. Nevertheless, beneficial changes in FA profiles of AT have been observed in response to weight loss, that can potentially contribute to weight maintenance, as well as to modifications of the dietary pattern. Some rather old studies, carried out in small cohorts of subjects, first observed that weight loss, promoted in obese subjects by different low calorie diets (LCD), results in a reduced accumulation of α linolenic acid in SAT while the content of other FA remained unchanged^[34,35]. This preferential reduction also occurred when LCD were supplemented with canola or linseed oils^[36]. These findings strongly suggest that the relative amount of individual FA in AT depends, not only, on

the dietary intake but also on the different rates of metabolic reaction that utilize FA as substrates. Subsequent analysis of FA composition after LCD-induced weight loss and 6-month weight maintenance showed a reduction in total SFA, palmitic and palmitoleic acid content that paralleled a concomitant increase of stearic and oleic acids^[37]. Likewise a significant increase in some $\omega 3$ (i.e., eicosapentaenoic and docosahexaenoic acids) and $\omega 6$ (i.e., linoleic, dihomo- γ -linolenic, arachidonic and docosatetraenoic acids) PUFA was also observed. Furthermore, a specific role of MUFA in weight management and as predictors of weight change was suggested. In fact lower baseline content of MUFA in SAT predicts a better weight maintenance while lower oleic acid accumulation predicts lower weight decrease^[37]. Potentially beneficial modifications in FA composition of SAT have also been observed in a Finnish study upon obesity surgery - a procedure that leads to changes in gut anatomy and diet as well as to weight loss - in combination with rapeseed oil- and fatty fish-enriched diet^[38]. The main findings were a decreased content in SFA, mainly due to palmitic acid decrease, paralleling an increased accumulation of oleic acid in SAT. The metabolic improvement observed after surgery has been attributed not only to weight loss but also to diet-induced changes of endogenous lipid metabolism enzymes. In fact the increased activity of elongase 5 and delta 6 desaturase (D6D), as well as the decreased activity of SCD1 and D5D were strongly associated with weight loss^[38]. Interestingly, with the exception of $\omega 3$ PUFA, comparable modifications in FA content were induced by weight loss or lifestyle intervention without surgery^[38].

DIETARY FATTY ACIDS AS MODULATORS OF ADIPOSE TISSUE INFLAMMATION

The primary event in the sequence leading to chronic inflammation in AT is metabolic dysfunction of adipocytes, that promotes inflammation via the expression of inflammatory response genes. In spite of the well-known capacity of FA to modulate inflammatory processes and of the observation that specific FA profiles in the AT are associated with a pro-inflammatory condition, only few studies investigated the effects of direct exposure of adipocytes rather than of other cell types within the stromovascular fraction, to different FA on AT inflammation. Contrasting results were some time achieved likely reflecting differences in AT microenvironment, as the stromovascular fraction of AT strongly contributes to shape inflammation being a major source of immune mediators. Nevertheless, these studies shed light on the molecular pathways triggered specifically in adipocytes underlying the pro- or anti-inflammatory effects of FA^[4,6].

In vitro studies have reported that SFA stimulate an inflammatory response by the activation of Toll-like receptor (TLR)/NF- κ B pathways. Abdominal SAT and VAT adipocytes from obese subjects exposed to free FA (i.e., oleic, linoleic, arachidonic, lauric and myristic acids or palmitic and stearic acid mixtures) show an increased expression of pro-inflammatory cytokines (TNF- α , IL-6 and CCL2)^[39,40]. Interestingly the extent of SFA response does not differ greatly between AT explants and isolated adipocytes, highlighting the capacity of the latter to mount an autonomous inflammatory response to environmental factors^[40]. However, the reported activation of TLR pathways by dietary SFA (lauric and palmitic acids) was not confirmed in other studies in SAT explants and adipocytes under different experimental conditions (i.e., FA concentration or degree of endotoxin contamination)^[41].

While SFA were found to stimulate pro-inflammatory responses, $\omega 3$ PUFA, in particular docosahexaenoic and eicosapentaenoic acids, have been reported to exert an anti-inflammatory action in whole SAT and VAT as well as in isolated adipocytes from obese subjects by down-regulating the expression of pro-inflammatory mediators (IL-18, CASP-1, IL-1 β , CX3CL1, CCL2, TNF- α and IL-6)^[41-43]. Likewise, our studies demonstrated that docosahexaenoic acid attenuates the VAT adipocyte inflammatory status by reducing STAT3 activation and IL-6 secretion, and up-regulating adiponectin expression, regardless the body weight^[26,27]. Conversely, exposure of VAT adipocytes from lean and obese subjects to arachidonic acid results in a significant up-regulation of phospho-STAT3 and concomitant down-regulation of PPAR γ expression as compared to the untreated paired individuals^[26,27]. The observation that obesity-associated FA profile of VAT parallels alterations of the immune cell repertoire in the tissue microenvironment suggests

a role for VAT PUFA composition in shaping immune phenotypes^[44]. $\omega 3$ and $\omega 6$ PUFA, in particular decosahexaenoic and arachidonic acids, have also been reported to differently influence adipocyte transcriptional program in lean and obese subjects^[42,43,45]. Among the genes modulated by decosahexaenoic acid are those involved in AT inflammation and metabolism such as *FADS1* and *FADS2* genes that code for D5D and D6D, respectively, and thus control AT PUFA metabolism^[45,46]. The regulatory action of dietary PUFA on adipocyte genomics adds further evidence for a role of diet in the modulation of obesity-associated AT inflammation.

The role of FA in the modulation of obesity-associated AT inflammation has also been investigated in several clinical trials aimed at assessing the effect of consumption of different FA on the expression of inflammation-related genes in AT. However, some conflicting results have been reported probably due to the large variability in concentration and type of FA used in each intervention, differences among the specific populations investigated, variability in intestinal microbiota among individuals. Moreover, recent studies suggest that the AT response to FA is more complex than originally anticipated, and that the gene expression in AT is site specific suggesting that not all fat depots in the body are controlled in the same manner. The analysis of inflammatory responses in different sites (SAT or VAT, whole AT or isolated adipocytes) may therefore have generated discordant results.

As described for the *in vitro* studies, the consumption of a SFA-rich diet results in a pro-inflammatory gene expression profile in SAT (i.e., CD16a, IL-1 β , IL-6, IL-6R and TNF- α), while MUFA-rich diet or acute post-prandial MUFA intervention lead to different profiles depending on the category of subjects. Indeed in abdominally obese^[32] or healthy subjects the majority of regulated genes show anti-inflammatory features, whereas in subjects at higher risk of T2D the post-prandial consumption of MUFA-containing macadamia nut oil evokes an inflammatory response with up-regulation of several inflammatory genes (CCL2, IL-1 β , IL-6, IL-6R, TNF- α and TNFRSF1A)^[47]. These results indicate that MUFA can also exert a pro-inflammatory response, which is greater among subjects with obesity-related diseases as compared to healthy individuals. In line with this evidence, an exacerbated AT post-prandial inflammatory response (NF- κ Bp65, CCL2, IL-6 and IL-1 β) occurs in SAT of metabolic syndrome patients^[48].

The potential benefits of $\omega 3$ PUFA consumption on a wide range of AT inflammatory responses have been highlighted in recent studies^[49]. In a randomized controlled trial involving severely obese patients (≥ 40 kg/m²), $\omega 3$ PUFA supplementation over an eight-week period results in down-regulation of inflammatory genes (CCL2, CCL3, HIF1A, CD40 and IL-6), and up-modulation of the anti-inflammatory adiponectin in SAT, but not in VAT, in comparison with the control group^[50]. Additionally, consumption of different sources of fatty fish reduces the expression of AT inflammatory genes including inflammasome-associated IL-18, IL-1 β and IL1RN or impairs fasting glucose in obese subjects^[43,51]. Moreover, in a clinical trial (FFAME) involving healthy volunteers and based on eicosapentaenoic and decosahexaenoic acid supplementation, $\omega 3$ PUFA showed immune-modulatory and anti-inflammatory capability through the modulation of several inflammatory and specific immune genes during evoked AT inflammation induced by experimental endotoxemia^[52,53]. In contrast to these results, no effect on SAT inflammation was observed neither in overweight to moderately obese adults consuming a diet rich in $\omega 3$ PUFA^[54], nor in obese postmenopausal women after decosahexaenoic acid supplementation^[55]. Finally, $\omega 3$ PUFA (i.e., eicosapentaenoic acid and/or α -lipoic acid) dietary supplementation in addition to weight loss and dietary interventions unravels differences in the expression of genes related to inflammation and immune response in SAT from overweight/obese women^[56]. Other clinical evidence demonstrates that weight loss, known to improve obesity-associated low grade inflammation, is linked to changes in AT FA composition, suggesting a specific role of different FA in weight management and control of inflammation^[57].

A strong association between SFA and MUFA AT profiles and AT inflammation was demonstrated by the KOBS study. The results achieved showed that surgery-induced weight loss of obese individuals

reduces the expression of IL-1 β and NF- κ B pathway and unravels a positive/negative association between inflammation and SFA and MUFA content, respectively, in both SAT and VAT^[16]. These correlations were modified by the FADS1/FADS2 genotype, both in KOBS and DIOGENES studies, indicating that variants in this gene cluster may influence the interaction between AT FA and tissue inflammation^[16,58]. In the same KOBS cohort, Walle *et al.*^[38] measured serum and AT FA composition and AT expression of genes regulating FA metabolism before and one year after obesity surgery, combined with dietary counseling. Interestingly, some of the changes observed in AT FA profile are associated with the expression of elongases and desaturases, key enzymes in FA metabolism^[38]. According to these results, weight loss after bariatric surgery in morbidly obese women^[59] or after LCD in obese premenopausal women^[60] significantly improves the SAT inflammatory and immune profile by decreasing the expression of pro-inflammatory factors while increasing that of anti-inflammatory molecules as well as of genes involved in the regulation of lipid metabolism. Furthermore, the effects of two different LCD among obese women, within the NUGENOB study trial, were determined on SAT mRNA expression level, according to energy deficit and fat to carbohydrate ratio^[61]. Among the transcripts deregulated during the diets are genes involved in lipid, carbohydrate and energy metabolism^[61,62]. In contrast to these results, no changes in adipocyte gene related to inflammation and metabolism were reported in SAT in two cohorts of obese pre- and post-menopausal women after energy-restricted diets^[63,64], although inflammation related biomarkers were reduced systemically^[63].

ADIPOSE TISSUE INFLAMMATION, FATTY ACID PROFILE AND COLORECTAL CANCER

AT-associated inflammation promoted by incorrect dietary habits and obesity, under the influence of signals derived from the gut microbial flora, is a main mechanism that may favor CRC development and progression^[65]. Inflamed AT may contribute to tumorigenesis by releasing pro-inflammatory mediators and by modifying the lipid metabolism, which is part of the reprogrammed energy metabolism that characterizes cancer^[66]. In turn, cancer cells have the ability to induce metabolic changes in neighboring adipocytes and to use AT released FA as substrates for their proliferation^[67,68].

A number of studies have analyzed the profiles of FA in AT of CRC patients, in association or not with dietary intake, or have correlated specific FA profiles with CRC risk^[69,70]. Some old studies reported a similar FA profile in healthy and CRC affected subjects^[71,72]. However, despite a comparable consumption of the major dietary components and fat intake between the study groups, an age-associated decrease in unsaturated fat intake was observed only in the CRC group. Conversely, correlation between dietary and AT PUFA:SFA ratio was found in the control but not in the cancer group^[72].

More recently, a general agreement has been achieved on the increased accumulation of pro-inflammatory ω 6 PUFA (mostly dihomo- γ -linolenic and arachidonic acids) as well as on the unbalanced ω 3/ ω 6 PUFA ratio in the AT of CRC patients, regardless of tumor subsite, even though differences were reported among studies on the relative abundance of individual PUFA and type of AT involved^[27,73-75]. In particular, a comparison of VAT and SAT FA profiles in colon cancer (CC) patients highlights a decrease of the ω 3 PUFA α linolenic and stearidonic acid content, coupled to increased dihomo- γ -linolenic and arachidonic acid content in VAT of CC patients as compared to controls. Conversely, FA composition of SAT in CC subjects is only marginally altered, with increased γ linolenic acid levels^[74]. Although no dietary information was available for the study population, the low ω 3/ ω 6 PUFA ratio generally observed in AT likely reflects Western dietary habits^[74]. Changes in the ω 3/ ω 6 PUFA profile (higher dihomo- γ -linolenic and docosapentaenoic acids, *vs.* lower α linolenic acid) were also reported in SAT from CRC patients, in association with markers of systemic inflammation^[75]. The altered ω 3/ ω 6 PUFA balance in cancer patients can markedly affect tissue composition and function as a result of the reduced protective effect of ω 3 PUFA. Moreover, a CRC-associated increase of total SFA and MUFA content in VAT and SAT, respectively, was also described in some studies^[74].

Although the influence of BMI on the FA changes in CRC-affected subjects was not considered in the above studies, its relevance emerged in our subsequent studies in which the VAT FA composition was analyzed in newly diagnosed CRC patients, sub-grouped in lean and obese according to BMI^[26,27,29]. A reduced $\omega 3/\omega 6$ PUFA ratio as well as an increased content of dihomo- γ -linolenic and docosatetraenoic acids, coupled with STAT3 activation characterize CRC subjects, irrespective of BMI^[26,27]. However, compared to healthy individuals, obese CRC patients show a selective accumulation of arachidonic acid in VAT, whereas a lower SFA content is specific of lean CRC subjects despite a higher dietary intake^[27,29]. The lack of correspondence between SFA intake and storage in the latter subjects could in part rely on an accelerated SFA to MUFA conversion as suggested by the increased estimated activity of SCD1 $\Delta 9$ -18^[29]. Interestingly, the increased content of pro-inflammatory FA in AT from CRC-affected individuals is associated to an enhanced adipocyte release of inflammatory cytokines and chemokines (IL-6, CCL2, CXCL8) and with the establishment of an immunosuppressive VAT microenvironment^[27].

AT and its FA content have also been related to tumor progression by Mosconi *et al.*^[76], who analyzed VAT peri-tumoral fat composition in CRC patients at different tumor stages. The total content of MUFA in close proximity of, but not far from the tumor lesion, was found significantly increased in patients at higher tumor stage, especially stage IV. Comparable levels of SFA and PUFA were instead found in the same patients, irrespective of the tumor stage, suggesting a selective involvement of MUFA in cancer progression^[76]. Some food processing techniques (industrial hydrogenation of fats) and cooking methods (heating and frying at temperatures > 220 °C) convert FA, naturally present in food in the *cis* form, into their *trans* form, and the latter configuration has been associated with increased risk of cancer and cardiovascular diseases^[77]. In the EURAMIC epidemiological study the FA composition of SAT samples was used as a measure of FA intake and associated with CC risk^[78]. Statistically significant inverse correlations were found between *cis*-MUFA concentration and the incidence of CC. Conversely, CC incidence positively correlates with the content of *trans*-MUFA^[78]. As it stems from these results, the same category of FA (e.g., MUFA) can exert a protective or promoting effect on CRC onset/progression, depending on their configuration, the fat depot in which they are enriched, and the tumor stage^[76,78].

As stated above, AT FA profiles are influenced also by the activity of enzymes driving FA synthesis and/or conversion. The expression/activity of these enzymes have been found dysregulated in tumor and AT from cancer patients. In particular, FA synthase (FASN), a key anabolic enzyme, increases in the intestinal mucosa of subjects with CRC or with pre-cancerous lesions according to the increased metabolic demand of cancer cells^[79]. In contrast, FASN expression and activity are significantly reduced in VAT adjacent to the tumor lesion, resulting in impairment of FA synthesis and AT storage^[80]. The inflammatory VAT microenvironment might account for this reduction as a comparable FASN downregulation was described in obese and CRC-affected individuals^[27,45,81].

Altogether, the alterations of FA metabolism and profiles in different fat depots, in proximity or far from the tumor lesions, contribute to maintain a pro-inflammatory microenvironment in CRC patients, and strongly support a role for both unbalanced dietary intake and changes in FA metabolism and storage in colon and rectal carcinogenesis.

CONCLUSION

The type of daily consumed dietary fat together with metabolic activities influence the FA profile in AT and can alter its functionality thus contributing to the onset of meta-inflammation that characterizes obesity and metabolic diseases [Figure 1]. The promotion of inflammatory processes within AT determines an inflamed microenvironment that can represent a trigger for the development of a number of obesity-related pathologies including CRC. All together these findings highlight the relevance of maintaining healthy dietary habits and the central role of AT in preserving health and well-being. However, the studies,

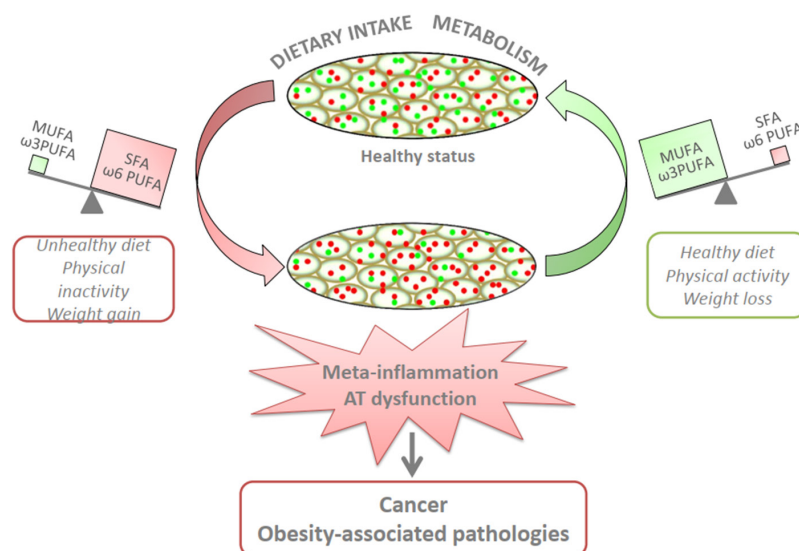


Figure 1. A schematic representation of the interplay between diet and metabolism in determining the fatty acid profile of adipose tissue. The consequences of adipose tissue dysfunction in the pathogenesis of obesity-related morbidities including cancer are depicted

especially the clinical trials, carried out until now provide often contrasting results. This should drive to implement new researches specifically addressed to clarify the relationship among the various molecular pathways involved in FA metabolism and inflammation establishment. The improvement of knowledge on the real role that dietary habits play in modulating key metabolic processes, will allow to provide personalized nutritional advices that take in account the inter-individual differences, including those gender-related, in the response to diet.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the review: Conti L, Del Cornò M, Gessani S
Contributed to the literature search, revision and editing of this manuscript: Scazzocchio B, Vari R, D'Archivio M, Varano B
Made substantial contribution to discussion: Masella R

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Membrane lipid binding molecules for the isolation of bona fide extracellular vesicle types and associated biomarkers in liquid biopsy

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Abstract

Cancer exacts a heavy socioeconomic cost. Earlier detection and treatment are likely to mitigate this cost. Unfortunately, conventional tissue biopsy, the gold standard in cancer diagnosis cannot fulfill the goal of earlier detection. While liquid biopsy is a promising alternative to tissue biopsy, it has its challenges and limitations. A major challenge is the isolation of bona fide lipid membrane vesicles from biological fluids. In this review, we presented a new perspective of isolating different types of extracellular vesicles (EVs) by their affinity for membrane lipid binding ligands for liquid biopsy. EVs are lipid membrane particles naturally released by almost all cells and are found in almost all biological fluids suitable for liquid biopsy. They carry materials from the secreting cells that could affect the biology of the recipient cells and could thus inform on the state and progress of the disease. However, isolating bona fide EVs is a technical challenge as biological fluids have a complex composition and contain particles or aggregates that are physically similar to EVs. Here we review the use of membrane lipid-binding ligands to isolate different bona fide EV subtypes, and to circumvent the problem of co-isolating physically similar non-EV complexes in current EV isolation protocols. We will discuss the advantages of this technique and its potential for accelerated biomarker discovery and validation through examples of pre-clinical studies. We propose that isolating EV subtypes is a technically viable and robust strategy to overcome the current bottleneck of isolating EVs for liquid biopsy.



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Keywords: Membrane lipid binding molecules, extracellular vesicle, cholera toxin B, shiga toxin B, annexin V, biomarker

INTRODUCTION

Cancer is the second leading cause of death in the world according to WHO (<https://www.who.int/news-room/fact-sheets/detail/cancer>). In 2018, there was an estimated 18.1 million new cancer cases and 9.6 million cancer deaths worldwide with 70% occurring in low and middle-income countries^[1]. This creates a devastating socioeconomic burden. To reduce this cancer burden, WHO has recommended cancer prevention through avoidance of risk factors and implementation of existing evidence-based prevention strategies. In addition, this burden can be reduced through early detection of cancer as many cancers could be cured if diagnosed early and treated adequately.

The gold standard in cancer diagnosis is tissue biopsy. However as this is an invasive procedure, it is often done when cancer is suspected and when the cancer is usually at an advanced stage. Also, the suspected cancer may be in tissues that are hard to access such as the brain and tissue biopsy will be difficult with significant clinical risks such as bleeding or infection. As such, surrogate methods for detecting the presence of cancer such as liquid biopsy are being aggressively developed. Liquid biopsy was originally defined by the US. National Cancer Institute as “a test done on a sample of blood to look for cancer cells from a tumor that are circulating in the blood or for pieces of DNA from tumor cells that are in the blood” (<https://www.cancer.gov/publications/dictionaries/cancer-terms/def/liquid-biopsy>). Although tissue biopsy remains the gold standard for cancer diagnosis, liquid biopsy is gaining traction as it is less invasive and could be used to screen for cancer prior to disease manifestation when the cancer is in its early stage. Biomarkers used in liquid biopsy have also expanded beyond circulating tumor cells or DNA to include circulating exosomes and miRNAs.

This review will focus primarily on the use of exosomes or more precisely, extracellular vesicles (EVs) in liquid biopsy. As recommended by Minimal Information for Studies of EVs (MISEV2014)^[2] and the recently updated and expanded MISEV2018^[3], the term “Exosomes” is presently used to describe small (50-200 nm) extracellular vesicle that has an endosomal biogenesis while the term “EV” is a more generic term to describe membrane vesicles secreted by cells. Since the EV types and biogenesis of most EVs in bodily fluids are presently not known, the term EV is, therefore, more appropriate when referring to secreted membrane vesicles in liquid biopsy.

EVS AS SOURCES OF BIOMARKERS

MISEV2018 defines EVs as “particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate, i.e., do not contain a functional nucleus”^[3]. They are produced by nearly all cell types from bacteria to yeast, plants and animals. EVs are known to carry a complex cargo of proteins, lipids, RNA, DNA and other metabolites. As the cargo is derived from the secreting cell, the cargo reflects the type and biology of the cells and could potentially inform on the state of health in the cells. Furthermore, as EVs are generally thought to mediate intercellular communication through the conveyance of biological materials from one cell to another, EVs could potentially propagate disease or modulate host response. Hence the cargo of EVs could also predict the progress or resolution of the disease. Together, these EV features provide a strong rationale for the use of EVs in bodily fluids such as blood, urine, saliva, tears or sweat in liquid biopsy assays. The reader is referred to several recent comprehensive reviews in this area^[4-8].

ADVANTAGE OF PLASMA EV OVER WHOLE PLASMA FOR BIOMARKER DISCOVERY AND ANALYSIS

EV-based liquid biopsy is essentially a transformation of the traditional assay for biomarkers in plasma or other bodily fluids into an assay for biomarkers in EVs isolated from either plasma or other bodily fluids. This transformation is critical as using EVs from bodily fluids as the source of biomarkers as opposed to unfractionated bodily fluids could greatly enhance the signal to noise ratio in detecting a biomarker. For example, plasma, a commonly used bodily fluid in diagnosis has a wide dynamic concentration range of at least 10 orders of magnitude between the most abundant plasma protein, albumin and rare biomarker proteins^[9]. In fact, > 95% of the protein mass in human plasma is dominated by a dozen proteins with albumin and the globulins constituting about 50% and 25%, respectively^[10]. As such, disease-associated protein biomarkers are likely to be rare and masked by the highly abundant proteins, making plasma biomarker discovery and detection challenging. Specific methods to Isolate EVs from plasma could potentially remove the highly abundant proteins from the plasma and enhances the discovery and detection of rare disease-associated biomarkers.

TECHNOLOGIES FOR ISOLATING EVS FROM BODILY FLUIDS

As extensively discussed in the MISEV2018^[3], EVs could be isolated from different bodily fluids such as serum/plasma, urine, tears, milk, spinal fluids using a large variety of techniques. As each of the fluids have unique features that could interfere with EV purification and analysis, MISEV emphasized the need for these fluids to be appropriately processed prior to EV isolation and analysis. For example, blood plasma is routinely treated with anti-clotting agents such as EDTA, citrate or heparin to prevent clotting while the urine is often depleted of Tamm-Horsfall protein, a major urinary protein. However, MISEV2018 does not specifically recognise any method as the gold standard for EV isolation but noted that the choice is dependent on the end use of the EVs. Presently, the most commonly used methods for EV isolation as described in MISEV2018 are generally based on biophysical parameters such as size (e.g., filtration, size exclusion chromatography, precipitation), density (e.g., differential ultracentrifugation, sucrose gradient ultracentrifugation) and charges (e.g., electrophoresis, ion exchange chromatography). EVs are also isolated according to surface proteins (e.g., immunoaffinity chromatography). Combination of these technologies, e.g., filtration with density ultracentrifugation are also being used or developed to improve the efficiency, specificity and purity of the EV preparation.

As noted by several groups, the desired end use of the purified EVs is greatly affected by the choice of isolation techniques or combination of techniques. For example, precipitation or filtration techniques could process large volumes of fluids and while this is critical in preparing sufficient therapeutic EVs, these methods may not be as stringent as size exclusion chromatography in isolating highly enriched EV preparation^[11,12]. As many of the current EV isolation techniques require specialized equipment and many procedural steps, microfluidic chips are increasingly being used in the isolation of EVs as a means to reduce the hands-on time and the number of procedural steps. Most of the microfluidic chips to isolate EVs are also based on the same fractionation parameters as the conventional EV isolation techniques namely immune-capture by antibodies against surface proteins of EVs^[13], size fractionation by membranes^[14,15], ciliated micropillars^[16], acoustics^[17] or viscoelasticity^[18].

Generally, most EV isolation technologies enriched for EVs on the basis of size or densities. Hence most EV preparations, especially those from biological fluids, are also significantly enriched in similar sized non-EV complexes such as HDL and LDL lipoprotein complexes and/or protein aggregates, and two or more isolation techniques often have to be used in tandem to reduce non-EV components^[19-23]. In addition, most of the current EV isolation technologies require relatively large volume of sample fluid, typically 250 μ L to 1 mL or long preparation time to isolate sufficient EVs for diagnostic applications.

Hence, development of EV-based liquid biopsy is hampered by two technical challenges, isolation of bona fide lipid membrane vesicles and EV isolation from small sample volumes.

ISOLATING EVS THROUGH THEIR UNIQUE DEFINING FEATURE, LIPID MEMBRANE

The unique defining feature that differentiates EVs from other secreted cellular products is their lipid membrane. Therefore, isolating EVs via their lipid membrane would enhance the specific enrichment of EVs.

Although the lipid membrane of EVs originates from the secreting cells, the EV membrane is not representative of the outer or inner cell membranes per se but rather of specific microdomains in cellular membranes. For example, the membrane of exosomes derived from Daudi cells is enriched in lipid rafts as evidenced by the presence of GM1 gangliosides which are enriched in lipid rafts^[24]. It was further reported using annexin V (AV) binding studies that phosphatidylserines are exposed on the outer surface of exosomes^[25]. However, it is not clear if exosomes with exposed phosphatidylserines are exosomes or apoptotic bodies as membrane vesicles from healthy cell populations are likely to contain apoptotic bodies from apoptotic cells that are inevitably present in a healthy cell population^[26]. In addition, it was also observed that in platelets, AV binding activity was restricted mainly to microvesicles, and not exosomes^[27]. Finally, we observed that small EVs produced by mesenchymal stem/stromal cells (MSCs) consist of at least 3 sub-types that are distinguished by their mutually exclusive binding affinity for cholera toxin B (CTB) chain, AV and shiga toxin B (ST) chain^[28]. The latter three proteins bind membrane lipids, GM1 gangliosides, phosphatidylserines and globotriaosylceramides, respectively. The extraction of small EVs by these three proteins was confirmed by scanning electron microscopy images of nanosized, spherical structures of approximately 100-200 nm. Protein analysis of the three EVs revealed an enrichment of exosome-associated proteins such as Alix, Tsg 101, CD9, CD81 in the CTB-binding EVs which were much reduced in the AV-binding EVs and not detectable in the ST-binding EVs. RNA was detectable only in the ST-binding EVs^[28]. It was also established through pulse-chase studies that the CTB-binding EVs have an endosomal biogenesis, i.e., exosomes while the biogenesis of the AV- and ST-binding EVs is not known^[28,29]. In the pulse-chase studies, the MSCs were pulse-fed with either biotinylated transferrin or biotinylated CTB and the biotinylated proteins was then “chased” to determine if the biotinylated proteins re-appear in the culture medium as EVs.

CTB, AV and ST are multi-meric with at least five lipid-binding monomers per molecule. It was reported that these monomers bind their lipid targets in a positive co-operative manner such that the lipid-bound ligand is thermodynamically most stable when all the lipid-binding monomers are bound by the target lipids^[30-32]. Therefore, the binding of an EV type by any of the three ligands implies that the targeted lipids for the ligands are exposed on the EV membrane surface and are also present in high localized concentration to enable multimeric binding^[28]. This thermodynamic preference of each ligand to bind multiple lipid molecules ensures that the ligand binds lipid-rich macromolecules or complexes such as EVs.

As a consequence of this preference, the lipid-binding ligand cannot be immobilized on a solid support and must be in solution to minimize steric hindrance and facilitate ligand binding to multiple lipids (unpublished observation, SKL). This thus minimizes the versatility in designing the procedural protocol for isolation.

Together, the above observations suggest that EVs could be isolated through their lipid membrane via specific membrane lipid-binding ligands as shown [Figure 1].

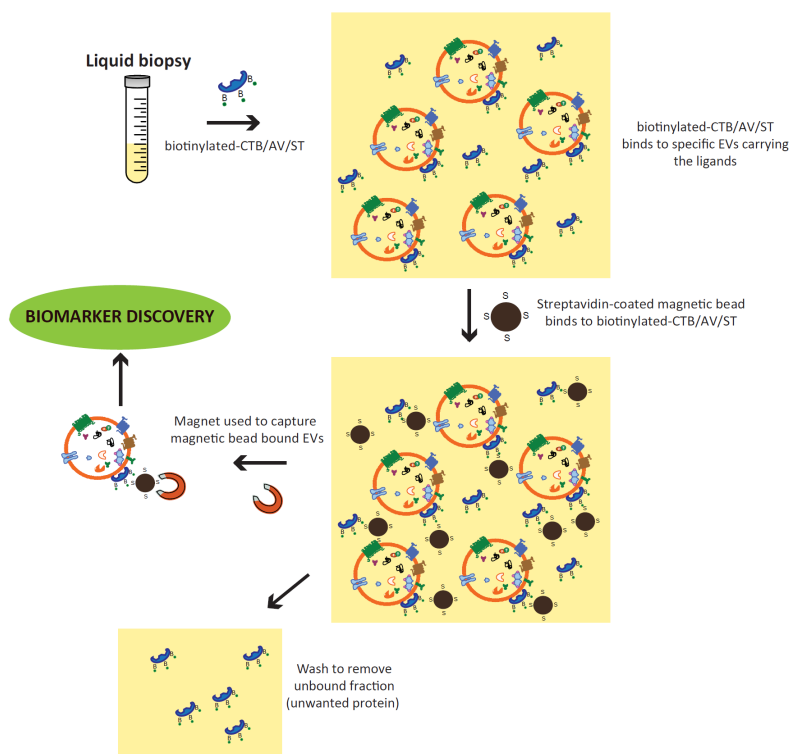


Figure 1. Isolation of CTB/AV/ST-binding EVs from liquid biopsy. Biotinylated-CTB, AV or ST is added to the liquid biopsy. These ligands, CTB, AV or ST will bind exposed membrane lipids, GM1 gangliosides, phosphatidylserines or globotriaosylceramides on the EV membrane, respectively. After binding, the ligand-bound EVs can be readily extracted using either streptavidin-coated magnetic beads and magnets, or streptavidin-coated polystyrene and membrane filtration. These extracted CTB/AV/ST-binding EVs can be used directly for biomarker discovery or assay. CTB: cholera toxin B; EVs: extracellular vesicles; AV: annexin V; ST: shiga toxin

ISOLATING PLASMA EVS THROUGH THEIR AFFINITY FOR MEMBRANE LIPID-BINDING

LIGANDS

The binding affinity of EVs for CTB and AV in conditioned medium from cell cultures has also been observed in EVs in biological fluids such as plasma and ascites fluid^[33,34]. However, it was observed that less than 1% EVs in the plasma have exposed phosphatidylserines^[35]. Furthermore, the CTB chain- and AV-binding EVs in the plasma and ascites fluid are distinct populations of EVs with each subtype having a cargo of proteins that is unique to EV subtype and pathological state of the patient^[33,34].

DIFFERENTIAL DISTRIBUTION OF DISEASE BIOMARKERS IN EV SUBTYPES OF BIOLOGICAL FLUIDS

When plasma EVs were isolated according to their affinity for lipid-binding ligands, it was observed that the distribution of a protein across the different EV subtypes is variable and the level of some proteins in one EV subtype but not the other EV types or the bodily fluid correlate with the disease state of the patient. This was evident even when the proteome of plasma and plasma EV sub-types from normal healthy pregnant woman and pre-eclampsia pregnant women were analyzed in low-resolution 2D protein gels^[33]. When analyzed by more sensitive mass spectrometry and compared, there were differences within and between the healthy and pre-eclampsia populations of patients in each of the three fractions, namely plasma, CTB- and AV-binding plasma EVs. These differences may occur in only one or two of the fractions, and the differences could be an elevation in one fraction and a reduction in another. As such, a single source of biological fluid could yield many candidate biomarkers.

In addition, we also discovered that known proteins with weak or no correlation to a disease exhibited a strong correlation to disease when associated with a specific EV type of the biological fluid. Therefore, to facilitate the discovery of candidate biomarkers and their subsequent validation in population, we routinely screened known and well-characterized proteins for candidacy as biomarkers using antibody arrays or ELISAs^[33]. The rationale for this strategy was two-fold. Firstly, the candidate biomarker would have a well characterised ELISA. Secondly, the isolation technique could be readily coupled to the standard 96 well ELISA platform leading to a semi-automated and moderately high throughput procedure^[33].

As a proof of concept, we tested our strategy in a study to identify predictive biomarkers for pre-eclampsia using a prospective biobank^[36]. Known and well-characterised proteins were first screened as potential pre-eclampsia biomarkers in plasma, plasma CTB-binding EV and plasma AV-binding EV from patients. Three different candidate biomarkers were identified, namely placental growth factor (PlGF), Tissue inhibitor of metalloproteinase & Plasminogen activator inhibitor-1 in plasma (plasma PlGF), plasma CTB-binding EV (CTB-TIMP1) and plasma AV-binding EV (AV-PAI1), respectively^[36]. These three biomarkers were tested in a prospective biobank of 843 pregnant women. Pre-eclampsia was predicted about 7.3 (\pm 2.9) weeks before clinical diagnosis with a combined AUC of 0.96, sensitivity of 100%, specificity of 78.6%, and PPV of 9.9%. The cut-off concentration for predicting PE was at < 1235 , ≤ 300 or > 1300 and $< 10,550$ pg/mL plasma for plasma PlGF, CTB-TIMP1 & AV-PAI1 respectively.

Differences in the proteome of the different EV types were also observed in cancer patients. Using a similar strategy as in the pre-eclampsia study, it was observed that MMP9 was elevated in AV-, but not detectable in CTB- or ST-binding EVs from the ascites fluid of serous ovarian cancer patients. However, this MMP9 elevation was not observed in AV-binding EVs from ascites fluid of liver cirrhosis patients^[34]. More recently, a preliminary screen of plasma, plasma CTB-binding EV and plasma AV-binding EV revealed the presence of potential biomarkers that could prospectively predict the outcome of chemoradiation therapy in patients with head and neck squamous-cell carcinoma^[37].

The presence of EVs in biological fluids with different affinities for membrane lipid-binding ligands demonstrated that the presence of concentrated, exposed domains of specific membrane lipids in some EV types are not unique to EVs from cell cultures. Importantly, this is a physiological phenomenon and not a consequence of the unique nutrient composition of the culture medium. It is also notable that each EV subtypes have different proteomes and that the proteome of these EV subtypes is differentially enriched in disease biomarkers.

ADVANTAGES AND DISADVANTAGES OF USING MEMBRANE LIPID BINDING MOLECULES IN EV ISOLATION FOR DIAGNOSIS

There are several advantages of using membrane lipid binding molecules to isolate EVs for diagnosis. The most important advantage is that the principle for this isolation technique is based on the defining feature of EVs, namely the presence of a lipid membrane. Membrane lipids are generally amphiphilic, i.e., having both hydrophobic and hydrophilic ends, and in aqueous solution, they will form micelles or liposomes, i.e., vesicles. Therefore, it is likely that these membrane lipids especially when present in localized high concentrations in aqueous biological fluids, are likely to be minimally in a micelle configuration, and most possibly, a vesicular structure. The presence of membrane or cytosolic proteins will strongly corroborate the presence of membrane vesicles.

Based on affinity for membrane lipid-binding ligands, it is obvious that biological fluids contain several EV types with EV type-specific proteome. Importantly from the diagnostic perspective, this EV type-specific proteome is also dependent on the pathophysiological state of the donor and this pathophysiology-

associated change in EV proteome varies with the EV type. Practically, it transforms a biological fluid as a single source of biomarkers into multiple independent sources of biomarkers.

A third advantage is that as the different EV types are novel sources of biomarkers, the distribution of known and well-characterized biomarkers and proteins in these EVs are not known. As we have shown, examining their distribution in the different EV types as a function of the pathophysiological state is a viable strategy to identify candidate biomarkers. Furthermore, it is easier to validate these known and characterized biomarkers and proteins as the necessary assay reagents such as antibodies are available.

A fourth advantage is that the technique for EV isolation by lipid-binding ligands is akin to the commonly used antibody capture techniques, and can be easily adapted for direct coupling to an ELISA for biomarker assay. Hence isolating EV from biological fluids and assaying for biomarkers by ELISA can be readily adapted for the standard automated 96 well ELISA platform used in routine clinical laboratory settings. It is also amenable to the recent developments in nano-plasmonic technology^[38].

Finally, the volume of plasma required for extracting EVs by their affinity for membrane lipid binding ligands in sufficient quantity for a typical ELISA ranges from 10-30 μL biological fluids per assay^[34,36]. As each of the EV types identified so far are unique EVs^[28,33], the volume of biological fluid could be reduced further by using multiplexed EV isolation system where the different membrane lipid-binding ligands are added to the same sample and the bound EVs are then extracted according to the ligands bound to the EVs.

Despite the advantages of lipid binding ligands for isolation of EVs, there are significant limitations with this approach. The avidity of the ligands for the different lipids has turned out to be a double-edged sword. The dissociation constants (K_D) of CTB to GM1 is between 4.6×10^{-12} ^[39] and 7.3×10^{-10} ^[40], K_D of AV to phosphatidylserine is 5×10^{-10} M^[41,42] and K_D of ST to GB3 is $5-30 \times 10^{-10}$ M^[32]. These dissociation constants which are comparable to the median K_D of antibody to antigen is $5-30 \times 10^{-11}$ M^[43] illustrate the difficulty in dissociating the EVs from the ligands for subsequent characterisation. While this poses significant challenge for biological studies of the different EV types, biomarker discovery and assay can be readily performed on the ligand-bound EVs.

Another drawback of this approach is the limited number of suitable lipid binding ligands to isolate EVs. Also, it is highly conceivable that many other EV types may not exhibit sufficiently distinctive lipid molecules for EV stratification by this method. Also, the lipid binding characteristics of an EV is not likely to be a characteristic of a specific cell type and is thus not likely to provide information on the cell source of the EVs. Nevertheless, lipid binding ligands represent an additional tool to isolate and stratify EV types that could increase the yield and specificity of EV types when complemented with present EV isolation tools such as size exclusion fractionation or immunoaffinity isolation technology.

CONCLUSION

We propose that the use of membrane lipid binding ligands in EV isolation for liquid biopsy is a viable and robust strategy to circumvent the present technical limitations in isolating bona fide EVs from biological fluids for liquid biopsy. In addition, this isolation technique is versatile can be easily integrated into the current clinical analysis platforms using 96 well assay plates and even newer analytical platforms such as nano-plasmonics^[44] which uses the interaction of light and electronic oscillations in metallic substrates in a nanometer-dimension to detect the presence of single EV.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception: Lai RC, Lim SK

Provided initial drafts of the work: Lai RC

Revising the work critically for important intellectual content: Tan KH, Lim SK

Final approval of the version: Lim SK

Availability of data and materials

Not applicable.

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Conflicts of interest

Lim SK holds founder shares in Paracrine Therapeutics Pte Ltd.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Role of autophagy in therapeutic resistance of glioblastoma

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Abstract

Patients with glioblastoma (GBM), a malignant brain tumor, exhibit a mean survival of less than 1.5 years. Despite treatment, the disease eventually develops resistance, resulting in disease relapse. Autophagy is a process of degradation and clearance that is activated to maintain cellular homeostasis. Its roles in cancer disease course and the treatment response, however, are controversial. In GBM, accumulating evidence has indicated that autophagy can protect cells, especially those with stemness features, causing the development of cell resistance. In this review, we discuss the impact of the cell reaction to currently active treatments, including temozolomide, radiation, tumor treating fields, bevacizumab (Avastin), etoposide (VP-16), cisplatin (CDDP), and carmustine (BCNU). Most of these induce the up-regulation of autophagy through signaling pathways of DNA damage response, reactive oxygen species, hypoxia, retinoblastoma, AMP-activated protein kinase, AKT/mTOR and MST4 kinase affecting cell fate by altering cell metabolism, cell death, and DNA repair. Treatment-related autophagy may be modulated by combining autophagy inhibitors such as chloroquine or antioxidants to prevent the development of resistance, thus improving cancer treatment.

Keywords: Autophagy, resistance, glioblastoma



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INTRODUCTION

The term autophagy is derived from Greek words, “auto” and “phagy”, meaning self-eating^[1], representing a process of degradation and clearance that is activated in response to aggregated protein and dysfunctional organelles to maintain cellular homeostasis^[2]. The autophagy pathway is initiated by a series of active proteins, such as autophagy-related (ATG) proteins, UVRAG, Beclin 1, PIK3C3, LC3 and p62^[3]. Together, they contribute to the formation of double-membrane autophagosomes, and eventually, fusion with lysosomes for degradation of inner substances [Figure 1]. Hence, autophagy has been implicated in several diseases including cancer^[4]. In the past, autophagy was believed to be related to apoptosis^[5]. Recently, an opposite impact of autophagy, i.e., promoting cell survival, has been widely studied^[6]. This “double-edged sword” effect in tumorigenesis varies depending on the cellular reaction to specific stimulation as well as in different cancer types. High levels of autophagy in the tumor environment (with limited nutrient and oxygen conditions) allow cancer cells to survive, resuming proliferation and initiation^[7].

Glioblastoma (GBM) is a disease composed of extremely varied tumor microenvironments and heterogeneous cancer cells. It is a fatal disease known to have a poor prognosis, and is not considered curable. Despite advanced treatment strategies, a notable improvement was not observed in terms of the outcome. Hence, it is not surprising that the tumor cells in the apex of the hierarchy, or those that are capable of self-renewal and differentiation, display highly-activated autophagy signaling to survive and thrive from the given treatment, such as temozolomide (TMZ)^[8]. Moreover, the molecular characters of GBM involved in the growth and survival via intracellular pathways are distinct genetic aberrations in epidermal growth factor receptor (EGFR), PTEN, TP53, IDH1 and so on^[9]. Among these frequently reported genes in clinical disease, EGFR is well-known as a driving receptor tyrosine kinase (RTK) in GBM that dictates multiple oncogenic signaling^[10]. The signaling amplification of EGFR accounts for approximately 60% of GBM cases. The mutant form EGFRvIII receptor, which is constitutively active that is independent of the ligand binding condition, is also common. In association with EGFR signaling pathway, it was found that levels of MDA-9, a protein related to tumor cell behavior and stemness, were increased in glioma stem-like cells to regulate the protective autophagy^[11] [Figure 1]. Moreover, it was found that STAT3 levels were related to beclin 1 expression via EGFR amplification^[12,13] [Figure 1]. Other RTKs in GBM, such as vascular endothelial growth factor receptor (VEGFR)^[14], platelet-derived growth factor receptor (PDGFR)^[15] and discoidin domain RTK 1 (DDR1)^[16], also contributes to modulating the autophagy formation through AKT/mTOR^[16,17], RAF/MEK^[18] and HIF-1/BCL2 Interacting Protein 3^[19-21] signalings [Figure 1]. As thus, therapeutic agents targeting these factors, for example bevacizumab^[14] and PDGF neutralizing antibody^[15], has been reported to enhance autophagy signaling [Figure 1]. Despite of the implications by disease- or treatment-related autophagic alterations, clinical trials with autophagy inhibitors, such as chloroquine (CQ) or its analogs, showed only limiting benefits^[22]. So far, the exact role of autophagy in GBM remains ambiguous, especially regarding treatment resistance^[23].

The effect of autophagy on tumors is not universal. On one hand, autophagy protects cells by clearing damaged organelles, thereby promoting cell survival. On the other hand, it achieves damage control by eliciting self-eating, leading to the process of autophagic cell death. The pros and cons of this biologic reaction does not have a clear boundary and may be dynamic. Regarding drug treatment, the appearance of autophagy also had divergent effect. For example, in a study of PI3K/mTOR inhibitor BGT226, application of the drug led to autophagic cell death in head and neck cancer cell lines^[24]. In contrast, application of PI3K/AKT/mTOR inhibitors sensitized cells to radiotherapy as well as reduced autophagy formation^[25]. Various effects of autophagy in the cancer treatment have been demonstrated, making the reaction complicated in each circumstance. However, in the case of GBM, the appearance of autophagy during specific treatment seems to support the development of resistance^[26]. In this article, we will discuss the molecular significance of autophagy in the current treatment of GBM. We will also discuss the formation of the reaction and the potential benefit of inhibiting autophagy along with the treatment.

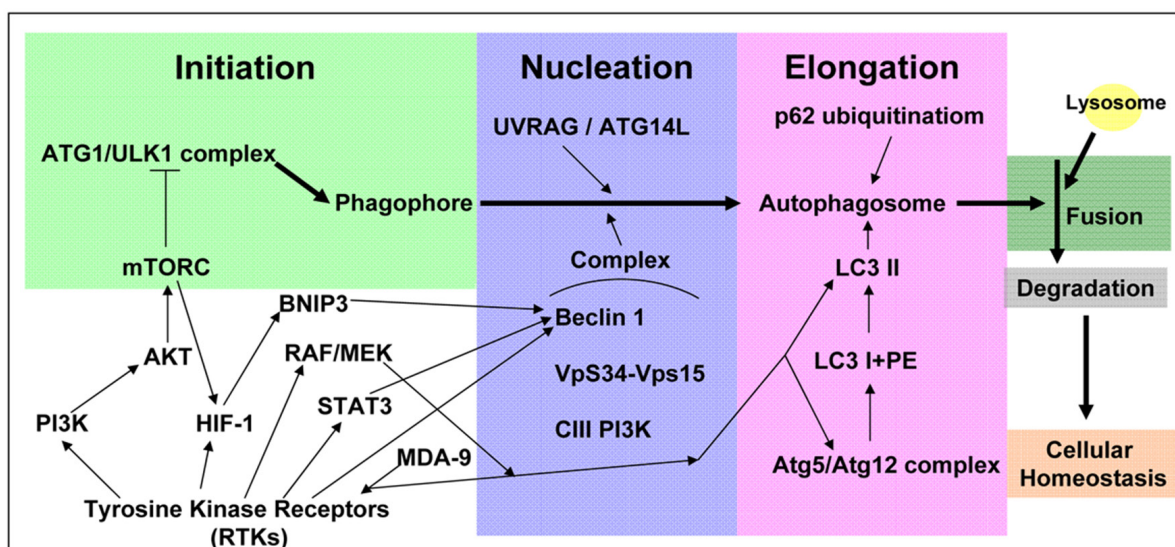


Figure 1. The schematic figure of the principal cell signaling pathways associated with the autophagic process. The process of autophagy pathway begins from the initiation step to the nucleation step, the elongation step, and to the formation of mature autophagosome which subsequently fuses with lysosome to perform the degradation process. The commonly activated pathways related to aberrant RTKs in GBM are also shown. Their roles to affect the individual steps of the autophagic process are indicated. RTKs: receptor tyrosine kinases; GBM: glioblastoma; HIF-1: hypoxia-inducible factor-1

TMZ

Being the most standard chemotherapy for GBM, it is mandatory to understand how autophagy is induced by TMZ. The first-line drug acts by inducing lethal DNA damage and subsequent reactive oxygen species (ROS) production^[27]. The effect is, however, mostly only transient and resistance is almost inevitable, with up to 90% of the patients experiencing early disease recurrence^[28]. Development of TMZ resistance in GBM is complicated and less understood, but till date, clear factors leading to resistance are still limited to pre-existing O⁶-methylguanine-DNA methyltransferase (MGMT)^[29]. According to previous studies, the DNA damage response (DDR) can induce autophagy in a yeast system^[30] and the enhanced autophagy can increase DNA repair ability to cause chemo-resistance^[31]. In GBM, TMZ-induced autophagy in glioma cell lines is dependent on MGMT expression, mismatch repair system and Rad51-mediated homologous recombination^[32]. The glioma cell lines resistant to TMZ cotreated with O⁶-benzylguanine, an O⁶-alkylguanine-DNA alkyltransferase (a DNA repair enzyme) inhibitor, can be re-susceptible to TMZ via autophagy regulation^[33]. The cascades of TMZ-induced autophagy showed that the phosphorylation of H2AFX at Ser139 following TMZ treatment initiates DDR [Figure 2], sequentially, leading to phosphorylation of PRKAA (Thr172), ULK1 (Ser555/575), MAPK14 (Thr180/Tyr182), RPTOR (Ser792) and suppressive phosphorylation of AKT (Ser473) and mTOR (Ser2448) to induce autophagy and inhibit apoptosis^[34]. However, further investigation is needed to examine whether there are other signaling pathways involved between autophagy and DDR.

In addition to MGMT that contributes to naïve TMZ resistance, the mechanism for tumor acquiring resistance is more complicated. In our previous studies, we identified transcriptional binding factor specific protein 1 and its downstream modulator, superoxide dismutase 2 (SOD2), as the critical factors in the tolerance of TMZ-induced ROS in models of resistance^[35,36]. In fact, it has also been reported that TMZ can induce autophagy via ROS signaling^[37] [Figure 2]. The detailed mechanism between autophagy and ROS, however, needs further investigation. SOD2 is known to specifically function in mitochondria to regulate oxidative stress and energy metabolism^[38]. Altered metabolic reprogramming by mitochondrial control in cancer may play a role in chemotherapy resistance^[39]. For instance, increased autophagy derived from

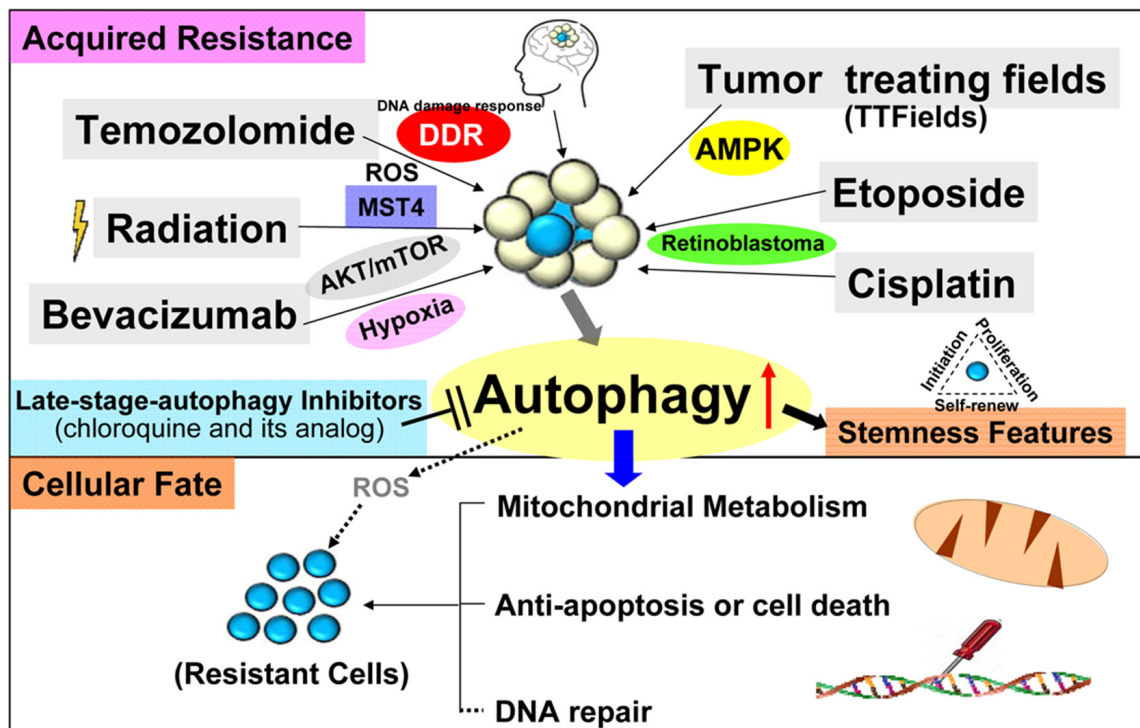


Figure 2. The schematic diagram demonstrates that in primary or recurrent GBM patients, the standard regimens consists of TMZ, radiation, TTFields, bevacizumab, etoposide and cisplatin, which enhance cell stemness, metabolism (energy), anti-apoptosis and DNA repair to acquire therapeutic resistance through the signaling pathways of DDR, ROS, hypoxia, AKT/mTOR, MST4 kinase, AMPK and RB. Nowadays, the inhibitors of late-stage autophagy, such as CQ or its analogs, have been tested in clinical trials of GBM patients. TMZ: temozolomide; GBM: glioblastoma; TTFields: tumor treating fields; ROS: reactive oxygen species; DDR: DNA damage response; AMPK: AMP-activated protein kinase; RB: retinoblastoma; CQ: chloroquine

glucose starvation condition causes GBM cell quiescence, survival and chemoresistance^[40] and complex I linked oxidative phosphorylation is required to maintain mitochondrial respiration (electron transfer system) through autophagy regulation^[41]. Furthermore, presence of glioma stem-like cells may have crucial role in acquiring resistance^[42]. An emerging concept in cancer biology suggests these specific subgroups with stemness features are responsible for acquisition of resistance^[42]. The stem cells are characterized by self-renewal and multipotent abilities^[43]. These allow cells to be resistant to standard therapy, and are associated with the treatment outcome^[42]. In glioma stem-like cells, autophagy-associated proteins, i.e., DNA Damage Regulated Autophagy Modulator 1 and p62, can regulate the cell migration and invasion by modulating energy metabolism (ATP) and affect mitochondrial morphology by regulating mitochondrial fusion^[44]. Some studies have indicated that autophagy can regulate epithelial-to-mesenchymal transition^[45] and the capabilities of migration and invasion in those cells under nutrient deprivation are damaged when autophagy is activated through regulation of ATG proteins or beclin 1^[46]. However, it was found that TMZ treatment plus autophagy inhibitor could suppress cell migration and invasion^[47], suggesting further investigation was needed to determine the role of TMZ-induced autophagy in this aspect. Moreover, stemness features in the glioma stem-like cells are reduced by autophagy inhibition and apoptosis is enhanced by blockage of autophagy^[48]. Recently, it is found that the blockage of autophagy up-regulates the TMZ-sensitivity in these stem-like cells through metabolic dysfunction-related ferroptosis^[49] in the stem-like cells, demonstrating the close correlation between autophagy and metabolism. In summary, application of these the explains how the disease is capable of surviving TMZ toxicity regardless of MGMT status.

RADIATION

Radiation can cause similar treatment response to TMZ. Radiation treatment in GBM cells can induce autophagy through the PI3K/AKT/mTOR pathway, ROS^[50,51] and MST4 kinase-ATG4B signaling^[52] [Figure 2]. Inhibition of autophagy makes these cells susceptible to stimulation^[53,54]. Moreover, it is reported that GBM cells are susceptible to radiation following inhibition of MDA-9 expression^[55]. Radiation has various effect to autophagy. Studies have indicated that the relatively radio-sensitive cells or small radiotherapy fraction (2 Gy) displays enhanced autophagic flux, while more radio-resistant cells or larger radiotherapy fraction exhibited an inhibition of autophagic flux^[56]. Regarding the glioma stem-like cells, it was reported the subsets were enhanced for autophagy after radiation to promote metabolism, anti-apoptosis and stemness^[57], while the other reports showed that CD133⁺ cells or sphere cells from one patient have a lower level of autophagy^[58,59]. Notably, it is also found that CD133⁺ glioma stem-like cells can be resensitized to radiation treatment by using autophagy inhibitors (bafilomycin) or down-regulation of ATG protein levels^[60]. Though the exact effect remains unclear, the association between autophagy, radiation, and the stemness features is intriguing.

Whether targeting autophagy will benefit radiotherapy effect is also controversial. On one hand, a study has found that autophagy in malignant glioma cells is a transition status promoting apoptotic cell death following radiation, which can be reversed by suppressing autophagy function^[61]. On the other hand, from the aspect of PI3K/AKT/mTOR signaling (it is active in most GBM patients^[62]), Cerniglia *et al.*^[63] and Gil del Alcazar *et al.*^[64] have indicated that NVP-BE235, a dual PI3K/mTOR inhibitor, can promote GBM cells sensitive to radiation and up-regulate autophagy by directly affecting the function of DNA damage repair (DNA-dependent protein kinase catalytic subunit and ATM kinase) and autophagy related proteins (ATG5 and Beclin1), respectively. The results demonstrated that there are different responses after administrating the inhibitor and radiation leading to cell death. Furthermore, Zhuang *et al.*^[65] also reported that rapamycin can lead to the differentiation of glioma stem-like cells, up-regulating the cell radiosensitivity and reducing tumorigenesis through activation of autophagy. However, the application of the autophagy inducers on mice experiment or clinical seems to be rare and limited in information^[62,66]. In addition, most clinical trials nowadays prefer the combination of autophagy inhibitors with radiochemotherapy but not radiotherapy only^[67]. Therefore, the directions of future research must focus on further exploration of how radiation induces autophagy (time point, tumor suppress genes, DNA damage repair and so on) and how the affected autophagy changes the cell fate.

TUMOR TREATING FIELDS

Optune, a tumor treating fields (TTFields) device, is used as an advanced GBM treatment with only minor side effects noted in newly diagnosed or recurrent GBM patients. The device in combination with TMZ increased the median overall survival by almost 5 months compared to the TMZ alone group^[68]. At 200 kHz frequency, TTFields treatment has been reported to cause damaged mitosis and up-regulation of autophagy^[69]. It is also found that the enhancement of autophagy is induced by TTFields treatment through AMP-activated protein kinase (AMPK) signaling^[70] [Figure 2] and down-regulation of the pathway inhibits the protective autophagy to reduce TTFields-induced acquired resistance. By combining with sorafenib, a multi-kinase inhibitor that can induce autophagy in prostate cancer cells through increased levels of LC3^[71], the device exhibited enhanced autophagy and more inhibited tumor behavior to promote a better therapeutic strategy^[72]. Regarding the mechanism, it was reported that TTFields can suppress DDR in non-small cell lung cancer cell lines^[73] and the combination of radiation can inhibit DNA damage repair in glioma cell lines^[74]. Furthermore, it is found that TTFields can cause ROS production to promote apoptosis in the cell line study^[72]. This may have impact in inducing the clearance of damaged mitochondria (mitophagy)^[75]. It is not clear, however, whether TTFields-increased autophagy is associated with inhibition of DNA damage repair. Further explorative experiments are thus needed to elucidate the mechanism and the role of autophagy in the disease-controlling mechanism of the device.

BEVACIZUMAB

Bevacizumab can directly promote apoptosis and antiangiogenesis by regulating the associated proteins in GBM cells^[19,76]. This drug has been reported to cause resistance in the cells through suppressing AKT-mTOR signaling^[76] or through inducing hypoxia-inducible factor-1 α /AMPK pathway^[19] to increase autophagy influx [Figure 2]. It was also reported that bevacizumab could cause a hypoxia microenvironment, which in turn, was related to enhanced autophagy^[77,78]. Thus, inhibition of the autophagy process was postulated to resensitize bevacizumab to GBM cells.

Up-regulation of glucose metabolism before autophagy induction was observed in bevacizumab-treated GBM cell lines^[79]. Further investigation is needed to link the mechanism between autophagy and the cell metabolism after the treatment. It was also noted that bevacizumab can induce autophagy in glioma stem-like cells by enhancing VEGF-independent angiogenesis (vasculogenic mimicry, an alternative vasculature) through KDR/VEGFR-2 phosphorylation, which the formation is closely related with GBM resistance^[80]. This highlights the therapeutic value of autophagy-inhibiting strategies in regard with the anti-angiogenesis therapy.

ETOPOSIDE

Etoposide (VP-16) treatment has been reported to induce autophagy, thereby increasing the therapeutic resistance in GBM cells^[81,82]. The drug is a topoisomerase II inhibitor that also enhances autophagy by modulating though retinoblastoma protein (RB)^[81] [Figure 2]. Down-regulation of RB can inhibit autophagy by preventing autophagosomes fusion from lysosome and promoting cell death in GBM cells and glioma stem-like cells^[81]. Furthermore, knockdown of RB can enhance apoptotic cell death and up-regulate the immunostaining intensity of γ -H2AX (a DNA double-strand break marker) induced by etoposide^[81]. Earlier studies have demonstrated a close link between etoposide-induced autophagy, RB, and DNA damage.

CISPLATIN

Cisplatin (CDDP) is a DNA alkylating agent that is widely used for human malignant tumors including GBM^[83]. CDDP-associated resistance often develops through autophagy induction in multiple cancer cell types^[84-87]. In cervical cancer cells, it is found that blockage of autophagy can increase CDDP-induced cytotoxicity via endoplasmic reticulum stress^[88]. One study indicated that autophagy was associated with CDDP resistance in GBM and down-regulation of RB suppressed autophagy induced by CDDP^[87] [Figure 2]. Moreover, it was found that CDDP could induce long non-coding RNA to suppress CDDP-induced autophagy, which promoted apoptosis in GBM cells^[89]. CDDP could also enhance the level of death receptor (DR5), directing glioma stem-like cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) apoptotic pathway^[90]. TRAIL, on the other hand, was reported to enhance the expression of autophagy leading to resistance of breast cancer cells, and blockage of autophagy could reduce the resistance^[91]. The association between autophagy and TRAIL in GBM has thus been inferred. In addition, the mechanism of DNA repair induced by CDDP could promote resistance of GBM cells via the Jun kinase/stress-activated protein kinase signaling pathway^[92]. Further experiments are required to verify whether autophagy is related to the DNA repair induced by CDDP.

CARMUSTINE

Another DNA damaging nitrosoureas agent carmustine (BCNU) is reported to promote ROS-mediated autophagic cell death in solid tumor cells in combination with arsenic trioxide^[93]. In terms of the role of ROS in the drug reaction, it was supportively reported that in human glioma cell line U98MG, the overexpression of Nrf 2, an anti-oxidant transcriptional factor, could reduce the carmustine-induced

cytotoxicity^[94]. How autophagy was utilized in carmustine-related oxidative stress and anti-oxidant reaction remains to be elucidated.

AUTOPHAGY INHIBITORS IN CLINICAL TRIAL

Traditionally, CQ is used for malaria treatment. Due to the basic property of CQ, it stays in lysosomes leading to enhanced lysosomal pH and inhibition of autophagy^[95,96]. Hence, CQ has been investigated in clinical trials for cancer therapy including GBM. An early clinical trial of CQ in a small population of GBM patients (NCT00224978) showed that the median survival increased in patients receiving CQ in combination with standard regimens^[97]. Currently, there are 3 ongoing clinical trials involving CQ in GBM or astrocytoma patients (NCT02378532 (Phase 1), NCT02432417 (Phase 2) and NCT03243461 (Phase 3)). In addition to CQ, more candidate autophagy modulators are expected with or without the standard drugs to be investigated in clinical trials.

CONCLUSION

The current standard treatments of primary or recurrent GBM can cause autophagy induction and the enhanced autophagy through DDR, ROS, hypoxia, AKT/mTOR, AMPK and RB pathways may result in cell resistance, resulting in enhancement of stemness features, increased abilities of metabolism, anti-apoptosis, and DNA repair. Therefore, in order to reduce the resistance caused by treatments, the usage of standard treatment plus autophagy inhibitors and signaling inhibitors may be a potential strategy for GBM therapy.

DECLARATIONS

Authors' contributions

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Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Liquid biopsy in lymphomas: a potential tool for refining diagnosis and disease monitoring

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Abstract

Liquid biopsy consists in a simple blood sampling that allows to analyze cell free DNA (cfDNA), containing specific genomic clues released by the tumor into the bloodstream. In this review, we shall focus on the analysis of cfDNA in lymphoma and, in particular, on its application in the genotyping and monitoring of two common types of B-cell lymphoma, i.e., diffuse large B-cell lymphoma (DLBCL) and classical Hodgkin lymphoma (cHL). From a diagnostic standpoint and based upon the current international guidelines, lymphoma diagnosis has so far relied on the analysis of the tissue biopsy. From a molecular viewpoint, though, the tissue biopsy does not reflect the entire molecular heterogeneity of lymphomas. In fact, in an individual patient, lymph nodes at different anatomical sites, as well as different areas of the same lymph node, may show different genetic profiles. Consequently, molecular analysis of genomic DNA extracted from a single lymph node biopsy may not recapitulate the whole mutational landscape of the disease. Liquid biopsy may overcome this hurdle, since cfDNA is released by all tumoral cells and can reveal the entire molecular complexity of lymphomas. From a translational perspective, liquid biopsy may also be used to evaluate clonal evolution, response to therapy and minimal residual disease. Consistently, in DLBCL as well in cHL, the drop of the mutational burden during the treatment course provides complementary information to conventional imaging techniques. The integration of liquid biopsy with imaging techniques may prove useful for a better prediction of patients' outcome and for a better treatment tailoring.

Keywords: Liquid biopsy, lymphoma, precision medicine



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GENERAL CONCEPT ON CIRCULATING CELL FREE DNA

Liquid biopsy consists in a simple and easy sampling of peripheral blood, that can be subjected to the molecular analysis of specific genomic clues released by the tumor^[1,2]. Because circulating tumor cells are very rare or absent in many types of lymphomas, liquid biopsy approaches have been focused on circulating tumoral DNA (ctDNA) released by lymphoma cells into the bloodstream^[3-5]. However, the ctDNA represents only a fraction of the total amount of cell free DNA (cfDNA), that is derived also from healthy cells. The cfDNA circulates in plasma at a low concentration as double-stranded DNA fragments with a dimension < 200 base pairs^[6]. In individuals without malignancies, plasma cfDNA derives mainly from the apoptosis of normal hematopoietic cells. cfDNA levels may also rise in para-physiological conditions, such as trauma, burns or high exercise. In healthy subjects, the cfDNA concentration ranges between 1 and 10 ng/mL and can reach 30ng/mL in lymphoma patients^[7].

CFDNA ISOLATION

It is possible to selectively isolate cfDNA from plasma in order to proceed with subsequent molecular analysis^[8]. To isolate cfDNA, a few very important technical precautions need to be observed in order to avoid white blood cell lysis that may contaminate the cfDNA with genomic DNA (gDNA)^[9]. If using standard collecting tubes containing ethylenediaminetetraacetic-acid (EDTA) as anticoagulant, plasma extraction is recommended within 3 h from the collection of peripheral blood^[9]. EDTA tubes, however, are not able to preserve the integrity of cfDNA and are not able to prevent the lysis of nucleated cells for more than 3 h. Therefore, in order to allow the shipment of biological material, specific tubes, namely cfDNA BCT Streck tubes (Streck), can stabilize cfDNA and can prevent the contamination by gDNA from white blood cells for up to 14 days at a temperature between 6 and 37 °C^[10]. After collection, plasma is separated from the corpuscular blood by centrifugation of blood samples at 800 rpm at 4 °C for 10 min. A second centrifugation at 13,000 rpm at 4 °C for 10 min allows to pellet and remove any remaining cells. Plasma samples are then stored in 1 mL aliquots at -80 °C until cfDNA extraction^[8].

cfDNA can be selectively extracted using two different methods. The first method, named QIAamp Circulating Nucleic Acid (Qiagen), relies on the use of ion-exchange resins that bind the plasma cfDNA^[11]. The second approach relies on an automatic method that utilizes the Maxwell RSC Instrument coupled with Maxwell RSC cfDNA Plasma Kit (Promega). This method consists in an automated nucleic acid purification platform that processes up to 16 samples simultaneously and allows to obtain high quality cfDNA starting from 1 mL of plasma^[12]. After extraction, cfDNA is usually quantified by a fluorometric assay. Approximately 30-40 ng of cfDNA are needed for subsequent molecular analysis^[8].

CAPP-SEQ ANALYSIS AS FOR DEFINING THE LYMPHOMA GENOTYPE ON CFDNA

Since lymphoid malignancies harbor a unique molecular marker, namely the immunoglobulin (Ig) gene rearrangement, the identification of presence of this biomarker in the cfDNA may be used to track minimal residual disease (MRD) during the course of treatment. Once the Ig gene rearrangement has been identified on the lymph node biopsy, it is possible to evaluate the amount of this rearrangement in the cfDNA using Next-Generation-Sequencing (NGS) or polymerase chain reaction based methods^[13,14]. Pivotal studies have shown that the proportion of cfDNA carrying the lymphoma-specific Ig rearrangement decreases rapidly in patients who respond to therapy and tends to remain negative in those who maintain the response^[13,14]. Conversely, the Ig rearrangement remains high in patients who do not respond to treatment^[13,14]. As expected, these patients experience both a worse progression-free survival and a worse overall survival compared to patients with undetectable Ig gene rearrangement^[13,14]. This method is very effective in monitoring MRD but has some pitfalls. First, this method does not work in a biopsy free manner since it requires, at the time of diagnosis, the identification of the individual patient's specific Ig

rearrangement on the lymph node tissue biopsy. Once the patient's unique Ig rearrangement has been identified on the tissue biopsy, it is then possible to track it on the liquid biopsy to evaluate MRD. Second, this method does not allow a comprehensive disease genotyping on the liquid biopsy^[15].

To overcome these limits, the use of Cancer Personalized Profiling by Deep Sequencing (CAPP-Seq) has been established as a reliable tool to genotype cancer patients^[16]. This molecular strategy utilizes disease specific probes that selectively capture a set of exonic and intronic regions known to be recurrently affected in a particular cancer type. The selected targets are then amplified and sequenced by NGS allowing the detection of the specific tumor mutational profile of a patient. This method, linked to a stringent bio-informatic analysis, allows to detect mutations in cfDNA with a high sensitivity, identifying mutations with allelic frequency as low as 0.02%. In addition, CAPP-Seq allows the identification of single nucleotide variants, as well as insertion/deletions, copy number alterations and rearrangements, thus covering a large variety of genomic alterations of human cancers^[16].

APPLICATION OF LIQUID BIOPSY IN LYMPHOMA

Lymph node biopsy is the gold standard for the diagnosis of lymphoma, but genetic lesions identified in the tissue biopsy may not reflect the entire molecular complexity of every single patient with lymphoma^[17,18]. Consistently, in an individual patient, lymph nodes at different anatomical sites, as well as different areas of the same lymph node, may show different genetic profiles^[19]. However, outside of clinical studies aimed at investigating the clonal heterogeneity of lymphomas, multiple biopsies are not routinely performed in lymphoma patients for both practical and ethical concerns. On these grounds, once a diagnosis of lymphoma is performed on a tissue biopsy, liquid biopsy may be used to explore the entire mutational landscape of lymphoma, since this approach has the potential to collect the tumor cfDNA deriving from most, or potentially all, of the different sites of tumor involvement in the body [Figure 1]^[15]. In particular, a liquid biopsy CAPP-Seq approach may be used to: (1) genotype lymphoma patients; and to (2) evaluate treatment response [Figure 2].

LIQUID BIOPSY AS A TOOL FOR GENOTYPING LYMPHOMA PATIENTS

In DLBCL, liquid biopsy allows to identify at least one somatic non-synonymous mutation per patient in over 70% of cases^[20,21]. The mutational profile identified by liquid biopsy reflects that revealed by DLBCL genomic studies performed on the lymph node biopsies^[22]. More precisely, the most common genetic lesions found by liquid biopsy reflect those detected on the tissue biopsy, and comprise alterations of *KMT2D*, followed by *TP53*, *CREBBP*, and *PIM1*. In addition, *EZH2* and *BCL2* mutations, as expected, are more frequently found in germinal center (GC) DLBCL, whereas *TNFAIP3* and *PIM1* mutations are more frequent in non-GC DLBCL^[20]. In addition, by comparing the mutations identified in cfDNA with those identified in gDNA extracted from the tissue biopsy, cfDNA appears to be representative of most of the mutations that occur in > 20% of the alleles of the tumor biopsy, with a sensitivity > 90% and a specificity of ~100%. Furthermore, a fraction of mutations has been found exclusively in cfDNA, conceivably because, due to spatial tumor heterogeneity, they are restricted to clones that are anatomically distant from the biopsy site^[20].

Regarding cHL, the rarity of neoplastic Hodgkin and Reed-Sternberg (HRS) cells in the biopsy has limited the assessments of the genetic landscape of the disease^[23]. In this context, liquid biopsy may be a reliable tool to overcome this limit. Analogous to DLBCL, it has been demonstrated that cfDNA analysis in cHL mirrors the genetics of HRS cells micro-dissected from the tissue biopsy. Consistently, most of cHL mutations are identified in the tissue biopsy as well as in the cfDNA and some more mutations are identified only in cfDNA, conceivably because they are derived from different anatomical sites^[23]. Genotyping of cHL on the liquid biopsy has allowed to refine the current knowledge of cHL genetics.

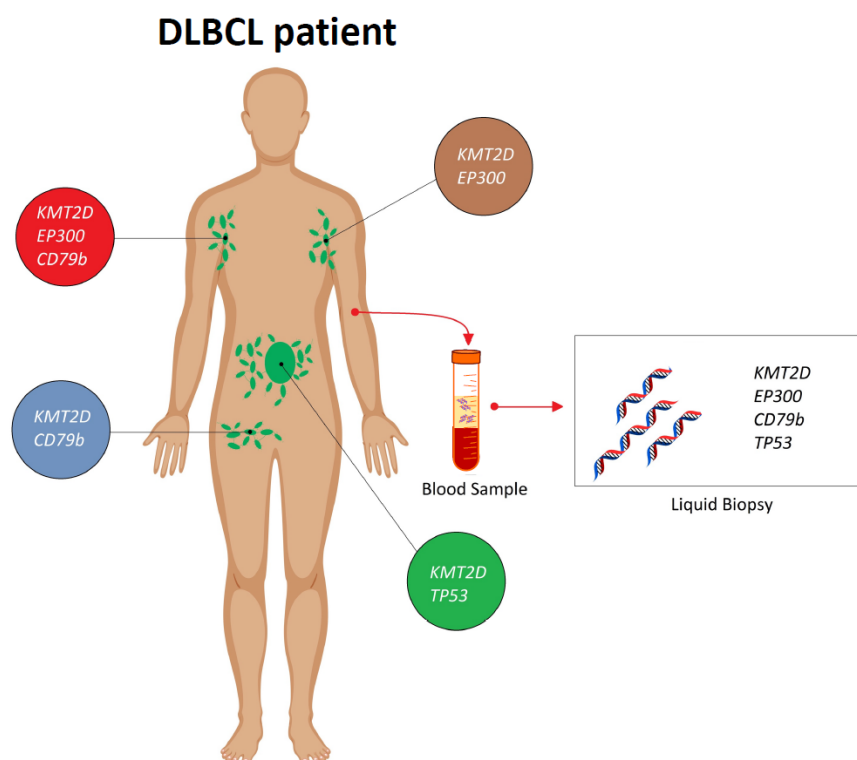


Figure 1. Liquid biopsy may recapitulate the entire mutational complexity of diffuse large B-cell lymphoma (DLBCL) patients. In every single DLBCL patient, the mutational profile of the lymphoma may vary in different anatomical sites. For instance, a DLBCL patient may harbor a *EP300* and *KMT2D* mutation in the left axillary lymph node (in brown), a *EP300*, *KMT2D* and *CD79b* mutation in the right axillary lymph node (in red), a *KMT2D* and *CD79b* mutation in the right inguinal lymph node (in blue), and a *KMT2D* and *TP53* mutation in a lymph node deep in the abdomen (in green). Consistently, if the biopsy targets a superficial lymph node (e.g., the left axillary lymph node; in brown), in order to avoid unnecessary surgical risks, a certain number of mutations (i.e., *CD79b* and *TP53* mutation) present in the lymphoma genome would go undetected. Importantly, those mutations, if detected, might serve as predictive biomarkers as well as molecular markers allowing the monitoring of the disease during treatment. Conversely, cfDNA analysis on the liquid biopsy may overcome these limitations, since it is representative of all the different anatomical sites of the disease

STAT6, not previously reported in exome sequencing studies of this lymphoma, has been identified as the most frequently mutated gene in cHL, underlying the importance of cytokine signaling in the biology of this tumor^[23]. Also, liquid biopsy has documented that different histologic subtypes of cHL are biologically distinct. *STAT6* and *TNFAIP3* mutations are enriched in nodular sclerosis cHL compared with mixed cellularity cases. Also, NF- κ B, PI3K-AKT, cytokine and NOTCH signaling pathways, are frequently deregulated by gene mutations, and might be relevant for the biology of the disease and for the identification of potential therapeutic targets^[23].

LIQUID BIOPSY AS A TOOL TO EVALUATE TREATMENT RESPONSE

In cHL, imaging techniques, such as interim positron emission tomography/computer tomography (PET/CT) scan after 2 cycles of chemotherapy, provide a powerful tool to predict cHL outcome before completion of chemotherapy^[24-26]. However, interim PET/CT results are inconsistent with the final outcome in 20% to 30% of patients^[24-26]. This lack of specificity and/or sensibility may be corrected, at least in part, by monitoring ctDNA concentration during the course of treatment. A recent study prospectively analyzed ctDNA during treatment in a cohort of 24 cHL patients homogeneously treated with adriamycin, bleomycin, vinblastine, dacarbazine (ABVD)^[23]. The study showed that ctDNA analysis may complement interim PET/CT in predicting patients' outcome^[23]. More precisely, patients achieving a complete response had a larger drop in the ctDNA load after 2 ABVD courses compared to refractory patients. Also, a 2-log drop

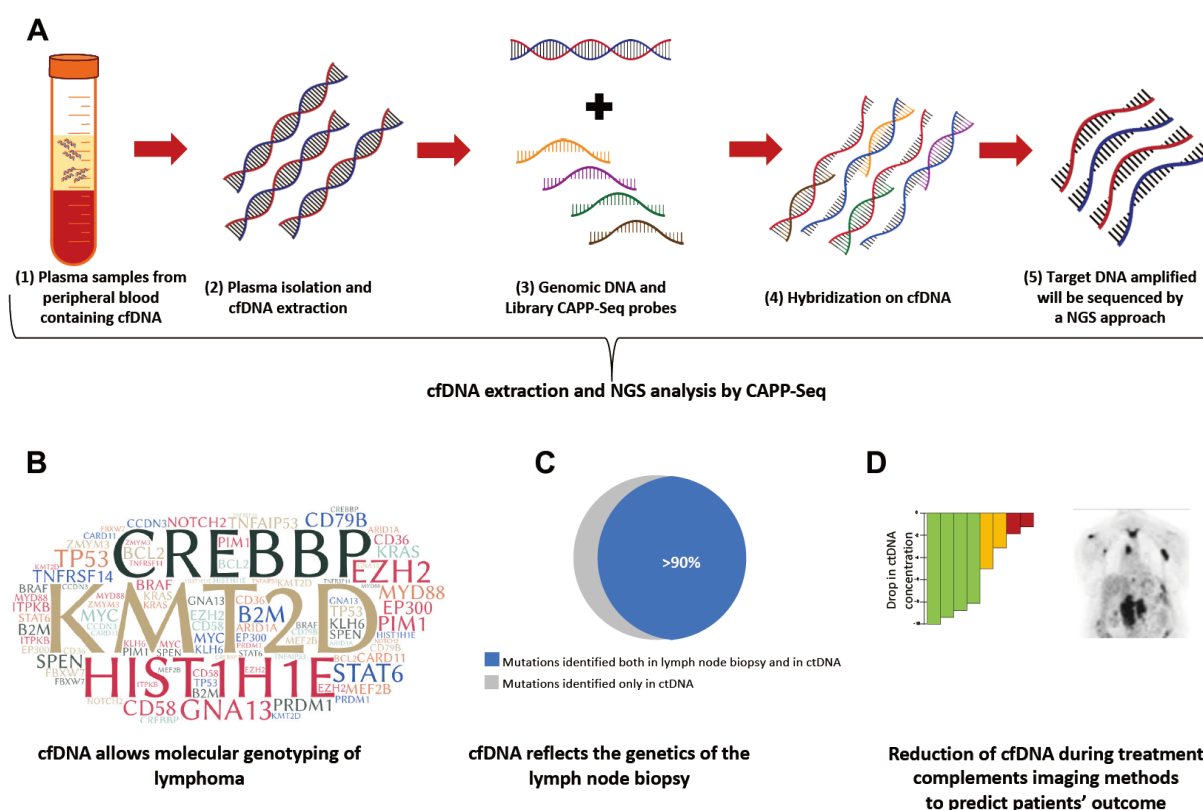


Figure 2. Applications of liquid biopsy in lymphomas. A: cell free DNA (cfDNA) can be selectively isolated and extracted from plasma samples. cfDNA may be analysed with the Cancer Personalized Profiling by Deep Sequencing (CAPP-Seq) method, that exploits magnetic probes that selectively capture and isolate specific genomic regions of interests, namely the target region. The target region is then subsequently sequenced by a Next-Generation-Sequencing (NGS) approach; B: consistently, cfDNA analysis on the liquid biopsy allows the genotyping of lymphomas and allows a better understanding of lymphoma pathogenesis; C: liquid biopsy mirrors the mutational landscape of the lymph node biopsy in most of cases and, in addition, allows the identification of mutations not identified in the tissue biopsy; D: the integration of imaging methods with the monitoring of the kinetics of the mutations identified by liquid biopsy on cfDNA may allow a more sensitive and specific prediction of patients' outcome. The colored bars indicate the various levels of cfDNA drop during therapy. A higher drop in the cfDNA concentration (in green) may predict a favorable response to therapy, whereas a smaller drop may predict resistance to therapy

in ctDNA after 2 chemotherapy courses sorted out as the best cutoff to predict progression. Indeed, cured patients who were inconsistently judged as interim PET/CT-positive achieved more than a 2-log drop in ctDNA, whereas relapsing patients who were inconsistently judged as interim PET/CT negative achieved less than a 2-log drop in ctDNA^[23].

Similarly, ctDNA analysis by CAPP-Seq methodology also allows to evaluate treatment response in DLBCL patients. ctDNA has been analyzed at baseline and during the course of treatment in a multicenter cohort of 217 patients with DLBCL treated with rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone (R-CHOP) or R-CHOP-like chemo-immunotherapy^[21]. Two different thresholds have been identified to optimally predict patients' outcome, namely early molecular response (EMR) and major molecular response (MMR). These thresholds, including a 2-log drop in ctDNA after one cycle (EMR) and a 2.5-log drop after two cycles (MMR), predict event free survival after front-line therapy in the training cohort and in two validation sets^[21]. Interestingly, the prognostic value of molecular response maintained an independent association with an increased risk of progression and death also in multivariate analyses^[21]. Recently, a new prediction tool for DLBCL patients, namely Continuous Individualized Risk Index (CIRI), has been documented to dynamically determine outcome probabilities for individual patients utilizing risk predictors acquired over time, including clinical, radiological and molecular markers identified with a liquid biopsy approach^[27].

CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, liquid biopsy is a non-invasive method that may be used in lymphomas to achieve several objectives: (1) better characterize the biology and pathogenesis of the lymphoma through the analysis of biological markers that reflect the entire molecular heterogeneity of the disease in its different anatomical sites; (2) identify new prognostic and predictive markers; and (3) prospectively evaluate the dynamics of ctDNA during treatment in order to predict the probability of response to treatment in a more sensitive and specific manner and, if necessary, the need for an early switch to a more efficacious treatment. In order to validate these initial results, several ongoing clinical trials aim at assessing whether cfDNA genotyping can improve outcome prediction in lymphoid malignancies and whether it might identify mutations predisposing to treatment resistance (NCT03280394^[28], NCT02883517^[29], NCT03702309^[30]).

In addition to the previously mentioned application of liquid biopsy in lymphoma, preliminary evidence also suggests that cfDNA analysis might reflect the methylation profile identified in the tissue biopsy^[31,32]. More precisely, global hypomethylation of the genome, as well as aberrant methylation of specific regions of interest detected on the liquid biopsy, namely DAPK1 promoter hypermethylation, might represent potential prognostic biomarkers in lymphomas^[31,32].

The liquid biopsy of lymphoma, as described in this review, aims at analysing mutations of somatic origin, and therefore does not pose major ethical issues beyond the conventional ethical requirements. If the patient plasma is stored in a biobank for subsequent analysis, the conventional guidelines for biobanking should be followed^[33,34].

DECLARATIONS

Authors' contributions

Writing, reviewing and editing the manuscript: Moia R, Favini C, Rasi S, Deambrogi C, Ferri V, Schipani M, Sagiraju S, Mokhtar Mahmoud A, Kodipad AA, Adhinaveni R, Patriarca A, Nassi L, Gaidano G

Availability of data and materials

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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The immunological regulation of cancer cachexia and its therapeutic implications

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Abstract

Cachexia affects the majority of patients with advanced cancer. It leads to poor surgical and oncological outcomes, and negatively affects quality of life. It has long been reported that components of the host immune system, including pro-inflammatory cytokines such as IL-1 α , IL-6, TNF- α and INF- γ , participate in the syndrome of cachexia. Yet therapeutic targeting of these pro-inflammatory factors has not yielded meaningful improvements in cachexia management. More recently, the impact of immune cells in the tumour mass (tumour-associated macrophages) and host circulation (myeloid suppressor cells) has garnered much interest with regards to their role in immune tolerance in cancer. However, their role in the generation of systemic inflammation and cancer cachexia is underexplored and outstanding questions remain. This review summarises the key mediators and targets of immune dysfunction in cancer cachexia. Here we describe the host response including skeletal muscle wasting; highlight the current knowledge gap areas; and report the results of previously trialled immunotherapies. A greater understanding of complex interaction between the tumour, immune system and peripheral tissues in the genesis and maintenance of cancer cachexia is a key step in identifying future therapeutic targets.

Keywords: Cancer cachexia, interleukins, macrophages, immunotherapy

INTRODUCTION

Cachexia is “a multifactorial syndrome defined by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive



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functional impairment”^[1]. Cachexia has a negative impact on a large proportion of patients with advanced cancer with it contributing to high levels of morbidity and mortality^[2]. Although there still remains some debate over the formal definition of cancer cachexia, it is characterized by unintentional weight loss, muscle wasting, anorexia and fatigue^[3]. Systemic inflammation is a key driver of cancer cachexia and has been advocated as a core nutritional assessment in patients with cancer^[4,5]. Pro-inflammatory cytokines are activated by the tumour mass, and act both centrally (through anorexia) and peripherally (by skeletal muscle wasting) to result in host nutritional depletion^[6]. Tumour and host-derived factors thus lead to a chronic inflammatory and impaired immune state^[7]. Immunosuppression is a large problem in cachectic cancer patients contributing to reduced responses to surgical and oncological outcomes^[8]. The dysfunction of the immune system is complex and involves multiple mechanisms characterised by a reduction in monocyte, macrophage, dendritic and natural killer (NK) cell function, ultimately leading to susceptibility to infections, and therefore, an overall increase in morbidity^[9].

In short, it has long been apparent that systemic inflammation plays a role in the pathogenesis of cancer cachexia. The successful therapeutic targeting of systemic inflammation requires a better understanding of the involved mediators and the link between tumour and immune tissues. This review aims to describe some of the key elements of immune dysfunction in cachexia and give an overview of previously trialled immunotherapies.

PRO-CACHECTIC CYTOKINES

TNF- α

TNF- α (previously known as “cachectin”^[10]) was initially held responsible for causing most of the metabolic derangements and clinical features of cachexia. TNF- α is released by many types of cell, including activated macrophages, CD4+, neutrophils, mast cells, eosinophils and neurons. In particular, it can be produced by tumour, immune and stromal cells to induce growth and survival advantage in the tumour microenvironment^[11]. Its expression can ultimately lead to anorexia, muscle and adipose wasting, loss of appetite, increased energy expenditure and insulin resistance in both patients with various types of cancer, and the Colon-26 carcinoma mouse model of cancer cachexia (C26)^[12]. Many of the effects of TNF- α arise through activation of NF κ B, which in turn leads to activation of the ubiquitin-proteasome pathway and skeletal muscle degradation^[13]. It also acts to induce oxidative stress and nitric oxide species (NOS). Experimental evidence suggests TNF- α can induce adipose wasting in white adipose tissue through inhibition of lipoprotein lipase (LPL), suppression of transcription and promotion of lipolysis^[14,15] as well as stimulation of thermogenesis through increased expression of UCP2 and UCP3 in skeletal muscle.

The role of TNF- α in mediating many of the effects of cancer cachexia was initially supported by evidence that intraperitoneal injection of soluble recombinant human TNF-receptor antagonist was able to improve food intake and thus lead to weight gain in tumour-bearing rats^[16]. Lewis lung carcinoma (LLC) mice deficient in TNF- α receptor protein type 1 showed a reduction in muscle wasting compared with LLC wild-type mice despite similar levels of TNF- α being detected in the serum^[17]. Treatment with antioxidants or NOS inhibitors was shown to increase body weight and prevent muscle wasting in mice^[18]. Despite this, TNF inhibition alone in animals has not been shown to be sufficient to reduce or reverse the cachectic process indicating that, although it is involved in the pathogenesis of cancer cachexia, it is not solely responsible^[19].

Studies in patients with cancer, have also not been successful. In particular adipocytes taken from cancer patients, showed no decrease in LPL messenger RNA (mRNA) or LPL enzyme activity^[20]. Some studies have shown raised TNF- α levels in the serum of patients with pancreatic cancer associated with weight loss, whereas other studies in patients with terminal cancer showed no association between TNF- α and weight loss^[21,22]. Others have shown that TNF- α correlates with stage of disease or tumour size rather than degree

of weight loss^[23]. These discrepancies between studies may be due to differences in measuring techniques, possible auto or paracrine roles for TNF- α in adipose tissue, or heterogeneity between patients, sexes and tumours.

In summary, TNF- α is involved in systemic inflammation, but as cachexia is likely to be multifactorial, it is difficult to implicate TNF- α as the sole cause. More clinical studies are required to fully isolate its effects in patients.

Interferon gamma

Interferons are multifunctional cytokines that block viral infections and affect cell proliferation and differentiation^[24]. Interferon gamma (IFN γ) is produced by activated T and NK cells, and is arguably the most potent monocyte-macrophage activating factor^[25]. In the context of cancer, tumour-infiltrating lymphocytes (TILs), which have shown to be of particular importance in tumour immunosurveillance, are the main source of IFN γ ^[26]. There is an overwhelming body of evidence for both beneficial and detrimental roles of IFN γ in a range of diseases, including cancer. However, its role in patients with cachexia is a relatively underexplored area.

Several animal studies have indicated a central role for IFN γ in the pathogenesis of cachexia. Central administration of rat interferon resulted in decreased food intake whereas peripheral administration failed to do so^[27]. Mice overexpressing IFN γ producing tumour cells developed loss of body weight, atrophy of adipose tissue, and reduced appetite, all of which were then reversed by pre-treatment of the mice with anti-IFN γ antibodies^[28]. Mice with LLC also demonstrated a reduction in weight loss after treatment with anti-IFN γ antibody, significantly reducing fat wasting in particular^[29]. In rats that had received transplants of MCG 101 sarcoma, anti-IFN γ antibody reduced weight loss, but the effect of treatment was short-lived^[30]. Similarly to TNF- α , IFN γ has been shown to inhibit LPL activity in adipocyte cells *in vitro*, as well as that of glycerol phosphate dehydrogenase in cultures of rat adipocytes^[31].

IL-1 α

Levels of IL-1 α have been shown to be increased in animal models of cachexia. It is thought to cause similar effects to that of TNF- α ^[32]. IL-1 α is a pro-inflammatory cytokine produced mainly by macrophages and endothelial cells and is known for being a trigger of the acute phase response, thus playing a role in cancer pathogenesis, as well as shock and autoimmune disorders^[33]. In a similar fashion to other cytokines discussed, it is also able to inhibit LPL activity and stimulate lipolysis in cultured adipocytes^[34]. The ability of IL-1 to induce anorexia is thought to be due to a central effect on appetite suppression^[35] involving blockade of neuropeptide Y. It also increases plasma concentrations of tryptophan and serotonin leading to early satiety and suppression of hunger^[36].

Again, there is evidence for the role of IL-1 α in animal models of cachexia, but little in humans. IL-1 α can induce cachexia and anorexia in rats. IL-1 α treated rats showed loss of body weight. Administration of IL-1 α receptor antagonist (IL-1 α) to tumourbearing rats however, did not result in any improvement in body weight^[37]. Following direct tumour injection with IL-1 α , C26 mice demonstrated significantly reduced weight loss (without an effect on tumour burden) compared with mice who had systemic injection^[38]. Cultures of C26 cells also demonstrated raised levels of IL-6 after stimulation with IL-1 α , which were suppressed by monoclonal antibody to IL-6^[38]. In tumour samples from patients undergoing surgical resection for upper gastrointestinal malignancy, IL-1 β and IL-6 were also significantly overexpressed in the cancer specimens, at both mRNA and protein levels, compared with control mucosa. Protein levels were seen to correlate with CRP, indicating that tumour may be the source of IL-1 β ^[39].

IL-1 β

IL-1 β is a proinflammatory cytokine released by macrophages. It regulates the expression of other cytokines including IL-6 and IL-12. Recently, the loss of p53 in cancer cells from breast cancer mouse models have been shown to induce the secretion of WNT ligands that stimulate tumour associated macrophages (TAMs) to produce IL-1 β , therefore helping to drive systemic inflammation. Macrophages were prevented from secreting IL-1 β by pharmacological and genetic blockade of WNT secretion in p53 null cancer cells. This blockade also resulted in decreased neutrophilic inflammation and metastasis formation^[40]. These findings therefore suggest an important potential future role for personalised immune therapy in patients with cancer cachexia.

IL-1 β has also been better associated with the clinical features of cachexia such as anorexia, weight loss and sarcopenia than other cytokines such as IL-6 in a study of 83 advanced cancer patients^[41]. Patients with gastric cancer cachexia have also been shown to have a higher prevalence of IL-1B+3954 T allele than those without indicating that patient genotype plays a role on immunological regulation of cancer cachexia^[42].

IL-6

IL-6 can target adipose tissue, skeletal muscle, gut, and liver tissue, which can all affect cachectic patient body composition. It signals through the membrane bound receptor gp130 found in most tissues in the body^[43]. Once bound to its receptor, it activates JAK tyrosine kinase leading to phosphorylation of tyrosines and the binding of STAT proteins. STAT proteins can translocate to the nucleus and increase the transcription of genes involved in immune function, cell proliferation, differentiation and apoptosis^[43].

Several mouse cancer models have clearly demonstrated that blocking IL-6 and associated signalling can attenuate cachexia progression. Deletion of the *IL-6* gene in the APCMin/+ mouse prevented the development of cachexia^[44]. IL-6 when secreted by tumour cells can also increase autophagy in myotubes when joined with soluble IL-6 receptor^[45]. IL-6 trans-signalling through the soluble IL-6R has the potential to amplify IL-6 signalling in the cachectic patient and has been shown to be involved in cross-talk between tumour, muscle and adipose tissue in genetic mouse models of pancreatic cancer cachexia^[46]. Autophagy and increasing IL-6 levels have been associated with poor prognosis and weight loss in lung cancer patients^[45]. It has also been shown to be the key cytokine that regulates the hepatic acute phase response in patients with pancreatic cancer cachexia^[47]. IL-6 remains a promising therapeutic target in cancer cachexia but a better understanding of its direct and indirect effects, as well as tissue specific actions, is required.

CYTOKINE GENOTYPE

The presence and concentration of (potentially) pro-cachectic cytokines in cancer patients appear to be dependent not only on the type of tumour, but also on the burden of disease present, and patient specific factors such as age, sex and genotype. It is still not fully understood why patients with the same histological disease may vary with regards to the presence and severity of cachexia. Genetic variation in immunity is one possible reason. Specific single nucleotide polymorphisms in the *IL-1*, *IL-6* and *IL-10* genes have been associated with cachexia in gastrointestinal cancers^[48]. The 1082G allele in the IL-10 promoter was validated in an independent cohort. This was shown to be more prevalent in Myc/mTOR-driven mouse models of cachexia as well as cachectic colorectal cancer patients^[48]. The C allele of the rs6136 polymorphism in the P-selectin gene has also been associated with weight loss and low CT muscularity in a large group of cancer patients^[49]. These results suggest a role for the immune system in the complex presentation of cachexia.

CELLS

Myeloid derived suppressor cells

Many studies have now suggested that tumour infiltrating immune cells (those which are mainly of myeloid origin) are able to differentiate into cells which then promote tumour growth and metastasis

through their ability to induce systemic inflammation^[50]. Tumours can grow through myelopoiesis and the successful evasion of tumour cells from both the innate and adaptive immune systems^[51]. However, the progression of cancers appears dependent on tumour-associated myeloid cells through their ability to promote angiogenesis and tissue remodelling^[52]. This apparent immunosuppression has been linked to the development of cachexia in very few studies, despite tumour-induced immunosuppression being well documented in the literature^[53].

Myeloid derived suppressor cells (MDSC) may play a role in tumour-related immunosuppression. Tumour-induced amplification of the myeloid compartment leads to the expansion of myeloid-derived suppressor cells. MDSCs are immature myeloid cells in various stages of differentiation, but are not fully differentiated neutrophils, monocytes/macrophages or dendritic cells. They are found in the bone marrow, spleen, lymph nodes and tumours^[50]. Their mechanisms of action are not fully understood but they are thought to be immunosuppressive and to play a role in the over production of cytokines and inflammatory mediators, which may contribute to cachexia.

MDSC expansion in 4T1 breast carcinoma-bearing mice is associated with the induction of the hepatic acute phase protein response and altered fat metabolism^[53]. This response is also seen in the C26 and LLC mouse models. The pro-cachectic acute phase response is not seen, however, in 66C4 subclone of 4T1 mice in which MDSC expansion does not occur^[53]. Defects in myeloid cell-mediated inflammation has also been shown to result in reduced expression of pro-inflammatory cytokines in the serum of mice with hepatocellular carcinoma^[54]. Interestingly, this led to enhanced loss of adipose tissue and decreased macrophage number in visceral adipose tissue, suggesting a possible local role for macrophages in the regulation of cancer-induced fat loss^[54]. These findings imply that myeloid cell-mediated inflammation confers a beneficial function in these rodents, and may provide a potential explanation for the failure of several anti-inflammatory drugs in treating cachexia. Although a direct link between the development of cachexia and MDSCs has not been proven, the above studies have suggested that the development of cancer cachexia is partly explained by the expansion of immature myeloid populations associated with the tumour.

TAMs

TAMs increase tumour progression and metastasis and suppress anti-tumour immune functions^[55]. Monocytes from blood infiltrate the tumour and are primed by the tumour microenvironment to exert these effects^[55]. The immune cells within the tumour's microenvironment consist of myeloid-derived suppressor cells, NK cells, dendritic cells, T cells and macrophages^[56]. It is this infiltrate that contributes to tumour growth and the release of cytokines that promote the pro-cachectic environment. TAMs are recruited via cytokines and chemokines and suppress the activity of cytotoxic T-lymphocytes via programmed cell death 1 ligand 1 (PD-L1) or B7-H4 and other receptors^[57]. Activated macrophages also secrete cytokines leading to the activation of several complex cascades, thereby increasing inflammatory status^[58]. The chemokine monocyte chemoattractant protein-1 is possibly responsible for the migration of monocytes to adipose tissue in chronic inflammation^[59]. The mechanisms by which macrophages modulate adipocyte function in cachexia are still unclear.

TILs

TILs are often found in tumours and are thought to reflect an immune response against the tumour. Many studies report a survival benefit associated with the presence of TIL, suggesting they may delay tumour progression. CD3+ and CD8+ TILs in particular have been identified as having a positive effect on prognosis^[60].

IMMUNE SYSTEM BIOMARKERS

The Neutrophil: Lymphocyte ratio is a prognostic indicator in cancer. Neutrophils increase the inflammatory reaction to pathogens but also interact with cancer cells to produce cytokines and effector

molecules (e.g., VEGF) that are able to stimulate angiogenesis and promote tumour growth^[61]. Activated neutrophils can move from the circulation to the tumour site to release reactive oxygen species that in turn can lead to further DNA damage. They also have anti-tumour roles through antibody-mediated cytotoxicity of tumour cells^[62].

C-reactive protein is an acute phase non-specific inflammatory marker that can be elevated in response to infection, surgery or malignancy. It is produced by the liver in response to increased levels of IL-6 released by activated macrophages, as well as IL-1 and TNF- α ^[63]. The Glasgow Prognostic Score utilises raised CRP and hypalbuminaemia to predict those patients with systemic inflammation as part of cancer cachexia and who have a poor outcome. It has been examined in more than 60,000 patients and has been shown to have independent prognostic value^[64].

IMMUNOTHERAPEUTIC AGENTS FOR CANCER CACHEXIA

Immunotherapeutic agents for cancer cachexia have yielded mixed results. The different forms of immunotherapy are discussed in detail below.

TNF α inhibitors

There are currently several TNF inhibitors in use for the management of diseases such as rheumatoid arthritis (RA), psoriasis and inflammatory bowel disease, namely etanercept, infliximab and adalimumab^[65]. These drugs have revolutionised the treatment of RA but have also offered insights into the role that TNF- α plays in cachexia. In RA patients, they attenuate the hepatic acute phase response and, importantly, improve patients quality of life^[65,66]. They have also been shown recently to prevent worsening of the disease and restore fat free mass^[67]. These drugs are now used to treat many thousands of patients and have been shown to be effective at blocking TNF- α , but are not effective in treating cancer-induced cachexia^[62-64]. The feature common to all of these diseases is chronic inflammation due to exaggerated production of pro-inflammatory cytokines. Etanercept has showed some promising results in improving fatigue in a small cohort of cancer patients^[68]. A phase I/II study was conducted on pancreatic cancer patients comparing etanercept and gemcitabine with gemcitabine alone. A small increase in progression free survival was seen associated with higher plasma IL-10 levels, but there was no significant improvement in 6 month progression-free survival compared with gemcitabine alone^[68]. A placebo-controlled double-blind trial was also undertaken in 63 patients with incurable malignancy and weight loss of > 2.27 kg over 2 months or daily intake of < 20 calories/kg body weight. Weight gain was found to be minimal in both arms with comparable survival times. Treatment was associated with higher neurotoxicity^[69]. In this trial, therefore, etanercept was not effective in the treatment of cachexia in patients with advanced disease.

Infliximab has been used in a phase II placebo controlled randomised study in patients with stage II-IV pancreatic cancer^[70]. Patients were given either 3 mg/kg or 5 mg/kg infliximab with gemcitabine or placebo with gemcitabine. The mean change in lean body mass was + 0.4 kg for those on placebo, + 0.3 kg for those receiving 3 mg/kg of infliximab, and + 1.7 kg for those receiving 5 mg/kg of infliximab. No statistically significant differences were seen, however^[70].

Another agent with anti-inflammatory activity is OHR/AVR118, a broad-spectrum peptide-nucleic acid immune modulator that targets both TNF- α and IL-6^[71]. A phase II study involving patients with advanced cancer and cachexia showed an improvement in anorexia, dyspepsia, strength, and depression^[71].

Anti-IFN γ treatments

Anti IFN γ treatments have been effective in reverting cachexia in the LLC mouse model^[29]. There have been no trials undertaken in cancer patients, mainly due to the fact that this type of therapy requires a dose to completely block the action of IFN γ , and at present, such a treatment programme would be very expensive. The only trial conducted in patients with cachexia due to sepsis showed no benefit^[72].

IL-1/6 inhibitors

The IL-1 pathway has been previously targeted in humans with the recombinant human IL-1 receptor antagonist Anakinra and the neutralising monoclonal anti-IL-1 antibody Canakinumab. Anakinra, as previously discussed, has had success in rheumatoid patients but has yet to be trialled in patients with cancer^[65]. However, a more specific IL-1a human monoclonal antibody, MABp1, has shown promising results in cancer. An initial dose escalation and expansion study was designed using MABp1^[73]. The first dose escalation study was performed in patients with refractory cancer to assess its safety and tolerability. It identified an optimal intravenous dose which was then used in the following phase II study of forty-two patients^[73]. Median plasma IL-6 concentrations decreased from baseline to week 8 ($P = 0.08$). Of those 30 patients who had an assessment of body composition, lean mass increased significantly by 1.02 ± 2.24 kg ($P = 0.02$)^[73]. It was then compared to megestrol acetate in patients with advanced colorectal cancer and > 5% weight loss. Those in the MABp1 treatment arm showed a trend towards increased survival^[74]. A placebo-controlled, double blind phase III study in 333 patients with advanced colorectal cancer was then undertaken which resulted in increased lean body mass as well as symptom relief (pain, anorexia, fatigue)^[75].

IP1510 is a synthetic peptide IL-1 receptor antagonist. Pre-clinical studies found it to have low toxicity, and to be a potential effective treatment for cachexia. It was then trialled in advanced gynaecological cancer patients where it was well tolerated, and it significantly improved patient anorexia, depression and physical performance. Weight stabilisation or gain was seen in 17 of the 26 enrolled patients^[76]. Interpretation of the current data is limited because the study was neither randomised nor controlled. However, further larger trials are to be initiated targeting IL1 in cancer cachexia^[74].

Studies involving IL-6 antibodies have been undertaken in patients with advanced non-small cell lung cancer. The humanised monoclonal IL-6 antibody Clazakizumab [ALD518] has shown beneficial results in increasing haemoglobin levels and preventing loss of lean body mass. Fatigue scores were also improved compared with controls^[77]. There are, however, no phase III trials underway.

Immunotherapeutic agents continue to be a promising treatment for cancer cachexia and may be added to the multi-modal management approach for this complex syndrome.

Standard cancer immunotherapy

Anti-cancer immunotherapy including pembrolizumab above has been shown to improve outcomes in a range of tumour types, including melanoma, lung and bladder cancers, many of which respond poorly to traditional agents. Some of these therapies are, however, poorly understood. Patients with cancer cachexia have been shown to respond poorly to some immunotherapies such as immune check point inhibitor therapy, likely due to elevated clearance or the establishment of primary resistance^[78]. In two large clinical trials involving patients with melanoma and non-small cell lung cancer, there was a paradoxical association between plasma clearance of Pembrolizumab [a programmed cell death protein inhibitor (PD-1)] and poor overall survival. Those patients who responded poorly were noted to have reduced body weight and low albumin, suggesting that the presence of cachexia rendered these patients unable to respond to Pembrolizumab^[76]. The hypoalbuminaemia was hypothesised to explain the elevated plasma clearance, and therefore the dose was increased in these patients to counteract this apparent resistance, but it did not result in improved outcomes. Studies in lung cancer patients treated with immunotherapy have shown that decreases in pre-treatment BMI, weight loss and high neutrophil:lymphocyte ratio were associated with significantly shorter progression-free survival^[79]. However, other studies in lung cancer patients have also shown that two thirds of those receiving PD-1 and PD-L1 immune check point inhibitors experienced stability or an increase in their skeletal muscle index^[80]. Thus, although at first glance immunotherapy would seem to be a natural antidote to cancer cachexia, it is difficult to unravel the clinical impact of immunotherapy from the known adverse outcomes of any cancer patients with poor nutritional status.

These studies therefore raise the possibility that immunotherapies could represent effective anti-cachexia agents but that synchronous multimodal and nutritional anti-cancer treatments may be required to establish or enhance their overall effectiveness.

A key consideration in using immunotherapy may be the inflammatory status of the host. It has been demonstrated that the host inflammatory status influences the efficacy of therapy with inflamed patients most likely to benefit from therapies with an anti-inflammatory mode of action^[81]. Similar to the call to “stage the tumor, stage the host”^[82] it is now key that treatment stratification is based on the inflammatory status of the patient and this is now being used as a mandatory measure in clinical trials in some tumour groups^[83]. It is clear that whilst immunotherapies as a treatment for cancer cachexia, there is a necessity to ensure patients who receive these are those who are most likely to benefit.

CONCLUSION

The pathogenesis of cancer cachexia is highly dependent on the patient's immune response. The interplay between inflammatory cytokines (such as TNF- α , IFN γ and interleukins) and pro-cachectic factors contributes to the complex aetiology. These cytokines are produced by the host in response to the tumour, as well as by the tumour itself. Many treatments have tried to regulate the immune response in cachexia but have largely been unsuccessful, perhaps in part due to the multifactorial nature of cachexia, and the observed heterogeneity of patient factors. Large-scale clinical studies are needed to prove whether neutralisation of deleterious cytokines or direct receptor antagonism in combinatorial treatment regimens is an effective therapeutic approach to improve patient outcomes or to reverse muscle loss in cancer cachexia.

DECLARATIONS

Authors' contributions

Drafted the manuscript: Miller J

Critically revised the manuscript and gave final approval for the version to be published: Laird BJA, Skipworth RJE

Availability of data and materials

Not applicable.

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Conflicts of interest

The authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

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Consent for publication

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Review

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Micromanaging autophagy with microRNAs to drive cancer metastasis

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Abstract

While we made great strides in the early detection of a handful of cancers, many other cancers are still detected at fairly later stages, thus hindering the deployment of effective surgical or therapeutic intervention to change their dismal clinical outcomes. The arduous journey of cancer cells from the primary tumor to colonize distant secondary organs or tissues begins with their ability to activate or deactivate various cellular processes at will, including the autophagy machinery. In this review, we discuss how circulatory cancer cells from primary tumors could selectively mobilize different subsets of microRNAs (miRNAs) to enable autophagic recycling of nutrients during their search for secondary sites to colonize and to disable such cell survival programs once they have successfully established at distant organs or tissues. We also discuss how this new miRNA-autophagy-metastasis axis can be targeted by the emerging RNA Medicine toolkit.

Keywords: Autophagy, miRNAs, cancer, metastasis

CANCER METASTASIS: KNOWING TOO LITTLE, TOO LATE

Metastasis remains one of the key reasons for the poor prognosis of cancer patients. The one-year survival of patients with stage 1 localized lung cancers is 87.3%, but plummets to merely 18.7% for stage 4 metastatic lung cancers^[1]. Similar trend is observed for colorectal cancer, where one-year survival is 97.7% if detected at stage 1 and rapidly decline to 43.9% if detected at stage 4. These depressing statistics underscores our



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current knowledge gap in understanding the metastatic process, which impacts our ability to offer curative treatment options to cancer patients.

In the early stages of cancer, tumors are typically benign and remain confined within the normal boundaries of a tissue. As tumors grow and become malignant, however, they acquire the capacity to break through these boundaries and invade adjoining tissues. Notably, dissemination of tumor cells can occur relatively early in cancer progression^[2]. During the process of metastasis, only a rare population of invasive cancer cells escape the primary tumor, survive the treacherous transit through the circulatory system, and subsequently establish secondary tumors in distant organs/tissues^[3]. These invasive cancer cells, which exhibit extensive cytoskeletal remodeling, secrete proteases like extracellular-matrix-degrading metalloproteinases and cathepsins to drive their invasion and migration through the stroma, a network of supportive, connective tissue cells at the basement membrane^[4,5]. Furthermore, the stroma can actively promote the initiation of metastasis by releasing transforming growth factor- β and other cell-signalling proteins to trigger cancer cells to undergo epithelial-to-mesenchymal transition (EMT) - a reversible phenotypic change in which cells lose intercellular adhesion and epithelial polarization and gain motility and invasiveness^[6]. Transition into a mesenchymal cancer cell state allows for efficient intravasation of the lymphovascular system and acquisition of a stem-cell phenotype to promote survival during transit. These circulatory cancer cells can be arrested at distant organs/tissues, where they extravasate into the parenchyma of target organs to commence colonization. After extravasation, the invading cancer cells undergo mesenchymal-to-epithelial transition (MET) at the new settlement sites. The conduciveness of these niches for colonization can dictate the period of dormancy of these micrometastatic cancer cells. Long period of latency often exists between the development of a primary tumor and clinical manifestations of metastasis, indicating that metastatic colonization is a highly inefficient process in which most of these disseminated tumor cells (DTCs) die. To survive and establish macrometastases at distant organs/tissues, the minor subset of DTCs must overcome immune surveillance and other host-tissue defenses to achieve overt colonization at secondary sites^[7]. Although breast-to-lung metastases have been shown to arise mainly from cells that have not undergone EMT in mouse models of breast cancer^[8], it could be interpreted as rapid conversion into the epithelial cell state of DTCs, via MET, as soon as they arrive at supportive niches in the lung. Notably, the breast cancer cells that have undergone EMT are found to be resistant to chemotherapy and this could be attributed to their acquisition of a stem cell-like phenotype^[9].

AUTOPHAGY: A CRUCIAL CANCER CELL SURVIVAL MECHANISM DURING METASTASIS

Autophagy is a cellular housekeeping mechanism which degrades and recycles bulks of cytoplasmic materials for the synthesis of essential cellular components^[10]. Highly conserved from yeast to mammals, autophagy acts as an adaptive response to environmental stresses such as nutrient deprivation. The autophagy machinery has multiple distinct stages: initiation, nucleation, elongation, fusion and cargo recycling^[10]. During stress, shifts in nutrient availability can be detected by proteins involved in energy homeostasis, such as the 5' AMP-activated protein kinase (AMPK)^[11] and the target of rapamycin complex 1 (TORC1). The activation of AMPK and the corresponding inhibition of TORC1 will lead to the assembly of the Atg1/ULK complex, thereby kickstarting autophagy.

During autophagy, cytoplasmic cargoes are encapsulated by double-membrane vesicles known as autophagosomes and thereafter sent to the lysosomes to be degraded^[12]. The intricate, multistep processes of autophagy are facilitated by numerous autophagy-related proteins (Atg). Detailed characterization of these proteins can be found in the review by Feng *et al.*^[13]. At the initiation stage, the Atg1/ULK complex is assembled. This complex consists of Atg1/ULK, the regulatory subunit Atg13 and the scaffold complex Atg7-Atg31-Atg29 [Figure 1]. Importantly, the assembly of the Atg1/ULK complex is critical for the

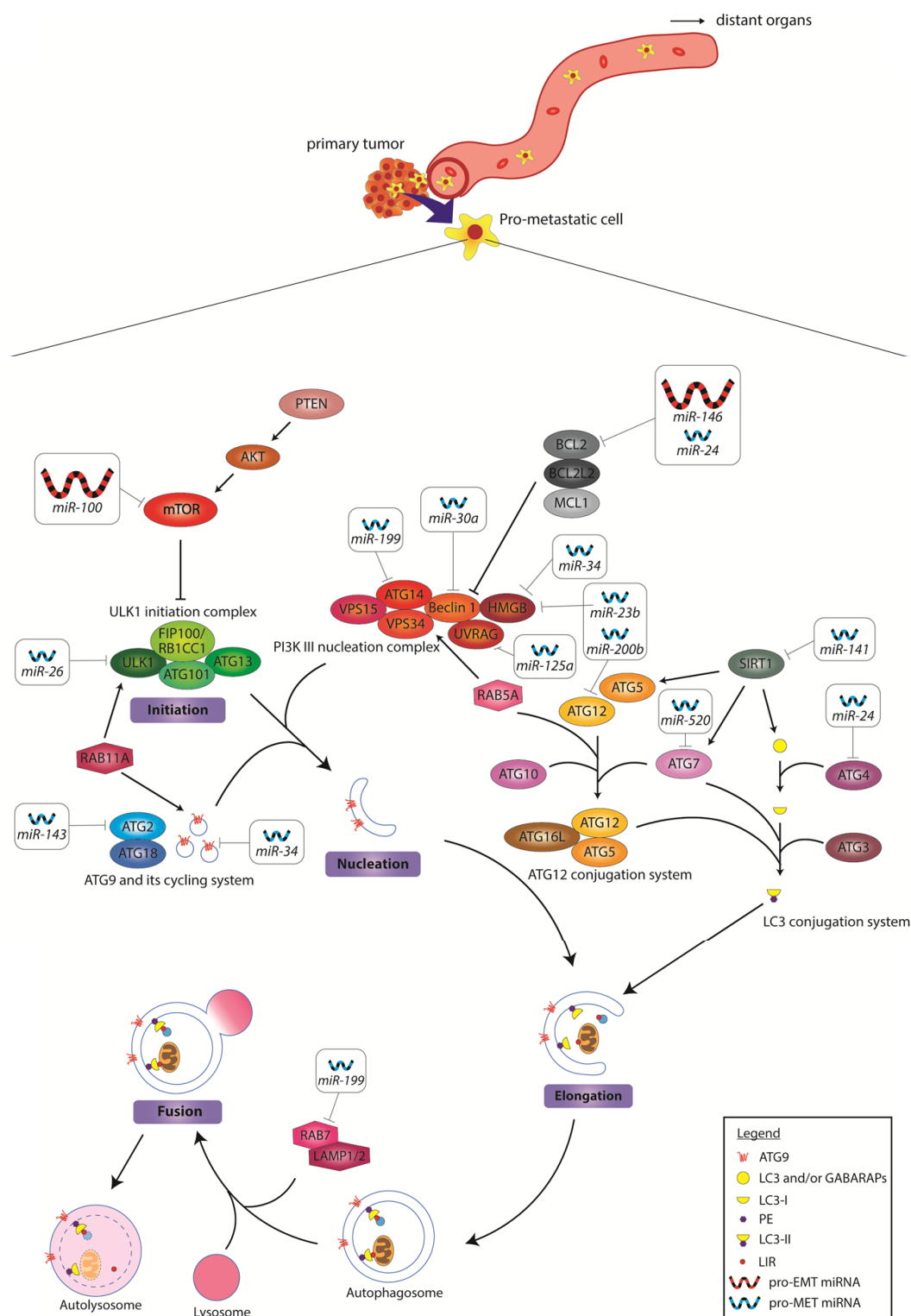


Figure 1. MiRNA regulation of autophagy during cancer invasion. The key components of macroautophagy is shown in this diagram. In the invading pro-metastatic cancer cell, autophagy is upregulated by gain of oncomiRs and loss of tumor-suppressive miRNAs. The oncomiRs target the inhibitors of autophagy to increase autophagic flux. The downregulated miRNAs on the other hand, are required for the activation of autophagy. Upregulated pro-EMT miRNAs are depicted as large red icons, while downregulated pro-MET miRNAs are depicted as small blue icons. The miRNA targets and mechanisms of actions are summarized in Table 1. EMT: epithelial-to-mesenchymal transition; oncomiRs: oncogenic miRNAs; MET: mesenchymal-to-epithelial transition

recruitment of downstream proteins to the phagophore assembly site. Next, Atg9 and its cycling system (Atg2, Atg18) are activated to facilitate membrane delivery to the expanding phagophore. This is followed by the assembly of the PI3K complex consisting of Vps34, Vps15, Vps30/Atg6, Atg14, and Atg38 [Figure 1], a process responsible for recruiting more proteins that are required for further phagophore expansion. Subsequently, two ubiquitin-like conjugation systems are activated for phagophore expansion, namely the Atg12 and the Atg8 conjugation systems. A detailed mechanism in which these conjugation systems function has been reported by Yin *et al.*^[10]. The human homologs of Atg8 are categorized into two families - LC3 and GABARAP. Importantly, LC3 is employed during the phagophore elongation stage, whereas GABARAP proteins are only activated at a later stage of maturation. Upon induction of phagophore elongation, LC3 is cleaved at the C-terminal by ATG4B protease to form cytosolic LC3-I. This is followed by its subsequent conjugation and recruitment to the membrane to form LC3-II^[13]. The level of LC3-II is often known to correlate with the number of autophagosomes due to its association with autophagosome maturation^[14].

Fundamentally, LC3-II acts as an adaptor protein that selectively recruits cargoes to the autophagosome to be degraded^[14]. Following cargo recruitment and closure of the autophagosome, the autophagosome directly fuses with a lysosome to form an autolysosome^[12]. The cytosolic cargoes within the autolysosome are then broken down by lysosomal hydrolases, liberating building blocks for future synthesis of other macromolecules.

The importance of autophagy as a cellular homeostatic mechanism is underscored by the prevalence of autophagic defects in a myriad of diseases. Besides being a protein and organelle quality control mechanism that prevents the accumulation of aggregated and dysfunctional proteins, basal autophagy is also a highly regulated catabolic process that supports cellular metabolic and biosynthetic programs in response to nutrient deprivation and other forms of stress. In cancer pathogenesis, however, autophagy is hijacked by rapidly proliferating cancer cells as they adapt to the perturbations of the cancer microenvironment^[15].

Autophagy is finely regulated in response to the specific energy demands or stress cues emitted throughout the different stages of metastasis - local invasion, intravasation, dissemination, extravasation and colonization at distant sites. Several studies suggested that autophagy is associated with EMT in cancer and activation of autophagy promotes metastasis^[16]. This is corroborated by the high levels of autophagy detected in EMT-activated cancer cells, hence strongly supporting its role in promoting early metastasis^[17]. Additionally, cell detachment from the extracellular matrix typically has been shown to induce anoikis, a self-defense strategy that eliminates misplaced cells through apoptosis^[18,19]. It then becomes crucial for cancer cells to evade anoikis so as to survive while traversing to distant organs/tissues. Importantly, several studies have postulated the pro-metastatic role of autophagy in overcoming anoikis^[20-22]. Although the underlying mechanisms remain largely unknown, anoikis resistance conferred by autophagy has been shown to enhance the survival of cancer cells when they disseminate into the lymphovascular system, leading to their successful metastasis to distant organs.

Given that autophagy is a key cellular process that confers stress tolerance under adverse conditions, its activation in cancer cells that intravasate into the blood or lymphatic vessels enables them to withstand extreme metabolic stress induced by the changing environment^[23]. To compensate for the sudden change in energy demands of the circulating DTCs, autophagy is activated to retain pools of functional mitochondria to sustain viability of the DTCs. In addition, autophagy-mediated degradation of proteins provides a sustainable source of nutrients for the DTCs to fuel ATP production and biosynthesis of other macromolecules that are essential for growth and survival^[23,24].

Successful metastatic colonization is not only limited to the ability of DTCs to overcome stress that they are exposed to during circulation. Following extravasation from the blood vessel, the DTCs have been shown

to switch from EMT to MET with concurrent inactivation of autophagy in supportive niches^[25]. However, if the new environment is not permissive in situations such as cellular stress or a lack of available growth factors, the DTCs could exist in a dormant state, possibly by retaining elevated autophagic functions and delaying MET. Notably, autophagy inhibition in the neoadjuvant setting has also been shown to reduce pro-metastasis stromal cells at the premetastatic niche^[26]. Future work could be performed to determine whether inactivation of autophagy is necessary to drive MET of invading tumor cells at distant organs/tissues, thus providing new impetus for the development of anti-metastasis autophagy inhibitors.

MIRNAS: NONCODING REGULATORS OF AUTOPHAGY AND CANCER METASTASIS

MicroRNAs (miRNAs) are endogenously expressed small non-coding RNAs of 19-25 nucleotides in length, which negatively regulate gene expression post-transcriptionally. They regulate multiple cellular processes including proliferation, differentiation and apoptosis. They have been implicated in a growing list of human diseases, such as diabetes and cancer^[27].

In the canonical miRNA biogenesis pathway, the primary transcript (pri-miRNA) in the nucleus forms a characteristic stem loop structure, which is recognized and cleaved by the Drosha/DGCR8 heterodimer into pre-miRNA. The pre-miRNA hairpin loop is transported into the cytoplasm by Exportin-5, where its terminal loop is further cleaved by RNA endonuclease III Dicer to generate the mature duplex miRNA. Either strand can be processed into the final mature single-stranded miRNA, with the strand originating from the 5' end of the transcript known as 5p, while the strand originating from the 3' end is known as 3p. Association of the mature miRNA duplex with Argonaute proteins (Ago) forms the RNA-induced silencing complex (RISC), where the guide strand of ~22 nt remains in Ago while the passenger strand gets degraded^[28,29]. Due to the short sequence length of miRNAs, and the fact that their partial complementary binding to multiple regions of target messenger RNAs (mRNAs) is sufficient to guide the RISC to its target genes, miRNAs are able to regulate the expression of a myriad of genes that play critical roles in controlling cellular processes, including autophagy and cancer metastasis.

AUTOPHAGY-REGULATING MIRNAS IN CANCER METASTASIS

Owing to the complexity and dynamic nature of autophagy, past literature that implicated a role of miRNAs in the regulation of autophagy have been highly discordant even in the same cell type. To reconcile and put things into perspective, we examined and discuss the opposing roles of miRNA-regulated autophagy during the invasion phase versus the distant organ/tissue colonization phase of cancer metastasis.

At the onset of metastasis, cancer cells in the primary tumor first undergo EMT to enable the attached cancer cells to gain motile traits for invasion of the lymphovascular system. The role of autophagy in cancer cell motility and invasion was comprehensively reviewed by Mowers *et al.*^[23]. Suffice to say that many reports have now demonstrated the elevation in autophagy in cancer cells drives tumor dissemination, and this may in part be regulated by miRNAs. miRNAs that have been shown to regulate autophagy and cell migration/invasion in the context of cancer have been summarized in [Table 1](#).

Upregulation in oncogenic miRNAs (oncomiRs) is known to promote tumorigenesis^[29] via modulation of gene expression of a variety of growth and survival pathways in cancer cells. For instance, elevated levels of miR-100 and miR-146 are found in cancer cells. Notably, these oncomiRs have been shown to be critical for the activation of pro-survival autophagy via suppression of the expression of autophagy inhibitory effectors like the mammalian target of rapamycin (mTOR) and Bcl2, respectively [\[Figure 1\]](#)^[30,32,33]. miR-100 and miR-146 have also been shown to be key EMT effectors that promote metastasis^[31,34], with miR-100 proposed to act through HOXA inhibition^[83], and miR-146 via Notch2 inhibition^[84]. Overexpression of

Table 1. MiRNAs and their effects on autophagy and metastasis

miRNA	Action	Gene target	Effect in Autophagy		Effect in metastasis
			Mechanism of action	Expression status in cancer	
<i>miR-100</i>	Activator ^[30]	<i>mTOR</i>	Activates ULK1 initiation	Upregulated ^[30]	Activator ^[30,31]
<i>miR-146a</i>	Activator ^[32]	<i>BCL2</i>	Activates PI3K III nucleation	Upregulated ^[33]	Activator ^[33,34]
<i>miR-23b</i>	Inhibitor ^[35]	<i>HMGB2</i> <i>ATG12</i>	Inhibits PI3K III nucleation Inhibits ATG12 conjugation	Downregulated ^[35,36]	Inhibitor ^[36]
<i>miR-24</i>	Inhibitor ^[37]	<i>BCL2</i> <i>ATG4</i>	Inhibits PI3K III nucleation Inhibits LC3 conjugation	Downregulated ^[38]	Inhibitor ^[38]
<i>miR-26a/b</i>	Inhibitor ^[39]	<i>ULK1</i>	Inhibits ULK1 initiation	Downregulated ^[39,40]	Inhibitor ^[40]
<i>miR-30a</i>	Inhibitor ^[41]	<i>Beclin1</i>	Inhibits PI3K III nucleation	Downregulated ^[42]	Inhibitor ^[42]
<i>miR-34</i>	Inhibitor ^[43,44]	<i>HMGB1</i> <i>ATG9a</i>	Inhibits PI3K III nucleation Inhibits ATG9 cycling	Downregulated ^[45]	Inhibitor ^[46]
<i>miR-125a</i>	Inhibitor ^[47]	<i>UVRAG</i>	Inhibits PI3K III nucleation	Downregulated ^[48]	Inhibitor ^[48]
<i>miR-141</i>	Inhibitor ^[49]	<i>SIRT1</i>	Inhibits ATG12 and LC3 conjugation	Downregulated ^[50]	Inhibitor ^[50,51]
<i>miR-143</i>	Inhibitor ^[52]	<i>ATG2B</i>	Inhibits ATG9 cycling	Downregulated ^[53]	Inhibitor ^[53]
<i>miR-199</i>	Inhibitor ^[54,55]	<i>ATG14</i> <i>Rab7A</i>	Inhibits PI3K III nucleation Inhibits autophagosome and lysosome fusion	Downregulated ^[56]	Inhibitor ^[56]
<i>miR-200</i>	Inhibitor ^[57]	<i>ATG12</i>	Inhibits ATG12 conjugation	Downregulated ^[58]	Inhibitor ^[58,59]
<i>miR-520</i>	Inhibitor ^[60]	<i>ATG7</i>	Inhibits ATG12 and LC3 conjugation	Downregulated ^[60]	Inhibitor ^[61]
<i>miR-21</i>	Inhibitor ^[62,63]	<i>Rab11A</i> <i>PTEN</i>	Inhibits ULK1 initiation and ATG9 cycling	Upregulated ^[64]	Activator ^[64]
<i>miR-29a</i>	Inhibitor ^[65]	<i>ATG9A</i>	Inhibits ATG9 and its cycling	Upregulated ^[66]	Activator ^[66]
<i>miR-93</i>	Inhibitor ^[67,68]	<i>PTEN</i> <i>ATG16L1</i>	Inhibits ULK1 initiation Inhibits ATG12 conjugation	Upregulated ^[67]	Activator ^[67,69]
<i>miR-214</i>	Inhibitor ^[70]	<i>PTEN</i> <i>ATG12</i>	Inhibits ULK1 initiation Inhibits ATG12 conjugation	Upregulated ^[71]	Activator ^[71]
<i>miR-221/222</i>	Inhibitor ^[72]	<i>ATG12</i>	Inhibits ATG12 conjugation	Upregulated ^[72,73]	Activator ^[73]
<i>let-7</i>	Activator ^[74]	<i>mTOR</i>	Activate ULK1 initiation	Downregulated ^[75]	Inhibitor ^[75]
<i>miR-7</i>	Activator ^[76]	<i>mTOR</i>	Activates ULK1 initiation	Downregulated ^[77]	Inhibitor ^[77]
<i>miR-16</i>	Activator ^[78]	<i>mTOR</i>	Activates ULK1 initiation	Downregulated ^[79]	Inhibitor ^[79]
<i>iR-205</i>	Activator ^[80,81]	<i>PTEN</i>	Activates ULK1 initiation	Downregulated ^[82]	Inhibitor ^[82]

these oncomiRs provides several advantages to the invading cancer cells. Firstly, an increase in autophagy flux helps DTCs to resist anoikis following detachment from the primary tumor as well as to enable DTCs to gain access to nutrients to survive the arduous journey of traversing to distant organs/tissues. In addition, they concurrently change gene expression patterns in the cancer cells to allow these cells to become mesenchymal and thus more motile to invade distant sites.

Apart from oncomiRs, the loss of tumor-suppressive miRNAs has also been shown to drive cell invasion and migration [Figure 1]. Several autophagy-inhibiting miRNAs, namely, miR-23b, miR-24, miR-26, miR-30a, miR-34, miR-125a, miR-141, miR-143, miR-199, miR-200 and miR-520 are found to be downregulated in cancer. Notably, these miRNAs are also known MET effectors^[35-43,45-61,85,86]. As opposed to the primary role(s) of oncomiRs, we speculate that the loss of these miRNAs allows for higher autophagy flux and suppression of MET in the DTCs to help them adapt to unfavorable settlement sites.

Intriguingly, there exists a group of miRNAs that appear to block autophagy and yet promote EMT. These include oncogenic miR-21, miR-29a, miR-93, miR-214 and the miR-221/222 cluster that are highly expressed in cancer [Figure 2]^[62-73,87]. It seems counter-intuitive that successful distant organ/tissue invasion can be achieved for cancer cells without autophagy, but it remains plausible that the effects of these miRNAs might be context-dependent. For example, overexpression of miR-21 and miR-93 have been shown to induce metastasis by targeting PTEN^[64,69], which in turn may promote the blockade of autophagy via activating the PI3K-Akt-mTOR signaling cascade. Since one miRNA can target many different genes, the biological

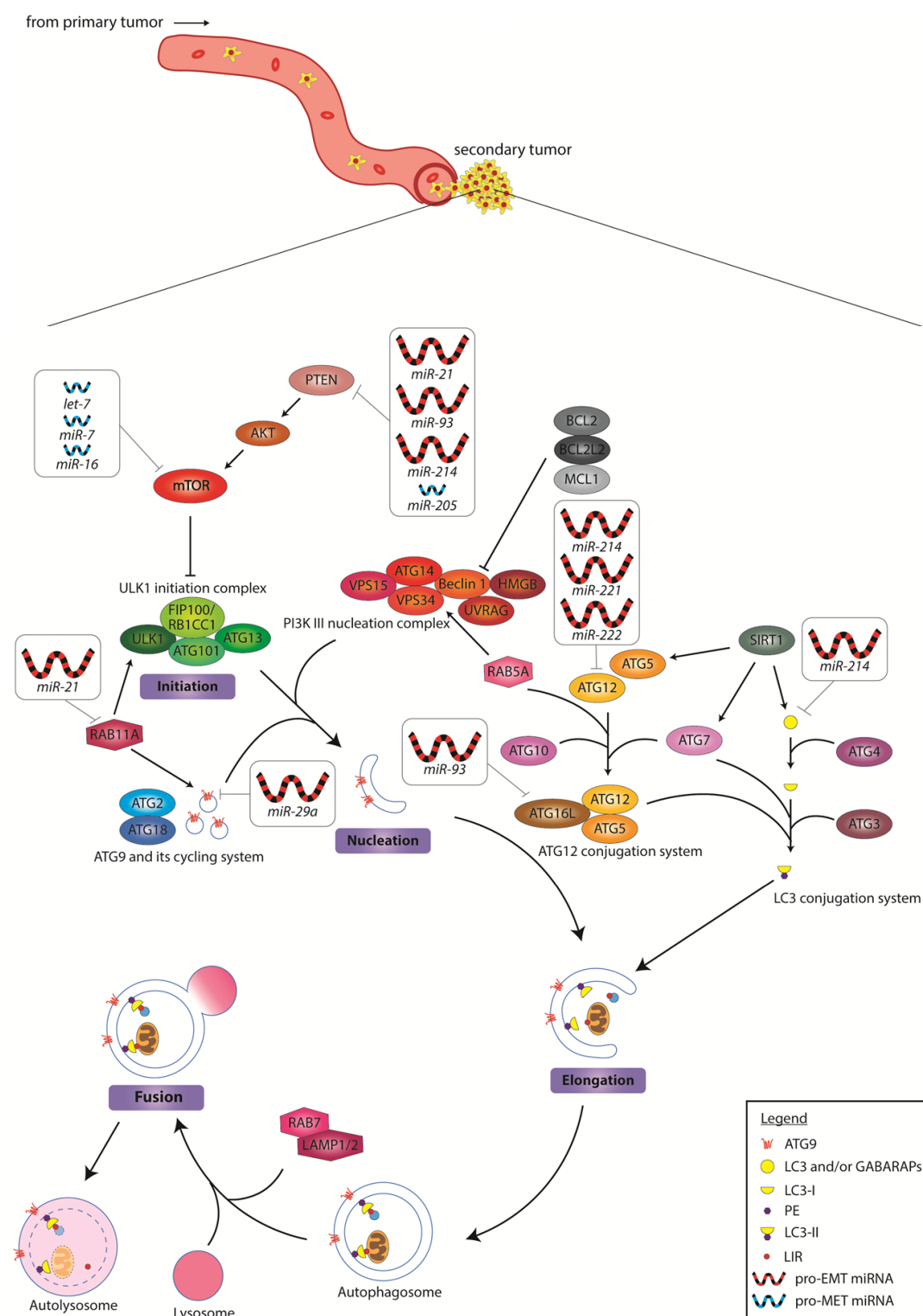


Figure 2. MiRNA inhibition of autophagy during secondary tumor formation. In the colonization process, autophagy is inhibited either by upregulation of anti-autophagy miRNAs, or the downregulation of the pro-autophagy miRNAs. This process promotes the re-establishment of an epithelial cell state for developing into macrometastasis. Upregulated pro-EMT miRNAs are depicted as large red icons, while downregulated pro-MET miRNAs are depicted as small blue icons. EMT: epithelial-to-mesenchymal transition; MET: mesenchymal-to-epithelial transition

context should also be taken into consideration in reconciling the opposing EMT-promoting and anti-autophagy effects of miRNA-dependent loss of PTEN. Could miR-21 and miR-93 also regulate other targets concurrently to yield the phenotype observed? We also noted that several of these studies demonstrate induction of cell migration/invasion by overexpressing miR-21 or miR-93 in cancer cells grown in the tissue culture setting^[64,67]. Unlike the circulating DTCs in a living organism, cancer cells cultured *in vitro* are never short of nutrient supply. It is perhaps not surprising that the need to trigger pro-survival autophagy in these cancer cells might be over-written by external culture conditions *in vitro*. Alternatively, miRNAs like miR-21 and miR-93 may serve as sensors of favorable distant niches. As the DTCs extravasate at their destination sites, these miRNAs could respond to the new environment that provides nutrients to fuel the transition from micro- to macro-metastasis. Similarly, there exists tumor suppressive miRNAs that have been implicated in autophagy activation but yet inhibit cancer growth and metastasis. These miRNAs, including let-7, miR-7, miR-16 and miR-205, have been shown to be less abundant in tumors^[74-82]. Their absence in DTCs at micrometastasis sites might be crucial to cell survival, as autophagy genes will remain activated to ensure nutrient supply until conditions of the new environment turn favorable for macrometastasis.

PRECISION TARGETING OF MIRNA-AUTOPHAGY SIGNALING NODES BY SMALL MOLECULE AND RNA-BASED THERAPEUTICS

To date, cytotoxic chemotherapy remains the most widely used neoadjuvant or adjuvant treatment modality against cancers despite its association with a host of unpredictable side effects^[88]. In addition, a recent study demonstrates that cytotoxic chemotherapeutics broadly used in neoadjuvant cancer therapy elicit tumour-derived extracellular vesicles with enhanced pro-metastatic capacity^[89]. Hence, precise targeting of metastasis-enabling cellular processes, such as autophagy, is warranted and could synergize with chemotherapy to maximize therapeutic efficacy. For instance, small molecule inhibitor targeting of focal adhesion kinase (commonly known as FAK) activates the SRC kinase and thereby inhibits autophagy, which in turn, prevents the migration of SRC-driven metastatic tumor cells^[90,91]. Given the complexity of signaling pathways driving metastasis, a systematic framework for the development of effective anti-metastatic drugs has recently been proposed^[92].

Of late, RNA-based therapies (antisense, siRNAs, aptamers, miRNA mimics/anti-miRs and synthetic mRNA) have shown great promise as powerful adjuncts to the drug developer's existing toolbox of small molecules and biologics^[93]. Notably, miRNA-based therapeutics gained much attention in translational medicine and are currently tested in various Phase I and II clinical trials. miRNA-based therapeutics is broadly categorized as miRNA mimics and inhibitors of miRNAs (also known as anti-miRs or antagomiRs). miRNA mimics, in the form of synthetic double-stranded small RNA molecules that match the corresponding miRNA sequence, are aimed at replenishing the lost miRNA expression in diseases. Most of the commercial miRNA mimics are often modified by methylation of the passenger strand to increase their stability. AntagomiRs, on the other hand, are antisense oligonucleotides that are single-stranded and can be chemically-modified (with locked nucleic acids or with 2'-O-methoxyethylribose nucleoside) to increase affinity for their miRNA targets to block their functions^[94]. To prevent degradation by RNases in biofluids or in the endocytic compartment of cells, these miRNA therapeutics are frequently stabilized by oligonucleotide chemistry (as mentioned above or by adding phosphorothioate-like groups) or encapsulated in delivery vehicles, such as poly(lactide-co-glycolide) particles, neutral lipid emulsions, synthetic polyethylenimine, cyclodextrin, chitosan, etc.,^[95]. For instance, epidermal growth factor (EGFR)-targeted, EnGeneIC delivery vehicle-packaged miR-16 mimics have been tested in a Phase I clinical trial (NCT02369198) in which acceptable safety profile and on-target activity of these miR-16 mimics have been demonstrated in patients with malignant pleural mesothelioma^[96]. In addition, two other clinical trials (NCT02862145 and NCT01829971), involving the use of miR-34a mimics to treat primary liver cancer, small

cell lung cancer (SCLC), lymphoma, multiple myeloma, renal cell carcinoma and melanoma, also showed promising anti-cancer results^[97]. Given that a subset of miRNAs mediate the crosstalk between autophagy and metastatic states of cancer cells, we postulate that the miRNA-autophagy-metastasis axis of cancer could be effectively targeted by the precise *in vivo* delivery of specific combinations of miRNA therapeutics (involving miRNAs depicted in [Figures 1 and 2](#)), which could improve the clinical trajectory of late stage cancers. Alternatively, blockade of the biogenesis of oncomiRs via small-molecule RNA ligands has also been reported to be a feasible anti-cancer approach^[98]. These small molecules consist of high affinity RNA binding motives, including the aminoglycoside neomycin and different natural and artificial nucleobases, that target pri-miRNAs of oncogenic miR-372/miR-373 and inhibit Dicer-mediated maturation of these miRNAs to elicit anti-proliferative effects on gastric cancer cells.

Apart from miRNA therapeutics, siRNA therapeutics that target a number of driver oncogenes like *c-Myc*, *PD-L1/2*, mutant *KRAS*, *PKN3*, *EphA2*, *LMP2/7*, *MECL1*, *APN401* and *PLK1* have also been shown to exhibit on-target anti-cancer effects and elicit little or no overt toxicities in humans in a number of Phase I clinical trials (NCT00306904, NCT02314052, NCT02528682, NCT01676259, NCT01808638, NCT01591356, NCT00672542, NCT02166255 and NCT01437007). We envisage that delivery of siRNAs targeting genes that are important for autophagy and metastasis of cancer cells could also be a viable therapeutic route to combat late stage cancers.

CONCLUSION

Alterations in key cellular processes, including cell adhesive properties and autophagy, promote tumor cell dissemination and colonization at distant organs/tissues. These processes are mediated by a vast network of miRNAs that has the propensity to crosstalk with each other. Striking this Achilles heel of cancer precisely via specific combinations of miRNA therapeutics could dramatically improve the survival outcomes of cancer patients. Future work should be focused on tissue-specific targeting delivery vehicles, which carry miRNA therapeutics or other payloads, so as to avoid potential toxicities and off-target effects.

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Authors' contributions

Manuscript writing: Eng GWL, Kok VJT, Cheong JK

Availability of data and materials

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

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Review

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Exploiting autophagy in multiple myeloma

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Abstract

Multiple myeloma (MM) is a plasma cell cancer characterized by sustained endoplasmic reticulum (ER) stress and unfolded protein response activation in the setting of high rates of immunoglobulin synthesis. Consequently, MM cells rely heavily on protein quality control pathways for survival as evidenced by the clinical efficacy of proteasome inhibitors (PI). Autophagy is an intracellular self-digestion mechanism that plays a role in the ER protein quality control process. Unsurprisingly then, basal levels of autophagy were recently found to confer a survival and drug-resistance benefit to MM cells. However, excessive induction of autophagy in MM cells leads to autophagic cell death, highlighting the double-edged nature of autophagy modulation in MM. This review provides an overview of the role that autophagy plays in MM pathogenesis, survival, and drug-resistance. We highlight the potential utility of therapeutically targeting autophagy in MM, focusing on preclinical data of autophagic modulators in combination with alkylators, anthracyclines, PI, and immunomodulatory drugs.

Keywords: Multiple myeloma, autophagy, drug resistance, hematopoiesis, immunoglobulin, antibody



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MULTIPLE MYELOMA

Multiple myeloma (MM) is a neoplastic disorder characterized by the dysregulated proliferation of a plasma cell clone that typically produces a monoclonal immunoglobulin, ultimately resulting in end-organ damage^[1-3]. Clinical suspicion for active MM is often based on the presence of one or more laboratory/imaging abnormalities, termed the CRAB criteria [hypercalcaemia (C), renal impairment (R), anaemia (A), and osteolytic bone lesions (B)], particularly if occurring in a patient with a precursor plasma cell disorder such as monoclonal gammopathy of undetermined significance (MGUS) or smoldering MM (SMM)^[4]. A diagnosis of active MM requires the presence of greater than 10% clonal bone marrow (BM) plasma cells in association with either one or more of the CRAB features or a biomarker of malignancy (BM plasmacytosis equal or greater than 60%, ratio of involved vs. uninvolved light chain equal or greater than 100 or the presence of more than 1 focal lesion on magnetic resonance imaging)^[5,6]. MM is generally preceded by the asymptomatic precursor conditions MGUS and/or SMM^[6]. MGUS is characterized by low levels of monoclonal protein (< 3 g/dL) and less than 10% clonal plasma cells in the BM while SMM is characterized by the presence of > 3 g/dL of monoclonal protein with BM plasmacytosis exceeding 10% but less than 60%^[6]. Evidence of end organ damage related to the plasma cell disorder is an exclusion criteria for MGUS/SMM diagnosis. Patients with MGUS and SMM progress to active MM at a rate of 1% and 10% per year, respectively^[7].

While single driver mutations have not been identified in MM, marked genomic instability is a hallmark of the disease and contributes to elevated proteotoxic stress^[8]. The high frequency of genomic mutations may confer a survival advantage by enabling MM cells to quickly adapt to stresses in the environment. However, this comes at a cost. This deregulation of gene expression results in the accumulation of toxic misfolded proteins that exerts additional stress on MM cells^[9-13]. Furthermore, MM are highly secretory cells, characterized by staggering rate of synthesis of clonal immunoglobulins which further contributes to baseline ER stress. Therefore, protein quality control pathways are essential for MM survival^[8].

AUTOPHAGY

Autophagy is a tightly regulated self-digestion mechanism that promotes the lysosomal degradation of organelles, intracellular pathogens, and misfolded proteins. It is a key cellular mechanism to maintain homeostasis and guarantee energy supply as products of autophagic digestion can be re-utilized in anabolic processes^[14-16]. Therefore, nutrient and energy deprivation, ER stress, and hypoxia can all induce autophagy as a means to enable cell survival^[17]. In mammalian cells, there are three main types of autophagy, namely macroautophagy, microautophagy, and chaperone-mediated autophagy^[15].

Macroautophagy

Macroautophagy is a type of autophagy that delivers cellular contents to the lysosome via the formation of double-membrane structures called autophagosomes which then fuse with lysosomes to form autolysosomes^[18,19]. Macroautophagy can be subdivided into non-selective (bulk) and selective autophagy^[16]. During non-selective autophagy, bulk cytoplasm is randomly engulfed by a phagophore [Figure 1]. Notably, the mammalian target of rapamycin (mTOR) pathway is a key inhibitor of autophagy^[20]. Subsequently, the phagophore matures into an autophagosome and this process is mediated by autophagy-related protein 7 (ATG7), ATG8 (LC3), and ATG12^[21,22]. ATG7 functions as an E1-like enzyme by binding and activating ATG12 and ATG8 to facilitate the transfer of ATG12 to ATG5 via the E2 enzyme ATG10^[23-27]. The resultant ATG12-ATG5 conjugate forms a large multimeric complex together with ATG16 (ATG12-ATG5-ATG16) which acts as an E3 ligase to facilitate phosphatidylethanolamine (PE) and LC3 conjugation and conversion of LC3-I to LC3-II. LC3-II stably associates with the autophagosome membrane and regulates autophagic membrane expansion, recognition of autophagic cargo, and autolysosome formation^[21,22]. Finally, autophagosome and lysosome fusion occurs and the autophagic cargo is degraded by lysosomal hydrolases^[21,22]. Selective

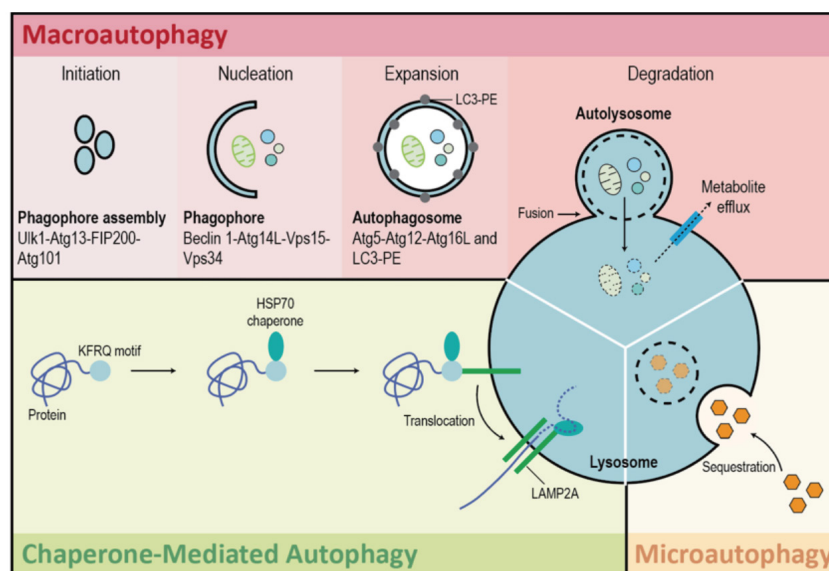


Figure 1. Autophagy. There are three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy is a type of autophagy that delivers cellular contents to the lysosome via the formation of double-membrane structures called autophagosomes which then fuse with lysosomes to form autolysosomes. Macroautophagy takes place in five main steps. Initiation of autophagy occurs in response to metabolic or therapeutic stress and is mediated by ULK1, ATG13, FIP200, and ATG101. During the nucleation step regulated by BECLIN-1, ATG14L, VPS15, and VPS34, the formation of the phagophore occurs. Expansion results in the sequestration of cytosolic contents within the autophagosome and is facilitated by ATG5, ATG12, ATG16L, and LC3-PE. Degradation is the breakdown of autophagosomal contents upon formation of the autolysosome (fusion of autophagosome and lysosome). Microautophagy is a largely non-selective process that facilitates the direct uptake and breakdown of cytosolic cargo by lysosomes. Chaperone-mediated autophagy refers to the chaperone-dependent targeting of specific cytosolic proteins to lysosomes for proteolysis. HSC70 binds to the consensus motif of specific proteins to target them to the lysosome-associated membrane protein type 2A (LAMP-2A) receptor on the lysosomal membrane. Once internalized by the lysosome, these cytosolic proteins are degraded

macroautophagy, on the other hand, specifically targets damaged or redundant organelles such as mitochondria (mitophagy), peroxisomes (pexophagy), ribosomes (ribophagy), aggresomes (aggrephagy), etc.^[28]. Specifically, mitophagy is the selective degradation of mitochondria by macroautophagy in a PTEN-induced kinase 1 (PINK1)- and Parkin-dependent fashion^[29,30]. Type 1 mitophagy sequesters and removes mitochondria in response to nutrient deprivation, whereas type 2 mitophagy removes damaged mitochondria^[31]. Type 3 mitophagy (micromitophagy), on the other hand, eradicates damaged mitochondrial components through the formation of mitochondria-derived vesicles that are subsequently degraded by lysosomes^[31].

Microautophagy

In eukaryotic cells, microautophagy is a largely non-selective process that facilitates the direct uptake and breakdown of cytosolic cargo by lysosomes. Specifically, cytosolic material is sequestered by direct invagination of the vacuolar/lysosomal membrane, forming autophagic tubes that pinch off into the lysosomal lumen^[32-34].

Chaperone-mediated autophagy

Chaperone-mediated autophagy (CMA) refers to the chaperone-dependent targeting of specific cytosolic proteins to lysosomes for proteolysis^[35-37]. This is a mechanistically distinct process that occurs only in mammalian cells. Unlike the other types of autophagy, CMA does not require the formation of vesicles^[37]. Instead, HSC70 binds to the consensus motif of specific proteins to target them to the lysosome-associated membrane protein type 2A receptor on the lysosomal membrane^[35-37]. Once bound, the targeted proteins start to unfold as they are internalized into the lysosomal lumen and then degraded^[35-37].

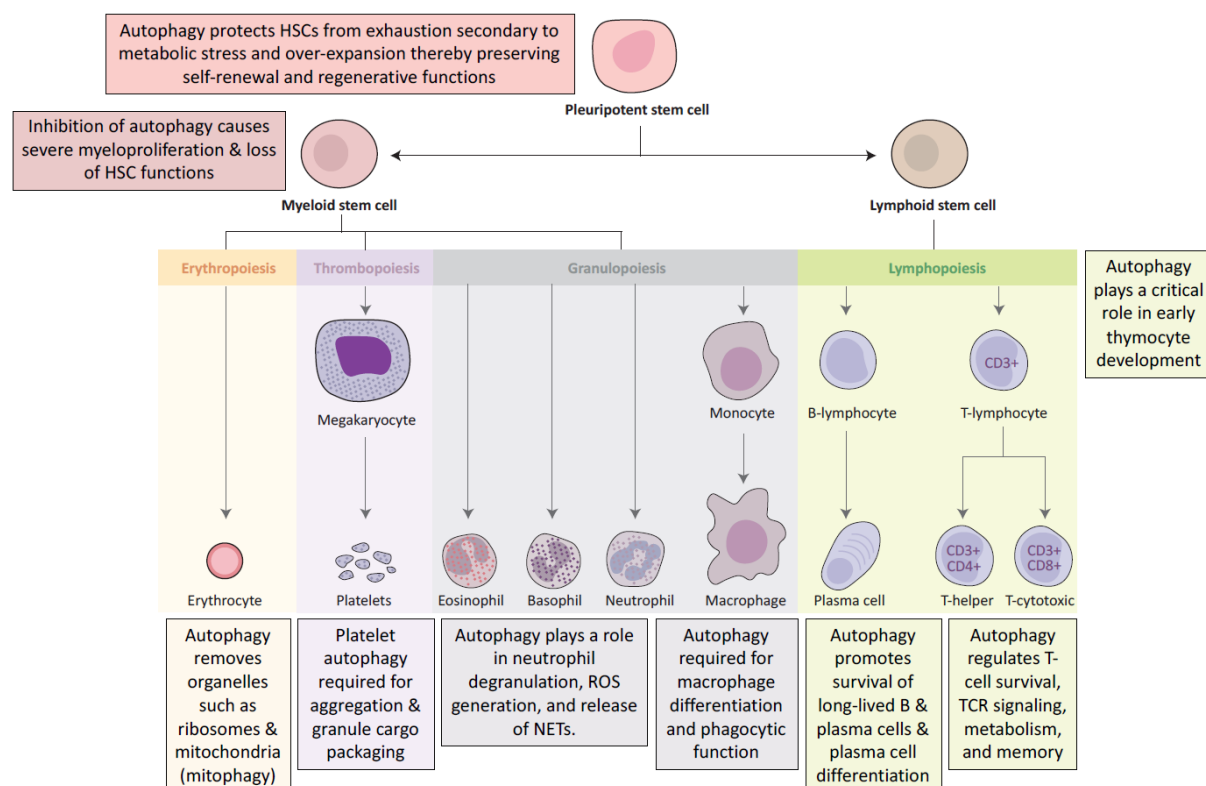


Figure 2. Examples of key functions that autophagy plays in the maintenance and differentiation of hematopoietic stem cells. Image adapted from First Aid for the USMLE Step 1^[63]

IMMUNE FUNCTIONS OF AUTOPHAGY

Autophagy as an immune effector

Autophagy plays a key role in eukaryotic cells as a stress response mechanism activated to recycle intracellular organelles in the face of nutrient deprivation^[38]. However, advances in our understanding of autophagy reveal also an intricate reciprocal relationship between autophagy and immunity^[38]. Specifically, autophagy acts as an immune effector by: (1) facilitating the direct elimination of microbes through xenophagy and LC3-associated phagocytosis^[39]; (2) modulating the inflammatory response by amplifying PAMP-TLR signaling whilst inhibiting type I IFN signaling and inflammasome activation^[40]; (3) enhancing MHC class II-mediated antigen presentation and cross-presentation^[39,40]; and (4) contributing to secretion of pro-inflammatory cytokines such as IL-6, which could be relevant to MM pathogenesis^[39].

Autophagy in the maintenance of hematopoiesis

Apart from regulating immune effector functions, autophagy is a key adaptive mechanism critical to the maintenance of hematopoietic hemostasis [Figure 2]^[41]. Hematopoietic stem cells (HSCs) thread a tight, but dynamic and demand-adapted, balance between quiescence and proliferation throughout the entire life of the organism^[41]. Studies have shown that autophagy protects HSCs from exhaustion secondary to metabolic stress^[41-43]. Firstly, basal autophagy removes activated mitochondria, regulates the metabolism, and maintains the self-renewal and regenerative capabilities of HSCs^[44]. Research shows that conditional deletion of ATG12 in transplanted murine HSCs severely impairs their ability for BM engraftment and self-renewal^[45]. Secondly, when subjected to *ex vivo* cytokine withdrawal or *in vivo* nutrient deprivation, HSCs robustly upregulate autophagy to circumvent an energy crisis to ensure HSC longevity^[43].

Deletion of ATG7, a critical component of autophagosome formation, in murine HSCs results in accumulation of dysfunctional mitochondria, upregulation of oxidative stress and DNA damage, and ultimately cell death^[46]. Interestingly, aged HSCs were found to maintain a low metabolic state by upregulating autophagy in order to sustain robust long-term self-renewal potential comparable to young HSCs^[44]. Downstream of HSCs, autophagy also plays a key role in the development, differentiation, and function of erythrocytes, platelets, granulocytes, macrophages, and T cells as summarized in [Figure 2](#)^[41].

Since malignant transformation of HSCs or early progenitors results in leukemia, homeostatic mechanisms; such as autophagy, that protect HSCs from metabolic, oxidative, and genotoxic stress; are crucial to prevent hematopoietic malignancies^[47]. Indeed, ATG7 deletion in myeloid cells results in dysregulated and invasive myeloproliferation resembling acute myeloid leukemia^[42].

Autophagy in plasma cell ontogeny

Autophagy plays a key role in plasma cell (1) differentiation; (2) survival; and (3) protein quality control.

Autophagy in plasma cell differentiation and survival

B lymphocyte to plasma cell differentiation is controlled by a complex genetic reprogramming system leading to downregulation of genes involved in the maintenance of B-cell identity [e.g., paired box protein 5 (PAX5), transcription regulator protein BACH2 (BACH2), B-cell lymphoma protein 6 (BCL6)] and upregulation of genes involved in terminal differentiation of Ig-secreting plasma cells [e.g., B-lymphocyte-induced maturation protein 1 (BLIMP-1), interferon regulatory factor 4 (IRF4), and X-box binding protein 1 (XBP1)]^[48]. Specifically, BLIMP-1 acts as a molecular switch to repress PAX5 and BCL6, and induces XBP1 to promote antibody production and plasma cell differentiation^[49,50]. Interestingly, while BLIMP-1 and IRF4 are essential for plasma cell differentiation, only IRF4 is essential for plasma cell survival^[51]. Consistent with this, BLIMP-1 deficient plasma cells remained viable and retained their transcriptional identity but lose the ability to secrete Ig^[51].

Beyond epigenetics, autophagy also plays an essential role in plasma cell differentiation and survival [\[Figure 3\]](#). Studies have found increased expression of autophagic genes in differentiating plasma cells. Conditional deletion of ATG5 in murine B cells results in reduced IgM and IgG responses in the setting of both T-cell dependent and independent immunizations; further suggesting that autophagy is required for B lymphocyte to plasma cell differentiation^[52]. Notably, ATG5 was also essential for the homing and/or survival of long-lived plasma cells in the BM^[52]. Consistent with this, long-lived plasma cells were found to highly express autophagic genes and display high basal levels of autophagy^[53].

Autophagy as a mechanism of protein quality control

Plasma cells are professional antibody secreting cells (ASCs) uniquely optimized towards large-scale immunoglobulin synthesis, folding, assembly, and secretion^[54]. Not unique to plasma cells, however, is the fact that the protein synthesis process is intrinsically error prone. In fact, up to 30% of newly-synthesized proteins are defective and need to be degraded^[47,54]. Thus, an intricate balance between protein synthesis, folding, and clearance must be maintained to prevent the accumulation of potentially toxic misfolded proteins^[47,54]. This is especially crucial for ASCs that cope with increased Ig synthesis by upregulating folding capacity through the induction of unfolded protein response (UPR)-driven ER expansion^[11]. However, when Ig synthesis exceeds folding capacity, the integrity of the proteome is preserved through an interconnected network of protein quality control pathways which include the proteasome, autophagy, aggresome, and UPR pathways^[47]. These pathways are so important to plasma cells that the amount of newly-synthesized proteins degraded by the proteasome is 15-folds higher in plasma cells compared to resting B-cells^[55]. Perhaps not surprisingly then, that MM, a cancer of plasma cells, exhibits the same reliance on the protein quality control as evidenced by the clinical efficacy of proteasome inhibitors (PI).

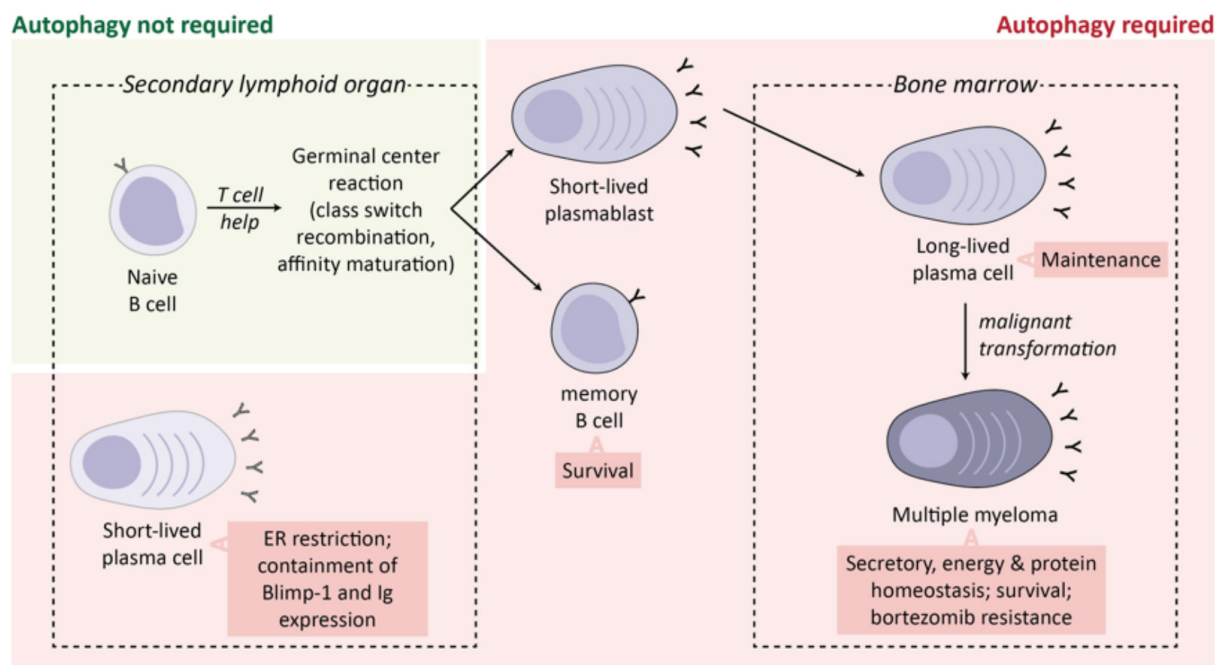


Figure 3. Role of autophagy in plasma cell ontogeny and malignancy. Differentiating plasma cells and MM cells induce autophagy to restrict the ER and downregulate BLIMP-1 expression to decrease immunoglobulin synthesis and deal with excessive proteotoxic stress. Autophagy is also essential for the survival and maintenance of memory B cells and BM long-lived plasma cells. In MM, induction of autophagy has been linked with bortezomib resistance. Image adapted from Milan *et al.*^[60]

Indeed, sensitivity of MM to PI is determined by a combination of Ig secretory load, protein degradation capacity, and commitment to plasma cell maturation; which, taken together, suggests that being an ASC confers this unique susceptibility to MM^[56-59].

While autophagy does not directly dispose of misfolded immunoglobulins, plasma cells deficient in autophagy had greater energy imbalance, enhanced immunoglobulin synthesis, reduced intracellular ATP, and elevated ER stress^[52]. Consistent with this, autophagy-deficient plasma cells displayed higher expression of BLIMP-1 and XBP1 with corresponding increase in ER size [Figure 3]^[52]. These findings suggest that autophagy not only facilitates the acquisition of an Ig-secretory phenotype in the in pre-plasma cells, but also serves to limit the overproduction of Ig and maintain cellular ATP levels to ensure survival in differentiated plasma cells [Figure 3]^[60]. Autophagy also plays an important role in the removal of misfolded protein aggregates which, due to steric hindrance, are unable to be efficiently degraded by the proteasome^[61]. Failure to dispose of misfolded protein aggregates results in proteotoxic stress and induction of terminal ER stress^[62].

AUTOPHAGY IN MM

Autophagy is necessary for the differentiation and maintenance of antigen-specific long-lived plasma cells, the physiologic counterpart to MM cells. As MM cells retain many characteristics of the original plasma cell clone such as Ig secretion and an enlarged cytoplasm and ER, it is reasonable to hypothesize that autophagy might also play a role in MM proteostasis and survival.

Indeed, MM cells exhibit higher levels of basal autophagy compared to other tumors, including lymphoma derived from earlier B-cell progenitors, and autophagy is necessary for the survival of MM cells^[64]. Disruption of autophagy through BECLIN-1 knockdown or pharmacologic inhibition (with 3-methyladenine and/or chloroquine) causes MM cell apoptosis^[65,66]. Basal autophagy is hypothesized to

alleviate proteotoxic stress and promote MM survival by (1) limiting the secretion of immunoglobulins and (2) providing an alternative proteolytic pathway for the clearance of ubiquitinated proteins through a p62-dependent mechanism^[60]. Despite this, concomitant inhibition of the proteasome and autophagy in pre-clinical studies have reported inconsistent results, ranging from synergism to antagonism^[60]. This may partly be explained by the observation that while basal autophagy is protective, persistent and uncontrolled autophagy results in autophagic cell death^[67,68]. Autophagic cell death in MM can be induced by caspase-10 or caspase-8 inhibition^[67,68]. Specifically, caspase-10 cleaves and inhibits BCL2 associated transcription factor 1, a BECLIN-1 activator, to temper the autophagic response and avoid cell death^[67]. MM cells therefore need to tightly regulate autophagy to maintain viability as dysregulation either way leads to deleterious effects in MM.

Mitophagy has been reported to play conflicting roles in carcinogenesis, survival, and drug-resistance. Some studies report that mitophagy protects untransformed cells from excessive reactive oxidative species damage and genetic instability, and that suppression of mitophagy favors carcinogenesis^[69,70]. Conversely, other studies suggest that mitophagy promotes cancer cell survival and drug resistance by protecting cells from apoptosis^[71,72]. In MM, while the suppression of mitophagy is associated with bortezomib resistance; doxorubicin, a widely used anti-MM chemotherapy, is a classical inhibitor of mitophagy^[73]. This dichotomy highlights the need for a better understanding of the role mitophagy plays in MM.

DNA damage and autophagy

Apart from ongoing proteotoxic stress, other hallmarks of MM are genetic instability and abnormalities (e.g., aneuploidy, translocations), and oxidative stress which lead to replicative stress and constitutive DNA damage^[74,75]. Recent studies have shown that DNA damage can activate autophagy through a number of interconnected pathways^[76]. Examples of DNA damage repair (DDR) pathway proteins that regulate autophagy are ataxia telangiectasia-mutated (ATM), poly (ADP-ribose) polymerase 1 (PARP1), c-Jun N-terminal kinase (JNK), and p53^[76]. Specifically, autophagy can be induced by ATM-mediated phosphorylation of AMP-activated protein kinase (AMPK)^[77,78]. AMPK in turn activates both tuberous sclerosis complex 2 (TSC2), to remove the inhibitory effect of mTOR complex 1 (mTORC1) on autophagy, and unc-51 like autophagy activating kinase 1 (ULK1) to promote autophagosome formation^[77-79]. ATM also phosphorylates and activates CHE-1 which in turn upregulates the transcription of two mTOR inhibitor genes [regulated in development and DNA damage responses 1 (REDD1) and DEP domain-containing mTOR-interacting protein (DEPTOR)]^[80]. Nuclear factor-kappa B, a well-known pro-myeloma transcription factor, can be activated by ATM and as a result, upregulate BECLIN-1^[81]. Induction of PARP1 promotes autophagy via AMPK activation on the background of ATP depletion and elevated AMP levels^[82]. DNA damage-induced JNK phosphorylates BCL-2 resulting in BCL-2 dissociation, relief of BECLIN-1 inhibition, and induction of autophagy^[83]. Nuclear p53 activates autophagy through a number of distinct signaling pathways. Firstly, p53 upregulates phosphatase and tensin homolog (PTEN) which leads to PI3K-Akt-mTORC1 inhibition and autophagy induction^[84,85]. Secondly, p53 influences AMPK activity (1) directly through transcriptional upregulation, and (2) indirectly by activating Sestrin1 and Sestrin2 which in turn activate AMPK^[85,86]. Thirdly, death-associated protein kinase (DAPK) is transcriptionally upregulated by p53 and triggers autophagy by phosphorylating BECLIN-1 to facilitate its dissociation from BCL-2 and BCL-X_L^[87-89]. DAPK also phosphorylates protein kinase D which activates the VPS34 class III PI3K complex leading to induction of autophagy^[87-89]. Lastly, p53 also upregulates damage-regulated autophagy modulator, a lysosomal protein involved in the degradation step of autophagy^[90]. However, unlike nuclear p53, cytoplasmic p53 can also activate mTOR to inhibit autophagy^[91]. Functionally, autophagy is essential for homologous recombination and nucleotide excision repair and cells deficient in autophagy rely chiefly on the error-prone non-homologous end joining repair pathway, which may explain the genomic instability observed in autophagy-deficient cells^[92-98].

EXPLOITING AUTOPHAGY IN MM

Preclinical studies of autophagy modulators in MM

HDAC6 inhibitors

Histone deacetylases (HDACs) catalyze the removal of acetyl groups on lysine residues in target proteins^[99]. Furthermore, HDACs also deacetylate non-histone proteins, thereby providing an additional layer of control over protein function, stability, and protein-protein interaction^[99]. HDAC6 is a class IIb HDAC that is mainly localized to the cytoplasm, unlike class I and IIa HDACs which shuttle between the cytoplasm and nucleus, suggesting that HDAC6 functions mainly to regulate non-histone proteins^[99]. Indeed, HDAC6 possess intrinsic ubiquitin-binding activity and co-localizes with the microtubule network to transport misfolded polyubiquitinated proteins to aggresomes/autophagosomes for subsequent lysosomal degradation^[99-101]. HDAC6 therefore promotes ubiquitin-dependent or ubiquitin-independent aggresome formation and while HDAC6 is not required for the initiation of autophagy per se, it is necessary for the targeted delivery of the autophagy machinery to aggresomes^[62,100-105]. Importantly, HDAC6 also promotes the formation of an F-actin network essential for autophagosome-lysosome fusion^[105]. The development of HDAC6 inhibitors therefore presents an opportunity to exploit autophagy to destabilize protein homeostasis in MM.

Preclinical studies have shown that the HDAC6-selective inhibitors WT161 and Tubacin trigger the accumulation of acetylated tubulin of and inhibits MM cell growth *in vitro*^[106,107]. Additionally, combinatory treatment using WT161 or Tubacin with the PI bortezomib not only induces synergistic cytotoxicity but was also able to overcome bortezomib resistance^[106,107]. Mechanistically, the addition of WT161 to bortezomib resulted in further accumulation of polyubiquitinated proteins which led to a further increase in ER stress signaling and the UPR, evidenced by the upregulation of ATF4 and the pro-apoptotic protein CHOP^[106]. Interestingly, the ER stress sensor proteins inositol-requiring enzyme 1 (IRE1 α) and PERK-like endoplasmic reticulum kinase (PERK) were found to be downregulated by WT161, suggesting that HDAC6 inhibition also suppresses the UPR, preventing it from functioning as an ER stress mitigator^[106].

Another HDAC6-selective inhibitor, ACY-1215, demonstrated synergistic cytotoxicity when used in combination with carfilzomib, an irreversible second generation PI^[108]. The addition of ACY-1215 resulted in increased LC3-II consistent with the disruption of autophagic flux secondary to HDAC6 inhibition^[108]. Mechanistically, the addition of ACY-1215 to carfilzomib inhibited both aggresome formation and autophagosome-aggresome association^[108]. Consistently, a novel HDAC6 inhibitor MPToG413 was shown to both disrupt bortezomib-induced aggresome formation and exhibit synergism with bortezomib^[109]. The combination of MPToG413 and bortezomib could also overcome cell adhesion-mediated drug resistance in the MM-BMSC co-culture setting^[109]. MPToG413 was further found to decrease MM-BMSC adhesion and downregulate the pro-myeloma cytokines VEGF and IL-6 in the context of the MM BM microenvironment^[109].

Autophagy inhibitors + DNA-damaging chemotherapy

DNA damaging agents such as melphalan are highly cytotoxic to MM cells and are a mainstay of treatment in MM, particularly as a conditioning regimen ahead of autologous hematopoietic stem-cell transplantation^[110]. Consistent with the aforementioned link between DDR and autophagy, a recent study found that melphalan and doxorubicin induce cytoprotective autophagy as a pro-survival mechanism in MM cells through the upregulation of BECLIN-1-dependent autophagosomes^[111]. knockdown of autophagy genes (BECLIN-1 and ATG5) and pharmacologic inhibition of autophagy (via hydroxychloroquine and 3-methyladenine) significantly enhanced the cytotoxicity of melphalan and doxorubicin *in vitro* and *in vivo*^[111].

High mobility group protein B1 (HMGB1) is a critical regulator of autophagy that is often upregulated in MM^[112,113]. Specifically, HMGB1 binds competitively to BECLIN-1 causing BCL-2 displacement and

autophagy induction^[112]. A study found that HMGB1 overexpression in MM was associated with mTOR inhibition, autophagy induction, and reduced sensitivity to dexamethasone^[113]. Conversely, knockdown of HMGB1 increased apoptosis in MM cells exposed to dexamethasone^[113].

HMGB1 has also been recognized a marker of induction of immunogenic cell death (ICD). ICD is triggered by exposure to certain cytotoxic agents (e.g., anthracyclines, oxaliplatin, bortezomib)^[114,115]. ICD involves the release of soluble mediators along with alterations in the cancer cell surface composition in a way that converts dying cancer cells into therapeutic vaccines that are phagocytosed by antigen presenting cells (APCs) for the cross-priming of CD8⁺ T-cells to stimulate anti-tumor specific T-cell immune responses^[114,115]. HMGB1 is a nuclear nonhistone chromatin-binding protein secreted by dying tumor cells exposed to cytotoxic agents that binds to toll-like receptor 4 on APCs to increase the rate of antigen processing and presentation by APCs to T cells^[116]. Interestingly, studies have shown that autophagy in tumor cells regulates HMGB1 secretion and that inhibition of autophagy leads to intracellular sequestration of HMGB1 and the induction of caspase-mediated cell death^[117]. Macroautophagy therefore enhances antigen cross-priming after ICD which results in the following conundrum. From a cytotoxicity point of view, it makes sense to combine DNA-damaging agents with autophagy inhibitors. However, from an ICD perspective, the addition of autophagy inhibitors to DNA-damaging agents may be counterproductive to antigen cross-priming.

Autophagy modulators + PI

Bortezomib, a first-in-class PI, was approved for use in MM by the FDA in 2003 and has become a mainstay of therapy at every disease stage ever since. Despite significant clinical efficacy in most naïve patients, most of the patients ultimately become refractory to bortezomib therapy^[118-120]. This has prompted the development of second-generation PIs (e.g., carfilzomib and ixazomib) currently in clinical use for relapsed or refractory MM; and next-generation PIs (e.g., marizomib and oprozomib) currently in advanced clinical trials^[121]. Based on evidence that susceptibility to PI depends on the ability of MM cells to remove misfolded proteins through protein quality control pathways, novel therapeutic approaches targeting alternative pathways in protein homeostasis are also actively being studied^[122].

Research has shown that crosstalk exists between the proteasome, UPR, and autophagy^[65,123,124]. Specifically, whenever the proteasome is overwhelmed and/or inhibited, polyubiquitinated and misfolded proteins co-aggregate in the cytosol to form aggresomes which are then degraded through macroautophagy^[101,125,126]. Mechanistically, proteasome inhibition induces ER stress and PERK-eIF2 α and IRE1-JNK activation, leading to the induction of autophagy^[127,128]. Other mechanisms of bortezomib resistance, in the context of autophagy, include the upregulation of PROFILIN-1 which enhances autophagy through BECLIN-1 interaction and increased expression of CIC5, a chloride channel that enhances bortezomib-induced autophagy via AKT-mTOR inhibition^[129]. PIs also rapidly induce the expression of SQSMT1/p62 to upregulate p62-dependent autophagy to compensate for proteasome insufficiency^[64].

Along the same line, preclinical studies have reported synergistic cytotoxicity with dual blockade of the proteasome and autophagy in MM. MG132, a PI, induced cytoprotective autophagy that can be inhibited, by 3-methyladenine, to enhance apoptotic cell death^[128]. Notably, 3-methyladenine resulted in the accumulation of polyubiquitinated proteins and exacerbation of ER stress in cells treated with MG132^[128]. Autophagy inhibitors that have demonstrated synergy and enhanced MM cytotoxicity with bortezomib *in vitro* include Elaiphyllin (macrolide antibiotic) and Metformin^[130,131]. Elaiphyllin, in particular, had significant cytotoxic activity even when used alone in mutant p53 MM^[130]. Metformin, on the other hand, inhibits GRP78 which is crucial mediator of bortezomib-induced protective autophagy^[131].

However, other conflicting studies have also shown that Metformin inhibits MM proliferation by inducing autophagy (and cell cycle arrest) through AMPK activation and dual repression of mTORC1 and

mTORC2^[132,133]. In line with this, it has been reported that when used in combination with bortezomib, the autophagy inhibitors 3-methyladenine or chloroquine can also have an antagonistic effect^[65]. One potential explanation could be that bortezomib induces apoptosis partially through autophagic cell death. Consistent with a role for autophagic cell death in MM cells treated with bortezomib, a novel SCF (Skp2) inhibitor CpdA stabilizes p27 to induce caspase-independent autophagic cell death in MM cells resistant to bortezomib; and also synergized with bortezomib^[134]. Another compound, betulinic acid (BetA), activates protein phosphatase 2A (PP2A) to trigger DAPK-dependent autophagic cell death in MM cells with high BCL-2 expression^[135].

Autophagy inhibitors have also shown potency in bortezomib and carfilzomib-resistant MM cells^[136]. The non-selective HDAC inhibitor SBHA upregulates the BH3-only protein BIM, which in turn sequesters BECLIN-1 to inhibit cytoprotective autophagy, thereby overcoming acquired bortezomib resistance^[136]. Chloroquine potentiated carfilzomib cytotoxicity and was able to overcome carfilzomib resistance *in vitro*^[137]. Lastly, pharmacologic inhibition of thioredoxin with PX12 upregulates mitophagy to re-sensitize bortezomib-resistant cells to bortezomib^[73]. Combination treatment of MM cells with PX12 and bortezomib led to synergistic toxicity and inhibition of ERK1/2 and mTOR signaling, suggesting the involvement of ERK1/2 and mTOR in mitophagy suppression^[73].

PI3K-AKT-mTOR inhibitors

While activating mutations in PI3K and AKT have not been reported in MM, the PI3K-AKT-mTOR pathway is commonly activated in MM through juxtacrine and paracrine signaling within the MM tumor microenvironment^[138,139]. Interactions between MM and the stromal and endothelial compartments result in the secretion of IL-6, VEGF, and IGF-1, which in turn activate pro-survival and proliferative pathways such as PI3K-AKT-mTOR, JAK/STAT3, NFκB, and MEK/ERK^[139]. In line with this, efforts to inhibit PI3K-AKT-mTOR signaling have led to the study of mTORC1 inhibitors (e.g., rapamycin) in MM. However, results from preclinical studies were disappointing as rapamycin and the other rapalogs demonstrated a cytostatic, but not cytotoxic, response in MM^[140]. This lack of potency could be due, in part, to the activation of cytoprotective autophagy. Nonetheless, mTORC1 inhibitors have shown synergistic activity in other cancers when used in combination with clinically approved anti-MM agents such as dexamethasone, lenalidomide, and panobinostat, and pre-clinical agents such as sorafenib (tyrosine kinase inhibitor), 17-AAG (HSP90 inhibitor), NVP-AEW541 (IGF1R inhibitor), and MK2206 (AKT inhibitor)^[141-148].

Upstream of mTOR, AKT inhibitors have also been studied in MM. In preclinical studies, perifosine, an AKT inhibitor, was cytotoxic to MM cells as a single-agent and also synergized with bortezomib, dexamethasone, doxorubicin, melphalan, and U0126 (MEK1/2 inhibitor)^[149]. Another AKT inhibitor TAS-117 enhanced ER stress and MM apoptosis when added to bortezomib or carfilzomib^[150]. These studies once again highlight the duality of autophagy in MM, suggesting that excessive induction of autophagy, rather than protecting cells from excessive ER stress, pushes cells towards autophagic cell death.

The next class of PI3K-AKT-mTOR pathway inhibitors are the dual PI3K-mTOR inhibitors. One such inhibitor, BEZ235, demonstrated good preclinical single-agent activity in MM and also synergized with bortezomib, doxorubicin, and melphalan^[151,152]. Another study reported a pro-survival function of autophagy in MM cells treated with PI-103, a competitive dual PI3K and mTOR inhibitor, which inhibits the proteasome, induces UPR, and upregulates autophagy^[153]. Importantly, Bafilomycin-A, an autophagy inhibitor, enhanced apoptosis in cells treated with PI-103^[153].

Heat-shock protein inhibitors

Heat shock proteins are molecular chaperones that play indispensable roles in protein folding/unfolding, multiprotein complex assembly, and protein sorting^[154]. As a function of protein sorting, HSP70 and HSP90

also participate in chaperone-mediated autophagy^[155-157]. Heat-shock proteins (HSPs) therefore help alleviate proteotoxic stress to prevent apoptosis in MM^[158]. Consistent with this, HSP70 and/or HSP90 inhibition induces UPR and apoptosis in MM^[159-161]. In preclinical studies, combination of HSP90 inhibitors KW-2478, Retaspimycin, and 17-AAG with bortezomib demonstrated synergistic cytotoxicity^[162-164]. Another HSP90 inhibitor, NVP-HSP990, displayed potent, *in vitro* anti-myeloma activity and synergism with melphalan, HDAC inhibitors, and PI3K/mTOR inhibitors^[165,166]. Other HSP90 inhibitors such as PU-H71, SNX5422, and NVP-AUY922 have also shown promising pre-clinical results in MM^[158,167-169]. Apart from HSP90, HSP70 has recently emerged as a promising therapeutic target in MM and a number of HSP70 inhibitors (e.g., PET-16, Ver-155008, MAL3-101) have shown good pre-clinical anti-myeloma activity^[160,161,170,171].

Interestingly, treatment of MM cells with HSP90 inhibitors (e.g., 17-AAG, NVP-AUY922), bortezomib, or dexamethasone results in compensatory upregulation of HSP70 which confers a degree of drug-resistance and protects MM cells from apoptosis^[172,173]. HSP70 is a molecular chaperone of HSP90. Consequently, inhibition of HSP70 leads to the downregulation of HSP90 while inhibition of HSP90 results in upregulation of HSP70^[160,171,174]. It has been shown however, that simultaneous inhibition of both HSP70 and HSP90 leads to greater MM cytotoxicity compared to inhibiting HSP90 alone^[160,171,174]. To this end, there has been increasing interest in developing inhibitors against heat shock factor 1 (HSF), the “master regulator” of the heat shock response that controls the expression of both HSP90 and HSP70^[158]. In preclinical studies, several novel HSF1 inhibitors (e.g., CCT251236, KRIBB11) were found to induce MM cell death that was associated with the induction of the UPR^[175].

Autophagy modulators currently in clinical trials

Several clinical trials have highlighted the potential role of autophagy modulators in the treatment of MM. For the purpose of consistency while discussing these trials, overall response rate (ORR) is defined as a partial response (PR) or better, and clinical benefit rate (CBR) is defined as stable disease (SD) or better.

Hydroxychloroquine/Chloroquine

Hydroxychloroquine (HCQ)/Chloroquine (CQ) have been clinically studied for their potential role in inhibiting autophagy. Although the exact mechanism of HCQ/CQ has not been elucidated, it is thought to function by alkalinizing intracellular compartments which disrupts the autophagic proteolytic process^[176]. HCQ/CQ have been extensively studied in myeloma to potentiate the effects of other anti-myeloma drugs, particularly PI.

A phase I clinical trial assessed the efficacy of HCQ in combination with bortezomib in patients with relapsed or refractory MM^[177]. Patients were given a 2-week run in with HCQ alone, followed by combination therapy with bortezomib. The combination of HCQ and bortezomib was well tolerated, with no adverse events meeting the criteria of a dose-limiting toxicity. Of the 22 patients assessed at the end of the study, 13.6% had a very good partial response (VGPR), 13.6% had minimal response (MR), and 45.5% had stable disease (SD). Interestingly, all of the patients who had a VGPR were bortezomib-naïve. Twenty-seven percent of patients in the study had progressive disease (PD). Of these, two thirds were bortezomib-refractory. This study also found a therapy-associated increase in autophagic vesicles in BM plasma cells. However, due to the small sample size, the authors were unable to correlate number of autophagic vesicles with clinical response.

A smaller phase II trial looked at CQ in combination with bortezomib and cyclophosphamide in refractory MM^[178]. Of the 11 patients enrolled, 8 completed at least 2 cycles and were assessed for clinical response. The most common side effects included fatigue, constipation, myalgia, anorexia, anemia and thrombocytopenia, but was generally well tolerated. 37.5% of patients achieved a PR with a median duration of response of 4 months. One patient (12.5%) had SD, and 50% of patients experienced PD, with

an overall clinical benefit rate of 40%. These studies demonstrate that HCQ/CQ are well tolerated in combinations with PI and may help potentiate the effects of existing myeloma therapies.

A recent study looked at dual autophagic inhibition via HCQ and rapamycin with cyclophosphamide and dexamethasone in patients with relapsed or refractory MM based on their preclinical, synergistic anti-tumor activity^[179,180]. The quadruple therapy was generally well-tolerated with one case of hematologic dose limiting toxicity (thrombocytopenia) occurring at a HCQ dose of 800 mg, which was then established as the maximum tolerated dose. Of the 18 patients enrolled in the trial, 1 had a VGPR and 3 had a PR, for an ORR of 22%. Seven patients had MR, and 5 had SD, for a CBR of 89%. Median duration of a response was 4.5 months, and median time to best response was 1.9 months. Two patients had immediate progression of disease. Unfortunately, correlative studies were unable to predict the depth of treatment response based on the number of autophagic vesicles in BM-derived MM cells. The promising results of this study warrant further investigation of dual autophagy modulation in MM.

HDAC6 inhibitors

HDAC inhibitors represent an important new group of anti-cancer drugs. There are 18 different isoforms of HDACs in human cells that are subdivided into 4 classes based on subcellular localization and non-cell-based enzymatic activity^[181]. Clinical studies with pan-HDAC inhibitors vorinostat and panobinostat outlined a narrow therapeutic window due to frequent and often severe hematologic and non-hematologic (particularly gastrointestinal/GI) toxicities, eliciting clinical interest in isoform-specific HDAC inhibition^[182].

Ricolinostat is an oral, selective HDAC6 inhibitor that has been extensively studied in MM. A phase 1b multicenter trial of escalating doses of ricolinostat with lenalidomide and dexamethasone in patients with relapsed or refractory MM showed 55% ORR^[182]. In lenalidomide-naïve and lenalidomide-sensitive patients, the ORR was 69%, compared to 25% in lenalidomide-refractory patients. In patients that responded, the median time to response was 7 weeks with a median duration of response of 24 months. Treatment with ricolinostat was overall well tolerated with 2 patients experiencing a dose-limiting toxicity at the highest tested dose which included syncope and myalgia.

Similarly promising results emerged from a phase 1/2 clinical trial assessing the safety and efficacy of ricolinostat with bortezomib and dexamethasone in patients with MM relapsed or refractory to PI, immunomodulatory drugs, or stem cell transplant^[183]. In this study, 15 patients were recruited for dose escalation monotherapy with ricolinostat, and 57 patients were given combination therapy. Monotherapy with ricolinostat was well tolerated with no dose-limiting toxicities up to 360 mg Q.D. Patients on combination therapy did not have any dose-limiting toxicities during escalation studies. Most common side effects associated with combination therapy were gastrointestinal toxicities, cytopenia, and fatigue. Of the patients treated with monotherapy, 6 patients (6/15, 40%) had stable disease with a median response duration of response of 11 weeks. Patients on combination therapy had an ORR of 29%, and a clinical response rate of 39%. Interestingly, patients on combination therapy who were previously refractory to bortezomib had an ORR of 14%. The response seen in patients with bortezomib-refractory MM suggests that HDAC6 inhibitors can overcome resistance to PI.

Taken together, these results demonstrate a promising role for HDAC6 inhibitors in the treatment of relapsed and/or refractory MM. There are currently ongoing trials assessing the role of HDAC6 inhibitors in combination with anti-myeloma treatments such as pomalidomide and dexamethasone (NCT01997840, NCT02189343).

HSP90 inhibitors

A phase 2 clinical trial looked at the HSP90 inhibitor tanespimycin in combination with bortezomib in patients with relapsed and/or refractory MM^[184]. All patients enrolled in the study experienced at least one side effect, with most common grade 3/4 toxicities being fatigue, thrombocytopenia, neutropenia, and abdominal pain. Four patients had significant liver toxicity with higher doses of tanespimycin, however this was manageable and reversible. The best responses observed to treatment were 1 MR in the 340 mg/m² group, and 2 PRs in the 175 mg/m² group. An additional 10 patients across all treatment groups had stable disease. A later phase 3 study evaluating tanespimycin and bortezomib has been completed, however results are not yet available.

KW-278 is a novel, non-anisomycin, non-purine based HSP90 inhibitor that has a more favorable pharmacokinetic and safety profile compared to tanespimycin^[185]. A phase 1 trial examined the safety and efficacy of KW-278 in B-cell malignancies, including 22 MM patients^[186]. The most common side effects included diarrhea, headache, rhinitis, and fatigue. Ten patients experienced grade 3 and 4 toxicities such as lethargy, syncope, QT prolongation, and neutropenia. Six patients experienced eye disorders (decreased visual acuity, blurry vision, dry eyes) that were deemed related to KW-278. All of the eye disorders were reversible with the exception of dry eyes. Two patients died during the trial and both deaths were determined to be unrelated to the study medication. Of the 21 MM patients evaluated, 20 patients (95%) had stable disease, and 1 patient had progressive disease.

A subsequent phase 1/2 study evaluated KW-278 with bortezomib in patients with relapsed or refractory MM who had failed at least 1 previous treatment^[187]. The most common side effects were fatigue and gastrointestinal toxicity, while the most common grade 3-4 side toxicities were cytopenias. There was no sign of significant ophthalmologic side effects in the 95 patients enrolled in this study. There were 28 serious treatment-related adverse events such as lung infections, GI toxicity, anemia, hematuria, syndrome of inappropriate diuretic hormone, pancreatitis, and transient ischemic attack. Efficacy of the treatment was based on a KW-278 dose of 175 mg/m² with bortezomib at the standard concentration. The ORR was 39%, the CBR was 92%. Median progression-free survival was 6.8 months, and median duration of response was 5.6 months. Patients who were lenalidomide-naïve had an ORR of 45.5 as compared to 25% for patients previously exposed to lenalidomide. Bortezomib-naïve patients had an ORR of 44% vs. 33% in patients previously exposed to bortezomib. These findings suggest a potential therapeutic benefit of KW-278 with bortezomib in the treatment of relapsed or refractory MM.

PI3K-AKT-mTOR inhibitors

The PI3K-AKT-mTOR inhibitor everolimus was tested as a single agent in a phase 1 trial in patients with relapsed and/or refractory MM^[188]. Most side effects were mild to moderate with gastrointestinal upset, elevated muscle and liver enzymes, and cytopenias being the most common. One patient developed an atypical pneumonia that was thought to be drug-related. Of the 15 patients assessed, 10 (67%) experienced clinical benefit with one patient achieving a partial remission after 4 cycles.

A phase I trial assessed the safety and efficacy of everolimus in combination with lenalidomide in patients with relapsed or refractory MM^[189]. Most common side effects were fatigue, cytopenia, diarrhea and neuropathy. One patient discontinued treatment due to non-infectious pneumonitis that was related to everolimus, and another discontinued due to grade 3 myalgia. Of the 23 patients that completed 2 cycles of treatment, the CBR was 74% with 1 CR and 4 PR. In patients who were lenalidomide-naïve, the ORR was 90%, compared to 37.5% for patients who had previously received lenalidomide. Median progression-free survival was 5.5 months, and median overall survival was 29.5 months. Promising results from these studies demonstrate the potential for mTOR inhibitors to be used the treatment of relapsed or refractory MM. A clinical trial looking at the role of mTOR inhibitors in combination with pomalidomide and dexamethasone is currently enrolling (NCT03657420).

Repurposing of FDA-approved drugs for use in MM

Metformin, the oral biguanide drug used in the treatment of diabetes, has been shown to exert anti-MM effects both *in vitro* and *in vivo*. The exact mechanism of metformin's anti-tumour activity has not been elucidated, but it is hypothesized to induce autophagy through the inhibition of STAT3 and BCL-2^[132]. The anti-retroviral drug nelfinavir has also shown anti-MM effects *in vivo* by triggering the UPR, and has been studied extensively clinical trials for relapsed MM^[190,191]. A new phase 1 clinical trial is assessing the safety and efficacy of metformin, nelfinavir and bortezomib in with relapsed or refractory MM (NCT03829020). This is only one example of the potential utility of drug repurposing strategies in cancer, rationally designed based on theoretical synergistic mechanisms of activity.

CONCLUSION

Autophagy plays a crucial pro-survival role in MM. Increased protein synthesis and proteotoxic stress are hallmarks of cancer, and MM is the prototypic cancer with impaired protein homeostasis based on its staggering rate of immunoglobulin synthesis and baseline level of proteotoxicity. Importantly, autophagy protects MM cells from excessive ER stress by limiting the secretion of immunoglobulins and providing an alternative proteolytic pathway for the clearance of ubiquitinated proteins. Consistent with a therapeutic role in targeting protein homeostasis, PI are highly effective anti-MM agents that are FDA approved for the treatment of MM in all its stages. However, clinical resistance to PI is inevitable, leading to research interest in targeting alternative pathways contributing to protein quality control such as autophagy, alone or in combination with PI. As with other mechanisms of protein homeostasis, such as the UPR; while a basal level of autophagy is cytoprotective and inhibiting autophagy to blunt non- proteasomal proteolysis has also been shown to have therapeutic benefit, sustained autophagy as in the setting of persistent proteasome inhibition, may result in autophagic cell death. This highlights the “Janus-faced” role of autophagy in MM. Autophagy therefore represents an opportunity to therapeutically exploit MM's unique “Achilles' heel”: its dependence on protein quality control. With a better understanding of the molecular sequelae of autophagy inhibition/induction in MM, we are eagerly awaiting the development or repurposing of drugs to target autophagy in an effort to overcome PI resistance and improve outcome for our patients with MM.

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Review

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Recent advances in the management of hyponatremia in cancer patients

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Abstract

Hyponatremia is the most frequently encountered electrolyte disorder in cancer patients and is usually multifactorial in its origin. In this review, we discuss the predisposing factors, pathophysiology, clinical symptomatology, and currently available diagnostic and therapeutic options for the management of hyponatremia. In addition to paraneoplastic syndromes, concurrent chemotherapy and comorbidities predispose oncology patients to the risk of hyponatremia. Initial symptoms and signs can be subtle and the prompt evaluation and initiation of treatment is of paramount importance to prevent neurocognitive and other complications. The syndrome of inappropriate antidiuresis (SIAD) is the most common cause of hyponatremia, and the use of serum and urine parameters that distinguish SIAD from other etiologies is discussed. Individualized treatment is preferred depending on the underlying cause and severity of hyponatremia. The treatment of hyponatremia is reviewed and the importance of avoiding rapid overcorrection of the sodium level to reduce the risk of osmotic demyelination syndrome is emphasized. Vasopressin receptor antagonists (vaptans) offer a direct approach to the management of euvoletic and hypervolemic hyponatremia, but the indications for their use and long-term safety need to be clarified. The treatment of hyponatremia is likely to reduce complications and improve survival in cancer patients.

Keywords: Hyponatremia, electrolyte abnormalities, dysnatremias, cancer



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INTRODUCTION

Dysnatremias are common electrolyte imbalances in cancer patients^[1]. Hyponatremia (serum sodium < 135 mEq/L) is the most common electrolyte abnormality encountered in clinical practice. Oncology patients are particularly at risk of developing hyponatremia due to their underlying cancer (including associated paraneoplastic syndromes), predisposition to infections, antibiotic use, concurrent chemotherapy, and nausea and pain. Hyponatremia in cancer patients is a predictor of outcome, hospital length and cost of stay, and an independent predictor of mortality in the intensive care unit^[2-5]. The syndrome of inappropriate antidiuresis (SIAD) is a frequent cause of cancer related hyponatremia^[6]. Hyponatremia due to ectopic arginine vasopressin (AVP; also known as antidiuretic hormone or ADH) production has been identified in 24.6% of small cell lung cancer patients and is associated with shorter survival^[7]. After brain metastasis, hyponatremia is the second leading cause of neurologic disorders in patients with small cell lung cancer^[8]. Other malignancies with a high prevalence of hyponatremia include gastrointestinal, hematological, breast and urological malignancies^[9]. Many chemotherapeutic agents coupled with hydration protocols can cause hyponatremia. Concomitant use of thiazide diuretics^[10] and opioid derivatives used for the management of cancer-related pain increase AVP release and contribute to hyponatremia^[5]. Vincristine and vinblastine have neurotoxic effects on the hypothalamic-pituitary-thyroid axis and cause hyponatremia while cyclophosphamide enhances the effect of AVP on the kidneys. Furthermore, cisplatin and carboplatin stimulate production of AVP and inhibit absorption of sodium by renal tubules. Treatment strategies with targeted therapies (inhibitors of angiogenesis, anti-epithelial growth factor tyrosine kinase receptor inhibitors and monoclonal antibodies) in cancer patients increases the incidence of hyponatremia (25.5%) in the treatment group. Higher incidence of hyponatremia is observed with a treatment combination of cetuximab and brivanib (63.4%) as well as with pazopanib (31.7%)^[11]. Patients receiving chemotherapy generally increase their free water intake. In small cell lung cancer patients receiving chemotherapy, tumor lysis syndrome increases the risk of hyponatremia due to increased release of AVP. In this clinical situation hyponatremia could be prevented by water restriction before the start of chemotherapy.

In cases of symptomatic hyponatremia, pharmacologic intervention may be necessary to increase serum sodium levels^[8]. The management of hyponatremia is becoming increasingly important in oncology due to the negative correlation of hyponatremia with performance status and the prognosis of cancer^[7,11]. Treatment of hyponatremia in small cell lung cancer patients is associated with improved survival^[12]. Sodium normalization is an independent prognostic factor for improved overall survival and progression free survival in patients with advanced non-small cell cancer with hyponatremia treated with first line chemotherapy or targeted therapy^[11]. Failure to identify the potential cause of hyponatremia and delay in appropriate treatment plan places the patient at risk of an adverse outcome. In this review, we discuss the initial evaluation, diagnostic algorithm and treatment of hyponatremia in cancer patients.

CLINICAL PRESENTATION

Symptoms of hyponatremia have variable clinical presentations, can be nonspecific and confounded by other comorbidities, and can range from mild cognitive deficits to severe neurologic symptoms. Symptoms of mild hyponatremia are nonspecific and may manifest as malaise, anorexia, muscle cramps, nausea, confusion, lethargy, and headache. More severe neurologic manifestations such as vomiting, somnolence, seizures, respiratory arrest, coma, and death are due to cerebral edema. Symptoms are often subtle, if present at all, in patients with chronic hyponatremia in which the brain has had time to adapt to the hypotonic state. Chronic hyponatremia is associated with falls and increased risk of fracture^[13]. A rapid decrease in serum sodium induces symptoms at a higher sodium level and the severity of symptoms reflect the degree of brain edema and guide the urgency of management. Patients with acute hyponatremia are at risk of permanent neurologic impairment.

PATHOPHYSIOLOGY OF HYPONATREMIA

Hyponatremia denotes a state of relative excess free water in relation to sodium in conjunction with impaired free water excretion. AVP regulates water balance in the body. AVP release is regulated by changes in effective osmolality or hypotonicity. Sodium is the major determinant of serum osmolality. Osmoreceptors, located in the anterior hypothalamus, detect changes in effective serum osmolality and regulate the release of vasopressin and thirst. An increase in serum osmolality activates osmoreceptors leading to secretion of vasopressin from the posterior pituitary. The threshold for releasing vasopressin is lower than for triggering thirst to avoid persistent thirst^[10]. Hyponatremia is a state of low effective serum osmolality or hypotonicity. Effective serum osmolality refers to the number of osmoles that contribute water movement between intracellular and extracellular compartments determined by the relative solute permeability properties of the membranes separating the compartments. Total osmolality reflects the concentration of all solutes in a given weight of water (mOsm/kg) regardless of the permeability properties of the separating membrane. A calculated serum osmolality [calculated serum osmolality (mOsm/kg) = $2 \times \text{Na (mEq/L)} + \text{glucose (mg/dL)}/18 + \text{urea (mg/dL)}/2.8$] < 275 mOsm/kg could refer to isotonic, hypotonic or hypertonic hyponatremia, depending on which osmoles are included in the formula, whereas a measured serum osmolality < 275 mOsm/kg always indicates hypotonic hyponatremia.

Clarification of tonicity is important because isotonic hyponatremia does not cause cerebral edema. The basolateral membrane of the renal collecting duct is permeable to water (aquaporin-3 and aquaporin-4 water channels). Vasopressin acts at the vasopressin-2 receptor on the cells of the renal collecting duct leading to fusion of aquaporin-2 in the apical membrane allowing water to enter into the cells of the collecting duct and ultimately to the circulation. Reabsorption of water from the collecting duct increases the concentration of sodium and other electrolytes in the urine, and concentrates urine. Low serum osmolality (normal range 285-295 mOsm/kg) decreases vasopressin release leading to excretion of free water from renal tubules. Patients with a reset osmostat, as seen in hypovolemia (baroreceptor stimulation), quadriplegia, and psychosis, have mild hyponatremia that is unresponsive to changes in fluid and water intake. Patients with a reset osmostat excrete 80% of a water load (10-15 mL/kg given orally or intravenously) within four hours, while patients with SIAD have impaired water excretion. Fractional excretion of urate [FEurate (%) = $(\text{urine urate} \times \text{serum Cr})/(\text{serum urate} \times \text{urine Cr}) \times 100$] is also normal (4%-11%) in patients with a reset osmostat while patients with SIAD have elevated FEurate (> 11%)^[14]. Identification of a reset osmostat is important because the risk of severe hyponatremia is low. In addition, treatment is not necessary and likely to be ineffective, since bringing sodium to the normal range will increase thirst^[15]. Since AVP release is controlled by hemodynamic and nonhemodynamic stimuli, cancer patients are at risk of hyponatremia due to hemodynamic (hypovolemia, hypotension, congestive heart failure, and nephrotic syndrome) and non-hemodynamic stimuli (pain, stress, nausea, vomiting, hypoxemia, hypercapnia, perioperative state and infections)^[16].

Pseudohyponatremia (a lab artifact) is due to an inaccurate measurement of sodium in the presence of abnormally high concentration of proteins and lipids in the blood. Larger relative proportion of plasma is occupied by excessive lipids and proteins. In this situation, serum osmolality is actually in the normal range since total number of solutes in the plasma remain the same. Monoclonal gammopathies can also cause pseudohyponatremia^[10]. Patients undergoing urological and gynecological surgery can have high serum osmolality due to mannitol and glycine absorbed in the circulation during irrigation. Effective osmoles, e.g., mannitol, glycine and glucose (seen in uncontrolled diabetes), increase serum osmolality by shifting water from intracellular compartments.

CLASSIFICATION OF HYPONATREMIA

Hyponatremia is classified on the basis of biochemical severity, rapidity of onset, development of symptoms, measured serum osmolality, and volume status. Acute hyponatremia is defined as hyponatremia that is

documented to exist < 48 h. The risk of cerebral edema increases in patients with acute hyponatremia since brain cells have less time to adapt to a hypotonic environment. Conditions predisposing cancer patients to acute hyponatremia include a postoperative state (especially after prostate and uterine surgery), polydipsia, thiazide diuretics, and infusion of cyclophosphamide. Brain cells adapt to hyponatremia by extruding sodium, chloride, and organic osmoles from cells. After adaptation, a rapid increase in serum sodium, as potentially seen after hypertonic saline administration, can cause damage to the myelin sheath leading to osmotic demyelination. In most clinical situations in which the duration of hyponatremia is less clear, hyponatremia should be considered chronic and managed accordingly to avoid the risk of osmotic demyelination^[10]. Mild hyponatremia refers to serum sodium levels ranging from 130 mEq/L to 135 mEq/L; moderate hyponatremia refers to serum sodium levels ranging from 125 mEq/L to 129 mEq/L; profound hyponatremia refers to serum sodium levels < 125 mEq/L. Assessment of volume status in hyponatremia is challenging because of the variability in individual clinical assessment. Furthermore, in this context it should be clarified whether volume status refers to effective circulating volume, total body water or extracellular fluid^[10]. In addition to absolute serum sodium levels, other factors that guide treatment of hyponatremia include symptoms and the rate of development of hyponatremia.

EUVOLEMIC HYPONATREMIA

Euvolemic hyponatremia results from low solute intake or SIAD and is a major cause of hyponatremia in cancer patients. Several malignancies are associated with SIAD. SIAD results from the abnormal production (increased release from the neurohypophysis or ectopic production) or action of AVP resulting in an excess of total body free water relative to sodium. SIAD is defined by hyponatremia (serum Na < 135 mEq/L) and an inappropriately concentrated urine (urine osmolality > 100 mOsm/kg, usually > 300 mOsm/kg) in the presence of low serum osmolality (< 275 mOsm/kg), euvolemia, and normal renal function. Renal sodium excretion is increased (usually > 30 mEq/L in a random urine sample) due to decreased aldosterone production and possibly increased secretion of natriuretic peptides^[17,18], which helps to distinguish SIAD from hypovolemic and hypervolemic hyponatremia. Urine sodium is typically < 30 mEq/L in hypovolemic and hypervolemic hyponatremia, unless there is an underlying process contributing to renal sodium loss such as diuretic use^[19]. Additional criteria that help to diagnose SIAD include serum osmolality less than the urine osmolality, low serum uric acid < 4 mg/dL and serum blood urea nitrogen (BUN) < 21.6 mg/dL, elevated fractional sodium excretion > 0.5%, fractional urea excretion > 55%, and fractional uric acid excretion > 11%.

Causes of SIAD are outlined in Table 1. SIAD is a diagnosis of exclusion. Other causes of euvolemic hyponatremia include adrenal insufficiency and hypothyroidism. Hematocrit levels and serum concentrations of uric acid and BUN may be decreased in SIAD^[20] due to hemodilution. These analytes could be elevated in hypovolemic hyponatremia^[21]. In the diagnosis of SIAD, diagnostic tools that are less useful, and potentially dangerous in the latter case, include AVP levels and water loading tests [Table 2]. Cancer patients can have a combination of SIAD and other processes that contribute to hyponatremia. Therefore, the management of these patients is often complicated and relies on the recognition of such comorbidities. Hyponatremia could be a marker of occult malignancy^[22]. Patients with an unidentifiable cause of SIAD should have a chest X-ray. Furthermore, a chest CT should be considered in patients with a history of smoking and a MRI of the brain in patients with persistent neurologic symptoms and signs despite correction of hyponatremia^[23].

HYPERVOLEMIC HYPONATREMIA

Patients with congestive heart failure, nephrotic syndrome, liver and kidney disease can have hypotonic hyponatremia with increased extracellular fluid volume due to reduced effective arterial volume that activates baroreceptor-mediated neurohormonal release of vasopressin. Concomitant use of diuretics,

Table 1. Causes of syndrome of inappropriate antidiuresis

Tumors	Infections	Drugs	Other
Extrapulmonary small cell carcinoma	AIDS	Chemotherapy agents	Postoperative state (major abdominal or thoracic surgery; pituitary surgery or other neurosurgery)
Lymphoma	Encephalitis	Cisplatin	Hydrocephalus
Meningeal carcinomatosis	Hydrocephalus	Cyclophosphamide	Cavernous sinus Thrombosis
Metastatic brain and spine tumors	Idiopathic, particularly in the elderly	Ifosfamide	Multiple sclerosis
Olfactory neuroblastoma	Meningitis	Vinristine	Guillain-Barre Syndrome
Ovarian teratoma	Pneumonia (bacterial and viral)	Vinblastine	Delirium Tremens
Endometrial carcinoma	Pulmonary abscess	Melphalan	Acute intermittent porphyria
Pancreatic carcinoma	Aspergillosis	Methotrexate	Acute respiratory failure
Primary brain tumors	Tuberculosis	Targeted Therapies:	Acute Psychosis
Prostate carcinoma	Brain abscess	Afatinib	Stroke
Bladder carcinoma	Rocky mountain spotted fever	Brivanib	Subarachnoid hemorrhage and other intracranial hemorrhages
Small cell lung carcinoma and other pulmonary tumors	Malaria	Cetuximab	Traumatic brain injury
Thymic tumors		Geftinib	General anesthesia
Sarcomas		Linifanib	Nausea
		Pazopanib	Pain
		Sorafenib	Stress
		Vorinostat	
		Non-chemotherapy agents	
		Desmopressin/Vasopressin	
		Methylenedioxymethamphetamine	
		NSAIDs	
		Opiates	
		Oxytocin	
		Phenothiazines	
		Prostaglandin-synthesis inhibitors	
		Rosiglitazone	
		Selective serotonin reuptake inhibitors (SSRIs)	
		Selective norepinephrine reuptake inhibitors (SNRIs)	
		Thiazide diuretics	
		Ciprofloxacin	
		Tricyclic antidepressants	
		Chlorpropamide	

e.g., thiazides, loop diuretics and spironolactone, may also contribute to hyponatremia. Reduced oncotic pressure in nephrotic syndrome has a similar effect due to baroreceptor-mediated neurohormonal activation. Chemotherapy induced tubulopathy decreases the ability of the kidney to excrete free water. Increased free water intake in the presence of kidney disease causes hypervolemic hyponatremia^[10].

HYPOVOLEMIC HYPONATREMIA

Reduced effective circulatory volume (as seen in pancreatitis, bowel obstruction, sepsis, diarrhea, and sweating) increases vasopressin release leading to increase water retention and hyponatremia that worsens with hypotonic volume repletion^[10].

CEREBRAL SALT WASTING SYNDROME

Cerebral salt wasting (CSW) syndrome is a clinical entity primarily but not exclusively associated with intracranial disease that leads to hyponatremia and decreased extracellular fluid volume^[24]. Although most commonly described in neurosurgical patients with subarachnoid hemorrhage, CSW is also seen in cerebral neoplastic dissemination in brain metastasis^[8]. However, CSW syndrome remains controversial and incompletely understood.

CSW is associated with primary overproduction of atrial natriuretic peptide and brain natriuretic peptide resulting in a decrease in sodium and water reabsorption in the kidney while reduced sympathetic outflow in intracranial disease reduces activity of renin-angiotensin system and increases natriuresis^[25-27]. CSW syndrome may lead to a clinical picture similar to SIAD^[28]. Laboratory findings common to SIAD and CSW syndrome include hypotonic hyponatremia, increased fractional excretion of urate (FEurate > 11%), high urine osmolality (> 100 mOsm/kg), and urine sodium (> 30 mEq/L) in the presence of normal thyroid,

Table 2. Criteria for diagnosis syndrome of inappropriate antidiuresis^[10,19]

Essential criteria	Supplemental criteria
Serum osmolality < 275 mOsm/kg	Blood urea nitrogen < 10 mg/dL
Urine osmolality > 100 mOsm/Kg	Serum uric acid < 4 mg/dL
Clinical euolemia:	FEurate [‡] > 11%
Absence of signs of hypovolemia* and hypervolemia**	FENa [‡] > 1%
Central venous pressure 6-10 cm H ₂ O	FEurea [‡] > 55%
Urine sodium > 30 mEq/L	Correction of hyponatremia through fluid restriction
No evidence of hypothyroidism, adrenal insufficiency	Failure to correct hyponatremia after 0.9% saline infusion
No diuretic use	
No renal insufficiency	

*Signs of hypovolemia (decreased skin turgor, orthostasis, dry mucus membranes, tachycardia). **Signs of hypervolemia (edema, ascites). [‡]FEurate (%) = (urine urate × serum Cr)/(serum urate × urine Cr) × 100. [‡]FENa (%) = (urine sodium × serum Cr)/(serum Na × urine Cr) × 100. [‡]FEurea (%) = (urine urea nitrogen × serum Cr)/(blood urea nitrogen × urine Cr) × 100

adrenal, and renal function. After normalization of hyponatremia, FEurate normalizes in patients with SIAD (FEurate 4%-11%) but remains persistently elevated in CSW (FEurate > 11%)^[14]. Clinical symptoms and signs of hypovolemia (decreased skin turgor, orthostasis, dry mucus membranes, tachycardia) and low central venous pressure in the setting of normal kidney function denote CSW. Serum AVP levels are elevated as seen in patients with hypovolemia^[21]. Identification of CSW from SIAD is of paramount importance to guide appropriate therapy; aggressive volume and salt repletion in CSW versus fluid restriction in SIAD.

After correction of hypovolemia, sodium loss is repleted with sodium chloride tablets and fludrocortisone with close monitoring of serum electrolytes^[29]. Rarely, hypertonic saline is required to correct hyponatremia^[29].

APPROACH TO THE DIAGNOSTIC EVALUATION OF HYPONATREMIA

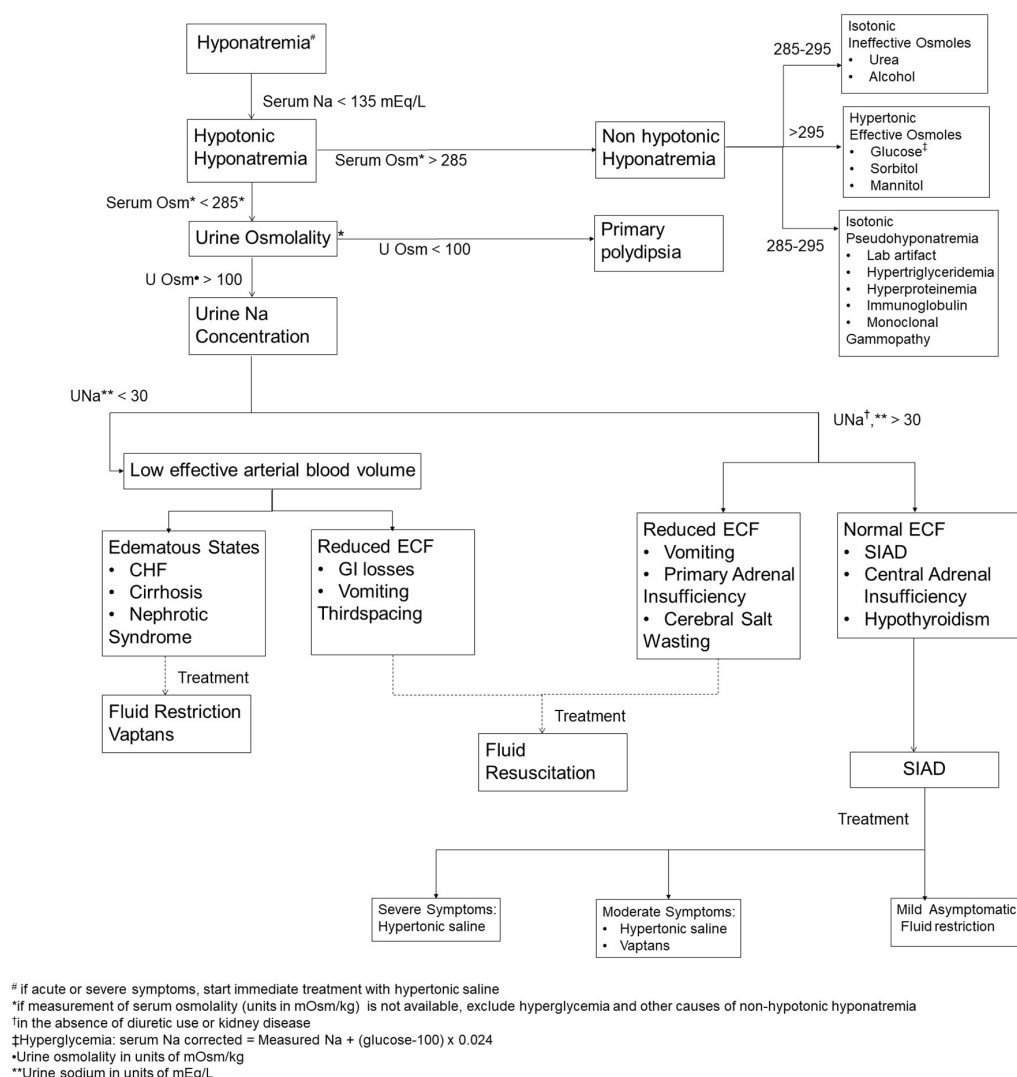
The initial step in the evaluation of hyponatremia is to measure serum osmolality. If hyponatremia is associated with hypoosmolality, the next step will be to measure urine osmolality and urine sodium. SIAD is a diagnosis of exclusion after ruling out other causes of euolemic hyponatremia such as hypothyroidism (free T₄, thyrotropin stimulating hormone) and adrenal insufficiency (total cortisol, adrenocorticotrophic hormone). Other causes that could be excluded in cases of hypotonic hyponatremia are decreased effective circulating blood volume and renal insufficiency^[16] [Figure 1].

Copeptin is secreted from the posterior pituitary along with AVP. There is a strong correlation between plasma AVP levels and copeptin levels. Although copeptin levels tend to be highest in small cell lung cancer, evaluation of copeptin levels between cancer-related versus non cancer-related SIAD show no significant difference. Multiple other factors (e.g., pain, nausea, stress, medications) contribute to elevated copeptin levels. Copeptin levels > 84 pmol/L indicate hypovolemic hyponatremia whereas low levels of copeptin < 3.9 pmol/L indicate primary polydipsia^[30].

CLINICAL MANAGEMENT/TREATMENT OPTIONS

Definitive treatment for hyponatremia involves treating the underlying cause. Medical therapy [Table 3] improves the serum sodium level and prevents potential neurologic complications. In patients with severe symptomatic hyponatremia, a 5 mEq/L increase of serum sodium is likely to improve clinical symptoms^[10]. The serum sodium should be acutely increased 1-2 mEq/L/h over 3-4 h using hypertonic saline (3% NaCl). To avoid the risk of over correction, current guidelines recommend i.v. infusion of 150 mL of 3% hypertonic saline over 20 min, rechecking serum sodium concentration and then, if necessary, repeating the 150 mL of 3% hypertonic saline infusion^[31]. Frequent monitoring of serum sodium along with 150 mL of hypertonic saline avoids rapid increase in serum sodium. Hypertonic saline can be administered at an

Treatment of hyponatremia in small cell lung cancer patients is associated with improved survival [10].

**Figure 1.** Evaluation of hyponatremia^[10,19,46]

initial rate of 1-2 mL/kg/h, although patients with seizure or coma may require a higher initial infusion rate of 2-4 mL/kg/h. A total volume of 500 mL hypertonic saline is sufficient to increase serum sodium 5 mEq/L. Hypertonic saline infusion is stopped when symptoms improve, serum sodium concentration increases by 10 mEq/L in total, or the serum sodium concentration reaches 130 mEq/L. If symptoms do not improve despite an increase of serum sodium by 10 mEq/L, or a serum sodium level of 130 mEq/L, other etiologies for these symptoms should be explored^[10]. In patients with mild symptoms, 3% saline can initially be administered at 0.5 mL/kg/h^[32]. Furosemide in combination with hypertonic saline infusion promotes electrolyte-free water excretion^[33]. The use of normal saline in the treatment of SIAD should be avoided due to the concern of net free water retention and worsening hyponatremia^[33].

In the asymptomatic patient, SIAD is initially treated with fluid restriction to 500-1000 mL daily (1 L/m²/day or 2/3 maintenance in children) so that free water intake is below the capacity for kidney water excretion plus insensible losses. Intake and output should be monitored closely to ensure compliance with fluid restriction and to maintain an oral intake of at least 500 mL/day below the average urine volume (in adults). In patients with mild asymptomatic hyponatremia fluid restriction could be started at 1200-1500 mL. A commonly

Table 3. Management of hyponatremia^[10,19,46]

Medical therapy	Indication	Dose	Adverse effects
Demeclocycline	Euvolemic and hypervolemic hyponatremia	300-600 mg bid	Slow onset of action, acute kidney injury
Lithium	Euvolemic hyponatremia	600-1800 mg/day	Narrow therapeutic window, confusion and somnolence
Fludrocortisone	Cerebral salt wasting SIAD	0.1-0.3 mg/day	Hypokalemia, hypertension
NaCl tab	Cerebral salt wasting SIAD	1-3 g/day in divided doses	Non-adherence to therapy for chronic use
Urea	Euvolemic hyponatremia	30 g/day	Lack of palatability, slow onset of action
Vaptans	SIAD hypervolemic hyponatremia	Conivaptan 20 mg i.v. bolus over 30 min and then 20 mg i.v. infusion over 24 h. Infusion rate can be increased to 40 mg daily; maximum duration of treatment is 4 days. Tolvaptan 15 and 30 mg tab for oral use. Maximum dose 60 mg. Therapy should be initiated in the hospital with monitoring of serum sodium	Dry mouth, increased thirst and polyuria; risk of rapid increase in serum sodium; liver injury associated with higher doses; concern for increase in mortality
Hypertonic saline 3%	Euvolemic hyponatremia and hypervolemic hyponatremia	Hypertonic saline 3% 150 mL i.v. bolus over 20 min × 3. Check serum sodium after every bolus. Hypertonic saline 3% continuous infusion 1-2 mL/kg/h*	Osmotic demyelination syndrome
Fluid restriction	Euvolemic and hypervolemic hyponatremia	Fluid restriction (500-1000 mL) includes all fluids including intravenous fluids. Degree of fluid restriction depends on serum sodium level, urine output and insensible fluid losses. Fluid restriction to < 500 mL/day of 24 h urine output is recommended	Rapid correction in case of primary polydipsia. Less likely to be effective if 24 h urine output is < 1500 mL
Isotonic saline	Hypovolemic hyponatremia	Fluid resuscitation with 0.9% normal saline	Ineffective and potentially dangerous in euvolemic hyponatremia. Should not be used in hypervolemic hyponatremia
Electrolyte free water	Management of overcorrection	Electrolyte free water 10 mL/kg over one hour. Desmopressin 2 mcg i.v. every 8 h	Hyponatremia

*Total amount of 3% NaCl to infuse (mL) = (desired change in serum Na × 1000)/ΔNa[†]. [†]ΔNa (change in serum sodium per liter infused) = (513[#] - serum sodium)/[TBW (L)** + 1]. [#]Amount of sodium (mEq) in 1 L of 3% NaCl. **TBW (L) = weight (kg) × 0.6 (children, nonelderly men); 0.5 (nonelderly women, elderly men); or 0.45 (elderly women). SIAD: syndrome of inappropriate antidiuresis; TBW: total body water

used medication to treat SIAD, especially chronic disease, is the tetracycline antibiotic demeclocycline, which has the side effect of causing nephrogenic diabetes insipidus^[34]. A few of the other side effects of demeclocycline include GI intolerance, renal toxicity, and photosensitivity. One should note, however, that its onset of action is delayed (typically 5 days or more), so it is not appropriate for the acute management of severe SIAD. Urea (available as prescription and over the counter) at a dose of 30-60 g/day has been used to induce osmotic diuresis in chronic hyponatremia. Side effects of urea include headache and GI irritation. Combining urea with NaHCO₃, sucrose and citric acid (urea 10 g + NaHCO₃ 2 g + citric acid 1.5 g + sucrose 200 mg) improves the taste and makes it palatable. Addition of urea to orange juice could also help reduce the bitter taste. Fludrocortisone can increase serum sodium up to 5 mEq/L and salt supplementation (NaCl 1 g po TID) can also be considered^[35].

Fluid restriction is preferred in patients with expanded extracellular volume. Intake and output of urine should be monitored closely. Free water intake should be 500 mL less than the urine output. Patients with reduced extracellular volume and hemodynamic instability require rapid fluid restoration. Extracellular volume is restored with i.v. infusion of 0.9% saline or a balanced crystalloid solution. As volume repletion suppresses vasopressin release in hypovolemia, close monitoring of serum sodium and urine output is required to avoid hypernatremia in the presence of increased free water clearance^[10]. Potassium deficiency should be replaced. Since potassium is osmotically active and can be exchanged with sodium, overcorrection of sodium is a concern in case of potassium repletion.

CENTRAL PONTINE MYELINOLYSIS

The rapid correction of hyponatremia by any means can cause central pontine myelinolysis, also known as osmotic demyelination syndrome^[36]. It is characterized by severe and often irreversible neurologic sequelae such as dysarthria, dysphagia, psychiatric disturbances, spastic paraplegia or quadriplegia, seizures, pseudobulbar palsy, and altered mental status. Symptoms of osmotic demyelination occur 4-6 days after rapid overcorrection^[37]. Therefore, the overall goal of treatment should be to avoid increasing the serum sodium levels by more than 0.5 mEq/L/h (or 10 mEq/L/day) and 8 mEq per subsequent day^[10,38]. However, some experts recommend an even more conservative therapeutic goal of an increase of no more than 8 mEq/day^[33,39]. Serum sodium levels should be assessed every 2-4 h during the initial stages of treatment for severe symptomatic hyponatremia. Desmopressin, 1-2 µg parenterally every 6-8 h, has been used along with hypertonic saline to reduce the risk of rapid correction, although further studies are needed to confirm the effectiveness of this approach^[40]. In addition to the degree of hyponatremia and rate of hyponatremia development, factors that also increase the risk of osmotic demyelination syndrome include liver disease, concurrent use of thiazide diuretics, and antidepressants. Suppression of AVP release with volume repletion in patients with reduced extracellular volume increases the risk of rapid correction of sodium due to increase free water clearance. In addition to close monitoring of urine output, serum sodium should be monitored every 2 h^[10].

VAPTANS: MECHANISM OF ACTION, CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

Vaptans are AVP receptor antagonists that are FDA-approved for clinical use in patients with euvolemic and hypervolemic hyponatremia. AVP antagonists block vasopressin receptors on principal cells in the renal collecting tubules. Inhibition of AVP receptors blocks increase in cAMP and protein kinase A that leads to reduced translocation of aquaporin 2 (water channels) to the luminal membrane. Binding of vaptans decreases the permeability of the apical membrane to water. Vaptans are aquaretic agents because they enhance electrolyte free-water clearance without associated natriuresis^[13]. Vaptans are utilized to reduce fluid restriction in patients with symptomatic mild to moderate chronic hyponatremia due to SIAD and heart failure^[39]. Conivaptan is a non-peptide dual V1A/V2 AVP receptor antagonist. Conivaptan, given as an initial i.v. bolus followed by a continuous infusion, increases serum sodium up to 6 mEq/L as compared with placebo. Use of i.v. conivaptan can be associated with rapid correction of hyponatremia, transient symptomatic hypotension, and peripheral i.v. site reaction. Patients receiving conivaptan through a central venous catheter did not have any adverse reactions^[41]. The most common reason for discontinuation of conivaptan is infusion site reaction^[42]. Tolvaptan is an oral V2-receptor antagonist also approved for use in euvolemic or hypervolemic hyponatremia. The most common adverse effects reported during tolvaptan use are thirst and dry mouth. Although it is an oral medication, the use of tolvaptan should be limited to the inpatient setting to monitor for overcorrection of serum sodium following administration.

Vaptan use has been associated with alanine aminotransferase elevation and hepatotoxicity with long term use. During treatment with vaptans, in addition to close monitoring of serum sodium, urine osmolality should be monitored. The dose of vaptan should be increased if the urine osmolality remains high. If urine osmolality is low, fluid intake should be restricted^[13]. The clinical trials evaluating these agents did not include patients with serum sodium less than 115 mEq/L^[43]. Caution is advised in patients with acute symptomatic hyponatremia, underlying liver disease, and volume depletion^[39]. Post hoc analysis from the trial ACTIVE showed a reduction in mortality ~50% in patients with congestive heart failure after the correction of hyponatremia with the use of tolvaptan. The EVERST trial evaluated the role of tolvaptan (30 mg Po QD) in cardiac failure patients for 2 years. There was no difference in all cause mortality in tolvaptan treated patients versus placebo^[41].

In the SALT WATER trial, long term use of tolvaptan (2 years) was evaluated for safety and efficacy in patients with euvolemic and hypervolemic hyponatremia. Hypernatremia was reported in only one patient.

As compared with fluid restriction, lithium and urea, vaptans offer a therapeutic option that is pathophysiologically focused, potent and effective^[41]. Despite efficacy of vaptans in improving serum sodium, clinical practice guideline on the diagnosis and treatment of hyponatremia do not recommend the use of vaptans due to concern for overcorrection of sodium and lack of mortality benefit^[23]. The safety and efficacy of tolvaptan was evaluated in a post hoc subgroup analysis of the patients with cancer in SALT-1 (study of ascending levels of tolvaptan in hyponatremia) and SALT-2 clinical trials. Baseline sodium in the cohort of the SALT trials was 130 mEq/L. A 15 mg dose of tolvaptan was titrated up to 30 or 60 mg over 4 days if the increase in serum sodium was < 5 mEq/L in the prior 24 h. Tolvaptan was withheld or reduced and fluid intake was increased if serum sodium was > 145 mEq/L. Serum sodium normalized to > 135 mEq/L in 66.6% of the patients by day 30. Serum sodium exceeded the desired rate of correction (> 8 mEq/L over the first 8 h of therapy or > 12 mEq/L over 24 h) in 1.8% of patients. None of these patients developed any symptoms of osmotic demyelination syndrome^[6].

In our experience, vaptans are well tolerated by cancer patients and offer a therapeutic option in patients with euvoletic and hypervolemic hyponatremia when fluid restriction alone is insufficient to increase serum sodium levels or to avoid the discomfort associated with fluid restriction. Although the vaptans are well tolerated and effective, their high cost continues to be a limitation for routine use. In a post hoc analysis, economic models from SALT-1, SALT-2 and EVERST reported decrease in length of stay in the hospital and hospital cost savings with tolvaptan use^[44]. To reduce the risk of hyponatremia all i.v. medications should be concentrated and hypotonic i.v. fluids should be avoided in hospitalized patients, e.g., perioperatively, during cancer chemotherapy, bronchitis, pneumonia or lung disease^[45].

CONCLUSION

Oncology patients with hyponatremia need close monitoring of fluid intake and output to prevent derangements in salt and volume balance. Hyponatremia in cancer patients is associated with advanced disease and poor prognosis. Improvement in serum sodium correlates with improved survival^[9]. Whether the treatment of hyponatremia improves overall survival needs to be investigated. Proposed algorithms for the management of hyponatremia could be utilized in patients with cancer, recognizing that hyponatremia is of diverse etiology in this population. Use of therapeutic agents such as the vaptans offer a convenient, effective, and targeted approach to the management of hyponatremia. Further studies are likely to clarify cost effectiveness and safety concerns related to vaptan use in cancer patients.

DECLARATIONS

Authors' contributions

Wrote the first draft of the manuscript: Khan MI

Provided critical edits to the manuscript: Waguespack SG, Ahmed I

Approved the final version of the manuscript: Khan MI, Waguespack SG, Ahmed I

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Review

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Head and neck cancer and immunotherapy: current knowledge and perspective

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Abstract

As with many types of cancer, immunotherapy is changing the management of squamous cell carcinoma of the head and neck (HNSCC). In a locally advanced or metastatic setting, treatment options have long been curtailed, but this paradigm is currently changing. Checkpoint inhibitors were the first to be validated in second-line treatment with PD-1 and PD-L1 inhibitors and these treatments are available in USA for first-line use. In addition, many studies are underway to use its molecules earlier in the care or try to increase their effectiveness with associations. The issues of patient selection that would benefit the most from immunotherapy and the evaluation of the response to these treatments are not completely solved. The goal is here to update the possibilities of current treatment by immunotherapy in HNSCC as well as on various development pathways in progress.

Keywords: Head and neck squamous cell carcinoma, checkpoint inhibitors, immunotherapy, clinical trial

INTRODUCTION

Head and neck cancers are currently responsible for more than 700,000 cases and more than 350,000 deaths per year^[1]. After a heavy treatment combining surgery, radiotherapy and chemotherapy, more than half of the patients relapse locoregional or metastatic^[2]. With chemotherapy and anti-epidermal growth factor receptor, advanced treatment was relatively limited before the arrival of immunotherapy in



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the therapeutic arsenal. Head and neck cancers are cancers inducing immunosuppression and therefore particularly likely to respond well to immunotherapy. The purpose of this article is to review studies validating the use of first-line and second-line checkpoint inhibitors (CPIs) in HNSCC, as well as studies evaluating new indications. The aim is also to evaluate the new immunotherapy modalities under evaluation and to discuss the difficulties of using these new treatment.

VALIDATED TREATMENT IN SECOND LINE

Nivolumab was the first molecule to be approved by the Food and Drug Administration with the Checkmate 141. This showed an overall survival improvement of 7.7 months *vs.* 5.1 months (HR = 0.68, 95%CI: 0.54-0.86) compared with monotherapy with docetaxel, methotrexate or cetuximab in HNSCC progressing after a first platinum-based line. This difference was greater in PD-L1 patients > 1% with an OS of 8.2 months *vs.* 4.7 months, but the curves were also separated for PD-L1 negative patients (< 1%). Nivolumab was therefore approved regardless of PD-L1 expression^[3].

Pembrolizumab was studied in the same situation with the Keynote 0-40 Phase III study, but this time docetaxel was given every 3 weeks at a dose of 75 mg/m². While the first analysis was not significant, the discount reached statistical significance with a median overall median survival of 8.4 months (95%CI: 6.4-9.4) *vs.* 6.9 months (95%CI: 5.9-8.0), HR = 0.80 (95%CI: 0.65-0.98, *P* = 0.0161). The reduction in HR relative to nivolumab can be explained by several factors. First, a cross-over effect in the chemotherapy group with many patients receiving immunotherapy secondarily, secondly because a majority of patients received docetaxel, which is the most effective chemotherapy and also by the use of docetaxel every three weeks used in this study which is probably more effective than the weekly rhythm used with nivolumab. In the subgroup analyzes, survival was significantly increased from 7.9 to 11.6 months in favor of pembrolizumab in patients with PD-L1 > 50%. There was no difference in the PD-L1 population < 50%. These results led to the approval in Europe of pembrolizumab in PD-L1 patients > 50%^[4].

A third phase 3 trial, the EAGLE study, compared an anti-PD-L1 (durvalumab) alone or with an anti-CTLA4 (Tremelimumab) to a standard chemotherapy. This study was negative for the overall survival, but the durvalumab arm as monotherapy was consistent with nivolumab and pembrolizumab with a median overall survival at 7.6 months and a response rate of 17.9%. In addition, the control arm was superior to what was expected because weekly paclitaxel was predominantly used (which is probably the best treatment option for these patients) and there was an important crossover with control arm patients receiving immunotherapy^[5].

FIRST LINE TREATMENT

The Keynote 048 trial is an open-label, randomized, phase 3 trial comparing 887 patients in first line with pembrolizumab to the extreme protocol [cisplatin or carboplatin, 5-fluorouracile (5FU) and cetuximab]^[6]. Pembrolizumab was administered at a fixed dose of 200 mg every 3 weeks alone or in combination with cisplatin and 5FU chemotherapy. The stratification factors were the expression of PD-L1 by tumor proportion score (TPS: expression of PD-L1 by tumor cells ≥ 50% *vs.* < 50%), p16 status for oropharyngeal tumors (positive *vs.* negative) and Eastern Cooperative Oncology Group status (0 *vs.* 1). Pembrolizumab showed a benefit in overall survival over chemotherapy and cetuximab in the combined positive score (CPS) population ≥ 20 (a score that studies expression on both tumor cells and immune cells in the microenvironment) with a median of 14, 8 months *vs.* 11 months; HR 0.58 (95%CI: 0.44-0.78). This advantage was found in the group of CPS ≥ 1 with a median of 12.3 months *vs.* 10.4 months; HR 0.74 (95%CI: 0.61-0.90). But it is important to note that if survival is better with pembrolizumab, at the beginning immunotherapy is deleterious for some patients with more deaths in the first six months. In terms of tolerance, treatment-related adverse event levels of Gr 3-5 were 17% with pembrolizumab against 69% with

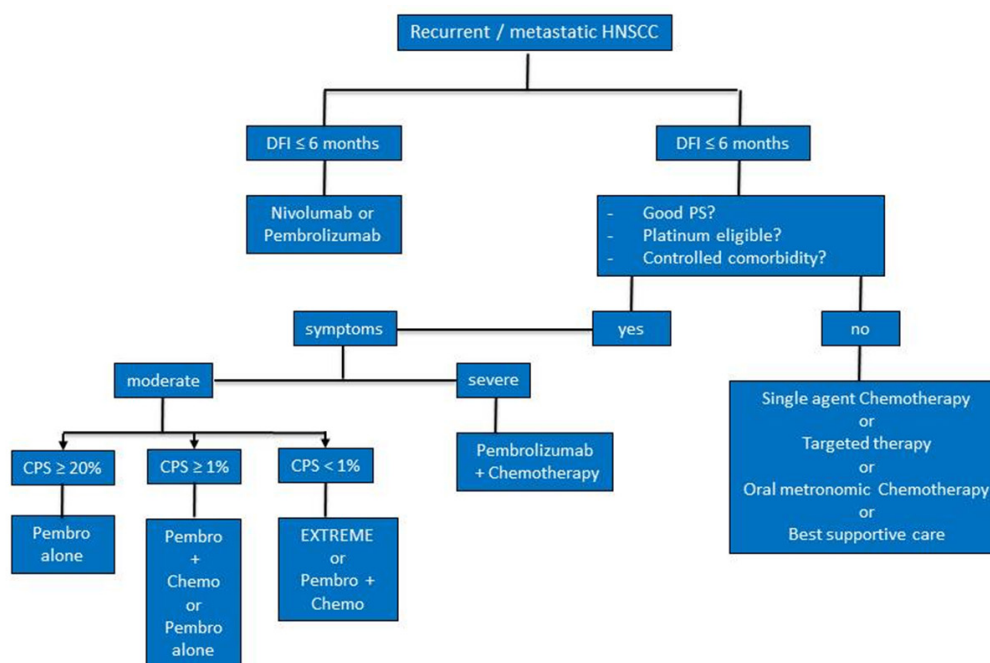


Figure 1. Proposed therapeutic decision tree in the recurrent/metastatic head and neck squamous cell carcinoma (HNSCC)

the Extreme protocol. The update during the American Society of Clinical Oncology (ASCO) 2019 also showed a benefit in overall survival in the pembrolizumab group associated with chemotherapy compared with the Extreme group, regardless of the subgroup of patients. The median OS was 14.7 months vs. 11 months, respectively; HR 0.60 (95%CI: 0.45-0.82) for the CPS group ≥ 20 , 13.6 months vs. 10.4 months; HR 0.65 (95%CI: 0.53-0.80) for the CPS group ≥ 1 and 13 vs. 10.7; HR 0.72 (95%CI: 0.60-0.87) months in total population.

It is therefore the first randomized trial that demonstrates an improvement in overall survival compared to standard platinum and cetuximab treatment in the first line. This trial also establishes the CPS as a valid marker for selecting patients most likely to benefit from pembrolizumab monotherapy. In CPS ≥ 20 , the toxicity profile favors treatment with pembrolizumab monotherapy, whereas the combination of pembrolizumab with chemotherapy seems the best alternative for CPS < 20 that can benefit. Pembrolizumab in monotherapy may also be discussed in slowly progressive patients with CPS < 20 . Be that as it may, it is likely that this study will upset the frontline management of HNSCC cancers in the front line.

There are currently two other studies currently underway evaluating first-line CPIs. Durvalumab alone or in combination with tremelimumab is compared to the EXTREME protocol in the KETREL trial and the nivolumab and ipilimumab combination is still compared to the same protocol in the CA609-651 trial.

Finally, these new studies will change the management of HNSCC cancers. We propose in [Figure 1](#) a decisional tree for the treatment of recurrent or metastatic HNSCC.

IMMUNOTHERAPY MORE PRECOCIOUS

Because of the improvement in overall survival provided by the inhibitors of checkpoint, the question arises of introducing these treatments earlier in curative intent for localized disease, in neoadjuvant or adjuvant treatment.

Table 1. Recent immunotherapy approaches in head and neck squamous cell carcinoma

Immunotherapy	Mecanism of action	Stage of clinical development	Setting
M7824	Bispecific anti-PD-L1 and anti-TGF- β antibody	Phase I	Recurrent/metastatic HNSCC in second therapeutic line
Monalizumab with cetuximab	New checkpoint inhibitor targeting NKG2A + anti EGFR	Phase II	Recurrent/metastatic HNSCC
Danvatirsen with durvalumab	Inhibitory antisense oligonucleotide of STAT3 + anti-PD-L1	Phase Ib/II	Metastatic HNSCC naive of anti-PD-L1
SD101 with pembrolizumab	TLR9 agonist + anti-PD-1	Phase I/II	Recurrent/metastatic HNSCC, anti-PD1 naive
Epacadostat	Oral IDO1 inhibitor	Phase III failures	Recurrent/metastatic HNSCC
Lenvatinib	Broad-spectrum TKI	Phase Ib/II	Metastatic HNSCC, after at least one line of chemotherapy
MEDI6974 with durvalumab	Ox40 agonist antibody + anti-PD-L1	Phase Ib	Before surgery with stage III-IVA HNSCC
Dendritic cell vaccination with conventional chemotherapy	Mature CD loaded with WT1 peptides + OK-432 (TLR-4 ligand)	Phase I/II	Recurrent/metastatic HNSCC

HNSCC: head and neck squamous cell carcinoma; EGFR: epidermal growth factor receptor; TGF: transforming growth factor

In adjuvant, trials are in progress with comparative immunotherapies or added to cisplatin or cetuximab during radiotherapy, but there is currently no efficacy data. There is no sign of toxicity so we expect a better tolerance than current treatments. A randomized Phase II trial compared potentiated radiotherapy with cetuximab or pembrolizumab in inoperable patients not eligible for cisplatin. Tolerance was better in the pembrolizumab group with a 3-4 grade toxicity of 69% vs. 88% with cetuximab. There was particularly less mucositis and radiodermatitis with pembrolizumab^[7].

In neoadjuvant, a phase I is in progress that combines durvalumab with TPF (cisplatin docetaxel 5FU) to potentiate induction chemotherapy.

A phase 2 study also studied still in neoadjuvant 23 patients with high-risk HNSCC cancer (probable R1 resection or capsular rupture) who received pembrolizumab 1 to 3 weeks before surgery. There was 47% response with an anti-tumor effect on more than 10% of the surface, including 32% major response on more than 70% of the surface and 1 complete response^[8]. Similar results have been observed with nivolumab^[9].

It is also possible that immunotherapy may make subsequent chemotherapy more effective, which would favor earlier administration of immunotherapy.

NEWS IMMUNOTHERAPY

Many immunotherapy molecules with different mechanisms of action are currently under development. A review of the 2016 literature presented the latest insights into the field of immunotherapy for HNSCC^[10]. Many immunotherapy associations were already being tested. The goal here is to present some of these innovations with a promising future to show the diversity of this one. Table 1 summarizes the molecules presented.

A phase 1 study showed promising results with a bispecific anti-PD-L1 and anti-transforming growth factor- β (TGF- β) antibody (M7824). The goal is to inhibit TGF- β , which increases tumor immunodepression. In addition, inhibition of the TGF- β pathway could increase the activity of anti-PD-L1. Among 32 patients, 7 had partial response, 6 had tumor stability, and grade III was found among 10 patients (31.3%). Further development will be interesting to follow^[11].

Monalizumab is a new CPI targeting NKG2A. This is expressed on CD8+ lymphocytes and on NK lymphocytes. The ligand of NKG2A, human leucocyte antigens (HLA)-E, is overexpressed in HNSCC.

Blocking this interaction stimulate CD8+ and NK lymphocytes and increase antibody-dependent cell-mediated cytotoxicity (ADCC). Since Cetuximab acts mainly via an ADCC mechanism, it could be potentiated by Monalizumab. A phase II study included 40 patients to receive weekly cetuximab combined to monalizumab every two weeks after failure of platin. The response rate, which was the primary endpoint, was 27.8%, or 11 out of 40 patients (compared to 13% with cetuximab alone according to historical data). The tolerance was particularly good, making this association a promising issue^[12]. It is interesting to note that monalizumab seems to be effective after failure of an anti-PD-1.

Another phase Ib/II study investigated the combination of danvatirsen with durvalumab among patients with metastatic HNSCC naive of anti-PD-L1. Danvatirsen is an inhibitory antisense oligonucleotide of STAT3 (that stimulates the immunosuppressive function of the microenvironment). Among 44 treated patients, 23% of responses (higher than those found with durvalumab monotherapy) were found with 3 complete responses. An increase of transaminases and transient thrombocytopenia were the major toxicities and it seems there is no additive toxicity of the two treatments^[13].

SD101 is a TLR9 agonist stimulating the activity of dendritic cells. This later became antigen-presenting cells to activate an anti-tumor response via T lymphocytes. A phase I/II study investigated the combination with pembrolizumab. Patients received pembrolizumab every 3 weeks and SD-101 injected into a single tumor site each week for 4 injections and then every 3 weeks up to 7 injections. Among the 26 patients included, the median injections of SD-101 administered were 5. Patients experienced injection site reactions and influenza-like illness with 28% of toxicities \geq grade 3. The immune-induced effects of pembrolizumab do not appear to have increased. The response rate in mITT (that is to say, among the 23 patients who had reached at least the first tumor evaluation) was 30.4% with 7 partial responses, 3 stabilities, 10 progressions in imaging and 3 clinical progressions before the scan. Tumor size reductions \geq 30% were observed in injected lesions (8/18 or 44.4%) as well as in non-injected lesions (8/14 or 57.1%). At the time of the analysis, 5 of the 7 responses were ongoing (1 to 27 weeks). Therefore, stimulation of dendritic cells seems promising with activity in the injected site, but also at a distance, at the cost however of a significant toxicity^[14].

Epacadostat is an oral IDO1 inhibitor (indoleamine 2,3-dioxygenase) showing good phase I and II in combination with pembrolizumab^[15]. IDO1 is an enzyme expressed in the tumor microenvironment that has an immunosuppressive effect by decreasing tumor infiltration by cytotoxic T lymphocytes (TILs). But in the face of phase III failures in melanoma, development has unfortunately been halted^[15].

Associations with tyrosine kinase inhibitors (TKIs) have also been studied in a phase Ib/II study. Lenvatinib, a broad-spectrum TKI, has been associated with pembrolizumab in 22 progressive metastatic patients after at least one line of chemotherapy. The 24-week response rate was 36% with a median response time of 8.2 months, a progression-free survival (PFS) of 8.2 months, and one year progression-free survival rate of 37%. But the toxicity is important with 91% of side effect, of which 14% of grade 4 and 18% of patients who had to stop the treatment. The main symptoms were fatigue, loss of appetite, hypertension and digestive disorders. Associations with other less toxic tyrosine kinase inhibitors may be considered^[16].

Another trial associated durvalumab with an activating agonist of the immune response, MEDI6974 (Ox40 agonist antibody). The treatments were administered for a short time before surgery. An increase and activation of cytotoxic lymphocytes within the tumor has been demonstrated. This association could potentially be interesting^[17].

A phase I/II study investigated dendritic cell vaccination with conventional chemotherapy in patients with head and neck cancer^[18]. Eleven patients with relapsed or refractory disease were recruited (with HLA-A2 or HLA-A24 positive as well as WT1 expression, a tumor antigen). Mature dendritic cell (DC) loaded

with WT1 peptides were injected intradermally approximately every 2-3 weeks. OK-432 (TLR-4 ligand) was administered subcutaneously near the vaccination sites to activate the DCs. Treatment was generally well tolerated with none of the patients with grade 2 adverse events except for hematologic adverse events with leucocytopenia and grade 2 anemia. Non-hematologic adverse events were consistent with the classic adverse effects of chemotherapy (discomfort, nausea, anorexia, and constipation except transient erythema at injection sites and low-grade fever). 5 patients presented a stabilization of the disease and 6 patients a progression of the disease. The median PFS and OS were 6.4 months and 12.1 months, respectively. But the median duration of PFS and OS of patients with stable disease was significantly longer than that of patients with progressive disease. The median PFS was 13.0 months *vs.* 2.8 months ($P = 0.00136$) and the median OS was 30.3 months *vs.* 8.1 months ($P = 0.0126$). Two patients had a long-term survival of 30.3 m and 44.4 m. Furthermore, all patients with stable disease showed improvement of WT1-specific immune responses after vaccination in all tests performed [HLA tetramer, interferon gamma (IFN γ) ELISPOT and mobilization CD107a] as well as an increased Th1/Th2 ratio of 1.36 times on average. Dendritic cell vaccination is therefore an interesting research path because of its efficacy in some patients and its good tolerance^[18].

In conclusion, many promising new immunotherapies are being developed with different mechanism of action. Some may be used alone and other combined with CPIs or other conventional therapy.

DIFFICULTY OF ASSESSING IMMUNOTHERAPY

Evaluation of immunotherapies may be different and more difficult than conventional anti-tumor treatments. In fact, patients treated with CPI observed phenomena of pseudo-progression (tumor response after an initial progression) and, on the contrary, hyperprogression phenomena with a rapid and significant increase in tumor volume.

These pseudo progression are probably due to infiltration of immune and inflammatory cells and/or a delayed clinical response. These phenomena are relatively rare but may explain the benefit of immunotherapy treatment beyond progression. For example, in the Checkmate 141 study, of 240 patients randomized to receive nivolumab, 146 showed progression according to RECIST. Sixty-two of these patients continued nivolumab and 84 discontinued treatment. Of the 60 patients who continued nivolumab evaluable for the response, 15 (25%) had not changed their tumor burden and 15 (25%) had a reduction in the size of the target lesion (3% of patients had responded by RECIST after progression). This attests the interest to continue the treatment beyond the progression if the general state remains correct and in the absence of rapid progression. Note that the expression of PDL1 does not seem to differentiate patients who will be treated or not beyond progression^[19].

This pseudo progression can in part explain the possible increase of efficacy of subsequent chemotherapy after failure of anti-PD-1. Indeed a French retrospective study in 82 patients receiving chemotherapy (45% monotherapy and 55% combination therapy) after failure of immunotherapy showed a 30% responses and a 57% disease control, higher than those usually observed in the 3rd line. Chemotherapy could also activate the immune system by releasing tumor antigens, with check point inhibitors known to remain in the body for a long time.

Hyperprogressions are associated with poorer survival. A French retrospective study suggested that hyperprogression can be observed in 29% of patients treated by immunotherapy^[20]. This controversial phenomenon could be induced by an excessive immune reaction, a paradoxical action of anti-PD-1/PD-L1 promoting tumor development, or ultimately only the natural history of advanced phase cancer.

Finally, unlike conventional chemotherapy, the occurrence of autoimmune adverse events seems to correlate with the effectiveness of the CPIs. In an analysis of 114 patients treated with anti-PD-1: 43% exhibited

immunological toxicity. These patients had higher response rates (30.6% *vs.* 12.3%), better progression-free survival (6.9 months *vs.* 2.1 months) and improved overall survival (12.5 months *vs.* 6.8 months). This analysis may of course be biased since patients who benefit from immunotherapy pursue it longer and are therefore more likely to develop immunological toxicity^[21].

PREDICTIVE FACTOR OF EFFICACY OF IMMUNOTHERAPY

One of the major challenges of immunotherapy is to select patients responding to immunotherapies with predictive biomarkers of effectiveness. Currently, the only one used is the expression of PDL1 but it is very imperfect, especially with excellent results in PDL1 negative patients.

The expression of PD-L2 and the signature IFN γ have been explored, but do not seem particularly convincing.

HNSCC have been classified into 6 groups: immunoreactive, inflammatory, Human Papilloma Virus (HPV)-like, classical, hypoxemic and mesenchymal^[22]. The inflammatory and immunoreactive profiles are probably more sensitive to immunotherapies because they are strongly infiltrated by cells of the immune system. The important tumoral infiltration by the CD8 lymphocytes could characterize this inflammatory type.

In a cohort of 34 patients with significant CD8+ lymphocyte infiltration and a high PD-1/TIM3 expression ratio (regardless of HPV status and smoking status) showed an increase in overall survival (84 months *vs.* 13 months) with anti-PD-1 treatment, compared with non-inflammatory tumors^[23].

The estimation of the mutational load (TMB) in the primary tumor, but also from the circulating DNA could also become a predictive biomarker^[24].

There is also evidence that HPV-positive tumors may be better able to respond to anti-PD1. There is indeed a biological rationale for better efficacy of anti-PD1 in HPV + tumors since they show more immune infiltrates and more markers of activated T cells. A retrospective analysis of 54 patients treated after platinum failure with pembrolizumab (32 patients) or nivolumab (22) overall survival was significantly better for HPV positive patients: 17 months *vs.* 4.5 months. Similarly, the duration of anti-PD1 treatment was significantly increased in positive HPV: 7 months *vs.* 3 months^[25].

Much simpler, a study presented at ASCO 2018 has recently been shown to have a high lymphocyte neutrophil count associated with tumor inflammation and poor prognosis^[26]. The 114 patients were treated with anti-PD1 classified in 4 quartiles according to this ratio. Patients with the lowest ratio had an overall survival well above 12.5 months compared to those with the highest ratio (4.1 months, $P < 0.0001$). In this cohort, PD-L1 expression did not discriminate patients. Thus, a very simple and clinical marker proves to be a powerful prognostic factor for the efficacy of immunotherapies.

CONCLUSION

Squamous cell carcinoma of the head and neck has not been spared by the revolution of immunotherapy. Indeed, the CPIs are now an integral part of the therapeutic arsenal and new indications are emerging either as monotherapy or in combination. In addition, many other classes of molecules are currently under development. The selection of patients who will benefit most from immunotherapy with new biomarkers remains an important challenge in the use of these new therapies. The question of the optimal duration and determination of the best combinations of these immunotherapy treatments remains unresolved. The therapeutic management of HNSCC cancers will continue to be disrupted in the coming years.

DECLARATIONS

Authors' contributions

Wrote and reviewed the manuscript: Gauduchon T, Reverdy T, Gau M, Karabajakian A, Collet L, Neidhardt EM, Fayette J

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All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

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Review

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High-risk HPVs, microbiota and epithelial carcinogenesis: state of the art and research contribution of *in vitro* 3D models

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Abstract

Persistent high-risk human papillomavirus (HR-HPV) infection is associated with anogenital and head & neck squamous epithelial (HNSCC) tumors, which altogether cause about 550,000 new cases every year. Several evidences suggest that the microbiota could have a role on the inflammatory, epithelial mesenchymal transition and tumorigenesis processes promoted by HR-HPV infection, yet the mechanisms involved remain to be clarified. In this review we report the state of the art on this topic and on the most promising *in vitro* developed models for studying the host-pathogen interactions. Using MEDLINE, several terms were searched and combined to select the most pertinent papers. The investigation was limited to the international indexed articles published in PubMed in the last 10 years. This review reports the latest knowledge in the field of the microbial-associated anogenital tumors and HNSCC. In addition, we also discuss the *in vitro* epithelial culture systems that reproduce the pathophysiological features of the tumoral microenvironment and the *in vivo* response to microbial agents, thus representing a useful tool for analyzing at cellular and molecular levels the role played by infective agents in tumorigenesis.



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Keywords: High risk human papillomavirus, microbiota, *Fusobacterium nucleatum*, epithelial-mesenchymal transition, anogenital cancer, head & neck squamous cell carcinoma, colorectal cancer, transepithelial electrical resistance

INTRODUCTION

As referred in the last human papillomavirus (HPV) cancer report of the HPV Information Centre^[1], unremitting HPV infection, mainly promoted by high-risk HPV (HR-HPV) 16 and 18 E6 and E7 oncoproteins, is the main microbial etiological factor till now recognized that is necessary, yet not sufficient, for the development of anogenital and of head & neck cancers (namely of the tonsil, tongue and oropharynx).

Despite the progresses in vaccination and screening prevention led to the reduction of cases number^[2,3], these tumors are still diffuse among the general population, with the highest mortality rate in countries with a low socio-economic level^[3].

Cervical cancer is the 3rd most frequent female tumor in the world (2nd in the age range 15-44) with about 600,000 new cases/year and a 50% mortality rate^[4,5].

In the last three decades oropharyngeal tumors, which include some 450,000 new cases/year (mostly affecting older tobacco and alcohol users worldwide), have increased also in young people, due to sexual habits changes predisposing to HR-HPV infection^[6].

It has been reported that oncogenic HPV-related oropharyngeal tumors have a better prognosis than those HPV negative^[7,8]. On the other hand, it is known that HR-HPV-mediated cervical oncogenesis generally develops after a long latency (about 10-30 years from infection). Thus, it remains a conundrum the fact that tumor latency and prognosis differ in the two populations of patients, regardless of the treatment and of the fact that both types of cancer share a common infective cause^[9]. Seemingly, HR-HPV infection is not *per se* enough to cause these cancers, though viruses are classified as Class 1 carcinogens by the International Agency for Research on Cancer^[4]. Recent evidences suggest that the microbiota of the cervical and oropharyngeal niches (that includes bacteria, protozoa, viruses and fungi, although sometimes the term specifically refers only to bacterial communities) can have both a harmful and a protective role. Further, the microbiota can influence persistent HPV-mediated inflammation, epithelial mesenchymal transition (EMT) and tumorigenesis, although the mechanisms involved are still largely unknown and controversial^[10]. In fact, just as a balanced bacteriota may be constituted by both pathogens and commensals, the human virota can include, among the others, both low risk and HR-HPVs, without inducing morbidity for its host if the immune surveillance and the whole microbiota itself are able to control its load and persistence^[11].

Therefore, an open question is if commensal HPV types, belonging to the human virota, play a protective role against the oncogenic ones or not^[12-15]. What is sure is that cancer often arises from a metabolic imbalance. Given this assumption, the metabolic profile of the microenvironment, in which host cells directly cooperate with viruses and bacteria, could reveal a snapshot of their functional and complex interactions that are at the basis of HPV infection, persistence susceptibility, inflammation, EMT, uncontrolled cell proliferation and cancer progression.

To learn more about it, researchers have developed several *in vitro* co-culture systems that could mimic the physiological appearance of the tumoral microenvironment^[16].

On this basis, the purpose of this study was to show the state of the art on epithelial carcinogenesis and on the most suitable *in vitro* 3D models till now developed for the study of host-pathogens interactions.

By using MEDLINE, terms such as “microbiota”, “virota”, “virus”, “epitheliotropic virus”, “HPV”, “bacteriota”, “bacteria”, “*Fusobacterium nucleatum*”, “*Porphyromonas gingivalis*”, “EMT”, “eukaryotic cells”, “keratinocytes”, “epithelial cells” “epithelial infection”, “epithelial cancer”, “anogenital cancer”, “head and neck squamous cell carcinoma (HNSCC)”, “epithelial models”, “2D”, “3D”, “3D epithelial cultures”, “rafts”, “spheroids”, “reconstructed tissues”, both in single and mutually combined, were searched in titles and abstracts in order to find PubMed indexed most pertinent articles; only original research articles published in peer review journal were considered. Papers including EBV or other epitheliotropic viruses other than HPV were excluded.

The literature search was limited to the most recent scientific publications (10 years); six thousand and five hundred nine appropriate abstracts and full papers were carefully read and reviewed; those relevant (216) were reconsidered based on the above described inclusion and exclusion criteria and then 70 were reported in detail and included in the text.

Overall, our findings highlight the most recent advances in the field of host-pathogens interactions. We focused on the *in vitro* 3D modeling for the study of the microbial induced epithelial carcinogenesis and emphasized its ability in mimicking at best the role of bacteria and viruses within an *in vivo* microenvironment.

MICROBIOTA, IMMUNE RESPONSE AND EPITHELIAL CANCER

Several studies, among which that conducted by Kyrgiou *et al.*^[10], underlined that vaginal microbiota may drastically influence host innate immune response, infection susceptibility and cervical cancer development, but without proving causality and thus making necessary further longitudinal studies. In this direction, Ilhan *et al.*^[17], through a triple approach based on liquid chromatography mass spectrometry, 16S rRNA gene sequencing and immunoassay, evaluated and integrated the metabolic signature of the vaginal microbiota and the inflammatory status changes of 78 women, including HPV-negative/positive controls, low-/high-grade cervical-dysplasia or -cancer affected females. These authors found that unique cervicovaginal metabolites and microbes allow to discriminate clearly the affected patients from healthy subjects, thus providing novel cancer hallmarks. For example, the lipidic three-hydroxy-butyrate, eicosenoate, and oleate/vaccenate are distinctive of cancer patients, while sphingolipids, plasmalogens and linoleate positively correlate with cervicovaginal inflammation.

Regarding this niche, the absence of dysbiosis and a dominance of peculiar gram-positive bacteria belonging to the *Lactobacillus* genus may inhibit several pathogens, preserve aminoacidic and nucleotide metabolisms and avoid the inflammatory state. Conversely, *Fusobacterium* spp., *F. nucleatum* in primis, promotes cell survival, proliferation, dysregulated carcinogenesis and cervical cancer through the induction of the WNT/ Beta catenin signaling pathway, as emerged by research in colorectal cancer (CRC)^[12,18,19].

Since it has been demonstrated a significant correlation between bacteria belonging to the *Fusobacteria* genus, mainly *Sneathia sanguinegens*, and high-grade squamous intraepithelial lesions, they could be hired as microbiological markers of clinically significant cervical diseases^[10].

This evidence has also been observed in another study by Mitra *et al.*^[12], who found that a relative abundance of *Fusobacterium* spp. associates with higher levels of cytokines such as IL-4 and TGF- β , which have been shown to generate local immunosuppression, HPV immune evasion and cancer development.

Moreover *F. nucleatum* inversely correlates with CD3⁺-T cell counts, whose aberrant signaling and function is typical in cervical carcinogenesis.

Al-Hebshi *et al.*^[15] have reported an abundance of the periodontal pathogen *F. nucleatum*, selected by the tumor environment, followed to a lesser extent by *Campylobacter*, *Parvimonas* and *Prevotella* spp. in oral squamous cell carcinoma, while the *Streptococci* are frequently associated with a healthy status.

Although the results obtained from clinical studies vary in the oral cancer associated microbiota composition, partially due to differences in the methodological approaches, the functional data consistently show an enrichment of a virulent, chronic inflammatory bacteriota that, as a passenger within the tumor environment, contributes to the worsening of the tumoral disease.

HIGH-RISK HPVS, MICROBIOTA, EPITHELIAL MESENCHYMAL TRANSITION AND CANCER PROGRESSION

As known, EMT associates with cell migration, invasion, resistance to chemotherapy and anoikis. During this process, several signaling pathways, such as those involving TGF- β , Wnt, Hedgehog, β -catenin, Notch, Nanog, STAT3 and ERK, are activated in the cell leading to a pro-metastatic behavior^[20,21]. In this context, EMT can be also regulated by HR-HPVs^[22,23].

Despite anogenital and oropharyngeal squamous cell carcinomas (OPSCC) promoted by these viruses have a more favorable prognosis respect to HPV negative ones, EMT usually associates with an aggressive tumoral behavior, making HPV role an open issue. In a study on a cohort of 296 OPSCC-patients, EMT was confirmed as an adverse marker of evolution of HPV related carcinomas^[24].

In another research, the expression of five EMT markers was assessed in normal and tumoral cervical tissues, revealing that E-cadherin and β -catenin expression decreases gradually, while that of N-cadherin, vimentin and fibronectin increases during malignant progression^[25]. Moreover, Su *et al.*^[26] studied the role of DNA methylation of the ten-eleven translocation methyl-cytosine dioxygenase 1 (TET1), which oxidizes 5-methylcytosine to 5-hydroxymethylcytosine within cervical lesions. At a precancerous level, TET1 inhibits EMT, interacting with chromatin modifiers and downregulating mesenchymal genes such as *ZEB1* and *VIM*^[26]. *In vitro* culture of cervical cancer cells revealed that p68, an ATP-dependent RNA helicase, induces EMT through the transcriptional activation of the TGF- β 1 pathway, inducing cancer progression^[27].

Finally, Pang *et al.*^[28] reported that HPV18 E6 regulates YB-1 mRNA expression by promoting EMT and cervical lesion progression through the enhanced Snail expression.

Moreover, both HPV and EMT upregulate programmed cell death ligand-1 (PD-L1) expression, an immune checkpoint and therapeutic target in OPSCC, whose efficacy is currently under investigation^[29,30]. Precisely, PD-L1 is overexpressed in about half of OPSCC that seem to have a better response to anti-PD-1 therapy^[31].

Regarding the involvement of bacterial microbiota in EMT, *F. nucleatum* has been frequently found in stools and bioptic specimens of CRC affected patients, and more recently also of OPSCC patients. It has been suggested that this bacterium triggers EMT to malignant transformation by interacting with E-cadherin, as suggested by *in vitro* experiments on NCM460 cells^[32,33]. In a cohort of stage III/IV CRC affected patients, Yan *et al.*^[34] found a significant correlation between the presence of *F. nucleatum* and tumor progression also in relation to chemotherapy efficacy, suggesting its role as a biomarker for a

therapeutic approach improvement. Panebianco *et al.*^[35] also confirmed this link. Moreover, the same authors underlined that not only gut bacteria, but also the intratumor ones, may influence patients' response toward chemo- and immuno-therapeutic drugs and *vice versa*^[36].

More recently, it has emerged that EMT can also be promoted by *Porphyromonas gingivalis*, an anaerobe frequently found in chronic periodontitis. In fact, it has been observed that this bacterium causes an early EMT of chronically infected human oral epithelial cells (OECs) that, through PI3K/Akt activation, ultimately lead to the loss of E-cadherin and to the accumulation of β -catenin, together with an increased expression of Zeb1, vimentin, MMP-2, -7, and -9. Moreover, upon stimulation with this bacterium, OECs migratory capacity increases proportionally^[37-39].

Both *F. nucleatum* and *P. gingivalis* determine an enhanced transcription of mesenchymal markers, an increase of TGF- β 1, TNF- α and EGF and a downregulation of those associated to epithelial layer integrity, as evidenced by transepithelial electrical resistance measures^[40].

Recently, a new tumor metastasis model has been proposed. Till now it was supposed that epithelial-like cancer cells acquire mesenchymal features in order to produce metastasis by exploiting the vascular system, and then switch back to an epithelial phenotype to establish the new tumor. The existence of a stable population of hybrid epithelial/mesenchymal cells, possessing epithelial and mesenchymal characteristics with both tumorigenic and metastatic properties, has been hypothesized^[41,42]. Moreover, in predisposed individuals, with defects in the innate immunity response or with a specific epigenetic background, bacterial or viral infections could lead to EMT, causing a chronic inflammatory state, through the activation NF- κ B and MAPK^[43]. Considering this and the emerging role of the microbiota on EMT and cancer progression, more rational and shared laboratory models should be used to better investigate the microbiota composition^[44] and its relationship with the human host.


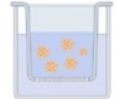

IN VITRO EPITHELIAL MODELS FOR STUDYING THE HOST-PATHOGENS INTERACTIONS

The first studies focused on host-pathogens connections started in the 1970s with Todaro^[45] and Taylor-Robinson^[46], who described the interactions between oncogenic viruses and human cells, and mycoplasma pneumonia and ciliated tracheal epithelium, respectively. Since then, *in vitro* models acquired popularity in the microbiology field, due to their reproducibility, higher versatility and high-throughput data acquisition respect to *in vivo* models^[47] [Table 1]^[5,16,38,45-62]. Undeniably, due to the improvement in imaging and screening techniques and to the *in vitro* models features, nowadays it is possible to directly analyse characteristics such as: specific surface-adhesion processes (i.e., biofilm formation), spreading capabilities, immune system escapes aptitudes, migration in 3D matrices, and host's cell-type-specific interactions in general.

The development of functional co-culture systems mostly depends on their ability to allow the physiological growth of cellular components in optimized culture media inside trans-well devices or microfluidic chambers. In this regard, Di Giulio *et al.*^[50] developed a model in which *Streptococcus mitis* was grown with human gingival fibroblasts in saliva sterilized stocks enriched with 1% sucrose as culture medium. With this system, the authors demonstrated that *S. mitis* can penetrate within fibroblasts, via a FAK-integrin β 1-vinculin-actin mediated process, which is modulated by the saliva and the microenvironment themselves.

Sterilized artificial saliva has also been used in more complex models such as the multi-culture trans-well system developed by Millhouse *et al.*^[51], in which a complex multispecies biofilm, composed by *S. mitis*, *F. nucleatum*, *P. gingivalis* and *Aggregatibacter actinomycetemcomitans*, was grown on a glass slide and co-cultivated in a trans-well together with immortalized oral keratinocytes (OKF6-TERT2). With this system, these authors validated the efficacy of commercially available oral hygiene products.

Table 1. Epithelial study models' overview (images created with BioRender.com)

Epithelial study models	Since	Pros	Cons	Ref.
2D cultures 	1920s	<i>In vitro</i> reproducibility; versatility; high-throughput data acquisition	Inability to reproduce the <i>in vivo</i> context; poor biological complexity; lack of the 3D; poor data reliability; no cell-cell interaction	Todaro <i>et al.</i> ^[45] Bergmann <i>et al.</i> ^[47]
<i>In vivo</i> models 	1980s	Suitability for: (1) new therapies and preventative treatments testing; (2) biological processes understanding	Inability to recapitulate the human tissue context; time-consuming procedures; high costs; requirement of specific manual skills; ethical issues	Taylor-Robinson <i>et al.</i> ^[46] Pasupuleti <i>et al.</i> ^[48]
3D spheroids 	1980s-90s	Fully humanized models; no animal sacrifice required	No tissue organization; unsatisfactory biological complexity; insufficient data reliability; lack of cell-microenvironment interactions	Melissaridou <i>et al.</i> ^[16] Bergmann <i>et al.</i> ^[47] Sawant <i>et al.</i> ^[61]
3D organotypic cultures 	1990s-2000	Fully humanized models; no animal sacrifice required; capacity to mimic the <i>in vivo</i> context: recapitulation of epithelial strata, cytochrome differentiation, EMT markers expression and carcinogenic process; possibility of implementation with the vascular counterpart, macrophages and T-cells; permissiveness to the physiological growth of cellular components; appreciable biological complexity; suitability for the study of pathogen-induced cells modification and host tissue-microbial agents interactions; more reliability and cheaper to antiviral drugs properties study than xenografts; reliability of the data	Model management complexity; inability to mimic long-term conditions	Squarzanti <i>et al.</i> ^[51] Sztukowska <i>et al.</i> ^[38] Riedl <i>et al.</i> ^[49] Di Giulio <i>et al.</i> ^[50] Millhouse <i>et al.</i> ^[51] Bradbury <i>et al.</i> ^[56] Genovese <i>et al.</i> ^[53] Banerjee <i>et al.</i> ^[54] Chow <i>et al.</i> ^[55] Spurgeon <i>et al.</i> ^[52] Hogervorst <i>et al.</i> ^[57] Fuller <i>et al.</i> ^[58] de Carvalho Dias <i>et al.</i> ^[59] Zhang <i>et al.</i> ^[60] Dabija-Wolter <i>et al.</i> ^[62]

EMT: epithelial mesenchymal transition

Despite these findings and the evidences on the cytoskeletal modifications, the impaired proliferation rate and the gene expression changes occurring in cells infected by bacterial or viral pathogens^[50,63-66], some limitations arise when more complex evaluations are required. According to the literature, the microenvironment highly modifies cells behaviour; therefore, the lack of the “3D” in many *in vitro* models reduces the transferability potential of the *in vitro* data^[52]. This is particularly true for epitheliotropic viruses, such as HPVs, for which the intracellular expression of viral proteins is conditioned by the epithelial renewal/stratification process that cannot be detected within 2D cultures.

In fact, most 3D models enable not only viral life cycle, but also virus induced cellular modifications. They are generally constituted by HPV infected keratinocytes, cultivated onto fibroblast repopulated matrices and let to stratify at the air-liquid interface in specific culture media. They can be easily analysed by western blot, histology, high throughput sequencing and protein-protein interaction assays.

These models made possible to identify novel pro-oncogenic co-factors, such as p130^[53] or 53BP1^[5], important for investigating novel antiviral targets.

3D RAFT (real architecture for 3D tissue) cultures also allowed to elucidate the effect of HPV chronic infection onto eukaryotic cells mitosis; as showed by Banerjee *et al.*^[54], HPV DNA amplification occurs during host's cell G2 phase, despite DNA eukaryotic amplification normally occurs in the S phase; in particular, they elucidated the key role of E7 in forcing host cells permanence in the G2 phase via cyclin B1 cytoplasmic accumulation.

Additionally, RAFT cultures are a more reliable and cheaper solution to study antiviral drugs respect to xenografts in SCID mice, since they are more suitable to be analysed by *in situ* hybridization and RT-qPCR techniques^[55].

Moreover, they can allow the study of host-virus interactions in absence of other cell types or tissues that could hide fundamental pathways promoted by the pathogen, especially when the studies require miRNA or EMT analysis.

As previously described, HR-HPVs E6 and E7 proteins interact and inhibit host p53 and pRb onco-suppressors respectively. Recently, the idea that E6 and E7 can also bind host miRNA or miRNA regulators has been evaluated^[67]. Indeed, despite HPV inability to encode for miRNAs, HPV mediated indirect regulation of both onco-suppressive or tumorigenic miRNAs may offer the opportunity to identify novel therapeutic targets.

RAFT CULTRES, DRUG RESISTANCE AND EMT RESEARCH

Regarding drug resistance and EMT markers expression analysis, RAFT cultures can overcome both 2D and spheroid cultures limitations. Melissaridou *et al.*^[16], working with 5 HNSCC-derived cell lines grown in both 2D and spheroids assays, observed that, while 2D eukaryotic cells and spheroid conformation changes the receptor topography and gene expression, and erases the drug resistance pathways that occur *in vivo*, 3D cultures can better mimic what happens during *in vivo* therapies and allow to study EMT expression markers such as CDH1, NANOG, SOX and EGFR.

However, when epithelial models are necessarily to be used, spheroids utility is limited, because of the less reliability of the data they produce respect to those obtained with the organotypic models. In fact, although they are both made up of epithelial cells, spheroids morphology and structure are too much simplified and do not reproduce the mucosal epithelium in such a representative way as the 3D epithelial RAFTs do. Therefore, compared to 3D RAFTs, the spheroids are less performant for studies on the specific interactions that occur in a well-defined *in vivo* environment.

On the other hand, RAFT cultures can be built with both isolated healthy or tumour/cancer associated keratinocytes and fibroblasts, able to better elucidate the interactions between stromal and epithelial compartments that are at the basis of the initial steps of the carcinogenetic process^[56]. Moreover, they recapitulate all the epithelial layers, the physiological cytokeratin differentiation and the carcinogenic process.

By using *in vitro* reconstructed epithelial systems, Hogervorst *et al.*^[57] highlighted the importance of the papillary-reticular fibroblasts switch. In fact, when epithelial cultures are grown in presence of switched fibroblasts, the expression of CAF associated markers (α -SMA and vimentin) and EMT markers (SNAIL2, N-cadherin and ZEB1) increase, thus suggesting that the fibroblasts switch could be considered an indicator of squamous cell carcinoma (SCC) progression. Similarly, Fullar *et al.*^[58] proved that HPV16 induces the modification of connective matrix that, by the action of epithelial Matrix Metalloproteinase MMP-7 and fibroblast-produced MMP-2, loses collagen and fibronectin in favour of laminin, thus facilitating EMT and carcinogenic events. Comparable effects on MMP super-families were also observed in other HR-HPV positive models^[68].

In agreement with the above, the last trend is to reproduce tissue models with the whole organ complexity^[69]; in fact, the models have been implemented with the vascular component, with macrophages and T-cells, thus allowing to further improve the knowledge in the microbiology field^[59-61].

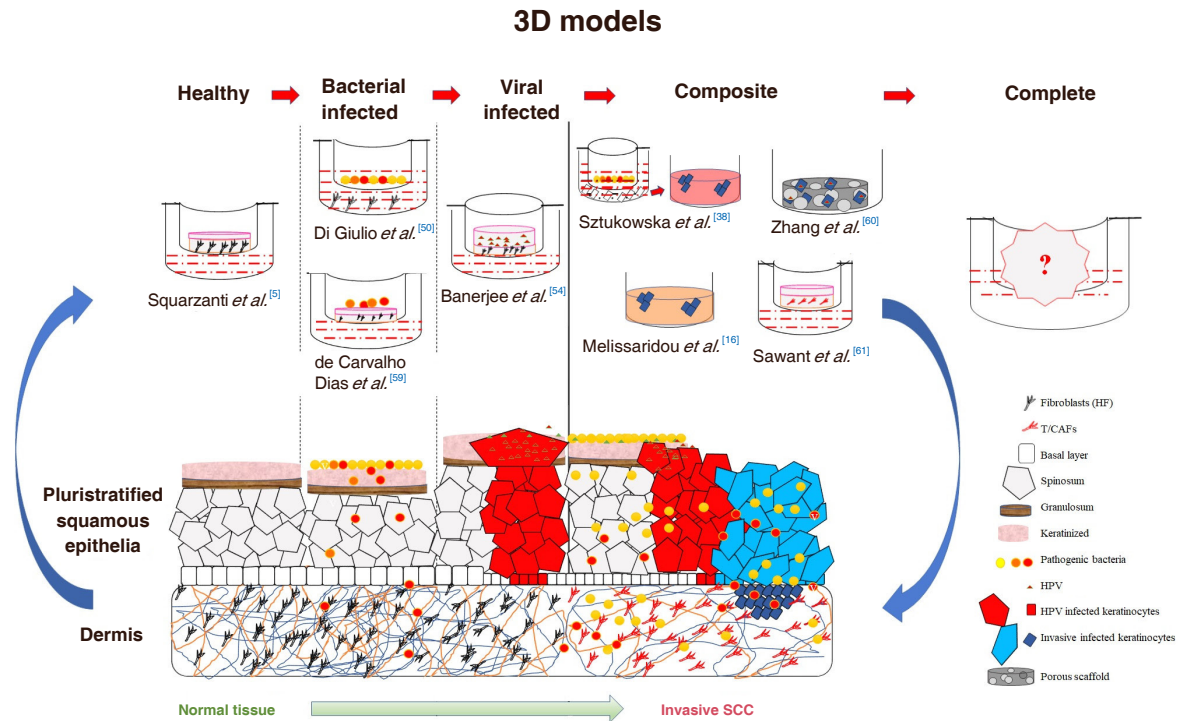


Figure 1. Schematic representation of SCC progression achievable by using *in vitro* 3D infected models. SCC: squamous cell carcinoma; HPV: human papillomavirus

In line with the technological progress, in the last few years several authors have focused their attention on the bacterial-induced EMT. For instance, Sztukowska *et al.*^[38] showed that *P. gingivalis*, associated with OPSCC development, is able in a gingival 3D equivalent to induce the expression and the nuclear re-localization of ZEB1, a well-known EMT transcriptional factor. This effect is reduced by the lack of the bacterial fimbrial FimA protein, while it is enhanced by the co-infection with *F. nucleatum* or *S. gordonii*. A similar study conducted by Lee *et al.*^[37] showed that the carcinogenic properties of *P. gingivalis* are unrelated to the presence of chronic inflammation periodontitis. In this study, the authors showed that in addition to ZEB1, also glycogen synthase kinase-3 beta (p-GSK3 β), Slug, Snail, and vimentin usually increase during EMT under the influence of *P. gingivalis*. Moreover, the co-infection with *F. nucleatum* induces the loss of adhesive molecules such as E-cadherin and β -catenin in cancer-committed cells and the overexpression of MMP-2, -7 and -9 as showed for HPV infection^[69]. These findings confirm those obtained in an established periodontitis-associated oral tumorigenesis murine model used to demonstrate that both *P. gingivalis* and *F. nucleatum* induce tumorigenesis independently from the inflammatory status^[70]. In this latter study, the interaction of bacteria with the keratinocytes Toll-like receptor and the consequent activation of the IL-6-STAT3 axis was possibly involved in the tumorigenesis process.

The above cellular models can be also used to quantify the microorganisms' migration potential, as made by Dabija-Wolter *et al.*^[62] who *in vitro* reconstructed a gingiva to evaluate the migration of *F. nucleatum* through a keratinized squamous epithelium without destroying it, while establishing a chronic infection [Figure 1].

Summarizing, in the last few years, thanks to their suitability to exceed the limitations of monolayer adherent cell cultures, as well as of *in vivo* models and spheroids, 3D epithelial co-cultures have become indispensable tools for investigating the interactions between microbial agents and their hosts.

Although several questions are still open, soon further improvements might help to generate microenvironments that also mimic the bacteria and virus migratory potential.

CONCLUSION

Overall this review highlights the importance of the *in vitro* 3D modeling research field for the study of microbial induced epithelial carcinogenesis. Yet, further improvements are still needed to reach the level of complexity of the real tissue. These models will hopefully help to better understand the respective roles of virus and bacteria in HR-HPV-related cancers.

DECLARATIONS

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Authors' contributions

Wrote part of the paper and prepared Table 1: Squarzanti DF

Wrote part of the paper and prepared Figure 1: Sorrentino R

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All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

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Review

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CXCR4 signalling, metastasis and immunotherapy: zebrafish xenograft model as translational tool for anti-cancer discovery

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Abstract

Cell-to-cell communication guarantees homeostasis in a multi-cellular organism. Cancer-to-microenvironment communication sustains malignant growth and dissemination. Whereas the accumulation of mutations is at the origin of malignant cell transformation and neoplasia onset, the interaction between cancer and the surrounding stroma, specifically immune cells, influences the balance between tumour regression and tumour progression. To study how the interaction between cancer and stromal cells is disadvantageous or beneficial for tumour progression, the use of a transparent *in vivo* model bears important research potentials. Zebrafish has been increasingly used as animal model to study tumour biology. The use of transparent zebrafish embryos, with fluorescent endothelial and immune cells, allows the visualization of cell-to-cell interaction, among host cells themselves and between zebrafish stroma and implanted human cancer cells. Here, we summarise our findings on the role of CXCR4 signalling in tumour progression, considering its signature both on cell autonomous and host dependent mechanisms. Finally, we address the translational impact of targeting CXCR4 signalling in cancer and the tumour microenvironment for the treatment of metastatic cancer.

Keywords: CXCR4, cancer, metastasis, neutrophils, zebrafish, immunotherapy



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THE TUMOUR MICROENVIRONMENT

Tumours are in constant interaction with the surrounding microenvironment. The tumour microenvironment consists of stromal cells such as cancer-associated fibroblasts (CAFs), endothelial cells, mesenchymal stem cells (MSCs), tumour-associated macrophages (TAMs) and neutrophils (TANs), adaptive immune cells and extracellular matrix (ECM)^[1]. The interaction between cancer and stroma cells results in either tumour promoting or inhibiting effects and the tumour microenvironment differentially contributes to the efficacy of cancer therapies^[2]. Tumour cells engage cells from the microenvironment, either educating resident stromal cells or inducing the recruitment of distal ones to further support malignant growth, motility and dissemination. Along with the angiogenic switch, where endothelial cells are educated by malignant cells to form new vasculature to provide oxygen and nutrients, the immunosuppressive switch phenomenon takes place: the polarization from pro-inflammatory to anti-inflammatory neutrophils and macrophages (N1 to N2 and M1 to M2), where the sub-type 2 associates with a tumour-promoting function, links to immunosuppression, characterized by reduced cytotoxic T cell and enhanced T regulatory (Treg) and myeloid-derived suppressor (MDSCs) cell infiltration^[3]. Interestingly, the cooperation between different subsets of leukocytes and its role in cancer metastases has been recently reported^[4]. The plasticity phenomenon in the microenvironment has been described also for fibroblasts, which respond to a neoplastic lesion in a similar fashion as to a never healing wound^[5]. The interaction between tumour and the microenvironment is controlled by a plethora of signalling molecules, such as chemokines, and their complex networking in cancer requires further understanding to inhibit tumour development.

CXCL12-CXCR4 AXIS IN CANCER AND THE TUMOUR MICROENVIRONMENT

Chemokines are chemotactic cytokines that guide directional cell migration in development and disease and more than 50 chemokine ligands and 18 chemokine receptors have been described in *Homo sapiens*^[5]. Chemokines are classified into four classes, depending on the presence and position of the conserved cysteine residues (CXC, CC, (X)C and CX3C) at the N-terminus, involved in the formation of disulphide bonds between the first and third or second and fourth cysteines^[6]. The chemokines belonging to the CXC subgroup are further classified into angiogenic ELR+ and angiostatic ELR-, whether they are positive or negative for the Glu-Leu-Arg (ELR) motif at the N-terminus^[7,8]. Chemokine ligands can bind multiple chemokine receptors, which possibly work in concert to control signalling activation and inhibition^[8].

CXCR4 is a seven-transmembrane, chemokine, G-protein coupled receptor. The chemokine CXCL12 binds both CXCR4 and CXCR7 receptors in order to guide a directional and collective migration of cell primordia, during the formation of sensory organs in zebrafish^[9-11]. CXCL12 binding to CXCR4 induces the dissociation of the G protein $\alpha\beta\gamma$ trimer and activation of PI3K/AKT/mTOR, MAPK, PKA and PLC/ Ca^{2+} pathways. Moreover, MAPK cascade activation and CXCR4 internalization occur via β -Arrestin, independently from G-proteins [Figure 1A]. In addition, CXCR4 can form homodimers, activating the JAK/STAT pathway and Ca^{2+} release from intracellular storage into the cytoplasm [Figure 1B]. CXCR4 can also form heterodimers with CXCR7. Whereas CXCR4 is internalized and degraded after CXCL12 binding, CXCR7 is internalized and recycled to the plasma membrane. Via β -Arrestin, CXCR7 has either CXCL12 scavenging functions or triggers MAPK signalling activation [Figure 1C]. CXCL12 signalling via CXCR4 and CXCR7 controls cell chemotaxis and migration as well as cell proliferation and survival^[12,13].

In cancer, malignant cells acquire higher CXCR4 levels, compared to normal tissues, and are found to preferentially metastasise in organs where CXCL12 is secreted, in line with the “seed and soil” theory^[14]. Enhanced CXCR4 signalling has been identified in several malignancies such as gastrointestinal tumours^[15], melanoma^[16], basal cell carcinoma^[17], head and neck squamous cell carcinoma^[18], lung cancer^[19], breast^[20] and ovarian^[21] tumours, renal cell carcinoma^[22], prostate cancer^[23], glioblastoma multiforme

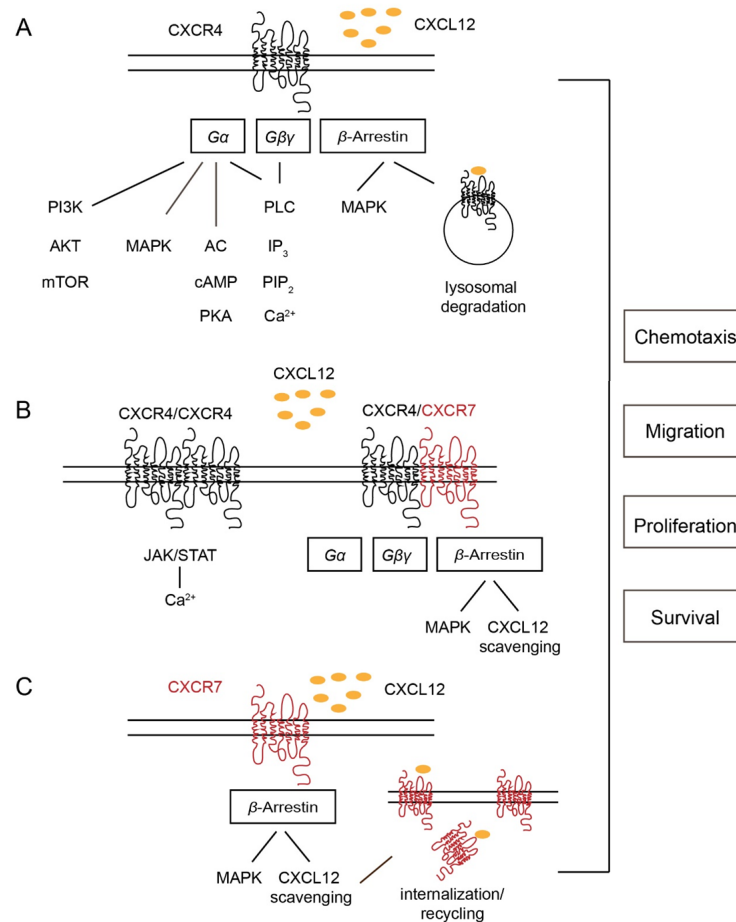


Figure 1. CXCL12-induced signalling via CXCR4 and CXCR7. (A) CXCL12 binds to CXCR4, inducing Gα and Gβγ dissociation and activation of PI3K, MAPK, AC, and PLC signalling pathways. CXCL12 binding to CXCR4 activates β-Arrestin, leading to MAPK signalling pathway activation or receptor internalization. (B) CXCR4 can form homo- and hetero-dimers with CXCR7. (C) CXCL12 binding to CXCR7 induces, via β-Arrestin, MAPK signalling activation, or CXCL12 scavenging, through receptor internalisation and recycling to the plasma membrane. CXCL12-mediated signalling plays a role in cell chemotaxis, migration, proliferation and survival. PI3K: phosphatidylinoside 3-kinase; MAPK: mitogen-activated protein kinases; AC: adenylyl cyclase; PLC: phospholipase C

(GBM)^[24], Ewing sarcoma^[25] and leukemia^[26]. Elevated CXCR4 levels result in increased cell proliferation, dedifferentiation, migration and metastatic spreading of tumour cells, cancer stem cell (CSC) maintenance and it has been associated with the development of tumour resistance towards conventional therapies, leading to poor patient prognosis^[27].

CXCR4 is expressed by both cancer cells and surrounding stromal cells [Figure 2]. The recruitment of stromal cells expressing CXCR4 can be guided by the secretion of CXCL12 by cancer cells themselves or other stromal cells, such as MSCs and CAFs^[28]. Moreover, CXCL12 secreted by CAFs displays effects on tumour cells, enhancing invasive potential^[29] and functioning as a protective shield against T cells, boosting immune escaping mechanisms^[30]. In this context, pharmacological inhibition of CXCR4, resulted in redistribution of CD3+ T cells within the “cancer cell nest”, as defined by the authors, causing reduced cancer cell growth and improved response to check-point inhibitors^[31]. CXCR4 is involved in leukocyte trafficking, hematopoietic stem progenitor cells homing and neutrophil retention in the bone marrow during homeostasis, inflammation, infection and cancer^[12,32-35]. Infiltration of CXCR4hi neutrophils associates with faster tumour growth and angiogenesis in IFNβ deficient mice, injected with melanoma and fibrosarcoma^[36]. CXCR4hi macrophages have been identified in CXCL12-enriched tumour

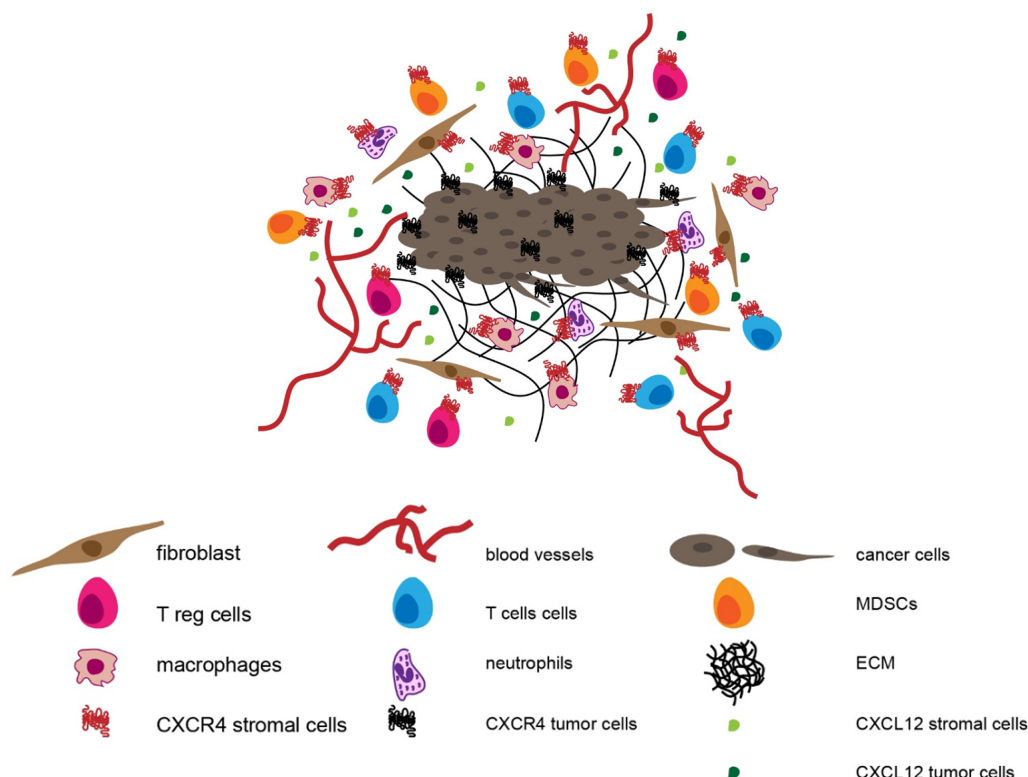


Figure 2. CXCR4 drives the interaction between cancer and stromal cells. The CXCR4-CXCL12 axis signals in a bi-directional fashion. CXCR4 is expressed by both tumour cells and cells that form the surrounding stroma [fibroblast, T cells, T reg cells, myeloid derived suppressors cells (MDSCs), macrophages and neutrophils], embedded in the extracellular matrix (ECM). The CXCR4 cognate ligand CXCL12 is secreted by both cancer cells and cells in the microenvironment

areas after chemotherapies and are suggested to display pro-angiogenic functions that drive tumour-relapse^[37]. Moreover, CXCL12 expressing glioblastoma cells induce VEGF production and angiogenesis in microvessel enriched areas with high CXCR4 levels^[38]. In addition, CXCR4-expressing peripheral blood monocytes respond to CXCL12-secreting multiple myeloma (MM) tumour cells and acquire M2 associated properties^[39]. Finally, the inhibition of CXCR4 signalling by oncolytic virotherapy limits the infiltration of Treg, decreasing immunosuppression^[40].

Considering the major and intricate role of this chemokine receptor in cancer, its targeting represents an important pharmacological approach that is currently under development, through the use of CXCR4 antagonists, antibodies and CXCL12 binding agents. Importantly, the role of the stromal CXCR4 signalling needs to be considered in drug treatments that target CXCR4 to inhibit cancer spreading.

In 2018, the Nobel prize in Physiology and Medicine was awarded to J.P. Allison and T. Honjo for the development of immune-checkpoint blockade^[41]. This revolutionary discovery clearly underlines the well-known pivotal role of the immune system in cancer. Inhibition of CXCR4 signalling has been found to improve the efficacy of immunotherapies in metastatic breast cancer, by alleviation of desmoplasia and increased T cell infiltration in preclinical *in vivo* models^[42].

Limiting cancer spreading by targeting CXCR4 signalling in the tumour microenvironment is a promising approach that requires further investigations to become an alternative therapeutic form of intervention.

ZEBRAFISH XENOGRAFT AS A MODEL TO STUDY CANCER

Research performed in pre-clinical *in vivo* models is constantly under development to provide further insights into the communication between tumour and the surrounding microenvironment. Zebrafish (*Danio rerio*) is a tropical freshwater teleost, increasingly used to study a range of disease processes^[43] as well as being an excellent tool for the study of development. Several important advances in understanding of cancer and inflammation have arisen from studies in zebrafish^[44-46]. The rapid and external development of transparent embryos^[47], availability of reporter lines with traceable fluorescent cells^[48-50], ease of genetic manipulation^[51,52] and pharmacological approaches^[53] make the zebrafish an excellent *in vivo* model to visualise single cell interactions in real time and to uncover the signalling mechanisms involved, on a whole organism level. Zebrafish is increasingly used as a model organism to study cancer^[54]. There is high conservation of oncogenes and tumour-suppressor genes between zebrafish and human therefore data collected in zebrafish are relevant for humans^[55]. The histology of zebrafish tumours has been shown to be highly similar to tumours found in human cancers^[56]. Moreover, zebrafish is a valuable tool to study drug discovery in the context of cancer research^[57,58]. Zebrafish larvae can absorb small molecular weight compounds from water, which is advantageous when screening for anti-cancer compounds^[59]. The experimental costs are low and procedure are simple and fast. This accounts for the experimental increase in the use of zebrafish in drug discovery during the last two decades in a time- and cost- effective manner. For melanoma, a presently on-going phase I/II clinical trial of Leflunomide combined with vemurafenib is the first to arise from initial screen in zebrafish. To study human cancer metastasis, our group generated a xenotransplantation model of experimental micrometastasis^[60,61]. Human tumour cells engrafted into the blood circulation of 2-day-old zebrafish embryos induce angiogenesis and form micrometastasis sustained by neutrophils and macrophages, nearby hematopoietic sites^[60]. In particular, tumour-induced angiogenesis, metastasis formation and relative chemical approaches to inhibit these processes have been studied using zebrafish as a xenotransplantation model, complementing current knowledge developed through the use of *in vitro* and other *in vivo* models^[62]. Upon localised or haematogenous engraftment of cancer cells, zebrafish xenografts allow qualitative and quantitative assessment of tumour burden and tumour-microenvironment interaction, representing a powerful pre-clinical model to unravel cancer mechanisms and to develop new therapeutic strategies^[61]. In particular, alongside murine models, the use of PDXs in zebrafish has the potential to be used in personalised medicine^[63-66], with the advantage of requiring less tumour material and shorter times for the monitoring of tumour development^[57]. Several studies have shown that the combined use of zebrafish and murine models paves the way towards important insights to elucidate the biology of metastatic cancers and the development of new treatments^[67-71]. Therefore, the zebrafish xenograft model bears the potential to elucidate crucial kinetics and key mechanisms that regulate tumour-microenvironment interaction and ultimately support tumour spreading.

CELL-AUTONOMOUS CXCR4 SIGNALLING: THE CXCR4 ANTAGONIST IT1T IMPAIRS EARLY HUMAN METASTATIC EVENTS, IN A ZEBRAFISH XENOGRAFT MODEL WHERE THE INTERSPECIES CROSS-TALK TAKES PLACE

Chemokines direct tumour and stromal cell bidirectional migration^[72]. CXCR4 plays a physiological role in hematopoiesis^[73,74], leukocyte trafficking^[75-77], cell migration and embryo development^[78], as well as a pathological function in HIV pathogenesis^[79], WHIM syndrome^[80] and cancer^[81,82]. In addition to its cognate ligand CXCL12, CXCR4 can bind ubiquitin^[83], macrophage migration inhibitory factor^[84-86] and CXCL14^[87]. The CXCR4-CXCL12 signalling axis is known to play a critical function in cancer cell spreading, when tumour cells expressing high levels of CXCR4 communicate with CXCL12-secreting stromal cells of distant organs that function as metastatic and secondary growth “soils”^[88].

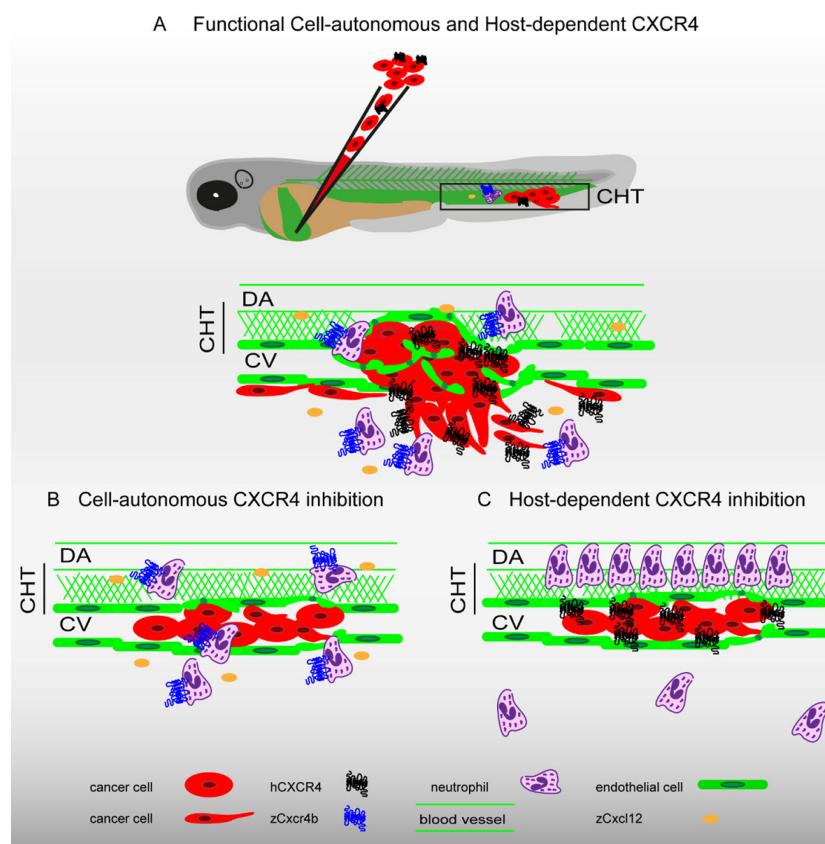


Figure 3. Role of cell-autonomous and host-dependent CXCR4 signalling in experimental metastasis formation in the zebrafish xenograft model. A: inoculation of human tumour cells into the blood circulation of zebrafish embryos results in experimental metastasis formation, characterized by tumour cell aggregates in the blood vessels, and extravasation and invasion in the surrounding tissue, in the region of the caudal hematopoietic tissue (CHT). During early metastatic events, endothelium alteration takes place and neutrophils localize in the surrounding of the tumour. The CHT is a vascular plexus in the tail fin between the DA and the CV and is a hematopoietic site; B: upon disruption of the tumour cell-autonomous CXCR4 signalling, cancer cells are unable to initiate early metastatic events, while surrounded by immune cells; C: the same inhibition of experimental metastasis formation occurs upon disruption of the host-dependent CXCR4 (Cxcr4b) signalling. Neutrophils are preferentially retained in the CHT and their recruitment at the metastatic site is impaired upon Cxcr4 signalling inhibition

We have previously shown that the impairment of the cell autonomous CXCR4 signalling blocks triple-negative breast cancer (TNBC) early metastatic events in the zebrafish xenograft model [Figure 3A and B]. In our model, human triple-negative breast cancer cells, derived from bone metastases developed in a mouse model, were implanted directly into the blood circulation of zebrafish embryos. Using this model, the formation of the primary tumour and the initial steps of metastasis (local invasion and intravasation into the blood circulation) were by-passed. Tumour cells, inoculated into the blood circulation, were found to form early metastases, by adhering to the endothelial wall, forming aggregates and invading the local tail fin tissue. Experimental metastases occurred in proximity of the caudal hematopoietic tissue, an intermediate site of hematopoiesis and a functional analogue of the fetal liver during mammalian development. This observation was in line with breast cancer metastasis formation in the bone^[89,90]. In addition, others have also shown that tumour-derived CXCR4 signalling, in concert with the transcription factor Pit-1, drives tumour growth, in a zebrafish model^[91,92]. Moreover, we demonstrated that the CXCR4 signalling functions across human and zebrafish systems, because CXCR4-expressing human cells respond to zebrafish Cxcl12 ligands and Cxcr4-expressing zebrafish cells migrate towards human CXCL12, showing that the zebrafish xenograft model is a valid approach to study human tumours. Taking advantage of the same *in vivo* model, where the interspecies crosstalk is validated, we propose a recently described CXCR4 antagonist, IT1t, as a possible therapeutic to inhibit early metastasis of TNBC^[93]. In particular, breast

cancer cells pre-treated *in vitro* with the CXCR4 antagonist IT1t displayed reduced metastatic potential in zebrafish. Impaired tumour burden *in vivo* was also observed upon genetic inhibition of tumour-derived CXCR4 or microenvironment-dependent Cxcl12. In conclusion, we showed that the xenograft approach in zebrafish is a valuable model to study human tumours as the CXCR4 signalling functions in human cells upon zebrafish CXCL12 stimulation and vice versa CXCR4-expressing zebrafish cells respond to the human cognate chemokine.

HOST-DEPENDENT CXCR4 SIGNALLING: CXCR4 CONTROLS THE TUMOUR METASTATIC NICHE PREPARATION, BY REGULATING INTRINSIC NEUTROPHIL FUNCTION AND RESPONSE TO CANCER CELLS

Immune cells are programmed to recognise and eliminate transformed cells. However, cancer cells have evolved mechanisms that reprogram the immune defence and make the foe-to-friend switch an important support for survival and progression. The combination of chemotherapy and immunotherapy is a current strategy in the clinic^[94]. Galluzzi *et al.*^[95] have recently reviewed anti-cancer therapies that re-activate the immune system, such as tumour-targeting antibodies, adoptive cell transfer and oncolytic viruses (all classified as passive immunotherapy), dendritic cell-based immunotherapies, anti-cancer vaccines, immune-stimulatory cytokines, immunomodulatory antibodies, inhibitors of immunosuppressive metabolism, pattern recognition receptor agonist, and immunogenic cell death inducers (all classified as active immunotherapy). Antibodies against CXCR4 are included in immunotherapeutic agents that skew the balance between M2/M1 TAMs toward the pro-inflammatory and anti-tumour M1 phenotype^[95].

We have recently shown the role of the host dependent CXCR4 signalling in supporting early metastatic events in the zebrafish xenograft model. Previous work from our group has shown that neutrophils are involved in the metastatic niche preparation by conditioning the ECM during their apparent random walk in the transmigration from the CHT (caudal hematopoietic tissue, transient hematopoietic site) to the tail tissue of zebrafish embryos^[60]. Because CXCR4 is known to regulate the retention of hematopoietic stem progenitor cells (HSPCs) and differentiated leukocytes in the bone marrow in mammals^[96], and is highly expressed in zebrafish myeloid cells^[97], we hypothesised that CXCR4 signalling plays a role in controlling intrinsic neutrophil motility in physiological conditions. We found that neutrophils display altered motility and their number fluctuates during embryo development, leading to the conclusion that CXCR4 regulates neutrophil development in zebrafish. Moreover, a link between CXCR4 signalling and neutrophil response during inflammation has been recently described^[98]. In our model, the neutrophilic response towards cancer cells was also altered in zebrafish mutants with a non-functional *Cxcr4* (*Cxcr4b*). We identified a population of neutrophils that was mainly retained in the CHT and a population of neutrophils that even if moving in the tissue, displayed the inability to infiltrate tumour cell aggregates in the tail fin of *Cxcr4b*-null mutants. In the surrounding of cancer cells, *cxcr4b*-expressing neutrophils reduced their speed in motility, while *Cxcr4b*-null neutrophils maintained similar speeds as in neutrophils that had not been challenged by cancer cells. Therefore, we propose that *Cxcr4* controls neutrophil development and response to tumour cells, initiating early metastatic events [Figure 3A and C]. RNA sequencing performed on sorted neutrophils from wild-type or *cxcr4b*^{-/-} zebrafish larvae supported our conclusion that motility and adhesion are altered when neutrophils lack a functional *Cxcr4* signalling^[99]. In conclusion, we propose that these alterations are responsible for the impaired tumour niche preparation and inhibition of early micrometastasis formation in different types of cancer.

CONCLUSION

Cancer is a complex, multi-step disease and the second leading cause of death worldwide [1 in 6 deaths is due to cancer, 9.6 million cancer-related deaths in 2018 (www.who.int, October 2019)]. Patients diagnosed

with primary tumours are treated, when possible, with surgery. However, metastasis can occur years after surgical intervention^[100]. Metastatic cancer associates with poor patient prognosis and represent a major challenge for clinical research. Chemotherapy is often the pharmacological choice to treat cancer, although side effects alter normal cell physiology and affect patient life quality. Moreover, cancer relapse and therapy resistance associate with poor prognosis. Progress in biomedical research has shown that targeting cancer cells is not the only therapeutic option. The interaction between tumour and surrounding stroma supports cancer survival and spreading, representing therefore a possible new treatment strategy^[101]. Here, we describe the use of the zebrafish xenograft model to study early stages of experimental micrometastasis formation, engrafting fluorescent tumour cells in transparent zebrafish embryos with fluorescent endothelial and immune cells. We propose that targeting CXCR4 signalling on cancer cells or in the tumour microenvironment is a valid approach to inhibit metastatic cancer and suggest that anti-CXCR4 therapy might have double treatment benefits. In addition, therapeutic modulation of the immune system might result in the reinforcement of the immune defence against cancer. However, we suggest that treatments designed to target malignant cells might affect tumour microenvironment intrinsic functions. Specifically, the intrinsic physiological role of myeloid cells can be affected by cancer treatment, resulting in an inability to mount a functional anti-cancer response or, on the other hand, in the ability to mount a tumour-supportive response.

DECLARATIONS

Authors' contributions

Wrote and reviewed the manuscript: Tulotta C, Snaar-Jagalska BE

Availability of data and materials

Not applicable.

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Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Monoclonal antibody pharmacogenomics in cancer treatment

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Abstract

Conventionally, in the pharmacokinetic/pharmacodynamic analysis of small molecule compounds such as cytotoxic anticancer drugs, polymorphism analysis of genes related to absorption, distribution, metabolism, and excretion has been performed in addition to the analyses of blood concentrations of drugs. Such pharmacogenetic factors play an important role in predicting therapeutic effects and adverse events and in the proper use of drugs. With the recent launch of immune checkpoint inhibitors (ICIs) and the rapid development of antibody-drug conjugates (ADCs) currently underway, there is no doubt that antibody drugs, which are large molecule compounds, will become key drugs in anticancer drug treatment. However, the pharmacokinetic and pharmacodynamic analysis of antibody drugs is still not sufficient, and further elucidation of factors and mechanisms affecting their dynamics in the human body is necessary. Moreover, the pharmacogenomic factors of antibody drugs have not yet been fully studied. There are many factors that should be clarified, such as factors that regulate the host immune response in ICI therapy and the effects of ATP-binding cassette transporter and cytochrome P450 on the payload of ADCs. This review provides an outline of antibody drugs in cancer treatment and summarizes the pharmacogenomic factors of antibody drugs known to date.

Keywords: Pharmacogenomics, antibody drug, antibody-dependent cellular cytotoxicity, immune checkpoint inhibitor, antibody-drug conjugate



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INTRODUCTION

An antibody is a humoral immunity factor produced by B cells as a biological defense against foreign antigens in the living body. Antibodies, i.e., immunoglobulins, are composed of a light chain and a heavy chain, and there are 2 types of light chains and five types of heavy chains^[1]. Immunoglobulins are classified into IgA, IgD, IgE, IgG, and IgM according to heavy chain subtypes, and in human organisms, IgG accounts for 80%. The IgG family includes IgG1, IgG2, IgG3, and IgG4 according to heavy chain subtypes. On the other hand, immunoglobulin receptors are expressed on neutrophils and macrophages and are composed of glycoproteins called Fc receptors. The Fc receptor has subtypes depending on the immunoglobulin to be bound, IgA binds to Fc α receptor, IgD binds to Fc δ receptor, IgE binds to Fc ϵ receptor, and IgG binds to Fc γ receptor. The structure of the heavy chain of IgG differs in its binding affinity to Fc γ receptors, resulting in specific functional properties for each IgG antibody subtype.

For the immunoglobulin function, four major types of mode of action are known: (1) the neutralizing function by binding of antibody to the antigen; (2) the function of opening the cell membrane by activating complement [complement-derived cellular cytotoxicity (CDC)]; (3) the opsonizing effect taken up by phagocytes through binding of antibody to the antigen; and (4) antibody-dependent cellular cytotoxicity (ADCC) that occurs by binding the antibody to NK cells and releasing cytokines. All currently approved antibody drugs are of the IgG subtype and produce their antitumor effects through these functions.

The application of antibodies to cancer treatment was proposed by the bacteriologist Paul Ehrlich around 1900, but their clinical application became practical since the discovery of monoclonal antibody production technology by the hybridoma of Köhler and Milstein^[2] in 1975. Monoclonal antibodies are characterized by having high antigen specificity, producing almost infinite antibodies by hybridomas, and carrying out the same analysis by producing the same antibodies. They also have advantages for anticancer treatment, such as a long half-life, high potency, and low off-target effect.

As a result, monoclonal antibody therapy has increased hope as a “magic bullet”. However, the initial development of monoclonal antibodies using mice or rats has been abandoned one after another because of their short half-lives and high immunogenicity. With such a background, several techniques for modifying antibody formulations have been developed such as chimeric antibody that genetically substitutes the highly antigenic constant region of mouse antibody, humanized antibody that substitutes human immunoglobulin except for the complementary-determining region (CDR) site, and fully humanized antibody. Currently, about 26 antibody drugs have been approved against cancer, and the monoclonal antibodies for cancer treatment will be reviewed from a pharmacogenomic perspective.

CANCER THERAPEUTIC ANTIBODY

As of August 2019, a total of 26 antibody drugs have been approved for cancer treatment by the US Food and Drug Administration (FDA) [Table 1].

The antitumor efficacy of antibody drugs is brought about by any of the 4 functions mentioned above. Of the 26 FDA-approved drugs, 20 are IgG1, one is IgG2, five are IgG4 isotype, and there are no drugs of IgG3 isotype. This is considered to be due to the fact that IgG1, 2, and 4 have a half-life of about 21 days, while IgG3 has a short 7-day half-life. The main features of each IgG subtype are shown in Table 2. ADCC activity, which is dependent on the avidity of IgG and Fc γ R, is strongest in IgG3, moderate in IgG1, and weak in IgG2 and IgG4^[3]. Therefore, a drug that exerts ADCC activity is composed of an IgG1 isotype and an IgG4 or IgG2 isotype for the purpose of neutralizing action or signal inhibition.

Many of the antibody drugs that have been marketed are made to have ADCC activity or ADCC activity and a neutralizing effect as their main antitumor effects. In the 2010s, development of antibody-drug

Table 1. Therapeutic antibodies for cancer treatment

Class	Generic name	Brand name	Subtype	Target	FDA approval	Major indication
Chimeric Ab	Rituximab	Rituxan	IgG1κ	CD20	1997	CD20+ non-Hodgkin lymphoma
	Brentuximab vedotin	Adcetris	IgG1	CD30	2011	Hodgkin lymphoma
	Cetuximab	Erbitux	IgG1κ	EGFR	2004	Colon cancer
	Dinutuximab	Unituxin	IgG1κ	GD2	2015	Neuroblastoma
Humanized Ab	Trastuzumab	Herceptin	IgG1κ	HER2	1998	HER2+ Breast cancer
	Gemtuzumab ozogamicin	Mylotarg	IgG4κ	CD33	2017	Acute myeloid leukemia
	Bevacizumab	Avastin	IgG1κ	VEGF	2004	Colorectal cancer
	Mogamulizumab	Poteligio	IgG1κ	CCR4	2018	CCR4+ Adult T cell leukemia lymphoma
	Pertuzumab	Perjeta	IgG1κ	HER2	2012	HER2+ Breast cancer
	Trastuzumab emtansine	Kadcyla	IgG1κ	HER2	2013	HER2+ Breast cancer
	Obinutuzumab	Gazyva	IgG1κ	CD20	2013	Chronic lymphatic leukemia
	Pembrolizumab	Keytruda	IgG4κ	PD-1	2014	Non-small cell lung cancer
	Elotuzumab	Empliciti	IgG1κ	SLAMF7	2015	Multiple myeloma
	Atezolizumab	Tecentriq	IgG1κ	PD-L1	2016	Urothelial cancer
	Inotuzumab ozogamicin	Besponsa	IgG4κ	CD22	2017	Acute lymphatic leukemia
Human Ab	Panitumumab	Vectibix	IgG2κ	EGFR	2006	Colorectal cancer
	Ofatumumab	Aezerra	IgG1κ	CD20	2009	Chronic lymphatic leukemia
	Ipilimumab	Yervoy	IgG1κ	CTLA4	2011	Malignant melanoma
	Ramucirumab	Cyramza	IgG1	VEGFR2	2014	Gastric cancer
	Nivolumab	Opdivo	IgG4	PD-1	2015	Malignant melanoma
	Necitumumab	Portrazza	IgG1κ	EGFR	2015	Non-small cell lung cancer
	Daratumumab	Darzalex	IgG1κ	CD38	2015	Multiple myeloma
	Olaratumab	Lartruvo	IgG1	PDGFR	2016	Soft tissue sarcoma
	Avelumab	Bavencio	IgG1λ	PD-L1	2017	Merkel cell carcinoma
	Durvalumab	Imfinzi	IgG1κ	PD-L1	2017	Urothelial cancer
Unclassified Ab	Cemiplimab	Libtayo	IgG4	PD-1	2018	Cutaneous squamous cell carcinoma

conjugates (ADCs), in which cytotoxic anticancer drugs are bound to antibody drugs, and immune checkpoint inhibitors (ICIs) that cause binding inhibition of immune checkpoint molecules has been rapidly advancing.

PHARMACOGENOMICS OF CANCER THERAPEUTIC ANTIBODIES

Antibody drugs show anti-tumor effects by binding to antigens *in vivo*, but because they are proteins with a large molecular weight of about 150 kDa, they have complex pharmacokinetic and metabolic pathways that are completely different from small molecule compounds. Small molecule compounds generally have good membrane permeability and a large distribution volume (Vd) because they are distributed in cells^[4]. Since the effects of metabolism and excretion pathways are large for each drug, many pharmacogenetic studies have been conducted on the effects of polymorphisms of cytochrome P450 and ABC transporter on blood concentration levels of drugs. However, in the case of antibody drugs, the Vd is relatively small, they do not undergo metabolism such as by cytochrome P450, and the main elimination route is the digestion of amino acids in cells. For these reasons, they exhibit very different pharmacokinetics from small molecule compounds, and there are still many unknowns. A detailed description of the pharmacokinetics of antibody drugs was given by the critical review of Liming Liu, and this section outlines pharmacogenomic factors that affect the efficacy and pharmacology of antibody drugs^[5].

Neonatal Fc receptor

The neonatal Fc receptor (FcRn) encoded by *FCGRT* was assumed in the 1960s to be a receptor that protects IgG from catabolism by Roger Brambell^[6,7]. After cloning of FcRn by Simister & Mostov in 1989, analysis of knockout mice by Roopenian & Akilesh proved its function in 2007^[8,9]. The current understanding of FcRn is that blood circulating IgG is taken up by vascular endothelial cells and monocytes by pinocytosis and receptor-mediated endocytosis. Thereafter, IgG binds to FcRn in endosomes in an acidic environment (pH

Table 2. Characteristics of IgG isotypes

Heavy chain subtype	Isotype	ADCC activity	CDC activity	Half-life (days)
$\gamma 1$	IgG1	++	++	21
$\gamma 2$	IgG2	+	+	20
$\gamma 3$	IgG3	+++	+++	7
$\gamma 4$	IgG4	+	-	21

ADCC: antibody-dependent cellular cytotoxicity; CDC: complement-derived cellular cytotoxicity

< 6.0), escapes lysosomal degradation, and is released again into the blood. If the blood IgG concentration is high, binding of FcRn to IgG is saturated, then lysosome-mediated IgG degradation is enhanced, and if the IgG concentration is low, IgG is bound to FcRn and recycled, thereby reducing IgG degradation^[10].

Gene polymorphism of FcRn has been reported to be involved in FcRn expression, with VNTR of 37 bases occurring in the promoter region^[11]. The most common VNTR3/VNTR3 homozygous genotype is reported to have 1.66 times more FcRn expression than the VNTR3/VNTR2 heterozygous genotype^[11]. The levels of anti-TNF- α antibodies infliximab and adalimumab are lower in blood by 14% and 24%, respectively, among heterozygous cases compared to homozygous cases^[12]. On the other hand, as an antibody drug against cancer, in 94 cases that received cetuximab therapy, which is an EGFR antibody for colorectal cancer, a significant difference in distribution clearance and a tendency to a prolonged half-life were observed. There was no change in PK due to copy number variation (CNV) of *FCGRT* gene^[13]. In addition, in 476 patients who received farletuzumab, an anti-folate receptor α antibody against ovarian cancer, there was no difference between PK and the area under the curve (AUC) of VNTR genotype and steady-state, and no change in PK due to CNV^[14]. As described above, there are few reports on blood concentrations of antibody drugs against cancer based on the FcRn genotype, and the relationship with efficacy is unknown. Further evidence is needed.

Antibody-dependent cellular cytotoxicity

An immunoglobulin including an antibody drug binds to an antigen at the Fab region, and the Fc region binds to Fc γ Receptor expressed on immune cells in the body. There are six types of Fc γ R: I, IIA, IIB, IIC, IIIA, and IIIB. Of them, I, IIA, IIC, IIIA, and IIIB are activated forms, and they mainly activate immune cells by phosphorylation of immunoreceptor tyrosine-based activation motif, which transmits activation signals into cells. On the other hand, IIB causes inhibitory functions via phosphorylation of an immunoreceptor tyrosine-based inhibitory motif^[15]. The ADCC activity as a mechanism of action of antibody drugs for cancer is considered to be the apoptosis of target cells by the release of perforin and granzyme from NK cells, mainly by the binding of the antibody drug to Fc γ RIIA of NK cells. The binding strength of the antibody drug and Fc γ RIIA is considered to be correlated with the ADCC activity.

Factors affecting the binding strength to Fc γ R include structural problems with the antibody drug and genetic polymorphisms affecting host immunity. Normally, the Fc region of immunoglobulins is glycosylated, and variations with or without terminal galactose, bisecting N-acetylglucosamine, sialic acid, and fucose at the root have been reported. For the ADCC activity, it has been reported that aglycosylation reduces ADCC activity, and addition of bisecting N-acetylglucosamine and removal of fucose increase ADCC activity. It has been reported that the addition of N-acetylglucosamine to mAb increases ADCC activity by 10-20 times^[16]. Moreover, fucose removal has undergone many technological developments, such as PotelligentTM technology (BioWa, Japan) and GlycomabTM technology (Glycart, Switzerland) based around cell lines engineered with altered glycosylation machinery, that increase ADCC activity by more than 100-fold^[17,18]. In the PotelligentTM cell line, both FUT8 gene alleles, which encode α 1,6-fucosyltransferase, were disrupted by sequential homologous recombination. The GlycomabTM cell lines are stably transfected with the gene encoding 1,4-N-acetylglucosaminyltransferase III (GnIII), resulting in the

Table 3. FcγR polymorphisms and antibody drug efficacies

Drug	Author (year)	Disease treatment (n)	FCGR2A-131 Polymorphism (n)	FCGR3A-158 Polymorphism (n)	Genotyping method	Results
Trastuzumab	Musolino <i>et al.</i> ^[19] (2008)	Metastatic breast cancer (54) Trastuzumab + Taxane	H/H (10) H/R (34) R/R (10)	V/V (11) V/F (26) F/F (17)	Nested PCR-based allele-specific restriction analysis assay	FcγRIIIa-158 V/V genotype was significantly correlated with ORR and PFS. There was a trend to significance in ORR and PFS for the FcγRIIIa-131 H/H genotype
	Tamura <i>et al.</i> ^[20] (2011)	Neoadjuvant breast cancer (15) AC/ PTX+Trastuzumab	H/H (7) H/R (6) R/R (2)	V/V (7) V/F (6) F/F (2)	Goldgate Genotyping	The FcγR2A-131 H/H genotype was significantly correlated with the pCR and the objective response. The FcγR3A-158 V/V genotype exhibited a tendency to be correlated with the objective response
	Tamura <i>et al.</i> ^[20] (2011)	Metastatic breast cancer (35) Trastuzumab	H/H (15) HR (18) R/R (2)	V/V (15) V/F (17) F/F (3)	Goldgate Genotyping	Patients with the FcγR2A-131 H/H genotype had significantly longer PFS
	Hurvitz <i>et al.</i> ^[21] (2012)	Adjuvant breast cancer (1286) Trastuzumab combination	H/H (323) HR (597) R/R (298)	V/V (169) V/F (471) F/F (549)	Nested PCR plus Sanger sequencing	No correlation between DFS and FCGR3A/2A genotypes was seen for trastuzumab-treated patients
	Musolino <i>et al.</i> ^[22] (2016)	Neoadjuvant breast cancer (73) Chemotherapy, Trastuzumab, Lapatinib.	H/H (101) HR (143) R/R (56)	V/V (43) V/F (150) F/F (107)	PCR plus Sanger sequencing	FcγRIIIa V allele correlated with pCR in patients treated with chemotherapy plus trastuzumab plus lapatinib. No significant associations were observed between pCR and FcγRIIIa polymorphism
Rituximab	Cartron <i>et al.</i> ^[23] (2002)	Caucasian FL (49) Rituximab	H/H (13) HR (23) R/R (9)	V/V (10) V/F (22) F/F (17)	PCR followed by an allele-specific restriction enzyme digestion	An association between the FCGR3A genotype and clinical and molecular responses to rituximab
	Weng <i>et al.</i> ^[24] (2003)	Caucasian FL (87) Rituximab	H/H (20) HR (43) R/R (24)	V/V (13) V/F (40) F/F (34)	Nested PCR followed by allele-specific restriction enzyme digestion	FcγRIIIa 158 valine/valine and the FcγRIIIa 131 Histidine/histidine genotypes were found to be independently associated with the response rate and freedom from progression
	Kim <i>et al.</i> ^[25] (2006)	Asian DLBCL (113) R-CHOP	H/H (60) HR (40) R/R (8)	V/V (53) V/F (54) F/F (6)	Nested PCR followed by allele-specific restriction enzyme digestion	No difference was found in OS or EFS according to FCGR3A or FCGR2A alleles. The FCGR3A SNP is predictive of response to R-CHOP, but does not correlate with survival
	Carlotti <i>et al.</i> ^[26] (2007)	Caucasian DLBCL (94) R-CHOP	H/H (30) HR (46) R/R (18)	V/V (18) V/F (46) F/F (30)	PCR with fluorescent labeled probes followed by melt curve analysis	No correlation between FcγRIIIa-158V/V/VF and FcγRIIIa-131HH/HR polymorphisms and the OS, response, and the EFS
	Mitrović <i>et al.</i> ^[27] (2007)	Caucasian DLBCL (58) R-CHOP	H/H (23) HR (27) R/R (8)	V/V (16) V/F (32) F/F (10)	PCR followed by allele-specific restriction enzyme digestion	FcγRIIIa and FcγRIIIa polymorphisms had no impact on EFS and OS
	Dornan <i>et al.</i> ^[28] (2010)	Caucasian CLL (419) Chemotherapy +/- Rituximab	H/H (110) HR (218) R/R (91)	V/V (49) V/F (202) F/F (168)	Allele-specific PCR with SYBR Green	FCGR2A and FCGR3A polymorphisms do not significantly influence the outcomes of CLL patients treated with chemotherapy +/- rituximab
	Zhang <i>et al.</i> ^[29] (2010)	Asian DLBCL (34) R-CHOP	NA	V/V (11) V/F (18) F/F (5)	Allele-specific PCR	FcγRIIIA polymorphisms do not predict prognosis independently
	Prochazka <i>et al.</i> ^[32] (2011)	Caucasian FL (102) Rituximab combination chemotherapy	NA	V/V (8) V/F (51) F/F (43)	Nested PCR followed by allele-specific restriction enzyme digestion	FcγRIIIA polymorphisms have no effect on the outcomes of patients treated with risk-adapted chemotherapy with or without rituximab

Ahlgrimm <i>et al.</i> ^[30] (2011)	Caucasian DLBCL (263)	H/H (105) HR (235) R/R (64)	V/V (76) V/F (140) F/F (47)	TaqMAN SNP Assay	in patients treated with R-CHOP, the EFS and PFS, but not the OS, curves for FcγRIIIa 158 F/F showed a trend to be lower than of 158 V/F and 158 V/V
Fabisiewicz <i>et al.</i> ^[31] (2011)	Caucasian DLBCL (87) R-CHOP	H/H (27) HR (40) R/R (20)	V/V (15) V/F (35) F/F (37)	TaqMAN SNP Assay	Neither FcγRIIIa nor FcγRIIIA allele was statistically significantly related to OS and PFS
Varoczy <i>et al.</i> ^[35] (2012)	Caucasian DLBCL (51) R-CHOP	NA	V/V (12) V/F (29) F/F (10)	TaqMAN SNP Assay	The EFS data were less favorable in the F-allele carriers than in V/V homozygous patients, but not significantly, and OS was almost the same
Ghesquieres <i>et al.</i> ^[33] (2012)	Caucasian FL (460) Rituximab combination chemotherapy	H/H (127) HR (226) R/R (102)	V/V (68) V/F (215) F/F (177)	TaqMAN SNP Assay	FCGR3A and FCGR2A polymorphisms do not affect response rate and outcome when rituximab is combined with chemotherapy or used as maintenance treatment
Persky <i>et al.</i> ^[34] (2012)	Caucasian FL (142) Chemotherapy or Chemotherapy plus CD20 mAb	H/H (17) HR (40) R/R (9)	V/V (5) V/F (29) F/F (20)	TaqMAN SNP Assay	FcγRIIIa polymorphism status may be predictive of survival in FL patients receiving treatments containing an anti-CD20 antibody but not treatment with chemotherapy alone
Liu ^[37] (2014)	Asian DLBCL (164) R-CHOP		V/V (14) V/F (59) F/F (91)	Nested PCR	FcγRIIIa V/V allele was borderline significantly correlated with PFS, but it was not correlated with better OS
Ghesquieres <i>et al.</i> ^[36] (2017)	Caucasian DLBCL (1134) Rituximab combination chemotherapy	H/H (289) HR (579) R/R (266)	V/V (144) V/F (513) F/F (449)	TaqMAN SNP Assay Illumina Infinium array Pyrosequencing	Meta-analysis of two prospective studies. FCGR3A was not associated with EFS and OS. FCGR2A (per R allele) was associated with a better EFS and OS

DLBCL: diffuse large B-cell lymphoma; PFS: progression-free survival

expressed antibodies bearing bisecting N-acetylglucosamine. Now, the Potelligent™ technology is used for the development of mogamulizumab, and the Glycomab™ technology is used for obinutuzumab.

On the other hand, there are gene polymorphisms of *FcGR* as a host factor, and among them, polymorphisms of *FCGR2A* and *FCGR3A* are reported to be related to ADCC activity. A coding polymorphism in the extracellular domain of *FCGR2A* has been described where a C>T substitution (rs1801274) changes the amino acid at position 131 from histidine to arginine (H131R). A second important *FcGR* coding polymorphism occurs in extracellular domain 2 of *FCGR3A*; a T>G substitution changes valine to phenylalanine at position 158 (V158F, rs396991).

Although many studies have been conducted on trastuzumab and rituximab as to whether these gene polymorphisms affect the efficacy of antibody drugs, many conflicting reports have been published [Table 3]. For trastuzumab, FcGR2A H/H and FcGR3A V/V are reported to be correlated with prolongation of progression-free survival (PFS) in metastatic breast cancer, FcGR2A H/H and FcGR3A V/V were reported to be correlated with pathological complete remission in the neo-adjuvant setting, and no obvious correlation was found between FcGR and overall survival in the adjuvant setting; thus, there is still no unified view^[19-22]. Similarly, rituximab has been investigated for its effect on drug efficacy against follicular lymphoma and diffuse large B cell lymphoma (DLBCL), but there are no reports of statistically significant effects, except in the early 2000s^[23-37]. As described above, *FcGR* SNPs and the ADCC activity of antibody drugs are clear *in vitro*, but their relationship with clinical efficacy is not clear. The reasons for this include the validity of the SNP verification method, the possibility that the number of cases needed to verify the effect of SNP

on ADCC activity has not been collected, and the possibility that there are individual differences in the number or function of host immune cells that exert ADCC activity. The search for further mechanisms and factors involved in ADCC is desirable.

Immune-checkpoint inhibitors

In recent years, ICIs that activate the exhausted immune cells by inhibiting immune checkpoint molecules typified by CTLA4 and PD-1/PD-L1 pathways with antibody drugs have been key drugs for the treatment of malignant melanoma and non-small cell lung cancer. To date, 1 anti-CTLA4 antibody (ipilimumab), 2 anti-PD-1 antibodies (nivolumab and pembrolizumab), and 3 anti-PD-L1 antibodies (atezolizumab, durvalumab, and avelumab) have been marketed. Anti-PD-1 antibodies have IgG1 isoforms, and the other ICIs have IgG4 isoforms. All drugs have anti-tumor effects, mainly due to neutralization or signal inhibition, with dramatic therapeutic effects in many cancer types such as malignant melanoma, NSCLC, and merkel cell carcinoma^[38]. However, ICIs have unclear drug efficacy biomarkers, and they cause immune-related adverse events (irAEs) that are different from the adverse events of conventional anticancer drugs. IrAEs occur due to the activation of host immunity by ICIs, causing immune response disorders and hormonal abnormalities in organs such as the thyroid, lungs, and pancreas. Although myelotoxicity and gastrointestinal toxicity are less likely than with cytotoxic chemotherapy, irAEs can sometimes be life threatening. Moreover, various biomarker searches for PD-1 inhibitors, which are frequently used in clinical practice, have been investigated. Tumor PD-L1 expression, tumor mutation burden (TMB), T cell receptor (TCR) repertoire, human leukocyte antigen (HLA), microbiome, etc. are attracting attention^[39]. ICIs activate host immunity by blocking the co-stimulatory/inhibitory pathway with antibody drugs, but the major signaling pathway of antigen-presenting cells and T cells is the binding of major histocompatibility complex (MHC) and TCR. Antigen-presenting cells present peptides produced from tumors as antigens, which are recognized by TCRs. From the above, peptides presented as antigens are factors on the tumor side, and MHC and TCR are factors on the host side.

Tumor antigen peptides that are factors on the tumor side include cancer testis antigens such as the melanoma-associated antigens (MAGEAs) and New York esophageal squamous cell carcinoma 1 that are expressed in tumors regardless of gene mutations, and tumor antigen peptides generated by tumor gene mutations. The number of tumor non-synonymous mutations is called the TMB, and it is attracting attention as an index of tumor immunogenicity, especially in melanoma and NSCLC that typically have high mutation burdens due to the mutagenic effects of ultraviolet light and cigarette smoking, respectively^[40]. In 2014, Snyder *et al.*^[41] performed 64 whole-exome sequencing of melanoma patients treated with CTLA-4 antibody and showed significantly higher TMB in patients with stable or responsive disease for more than 6 months, and TMB more than 100 was associated with better overall survival^[41]. This result was confirmed by examination of CTLA-4 antibody against other malignant melanoma cohorts, and it was shown that the higher the TMB of non-small cell lung cancer, the better the response rate and PFS of PD-1 antibody^[42,43]. Since then, many cancer types have been shown to have a linear response rate with TMB, and it has become clear that TMB can help predict certain therapeutic effects^[44]. However, some cancer types do not follow this linear response. For example, merkel cell carcinoma and renal cell carcinoma are more sensitive than expected to TMB, whereas mismatch-repair (MMR)-proficient colorectal cancer is less sensitive than expected. That is, although TMB has some degree of correlation with the therapeutic effect, other factors are also assumed to be related to the therapeutic effect of ICIs. In addition, technical issues still remain, such as the fact that TMB does not have a confirmed calculation method and cut-off criteria, and whether analysis samples are performed with tumor samples or cell-free DNA.

Like TMB, DNA MMR defects (MMRds) are considered biomarkers of ICIs by causing somatic mutations. Le *et al.*^[45,46] showed that pembrolizumab had a response rate of 53% in 86 cases with 12 cancer types of

tumors with MMRds exhibiting micro-satellite instability (MSI), showing a robust efficacy regardless of the type of cancer^[45,46]. From these results, nivolumab and pembrolizumab were first approved by the FDA in 2017 with the genotype MSI-positive regardless of cancer type.

The HLA genotype is important as a host element. HLA class I is rich in sequence diversity of peptide binding sites. The HLA-I allele is encoded by three genes on chromosome 6 (HLA A, HLA B, and HLA C), and each of these variants constitutes a slightly different peptide. Chowell *et al.*^[47] examined the genotype of HLA-I in 1535 patients treated with ICIs. They found that patients with HLA-B44 supertype showed prolonged survival, and patients with HLA-B62 supertype or somatic loss of heterozygosity at HLA-I showed reduced survival^[48]. Chowell *et al.*^[48] analyzed the expression of MHC class I and II in tumor tissues in malignant melanoma treated with ipilimumab, nivolumab, or their combination. They found that 78 of 181 cases (43%) showed disappearance of MHC class I, which was correlated with initial tolerance of ipilimumab. In addition, MHC class II expression was observed in 55 of 181 cases (30%), and a correlation with the therapeutic effect of nivolumab was demonstrated^[48].

As described above, several pharmacogenomic factors on the tumor side and host side are attracting attention as ICI efficacy biomarkers. It is difficult to establish a single biomarker for ICIs because there are multiple factors, such as blocking efficiency of the signal pathway and immune environment, on the host side. In particular, the host immune system is considered to be a large factor, and identification of a pharmacogenomic factor that is an index of host immune responsiveness is required.

Antibody-drug conjugate

Currently, antibody-antibody drugs (ADCs), which are obtained by linking a cytotoxic anticancer drug (called “payload”) to the antibody with a linker, have rapidly developed as a new antibody treatment strategy against cancer. The ADC is an innovative drug design approach that increases the local concentration of payload only around the target due to the high target selectivity of antibody drugs (called “bystander effect”). Drugs such as KS1/4-methotrexate and BR96-doxorubicin were developed as the first-generation ADCs in the 1980s, but their efficacy was not satisfactory^[49,50]. This may be due to insufficient titer of the drug itself, wrong target antigen selection, poor internalization efficiency of the antibody, poor tumor accumulation, linker stability too high/low, and immune response to mouse antibodies. Gemtuzumab ozogamicin, an anti-CD33 ADC, received FDA approval for the first time as an ADC product in 2000^[51]. However, subsequent clinical trials found no clinical effect and increased fatal adverse events, and approval was withdrawn^[52,53].

In order to overcome the challenges of first-generation ADCs, appropriate target search and appropriate payload development are underway, and brentuximab vedotin (anti-CD30 ADC), ado-trastuzumab emtansine (anti-HER2 ADC), and inotuzumab ozogamicin (anti-CD22 ADC) were approved by the FDA as second-generation ADCs^[54]. However, the second-generation ADCs also have problems such as the presence of a few unbound types due to the instability of the linker, early clearance by becoming a free drug, and causing off-target toxicities. The development of these first- and second-generation ADCs showed that selections of appropriate tumor cell targets, payloads, antibodies, linkers, and payload binding sites were important points in drug discovery. In other words, if the appropriate target and an antibody with high binding power cannot be selected, the accumulation of ADC in the tumor will be reduced; if effective payload selection cannot be performed, sufficient cell killing effects cannot be achieved; if the stability of the linker is low, it will dissociate in the body and cause an off-target effect; and if the stability of the linker is too high, an effective bystander effect cannot be attained.

Based on the above, many third-generation ADC products are currently being developed [Table 4]. In particular, in June 2019, the FDA approved Genentech’s anti-CD79b ADC, polatuzumab vedotin (Polivy),

Table 4. Current status of antibody-drug conjugates

Generic name	Brand name/ investigational name	Company	Target	Payload	DAR	Major indication	Current status
Brentuximab vedotin	Adcetris	Seattle Genetics/ Takeda	CD30	MMAE	4	Hodgkin lymphoma	Approved
Gemtuzumab ozogamicin	Mylotarg	Pfizer	CD33	Calicheamicins	3	Acute myeloid leukemia	Approved
Trastuzumab emtansine	Kadcyla	Roche	HER2	DM1	3.5	Breast cancer	Approved
Inotuzumab ozogamicin	Besponsa	Pfizer	CD22	Calicheamicins	NR	Acute B-cell lymphoblastic leukemia	Approved
Polatuzumab vedotin	Polivy	Genentech	CD79b	MMAE	3.5	DLBCL	Approved
Telisotuzumab vedotin	ABBV-399	AbbVie	c-Met	MMAE	3.1	NSCLC, solid tumor	Phase 2
Samrotamab vedotin	ABBV-085	AbbVie	LRR15	MMAE	2	Sarcoma, HNSCC, Breast	Phase 1
Trastuzumab deruxtecan	DS-8201a	Daiichi Sankyo	HER2	DXd	8	Breast, Lung, Colon	Phase 3
-	DS-1062a	Daiichi Sankyo	Trop2	DXd	4	Solid tumor	Phase 1
-	U3-1402	Daiichi Sankyo	HER3	DXd	8	Breast, Lung	Phase 1
-	MORAB-202	Eisai	FOLR1	Eribulin	4	Solid tumor	Phase 1
Enfortumab vedotin	ASG-22CE	Astellas pharma/ Seattle genetics	Nectin-4	MMAE	NR	Urothelial	Phase 3
Tisotumab vedotin	HuMax-TF	Genmab/Seattle Genetics	TF	MMAE	NR	Solid tumor, Cervical	Phase 2
Enapotamab vedotin	HuMax-AXL- ADC	Genmab/Seattle genetics	AXL	MMAE	NR	Solid tumor	Phase 1/2
Ladiratuzumab vedotin	SGN-LIV1A	Seattle genetics	LIV-1	MMAE	NR	Lung, Breast	Phase 2
Mirvetuximab soravtansine	IMGN853	ImmunoGen	FOLR1	DM4	NR	FOLR1 positive solid tumor	Phase 3
Sacituzumab govitecan	IMMU-132	Immunomedics	Trop2	SN38	7.5	Breast, Urothelial, Glioblastoma	Phase 3
-	XMT-1536	Mersana Therapeutics	NaPi2b	AF-HPA	-12	Ovary, NSCLC	Phase 1
-	SAR408701	Sanofi	CEACAM5	DM4	NR	Solid tumor	Phase 1

MMAE: monomethyl auristatin E; AF-HPA: austatin F-HPA; DXd: exatecan derivative; NR: not reported

for relapsed/refractory DLBCL. This drug showed the surprising result that complete remission was obtained in 40% (16/40) in the polatuzumab vedotin and bendamustine plus rituzimab therapy group in the Phase 1b/2 trial (GO29365 study)^[55]. Currently, clinical trials are ongoing with hematological tumors such as follicular lymphoma and in combination with other drugs^[56]. Moreover, Daiichi Sankyo's novel anti-HER2 ADC, Ds8201a (Trastuzumab Deruxtecan), is under development. In breast cancer, multiple phase 3 studies for patients with HER2-positive cancer (DESTINY-Breast01: NCT03248492, DESTINY-Breast02: NCT03523585, DESTINY-Breast03: NCT03529110), a phase 3 study for HER2-low cancer (DESTINY-Breast04: NCT03734029), a phase 2 study for HER2-positive gastric (DESTINY-Gastric01: NCT03329690), lung (NCT03505710), and colon cancers (NCT03384940), and combined use with ICIs (NCT04042701, NCT03523572) are in progress. A recently reported Phase 1 trial for HER2-positive advanced breast cancer showed a surprising response rate of 59.5% despite a previous heavy treatment history with trastuzumab or T-DM1^[57]. On the other hand, as an adverse event, pneumonitis was reported in 20 cases, and 2 fatal cases also occurred. Currently, the FDA has granted Fast Track and Breakthrough Therapy designations, and early approval is expected.

As shown in Table 4, there are currently 14 ADCs showing relatively good progress. Each drug targets not only HER2, but also HER3, Trop2, FOLR1, c-Met, AXL, and other molecules that are specifically expressed in tumors. Of 14 ADCs, 10 drugs used tubulin inhibitors (including 6 MMAE, 2 Maytansinoid DM4, 1 Eribulin, and 1 AF-HPA), and 4 drugs used a DNA topoisomerase I inhibitor (3 DXd, 1 SN38). In previous generations of ADCs, tubulin inhibitors were used in about 60% or more of payloads, but ADCs using a topoisomerase I inhibitor are increasing. In addition to improving the payload, each company is improving

the drug-antibody ratio, the stability of the linker, and the cancer cell specificity, which have been issues with ADCs. In addition to the development of ADCs alone, their combination with ICIs is being actively developed.

Although it is expected that there will be a wider range of options for antibody therapy, the pharmacogenomic factors in ADCs are not clear so far. Since the antibody part is IgG1 in many ADCs, the above-mentioned FcγR polymorphism may have an effect. There is also concern about the effects of metabolic and excretory enzymes in the payload. For example, there are reports that ABCC1 overexpression is involved in drug resistance to trastuzumab emtansine, and that ABCB1 is associated with the efficacy of gemtuzumab ozogamicin^[58,59]. In addition, MMAE, frequently used as a payload in many ADCs, is a CYP3A4 substrate, and eribulin is also a CYP3A4 substrate and an ABCB1 substrate. In other words, classical pharmacogenomic factors such as ABC transporter and CYP may affect metabolism and excretion of payloads and thus drug efficacy even in ADCs.

Moreover, recent ADC adverse events in clinical trials raise new concerns about immunogenicity. It has been reported that Ds8201a has a high incidence of pneumonitis, and this was the same case with Morab-202, which showed pneumonitis in 3 of 19 cases (15.8%)^[60]. Since the frequency of pneumonitis is not high with antibody drugs alone, pneumonitis may be a characteristic adverse event of ADCs. It is desirable to examine the pharmacogenomic factors of ADCs that are expected to be used more frequently in the future, including the enhancement of immunogenicity due to the linkage between linker and payload, and the possibility of affecting the responsiveness of host immunity.

As described above, ADCs have developed rapidly in recent years, but pharmacogenomic factors have not been fully studied. In addition to analyzing classical ABC transporters and CYP polymorphisms, pharmacogenomic analyses including host factors for characteristic adverse events such as lung injury are greatly needed.

FUTURE DIRECTION OF RESEARCH

There are still many black boxes in the pharmacokinetics of antibody drugs. Despite confirming doses several tens of times for the target occupancy in preclinical research, antibody blood concentrations in clinical practice may vary widely. Until now, it has been said that the pharmacokinetics of antibody drugs are not related to renal function, liver function or metabolic pathway, however, increased catabolism associated with organ dysfunction may affect the pharmacokinetics of antibody drugs. Furthermore, it is not clear whether the blood concentration of antibody drugs is correlated with the intratumoral concentration. It is necessary to identify the detailed pharmacokinetics of antibody drugs and the factors that affect the pharmacokinetics.

Another issue that must be considered is biosimilars. Unlike generic drugs, biosimilars cannot prove the identity of active ingredients. Therefore, at the time of approval, structural similarity is shown in a comparative quality study, PD and toxicity are shown in a comparative preclinical study, and PK, safety and efficacy are confirmed in a comparative clinical study. However, comparative clinical trials are only specifically designed to rule out clinically relevant differences in safety or efficacy between the biosimilar and the reference medicine, and to confirm biosimilarity. Differences in sugar chain modification and activity between lots have also been pointed out in the previous products, and it is still unclear whether biosimilars can exhibit sufficient pharmacokinetics and antitumor effects in clinical practice^[61]. In addition, it will be necessary to verify immunogenicity and pharmacogenomic differences.

CONCLUSION

The pharmacogenomics of antibody drugs is a complex area with more relevant factors than small molecule compounds. It is expected that pharmacogenomic factors related to drug efficacy and adverse events will be identified by comprehensively analyzing not only PK and PD, but also multiple factors such as host immune environment and genetic factors.

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Authors' contributions

Wrote the manuscript and prepared the figures and tables: Yagishita S, Hamada A

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Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

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Review

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Novel immunotherapeutic approaches in head and neck cancer

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Abstract

Unresectable recurrent or metastatic head and neck cancer is an incurable disease with survival of approximately 12 months. Head and neck tumors exhibit numerous derangements in the tumor microenvironment that aid in immune evasion and may serve as targets for future therapies. Pembrolizumab is now approved as a first line therapy. Despite the promise of currently approved immunotherapies there continues to be low response rates and additional strategies are needed. Here, alterations in the immune microenvironment and current therapeutic strategies are reviewed with a focus on novel immunologic approaches.

Keywords: Immunotherapy, head and neck, immune evasion, immune derangement, checkpoint inhibitors, recurrent/metastatic

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) represents the 6th most common malignancy globally and accounts for 1%-2% of all cancer related deaths^[1]. HNSCC is comprised of a heterogenous group of tumors including those arising from the oral cavity, oropharynx, hypopharynx and larynx. Traditional risk



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factors include tobacco, alcohol, and more recently human papillomavirus (HPV). High risk strains of HPV (HPV 16, 18) now are responsible for 70%-80% of oropharyngeal squamous cell carcinoma^[2,3].

Treatment of HNSCC varies by tumor site and stage, however the mainstays of treatment include surgery, radiation, and cytotoxic chemotherapy. Despite advancements in surgical and radiation techniques, treatment failures occur in up to 50% of patients with HNSCC^[4,5]. In the unresectable recurrent or metastatic (R/M) setting, chemotherapy has previously been the main therapeutic option, with dismal outcomes and median survival times ranging from 6-10 months^[6]. Immunotherapy, particularly checkpoint inhibitors, have shown promising results in R/M HNSCC^[7,8]. In June of 2019, the United States Food and Drug Administration approved pembrolizumab as a 1st line treatment for patients based on PD-L1 expression in the tumor immune microenvironment^[9]. Despite these recent reports, overall response rates remain low with underwhelming improvements in long term survival. Hence there continues to be a need for novel therapeutic options.

Head and neck tumors display various derangements in anti-tumor immunity and detailed understanding of these changes has led to development of currently approved immunotherapies. Here, we discuss alterations in the tumor immune microenvironment, review the mechanism of current treatments and focus on approaches for development of novel immunologic therapies.

DERANGEMENT OF HEAD AND NECK TUMOR IMMUNE MICROENVIRONMENT

Tumor immunity cycle

Anti-tumor immunity requires a complex set of interactions between the tumor and host immune system. This process has been termed the cancer immunity cycle^[10,11]. Initial tumor cell lysis results in release of tumor specific antigens (TAs) and priming of antigen presenting cells (APCs). APCs then interact with host immune cells resulting in activation and trafficking of cytotoxic T cells (CTLs) into the tumor. Once in the tumor, CTLs identify malignant cells displaying the specified tumor antigen and target them for cell death. Tumor antigens also referred to as neoantigens have become an area of intense research. These can be derived from either driver or passenger mutations and generation of TAs is thought to be closely linked to mutational burden, with a higher mutational load correlating to increased TAs^[12,13]. HNSCC has been found to have 1 of highest mutational burdens of all malignancies, likely due to their relationship with carcinogen exposure (i.e., tobacco smoke) which results in significant mutagenesis^[13,14]. As sequencing techniques have advanced, more sophisticated modeling has allowed for identification of specific mutational profiles including smoking and APOBEC signatures as well as prediction of neoantigen load^[15]. Detailed review of neoantigen prediction modeling has previously been published and is outside the scope of this review^[16-19]. Finally, with targeted tumor cell death by CTLs there is further release of tumor antigens resulting in perpetuation of the cycle. Head and neck tumors have evolved multiple mechanisms of immune escape which will be reviewed below in context of the cancer-immunity cycle.

Inhibition of antigen processing and presentation and immune cell activation

While HNSCC is thought to be highly antigenic, further steps are required for activation of a TA-specific adaptive immune response. After being released from tumor cells, TAs are degraded, processed, and presented by professional APCs including dendritic cells. Normal processes allow for extracellular protein presentation through major histocompatibility complex (MHC) class II and CD4 interaction, however, for TA to activate a CD8 response, cross presentation occurs, requiring an additional set of processing machinery^[20]. Large scale sequencing studies as well as analysis of TCGA data have revealed that up to 20% of HNSCCs contain alterations in antigen processing machinery (APM) or downregulation of MHC class I^[20,21]. In HPV positive tumors, the latter is thought to be mediated through viral oncoproteins, E5 and E7, which have been shown to downregulate both MHC class I and class II^[22-24]. Additional studies show that patients with

alterations in these pathways had both decreased CD8 T cell infiltration and worse survival outcomes^[25,26], indicating inhibition of antigen presentation may play a key role in head and neck tumor immune escape.

Once primed, APCs interact with and activate CTLs. Activation of CTLs occurs through contact between the T cell receptors and MHC class I bound to TA. This process requires a co-stimulatory signal between CD80 (present on the surface of the APC) and CD28 (a surface receptor on the CTL). Conversely, CD80 may instead bind CTL associated antigen 4 (CTLA-4) leading to CTL inhibition^[11,27,28]. While in normal physiologic conditions, the CTLA-4 immune checkpoint prevents an exaggerated immune response, in the setting of malignancies, it is thought to be a major mechanism of immune escape^[27,28].

Immune cell trafficking and infiltration

After priming and activation, CTLs infiltrate the tumor where they identify malignant cells displaying the specific TAs. In order for successful immune cell trafficking to occur, there must be appropriate cytokine signaling as well as an optimized physical environment. Physical blockade of immune cell infiltration has been suggested to play a role in immune escape and is thought to be mediated through elevated vascular endothelial growth factor (VEGF) signaling. This results in increased angiogenesis and increased oncotic pressure within the tumor creating a physical barrier to infiltration^[29]. *In vivo* studies evaluating anti-angiogenic tyrosine kinase inhibitors have shown an increase in tumor infiltrating lymphocytes correlating with reduced angiogenesis^[30,31]. Tumors may also promote an immune deplete environment through recruitment of suppressive and regulatory immune cells. This is achieved through direct secretion of suppressive cytokines such as tumor growth factor beta (TGF- β), or through secretion of CCL, CXCL, or VEGF which recruit myeloid derived suppressor cells (MDSC)^[32-34]. MDSCs are able to directly induce T cell tolerance through arginase, nitric oxide synthase^[35] and indoleamine 2,3-dioxygenase (IDO)^[36] dependent mechanisms^[32,37,38]. In HNSCC, increased tumor lymphocyte infiltration is linked to improved prognosis^[39-41], while elevated levels of MDSCs have been linked worse prognosis^[38,42].

PD-1/PD-L1 axis inhibition

Once in the tumor, CTLs induce tumor cell death. However, tumor cells may inhibit cell killing via co-stimulatory signals through the PD-1/PD-L1 axis. PD-1 is a member of the CD28 superfamily and is expressed on dendritic cells, regulatory T cells (Treg), CD8 and CD4 T cells, MDSCs, and natural killer cells (NK)^[43]. PD-L1 is expressed on APCs and causes T cell anergy and apoptosis upon binding with PD-1 receptors, thereby serving as a check to prevent an overactive immune response^[44,45]. However, upregulation of PD-L1 or additional PD-1 ligands such as PD-L2 can also be seen on tumor cells and acts as a mechanism of immune escape in various malignancies^[46]. The interaction between PD-L1 and PD-1 is complex and has been previously explored in multiple reviews^[47,48]. In HNSCC, PD-1/PD-L1 expression has been reported in 46%-100% of tumors and with higher expression in HPV-positive tumors compared to HPV negative tumors^[46,49-51]. Multiple currently approved therapeutics have been developed to target the PD-1/PD-L1 axis, including Pembrolizumab and Nivolumab as well as additional therapeutics that are currently undergoing clinical investigation such as Atezolizumab, and Durvalumab among others [Figure 1]. Both Pembrolizumab and Nivolumab inhibit PD-1, while the latter 2 (Atezolizumab and Durvalumab) target PD-L1. As PD-1 can be activated by additional ligands such as PD-L2 there is a theoretical advantage to targeting PD-1 over PD-L1, however anti-PD-1 and anti-PD-L1 therapies have demonstrated similar response rates and toxicity profiles in clinical trials as discussed below.

IMMUNOMODULATORY MECHANISMS OF TRADITION THERAPIES

Prior to review of current immunotherapeutics, it is prudent to discuss the immunomodulatory role of traditional therapies. Both platinum based chemotherapy and radiation have been shown to alter the tumor immune microenvironment. *In vivo* studies revealed that cisplatin treatment increases expression of MHC class I and antigen presentation machinery in patient derived HNSCC cell lines^[52]. While these

Mechanism of Action of Checkpoint Inhibitors

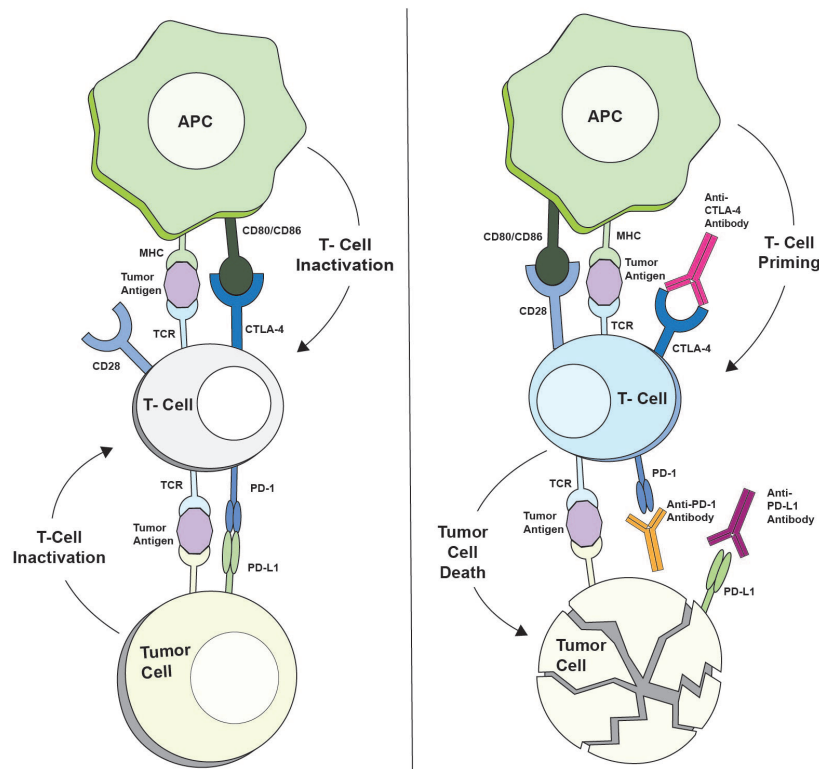


Figure 1. Mechanism of action of checkpoint inhibitors. APC: antigen presenting cell; MHC: major histocompatibility complex

changes may improve the anti-tumor immune response, this same study also demonstrated increased PD-L1 expression, decreased IFN- γ release by Tregs, and increased antigen specific T cell death with cisplatin treatment^[52,53]. These alterations in the tumor immune microenvironment suggest potential for dual treatment with cisplatin and anti-PD-1/PD-L1 immunotherapeutics, and *in vivo* studies have shown benefit of combination therapy^[52]. While cisplatin leads to increased Treg populations in HNSCC, similar studies performed in other types of malignancies have found that cisplatin treatment can actually lead to decreased Treg function and CTL activation by dendritic cells^[53,54]. These contradicting results suggest a complex mechanism by which cisplatin may affect the tumor microenvironment.

Radiation therapy (RT) is commonly used in the treatment of head and neck cancer and has also been shown to play a regulatory role in the tumor immune microenvironment. The specific mechanism by which radiation enhances the immune system is multifactorial. RT has been shown to increase production of TA^[55], increase expression of MHC class I and APM components^[56-58], and increase the number of tumor infiltrating lymphocytes^[59]. Similar to chemotherapy, RT can also suppress the immune response through recruitment of MDSCs and other suppressive immune cells^[60]. Further indication of the immunoregulatory effect of radiation is evident by the fact that radiation has been reported to induce tumor cell death in tumor deposits outside of the irradiated field. This is termed the abscopal effect and is thought to be an immune mediated response^[61,62].

MECHANISMS OF CURRENTLY APPROVED IMMUNOTHERAPEUTICS

In addition to traditional therapies, there are 3 currently approved biologics for HNSCC including Cetuximab, Pembrolizumab, and Nivolumab, which have also been shown to have immunomodulatory mechanisms.

Cetuximab, a monoclonal antibody against epidermal growth factor receptor (EGFR), was designed as a targeted therapy. However, recent studies have suggested that it may be tumoricidal via immune modulation^[63]. Accumulating evidence suggests that cetuximab increases antibody dependent cellular cytotoxicity (ADCC) of NKs^[63-65]. As Tregs inhibit NK mediated ADCC, through a TGF- β dependent pathway, it is postulated Cetuximab's mechanism of action may be through reduction or inhibition of Tregs. A recent study demonstrated a decrease in the number of Treg in peripheral blood samples in HNSCC patients treated with Cetuximab. This observed decrease in Treg population also correlated with improved overall survival in this cohort^[63]. Additional mechanisms of cetuximab have also been suggested, including increased crosstalk between dendritic cells and NK cells with further activation of the adaptive immune response, specifically targeting EGFR expressing cells^[66,67] and activation of the complement system^[68]. More recent studies have also indicated that cetuximab may increase CTLA-4 and PD-1/PD-L1 expression and suggest a need for combination therapies^[64,69].

Both Pembrolizumab and Nivolumab which target PD-1 have proven to have clinical benefit in patients with progressive disease after platinum based therapy^[7,70]. Initial approval for these drugs occurred in 2016 after the published results from multiple clinical trials were released. The phase III clinical trial, CHECKMATE-141, evaluated nivolumab *vs.* standard of care (SOC) chemotherapy in patients with platinum refractory R/M HNSCC. The overall response rate (ORR) for the nivolumab group was 13.3% compared to 5.5% in the SOC arm. Furthermore, nivolumab demonstrated an improved survival [7.5 months *vs.* 5.1 months (HR = 0.7, 97.7%CI: 0.51-0.96)] which was the 1st time a 2nd line agent demonstrated survival benefit^[7]. Similar results were seen in the KEYNOTE 040 trial, comparing Pembrolizumab to SOC in patients with R/M HNSCC who had failed platinum therapy. In this trial there was an ORR of 14.6% in the pembrolizumab group compared to 10.1% in the SOC group and a median OS of 8.4 months *vs.* 6.9 months (95%CI: 0.65-0.98)^[70]. Both studies had secondary endpoints evaluating survival outcomes stratified by PD-L1 status and revealed a greater benefit in patients with positive PD-L1 expression although not statistically significant. These results supported the use of nivolumab or pembrolizumab as a standard of care treatment for patients with platinum refractor R/M HNSCC.

Until recently, there has been no data supporting use of PD-1 inhibitors as a first line treatment option for R/M HNSCC. The results of the KEYNOTE 048 trial were presented at the 2019 American Society of Clinical Oncology meeting^[71]. This study evaluated the use of pembrolizumab alone, pembrolizumab with platinum and fluorouracil, *vs.* the EXTREME regimen in patients with R/M HNSCC. Results revealed an improvement in OS in all patients treated with pembrolizumab with a PD-L1 combined positive score $\geq 1\%$ by the 22C3 assay^[9].

While there are no mature data evaluating the effect of anti-PD-L1 therapeutics, such as Atezolizumab or Durvalumab, ongoing phase I and II trials have demonstrated excellent safety profiles with promising responses. A phase I trial published by Colevas *et al.*^[72] in 2018 enrolled 32 patients with advanced unresectable or incurable HNSCC who underwent treatment with Atezolizumab. The overall response rate in this cohort was 22% with a median progression free survival of 2.6 months and median overall survival of 6 months. Responses were found to be independent of PD-L1 expression level or HPV status. While 66% of patients experienced treatment related adverse events, only 4 patients (13%) had grade 3 or 4 toxicity. Given these results, a phase III randomized control trial is currently underway investigating the use of Atezolizumab in HNSCC (NCT03452137). Durvalumab has also been evaluated in Phase I/II trials in HNSCC. A study by Segal *et al.*^[73] published in 2019 examined 62 patients with unresectable and previously treated HNSCC treated with Durvalumab and found an ORR of 6.5% with a median overall survival of 8.4 months. Unfortunately, the results of the phase III EAGLE trial evaluating Durvalumab as single modality therapy in patients with progressive disease after platinum therapy was did not reveal any survival benefit alone or in combination with CTLA-4 inhibitor, Tremelimumab^[74]. An ongoing trial (NCT02551159)

Table 1. Checkpoint inhibitors in HNSCC

Immunotherapeutic	Target	Select ongoing trials
Pembrolizumab (Pembro)	PD-1	NCT03546582 (KEYSTROKE) - SBRT +/- Pembro in recurrent or secondary primary HNSCC NCT02641093 - Pembro + adjuvant RT or CRT (cisplatin) after surgery in HNSCC NCT02296684 - Neoadjuvant Pembro + SOC (surgery or RT) in high risk HNSCC NCT03765918 - Pembro before surgery and Pembro +/- Cisplatin and RT post-operatively in stage III-IV LA HNSCC
Nivolumab (Nivo)	PD-1	NCT03521570 - IMRT + Nivo for recurrent or second primary HNSCC after prior RT (resectable or eligible for curative re-irradiation) NCT03576417 - SOC (C/RT) +/- Nivo in HNSCC NCT03247712 - Nivo + RT prior to surgical resection of HNSCC
Atezolizumab	PD-L1	NCT03452137 - Atezolizumab or placebo as adjuvant therapy after definitive local therapy in high-risk LA HNSCC NCT03708224 - Effect of neoadjuvant Atezolizumab prior to surgical resection in HNSCC NCT03818061 - Atezolizumab + Bevacizumab in recurrent/metastatic previously treated HNSCC
Durvalumab	PD-L1	NCT03212469 - Durvalumab + RT or Durvalumab + Tremelimumab and SBRT in metastatic HNSCC (also includes lung and esophageal tumors) NCT02997332 (MEDINDUCTION) - Durvalumab + Docetaxel, Cisplatin and 5-FU for LA HNSCC NCT03635164 - Radiotherapy + Durvalumab before surgical resection for HPV negative HNSCC
Avelumab	PD-L1	NCT03498378 - Avelumab, Cetuximab, and Palbociclib in recurrent/metastatic HNSCC NCT02999087 - Avelumab-cetuximab-radiotherapy vs. SOC in LA HNSCC NCT03260023 - TG4001 and avelumab in patients with HPV-16 positive recurrent or metastatic malignancies (including OPSCC)
Ipilimumab	CTLA-4	NCT02812524 - Intratumoral injections of ipilimumab prior to surgical resection in HNSCC NCT03799445 - Ipilimumab, nivolumab, and RT for HPV positive OPSCC
Tremelimumab	CTLA-4	NCT03522584 - Tremelimumab + Durvalumab + hypofractionated RT in recurrent/metastatic HNSCC NCT03212469 - Durvalumab + RT or Durvalumab + Tremelimumab and SBRT in metastatic HNSCC (also includes lung and esophageal tumors) NCT02551159 (KESTREL) - MEDI 4736 +/- Tremelimumab vs. SOC in recurrent/metastatic HNSCC (active, not recruiting)
Relatlimab	Lag-3	NCT04080804 - Nivolumab +/- Relatlimab or Ipilimumab in head and neck cancer

HNSCC: head and neck squamous cell carcinoma; OPSCC: oropharynx squamous cell carcinoma; RT: radiation therapy; CRT: chemoradiation therapy; LA: locally advanced; SBRT: stereotactic body radiation therapy; SOC: standard of care; HPV: human papillomavirus

is evaluating Durvalumab with and without Tremelimumab in patients with R/M disease who have not received previous treatment for recurrent disease. A summary of select ongoing clinical trials investigating these therapies in addition to other checkpoint inhibitors is shown in [Table 1](#).

DEVELOPMENT OF NOVEL IMMUNOTHERAPEUTICS

Despite these encouraging results and updated treatment guidelines, it should be noted that overall response rates to these immunotherapies remains low for HNSCC and ongoing clinical trials are evaluating novel immunotherapeutic strategies [[Table 2](#)].

Oncolytic viruses provide potential for direct tumor cell lysis with further activation of an immune specific response. One of the most promising trials involving oncolytic vaccines in HNSCC to date evaluates the use of pembrolizumab in combination with Talimogene laherparepvec in patients with R/M disease. Final study results are pending, however initial updates suggest at least partial response in some patients (NCT02626000). Additional oncolytic viruses are also under early phase studies (NCT00625456, NCT03740256, NCT01584284).

Vaccinations represent a promising novel therapeutic strategy for various malignancies including HNSCC. Vaccines currently under investigation in HNSCC target patient specific TA (NCT03633110, NCT03548467), or TAs that are expressed in the majority of HNSCC such as mutant p53 (NCT02432963, NCT02955290, NCT02544880, NCT03946358). Additional vaccines are designed to enhance a general immune response such as those using the fowlpox-TRICOM vaccine (NCT00021424). For patients with HPV-mediated disease, the potential for vaccines targeting HPV specific peptides has gained enthusiasm

Table 2. Ongoing clinical trials

Category of therapy	Trial number (clinicaltrials.gov) - name/description
Oncolytic viruses	NCT02626000 (MASTERKEY232/KEYNOTE-137) - Talimogene Laherparepvec + Pembro in recurrent/metastatic HNSCC NCT00625456 - Safety study of recombinant vaccinia virus to treat refractory solid tumors NCT03740256 - Attenuated vaccinia virus (GL-ONC1) in combination with cisplatin and radiation therapy in locoregionally advanced HNSCC
Cancer vaccines	NCT03633110 - Safety, tolerability, immunogenicity, and antitumor activity of GEN-009 adjuvanted vaccine NCT03548467 - Safety, feasibility, efficacy of multiple dosing with VB10.NEO immunotherapy in patients with LA or metastatic solid tumors NCT02432963 - p53MVA vaccine (modified vaccinia virus ankara vaccine expressing p53) + Pembro in solid tumors after failed prior therapy NCT02955290 - Human EGF-rP64K/montanide ISA 51 vaccine (CIMAavax) + nivolumab in advanced HNSCC and non-small cell lung cancer NCT02544880 - PDE5 inhibition via Tadalafil to enhance anti-tumor mucin 1 (MUC1) vaccine efficacy in patients with HNSCC NCT03946358 - Combination of UCPVax vaccine and Atezolizumab for the treatment of human papillomavirus positive cancers (VolATIL) NCT00021424 - Recombinant fowlpox-TRICOM vaccine therapy in stage IV HNSCC NCT03418480 - Phase I/II vaccine dose escalation study with intradermal injections of HPV anti-CD40 RNA vaccine (HARE-40) in patients with advanced HPV 16 + cancers NCT03260023 - TG4001 and Avelumab in patients with HPV-16 positive recurrent or metastatic malignancies (including OPSCC) NCT00257738 - 0804 GCC: MAGE-A3/HPV 16 vaccine for squamous cell carcinoma of the head and neck NCT02002182 - DXS 11-001 vaccination prior to robotic surgery, HPV-positive OPSCC
Immunomodulatory	NCT03689192 - Arginase-1 peptide vaccine in patients with metastatic solid tumors NCT02752074 (Keynote-252/ECHO-301) - A phase 3 study of pembrolizumab + epacadostat or placebo in subjects with unresectable or metastatic melanoma NCT02740270 - Phase I/Ib study of GWN323 alone and in combination with PDR001 in patients with advanced malignancies and lymphomas NCT02274155 - Anti-OX40 antibody in head and neck cancer patients
T-cell therapy	NCT03578406 - HPV-E6-specific anti-PD1 TCR-T cells in the treatment of HPV-positive HNSCC or cervical cancer

HNSCC: head and neck squamous cell carcinoma; OPSCC: oropharynx squamous cell carcinoma; LA: locally advanced; HPV: human papillomavirus

and there are multiple ongoing trials investigating vaccines in this subset (NCT03418480, NCT03260023, NCT00257738, NCT02002182).

Both oncolytic viruses and vaccine therapy aim to promote a tumor specific adaptive immune response. Additional novel therapeutics have also been developed to try to remove the inhibitory signals that suppress an already active immune response.

One example of this strategy is the targeting of MDSCs, as multiple studies have shown these cells to be present in high abundance in HNSCC^[38,75]. *In vivo* studies have shown that elimination of MDSCs from the tumor microenvironment results in decreased Tregs and increased activity of CTLs^[76]. As discussed above, Arg, NOS, and IDO are thought to play a role in MDSC mediated Treg function; thus, drugs targeting these pathways are of particular interest. Phosphodiesterase-5 inhibitors are known to reduce both NOS and Arg production and a recent clinical trial leveraged the already FDA approved drug Tadalafil for use in HNSCC. In this randomized, double blinded, placebo controlled trial, patients with previously untreated (primary or recurrent) HNSCC received either tadalafil or placebo for 10 or more days prior to definite treatment. This study revealed decreased MDSC and Treg populations in the tadalafil treated cohort compared to placebo controls as well as increased CTL activity. Subgroup analysis of patients with available tumor specimens ($n = 6$) demonstrated increased tumor specific immunity in the Tadalafil treated group^[37]. No measures of survival outcomes were reported for this study. An additional clinical trial (NCT03689192) evaluating a vaccine targeting arginase is also currently underway.

Inhibition of IDO in combination with Pembrolizumab has also been under intense study in various malignancies^[77]. In HNSCC, a phase III clinical trial evaluating IDO inhibitor, Epacadostat in combination

with Pembrolizumab (NCT02752074), was unfortunately halted after a similar trial in melanoma revealed no improvement in overall or progression free survival with the addition of Epacadostat to pembrolizumab compared to the control arm^[36].

Additional therapies targeting immunosuppressive cytokines have also been developed^[78]. Current trials investigating antagonists of TNF receptor aims to inhibit Treg activity and are being tested both as monotherapy and in combination with anti-PD-1 therapies in HNSCC (NCT02740270, NCT02274155).

The final step in the cancer immunity cycle involves tumor cell death induced by activated CTLs. The ideal immunotherapy would bypass the preceding steps and provide T cells already primed to patient specific antigens. This has been successful in hematologic malignancies with therapies such as CAR-T and multiple ongoing clinical trials are investigating the use of T-cell therapies in solid malignancies as well^[79]. A clinical trial investigating the use of adoptive T cell transfer in HPV mediated disease in currently ongoing (NCT03578406). A previous study has shown some promise in seven patients with head and neck cancer (5 with SCC, 1 with melanoma and 1 with spindle cell sarcoma)^[80].

There are also ongoing trials assessing unique approaches that target multiple aspects of the immune cycle. MVX-ONCO-1 is a treatment that involves subcutaneous injection of capsules containing immune-modulatory granulocyte-macrophage colony stimulating factor and irradiated tumor cells with the aim of stimulating a tumor specific immune response (NCT02999646).

CONCLUSION

HNSCC represents a diverse group of diseases and exhibits varying degrees of immune dysregulation. Traditional therapeutic approaches are curative in 50% of patients and have proven to have immunomodulatory effects. Currently approved immunotherapies have shown some promise but unfortunately only a small fraction of patients benefit. This review summarizes the most common immune disruptions identified in head and neck cancer and discusses ongoing approaches aimed at targeting the tumor immune microenvironment.

DECLARATIONS

Authors' contributions

Conceptualization, data curation, writing, review/editing: Heft Neal ME

Writing, review/editing: Haring CT

Writing, review/editing: Mann JE

Supervision, review/editing: Brenner JC

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Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Original Article

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Loss of the Krüppel-like factor 4 tumor suppressor is associated with epithelial-mesenchymal transition in colorectal cancer

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Abstract

Aim: Colorectal cancer (CRC) is the third leading cancer-related cause of death due to its propensity to metastasize. Epithelial-mesenchymal transition (EMT) is a multistep process important for invasion and metastasis of CRC. Krüppel-like factor 4 (KLF4) is a zinc finger transcription factor highly expressed in differentiated cells of the intestinal epithelium. KLF4 has been shown to play a tumor suppressor role during CRC tumorigenesis - its loss accelerates development and progression of cancer. The present study examined the relationship between KLF4 and markers of EMT in CRC.

Methods: Immunofluorescence staining for KLF4 and EMT markers was performed on archived patient samples after colorectal cancer resection and on colonic tissues of mice with colitis-associated cancer.

Results: We found that KLF4 expression is lost in tumor sections obtained from CRC patients and in those of mouse colon following azoxymethane and dextran sodium sulfate (AOM/DSS) treatment when compared to their respective normal appearing mucosa. Importantly, in CRC patient tumor sections, we observed a negative correlation between KLF4 levels and mesenchymal markers including TWIST, β -catenin, claudin-1, N-cadherin, and



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vimentin. Similarly, in tumor tissues from AOM/DSS-treated mice, KLF4 levels were negatively correlated with mesenchymal markers including SNAI2, β -catenin, and vimentin and positively correlated with the epithelial marker E-cadherin.

Conclusion: These findings suggest that the loss of KLF4 expression is a potentially significant indicator of EMT in CRC.

Keywords: Krüppel-like factor 4, colorectal cancer, epithelial-mesenchymal transition

INTRODUCTION

Colorectal cancer (CRC) is the third leading cause of the cancer-related deaths often with metastasis to the liver, lung, and bone^[1,2]. Epithelial-mesenchymal transition (EMT) is a transdifferentiation process that allows a polarized epithelial cell to assume a mesenchymal cell phenotype, characterized by enhanced migratory and invasiveness capacity, elevated resistance to apoptosis, and increased synthesis of extracellular matrix (ECM) components^[3]. During the EMT process, epithelial cells lose apical-basal polarity that is accompanied by reorganization of cytoskeleton and reprogramming of the signaling pathways that allow for an increase in motility and the development of an invasive phenotype. This multistep complex process is characterized by modifications in the expression of a host of transcription factors and specific cell-surface proteins, as well as reorganization and expression of cytoskeletal proteins, and production of enzymes that degrade the ECM^[3]. A change in some of these factors, such as upregulation of TWIST, SNAI1, SNAI2, ZEB1, vimentin, and N-cadherin, and downregulation of E-cadherin and tight junction proteins such as ZO-1 is indicative of progression of EMT^[4,5]. In CRC, EMT has been strongly associated with the invasive and metastatic phenotype, thereby generating the life-threatening manifestations of metastatic disease cancer. The activation of the EMT program has been suggested to be the critical mechanism for the acquisition of malignant phenotypes by epithelial cancer cells^[3].

Krüppel-like factor 4 (KLF4) belongs to the family of zinc-finger transcriptions factors that play critical roles during development, proliferation, differentiation, and homeostasis, as well as development and progression of many diseases including inflammation and carcinogenesis^[6-8]. In the digestive tract, KLF4 is predominantly expressed in differentiated cells of the villus and surface epithelium of the small intestine and colon, respectively^[9-13]. Importantly, evidence indicates that KLF4 functions as a tumor suppressor that inhibits progression of CRC^[12]. It has been shown that loss of KLF4 expression is associated with the early stage of CRC development and that KLF4 is a prognostic indicator for CRC survival and recurrence^[14,15]. Recently, we demonstrated that KLF4 also plays a protective role against tumor formation during inflammation-induced colorectal tumorigenesis^[16-18]. The biochemical mechanisms triggering the acquisition of the invasive phenotype and the subsequent systemic spread of the cancer cell have been areas of intensive research. Here, we demonstrate that KLF4's role in colorectal tumorigenesis extends to its ability to regulate EMT.

METHODS

Samples from patients

Surgical specimens of resected colorectal cancer specimen obtained from Stony Brook University and State University of New York Downstate were used in this study. In total, 12 specimens were processed for immunofluorescence. All samples were of Caucasian origin, with 2 female and 10 male. One sample was qualified as stage I, one as stage 2, two as stage 3, and eight as stage IV [Table 1]. The protocol for the sample collection was originally approved by the Institutional Review Board by the State University of

Table 1. The characteristics of human samples

Sample ID	Age	Sex	Tissue type/area	Stage	Ethnicity
SB-036	64	Male	NSC TSC	IVB	Caucasian
SB-122	41	Male	NR TR	IV	Caucasian
SB-124	72	Male	NAC TAC	IV	Caucasian
SB-130	72	Female	NSC TSC	IV	Caucasian
SB-263	52	Male	NCE TCE	IV	Caucasian
SB-378	36	Male	NCE TCE	IV	Caucasian
SB-474	85	Female	NAC TAC	IV	Caucasian
SB-337	75	Male	NCE TCE	IIIC	Caucasian
SB-396	51	Male	NSC TSC	IIIA	Caucasian
SB-555	40	Male	NCE TCE	IV	Caucasian
SB-645	38	Male	NSC TSC	I	Caucasian
SB-670	65	Male	NSC TSC	IIA	Caucasian

NSC: normal sigmoid colon; TSC: tumor sigmoid colon; NR: normal rectum; TR: tumor rectum; NAC: normal ascending colon; TAC: tumor ascending colon; NCE: normal cecum; TCE: tumor cecum

New York at Stony Brook on 17 October 2014 (CORIHS 2014-2821-F) and qualified for a waiver under the Federal Law of Department of Health and Human Services per article 45CFR46.116.d.

Mice

All animal studies were approved by the Stony Brook University Institutional Animal Care and Use Committee and performed in accordance with institutional policies and NIH guidelines. Mice with the floxed *Klf4* gene (*Klf4^{fl/fl}*) were described previously^[12]. These mice were derived from a C57BL/6 background and are indistinguishable from the wild-type mice.

Azoxymethane and dextran sodium sulfate treatment

Azoxymethane (AOM) and dextran sodium sulfate (DSS) treatment was performed as described previously^[18]. Briefly, adult gender- and age-matched *Klf4^{fl/fl}* mice ($n = 16$) were injected intraperitoneally with 10 mg/kg of AOM working solution. After seven days, normal water was replaced with 2.5% DSS in the drinking water for five days, followed by two weeks of recovery (with normal water). This was followed by a second cycle of 2.5% DSS for five days, with two weeks of recovery (with normal water). The mice were euthanized at the end of the last recovery treatment, and samples were collected for pathologic analysis.

Tissue harvesting and tumor assessment, preparation, and immunostaining

Tissues were collected and prepared for immunofluorescence as described previously^[18]. Briefly, tissue sections were baked in a 65 °C oven overnight, deparaffinized in xylene, and rehydrated by incubation in a decreasing ethanol bath series (100%, 95%, and 70%), followed by antigen retrieval in citrate buffer solution (10 mM sodium citrate and 0.05% Tween-20, pH 6.0) at 120 °C for 10 min using a decloaking chamber (Biocare Medical) and 30 min incubation at 4 °C. The histological sections were incubated with blocking buffer (5% bovine serum albumin and 0.01% Tween 20 in 1 × Tris-buffered phosphate-buffered saline) for 1 h at 37 °C. The primary antibodies goat anti-KLF4 (1:200 for human sections and 1:300 for mice sections; R&D: AF3158), mouse anti-PanCK (1:200 for human sections; Biocare Medical: AE1/AE3), rabbit anti-β-catenin (1:500 for human sections and 1:150 for mice sections; Cell Signaling: 8480), rabbit anti-TWIST (1:500; Abcam: ab49254), rabbit anti-Claudin-1 (1:500; Cell Signaling: 13255), rabbit anti-N-cadherin (1:500; Cell Signaling: 13116), rabbit anti-E-cadherin (1:300 for mice sections, Cell Signaling: 3195), rabbit anti-Vimentin (1:500 for human sections and 1:100 for mice sections; Cell Signaling: 5741), and rabbit anti-SNAI2 (1:500 for human sections and 1:400 for mice sections, Cell Signaling: 9585) were added and incubated at 4 °C overnight. For KLF4, secondary unconjugated bovine anti-goat antibody was added at 1:500 dilution in blocking buffer for 30 min at 37 °C. For human sections to stain for PanCK, secondary unconjugated chicken anti-mouse antibody was added at 1:500 dilution in blocking buffer for 30 min at 37

°C. Appropriate Alexa Fluor-labeled antibodies (Molecular Probes) were added at 1:500 dilution in blocking buffer for 30 min at 37 °C. For mice sections, mouse anti-PanCK antibodies conjugated with Alexa 488 (1:100; ThermoFisher Scientific: 53-9003-82) were used. All slides were counterstained with Hoechst 33258 (ThermoFisher Scientific: H3569) and mounted with Fluoromount Aqueous Mounting Medium (Sigma-Aldrich: F4680). Slides were analyzed using a Nikon eclipse 90i microscope (Nikon Instruments Inc.) equipped with DS-Qi1Mc and DS-Fi1 CCD cameras (Nikon Instruments Inc.).

Immunofluorescence quantification

For each EMT/KLF4 co-stain, we quantified slides of 4-6 human specimen. For normal adjacent mucosa and tumor sections, we quantified four separate fields and counted 250-400 cells per each field. Each cell was labeled as positive or negative for KLF4 and positive or negative for each of the specific EMT markers. The statistical analysis was performed using a two-tailed Student *t* test.

Cell culture

SW480 colorectal cancer cell line (ATCC® CCL-228) was maintained in RPMI640 medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in atmosphere containing 5% CO₂. To overexpress Klf4-GFP and GFP-control in SW480 cell line, cells were transiently transfected with 3 µg plasmid DNA (per well in a six-well plate) using Lipofectamine 2000 reagent (ThermoFisher Scientific) according to manufacturer's instructions. The cell lysates were collected using Laemmli buffer and subjected to Western blot analysis with the following antibodies: rabbit anti-KLF4 (MBL: PM057), rabbit anti-ZEB1 (Cell Signaling: 3396), rabbit anti-SNAI1 (Cell Signaling: 3879), rabbit anti-SNAI2 (Cell Signaling: 9585), and mouse anti-actin (SigmaAldrich: A1978). Then, they were developed using secondary antibodies goat anti-rabbit HRP-conjugated (JacksonImmuno Research: 111-035-144) and goat anti-mouse HRP conjugated (SigmaAldrich: AP200P), respectively.

Statistical analysis

Student's paired or unpaired *t* test was used for statistical analyses. Differences between values were considered significant when $P < 0.05$. This analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Expression of KLF4 in human colorectal cancer is negatively correlated with markers of EMT

EMT is a precisely orchestrated multistep process regulated by several transcriptional factors including TWIST, SNAI1, SNAI2, and ZEB1^[3]. To determine the correlation between KLF4 expression and EMT in CRC, we performed immunofluorescence analysis of matched pairs of archived samples from patients after tumor resections. Firstly, we analyzed the expression pattern of KLF4 and TWIST. As shown in [Figure 1A](#) (SB396N), KLF4 is expressed in the nucleus of epithelial cells in the normal-appearing mucosa adjacent to the cancer tissues. These cells are positive for PanCK, an epithelial marker, and negative for the nuclear expression of the biomarker of EMT, TWIST. In contrast, in the tumor samples from the same patient [[Figure 1A](#), SB396T], the expression of KLF4 is downregulated in the epithelial cells, which is accompanied by a significant increase in the expression of TWIST. Our statistical analysis showed that there is a negative correlation between KLF4 and TWIST expression in normal-appearing mucosa and tumor tissues ($P < 0.001$). Several common signaling pathways regulate factors involved in EMT including HH, WNT, NOTCH, and TGF-β^[19]. WNT signaling plays an important role in the homeostasis of the intestinal epithelium and its deregulation leads to cancer formation which is accompanied by modification of the pattern and level of expression of its major effector, β-catenin^[20-22]. In the normal-appearing mucosa, β-catenin is predominantly localized to the membrane of the epithelial cells with a modest nuclear staining [[Figure 1B](#), SB474N]. Upon loss of KLF4 in the colorectal tumor [[Figure 1B](#), SB474T], the levels of the cytoplasmic and

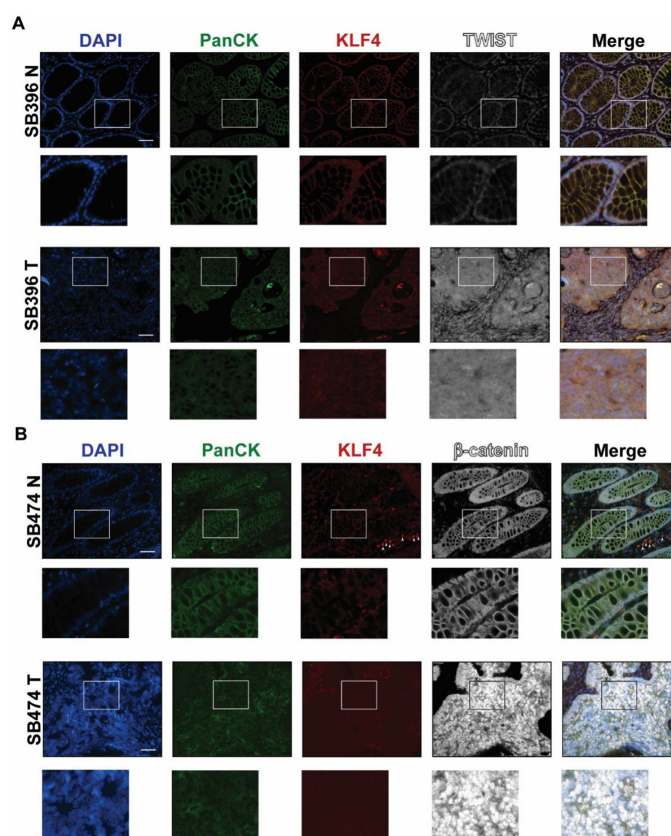


Figure 1. Immunofluorescence staining for PanCK, KLF4, TWIST, and β -catenin in human colonic tissues. Representative images of normal adjacent mucosa and tumor sections from two different human specimens: (A) TWIST (SB396N and SB396T); and (B) β -catenin (SB474N and SB474T). White arrowheads indicate KLF4 stain in stromal tissue. White boxes mark insets. Scale bar: 50 μ m

nuclear β -catenin are significantly increased while its membranous expression is decreased ($P < 0.001$). Loss of cell polarity and cell-cell junctions is another hallmark of EMT and is characterized by increased expression of claudin-1 and N-cadherin^[23-27]. Immunofluorescence staining of claudin-1 [Figure 2A, SB474N] and N-cadherin [Figure 2B, SB378N] within the normal-appearing mucosa shows little or no staining while there is pronounced nuclear KLF4 staining. In matching colorectal cancer tumor tissues with lack of KLF4 expression, both claudin-1 and N-cadherin levels are significantly increased [Figure 2A, SB474T and Figure 2B, SB378T, respectively]. Our analysis showed that there is a significant negative correlation between expression of KLF4 and claudin-1 and N-cadherin between the normal-appearing mucosa and tumor tissues ($P < 0.001$). Furthermore, staining for vimentin, another mesenchymal marker, showed a lack of expression within the epithelial component of the normal-appearing mucosa [Figure 3, SB474N], but a slight increase within the epithelial compartment upon loss of KLF4 in the tumor tissues [Figure 3, SB474T, white arrowheads]. Statistical analysis showed that the expression levels of KLF4 and vimentin are negatively correlated in normal mucosa ($P < 0.05$) and in tumor sections ($P < 0.001$).

KLF4 expression is negatively correlated with markers of EMT in a mouse model of colitis-associated cancer

Recently, in a mouse model, we demonstrated that KLF4 plays a protective role against progression of colitis-associated cancer and that decrease in KLF4 levels is associated with increased aggressiveness of the disease^[18]. To test if KLF4 expression levels correlate with the expression pattern of EMT markers, we performed immunofluorescence staining of KLF4 and select EMT markers on the mouse tissues (*Klf4*^{fl/fl} mice) after treatment with AOM/DSS, as described in the Material and Methods Section.

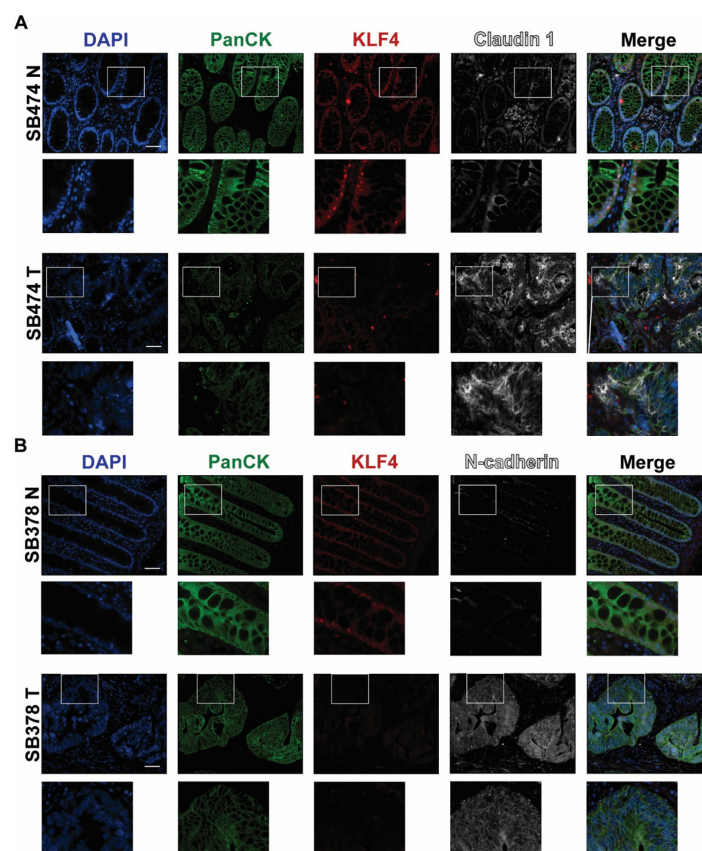


Figure 2. Immunofluorescence staining for PanCK, KLF4, claudin-1, and N-cadherin in human colonic tissues. Representative images of normal adjacent mucosa and tumor sections from two different human specimens: (A) claudin-1 (SB474N and SB474T); and (B) N-cadherin (SB378N and SB378T). White boxes mark insets. Scale bar: 50 μ m

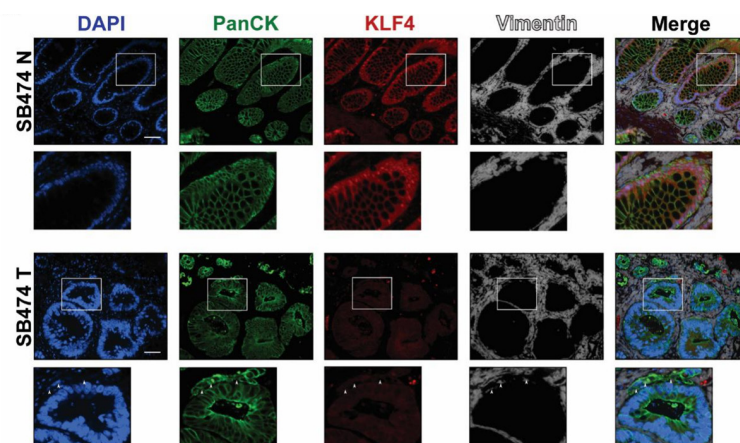


Figure 3. Immunofluorescence staining for PanCK, KLF4, and vimentin in human colonic tissues. Representative images of normal adjacent mucosa (SB474N) show that KLF4 and vimentin do not co-localize (top inset) and tumor sections (SB474T) that show loss of KLF4 and stain of vimentin in epithelial cells. White boxes mark insets. White arrowheads indicate vimentin stain within epithelial cells (bottom inset). Scale bar: 50 μ m

Immunofluorescence staining showed that in normal mucosa KLF4 is expressed in the nuclei of epithelial cells defined by staining for PanCK. Co-staining for SNAI2 showed that SNAI2 was predominantly expressed in the stromal section of the normal-appearing mucosa and tumor section of mice after AOM/DSS treatment [Figure 4A] and was absent from epithelial compartment. However, in the tumor section

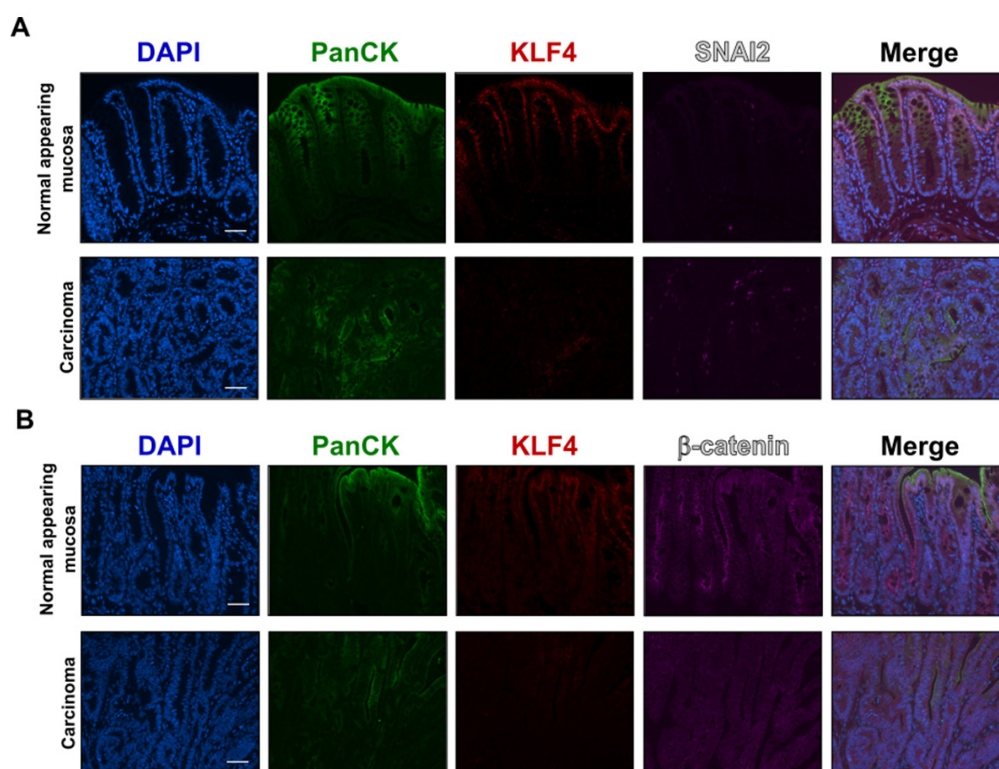


Figure 4. Immunofluorescence staining for PanCK, KLF4, SNAI2 and, β -catenin in mouse colonic tissues after AOM/DSS treatment. (A) Representative images of PanCK, KLF4, and SNAI2 in normal adjacent mucosa (top panel) and tumor sections (bottom panel). (B) Representative images of PanCK, KLF4, and β -catenin in normal adjacent mucosa (top panel) and tumor sections (bottom panel). Scale bar: 50 μ m. AOM/DSS: azoxymethane and dextran sodium sulfate

where KLF4 loss was observed, we noticed increased levels of SNAI2 in the nuclei of the epithelial cells that were defined by positive staining for PanCK [Figure 4A]. As in human specimens, we performed immunostaining analysis for β -catenin. However, we did not observe an increase in nuclear β -catenin staining in the tumor sections in comparison to the normal adjacent mucosa [Figure 4B]. With respect to adherens junction complexes, we observed a reduction in the expression levels of E-cadherin in the tumor section as compared to the normal adjacent mucosa [Figure 5A]. Expression of the mesenchymal marker vimentin in normal adjacent mucosa of mice after AOM/DSS treatment is confined to the stroma [Figure 5B] and does not correlate with KLF4 expression. However, in the tumor of mice after AOM/DSS treatment, we observed a slight increase in the staining of vimentin within the epithelial section, which suggests a change in the characteristics of these cells from epithelial toward mesenchymal phenotype [Figure 5B]. Furthermore, overexpression of KLF4 in SW480 CRC cell line resulted in decreased levels of the mesenchymal markers of EMT, namely ZEB1, SNAI1, and SNAI2 [Figure 6], confirming a suppressive role in the regulation of the epithelial-to-mesenchymal transition.

DISCUSSION

In this study, we investigated the correlation in expression between KLF4 and EMT markers in tissues obtained from patients with CRC and from a mouse model of colitis-associated cancer. The results from studies of epidermal cancer, hepatocellular carcinoma, breast cancer, pancreatic cancer, and prostate cancer with data predominantly originating from *in vitro* experiments show that KLF4 negatively regulates EMT^[28-33]. On the other hand, KLF4's ability to regulate the stemness of cancer cells has been shown as an important factor in stimulating EMT in pancreatic, ovarian, endometrial, nasopharyngeal, prostate, and non-small cell lung cancers^[34-40]. We demonstrated that KLF4 expression is positively correlated with

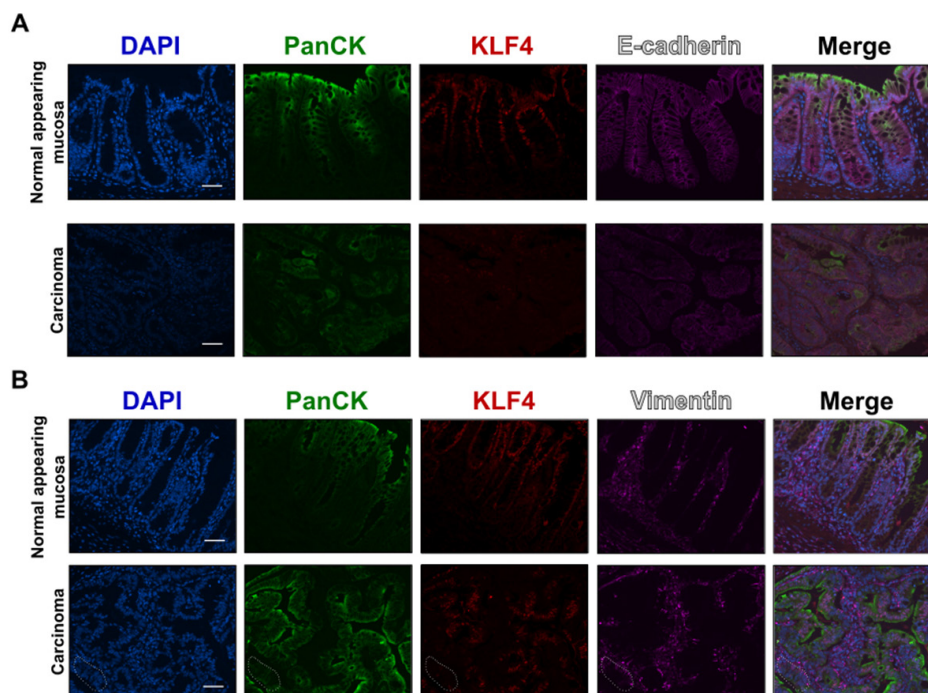


Figure 5. Immunofluorescence staining for PanCK, KLF4, E-cadherin, and vimentin in mouse colonic tissues after AOM/DSS treatment. (A) Representative images of PanCK, KLF4, and E-cadherin in normal adjacent mucosa (top panel) and tumor sections (bottom panel). (B) Representative images of PanCK, KLF4, and vimentin in normal adjacent mucosa (top panel) and tumor sections (bottom panel). Dotted area shows transition from epithelial (PanCK staining) to mesenchymal characteristic (vimentin staining). Scale bar: 50 μ m. AOM/DSS: azoxymethane and dextran sodium sulfate

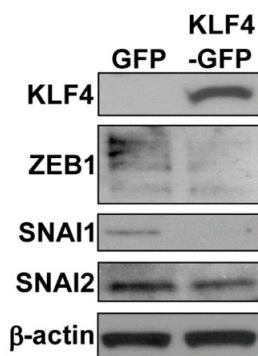


Figure 6. Overexpression of KLF4 in CRC cell line decreases the levels of mesenchymal EMT markers. Western blot analysis of KLF4 and EMT markers ZEB1, SNAI1, and SNAI2 in SW480 colon cancer cell line with EGFP or KLF4-EGFP overexpression. Actin is a loading control. EMT: epithelial-mesenchymal transition

epithelial markers of EMT in normal mucosa and negatively with mesenchymal markers in CRC. These results are in agreement with previous observations that KLF4 is a suppressor of EMT^[28-31,41]. This could be accredited to the role of KLF4 in the regulation of differentiation along the crypt-luminal axis in the intestinal epithelium^[40]. Importantly, KLF4's suppressive role in EMT regulation is not limited to the colonic epithelium but has been shown to play a crucial role in corneal epithelial cell fate. It was shown that downregulation of KLF4 promotes expression of mesenchymal markers and decreases the expression of epithelial markers^[42-45]. Furthermore, studies using conditional ablation of KLF4 from the intestinal epithelium showed a deficiency in goblet cell differentiation, thereby demonstrating that KLF4 plays a role in maintaining intestinal epithelial morphology and homeostasis^[9,12]. Additionally, KLF4 has been

shown to regulate apical-basolateral polarity in intestinal epithelial cells and to enhance their polarity by transcriptional regulation^[13]. Thus, loss of KLF4 expression during development and progression of CRC may lead to loss of cell polarity with progression toward EMT. Furthermore, it has been previously demonstrated that E-cadherin (*Cdh1*), N-cadherin (*Cdh2*), vimentin (*Vim*), and β -catenin (*Ctnnb1*) genes are direct transcriptional targets of KLF4^[30]. Yori et al.^[29,41] demonstrated that KLF4 is necessary for the maintenance of E-cadherin expression, repression of SNAIL1, and prevention of EMT in mammary epithelial cells. These results are consistent with our observations. We demonstrated a positive correlation between KLF4 and E-cadherin and a negative one between KLF4 and N-cadherin and vimentin in human and mouse tissues. However, we were not able to show increased nuclear levels of β -catenin in mouse tissues after AOM/DSS treatment^[18]. This could be due to technical differences, as we previously used immunohistochemical staining and the current analysis was based on immunofluorescence studies. In addition, we showed that KLF4 expression is negatively correlated to the expression of TWIST, SNAIL2, and claudin-1. Taken together, these results suggest that KLF4 may regulate EMT at several different stages of CRC progression, including suppression of expression of transcription factors (TWIST and SNAIL2), transcriptional co-activator (β -catenin), and regulation of cell polarity (E-cadherin, N-cadherin, and claudin-1). In conclusion, we showed a negative association between KLF4 and mesenchymal EMT markers in both human and mouse CRC tissues. Future studies are necessary to identify the mechanism by which KLF4 regulates EMT progression in CRC.

DECLARATIONS

Acknowledgments

We thank the Department of Pathology at Stony Brook University for technical assistance in histopathological analysis.

Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: Yang VW, Ghaleb AM, Bialkowska AB

Performed data acquisition and analyzed data: Agbo KC, Huang JZ, Ghaleb AM

Provided tissues samples: Williams JL

Provided assistance with histopathological analysis: Shroyer KR

Availability of data and materials

Not applicable.

Financial support and sponsorship

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

The protocol for the sample collection was originally approved by the Institutional Review Board by the State University of New York at Stony Brook on October 17th, 2014 (CORIHS 2014-2821-F) and qualified for a waiver under the Federal Law of Department of Health and Human Services per article 45CFR46.116.d.

Consent for publication

Not applicable.

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Review

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Histone chaperone FACT and curaxins: effects on genome structure and function

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Abstract

The histone chaperone facilitates chromatin transcription (FACT) plays important roles in essentially every chromatin-associated process and is an important indirect target of the curaxin class of anti-cancer drugs. Curaxins are aromatic compounds that intercalate into DNA and can trap FACT in bulk chromatin, thus interfering with its distribution and its functions in cancer cells. Recent studies have provided mechanistic insight into how FACT and curaxins cooperate to promote unfolding of nucleosomes and chromatin fibers, resulting in genome-wide disruption of contact chromatin domain boundaries, perturbation of higher-order chromatin organization, and global dysregulation of gene expression. Here, we discuss the implications of these insights for cancer biology.

Keywords: Genomics, transcription, nucleosomes, chromatin structure

INTRODUCTION

Facilitates chromatin transcription (FACT) is a highly conserved histone chaperone that participates in multiple physiological processes^[1] including transcription^[2-8], DNA replication^[9-13], DNA repair^[14-17],



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and centromere function^[18,19]. FACT is a heterodimer of the suppressor of Ty 16 (Spt16) protein and the structure-specific recognition protein 1 (SSRP1) in mammals or the polymerase one binding protein 3 (Pob3) in yeast^[1,8,9,20,21]. Unlike human SSRP1, the yeast Pob3 lacks the high mobility group B1 (HMGB1) DNA-binding domain; however, yeast FACT functionally interacts with the non-histone protein 6 (Nhp6) that consists of a single HMG box^[22-24]. Both Spt16 and SSRP1 are able to interact with H2A/H2B dimers and H3/H4 tetramers^[20,25-29]. FACT is involved in both assembly and disassembly of nucleosomes, as well as in nucleosome unfolding that is dependent on its interactions with other factors. Single-particle Förster resonance energy transfer (spFRET) microscopy studies demonstrated that in the presence of Nhp6 protein yeast FACT can induce large-scale, reversible, and ATP-independent unfolding (reorganization) of individual nucleosomes that involves uncoiling of nucleosomal DNA^[30,31]. In contrast, in the absence of Nhp6 protein, FACT can stabilize nucleosomes lacking the unstructured histone tails^[32]. The Spt16 and SSRP1 subunits of FACT also contain additional intrinsic DNA-binding sites^[33,34]. SSRP1 can bind the left-handed Z-form of DNA^[35]. Notably, SSRP1 is usually phosphorylated by casein kinase 2, which inhibits its DNA binding activity^[36,37]; the phosphorylated FACT can only bind destabilized nucleosomes or hexasomes^[22,38].

Curaxins are carbazole-based compounds that intercalate into DNA and alter the physical properties of both DNA and chromatin without causing structural damage (i.e., phosphodiester bond breaks or chemical modifications)^[34,39,40]. These drugs inhibit the activities of FACT, which has particularly severe consequences in cancer cells^[35]. The curaxins CBL0137 and CBL0100 have been demonstrated to have broad anticancer activity in many different models^[35,41-45]. Clinical candidate CBL0137 has higher metabolic stability and water solubility, and efficiently inhibits growth of various cancers in preclinical models^[35]. CBL0100 is more biologically active but is rarely used in animal studies due to its lower solubility and higher toxicity. Curaxins were first identified in a screen for small molecule compounds capable of simultaneously inhibiting NF- κ B, activating p53, and preferentially killing tumor cells^[35]. Curaxins induce chromatin trapping (c-trapping) of FACT in less than 1 min after addition to cells, and strongly inhibit normal human FACT activities *in vivo*^[35]. This c-trapping of FACT involves rapid formation of Z-DNA and binding of the SSRP1 subunit (z-trapping)^[34], as well as curaxin-dependent nucleosome unfolding resulting in binding of FACT to the disrupted nucleosomes (n-trapping)^[31]. The anticancer activity of curaxins is highly dependent on c-trapping of FACT^[31,34,35]. However, in addition to the c-trapping and activation of p53, curaxins also induce dramatic changes in chromatin structure (e.g., disruption of long-range chromatin interactions^[46], see below) and dysregulation of multiple cellular responses, including changes in transcription profiles^[31,40] and induction of transcription of repeats activates interferon (TRAIN)^[47].

This review focuses on recent studies of the roles of FACT and curaxins in gene expression and genome structure; other topics have been extensively covered in several excellent recent reviews^[3,39,48].

ROLES OF FACT IN TRANSCRIPTION, REPLICATION, AND DNA DAMAGE REPAIR

FACT is detected at transcribed regions of genes in multiple eukaryotes^[40,49-51]. FACT is associated with both promoters and coding regions of transcribed genes^[31,49,52,53]. Insertion of transposons (Ty elements) often interferes with the expression of nearby genes in *Saccharomyces cerevisiae*^[54]. Depletion of yeast *SPT16* causes cell cycle arrest in early G1 phase due to failure to transcribe cyclin genes, and less severe mutations cause the Spt- (suppressor of Ty) phenotype due to inappropriate activation of a cryptic promoter in Ty1^[54]. Yeast FACT and Nhp6 are also involved in maintaining nucleosome-depleted regions near many yeast promoters^[52]. The nucleosome unfolding activity of yeast FACT working together with Nhp6 protein that has been detected *in vitro* likely plays a role in nucleosome destabilization and/or histone removal at promoter regions^[2,30]. The *in vitro* studies of chromatin transcription by yeast RNA polymerase II (Pol II) have determined that FACT can facilitate Pol II transcript elongation through chromatin and nucleosome survival during this process by transiently interacting with the DNA binding surface of the H2A/H2B

dimers^[9]. However, depletion of FACT only minimally affects transcript elongation rates *in vivo*, while numerous studies have shown that FACT is critically involved in maintaining the nucleosomal structure of the genome^[6,9,55-59]. Thus, depletion of FACT in yeast causes accumulation of short transcripts from normal transcription start sites and cryptic transcripts initiated intragenically^[56-59]. Yeast FACT also plays important roles in facilitating efficient replication and nucleosome assembly/disassembly during this process^[14,15]. FACT occupancy is increased at DNA damage sites after different stresses, including UV-induced damage^[17,60,61], oxidative damage^[19], and single- and double-strand breaks^[62,63]. FACT also plays roles in recovery from transcription arrest^[61], replication fork stalling^[64], and R-loop formation^[65]. The FACT subunit SSRP1 is able to detect non-B form DNA structures, and this activity might be important for inducing cell signaling cascades in response to these perturbations^[34].

FACT is essential for normal embryonic development. *Ssrp1* knockout mice demonstrated early embryonic lethality at the blastocyst stage (3.5 dpc), and ES cells derived from the *Ssrp1*-null blastocysts could not be propagated *in vitro*^[66]. Defects in development were also observed in zebrafish with a mutant *Ssrp1*^[67] and in plants with reduced levels of SSRP1 and SPT16 proteins^[68,69]. Several studies also revealed that FACT subunits play specific roles in mammalian cell differentiation and considerably modify gene expression profiles during muscle cell differentiation^[70] and *in vitro* differentiation of human mesenchymal stem cells^[71]. Although FACT can be involved in gene transcription, it is not ubiquitously expressed and therefore is not a required core component of the transcription machinery. The levels of FACT expression are higher in undifferentiated and proliferating cells, but are very low in most differentiated mammalian cells^[72-75]. Importantly, both curaxin treatment and (CRISPR)-mediated deletion of the FACT subunit Spt16 block induction of pluripotency without affecting the viability or proliferation of fibroblasts^[76]. In tumor cells, both FACT subunits are highly expressed, and their higher levels correlate with poor prognosis in several tumor types^[35,74,77-80]. Thus, FACT plays a more important role in cancer cells than in normal cells, making it a target for cancer therapy. The FACT complex is also involved in HIV integration into the host genome^[81] and viral gene transcription^[82,83], suggesting that FACT could serve as a target for HIV therapy as well. The mechanistic studies of FACT activity described below therefore provide insights that could have clinical significance.

FACT-NUCLEOSOME INTERACTIONS

FACT does not bind stably to intact nucleosomes and does not affect nucleosome structure unless it is assisted by additional factors. The HMGB1-domain protein Nhp6 provides this nucleosome-destabilizing activity *in vitro* and *in vivo* for yeast FACT, and also supports nucleosome reorganization by human FACT *in vitro*^[2]. The HMGB1 domain of SSRP1 does not appear to be adequate to promote uncoiling of the DNA from intact nucleosomes, suggesting that it may function primarily during nucleosome assembly^[29,31,55]. Curaxins can distort DNA shape and this distortion promotes trapping of FACT in chromatin and changes chromatin compaction^[31,46]. We propose that either HMGB proteins or curaxins weaken the histone-DNA contacts near the entry/exit sites of nucleosomal DNA and thus initiate a series of binding events by FACT domains that can lead to full reorganization of the nucleosome [Figure 1]. In this model, DNA distortion exposes the initial set of binding sites for the CTDs of each subunit of FACT, resulting in similar reorganization of the nucleosome independent of the initiating factor (curaxin or HMGB binding). In the reverse reaction leading to nucleosome assembly, Nhp6 or the intrinsic HMGB domain of SSRP1 can assist in stabilizing bent forms of the DNA to promote formation of the canonical nucleosome, resulting in release of FACT. Curaxins do not provide this assistance, instead leading to persistent binding of FACT and trapping it in chromatin (see below).

CURAXIN-INDUCED TRAPPING OF FACT ON CHROMATIN

Computational modeling and circular dichroism studies suggest that curaxins intercalate between DNA base pairs and the side chains of the molecule bind to both major and minor grooves of DNA^[34,39]. Intercalation of curaxins in DNA causes an increase in the distance between the DNA base pairs, resulting

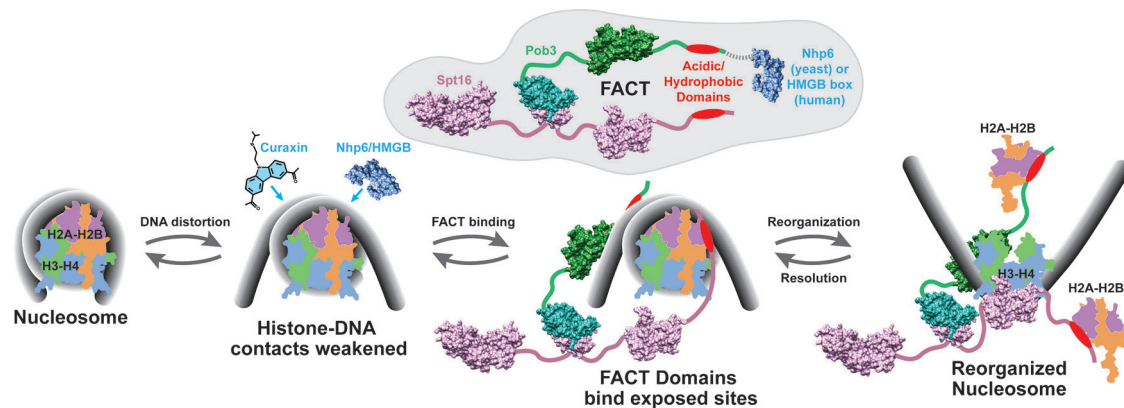


Figure 1. Nhp6 or curaxins can initiate uncoiling of nucleosomal DNA to promote reorganization by FACT. Either Nhp6/HMGB or curaxins can weaken the histone: DNA contacts near the entry/exit points of the nucleosomal DNA, exposing the binding site in H2A-H2B dimers for the acidic/hydrophobic anchor sequences found in the C-terminal domains of both subunits of FACT^[1]. The DNA distortion initiates a stepwise series of binding events by different domains of FACT that ultimately leads to uncoiling of the DNA to make the reorganized form of the nucleosome. Each step is reversible, providing a pathway for assembly of nucleosomes. Structures of the domains of FACT are shown, with the HMGB domain of SSRP1 represented by Nhp6 (modified from Figure 8^[2]). FACT: facilitates chromatin transcription; Nhp6: non-histone protein 6; HMGB: high mobility group B; Spt16: suppressor of ty 16; Pob3: polymerase one binding protein 3

in unwinding of the DNA helix and a change in the DNA topology^[34]. In the presence of curaxins, the nucleosome structure is destabilized in a dose- and time-dependent manner^[31,34,39,40]. In cells, CBL0137 does not cause histone loss or nucleosome disassembly at concentrations lower than 3 $\mu\text{mol/L}$ during a short time period (< 20 min in cancer cells). However, histone loss is detectable in cells treated with ≥ 3 $\mu\text{mol/L}$ CBL0137, and chromatin structure is destabilized when the concentration is > 5 $\mu\text{mol/L}$ ^[34]. Similarly, CBL0137 and CBL0100 cause only minimal disruption of nucleosomes in cell free conditions at concentrations ≤ 2.5 $\mu\text{mol/L}$ after incubation for several minutes^[31,34], while nucleosomes become unstable and are partially disassembled after incubation for a longer time period (> 1 h)^[31].

Although at concentrations ≤ 2.5 $\mu\text{mol/L}$ curaxins only minimally affect nucleosome structure, they significantly alter the structure of the internucleosomal linker DNA, increasing the average distance between the DNA regions entering and exiting a nucleosome^[46]. On defined nucleosomal arrays the change in the structure of linker DNA is translated into a considerable decrease in the probability of long-range interactions, impacting enhancer-promoter communication (EPC) *in vitro*. As a result, enhancer-dependent transcription is strongly inhibited by curaxins; no direct effect on the activity of the transcriptional machinery itself was observed^[46]. In agreement with the *in vitro* data, enhancer-dependent transcription is preferentially inhibited by curaxins in cells^[46].

Curaxin treatment has been shown to cause FACT (detected by fluorescence microscopy and chromatin immunoprecipitation) in cancer cells to disperse from transcribed regions and become trapped in other regions within 1 min^[31,34]. This FACT redistribution results in genome-wide changes in FACT-dependent transcription profiles^[31]. This phenomenon was recapitulated using a highly purified Pol II transcription system and positioned mononucleosomes *in vitro*^[31]. Curaxins inhibit human (hFACT) action during chromatin transcription *in vitro* by trapping FACT complexes on competitor nucleosomes. Under conditions where hFACT and curaxins alone had minimal effects on nucleosomes in spFRET assay, they induced extensive, reversible uncoiling of nucleosomal DNA (nucleosome unfolding or reorganization) when combined, demonstrating highly synergistic action^[31]. The non-transcribed regions of the genome are in excess over transcribed regions in a human cell, so FACT trapping by unfolded nucleosomes (n-trapping) causes depletion of hFACT from active regions of chromatin^[31]. Thus, FACT n-trapping is an important mechanism behind the short-term curaxin action in cancer cells.

Although hFACT induces extensive, reversible nucleosome unfolding in the presence of either Nhp6 protein or curaxins [Figure 1], the properties of these FACT-unfolded nucleosomes are different^[2,31]. The hFACT-γNhp6-nucleosome forms a reversible complex that is stable during electrophoresis, while the hFACT-nucleosome complex formed in the presence of curaxins is stable in solution, but falls apart during PAGE^[2,31]. Since Nhp6 and curaxins are expected to bind to similar, end-proximal regions of the nucleosomal DNA and produce similar unfolding of nucleosomes together with FACT^[2,31], the differences in stability of the complexes may be explained by the different structures/stabilities of Nhp6-nucleosome and curaxin-nucleosome complexes, or possibly by a direct effect of Nhp6 protein on the properties of hFACT.

Many DNA binding molecules other than curaxins also cause trapping of FACT on chromatin in cell-free systems^[40]. However, to be potent in chromatin destabilization, small molecules need to be able to get to the nuclei and compete with histones and other chromatin components for DNA binding. The curaxins that were selected and optimized in a cell-based assay for p53 activation^[84,85] easily enter into cell nuclei and are strong DNA binders. They were also selected in cells which were not responsive to DNA damage after p53 activation; thus, compounds capable of causing DNA damage were eliminated from the analysis. Curaxins can cause DNA damage in some systems; e.g., they cause frame shift mutations in some strains of *E. coli* in the Ames test. However, the much higher proofreading efficiency of mammalian DNA polymerases probably mitigates this effect at low concentrations of curaxins and blocks replication at high concentrations of the drug. Curaxins are also potent inhibitors of topoisomerases^[34], but, in contrast to many known drugs that inhibit resolution of topoisomerase cleavage complexes, do not prevent cleavage itself: curaxins inhibit initiation of topoisomerase action, preventing the induction of DNA breaks. Most importantly, the toxicity of curaxins for cells does not depend on DNA damage, since DNA damage is undetectable in many types of tumor cells as they are being killed by curaxin treatment^[35].

In addition to the c-trapping of FACT, curaxins globally affect the three-dimensional organization of the genome in living cells^[46]. Studies employing nucleosome arrays show that curaxins induce changes in the structure of internucleosomal linker DNA and unfolding of the chromatin fiber^[46]. Hi-C analysis demonstrated that addition of curaxins to cancer cells partially disrupted the boundaries of topologically associated domains (TADs), inhibited spatial interactions within the TADs (over distances less than 600 Kb), and enhanced spatial interactions over longer distances^[46]. These alterations of the three-dimensional genome organization strongly affected enhancer-promoter interactions: only 30% of the intradomain chromatin interactions annotated in control cells were maintained in CBL0137-treated cells. As a result, numerous new alternative, long-range interdomain interactions were detected^[46]. Furthermore, genomic studies demonstrated that curaxins caused partial depletion of CCCTC-binding factor (CTCF) from its binding sites^[46]. Since CTCF contributes to maintaining the spatial organization of the mammalian genome^[86], depletion of CTCF from domain boundaries by curaxins likely contributed to the drastic changes observed in genome topology. The changes in chromatin structure, in turn, are expected to dramatically change gene regulation and the pattern of gene expression. Indeed, after CBL0137 treatment, gene expression profiles were drastically changed. These changes included downregulation of essential genes necessary for cell survival, including both normal and translocated versions of MYC family genes, which could explain the observed drop in cancer cell viability^[46]. Thus, curaxins can be classified as epigenetic drugs that globally affect gene regulation and cause cancer cell death by targeting the three-dimensional organization of the genome within living cell nuclei.

THE MECHANISM OF CURAXIN ACTION

Taken together, the current studies suggest the following scenario for curaxin action in cancer cells [Figure 2]. In untreated cancer cells, FACT is present mostly in the nucleoplasm; only a small fraction of the complexes are transiently associated with nucleosomes perturbed by transcribing RNA polymerase.

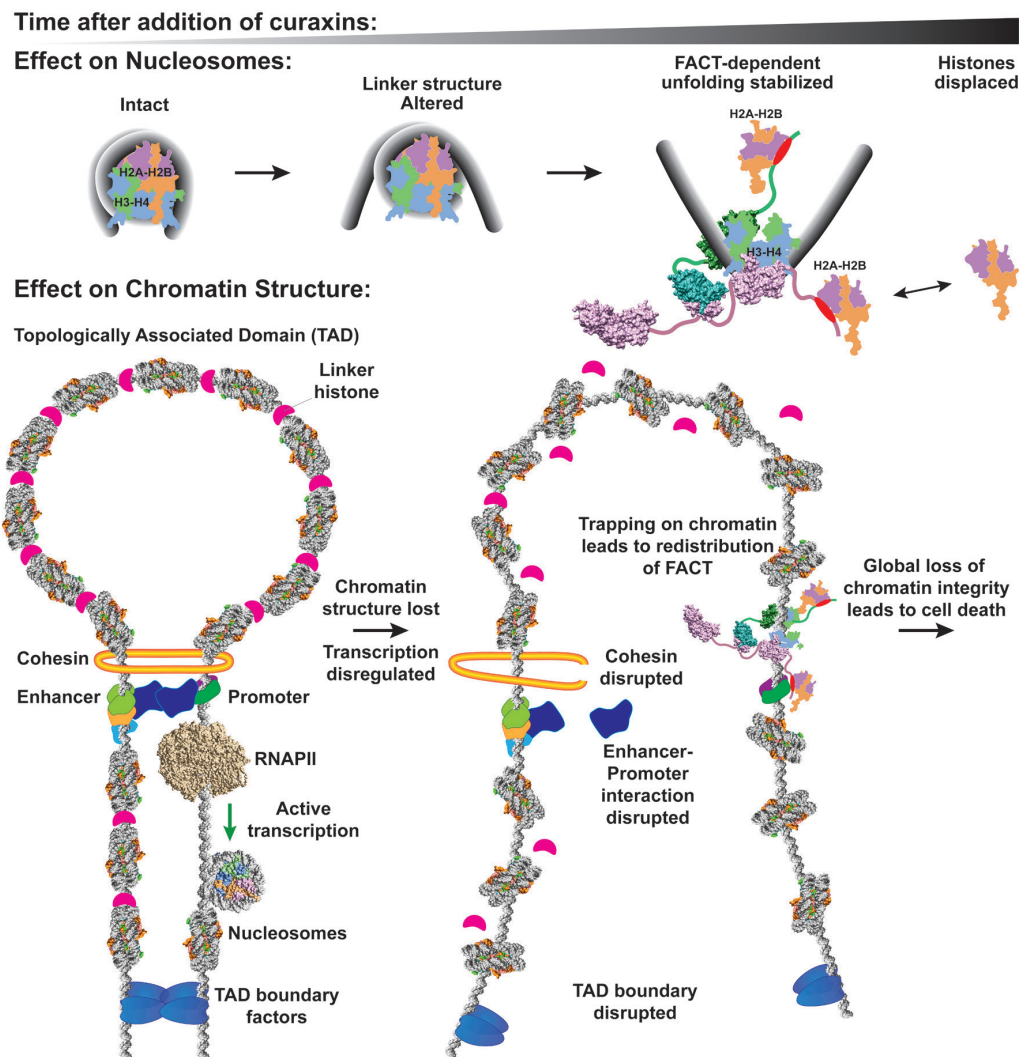


Figure 2. Curaxins affect different levels of chromatin structure. A proposed sequence of events during incubation of cancer cells with curaxins. Upper part: initially, curaxins induce time- and concentration-dependent FACT trapping on the unfolded nucleosomes. As curaxins intercalate into DNA, they induce progressive, FACT-dependent unfolding of nucleosomes that could eventually lead to eviction of core histones. Lower part: in parallel with the changes in the nucleosome structure, the higher-order chromatin structure is dramatically disturbed. Curaxins induce changes in the structure of internucleosomal linker DNA, releasing linker histones, unfolding the chromatin fiber, causing displacement of CTCF factor from DNA, and disrupting existing chromatin domain boundaries. Taken together, these changes in the structure of nucleosomes, chromatin fibers, and global three-dimensional chromatin organization lead to a large-scale loss of chromatin integrity and cancer cell death. Components are modeled as in Figure 1, with the addition of PDB 1Y1V (RNA polymerase II elongation complex structure). FACT: facilitates chromatin transcription; CTCF: CCCTC-binding factor

Curaxins rapidly induce changes in the structure of internucleosomal linker DNA and eviction of histone H1 from chromatin. The speed of this process is probably explained by the dynamic interaction of linker histones with chromatin^[13]. FACT trapping in bulk chromatin is initiated at the same concentrations of curaxins (0.5-1 $\mu\text{mol/L}$ of CBL0137) that induce displacement of H1, and both processes occur at about the same time (a few seconds after addition of CBL0137). It is therefore possible that FACT participates in H1 eviction or prevents reassociation of H1 with linker DNA. Higher curaxin concentrations also displace core histones from chromatin.

Binding of FACT to bulk chromatin occurs at multiple genomic locations, but there is some preference for regions enriched for tandem dinucleotide AC/TG repeats known as mini- and microsatellites. These occur mostly at non-coding regions, which are dispersed throughout the human genome^[87]. FACT binding and

displacement of histone H1 contribute to unfolding of the chromatin fibers and also to largely reversible uncoiling of nucleosomal DNA (nucleosome unfolding). Since FACT is tightly bound to (trapped by) and unfolds nucleosomes genome-wide, FACT-dependent gene expression is globally affected, preferentially decreasing the viability of cancer cells that have a higher requirement for FACT. After a longer curaxin treatment, FACT-bound unfolded/destabilized nucleosomes are prone to disruption, possibly evicting core histones from the DNA. Nucleosome unfolding and histone eviction are likely to result in the release of negative DNA supercoiling that was constrained in intact nucleosomes and in formation of alternative DNA structures, such as Z-DNA, resulting in further FACT trapping.

In addition to affecting the structures of nucleosomes and chromatin fibers, curaxins globally alter genome topology in cells. Curaxins induce eviction of CTCF from DNA, thus disrupting domain boundaries and dramatically decreasing the number and type of chromatin loops that are characteristic of cancer cells. The latter effect is likely explained by the global unfolding of the chromatin fiber due to the altered geometry and topology caused by curaxin treatment. In turn, the changes in chromatin topology result in less efficient EPC, and in preferentially reduced expression levels of enhancer-dependent genes (e.g., *Myc*) and global alteration of the profiles of gene expression. The suppression of enhancer-dependent oncogenes strongly decreases the efficiency of cancer cell survival. This combined mechanism of curaxin action explains the high efficiency of the compounds and suggests novel therapeutic strategies targeting chromatin structure and spatial organization of the genome to alter gene expression profiles, thereby suppressing cancer cell growth.

PERSPECTIVES

Mutations in enzymes that “write”, “read”, or “erase” chromatin marks have emerged as a recurring theme in multiple types of cancer^[88]. However, the mechanisms through which epigenetic changes benefit cancer cells remain poorly understood. Existing theories tend to focus on specific genes or pathways; that is, the effects of altering global properties of chromatin are usually attributed to altered expression of individual genes or groups of genes. However, discoveries such as “oncohistones”, where mutations in core histones are associated with cancer^[89], raise the possibility that carcinogenesis results from a broad combination of defects occurring genome-wide.

The role of FACT in cancer, and the use of curaxins to combat cancers, face similar questions. Neither FACT nor curaxins have clear gene-specific effects, and yet the former preferentially supports the viability of aggressive cancer cells and the latter has clear anti-cancer activity. In one scenario, FACT and curaxins could function through a common mechanism by destabilizing nucleosomes. In this model, FACT might enhance global expression of genes, including a set that is important for maintaining excessive proliferation, and curaxins extend this activity past the point where it is advantageous. However, recent results suggest instead that FACT and curaxins might have opposing effects on nucleosomes, with curaxins promoting unwrapping of DNA from the core^[34], while FACT promotes survival of nucleosomes by tethering the components together and promoting reassembly^[29]. As FACT is capable of both destabilizing and stabilizing nucleosomes, understanding how it promotes survival of cancer cells and how curaxins oppose this activity will ultimately require better understanding of how global nucleosome stability contributes to cancer cell progression and viability.

Pervasive cryptic transcription has been detected in multiple types of cancer^[90-92], suggesting that the DNA is more accessible to transcription machinery in tumor cells than it is in normal cells, as expected if the nucleosomal barrier is globally weakened. In this case, the role of FACT in stabilizing and restoring nucleosomes would be critical to allowing tumor cells to maintain chromatin. Curaxins would then interfere with this process, creating an excessive burden of disrupted chromatin, eventually leading to cell death. Then, the next questions to answer are: Why are nucleosomes less stable in tumors, thereby

creating greater need for FACT activity? Do elevated transcription and replication decrease the stability of nucleosomes, or do tumor cells promote destabilization through an unknown mechanism to provide advantages such as easier reorganization of the three-dimensional structure of the genome or easier transitions among gene expression profiles? What is the relationship between nucleosome stability and three-dimensional chromatin structure? While it might seem obvious that loss of chromatin structure is disadvantageous, is this because of toxicity of the free histones that are produced by chromatin disassembly or is it the result of inappropriate exposure of DNA? Cancer cells survive by unleashing the beasts of excessive proliferation and metastasis that are normally constrained within chromatin; how do FACT and curaxins alter the balance between taking advantage of these dangerous forces to become cancerous and being overtaken by them?

DECLARATIONS

Authors' contributions

Wrote the initial draft of the manuscript and finalized it: Chang HW

Wrote parts of the manuscript: Nizovtseva EV, Razin SV

Wrote parts of the manuscript and prepared the final figures: Formosa T

Wrote parts of the manuscript: Gurova KV

Designed the review and the figures, wrote parts of the manuscript and finalized it: Studitsky VM

Availability of data and materials

Not applicable.

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Conflicts of interest

Gurova KV obtained a research grant and consulting payments from Incuron, Inc. The remaining authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Electrolyte disorders in cancer patients: a systematic review

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Abstract

Electrolyte disorders are very common complications in cancer patients. They might be associated to a worsening outcome, influencing quality of life, possibility to receive anticancer drugs, and conditioning survival. In fact, they might provoke important morbidity, with dysfunction of multiple organs and rarely causing life-threatening conditions. Moreover, recent studies showed that they might worsen cancer patients' outcome, while a prompt correction seems to have a positive impact. Furthermore, there is evidence of a correlation between electrolyte alterations and poorer performance status, delays in therapy commencement and continuation, and negative treatment outcomes. These alterations usually involve sodium, potassium, calcium, and magnesium serum levels. Several causes might contribute to electrolyte disorders in cancer patients: cancer effects, such as paraneoplastic syndrome of inappropriate antidiuresis and tumor lysis syndrome; anti-cancer therapies; and other concomitant clinical conditions or treatments. However, the origin of the electrolyte disorder is often multifactorial, thus identifying and correcting the causes is not always feasible. Furthermore, they are often not recognized or not considered in clinical practice, worsening these alterations and patient condition. An improvement of knowledge about the physiological mechanisms underlying electrolyte disorders is necessary to strengthen their identification and set up a prompt, adequate, and effective treatment. The aim of this systematic review is to provide an analysis of the pathophysiological mechanisms of electrolyte abnormalities in cancer patients to facilitate their identification, management, and therapy to improve patient outcome.

Keywords: Cancer, electrolyte disorders, hyponatremia, syndrome of inappropriate antidiuresis, hyperkalemia, hypocalcemia, hypomagnesemia



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INTRODUCTION

Electrolyte disorders are very common conditions in cancer patients. They mainly concern changes in serum sodium, potassium, calcium, and magnesium levels. In most cases, these alterations are asymptomatic and therefore not always taken into consideration in clinical practice. However, they can sometimes be associated with clinical manifestations that can worsen patient's clinical condition up to more serious life-threatening events. Furthermore, several clinical studies showed an important impact of electrolyte disorders on cancer patients' journey. Firstly, they seem to correlate with a worsening quality of life and performance status, reduced probability of tumor response to anti-cancer treatment and treatment delays, and cause poorer outcomes and reduced survival^[1]. Electrolyte disorders in cancer patients might depend on several causes: cancer physiopathology, anti-tumor treatments, concomitant clinical conditions, or therapies. However, they often have a multifactorial origin and they might be both secondary and responsible for multiple organ systems' dysfunction. A prompt correction of electrolyte disorders is commonly associated with a better prognosis. Therefore, increasing attention towards electrolyte disturbances is emerging in the literature and clinical trials^[2]. Ingles Garces *et al.*^[3] published a review on phase I trials performed between 2011 and 2015. They showed elevated rates of hyponatremia (62%), hypokalemia (40%), hypomagnesemia (17%), and hypocalcemia (12%) in cancer patients treated with new anticancer-agents and that patients who developed adverse events in terms of electrolyte disturbances during follow-up had a poorer median overall survival (26 weeks *vs.* 37 weeks, hazard ratio = 1.61; $P < 0.001$). These data suggest the importance of monitoring and correcting electrolyte disorders in cancer patients. The relationship between electrolyte disorders and poor prognostic impact on cancer patients might depend on the fact that several ion channels and transporters are over- or under-expressed in cancer cells and implicated in phenomena of cell proliferation, apoptosis, migration, and invasiveness^[4]. Recently, it has been demonstrated that several channel genes are expressed in several cancer types, suggesting an important role in tumor development and progression. For example, a recent study proposed an ion channel gene signature for breast cancer as a prognostic and diagnostic biomarker, showing an association between different ion gene expression and p53 mutation status, estrogen receptor status, and grading^[5].

Our review aims to focus on electrolyte disorders in cancer patients, providing information for correct and prompt diagnosis, therapy, and monitoring, in order to improve the outcome of the patients.

SODIUM

Sodium is the major cation of extracellular fluid (ECF) and the main constituent of serum osmolality. Its concentration in ECF is essential for maintaining the circulating blood volume and it is strictly interdependent of water presence. Normal serum sodium level is 135-145 mmol/L and its imbalance may be caused by alteration of both solute and water intake, depletion, and dilution^[6].

Several integrated systems are involved in maintaining normal serum sodium concentration, such as regulation of water intake by thirst, control of free water renal excretion by antidiuretic hormone secretion, renal sodium excretion by glomerular filtration, peritubular conditions, and adrenal aldosterone secretion.

In particular, the angiotensin II arginine vasopressin (AVP) - atrial natriuretic peptide pathway is the most important mechanism of sodium balance^[7], and it is strictly associated to serum osmolality^[8].

Alterations in serum sodium concentration include hyponatremia and hypernatremia. Sodium channels have been described in cancer cells and associated with a more aggressive behavior^[4]. Voltage-gated sodium channels (VGSC) are a large group of trans-membrane proteins that allow the flow of sodium ions down the electrochemical gradient through cell membranes. In particular, overexpression of Nav 1.5, a VGSC, seems to promote cell proliferation, migration, invasion, and metastasis of oral squamous cell

carcinoma^[9], breast cancer^[10], and cervical carcinoma^[11]. The expression of a particular VGSC, SCN9A, in prostate cancer is associated with higher risk of metastasis due to its activity promoting cell migration. Furthermore, it is overexpressed in prostate cancer cells, compared to normal cells, representing a potential diagnostic biomarker^[12]. VGSCs are overexpressed in non-small-cell lung cancer cells and involved in cell invasiveness. Recent data show that the upregulation of SCN9A in non small cell lung cancer is regulated by EGFR signaling and it is crucial for the invasive behavior of cancer cells, suggesting a potential new biomarker and therapeutic target^[13]. Nav 1.5 (SCN5A) was found overexpressed also in colorectal cancer (CRC) cells compared to normal colon tissues. SCNA5 was demonstrated to regulate CRC invasion process. In fact, it is involved in activation of several pathways: Wnt signaling, steroid metabolic process, favoring cell migration, ectoderm development, and influencing cell cycle control^[14]. Finally, a recent multicenter study identified two single nucleotide polymorphisms of VGSC genes (the intron SNP SCN4A-rs2302237 and the SCN10A-rs12632942 SNP) that were associated with oxaliplatin-induced peripheral neuropathy development, which is interesting as the majority of patients with CRC are given oxaliplatin^[15].

Serum sodium concentration is also regulated by aquaporins (AQP), or water channels, a large family of membrane proteins involved in water transportation between cells. AQP1 might be expressed in breast cancer cells and it is associated with aggressive behavior. In fact, it correlates with higher grading, CK14 expression, smooth muscle actin expression, basal-like group, and poor prognosis^[16]. Aquaporin's expression has also been investigated in CRC. In particular, AQP1, AQP3, and AQP5 are expressed in CRC cell lines and in primary CRC. They have been described during CRC progression and in liver metastases^[17]. AQP5 over-expression was demonstrated to be associated with worse TNM stage, grading, and lymph node involvement^[18]. AQP3 expression is positively regulated by endothelial growth factor pathway and it is associated with lymph node involvement, metastasis, and tumor differentiation^[19]. Furthermore, a recent study showed that reduced AQP9 gene expression is associated with a lack of response to adjuvant chemotherapy^[20]. AQP3 and AQP5 are also over-expressed in esophageal cancer cells compared to normal tissue and their co-expression seems to have a negative prognostic role^[21]. The co-expression of AQP3 and AQP5, also described in gastric cancer, is associated with lymph node involvement and intestinal type^[22].

Although the effect of serum sodium concentrations' alterations on the functionality of VGSCs and AQP in tumor cells is still unknown, many data are emerging on the effect of dysnatremic conditions in neurons. In particular, a hyponatremic condition induces a modification of the axonal Na⁺ channels kinetics, resulting in a decreased Na⁺ current. Moreover, the damage is proportional to the severity of hyponatremia and concerns the recovery phase of VGSC. On the other hand, hypernatremia increases the excitability of the membrane, reducing the response time of the channel. However, the effect of hyponatremic condition seems to be less destructive compared to situation of hyponatremia on the activity of Na ion channel^[23]. Syndrome of inappropriate antidiuresis (SIAD) is a rare cancer paraneoplastic syndrome causing hyponatremia. It is associated with overexpression of AQP in renal cells. Even though no data are available on AQP expression in cancer cells, secondary to SIAD, dysnatremia could also play a role in AQP expression in cancer cells^[1]. These data suggest that alterations in serum sodium concentration might play an important role on the functionality of tumor cells that overexpress sodium channels and AQPs.

Finally, the importance of sodium in oncology is also underlined by another potential development field, sodium magnetic resonance imaging (²³NaMRI). ²³NaMRI seems to be able to provide information on cells' metabolism and their physiology, exploiting the different total tissue sodium concentration between healthy tissue and neoplastic cells. ²³NaMRI takes advantage of the abnormally high sodium concentration of proliferating cells, due to altered pH and Na⁺/H⁺ transport kinetics. Currently, the most promising application areas of ²³NaMRI are the early diagnosis of brain tumors and breast cancer^[24].

Hyponatremia

Definition and clinical implications

Hyponatremia is defined as a serum/plasma sodium concentration lower than 135 mmol/L. It can arise rapidly within 48 h (acute hyponatremia) or, more frequently, slowly (chronic hyponatremia). According to its serum level, it can be classified into three severity grades: mild (130-134 mEq/L), moderate (125-129 mEq/L), and severe (< 125 mEq/L)^[25].

It represents the most common tumor-related electrolyte disorder. Even though its accurate incidence is still unknown because different serum sodium cut-off levels were considered in several studies, a variable incidence of 4%-44% was reported, depending on cancer type and clinical setting^[26]. Higher incidences are associated with thoracic neoplasms, and, in particular, it occurs more frequently in patients with small-cell lung cancer, with a median estimated rate of 15%^[26].

Several studies evaluated the impact of hyponatremia on cancer patients, showing that, independently of causes, it negatively correlates with patients' outcome^[27]. In particular, hyponatremia seems associated to poorer performance status^[28] and reduced survival in patients with lung cancer^[29], renal cell carcinoma^[30], malignant pleural mesothelioma^[31], gastric cancer^[32], colon-rectal cancer^[33], and lymphoma^[34]. Recent evidence shows an important negative predictive role of hyponatremia, for patients receiving both chemotherapy and target therapy^[35], while a prompt correction of this electrolyte disorder improves patients' outcome, which takes into account the correct timing to avoid neurological damage^[36]. Furthermore, hyponatremia seems to also have a negative role in hospitalized patients, as it was demonstrated to be associated with a longer length of hospital stay, inducing a negative impact on quality of life and prognosis and an increase in hospitalization costs^[37].

Causes

In cancer patients, several causes might induce hyponatremia^[1]:

- (1) Cancer: paraneoplastic syndromes such as SIAD, brain metastasis, adrenal metastasis, and kidney metastasis can cause hyponatremia.
- (2) Cancer-treatment: it can cause hyponatremia as a direct effect of their mechanism of action (vinca alkaloids might induce SIAD; platinum derivatives are frequently associated to hyponatremia; and target therapies, in particular antiangiogenic agents, seem to induce hyponatremia, despite the underlying mechanism being unknown) or as a result of side effects such as gastrointestinal losses (vomiting and diarrhea caused by most of chemotherapeutic agents, target therapies, and immunotherapy), kidney loss, and heart failure (cardiotoxic drugs such as anthracyclines and target therapies such as anti HER-2, anti-ALK, and anti-MEK). Immunotherapeutic agents might cause direct damage to adrenal or pituitary gland, favoring hyponatremia development.
- (3) Concomitant drugs: diuretics, antibiotics, non-steroidal anti-inflammatory drugs (NSAIDs), opioids, antidepressants, and neuroleptics can induce hyponatremia.
- (4) Concomitant diseases: heart failure, kidney failure, thyroiditis, hypercortisolism, liver cirrhosis, pneumonia, and inflammatory lung or brain diseases can induce hyponatremia.

However, in most cases, more than one of the aforementioned factors might induce hyponatremia in cancer patients.

These causes can be traced back to two different basic mechanisms: excessive free water (for increased intake or reduced elimination) or, rarely, sodium loss (reduced intake or increased loss). The knowledge

Table 1. Causes of hyponatremia

Extracellular volume	Causes of hyponatremia
Hypovolemic: increased water loss	Gastrointestinal losses (vomiting, diarrhea) Renal losses (nephropathies, M. Addison, Diuretics) Cerebral salt wasting syndrome
Euvolemic: salt loss	Syndrome of inappropriate antidiuresis Endocrine dysfunctions (hypercortisolism, hypothyroidism)
Hypervolemic: water retention	Edematogenic syndromes (cirrhosis, heart failure, kidney failure, nephrotic syndrome)

of these two different mechanisms is fundamental for the differential diagnosis between the potential causes in order to set a correct therapeutic approach. Extracellular volume (ECV) status is fundamental to distinguishing the mechanism underlying hyponatremia.

According to ECV status, hyponatremia can be classified in [Table 1]:

- Hypovolemic, with reduced ECV.
- Euvolemic, with normal ECV.
- Hypervolemic, with increased ECV.

Hypovolemic hyponatremia is often due to water loss, namely gastrointestinal loss (vomiting, diarrhea), renal losses, bleeding, and cerebral salt wasting, caused by a dysfunction of hypothalamic-renal axis.

Euvolemic hyponatremia, despite being rare, is an important and frequent condition in cancer patients. Several mechanisms induce euvolemic hyponatremia such as adrenal insufficiency, hypothyroidism, and SIAD. SIAD is the principal cause of hyponatremia in oncological and hospitalized patients, occurring in 1%-2% of all subjects and in 30% of cancer patients^[26]. It is characterized by a deregulated AVP activity, which induces a lower free water excretion. The relative free water surplus leads to serum euvolemic hypo-osmolar hyponatremia. It is often due to a paraneoplastic syndrome, related to several kinds of tumors. It is most frequently reported in patients with small-cell lung cancer, but it is also described in patients with non-small-cell lung cancer, head and neck cancer, and, rarely, other malignancies^[26].

SIAD may be caused by^[38]:

- Inappropriate secretion of antidiuretic hormone (ADH) from cancer cells (paraneoplastic syndrome).
- Activating mutation of ADH receptor V2.
- Inappropriate or persistent release of AVP.

Although paraneoplastic syndrome is the most frequent cause of SIAD, it should be considered that many conditions might lead to an inappropriate release of AVP in cancer patients^[39,40]:

- Concomitant drugs: diuretics, analgesics, chemotherapies, anticancer target agents, antidepressants, antipsychotics, antiepileptics, and antiemetics.
- Central nervous system disease: expansive lesions such as neoplasms or hematomas, inflammatory diseases (e.g., encephalitis, meningitis, *etc.*), degenerative diseases (e.g., Guillain-Barré syndrome), and other rare conditions (e.g., hemorrhage, delirium tremens, hypophyseal peduncle section, transphenoidal adenectomy^[41], and hydrocephalus).
- Pulmonary disorders: infections, respiratory failure, chronic obstructive pulmonary disease, and active pressure ventilation^[1].
- Others: AIDS, senile atrophy, and idiopathic.

Hypervolemic hyponatremia is characterized by an excess of both total body sodium and water. It occurs in edematous conditions such as cirrhosis, chronic kidney disease, nephrotic syndrome, and congestive heart failure^[42].

According to serum osmolality status, hyponatremia can be divided into^[43,44]:

- Hypotonic is characterized by reduced serum osmolality, due to an excess of free water compared to the sodium. This condition might be induced by an excessive water intake (e.g., primary polydipsia) or by a compromised renal water excretory capacity (SIAD).
- Hypertonic is characterized by increased serum osmolality (e.g., in glycemic decompensation).
- Isotonic is characterized by normal serum osmolality, often secondary to an artificially hyponatremia (pseudohyponatremia) due to elevated serum solutes concentrations (e.g., hyperlipidemia).

Management

A correct and timely diagnosis of hyponatremia is essential to setting up a rapid therapy and improving the prognosis of cancer patients. Diagnosis can occur from clinical suspicion, but it is essential for clinicians monitoring serum/plasma sodium level in all cancer patients, in order to promptly correct this electrolyte disturbance, when present, even in the case of symptoms' absence. Hyponatremia symptoms are often absent or generic and closely related to hyponatremia grade and onset speed^[1].

Patients with mild and/or chronic hyponatremia are often asymptomatic or present blurred symptoms that can be misunderstood or imputed to other causes (e.g., dizziness, postural instability, and asthenia).

Patients with severe and/or acute hyponatremia can present different symptoms (from gastrointestinal symptoms such as lack of appetite, nausea, and vomiting to neurological disorders such as headache, irritability, attention deficit, confusion, gait disturbances, and muscle cramps), including life-threatening conditions (bulbar paralysis, lethargy, convulsions, encephalic brain herniation, coma, and cardio-respiratory arrest).

Diagnosis of hyponatremia requires routine laboratory tests. For a correct therapeutic approach, it is crucial to identify the underlying causes, thus lab assessment should also include plasma and urine osmolality, ECV status evaluation, and urinary sodium concentration to obtain a correct differential diagnosis [Figure 1].

In particular, due to different therapeutic options, it is fundamental to exclude SIAD. SIAD diagnosis is diagnosis of exclusion, for which the main criteria are:

- Presence of hyponatremia (< 135 mEq/L).
- Normal ECV.
- Lower serum osmolality (< 275 mOsm/kg).
- Altered urine osmolality (> 100 mOsm/kg).
- Elevated urine sodium concentration (> 30 mmol/L).
- Normal renal function.
- Normal adrenal and thyroid function.
- No use of diuretics.

The therapeutic approach depends on etiology, presence of symptoms, and grade of hyponatremia [Figure 1]. Regardless of hyponatremia's cause, it is important to remember that an effective and prompt correction of serum sodium concentration improves the outcome of cancer patients^[45].

Treatment options include fluid restriction, diuretics, saline solution administration, and vaptans (selective vasopressin receptor antagonists).

Fluid restriction is a difficult therapeutic choice since it is associated with poor compliance because cancer patients often need abundant hydration for oncological therapies. Furthermore, several days are required to correct serum sodium concentrations. In the case of ECV, isotonic saline infusion should be preferred.

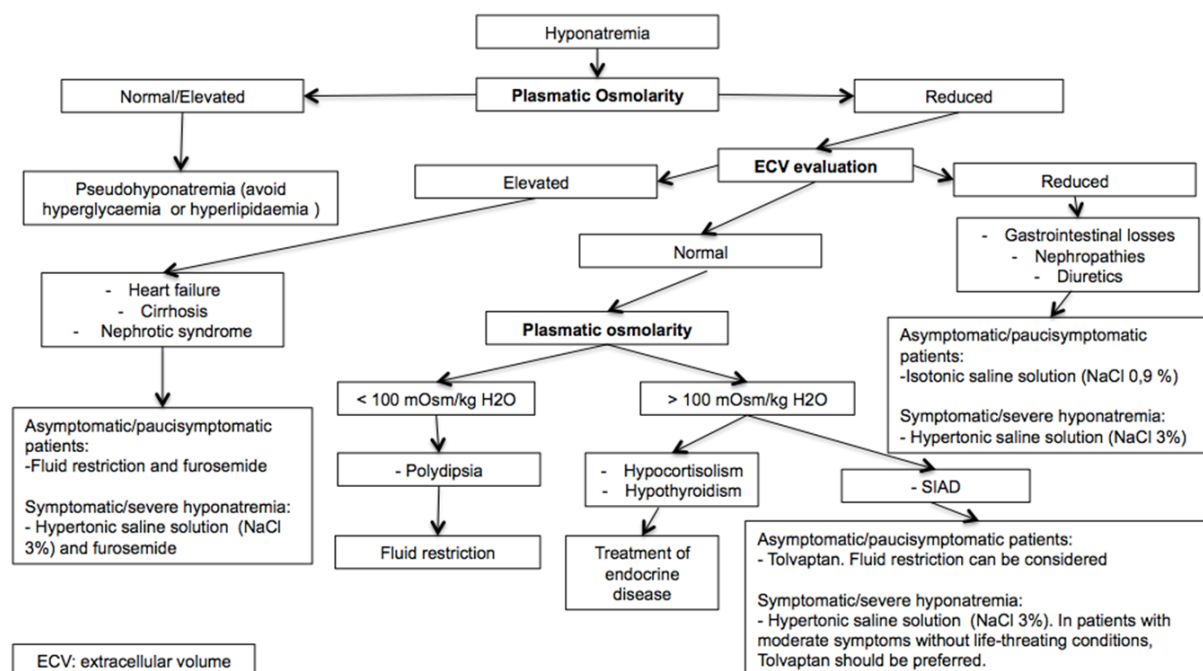


Figure 1. Hyponatremia management algorithm^[38,44-48]. The correct management of hyponatremia and its treatment require the detection of its cause. Plasmatic osmolarity and ECV evaluation are necessary to recognize the origin of hyponatremia. Symptomatic hyponatremia should be treated with hypertonic saline solution. Tolvaptan should be considered in hyponatremia due to SIAD. ECV: extracellular volume; SIAD: syndrome of inappropriate antidiuresis

In the case of hyponatremia secondary to SIAD, the use of Tolvaptan, a selective V2 receptor antagonist, should be considered^[46]. In fact, has shown an important efficacy to correct and stabilize serum sodium concentration, favoring the beginning and prosecution of anticancer treatments without delay. Furthermore, it seems to reduce the risk of hyponatremia development as chemotherapy adverse event^[47]. Tolvaptan schedule requires starting dose of 15 mg once daily and it should be administrated first in a hospital department to monitor the therapeutic response and any adverse event. It could be increased at 24-h intervals, when serum/plasma sodium levels are not improved, to a maximum of 60 mg once a day^[46].

Other approved therapeutic agents are urea and Demeclocycline. However, due to their toxicity and poor patient compliance, they are no longer employed in clinical practice.

In the case of presence of severe neurological symptoms or life-threatening conditions, serum sodium levels should be corrected rapidly and the appropriate therapy is the administration of 3% hypertonic saline^[48].

It is important to monitor the rate of correction of hyponatremia since an excessive speed of rising sodium levels might cause the development of central pontine myelinolysis, an irreversible condition that leads to death. The correction rate should not exceed 12 mmol/L/24 h. Therefore, it is recommended to monitor plasma sodium levels in the first 24 h at regular intervals of 4-6 h, in order to control the correction speed^[49].

Hypernatremia

Definition and clinical implication

Hypernatremia is defined as an elevated serum sodium level over 145 mmol/L. It is a frequent electrolyte disturbance occurring in 1%-5% of hospitalized patients. It represents a negative prognostic factor, related to an elevated mortality rate (40%-75%) compared to eunatremic patients^[50].

Table 2. Causes of hypernatremia

Causes of hypernatremia	
Euvoletic: loss of free water	Losses through skin and breath Reduced thirst stimulation Neurogenic or nephrogenic diabetes insipidus
Hypovolemic: loss of free water	Renal disorders (diuretics, tubular necrosis) Gastro-intestinal disorders (e.g., vomiting, nasogastric drainage, entero-cutaneous fistulae, diarrhea) Cutaneous diseases (burns, excessive sweating)
Hypervolemic: sodium accumulation	Hypertonic sodium bicarbonate or hypertonic saline infusion Excessive ingestion of NaCl Use of emetics rich in NaCl Enteral nutrition Urogenital injection of hypertonic saline Hypertonic dialysis Primitive hyperaldosteronism Cushing Syndrome

Causes

In cancer patients, several causes might induce hypernatremia [Table 2]^[51,52]:

- (1) Cancer: anorexia and cancer cachexia, kidney damage, brain metastasis inducing diabetes insipidus, and gastrointestinal disorders due to cancer infiltration (e.g., fistulae and nasogastric drainage due to bowel obstruction) can induce hypernatremia.
- (2) Cancer treatment: adverse events such as vomiting and diarrhea common to most anti-cancer agents (chemotherapy, TKIs, and immunotherapies) associated with reduced thirst stimulation might cause hypernatremia. Elevated serum sodium concentration might be induced also by bowel direct damage due to antiangiogenetic agents or immunotherapy. Furthermore, some chemotherapeutic agents such as ifosfamide might induce an iatrogenic diabetes insipidus.
- (3) Concomitant drugs: osmotic diuretics, corticosteroids, enteral or parenteral nutrition, and hypertonic saline infusion can induce hypernatremia.
- (4) Concomitant diseases: cushing syndrome might induce hypernatremia.

Two different basic mechanisms might be involved in hypernatremia development: water loss (for reduced introduction (euvoletic hypernatremia) or increased elimination (hypovolemic hypernatremia), or, rarely, accumulation of sodium (often on iatrogenic basis, hypervolemic hypernatremia). Understanding these mechanisms is crucial for a correct differential diagnosis among potential causes of hypernatremia.

The most frequent mechanism underlying hypernatremia is total body water loss due to impaired thirst stimulation. It is often associated with altered mental status conditions, such as older age, brain tumors, damage, or surgery, causing a deficit in thirst and osmoregulation^[51]. Water loss can also be due to renal or extra renal disorders^[52].

Renal water loss usually is caused by osmotic diuresis (e.g., hyperglycemia, increased serum urea concentration, or administration of mannitol or hypertonic solutions) and by a treatment with diuretics^[53].

Rarely, renal water loss can be induced by insipidus diabetes, a deficit of the vasopressin-ADH-receptor system, which can have a central or a nephrogenic origin. Central insipidus diabetes is characterized by a reduced secretion of AVP, often related to a central nervous system damage (e.g., pituitary or hypothalamus neoplasms, brain surgery, or irradiation)^[54]. Nephrogenic insipidus diabetes instead depends on renal resistance to the action of AVP. It is a rarely congenic condition, more frequently related to iatrogenic effect of amphotericin B, lithium, ifosfamide, foscarnet, and streptozocin on tubular reabsorption of water^[53].

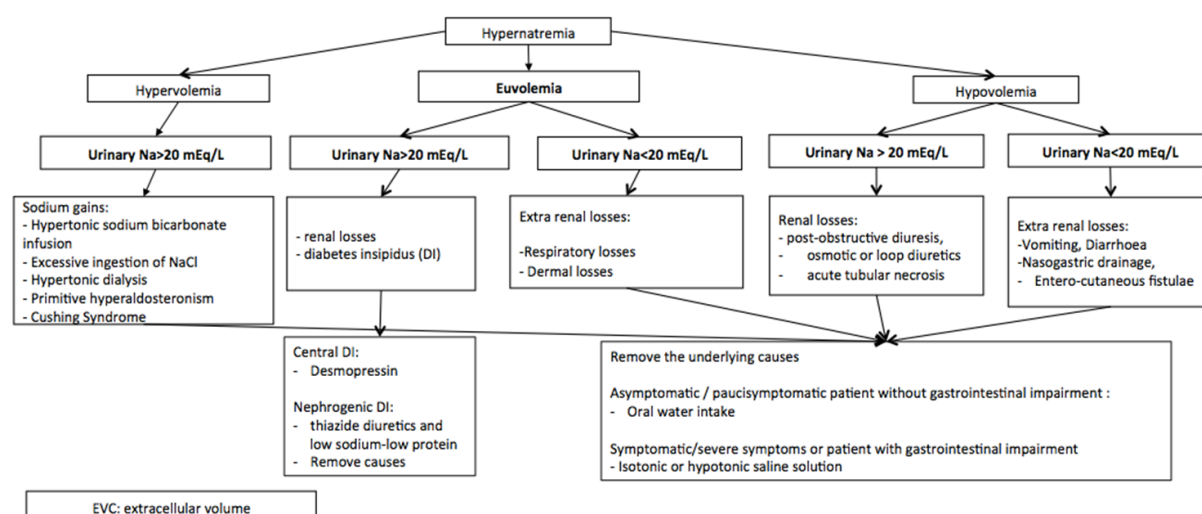


Figure 2. Algorithm of hypernatremia's management^[48,51-60]. The treatment of hypernatremia in cancer patients is based on the correction of the cause. For the differential diagnosis, the evaluation of volume and urinary sodium are fundamental. DI: diabetes insipidus; EVC: extracellular volume

Extra-renal water loss is often related to gastrointestinal diseases (vomiting, nasogastric drainage, and diarrhea).

Rarely, hypernatremia can be caused by excessive salt intake. This condition is often iatrogenic and induced by parenteral administering of hypertonic solutions or chronic nutrition support with hyperosmolar or high protein feeds^[55].

Management

Hypernatremia causes neurological symptoms, for which severity is correlated with both grade and onset speed. In most cases, patients refer non-specific symptoms such as thirst, anorexia, restlessness, nausea, muscle weakness, and confusion. In the case of rapid onset or severe hypernatremia, patients might present lethargy, hyperreflexia, until convulsions, and coma^[56].

Clinical suspicion of hypernatremia should be confirmed by laboratory exam. The correct diagnosis and the detection of specific causes or predisposing factors are crucial for a correct management [Figure 2].

For a correct differential diagnosis between hyponatremia caused by excess of sodium intake and hyponatremia caused by loss of free water, the assessment of urine osmolality, urine sodium concentration, and urine volume should be obtained^[57]. Concentrated urine is usually related to insufficient water intake or extra-renal losses.

Conversely, hypernatremia is associated to elevated serum osmolality and low urine osmolality renal damage with deficient capacity of urinary concentration^[56].

Hypernatremia associated to polyuria (e.g., 24 h urine volume exceeding 2.5 L), low urine osmolality, and urinary sodium are criteria for central insipidus diabetes diagnosis^[58].

Once hypernatremia diagnosis is confirmed, the optimal management requires the removal of the cause and the correction of the electrolyte disorder based on the total ECV, restoring intravascular volume and free water.

In collaborating and asymptomatic patients without gastrointestinal dysfunction, oral hydration is effective, and should be preferred. In patients with severe hypernatremia or unable to intake fluid orally (due to vomiting or neurological changes), intravenous hydration should be considered.

The infusion of free water (5% dextrose solution) should be considered in the case of loss of free water alone. Loop diuretics should be considered in the case of pure sodium gain natriuresis.

In patients with acute hypernatremia (within 24 h) or severe symptoms, treatment with isotonic or hypotonic solutions should be started immediately, since it was demonstrated that a prompt correction (up to 8-12 mmol/L per day) improves patients' prognosis without risk of convulsions or cerebral edema. A correction of 1 mEq/L/h should be considered safe^[59].

In patients with chronic hypernatremia or when time of onset is unknown, the correction should be obtained within 48 h, with a reduction of serum osmolality of no more than 0.5 mOsm/L/h to avoid cerebral edema onset. A reduction of serum sodium concentration of 8-10 mmol/L per day should be considered safe. Monitoring serum sodium levels at regular intervals of 4 h is highly recommended to control the correction speed^[59].

Patients experiencing central insipidus diabetes should receive nasal or oral desmopressin. Nephrogenic insipidus diabetes should be treated with a combination of thiazide diuretics and low sodium-low protein, removing potential precipitation factors^[60].

CALCIUM

Calcium is an extracellular cation and the normal serum calcium concentration range is 2.1-2.5 mmol/L (8.5-10.5 mg/dL) or ionized calcium of 1.1-1.4 mmol/L (4.5-5.6 mg/dL)^[61]. Most of the calcium content is deposited in the organic matrix by hydroxyapatite crystals of bones. Calcium appears in three different forms: free ion (50%), bound to plasma proteins, and in diffusible complexes. Acid-base status influences the binding between calcium and serum proteins. In particular, alkalosis favors the binding while acidosis induces the ionized calcium form. Calcium derives from diet and it is excreted by kidney.

Calcium reabsorption in kidney occurs mainly in the proximal tubules, and a small share in the ascending loop of Henle, thus loop diuretics decrease tubular calcium resorption, whereas thiazide diuretics improve its resorption^[62]. Calcium metabolism requires a steady interaction between bone and ECF. Several hormones are involved in calcium homeostasis. Parathyroid hormone (PTH), whose secretion is mediated by reduced serum calcium levels, acts on bone, favoring osteoclastic-mediated bone resorption and promoting calcium leakage and it induces the synthesis of active vitamin D and calcium intestinal absorption. Calcitonin instead, whose secretion is mediated by increased serum calcium levels, reduces the transfer of calcium from bone calcium pool to the ECF, and it decreases bone resorption inhibiting osteoclasts' activity and increases calcium renal excretion^[63].

Vitamin D also plays a crucial role in serum calcium homeostasis, favoring increased intestinal calcium absorption and bone calcium storage^[64].

Several studies demonstrated a crucial role of calcium-mediated signaling pathways in carcinogenesis, dedifferentiated into cancer stem cells, cellular motility favoring tumor invasion and metastasis, and the regulation of apoptosis^[65].

Several calcium channels are involved and expressed in cancer cells. Ca^{2+} ATPase I isoforms (SPCA1 and ATP2C1) are described in basal-like breast cancer, favoring cell proliferation, while calcium efflux pump

Table 3. Causes of hypocalcemia

Causes of hypocalcemia	
Reduction of serum calcium	Not ionized calcium: hypoalbuminemia, nephrotic syndrome, liver disease, malnutrition Ionized calcium: hyperlipidemia, parenteral nutrition enriched of free fatty acid Extravascular deposition: osteoblastic metastases, pancreatitis
Renal failure	Iatrogenic, post-renal obstruction, compression and infiltration by malignancy, tumor lysis syndrome, hyperuricemia, sepsis, contrast agent nephropathy
Endocrine disorders	Vitamin D deficiency or resistance: inadequate dietary intake, reduced absorption due to hepatobiliary or intestinal malabsorption, liver disease PTH deficiency or resistance: parathyroidectomy, autoimmune disorders, hungry bone syndrome
Concomitant electrolyte disorders	Hypomagnesemia Hyperphosphatemia
Drugs	Antiepileptics: phenytoin, phenobarbital Anticancer agents: fluorouracil, leucovorin, nab-paclitaxel, estramustine, octreotide, imatinib, axitinib, panitumumab, cetuximab, cisplatin Others: bisphosphonates, denosumab, rifampicin, calcium chelators, radiographic contrast agent, furosemide, foscarnet, EDTA, cinacalcet

PTH: parathyroid hormone

PMCA2 (ATP2B2) is most frequently overexpressed in HER2-receptor-positive breast cancer^[66]. Calcium channels are also described in androgen-responsive prostate cancer. In fact, they mediate androgen-induced effects^[67]. Transient receptor potential cation channel (TRPC), subfamily C, is a group of channels expressed in cancer cells. In breast cancer, TRPC1 correlates with low proliferation and TRPM8 overexpression correlates with ER-positive and well-differentiated lower-grade breast cancer^[68]. In prostate cancer, TRPM8 expression is regulated by androgens. Decreased expressions of TRPM8 and TRCP1 are associated with the progression to androgen-independent phase and poor prognosis^[67]. Contrariwise, TRPV6 ion channel's expression seems to be controlled by androgen receptors and it is highly expressed in cancer prostate cells, regardless of androgen dependence, representing a potential biomarker of cancer. In fact, it is not expressed in the healthy prostate cells and benign prostatic hyperplasia. Furthermore, its expression correlates with Gleason score and presence of metastases^[69].

Hypocalcemia

Definition

Hypocalcemia is defined as a lower serum calcium concentration (total serum calcium of < 2.1 mmol/L or < 8.5 mg/dL or ionized form of < 1.1 mmol/L or > 4.5 mg/dL)^[70].

Causes

Several causes can induce hypocalcemia in cancer patients [Table 3]:

(1) Cancer: malnutrition due to anorexia, cancer cachexia or bowel obstruction, malabsorption related to bowel tumor infiltration or previous intestinal surgery, abnormal liver function due to liver metastasis might promote the development of hypoalbuminemia and subsequent hypocalcemia^[70]. Furthermore, malabsorption and malnutrition might frequently cause vitamin D deficiency and then hypocalcemia in cancer patients^[70]. Another condition leading to hypocalcemia is PTH deficiency. It is a common condition of patients undergoing total thyroidectomy with subtotal or total parathyroidectomy for cancer^[71]. Paraneoplastic disorders are also involved in hypocalcemia in cancer syndrome. Such as tumor lysis syndrome or the hungry bone syndrome. The “hungry bone syndrome” is frequent in metastatic parathyroid and prostate cancer and it is characterized by osteoblastic metastases causing an increased deposition of calcium and phosphate in bone and decreased serum calcium and phosphate concentrations^[72].

(2) Cancer treatment: hypocalcemia is also reported in cancer patients receiving bisphosphonates or denosumab, an anti-RANKL (receptor activator of nuclear factor kappa B ligand) monoclonal antibody, employed in cancer patients with bone metastasis in order to delay or prevent skeletal-related events. In

fact, they promote calcium deposition in the bones, reducing blood calcium concentration^[73]. Therefore, checking calcium serum level before these treatments and implementation of calcium and vitamin D oral intake are recommended^[74]. Furthermore, several drugs, such as chemotherapeutic agents, target therapies, immunotherapies can induce hypocalcemia in cancer patients, through different mechanisms: kidney injuries, iatrogenic magnesium-deficiency, gastrointestinal damage, pancreatitis^[75]. In particular, monoclonal anti EGFR antibodies can cause hypomagnesemia with consequent hypocalcemia^[75].

(3) Concomitant drugs: diuretics and parenteral nutrition can induce hypocalcemia^[70].

(4) Concomitant diseases: kidney failure, autoimmune disorders causing PTH deficiency, sepsis, and pancreatitis can induce hypocalcemia^[70].

Management

Clinical manifestations of hypocalcemia are closely related to severity and time of onset. Symptoms and signs are influenced by other factors such as acid-base status, hypomagnesemia, and over-activity of sympathetic system^[76].

Clinical disorders due to hypocalcemia depend on altered electrical potential of cell membrane, and it appears as an imbalanced neuromuscular excitability. Chronic and mild hypocalcemia are often asymptomatic or they can present with muscle cramps, ectopic calcifications, parkinsonism, dementia, depression, psychosis, dry skin, and cataract.

Severe or acute hypocalcemia might cause tetanic spasms, laryngospasm until generalized convulsions, and coma^[76].

Severe hypocalcemia might also provoke cardiac alteration such as arrhythmias or heart block. ECG shows typical alteration such as prolongation of the QTc and ST interval, altered repolarization, T-wave pointed shape, or inversion^[77].

Diagnosis of hypocalcemia is based on symptoms' presence and it needs to be confirmed through laboratory exam. Since serum calcium is partially bound to proteins, it is suggested to correct total serum calcium concentrations with albumin levels [e.g., serum calcium (mg/dL) + 0.8 × (4-patient's albumin)]. Alternatively, ionized calcium can be evaluated. For a correct differential diagnosis, serum albumin, total protein, urinary calcium, phosphate, vitamin D, plasma PTH, and parathyroid, renal, and liver function should be evaluated [Figure 3]^[78].

Treatment of hypocalcemia depends on severity, clinical manifestation, and underlying causes. When possible, it is always advisable to correct the cause of hypocalcemia^[78].

In the case of acute and/or symptomatic hypocalcemia, patients should receive intravenous calcium. To avoid adverse events, calcium gluconate should be infused slowly (e.g., 10 mL of a formulations of 10% calcium gluconate should be diluted in 50-100 mL of 5% dextrose and infused over 5-10 min), and administered via a central venous catheter to prevent extravasation's complications^[79]. In fact, a rapid correction of hypocalcemia might increase the risk of cardiac arrhythmias, especially in patients receiving digoxin, thus cardiac activity should be monitored with ECG and correction rate of hypocalcemia should be checked every 1-2 h during intravenous calcium gluconate infusion^[79].

In the case of tetanic signs due to severe and/or acute hypocalcemia, the treatment initially requires a 10-min bolus of calcium gluconate intravenous infusion (10 mL of 10% solution) followed by the aforementioned formulation. Moreover, concomitant hypomagnesemia or alkalosis should be corrected^[80].

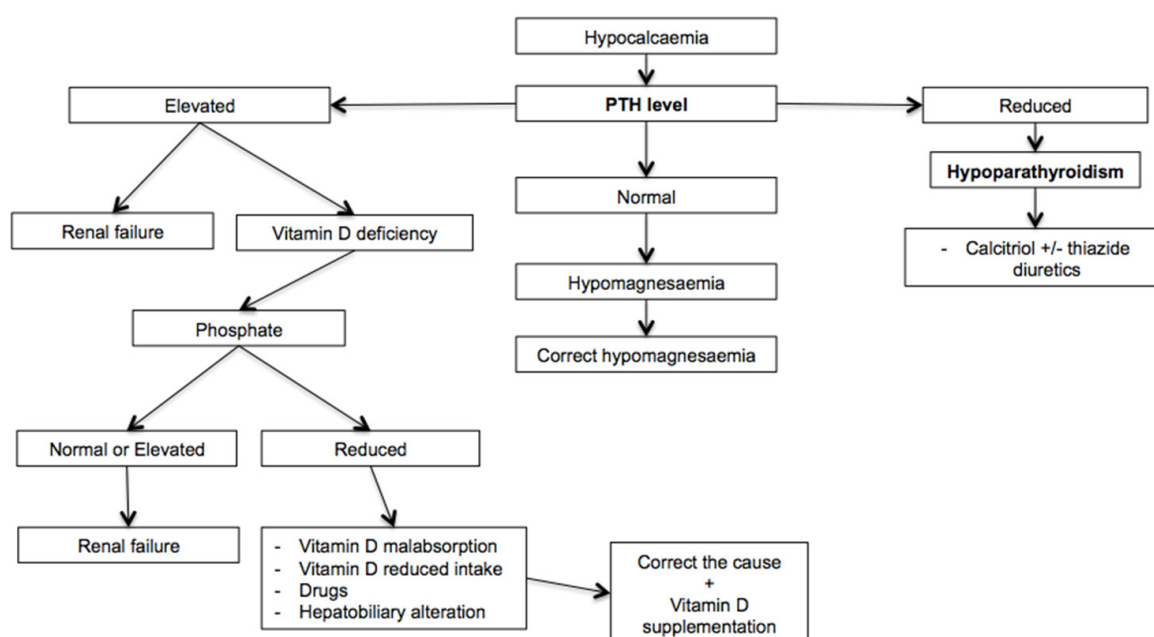


Figure 3. Algorithm of hypocalcemia management^[48,78-86]. For a correct diagnostic classification of hypocalcemia, it is essential to distinguish between hypoparathyroidism and other causes through the dosage of blood parathyroid hormone. In the case of high parathormone concentrations, dosage of vitamin D is useful to exclude deficiency. PTH: parathyroid hormone

In the case of hypoparathyroidism, the treatment aims to control symptoms, maintaining adequate serum calcium levels (2.00-2.12 mmol/L), and a calcium-phosphate ratio below 4.4 mmol/L, in order to prevent hypercalciuria and precipitation of calcium salts in soft tissues^[81]. Calcitriol, a vitamin D analog, is usually used with a starting dose of 0.5 mcg/day, which might be increased until adequate serum calcium concentrations are reached. Thiazide diuretics associated with a low phosphate diet could be considered^[82].

It is recommended to monitor weekly serum calcium, phosphorus concentration, and creatinine during initial administration to obtain a correct stabilization of the dose^[83].

In the case of chronic hypocalcemia, oral supplementation of calcium (calcium carbonate or calcium citrate) and vitamin D is recommended. In the case of hypomagnesemia, it should be corrected^[84].

Vitamin D insufficiency requires supplementation with oral or intramuscular ergocalciferol (vitamin D2) or oral cholecalciferol (vitamin D3). When hypocalcemia is secondary to vitamin D malabsorption, it is important to correct the underlying cause (e.g., celiac patients should receive a gluten-free diet)^[85].

Patients receiving bisphosphonates or anti-RANKL should receive oral calcium and vitamin D supplementation to prevent hypocalcemia^[86].

Hypercalcemia

Definition and clinical implication

Hypercalcemia is defined as a higher serum calcium concentration (total serum calcium over than 10.5 mg/dL)^[87]. It is a common electrolyte disorder in patients with advanced malignancies and it correlates with poor prognosis^[88].

Causes

Several causes might contribute to the development of hypercalcemia in cancer patients [Table 4]:

(1) Cancer: the main cause of hypercalcemia in cancer patients is hyperparathyroidism. It can be divided

Table 4. Causes of hypercalcemia

Causes of hypercalcemia	
Primary hyperparathyroidism	Parathyroid adenoma (MEN1 and 2a), parathyroid carcinoma, familial hypocalciuric hypercalcemia, isolated familial hyperparathyroidism
Secondary hyperparathyroidism	Renal failure, lithium, tumors, bone metastasis
Drugs	Lithium, thiazide diuretics, calcium containing antacids, vitamin A, estrogens, growth hormone, vitamin D intoxication, theophylline
Immobilization	Spinal cord injury, neurological diseases, pathological fracture, orthopedic surgery
Miscellanea	Chronic granulomatous disorders, hyperthyroidism, acromegaly, pheochromocytoma, adrenal insufficiency, parenteral nutrition

into primary and secondary hyperparathyroidism. Primary hyperparathyroidism, the most common cause of hypercalcemia in the general population, is characterized by inappropriate secretion of PTH provoking elevated serum calcium concentrations. Single parathyroid carcinoma is a frequent cause of primary hyperparathyroidism, sometimes inducing a rare but life-threatening condition, hyperparathyroidism-induced hypercalcemic crisis characterized by elevated PTH concentrations (3-10 times higher than normal values) and serum calcium-levels^[89]. Secondary hyperparathyroidism, instead, is characterized by elevated quantities of PTH, secreted by parathyroids. Several causes might contribute to this mechanism^[90], in particular malnutrition and cancer anorexia are the most common cancer related causes. Malignancies are an important cause of hypercalcemia. It was described as occurring in 20%-30% of cancer patients, especially those hospitalized, and it represents one of the most common life-threatening metabolic disorders^[91]. Even though several mechanisms underlay hypercalcemia in cancer patients, it seems to be correlated, especially in some kinds of tumors (head and neck, lung, renal cell, ovarian, thyroid, endometrial, colorectal, breast cancer, hepatocarcinoma, cholangiocarcinoma, thymomas, neuroendocrine tumors, gastrointestinal stromal tumor, and leukemias), with the ectopic production of PTH or parathormone-related peptide (PTHrP). These factors seem responsible for osteoclastic activation, through an increased synthesis of RANKL, provoking bone destruction and calcium release. Furthermore, they determine an increased renal calcium reabsorption, favoring the development of metastatic calcification involving multiple organs, especially lungs, potentially resulting in pulmonary edema^[92]. Moreover, bone metastases, in particular osteolytic ones, are often associated to hypercalcemia due to calcium release from bone. It represents a common cause of hypercalcemia, occurring in approximately 20% of patients with malignancy-related hypercalcemia. Bone metastasis releases several local factors, e.g., transforming growth factor β , RANKL, lymphotoxin, interleukin-1, interleukin-6, hepatocyte growth factor, and macrophage inflammatory protein (MIP-1 α), that favor the release of PTHrP and bone remodeling resulting in hypercalcemia^[93]. Rarely, hypercalcemia might be due to ectopic activity of 1- α -hydroxylase resulting in calcitriol production that promotes increased bone resorption with calcium release and intestinal calcium absorption. This mechanism is described in some kinds of tumors such as lymphomas (lymphoma-associated calcitriol production) and ovarian germ cell tumors^[88]. Finally, immobilization due to bedridden patients, a common condition of advanced cancer, can favor an acceleration of bone resorption resulting in hypercalcemia^[94].

(2) Cancer treatment: antineoplastic drugs can indirectly cause hypercalcemia, for example through kidney damage^[88].

(3) Concomitant drugs: several drugs might cause hypercalcemia. Thiazide diuretics, vitamin D intoxication, and parenteral nutrition are the most common agents involved in this electrolyte disorder in cancer patients.

(4) Concomitant diseases: several pathological conditions might cause hypercalcemia. It may depend on excess of PTH (primary hyperparathyroidism due to parathyroid adenoma, familial hypocalciuric hypercalcemia, isolated familial hyperparathyroidism, or most commonly secondary hyperparathyroidism

due to renal failure or drugs such as lithium) or arise due to mechanisms independent of PTH (chronic granulomatous disorders, hyperthyroidism, acromegaly, pheochromocytoma, and adrenal insufficiency)^[88-90].

Management

To improve patients' outcome, a periodic monitoring of calcium serum levels and prompt correction of potential hypercalcemia should be performed. To define an effective hypercalcemia, it is important to avoid the presence of concomitant factors that can influence the share of bound and free calcium (e.g., hyperglobulinemia might increase total calcium levels without modifying ionized concentrations; acidemia and reducing albumin-calcium affinity might increase the level of the ionized form, without altering total calcium levels)^[87]. Diagnosis is often incidentally during routine laboratory investigations, as most patients with mild hypercalcemia are asymptomatic^[88]. Chronic hypercalcemia, due to hyperparathyroidism, is often asymptomatic; however, in some cases, this long-lasting electrolyte disorder might cause nephrolithiasis. Instead, chronic hyperparathyroidism secondary to renal failure and dialysis might cause bone pain related to bone remodeling process, fibrous degeneration, and formation of cysts and nodules of fibrosis.

Clinical presentation depends on grade and time of onset. Most common symptoms are general malaise, thirst, lethargy, and constipation often associated with abdominal pain. Renal symptoms and signs such as polyuria, polydipsia, nycturia, nephrolithiasis, and rarely renal failure and nephrocalcinosis should be also considered.

Neuromuscular disorders, starting from muscles weakness, emotional instability, and confusion, until development of delirium, psychosis, stupor, and coma, could be observed in acute or severe hypercalcemia (serum calcium levels > 12 mg/dL). Furthermore, hypercalcemia might cause cardiac arrhythmias. The most common electrocardiographic changes are absent or shortened ST segment, shortened QT interval, widened QRS complex, enlarged T wave, and prolonged PR interval. Rarely, it might cause branch block and arrhythmias until to cardiac arrest in the case of serum calcium levels over 15 mg/dL^[88].

Renal function and immunoreactive parathyroid hormone analysis are recommended for a complete diagnostic classification that allows making differential diagnosis and to consider potential serious conditions (cardiac arrhythmias).

Electrocardiography should also be performed in order to avoid presence of cardiac alterations^[95].

Generally, primary hyperparathyroidism is usually characterized by hypercalcemia (with high ionized free serum calcium levels), hypophosphatemia, PTH being inappropriately high (e.g., no hypocalcemia), and excessive bone turnover.

Differential diagnosis between primary and secondary hyperparathyroidism could be difficult in the case of renal failure, although hyperphosphatemia is often linked to secondary hyperparathyroidism and normal or low phosphorus levels are indicative of primary hyperparathyroidism^[96].

Furthermore, in the presence of secondary hyperparathyroidism, radiographic examination should be useful to detect bone cysts presence and bone reabsorption.

When hyperparathyroidism has been excluded, a serum calcium level above 12 mg/dL is suspicious of malignancies, mostly when associated with hypophosphatemia, hypoalbuminemia, and lower or undetectable PTH level. PTHrP is a peptide that shows homology with the N-terminal portion of PTH, mimicking the action. It can be produced from breast, lung, and kidney cancer cells. When possible, the individuation of PTHrP is useful for reach diagnosis of paraneoplastic hypercalcemia^[88].

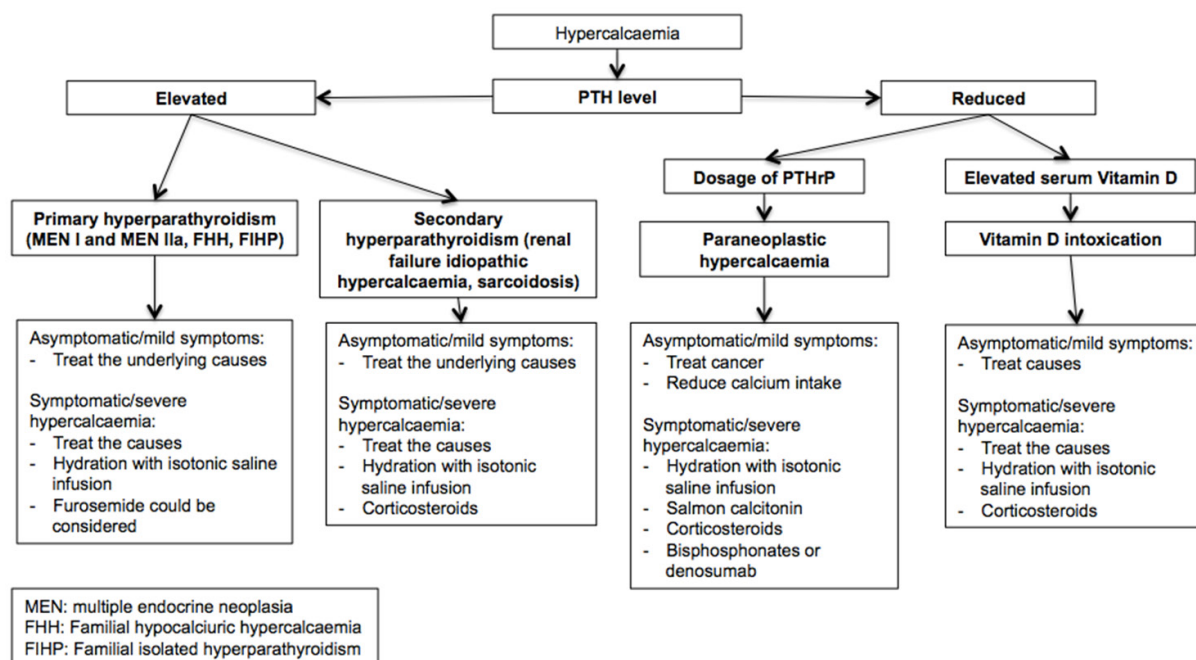


Figure 4. Algorithm of hypercalcaemia management^[48,88,94-104]. To treat hypercalcaemia, a correct diagnostic framework is essential, which is based on the parathyroid dosage to distinguish between hyperparathyroidism and other causes. In the case of low blood PTH concentrations, the dosage of PTHrP is useful to exclude paraneoplastic hypercalcaemia and vitamin D intoxication. PTH: parathyroid hormone; PTHrP: parathyroid hormone-related protein; MEN: multiple endocrine neoplasia; FHH: familial hypocalciuric hypercalcaemia; FIHP: familial isolated hyperparathyroidism

Treatment depends on clinical manifestation, grade of hypercalcaemia, and underlying cause, which should be correct whenever possible [Figure 4]. In the case of mild symptoms, serum calcium levels are below 11.5 mg/dL, which could sufficiently remove the cause of hypercalcaemia.

In the case of severe hypercalcaemia (total serum calcium over 15 mg/dL) or severe symptoms or signs, a treatment aimed to reduce serum calcium levels is recommended, in order to restore adequate intravascular volume and to improve glomerular filtration rate^[96]. Since hypercalcaemia induces polyuria, most patients are dehydrated. Therefore, intravenous isotonic saline solution (NaCl 0.9%) should be administered with an infusion rate of 100/120 mL/h, in order to obtain a urine output of 100-150 mL/h and a reduction of serum calcium concentration^[97]. Two to three liters of isotonic saline solution/day with a close monitoring of serum electrolyte levels and urinary volume is recommended^[98]. In patients with edematogenic syndromes (e.g., congestive heart failure) or anuria isotonic solution should be administered with caution in order to avoid fluid overload. However, only 30% of patients reach normocalcemia with fluids alone. Loop diuretics (e.g., furosemide) might be considered mostly in patients with edematogenic syndromes, in whom it is required to improve diuresis. The use of furosemide should be limited in dehydrated patients or in patients presenting other electrolyte abnormalities (magnesium and, potassium)^[99].

In patients with malignant hypercalcaemia, salmon calcitonin administration (4-8 IU/kg s.c. or i.m. every 12 h) was demonstrated to control serum calcium levels (estimated a maximal serum calcium drop of 2 mg/dL in 4 h after administration), and it can be used also in patents with renal injuries, in which intravenous saline is not recommended^[100]. Furthermore, calcitonin is more efficacious and quicker than bisphosphonates in normalizing calcemia and it can be used with bisphosphonates or in the case of bisphosphonates' resistance. However, due to the risk of tachyphylaxis, the duration of treatment with calcitonin should not exceed 48 h^[101].

The addition of corticosteroids (e.g., 20-40 mg of prednisone/day) is useful to control malignant hypercalcemia especially due to hematological tumors, through an increased renal calcium excretion and inhibition of bone reabsorption. However, since fewer than 50% of patients with malignant hypercalcemia respond to glucocorticoids after several days, in clinical practice, an alternative treatment is usually preferred. Corticosteroids are also used for the treatment of vitamin D intoxication, idiopathic hypercalcemia, and sarcoidosis^[102].

Finally, bisphosphonates, such as ibandronate, pamidronate, and mostly zoledronic acid, have been shown to be effective in reducing serum calcium in approximately 12 h^[103].

Recent evidence demonstrates the activity of denosumab in control malignant hypercalcemia, especially in patients with persistent hypercalcemia despite bisphosphonates. Furthermore, it could also be used in patients with reduced renal function^[104].

POTASSIUM

Potassium is the second most abundant cation in the human organism. It is the main intracellular cation; in fact, only 2%-5% of total body potassium is restrained in extracellular fluids, including blood. Normal serum potassium concentration ranges between 3.5 and 5.0 mEq/L^[105]. The maintenance of this concentration is crucial for several physiological processes (maintenance of cellular membrane potential, cellular volume, and action potentials' transmission in nerve cells)^[105]. Many mechanisms act for preserving potassium homeostasis: oral intake, renal elimination, and balance between intracellular and extracellular concentration.

Renal active excretion of potassium in cortical collecting ducts is regulated by aldosterone, through the modification of the epithelial sodium channel into the open configuration and the increase of the number of epithelial sodium channel. This modification favors sodium reabsorption and increases potassium secretion^[106].

Potassium transit among intracellular and extracellular fluid compartments depends on Na⁺-K⁺-ATPase, a membrane pump ubiquitous in all cells^[107]. This ionic channel creates a concentration gradient across cell membrane, maintaining the potential of cell membrane. Several factors influence the transit of potassium through cell membrane: blood pH, in particular, alkalosis induces the potassium's input from extracellular to intracellular fluid compartments, while acidosis causes the leak of potassium from cells. Furthermore, insulin and β -adrenergic catecholamines favor potassium's input into cells^[108].

Several potassium channels are involved in cancer proliferation. Potassium channels (KCN) are a large group of proteins involved in potassium transfer. In breast cancer, KCNMA1, KCNJ3, KCNN4, and KCNK9 are associated with estrogen receptor's expression and brain and lymph-node metastasis^[109]. In prostate cancer, several potassium channels are involved. In particular, KCNMA1 represents a promising diagnostic biomarker of prostate cancer. In fact, it is over-expressed in cancer cells with Gleason score of 5-6, and in hormone sensitive phase. KCNK2 seems to be involved in the regulation of cell proliferation^[110]. KCNQ1, a pore K⁺ channels, is over-expressed in more than 35% of lung tumors and it favors tumor development, cell proliferation and migration, and resistance to hypoxia^[111]. Voltage-gated K⁺ channels seem to have an important role in colorectal cancer. In particular, over-expression of KCNH2 regulates cell invasion, giving an invasive phenotype to the tumor, and it represents a negative prognostic factor in early stages when associated with the absence of Glut-1. It also seems to confer different chemosensitivity to different drugs; in particular, cells with over-expression of KCNH2 are inhibited by paclitaxel, vincristine, and hydroxy-camptothecin, while they seem to have resistance to doxorubicin. The clinical implication is due to

the influence of KCNH2 in modulating VEGF-A secretion^[112]. Overexpression of KCNH2 has been also demonstrated to be associated with poorer prognosis in squamous-cell carcinoma of esophagus^[113]. Overexpression of KCNH2 seems to also have a role in pancreatic cancer. In particular, it is involved in EGFR pathway, conferring an aggressive behavior and poorer prognosis^[114]. KCNH2 has also been investigated in gastric cancer. In particular, it has been demonstrated to be negatively correlated with grading, stage of disease, venous invasion, and shorter survival^[115]. Otherwise, it has been investigated in surgical samples of patients undergoing radical tumor resection and it seem to correlate with Lauren's intestinal type, fundus localization, low grading, and early stages (TNM I and II)^[116]. Furthermore, it has been demonstrated to modulate VEGF-A secretion and cisplatin-induced apoptosis^[117].

Hypokalemia

Definition and clinical implications

Hypokalemia is defined as a low serum potassium concentration (< 3.5 mEq/L). Severe hypokalemia is defined as a potassium level lower than 2.5 mEq/L, representing a potential life-threatening disorder^[118].

Causes

Hypokalemia is a common electrolyte disorder in cancer patients. Several causes might induce hypokalemia in cancer patients^[108]:

- (1) Cancer: several conditions related to cancer might induce a reduced potassium intake (malnutrition, anorexia, and malabsorption due to cancer bowel infiltration or bowel obstruction). Some neuroendocrine tumors might cause hypokalemia through secretive diarrhea, favoring potassium losses. Other tumors induce renal potassium losses through the production of hormones such as adrenocorticotrophic hormone (ACTH), cortisol, and mineralocorticoids, or through kidney damage, such as multiple myeloma.
- (2) Cancer treatment: chemotherapeutic agents, target therapies and immunotherapies might cause hypokalemia secondary to diarrhea or vomiting.
- (3) Concomitant drugs: thiazide diuretics, insulin, granulocyte growth factors, beta-2 agonists, and glucocorticoids might cause hypokalemia.
- (4) Concomitant diseases: endocrine dysfunctions causing excess glucocorticoids or mineralocorticoids, toxic epidermal necrolysis, and inflammatory bowel diseases might cause hypokalemia.

Causes of hypokalemia might be resumed substantially in three mechanisms: an inadequate potassium intake, redistribution of potassium among intra- and extracellular compartments, and potassium losses [Table 5].

In cancer patients, inadequate intake is often related to malnutrition and anorexia due to drugs and/or tumor condition.

The passage of potassium into the intracellular compartment might depend on many mechanisms: uptake of potassium by tumor cells, alkalosis, hypothermia, and drugs. For example, granulocyte growth factors, often employed in cancer patients, provoke an acute hematopoietic cell formation, favoring rapid potassium intake by the new cells^[119]. Hypokalemia is similarly induced by rapid cell proliferation in acute leukemia^[120].

Potassium losses can be classified into renal and non-renal losses. The most common extra-renal losses are gastrointestinal losses due to drugs or cancer-induced diarrhea and vomiting, infections, radiation enteritis, and type of tumors (villous adenoma and neuroendocrine neoplasms)^[121]. In particular, neuroendocrine neoplasms, although rare, are represented with the carcinoid syndrome characterized by serotonin and

Table 5. Causes of hypokalemia

Causes of hypokalemia	
Reduced potassium intake	Malnutrition, anorexia, refeeding syndrome Gastrointestinal injuries Vomit (caused by anticancer therapies, intestinal obstruction, etc.) Diarrhea (caused by anticancer therapies, cancer, surgery)
Redistribution of the potassium into intracellular compartment	Drugs (catecholamine, nasal decongestants, insulin, granulocyte growth factors, beta-2 agonists, barium intoxication, theophylline, bicarbonate, verapamil) Alkalosis Hypothermia
Increased potassium losses	Non-renal losses: Losses (diarrhea, vomiting, fistula, laxative abuse, villous adenoma) Profuse sweating Extended burns Toxic epidermal necrolysis Renal losses: Metabolic alkalosis Use of diuretics Osmotic diuresis Renal tubular diseases (tubular acidosis, Liddle syndrome) Endocrine dysfunctions (excess of glucocorticoids or mineralocorticoids, primary hyperaldosteronism due to adenoma or adrenal carcinoma, renin-secreting neoplasms, ectopic secretion of ACTH) Concomitant electrolyte disorders (hypercalcemia, hypomagnesemia) Drugs (amphotericin B, cisplatin, ifosfamide, glucocorticoids, anti-EGFR agents, mTOR inhibitors, eribulin, abiraterone)

ACTH: adrenocorticotrophic hormone; EGFR: endothelial growth factor receptor

kallikrein hypersecretion inducing flushing, severe secretory diarrhea with cramps and hypokalemia, tachycardia, hypotension until heart failure, and bronchial constriction^[122]. Another rare syndrome due to tumor hypersecretion of vasoactive intestinal polypeptide induces important watery diarrhea with hypokalemia and achlorhydria^[123].

Renal losses have several potential causes. Endocrine disorders should be considered in cancer patients. For example, Cushing syndrome can be due in rare cases to ACTH-producing tumors, especially in patients with small-cell lung cancer, medullary thyroid carcinoma, islet cell adenoma or carcinoma, pheochromocytoma, and ganglioneuroma, inducing an excessive production of cortisol able to blind mineralocorticoid receptors inducing hypokalemia^[124]. Another rare cause is primary aldosteronism, due to the excessive and autonomous secretion of aldosterone by adrenal adenomas or carcinoma. This syndrome is characterized by polydipsia, polyuria, resistant hypertension, and severe hypokalemia^[125].

Furthermore, a common cause of potassium renal losses in cancer patients is drug-related tubular toxicity. Several chemotherapeutic agents, target therapies, and immunotherapeutic drugs [Table 5] might induce renal injury associated to hypokalemia. Renal function should be evaluated before drug administration to avoid further renal damage^[126]. Concomitant therapies such as thiazide diuretics and glucocorticoids can favor potassium renal losses.

Finally, some kinds of tumor induce renal damage. For example, patients with multiple myeloma producing Bence-Jones proteins develop a progressive renal injury leading to hypomagnesemia and hypokalemia. Acute myeloid leukemia, through secretion of lysozyme, induces renal tubular damage^[127].

In hematological patients, especially in those with marked leukocytosis (e.g., in acute myeloid leukemia), hypokalemia can be confused with pseudo-hypokalemia, due to potassium intake in the stored blood sample before the laboratory analysis^[128].

Management

Clinical presentation depends on severity of hypokalemia. Patients are often asymptomatic, especially those with mild hypokalemia^[129]. Symptoms and sign of hypokalemia are non-specific and due to

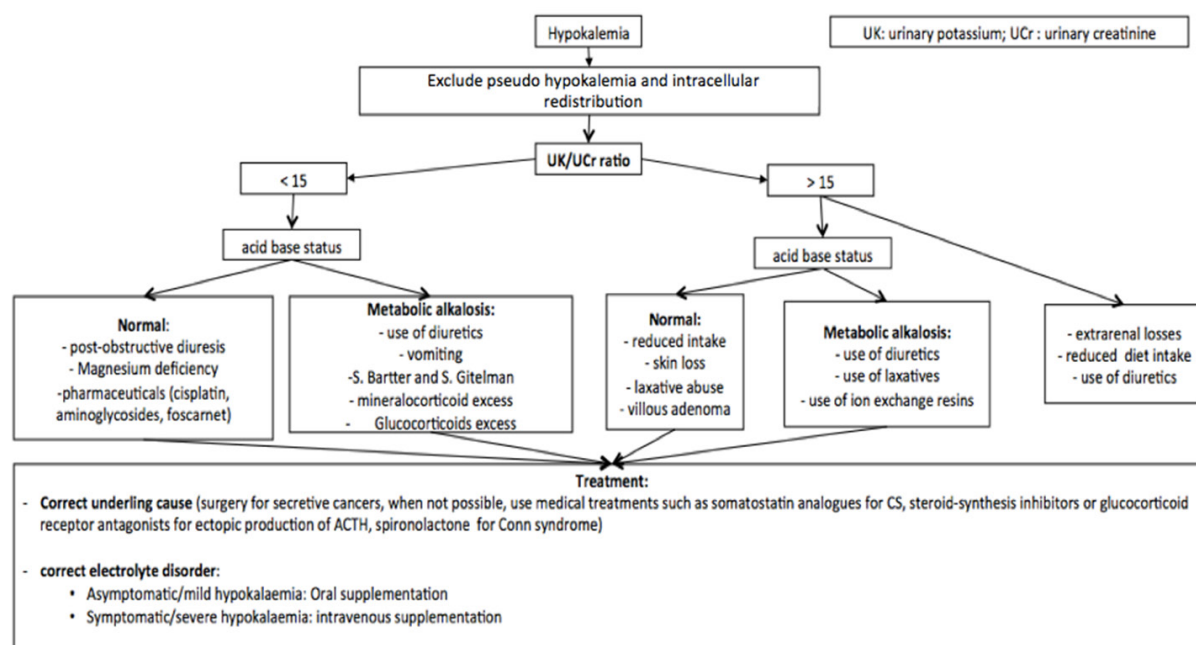


Figure 5. Algorithm of hypokalemia management^[48,129-139]. In the case of hypokalemia, it is useful to consider oral or intravenous supplementation based on the degree and symptoms. It is always useful to associate the identification and correction of the underlying cause. For a correct differential diagnosis, it is essential to evaluate UK and UCr. UK: urinary potassium; UCr: urinary creatinine

muscular, neurological, or cardiac dysfunction. The most common clinical manifestation is characterized by weakness, fatigue, myalgia, muscle cramps, and constipation. In the case of moderate or severe hypokalemia, neurological and psychiatric symptoms (e.g., psychosis, delirium, hallucinations, and depression), or cardiac signs (bradycardia) until acute respiratory failure with cardiovascular collapse, secondary to muscle paralysis, might occur. In particular, cardiac arrhythmias represent life-threatening complications requiring immediate diagnosis and adequate treatment. Therefore, ECG monitoring should be performed in patients with hypokalemia (typical alterations are inverted T waves, appearance of U wave, ST depression, and enlarged PR interval)^[130].

Diagnosis of hypokalemia is based on detection of low serum potassium levels. For a correct management of hypokalemia, it is important to understand the underlying causes. For a correct differential diagnosis, it is important investigate the patient's medical history, evaluating the concomitant therapies and clinical conditions that might cause potassium losses or intracellular redistribution (insulin, diuretics, nephrotoxic agents, anticancer agents, diarrhea, and vomiting). Presence of pseudo-hypokalemia should be excluded in patients with marked leukocytosis.

Furthermore, other laboratory exams should be performed for a correct differential diagnosis. Blood sugar, acid-base balance, creatinine, magnesium levels, and urine electrolytes concentration should be evaluated [Figure 5]^[131]. In particular, 24-h renal potassium concentration is useful to establish renal or extra-renal potassium losses.

When urinary potassium is > 30 mEq/L there is a renal potassium loss, while a urinary potassium concentration of < 25 mEq/L might be related to an extra-renal potassium loss. Another useful evaluation to distinguish between renal and extra-renal potassium losses is the urinary potassium concentration and urinary creatinine concentration ratio. Values > 15 are suggestive for renal potassium losses^[131].

The treatment of hypokalemia depends on symptoms and their severity. It is focused on preventing life-threatening complications and removing the underlying cause [Figure 5].

In patients with mild or moderate hypokalemia (serum potassium levels of 3.0-3.5 mEq/L), potassium oral supplementation should be preferred to intravenous, in order to avoid an iatrogenic hyperkalemia. A daily dose of 60-80 mEq of potassium chloride is recommended. However, it is important to monitor serum potassium concentration in order to adjust treatment doses accordingly. In the case of chronic hypokalemia refractory to oral supplementation, the addition of potassium-sparing diuretics (amiloride and spironolactone) should be considered^[132].

In asymptomatic patients with severe hypokalemia (serum potassium level of < 3.0 mEq/L), an oral potassium supplementation with potassium chloride 40 mEq every 3-4 h should be considered^[133]. Otherwise, in symptomatic patients with a life-threatening complication, or in patients unable to take oral drugs, intravenous potassium (10-20 mEq/h, able to increase serum potassium level an average of 0.25 mEq/h) is recommended^[134]. Continuous ECG monitoring is recommended in patients with arrhythmias, digitalis toxicity, and history of cardiac ischemia. In patients with renal failure, the doses should be reduced and serum potassium level monitored frequently. Refractory hypokalemia might depend on the presence of concomitant electrolyte disorders, such as hypomagnesemia, which should be investigated and treated when present^[135].

When hypokalemia is secondary to tumor activity, treating the cancer is necessary, in addition to symptomatic therapy for hypokalemia, in order to prevent the recurrence of symptoms.

Patients with carcinoid syndrome should receive somatostatin analog in order to inhibiting hormonal hyper-secretion and improve symptoms^[136]. When hypokalemia depends on primary aldosteronism, unilateral adrenalectomy is preferred to mineralocorticoid receptor antagonists^[137].

In the case of ectopic ACTH secretion, a complete resection of the tumor should be preferred, when possible. Other treatment options, such as steroid-synthesis inhibitors (metyrapone, mitotane, trilostane, ketoconazole, and aminoglutethimide) and glucocorticoid receptor antagonists (mifepristone), are available to control severe hypercortisolemia^[138]. In patients with severe (Grades 2-3) diarrhea due to anticancer drugs or radiotherapy, a symptomatic treatment with probiotics, loperamide, and in refractory cases octreotide should be administered in order to prevent hypokalemia^[139].

Hyperkalemia

Definition and clinical implications

Hyperkalemia is defined as an increased serum potassium concentration (> 5.5 mEq/L). According to potassium level, three different grades of severity are described: mild hyperkalemia (5.1-6.0 mEq/L), moderate hyperkalemia (6.1-7.0 mEq/L), and severe hyperkalemia (> 7.0 mEq/L), which represents a life-threatening condition^[140].

Causes

Several causes might induce hypokalemia in cancer patients^[108]:

(1) Cancer: several conditions related to cancer might induce hyperkalemia. Tumors with high proliferative index such as leukemia and small-cell lung carcinomas can result in lysis syndrome after specific anticancer treatment, inducing hyperkalemia.

(2) Cancer treatment: chemotherapeutic agents, such as platinum derivatives, might cause renal injury, which can lead to hyperkalemia.

Table 6. Causes of hyperkalemia

Causes of hyperkalemia	
Increased intake	Iatrogenic (excessive potassium infusion, parenteral nutrition, <i>etc.</i>)
Redistribution into extracellular compartment	Massive tissue catabolism (hemolysis, sepsis, diffuse trauma, rhabdomyolysis, chemotherapies, lysis syndrome) Drugs (beta-blockers, arginine, digital) Metabolic acidosis Insulin deficiency and hyperglycemia Severe muscular exercise Pseudo-hyperkalemia (<i>in vitro</i> hemolysis, leukocytosis, thrombocytosis)
Reduction of renal excretion of potassium	Renal injuries: Acute or chronic renal failure Depletion of effective circulating volume Tubulopathies Selective alteration of potassium excretion (acute transplant rejection, lupus nephritis, cyclosporine, analgesic nephropathy, lead poisoning) Nephrotoxic drugs (cisplatin, ifosfamide, mitomycin C, gemcitabine, methotrexate, bisphosphonates, interferon, somatostatin analogs) Corticosteroid insufficiency: Iopreninemic hypoaldosteronism (diabetic nephropathy, chronic interstitial nephritis, drug nephropathy) Primitive hypoaldosteronism (M. Addison) Use of potassium-sparing diuretics

(3) Concomitant drugs: diuretics, potassium-sparing diuretics, angiotensin-converting enzyme, inhibitors, and NSAIDs might induce hypokalemia.

(4) Concomitant diseases: renal failure, diabetes mellitus, sepsis, and parenteral nutrition might induce hypokalemia.

However, causes of hyperkalemia might be resumed in different mechanisms, such as excessive intake, redistribution into extracellular compartment, or abnormal renal elimination, that might depend on aldosterone deficiency or on renal parenchyma damage [Table 6].

Rarely, hyperkalemia depends only on increased potassium intake, and it is often associated with other risk factors: renal failure, diabetes mellitus, and concomitant medications that inhibit potassium excretion (e.g., potassium-sparing diuretics, angiotensin-converting enzyme inhibitors, and NSAIDs). Furthermore, in cancer patients, parenteral nutrition rich in potassium might induce to life-threatening hyperkalemia^[141].

Tumor lysis syndrome (TLS) represents an important cause of acute hyperkalemia in cancer patients. It is a rare but serious oncological emergency characterized by hyperuricemia, hyperkalemia, hyperphosphatemia, hypocalcemia, and azotemia. It usually appears 48-72 h after the commencement of anticancer-therapy (chemotherapy, radiotherapy, and radiofrequency ablation) as a consequence of massive cell necrosis and acute release of intracellular factors into the systemic circulation^[142]. In particular, elevated uric acid and calcium phosphate release tend to precipitate into renal tubules, causing local damage with glomerular filtration rate reduction until acute kidney injury (AKI). AKI worsens metabolic disorders and hyperkalemia, which might induce severe cardiac arrhythmias until death; low serum calcium levels and acidosis may exacerbate this risk^[143]. In high-grade hematologic malignancies and childhood cancers, it might also occur spontaneously^[144].

Several predisposing factors to tumor lysis syndrome were detected, such as elevated tumor burden, rapidly-growing neoplasms, high tumor sensibility to anti-cancer treatment, concomitant renal failure, elevated lactate dehydrogenase serum level, hyperuricemia, and hyperazotemia. These factors should be investigated before starting treatment, in order to set up a preventive therapy^[145].

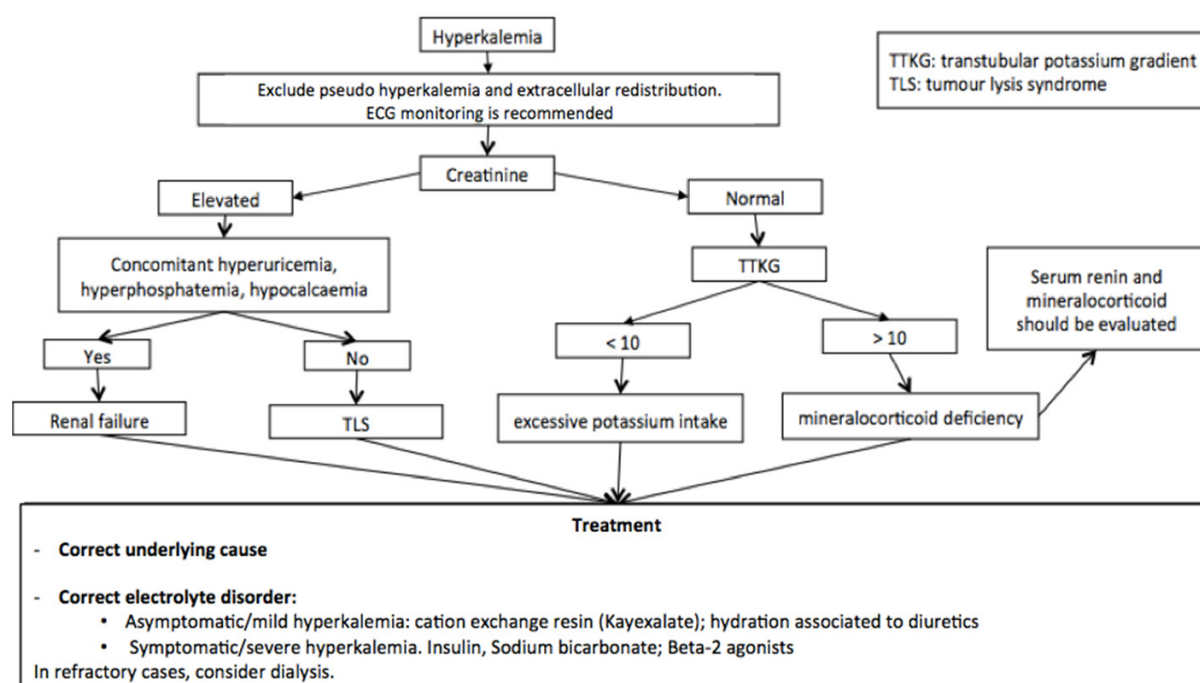


Figure 6. Algorithm of hyperkalemia management^[48,149-154]. In the case of hyperkalemia, the treatment is based on the degree and reported symptoms. When patients are symptomatic for hyperkalemia, insulin, beta-2 agonist, and sodium bicarbonate should be considered. It is always useful to associate the identification and correction of the underlying cause. For a correct differential diagnosis, it is necessary to evaluate renal function through blood creatinine level. If elevated, evaluate uricemia, calcemia, and phosphatemia to distinguish between renal failure and tumor lysis syndrome; TTKG: transtubular potassium gradient

Adrenal insufficiency secondary to metastasis involving both adrenal glands might cause hyperkalemia in cancer patients, especially with advanced lung and breast cancer or lymphomas. However, despite the high frequency of adrenal metastasis (40%-60% of patients), adrenal insufficiency is rarely described, thanks to concomitant administration of corticosteroids^[146]. Finally, in the case of elevated leukocytosis or thrombocytosis, hyperkalemia should be distinguished from pseudo-hyperkalemia, due cellular potassium escape after the blood sample is taken^[147].

Management

Diagnosis of hyperkalemia occurs with the detection of elevated serum potassium concentration. The diagnosis is often an incidental laboratory finding, as patients are often asymptomatic, especially in the case of plasma potassium concentration of < 5.5 mEq/L. When present, symptoms and signs are related to altered cellular resting membrane potential, causing muscle, neurological and cardiac dysfunctions, until life-threatening cardiac arrhythmias. Patients might experience, according to hyperkalemia grade, fatigue and weakness, fasciculation, cramps, and paresthesia, as well as muscular paralysis and palpitations in serious cases. Sometimes, the only signs of hyperkalemia are electrocardiographic specific alterations or cardiac arrhythmias such as pointed T-waves, decrease or absence of P waves, prolonged PR interval, enlarged QRS complex, reduced QT interval, ventricular fibrillation, or asystole^[148].

For a correct differential diagnosis, clinical history evaluating potassium intake and concomitant medications should be collected [Figure 6]. Furthermore, other laboratory exams should be performed: complete blood count, urinary potassium, sodium and osmolality, transtubular potassium gradient (TTKG), renal function, lactate dehydrogenase, and uricemia. Elevated serum creatinine and urea associated with hyperkalemia are suspicious for renal failure. Hyperkalemia, concomitant to the presence of hyperuricemia, hyperphosphatemia, and hypocalcemia, might be suggestive of TLS. In the case of $TTKG > 10$, hyperkalemia might depend on excessive potassium intake. Instead, a $TTKG < 10$ is generally due to a mineralocorticoid

insufficiency and serum concentrations of renin and mineralocorticoid should be evaluated to confirm the diagnosis^[149].

For a correct management of cancer patients with hyperkalemia, it is important to consider presence of ECG alteration, symptoms, and degree of hyperkalemia.

Hyperkalemia treatment requires first to eliminate all potassium exogenous sources and, when possible, discontinue treatment with drugs favoring hyperkalemia [Figure 6].

Furthermore, it is important to counteract cardiac effects of hyperkalemia. In the case of enlargement of QRS complex, intravenous infusion of calcium (e.g., 1fl calcium gluconate 10 mL) and electrocardiographic monitoring are required.

In the case of severe hyperkalemia and electrocardiographic alterations, immediate treatment should be set up. To obtain a rapid reduction of serum potassium level, the administration of medications able to bring potassium in intracellular compartment should be performed^[150]. Several treatment options can be considered:

- Insulin (e.g., 10 units of fast insulin associated to 500 mL of glucose solution at 10% or 250 mL of glucose solution infused in 30-60 min).
- Sodium bicarbonate (e.g., Sodium bicarbonate 1 mEq/kg in 10-20 min), which should be avoided in patients with heart or renal failure, because it might worsen fluid retention.
- Beta-2 agonists (e.g., 10-20 mg salbutamol to inhale in 10 min), which should be avoided in patients with ischemic cardiomyopathy or cardiac arrhythmias.

Although these treatment options are effective in rapidly correcting hyperkalemia, the redistribution of potassium into the intracellular compartment is temporary. Furthermore, sodium bicarbonate and beta-2 agonists should be used as adjuvant treatments, in combination with other therapies^[150].

Patients with moderate and asymptomatic hyperkalemia do not require an immediate serum potassium reduction and can be treated with medications that remove potassium excess but require several hours^[151]. Several treatment options can be considered. Cation exchange resins (Kayexalate) should be preferred. However, due to high sodium content, they have to be used with caution in presence of heart or renal failure, to avoid fluid retention.

Abundant intravenous hydration associated with diuretics (furosemide 40-80 mg) can be considered a valid treatment option; however, it should be avoided in patients with heart failure for fluid overload risk. Furthermore, diuretics have been demonstrated to control serum potassium level only in chronic hyperkalemia, and they should be reserved only for the management of these forms. Dialysis can be considered in the case of renal failure when resins and diuretics fail^[152].

In patients with severe hyperkalemia, plasma potassium levels and ECG should be frequently monitored during treatment (every 1-6 h) until symptoms' resolution, followed by monitoring every 4-12 h until normokalemia achievement^[153].

MAGNESIUM

Magnesium is an important intracellular cation, second to potassium for prevalence. It acts as an essential cofactor for several intracellular enzymes involved in cells replication and energy metabolism processes, including phosphate transfer. Furthermore, it has a crucial role in muscular contractility and neuronal transmission^[154].

Almost the complete amount (about 99%) of magnesium is placed in the intracellular compartment: about 50%-60% is in bone and 38% in soft tissues, while only 1% of total body magnesium is extracellular. Normal serum magnesium concentration is between 1.5 and 1.9 mEq/L (1.7-2.2 mg/dL). Dietary magnesium absorption takes place in intestinal tract, and it is negatively influenced by high protein, phosphate, and fat. Magnesium elimination is mainly controlled by kidney. Several factors (hormonal: PTH, calcitonin, glucagon, and vasopressin; and non-hormonal: acid-base balance, potassium reduction, and magnesium plasma concentration) are involved in renal elimination^[155].

Hypomagnesemia

Definition and clinical implications

Hypomagnesemia is an electrolyte disorder characterized by reduced magnesium serum level (< 1.5 mEq/L). Serum magnesium concentration lower than 1 mg/dL is defined as severe hypomagnesemia. It occurs frequently (7%-12%) in hospitalized patients, and it is correlated to increased risk of death^[156].

Causes

Hypomagnesemia in cancer patients might result from different causes:

(1) Cancer: several conditions related to cancer might induce hypomagnesemia. Anorexia, malnutrition, vomiting, intestinal injury, intestinal drainage/fistulae, diarrhea, and malabsorption syndrome are frequent conditions in cancer patients inducing reduced magnesium intake.

(2) Cancer treatment: several drugs commonly used in cancer patients induce kidney damage and secondary hypomagnesemia. Cisplatin might induce hypomagnesemia through distal renal tubular damage, and it persists after treatment discontinuation. Therefore, intravenous supplementation of magnesium is indicated to prevent hypomagnesemia and decrease risk of nephrotoxicity^[157]. Hypomagnesemia was also described in patients receiving EGFR inhibitors with an incidence of 34% in patients treated with monoclonal anti-EGFR antibodies. In fact, EGFR activation is crucial for magnesium renal reabsorption through TRPM-6 channel^[158].

(3) Concomitant drugs: diuretics, antibiotics beta-adrenergic agonists, foscarnet, and amphotericin B might induce hypomagnesemia.

(4) Concomitant diseases: hyperparathyroidism, hyperthyroidism, diabetes mellitus, dialysis, renal failure, and hereditary disorders (e.g., Bartter's syndrome and Gitelman's syndrome) might induce hypomagnesemia.

Three main mechanisms are involved: reduced intake, redistribution into intracellular compartment, and increased renal losses [Table 7].

The most common mechanism inducing hypomagnesemia is renal losses. However, reduced magnesium intake is also frequent in cancer patients, mainly due to altered absorption secondary to diarrhea, malabsorption syndromes, and extensive bowel resection, while rarely due to severe malnutrition.

Management

Treatment of hypomagnesemia depends on clinical manifestation and severity, and it should be focused on hypomagnesemia correction and causes removal.

Patients with hypomagnesemia are usually asymptomatic, until magnesium concentration falls below 1.2 mg/dL, although symptoms usually do not correlate with serum magnesium levels^[159].

The earliest manifestations of magnesium deficiency are usually non-specific neuromuscular and neuropsychiatric alterations such as weakness and muscle cramps. Severe hypomagnesemia might cause

Table 7. Causes of hypomagnesemia

Causes of hypomagnesemia	
Reduced intake	Insufficient introit (anorexia, malnutrition, vomiting) Altered intestinal absorption (intestinal injury, intestinal drainage/fistulae, diarrhea, malabsorption syndrome) Alcoholism
Redistribution from extracellular into intracellular compartment	Acidosis correction Re-feeding syndrome Catecholamine Accelerated ontogenesis Endocrine disorders (hyperparathyroidism, malignant hypercalcemia, hyperthyroidism, aldosteronism, diabetes mellitus)
Renal losses	Reduced sodium reabsorption, SIADH Infusion of saline diuretics Post-obstructive nephropathy Renal transplantation Dialysis Diuretic phase of acute renal failure Hereditary disorders (e.g., Bartter's syndrome, Gitelman's syndrome)
Other	Pancreatitis, burns, excessive sweating Drugs [diuretics, cytotoxic chemotherapy (cisplatin), EGFR inhibitors, antibiotics beta-adrenergic agonists, foscarnet, amphotericin B]

SIADH: syndrome of inappropriate secretion of antidiuretic hormone

cardiac arrhythmia, disorientation, irritability, tremors, tetany, athetosis, jerking, and confusion, as well as eventually hallucinations, depression, and epileptic crisis. Furthermore, hypomagnesemia is often associated with multiple biochemical alterations, such as hypokalemia, hypocalcemia, and metabolic acidosis that might confuse clinical manifestations^[160].

Hypomagnesemia might manifest with electrocardiographic alterations such as prolonged PR and QT intervals, T wave inversion, and ST elevation^[161].

The diagnosis is based on the detection of lower serum magnesium levels. For a correct differential diagnosis, data on potential gastrointestinal or renal losses and concomitant drugs should be collected. To distinguish between renal and extra-renal losses, magnesium fractional excretion, and urinary calcium and creatinine should be assessed [Figure 7]. Urine magnesium concentration > 2 mmol/day is due to renal wasting.

Treatment for hypomagnesemia depends on severity and clinical presentations. Underlying disorders causing hypomagnesemia should be corrected^[162]. Patients with anamnestic risk magnesium deficiency; laboratory tests and clinical symptoms should be monitored and, when indicated, a prophylactic treatment should be considered. For example, in patients receiving cisplatin-based chemotherapy, intravenous supplementation of magnesium on the day of cisplatin administration and 2-3 days after therapy is indicated.

In asymptomatic patients, oral supplementation of magnesium salts should be preferred (40-60 mEq/day); however, diarrhea might represent a dose-limiting adverse event.

In symptomatic patients, infusion of intravenous magnesium sulfate should be preferred. A total of 1-4 g should be administered with low infusion in 12-24 h (1 g/h), until magnesium levels rise to 1.2 mg/dL.

In the case of severe hypomagnesemia, patients should receive a prompt replacement therapy, in order to prevent cardiac arrhythmias and death^[161]. A total dose of 4-6 g of magnesium sulfate is usually required in these cases.

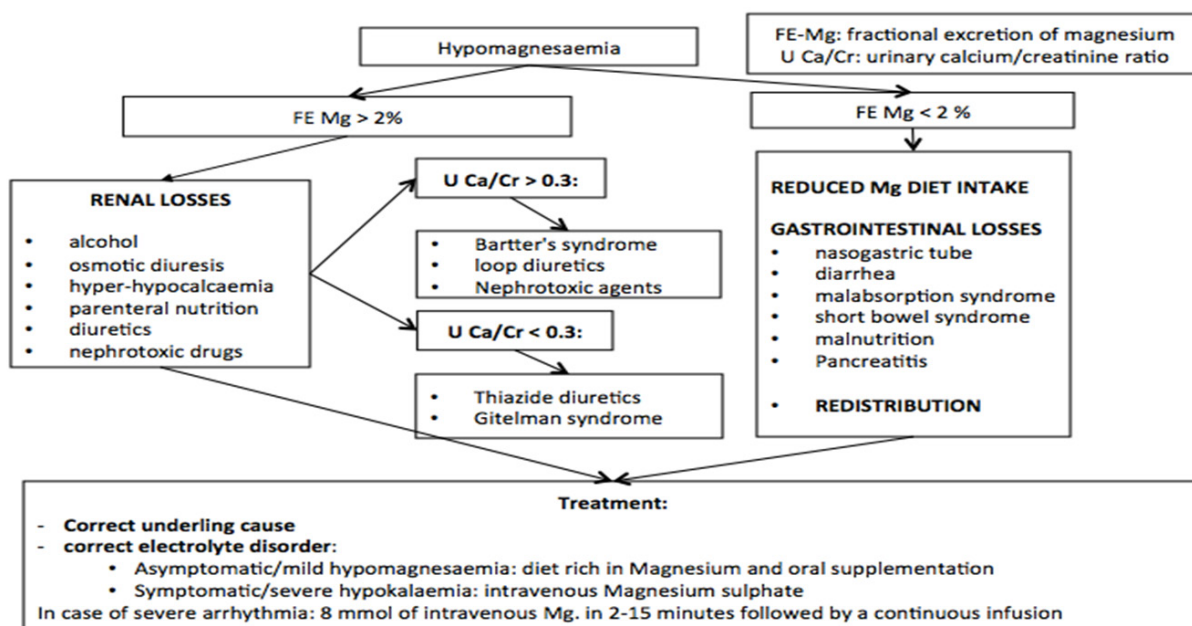


Figure 7. Algorithm of hypomagnesaemia management^[48,159-162]. The treatment of hypomagnesaemia is based on the correction of the underlying cause and the integration of magnesium, which can be oral or intravenous based on the degree and symptoms reported. The differential diagnosis of hypomagnesaemia is based on fractional excretion of magnesium: if it is > 2%, it might indicate renal loss, while, if it is < 2%, hypomagnesaemia might depend on gastrointestinal losses or reduced intake. FE-Mg: fractional excretion of magnesium; U Ca/Cr: urinary calcium/creatinine ratio

The maximum infusion rate should be 8 mEq/h in asymptomatic patients, since rapid intravenous administration of magnesium sulfate induces elevated serum magnesium levels, which favor magnesium renal excretion. Therefore, a slow infusion is crucial to obtain an adequate correction of hypomagnesaemia^[163]. Concomitant electrolyte disorder and vitamin D deficiency should be corrected^[164].

Hypermagnesaemia

Hypermagnesaemia is defined as a magnesium plasma level > 2.2 mEq/L. It is a rare electrolyte disorder and is usually iatrogenic (intravenous magnesium, magnesium-containing laxatives, or anti-acids).

Patient with hypomagnesaemia might complain of hypotension, respiratory depression, confusion, and ECG alterations such as bradycardia and complete AV-block until asystole. Treatment requires discontinuation of magnesium intake. In symptomatic patients presenting cardiac arrhythmias, respiratory depression, and hypotension, intravenous infusion of calcium gluconate 10% is suggested. In severe cases, hemodialysis may be necessary^[164].

CONCLUSION

Electrolyte alterations are common disorders in cancer patients and they are demonstrated to worsen prognosis. Altered electrolyte blood balance might depend on the presence of concomitant diseases and treatments, antineoplastic therapies, or paraneoplastic syndrome. Moreover, electrolytic alterations often cause important morbidity, negatively influencing quality of life and possibility of administration and response to the antineoplastic therapies. Furthermore, recent evidence shows the potential role of electrolyte channels in carcinogenesis, suggesting an important role of electrolyte balance in cancer patients. An improvement of knowledge about these conditions is necessary to monitor patients at risk for a prompt diagnosis and effective treatment, in order to improve patient outcome. Considering the lack of data in the literature supported by clinical trials enrolling cancer patients, the treatment recommendations

are based on retrospective or non-randomized studies such as case reports and case series, and based on clinical recommendations obtained by an Italian group of experts. A recent review analyzed dysnatremia, hypocalcemia, and hypercalcemia in cancer patients^[165]. Our manuscript deepens understanding of the consequences of electrolytic disturbances on the outcome of cancer patients as well as the roles of ion channels in carcinogenesis. In addition, in this review, the electrolytic disturbances of four cations are analyzed, namely Na, K, Ca, and Mg, to try to offer diagnostic and therapeutic algorithms useful for patient management in clinical practice. Prospective studies are needed to improve the management of electrolyte disorders in cancer patients.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the work: Berardi R

Performed acquisition and analysis of data and have drafted the manuscript: Torniai M, Lenci E, Pecci F, Morgese F, Rinaldi S

Revised the manuscript: Berardi R

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All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

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Consent for publication

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Review

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Lectin-like transcript 1 as a natural killer cell-mediated immunotherapeutic target for triple negative breast cancer and prostate cancer

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Abstract

Breast and prostate cancer are the leading causes of death in females and males, respectively. Triple negative breast cancer (TNBC) does not express the estrogen receptor, progesterone receptor, or human epidermal growth factor receptor 2, resulting in limited treatment options. Androgen deprivation therapy is the standard care for prostate cancer patients; however, metastasis and recurrence are seen in androgen-independent prostate cancer. Both prostate and breast cancer show higher resistance after recurrence and metastasis, which increases the difficulty of treatment. Natural killer (NK) cells play a critical role during innate immunity and tumor recognition and elimination. NK cell function is determined by a delicate balance of inhibitory signals and activation signals received through cell surface receptors. Lectin-like transcript 1 (LLT1, CLEC2D, OCIL) is a ligand of NK cell inhibitory receptor NKR1A (CD161). Several studies have reported higher expression of LLT1 is associated with the development of various tumors. Our studies revealed that TNBC and prostate cancer cells express higher levels of LLT1. In the presence of a monoclonal antibody against LLT1, NK cell-mediated killing of TNBC and prostate cancer cells were greatly enhanced. This review highlights the potential that using monoclonal antibodies to block LLT1 - NKR1A interactions could be an effective immunotherapeutic approach to treat triple negative breast cancer and prostate cancer.



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Keywords: Natural killer cell, lectin-like transcript 1, CLEC2D, CD161, breast cancer, prostate cancer, immunotherapy

INTRODUCTION

Breast cancer is one of the most serious diseases influencing the health of women, whereas prostate cancer threatens the health of men^[1]. In the 2019 Cancer Statistics report by Siegel *et al.*^[2], it was estimated that more than 170,000 men will be diagnosed with prostate cancer in the United States in 2019. Triple negative breast cancer (TNBC), a subtype of breast cancer, has the poorest prognosis and highest rate of recurrence and metastasis^[3]. Cancer recurrence and metastasis is the primary cause of cancer-related deaths and resistance to previous treatments is commonly seen. The critical functions of the immune system in cancer have been known for decades, but the transition to immunotherapy as a major method of cancer treatment has only occurred within the past few years. After the immunoediting hypothesis was suggested, the role of immune cells in cancer cell elimination, tolerance, and escape has been recognized^[4]. Although our immune system utilizes both innate and adaptive immune cells to recognize and eliminate abnormal cells, in many situations, cancer cells are still able to avoid immune surveillance. Cancer cells can adopt several mechanisms to evade immune surveillance; one mechanism is the upregulation of inhibitory signal pathways of immune cells to inhibit the immune response, such as upregulation of PD-L1 in TNBC^[5]. Lectin-like transcript 1 (LLT1) is a natural killer (NK) cell inhibitory ligand that has been described to contribute to the immunosuppressive properties of glioblastoma, prostate cancer, and triple negative breast cancer^[6-9]. This review highlights breast and prostate cancer, the function of NK cells, and NK cell-based immunotherapy. Additionally, this review explores LLT1 as a potential immunotherapeutic target for breast and prostate cancer elimination by NK cells.

BREAST CANCER AND PROSTATE CANCER

In 2019, there will be approximately 270,000 women diagnosed with breast cancer and 170,000 men diagnosed with prostate cancer in the United States^[2]. Unlike other cancers, breast cancer and prostate cancer are primarily gender specific in females and males, respectively. According to the expression level of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2), breast cancer is classified into the subtypes luminal A, luminal B, HER2 positive, and TNBC^[10,11]. Compared with the other subtypes of breast cancer, TNBC has a relatively worse prognosis owing to lack of recognizable molecular targets and high level of heterogeneity^[10,12-14]. Conventional treatments, including chemotherapy and radiotherapy, are still the primary way to treat TNBC patients. Beneficial results are observed when patients first begin chemotherapy; however, resistance to the chemotherapy and recurrence have been shown after later treatments^[15]. *BRCA1*, *BRCA2*, *ATM*, and *TP53* are essential genes in the DNA damage response signal pathways^[16]. Studies suggest mutations in these genes are found in TNBC^[16,17]. In TNBC, critical DNA damage response gene mutations lead to genomic instability, and a higher probability to produce neoantigens, which are termed “non-self” to differentiate from “self”^[18]. These neoantigens offer promising targets for immunotherapy.

In the United States, prostate cancer is the second leading cause of cancer-related death in males. Due to the vital role of the androgen receptor in the development of prostate cancer, androgen deprivation therapy has become the standard treatment for prostate cancer^[19-22]. Prostate cancer recurrence is usually androgen independent, which is termed castration-resistant prostate cancer^[23,24]. Therefore, new treatments are required for castration-resistant prostate cancer.

NK CELL FUNCTION

NK cells are an indispensable component of immune cells, but their role in immunotherapy has only been considered in recent years. NK cells were first suggested in tumor immunosurveillance due to studies

that showed people with higher incidence of cancers have defective NK cell functions caused by gene deficiency^[25,26]. Additionally, tumor growth and metastasis were also observed in NK mutant mice or after blocking NK cell activity by antibodies^[27,28]. Two types of receptors are expressed on NK cells: inhibitory receptors and activation receptors. Natural cytotoxicity receptor family, killer cell lectin-like receptor, and CD16 comprise the majority of activation receptors expressed on NK cells^[29,30]. The common characteristic of these NK cell activation receptors is having a cytoplasmic immunoreceptor tyrosine-based activation motif to mediate the activation signals. Unlike NK cell activation receptors, inhibitory receptors such as killer cell immunoglobulin-like receptors and the heterodimer CD94-NK group 2A recognize and bind to self MHC class I molecules and the inhibitory signals are mediated via cytoplasmic immunoreceptor tyrosine-based inhibition motifs^[31]. In contrast with CD8⁺ T cells, activation of NK cells does not require antigen presenting cell priming or MHC restriction^[32]. NK cell activation is regulated by the balance from activation and inhibitory receptor mediated signaling^[33]. Therefore, through multiple, simultaneous complex signaling pathways, NK cells recognize and kill a broad range of tumor cells. For example, tumor specific antigen interaction with activation receptors on NK cells, accompanied with lack of co-engagement of inhibitory receptors, will lead to secretion of perforin and granzyme from NK cells to target tumor cells^[34]. Additionally, it has been reported that some tumor cells have spontaneous loss of MHC class I expression as a mechanism for CD8⁺ T cell escape. NK cells, which do not require antigen presentation, are able to recognize and kill MHC class I low tumor cells^[35].

NK CELLS AND IMMUNOTHERAPY

Antibody dependent cell-mediated cytotoxicity (ADCC) is another primary function of NK cells and is currently being investigated to be used in NK cell-mediated immunotherapy. NK cells use the CD16 (FcγRIII) receptor to bind with the Fc portion of antibodies bound to specific antigens on target cells and induce NK cell cytotoxicity^[36]. Monoclonal antibodies (mAb) can also be used to block the interaction between ligands and receptors on NK cells. We have shown that activating NK cells via surface receptor CS1 (CD319, SLAMF7) enhanced the ability of NK cells to kill various tumor cells^[37,38]. Several other studies showed that CS1 is overexpressed on multiple myeloma (MM) and a humanized anti-CS1 mAb (Elotuzumab/Empliciti) was approved as a break through drug for treatment for MM patients^[39,40]. Our lab has found overexpression of LLT1 ligand on TNBC and prostate cancer, which interacts with the NK receptor NKR1A (CD161) and inhibits NK cell cytotoxicity^[9,41]. Thus, blocking inhibitory signals to NK cells using monoclonal antibodies to LLT1 could enhance the lysis of prostate cancer and TNBC cells by NK cells^[9,41].

Checkpoint inhibitors, which are traditionally used to promote CD8⁺ T cell function, have also been demonstrated to effect NK cell function. More specifically, NK cells express PD-1 and binding to PDL-1 on target cells results in inhibition of NK cell function and more aggressive tumors. Blockade of PD-1 and PDL-1 with mAb results in the strong NK cell responses required for full immunotherapeutic effect^[42].

In addition to checkpoint inhibitors, CARs (chimeric antigen receptor) can be engineered in NK cells or NK cell lines. Binding of a CAR to a tumor associated antigen leads to a strong activating signal, which triggers NK cell cytotoxicity^[43]. Currently, there are many clinical trials taking advantage of CAR NK cells. One such trial (NCT03056339) utilizes a CAR specific for the tumor associated antigen CD19 coupled to the costimulatory domains of CD28 and the zeta chain of the TCR/CD3 complex. Additionally, the retroviral vector expresses IL-15 for optimal NK cell activation and the inducible caspase 9 (iC9; iCasp9) suicide gene.

LLT1 AND NKR1A

LLT1, also known as CLEC2D (C-type lectin domain family 2 member D), OCIL (Osteoclast inhibitory lectin), and CLAX (Lectin-like NK cell receptor), belongs to the C-type lectin-like receptor superfamily and

was first described and cloned by our lab in human NK cells^[44]. Cross-linking of the LLT1 receptor on NK cells induces the production of IFN- γ without activation of the cytolytic pathway^[45]. Our lab, confirmed by others, also identified the NK cell inhibitory receptor NKR1A (CD161) as the ligand for LLT1 and implicated that LLT1 expressed on other cells could inhibit NK cell function^[46,47]. LLT1 is encoded by the *CLEC2D* gene, which produces five different isoforms through alternative splicing. Isoform 1 of LLT1 interacts with the NKR1A (CD161) receptor. Isoforms 2 and 4 stay in the endoplasmic reticulum and associate with LLT1 to form homodimers or heterodimers. Unlike LLT1 expressed on the cell surface, Isoforms 5 and 6 are soluble forms of LLT1^[48,49]. As a C-type lectin-like receptor, LLT1 is composed of three domains: a transmembrane domain, a stalk region, and the extracellular carbohydrate recognition domain, which is responsible for recognition^[7]. B cells, NK cells, T cells, and activated dendritic cells also express LLT1 on the cell surface^[48,49]. The crystal structure of LLT1 demonstrated it is highly glycosylated and when it forms a homodimer at the cell surface it serves as a ligand for the NKR1A receptor on NK cells^[46,47,50,51]. NKR1A is encoded by *KLRB1* and CD4⁺ T cells, invariant NKT cells, and $\gamma\delta$ -T cells have also been shown to express NKR1A^[49]. In mice, *CLEC2D* encodes the protein Oc1/Clr-b, which interacts with NKR-P1B/D^[52-54].

LLT1 AS AN IMMUNOTHERAPEUTIC TARGET FOR BREAST CANCER AND PROSTATE CANCER

NK cells, B cells, T cells, and activated dendritic cells express LLT1^[46,48]. Monoclonal antibody blocking of LLT1 could enhance the IFN- γ production of NK cells, but no differences in cytotoxicity have been seen^[45]. Increased expression of LLT1 was observed after Toll-like receptor induced activation of B cells and dendritic cells, which led to NK cell inhibition via LLT1 - NKR1A interaction^[49]. Inhibition of NK cell function through LLT1 has been observed on human malignant glioma cells, and immune crosstalk with B cells and monocyte-derived dendritic cells^[8,49,55]. Identification of a specific marker to enhance the ability of NK cells to recognize TNBC cells and prostate cancer cells provides a promising way for clearance of cancer cells. Previously, our lab studied the expression of LLT1 on TNBC and prostate cancer, and the results showed higher expression of LLT1 on TNBC cells compared to normal cells. Monoclonal antibody blocking of LLT1 enhanced NK cell cytotoxicity and TNBC cells lysis^[9]. In addition to higher surface expression of LLT1 on TNBC cells, our studies also demonstrated higher intracellular and surface expression of LLT1 in the prostate cancer cells compared with normal prostate cells. Blocking the interaction between LLT1 and NKR1A increased NK cell cytotoxicity against prostate cancer cells^[41]. In addition to blocking the inhibitory signal, anti-LLT1 mAb may activate ADCC (antibody dependent cell mediated cytotoxicity) function of NK cell against breast cancer and prostate cancer [Figure 1]. Pasero *et al.*^[56] showed that highly effective NK cells are associated with better prognosis in metastatic prostate cancer patients. Collectively, we demonstrate that monoclonal antibodies to enhance NK cell activation may provide a promising treatment to target and potentially prevent breast and prostate cancer metastasis. Santos-Juanes' newest study showed higher LLT1 expression was contributed to the risk of neck cutaneous squamous cell carcinoma (cSCC) nodal metastasis, which implicates LLT1 may also be a potential target to block cSCC nodal metastasis^[57]. Similar results have also been seen in mice; inhibition of NKR1B: Clr-b axis could enhance the NK cell-mediated immune surveillance to oncogenic transformation^[52]. Due to the important role of LLT1 in tumor progression and metastasis, monoclonal antibody blocking of LLT1 to enhance NK cell cytotoxicity may provide a novel treatment to prevent tumor metastasis.

CONCLUSION

Tumor recurrence and metastasis is associated with chemotherapy resistance, which greatly enhances the mortality in breast and prostate cancer^[3,58]. NK cell-mediated immunotherapy could provide specific recognition of tumor cells and overcome the resistance from chemotherapeutic drugs. LLT1, an inhibitory ligand, is widely expressed on several cancer cell lines. Our lab has demonstrated higher expression of LLT1 on TNBC and prostate cancer cells and increased lysis of cancer cells after blocking LLT1 with monoclonal antibodies. These results suggest LLT1 may offer a potential target for breast and prostate cancer treatment.

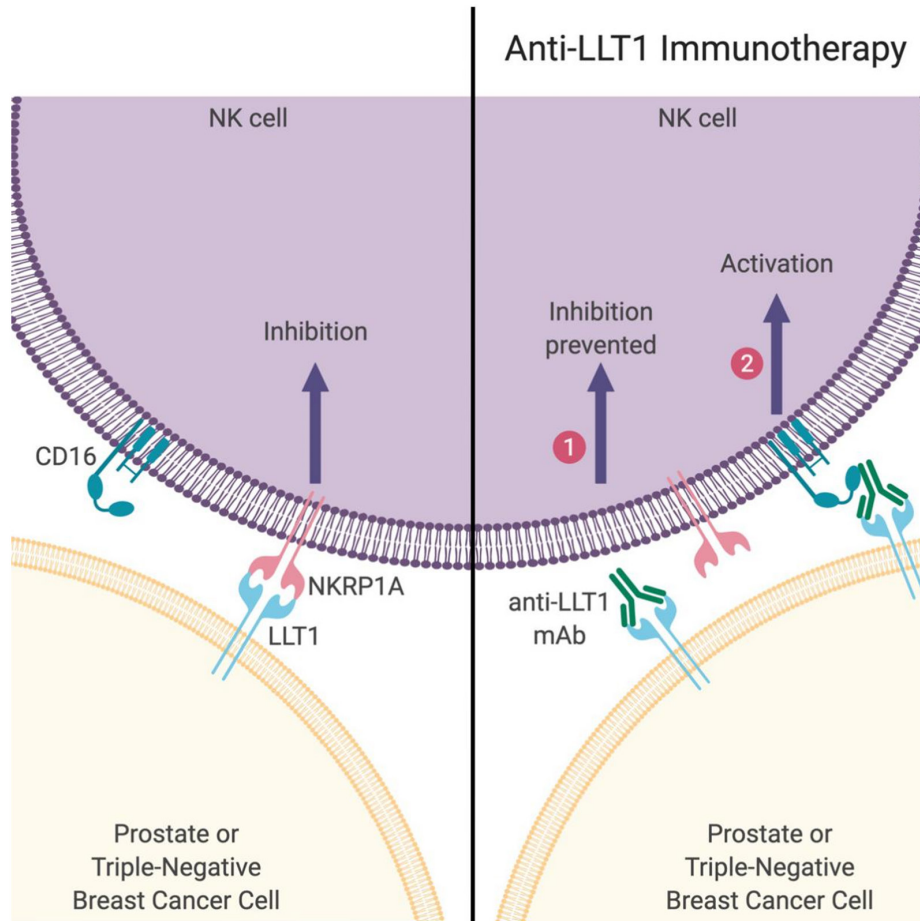


Figure 1. Activation of NK cell using anti-LLT1 monoclonal antibody. (Left) Prostate and triple-negative breast cancer cells express LLT1 which can bind to NCRP1A on NK cells leading to inhibition of NK cell function. (Right) However, in the presence of anti-LLT1 antibodies, NK cells can be activated via two mechanisms: (1) blocking of LLT1 prevents inhibitory signal transmission; and (2) activation when NK cell receptor CD16 binds to anti-LLT1 antibody bound to LLT1 on cancer cells. Image created using BioRender (www.BioRender.com). NK: natural killer; LLT1: lectin-like transcript 1; mAb: monoclonal antibody

DECLARATIONS

Authors' contributions

Designed and wrote manuscripts: Sun Y, Malaer JD, Mathew PA

Revised manuscript: Malaer JD, Mathew PA

Built figures: Malaer JD

Availability of data and materials

Not applicable.

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All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

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Case Report

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Physical exercise in locally advanced pancreatic adenocarcinoma: “If I walk, I live. Although one can die of cancer, now I am living”

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Abstract

The most widely used chemotherapeutic combinations in locally advanced (LA) or metastatic pancreatic ductal adenocarcinoma (PDAC) are Nab-Paclitaxel-Gemcitabine (Nab-PCT-GEM) and Fluorouracil, Folinic acid, Oxaliplatin, and irinotecan (FOLFIRINOX). Chemo-resistance, typical of PDAC, appears to be due to the negative influence of stroma population cells, namely regulatory T cells (Treg) and myeloid-derived suppressor cells, macrophages with inhibitory effects on the antitumor activity of the innate and adaptive immune systems, and resistance to cancer treatment. Among other factors that may influence immune surveillance, constant physical activity appears to reduce the risk of cancer-related mortality and cardiovascular risk. However, this does not seem to increase the survival of patients with PDAC. The exception is our young inoperable patient. For LA head PDAC, he was treated with seven cycles of Nab-PCT-GEM and RT 50 Gy/15 fractions combined to biweekly GEM and salvage FOLFIRINOX. The five-year surviving patient travelled 15,000 km on foot and continues inexorably his “walking therapy”.

Keywords: Pancreatic adenocarcinoma, depression, physical activity, Nab-paclitaxel, fluorouracil, regulatory T cells, myeloid-derived suppressor cells



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INTRODUCTION

The severity of pancreatic ductal adenocarcinoma (PDAC) is often linked to a late diagnosis with evidence of a locally advanced or metastatic disease, and the prognosis is poor. PDAC is one of the most chemoresistant tumors, which seems related to derived tumor-associated macrophages (TAMs) cells in the tumor's desmoplastic reaction^[1]. They are distinguished in M1 macrophages, which express inducible nitric oxide synthase, major histocompatibility complex class II (MHC-II) proteins, and CD80, CD86, and tumor necrosis factor α proteins, and they have immunostimulatory activity. In contrast, M2 macrophages expressing Th2 cytokines, arginase 1, CD206, and low amounts of MHC-II have immunosuppressive functions that allow tumor progression^[2]. Furthermore, the tumor microenvironment (TME), through Treg^[3] and myeloid-derived suppressor cells (MDSC)^[4] cells, has negative immunosuppressive effects.

Current treatments for advanced PDAC that improve prognosis and survival are Nab-Paclitaxel-Gemcitabine (Nab-PCT-GEM)^[5] and FOLFIRINOX^[6]. Furthermore, Nab-PCT-GEM regimen, in addition to its direct antitumor activity, seems to have immunoregulatory function. In fact, nab-PCT polarizes M1 macrophages by toll-like receptor 4 signaling^[2,7], whereas GEM has a dual action on MDSC and Treg^[8].

However, a cancer patient's immune system is affected by other factors, such as depression and impairment of physical activity. Depression due to a cancer diagnosis, as well as chronic stress and social isolation, suppresses immune function by the adrenergic and glucocorticoid pathways^[9]. The effects are especially strong on the lymphocytic stromal TME^[10], and are determinants in response to therapy.

Regarding physical activity, which could modify the depressive state, there are no conclusive data on the real impact on the immune system. Finally, although there is experience that correlates the amplitude of physical activity with the reduction of the risk of cancer-related and cardiovascular mortality^[11], this has not been reported on PDAC^[12].

The aim of this report is to analyze the characteristics of this long-term PDAC patient and the possible positive influence of long and constant physical activity on the global control of the disease.

CASE REPORT

A male 40-year-old patient has been recognized to have a locally advanced neoplasm of the pancreas head. After the appearance of obstructive jaundice, he was subjected to endoscopic retrograde cholangiopancreatography and positioning of biliary stenting with resolution of jaundice. Subsequently, he was subjected to exploratory laparotomy, which showed a locally advanced head pancreatic tumor that was judged inoperable. The intraoperative biopsy provided the diagnosis of PDAC. From December 2013 to July 2014, the patient underwent chemotherapy with Nab-PCT-GEM combination [Figure 1A and B] for seven cycles with radiological stability (not shown). Taking into consideration the persistence of non-operability, he performed RT 50 Gy/15 fractions combined to biweekly GEM with stabilization of the disease [Figure 1C and D].

After subsequent local progression, he was treated from April to October 2015 with FOLFIRINOX for six months with stabilization of the disease (not shown). This treatment resulted in bone marrow and peripheral neurological toxicity with negative repercussions on general conditions. After a slow and progressive clinical improvement, from February 2017, the patient undertook a constant course of physical exercise until he reached 15,000 km of walking. The reported daily route, carried out exclusively on foot, was on average 20 km and the total distance traveled was performed in 750 days.

During the five-year follow-up period, the stability status of the disease was confirmed [Figure 1E and F]. Furthermore, blood samples were taken the morning following a walk, periodically about every three months. The blood data are reported corresponding to the km traveled.

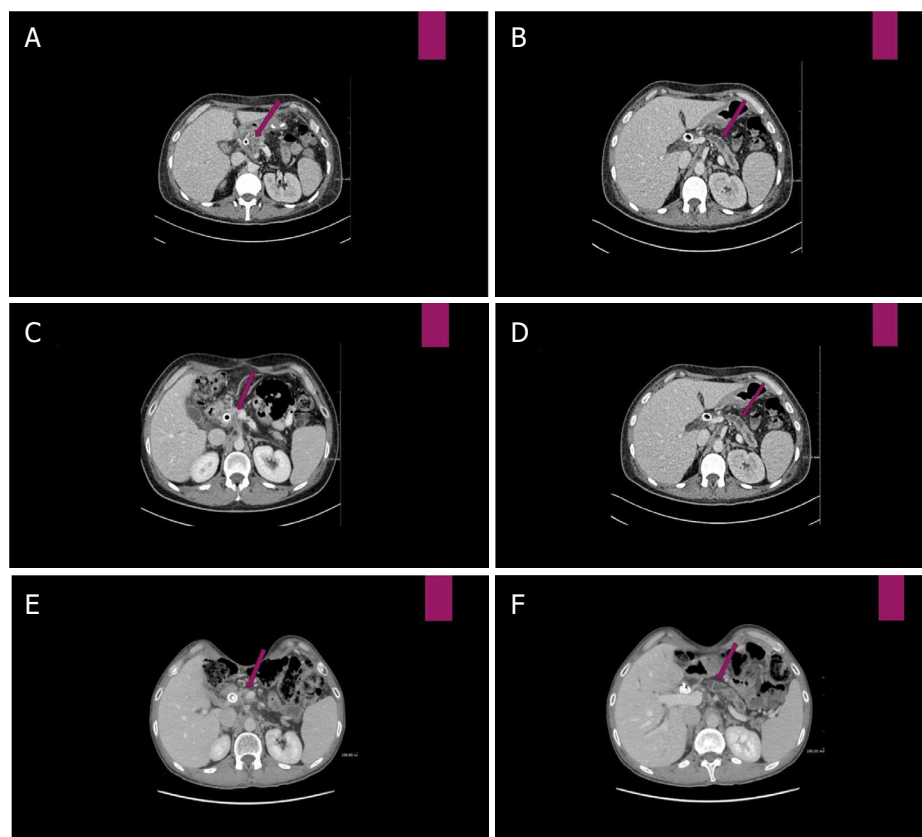


Figure 1. A: Computed tomography (CT) scan before Nab-paclitaxel-Gemcitabine therapy: hypodense lesion of 30 mm maximum transverse diameter, infiltrating the posterior lamina and presenting contact with the superior mesenteric artery; B: Wirsung duct dilatation of 8.42 mm; C: CT scan after Nab-paclitaxel-Gemcitabine therapy and consolidation radiotherapy: volumetric stability of the pancreatic lesion (29.76 mm); D: the Wirsung duct caliber (7.99 mm); E: 63 months from start therapy (after Nab-paclitaxel-Gemcitabine, radiotherapy and FOLFIRINOX salvage therapy) presence of slight reduction of the pancreatic lesion (25.38 mm maximum transverse diameter); and F: increase of the Wirsung caliber (10.67 mm) with atrophy of the pancreas body and tail

The hematological study has documented a count reduction of lymphocytes and platelets during chemo-radiotherapy followed by stabilization [Figure 2A]. Regarding the immune profile of lymphocyte subpopulations, a progressive reduction of CD3, CD4, and CD8 followed by an increase; a wave trend of CD16; and stability of HLA-DR and Treg were reported.

Moreover, after initial reduction of platelet/lymphocyte ratio followed by stabilization during therapy, an initial temporary increase tending to a fair final reduction after start of physical exercise was reported [Figure 2B and C]. Furthermore, after a decrease of Ca19.9 [Figure 2E] and C-reactive protein (CRP) and increase of albumin [Figure 2D], the stability over time of its level, as well as those of the other indexes of illness such as albumin [Figure 2D], CEA, and Ca19.9 [Figure 2E], was observed.

DISCUSSION

Current treatments for advanced PDAC represented by Nab-PCT-GEM and FOLFIRINOX combinations have improved prognosis and survival, which remain poor.

In addition to the aforementioned factors of a local nature, other factors of an immunological nature contribute to resistance to cytoreductive drugs and ultimately to neoplastic progression.

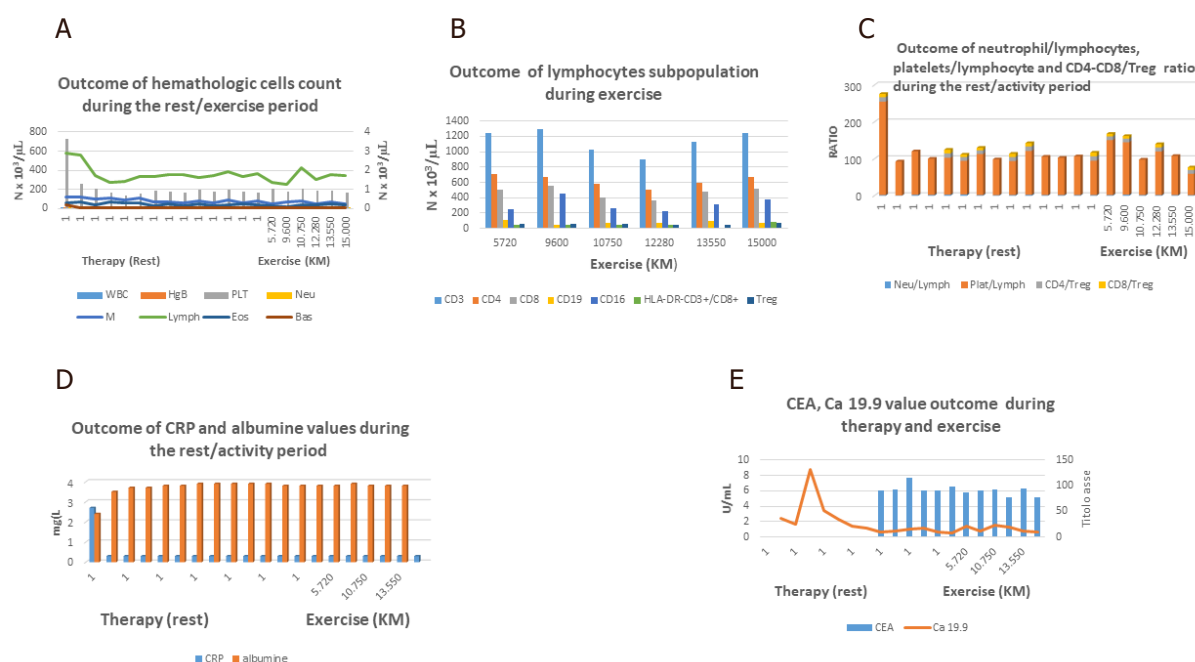


Figure 2. A: Hematologic outcome of population cells: decrease of platelets and lymphocytes count after therapy followed by stability of count cells during physical activity. Stability over time of hemoglobin level and white blood cells types; B: lymphocyte subpopulation during physical activity: progressive reduction of CD3, CD4 and CD8 followed by an increase, a wave trend of CD16 and stability of HLA-DR and Treg cells; C: initial reduction of Platelet/lymphocyte ratio followed by stabilization during therapy; it is followed by initial slight increase followed by stability and therefore slight reduction during exercise. Furthermore is shown a relative stability of neutrophil/lymphocyte, CD4/Treg, CD8/Treg ratios; D: recovery of albumin levels and decrease of CRP after therapy followed by stability during physical activity; E: decrease of Ca 19.9 after therapy followed by the stability over time together of CEA levels during exercise

It is known how the magnitude of the tumor mutation burden correlates with the probability of response to the new checkpoint inhibitory agents that have recently entered clinical practice in the treatment of tumors.

PDAC is also negatively influenced by the low tumor mutation burden, characterized by low prevalence of somatic mutations and of neo-antigens expression with consequent resistance to new immunotherapeutic agents, such as anti-PD1^[13]. One of the key points of immunosuppression also present in PDAC is represented by the CXCR4/CXCL12 axis. In fact, from its block derives the promotion of intratumoral lymphocyte infiltration and the synergism of action with anti-PD-1 agent^[14].

Among the external factors, physical activity is very important in the homeostasis of the organism. It exercises antitumor activity in animal models and high levels of post-diagnosis exercise reduce the risk of recurrence and cancer-specific mortality in common solid tumors^[15]. Such exercise must be undertaken gradually until a constant level is reached and maintained (inverted-J hypothesis), whereby regular moderate exercise improves immune system function while acute, intermittent efforts correlate with immunosuppression and increased susceptibility to cancer^[16].

Regarding the repercussions of physical activity and immune surveillance, the cells that are most stimulated are the natural killer (NK) cells, influenced by neuroendocrine^[17,18] and immunological mechanism such as IL-6 secretion resulting from muscle stress^[19].

However, discordant results have been reported in animal and clinical studies and were reiterated in a systematic literature review on the repercussions of physical exercise on NK cell counts and cytotoxicity. Indeed, definitive conclusions cannot yet be drawn because of the diversity in the intensity and duration of the exercise and in the time of blood sampling^[20].

The emerging difficulties derive from the possible hormonal role on the total NK count, which does not include the tissue one, and from the correlations with performance status, age, and disease status in acute and chronic exercise^[20].

Regarding innate immunity, immunological differences between healthy persons and cancer patients have been demonstrated^[21,22]. In healthy persons, exercise can positively influence the pro-inflammatory polarization of M1 macrophages following immune escape^[21], and neutrophil counts^[22], while, in a tumor animal model, exercise caused vascular normalization and hypoxia reduction with improvement of response to chemotherapy^[23] and reduction of TAMs and neutrophils infiltrating their tumors determining tumor volume reduction^[24].

In the adaptive immune response, Th1-polarized T cells and Tregs, influenced by adhesion molecules, are involved in maintaining peripheral tolerance and appear to have a prognostic role^[25-27]. Moderate-intensity exercise produced in healthy 65-year-old subjects a greater increase in lymphocyte response to immunogenic stimulation than in those who performed flexibility and toning exercises^[28]. In fact, a single bout of physical exercise also seems to determine in the healthy an increase of differentiated NK-cells, CD8+, and $\alpha\delta$ T-cells, with marked sensitivity to external stimulation. This could have an implication in hematopoietic stem cell transplantation and represent a valid aid in the conventional treatment of tumors^[29].

Reduction of leukocyte count, improvement of depressive symptoms, and increase in diurnal salivary cortisol rhythm can be obtained in patients with a six-month exercise program differently from in sedentary patients but without the lymphocyte functional finding^[18].

Finally, in low immunogenic tumors, such as pancreatic adenocarcinoma, chemotherapy with drugs such as anthracyclines can trigger an immune response through dendritic cells (DC)^[30].

Similar to chemotherapy, marathon running increases myeloid DC counts and the production of pro- and anti-inflammatory cytokines^[31]. This finding supports the role of immunomodulatory mechanisms in the response and adaptation to acute, excessive exercise. The possible clinical application of these results has found a clinical finding in pretreated, unresponsive patients and in progression of disease, obtaining a disease control rate of 41%, improvement of physical status, reduced risk of progression, and impact on increase of cytotoxic CD8+ CD28+ and decrease of suppressive CD8+ CD28- T cell^[32].

Regarding long-term survivors, the increased risk of developing second cancers and cardiovascular complications is known.

In this case, it also seems that the amplitude of physical activity reduced the risk of both cancer-related mortality and cardiovascular disease^[11]. However, this was not found in PDAC^[12].

Nevertheless, there is some evidence for correlations among antitlastic treatment, moderate but constant physical exercise, and immune response in the neoplastic patients, although knowledge about the impact of physical activity on the immune system is not conclusive.

Cell death by chemotherapy might not be the single modality of antitumor action; there may be other immunomodulation mechanisms, regulated and controlled by immunosuppressive cells, that can condition the therapeutic response. They could act as an additive to chemotherapy and could improve the therapeutic index if “physical conditioning exercise” has been administered in selected patients before starting chemotherapy or new innovative therapy with checkpoint inhibitors.

Therefore, if physical activity were recognized as a determining factor in the induction and maintenance of antitumor immune function, it could be used as adjuvant and maintenance therapies in the on and off phases of the active antineoplastic therapy, respectively.

Physical activity may have surprising positive results, especially in patients with a life-threatening disease, such as PDAC, who have undergone systemic treatments that cause long-term sequelae such as neurotoxicity with a negative impact on their quality of life. After a slow and progressive clinical improvement, our patient undertook a constant course of physical exercise until he reached more than 15,000 km of walking. These results are described in his book^[33], which deals with his manifold experiences, feelings, and personal reflections, as well as the clinical approach to his neoplastic disease. During the five-year follow-up period and walking activity, the stability of the disease was confirmed. Therefore, this could be linked to the contribution of the physical exercise as maintenance of response obtained after FOLFIRINOX. It is also worth noting the stability of the hematological and immunological profile, with a reduction trend of PLT/Lymph ratio, the normalization of CRP after treatment, and its maintenance over time during physical activity, which are indirect indices of immune surveillance. However, no statistical considerations can be made due to the lack of comparative data during an actual rest phase for the relative constant physical activity of the patient.

His unique experience should make us profoundly reflect on the effects of reactive depression to a cancer diagnosis, and to further study the relationships between physical activity and both treatment efficacy and immunosuppression. Prospective studies involving medical oncologists, immunologists, biologists, neurologists, and physiotherapists are needed to confirm these results.

DECLARATIONS

Authors' contributions

Conceptualization, data curation, formal analysis, investigation, methodology, project administration, software, supervision, validation, visualization, writing - original draft, writing - review & editing, and used in attributes: Lo Re G, Magnaldi S, Doretto P, Innocente R, Lo Re F

Availability of data and materials

We declare that the patient's data derive from personal treatment and clinical-instrumental follow-up. The scientific data reported derive from the international literature and it is listed in the references.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Journal articles not in English	Zhang X, Xiong H, Ji TY, Zhang YH, Wang Y. Case report of anti-N-methyl-D-aspartate receptor encephalitis in child. <i>J Appl Clin Pediatr</i> 2012;27:1903-7. (in Chinese)
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Books	Sherlock S, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub; 1993. pp. 258-96.
Book chapters	Meltzer PS, Kallioniemi A, Trent JM. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. <i>The genetic basis of human cancer</i> . New York: McGraw-Hill; 2002. pp. 93-113.
Online resource	FDA News Release. FDA approval brings first gene therapy to the United States. Available from: https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm . [Last accessed on 30 Oct 2017]
Conference proceedings	Harnden P, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer; 2002.
Conference paper	Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. <i>Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming</i> ; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. pp. 182-91.
Unpublished material	Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. <i>Proc Natl Acad Sci U S A</i> . Forthcoming 2002.

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