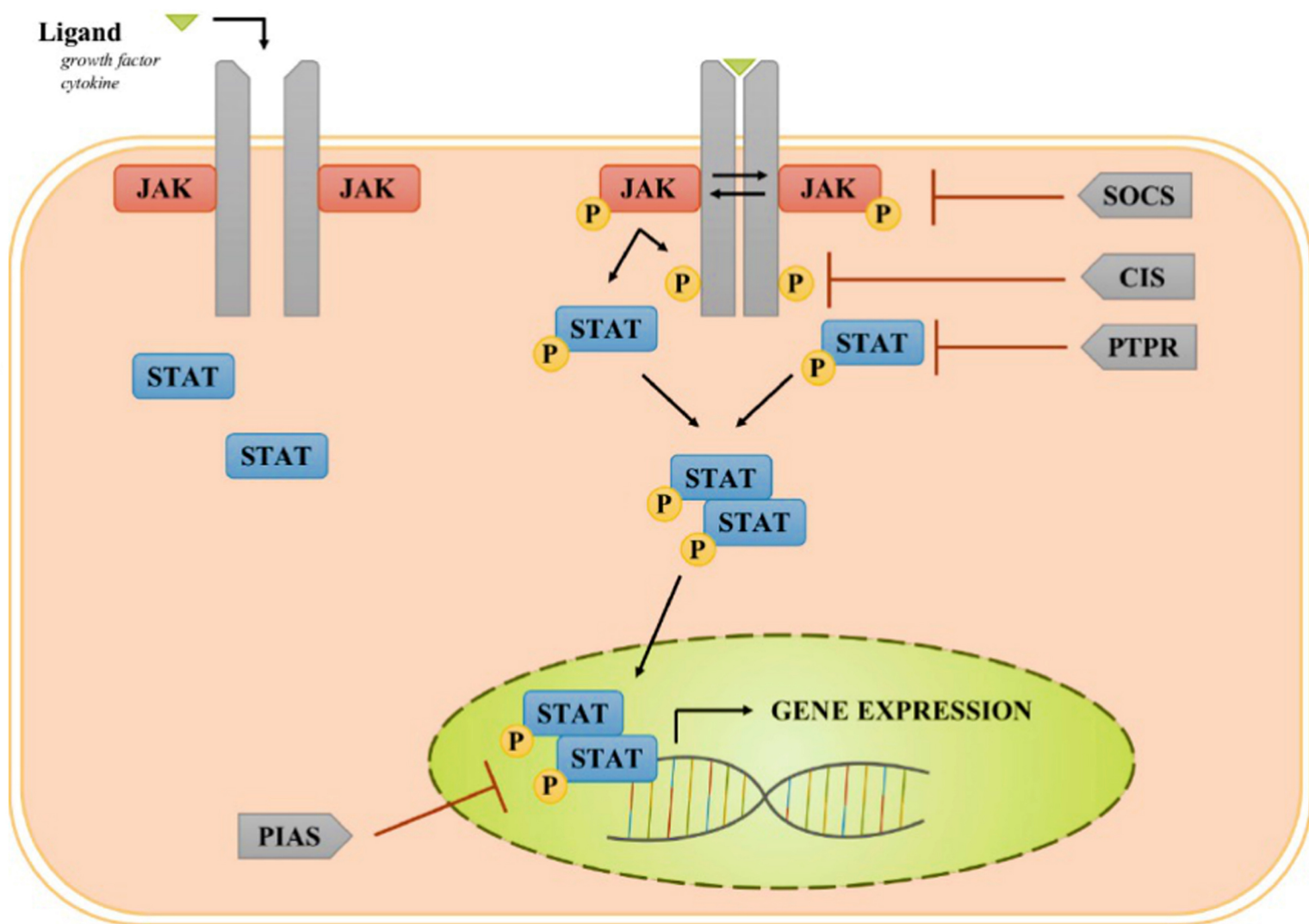


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Editorial

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Introduction to this Special Issue: “Biomarker Discovery and Precision Medicine”

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How to cite this article: Fang B. Introduction to this Special Issue: “Biomarker Discovery and Precision Medicine”. *J Cancer Metastasis Treat* 2020;6:1. <http://dx.doi.org/10.20517/2394-4722.2019.42>

Received: 4 Dec 2019 **Accepted:** 5 Dec 2019 **Published:** 7 Jan 2020

Science Editor: Judy S. Crabtree **Copy Editor:** Jing-Wen Zhang **Production Editor:** Tian Zhang

With advances in genomics, transcriptomics, proteomics, and metabolomics, blooming data have been available for exploring molecular alternations in cancers. Many of these molecular alternations have been investigated as biomarkers for cancer diagnosis, prognosis, and precision therapies. It is my privilege to introduce this Special Issue of the Journal of Cancer Metastasis and Treatment, which contains four review articles and four original articles that focus on the topic of biomarker discoveries for cancer diagnosis and precision therapy.

Solid tumors are known to shed their cellular components (proteins, nucleic acids, lipids, glycosaminoglycans, and metabolites) or malignant cells themselves into peripheral blood. Some of these molecules are already used as biomarkers for cancer screenings and follow up tests in clinics^[1,2]. The advent of new technologies in genomics, proteomics, metabolomics, and cell biology analyses has dramatically expanded the scope of circulating tumor biomarkers from traditional tumor-associated antigens to circulating tumor cells, circulating tumor nucleic acids (cell free DNA and miRNA), exosomes, and plasma proteomics. The tests on circulating tumor cells or tumor-specific nucleic acid in blood are also referred to as liquid biopsies^[3]. Three review articles in this Special Issue describe recent advances and challenges in liquid biopsy. Lai *et al.*^[4] reviewed the use of membrane lipid-binding ligands in isolating subtypes of exosomes or extracellular vesicles for improvement of discovery and detection of disease-associated biomarkers in peripheral blood. Huang *et al.*^[5] discussed advances in developing new devices, such as microfluidics and nanotechnology, for capturing and molecular characterization of circulating tumor cells. Bookland



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and Kolmakova^[6] reviewed current advances in searching for circulating biomarkers for pediatric brain tumors, including cell-free DNA, non-coding RNA, tumor metabolites, and proteins in body fluids, such as cerebrospinal fluid, blood, and urine. On the other hand, the review article by Farlow *et al.*^[7] discussed applications of biomarkers in design of clinical trials. These reviews and discussions on advances and challenges in biomarker discoveries stimulate new thinking on addressing the challenges encountered in the field of cancer biomarker discoveries and precision therapies.

The authors of four original articles reported results of their research projects on the discovery of new cancer biomarkers. Vander Borgh *et al.*^[8] described the generation and evaluation of monoclonal antibodies specific for exon 18 neural cell adhesion molecule for detecting small cell lung cancer cells. Ossoliński *et al.*^[9] reported their study on mass spectrometry-based metabolomics profiling of prostate cancer. They found over two hundred differentiating metabolites in urine, serum, and interstitial fluid of prostate cancer patients. The study presented by Zaichick *et al.*^[10] showed that contents of several chemical elements were drastically different between thyroid malignant tumors and normal thyroid tissues. Finally, Liu *et al.*^[11] reported the results of using visible resonance Raman spectroscopy for rapid skin cancer diagnosis.

I hope you enjoy reading the articles in this Special Issue on biomarker discoveries. I also want to thank Dina Li for her assistance in organizing this Special Issue.

DECLARATIONS

Authors' contributions

The author contributed solely to the article.

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

Not applicable.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Editorial

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Acknowledgement to reviewers of *Journal of Cancer Metastasis and Treatment* in 2019

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Correspondence to: OAE Publishing Inc., Rm. 968, 9th Floor, Xinhengji International Building, No. 3 Maizidian West Road, Chaoyang District, Beijing 100016, China. E-mail: editorialoffice@jcmtjournal.com

How to cite this article: Journal of Cancer Metastasis and Treatment Editorial Office. Acknowledgement to reviewers of *Journal of Cancer Metastasis and Treatment* in 2019. *J Cancer Metastasis Treat* 2020;6:2. <http://dx.doi.org/10.20517/2394-4722.2020.07>

Received: 19 Jan 2020 **Accepted:** 19 Jan 2020 **Published:** 21 Jan 2020

Science Editor: Lucio Miele **Copy Editor:** Jing-Wen Zhang **Production Editor:** Jing Yu

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If you are interested in becoming a reviewer for *JCMT*, you are welcome to apply at https://jcmtjournal.com/journal/vol_reviewer.

DECLARATIONS

Authors' contributions

Writing and revision of the article and approved the final version: Journal of Cancer Metastasis and Treatment Editorial Office.

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

Not applicable.

Consent for publication

Not applicable.

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Editorial

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Introduction to this special issue “Breast Cancer Metastasis”

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How to cite this article: Schieman WP. Introduction to this special issue “Breast Cancer Metastasis”. *J Cancer Metastasis Treat* 2020;6:3. <http://dx.doi.org/10.20517/2394-4722.2020.01>

Received: 6 Jan 2020 **Accepted:** 3 Feb 2020 **Published:** 14 Feb 2020

Science Editor: Bingliang Fang **Copy Editor:** Jing-Wen Zhang **Production Editor:** Tian Zhang

Breast cancer remains the most common malignancy and most frequent cause of cancer-related death in women, a devastating reality that annually claims more than 600,000 lives across the globe^[1]. The vast majority of deaths due to breast cancer are attributed to metastasis and its associated relapse^[2,3], which typically transpires in patients ~5-20 years after their initial diagnosis^[4]. Although metastasis is the most lethal characteristic of breast cancer, our understanding of the molecular mechanisms that govern this event remains incomplete, a stark reality reinforced by the finding that diagnosis of distant-stage disease has remained unchanged over the course of the last two decades^[5].

The metastatic cascade is a highly complex and inefficient process subject to regulation by a host of intrinsic and extrinsic cellular mechanisms^[6]. Breast cancers most frequently disseminate to the brain, bone, and liver^[7], doing so by undergoing a distinct sequence of events that involve: (1) local invasion through the epithelial basement membrane and migration through stromal connective tissue; (2) intravasation into blood or lymphatic vessels to facilitate dissemination; and (3) extravasation and infiltration into tissue parenchyma, followed by eventual colonization of secondary organ sites^[6,8,9]. Importantly, the rapid development of powerful new technologies and models to study breast cancer metastasis has greatly expanded our appreciation of the complexities associated with this last frontier of cancer biology. This Special Issue on Breast Cancer Metastasis is a compendium of 17 review articles, 13 original articles, and 3 case reports that collectively cover a wide spectrum of topics associated with the development and treatment of breast cancer metastases. Among the review topics presented herein are thought-provoking discussions on the potential impact of estrogen disrupting agents^[10] and *ESR1* mutations to engage the



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metastatic cascade^[11], as well as eloquent analyses of how fluidity^[12] and plasticity^[13] within the hierarchy of normal and malignant stem cells drives breast cancer development and metastatic progression. Similarly, Robinson *et al.*^[14] provide an elegant perspective on how alternations in telomere maintenance mechanisms influence the metastatic evolution of breast cancer stem cells. Likewise, thoughtful discussions on the regulation of epithelial-mesenchymal transition (EMT) programs and carcinoma plasticity also extend into new arenas of: (1) epigenetic modification of histones by lysine-specific demethylase (LSD1 or KDM1A^[15]); (2) spatiotemporal control of the transcriptome coupled to EMT by aberrant expression and activation of hnRNP E1^[16]; and (3) mRNA splicing factors and their essential contribution to the acquisition of EMT and metastatic phenotypes^[17]. Along these lines, Maisel and Schroeder^[18] present an intriguing and comprehensive discourse on how defects in retrograde trafficking of cytokine and growth factor receptors contribute to the acquisition of metastatic phenotypes in breast cancer.

Science and medicine currently lack the knowledge to understand how disseminated breast cancers escape clinical detection by remaining dormant for years before reemerging as chemoresistant and incurable secondary tumors^[19,20]. Indeed, the mysteries of metastatic dormancy have been identified as one of the ten most critical research gaps and translational priorities needed to be solved to alleviate breast cancer^[21]. Herein, the paradoxical role of autophagy in both suppressing and promoting breast cancer development and metastatic progression is discussed, particularly its importance in maintaining dormancy-associated phenotypes in disseminated breast cancer cells^[22]. Gooding *et al.*^[23] highlight the far-reaching impact of the noncoding genome and the long noncoding RNA BORG in regulating breast cancer metastasis, chemoresistance, and disease recurrence during metastatic dormancy. Not to be forgotten are the essential functions of the tumor microenvironment in regulating breast cancer biology and metastatic progression^[24]. Indeed, the therapeutic potential of disrupting breast cancer cell communication with stromal factors and cryptic peptides is presented^[25], as are intriguing discussions of how: (1) involution in the postpartum mammary gland engenders a pro-metastatic microenvironment reminiscent of those found in their tumorigenic counterparts^[26]; and (2) stress and inflammatory signals disrupt neuronal circuitry, thereby contributing to brain metastasis by breast cancers^[27].

This Special Issue also considers the translational and clinical aspects of therapeutic targeting metastatic breast cancers. Indeed, Kamal *et al.*^[28] present a comprehensive review comparing active *vs.* passive treatment strategies to alleviate breast cancer metastasis to the brain, while dos Santos *et al.*^[29] offer an equally far-reaching review on the molecular mechanisms underlying photodynamic therapy and its potential to treat metastatic disease. Additionally, the clinical utility of monitoring circulating tumor cells as a measure of disease progression and therapeutic effectiveness is also discussed^[30].

Finally, it is our hope that these timely and topical reviews will prove to be intellectually stimulating and highly thought-provoking. Likewise, we invite you to explore the 13 original articles and 3 case reports, which dovetail topically with the aforementioned reviews.

DECLARATIONS

Authors' contributions

The author contributed solely to the article.

Availability of data and materials

Not applicable.

Financial support and sponsorship

CA236273.

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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Original Article

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Brain metastasis as exclusion criteria in extensive-stage small cell lung cancer trials: a trend over decades

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How to cite this article: Komiya T, Chaaya G, Deshotels L, Powell E, Guddati AK. Brain metastasis as exclusion criteria in extensive-stage small cell lung cancer trials: a trend over decades. *J Cancer Metastasis Treat* 2020;6:4. <http://dx.doi.org/10.20517/2394-4722.2019.41>

Received: 18 Nov 2019 **First Decision:** 7 Jan 2020 **Revised:** 17 Feb 2020 **Accepted:** 17 Feb 2020 **Published:** 21 Feb 2020

Science Editor: Stephen J. Ralph **Copy Editor:** Jing-Wen Zhang **Production Editor:** Jing Yu

Abstract

Aim: To investigate the frequencies and trends of brain metastases (BMs) as exclusion criteria in extensive-stage small cell lung cancer (ES-SCLC) trials.

Methods: We conducted a comprehensive search to identify prospective clinical trials in patients with ES-SCLC. PubMed searches were conducted with the key words “small cell lung cancer” and “extensive”. The online archives of 20 oncology journals were also searched. Recent review articles in ES-SCLC were also investigated for additional articles. Eligible studies must have enrolled primarily ES-SCLC and been published in English. Studies involving brain/chest radiation and brain metastasis-specific trials were excluded. Studies were categorized into allowed/undefined, conditional, or complete exclusion of BM.

Results: In total, 491 published studies were identified by PubMed (240), journal websites (198), and review articles (53). Early publication year (1970-1999) and first-line/maintenance setting were associated with higher incidence of complete exclusion of cases with BMs ($P < 0.0001$ and 0.0233 , respectively). Incidence of complete exclusion was 27% in the 1990s, and then decreased to 12% in the 2000s and 8% in the 2010s.



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Conclusion: A significant number of ES-SCLC trials continues to exclude patients with BM. Future studies need to ease eligibility regarding BM according to ASCO/Friends recommendations.

Keywords: Brain metastasis, small cell lung cancer, clinical trials

INTRODUCTION

Despite the introduction of cancer prevention, screening, and new treatment modalities, cancer remains the leading cause of human mortality in both men and women worldwide. According to the National Cancer Institute Surveillance, Epidemiology, and End Results Program, more than 600,000 people die annually from cancer in the United States^[1]. To develop novel therapies and further improve outcomes, well-designed clinical trials recruit candidates in numerous clinical settings. Although they are intended to help physicians' decision-making in future oncology patients, these trials are often restrictive in patient selection. Patients with unfavorable risk factors are not permitted to participate due to fear of safety risks, resulting in a lack of generalizability to the typical patient population.

In the real world, oncologists often face clinical scenarios that have not been addressed in previous trials. Patients in oncology clinics may not have the same clinical characteristics that are required for participation in trials, indicating potential discrepancies in patient populations between clinical trial and non-clinical trial settings. In fact, a retrospective review of lung cancer cases at a Canadian institution showed that 73% of their consecutive patients would have been trial-ineligible for their hypothetical trials with common eligibility criteria^[2]. Due to lack of data in the trials, patients may develop unexpected outcomes with newly-developed treatments or be undertreated because of the fear of unknown risk.

To address the lack of generalizability in oncology clinical trials, the American Society of Clinical Oncology (ASCO) and Friends of Cancer Research proposed to the Food and Drug Agency (FDA) that clinical trials must ease eligibility criteria and suggested re-considering several items commonly listed in exclusion criteria^[3-6]. These include presence of brain metastases (BMs), history of HIV/Hepatitis B/C, minimum age, organ dysfunction, and prior and concurrent malignancies. These items have been frequently listed in exclusion criteria due to historical concerns without valid scientific analysis^[3-6]. Oncology patients with any of these clinical characteristics tend to be excluded from clinical trials even though they account for a significant proportion of all cancer cases in the real world.

In this study, we focused on the presence of BMs as an exclusion criterion in prospective clinical trials of extensive-stage small cell lung cancer (ES-SCLC). Frequency of trial exclusion due to presence of BMs was assessed to determine a trend over several decades.

METHODS

We conducted systematic screening to identify prospective clinical trials in ES-SCLC [Table 1]. The initial screening used PubMed search with key words "small cell lung cancer" and "extensive". Twenty journals commonly publishing these studies were identified during the PubMed search: *Journal of Clinical Oncology*, *British Journal of Cancer*, *New England Journal of Medicine*, *Lancet*, *Lancet Oncology*, *Cancer Research*, *Clinical Cancer Research*, *Annals of Oncology*, *Lung Cancer*, *Journal of Thoracic Oncology*, *Journal of National Cancer Institute*, *Clinical Lung Cancer*, *Cancer*, *International Journal of Cancer*, *American Journal of Clinical Oncology*, *European Journal of Cancer*, *Annals of Internal Medicine*, *The Oncologist*, *Cancer Chemotherapy and Pharmacology*, and *Oncotarget*. Online archives of these journals were investigated for additional

Table 1. Selection of ES-SCLC trials

Inclusion criteria	ES-SCLC
Exclusion criteria	Publication in English literature
	Publication by December 2018
	Including NSCLC
	Trials specific for BMs
Investigation period	Radiation therapy as primary intervention
	From January 2018 to June 2018
Search methods	Pubmed search
	Online journal website
	Citation in review articles

ES-SCLC: extensive stage small cell lung cancer; NSCLC: non-small cell lung cancer; BMs: brain metastases

articles. Recently published clinical reviews about ES-SCLC were also identified and investigated for more articles^[7-10].

Eligible studies must have enrolled primarily ES-SCLC and been published in English. Studies primarily investigating brain/chest radiation and BM-specific trials were excluded. Studies were categorized into allowed/undefined, conditional (e.g., only previously treated or asymptomatic BM are allowed), or complete exclusion of BM. Chi-square test was used for categorical group comparison. Two-sided *P*-values of less than 0.05 were defined as statistically significant.

RESULTS

The screening for clinical trials in ES-SCLC identified 240, 198, and 53 distinct articles by PubMed, journal website archives, and review articles, respectively. Characteristics of the trials are shown in Table 2. Most of the studies are published in journals with impact factor < 10, publication year 2000 or later, non-US sites, phases other than phase III, first-line setting, non-randomized, fewer than 100 patients, performance status other than 0-1, and no age limit. Radiographic screening of BM by magnetic resonance imaging (MRI) or computed tomography (CT) was mandated only in 32% (157 studies, data not shown). Anti-angiogenic agents were used only in 19 studies.

Early publication year (1970-1999) and first-line/maintenance setting were significantly associated with a higher incidence of complete exclusion of cases with BM ($P < 0.0001$ and 0.0233 , respectively). The complete exclusion was 20% in early vs. 10% in late publication years, and 16% in first-line/maintenance vs. 8% in others. There was no correlation between the complete exclusion of BMs and other clinical characteristics including journal impact factor, region, trial phase, randomization, sample size, patient performance status, and age limit.

Studies in the 1990s had the highest incidence (27%) of complete exclusion, which decreased to 8% in the 2010s [Figure 1]. The conditional group increased from 25% in the 1970s to 34% in the 1990s and 79% in the 2010s.

DISCUSSION

Lung cancer is the most common cause of cancer mortality in the United States. Small cell lung cancer (SCLC), which accounts for approximately 15%-20% of all lung cancer cases, commonly presents with BMs^[11-13]. Clinical trials in ES-SCLC focusing on systemic therapy often exclude patients with BMs primarily due to safety concerns; however, detailed incidence and trends in excluding BMs have not been investigated. The current study demonstrated that 8% of ES-SCLC trials in the 2010s still completely exclude patients with BM, whereas a recent analysis on non-small cell lung cancer trials at clinicaltrials.gov reported a complete exclusion rate of 14%^[14].

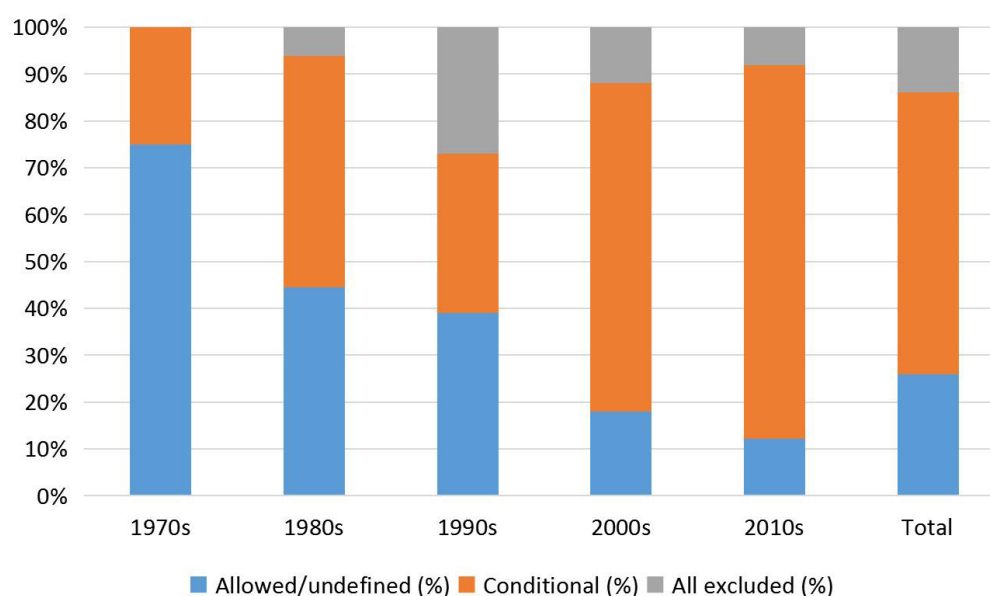


Figure 1. A trend of brain metastasis as exclusion criteria in extensive-stage small cell lung cancer. Each bar indicates percentage of each group regarding exclusion of brain metastasis

Table 2. Characteristics of studies in extensive-stage small cell lung cancer

Characteristics	Variables	Excluded all BM (%)	Others (%)	Total	P
Total		69 (14)	421 (86)	490	
Impact factor	< 10	43 (14)	271 (86)	314	0.875
	≥ 10	26 (15)	150 (85)	176	
Publication year	1970-1999	38 (20)	155 (80)	193	< 0.0001
	2000-2018	30 (10)	267 (90)	297	
Region	US included	27 (14)	169 (86)	196	0.957
	Non-US	41 (14)	253 (86)	294	
Study phase	III	11 (11)	88 (89)	99	0.373
	Others	57 (15)	334 (85)	391	
Study line	1st +/- M	53 (16)	283 (84)	336	0.027
	M	3 (19)	13 (81)	16	
	1st and 2nd	3 (14)	18 (86)	21	
	2nd	8 (7)	101 (93)	109	
	Unknown	1 (13)	7 (87)	8	
	1st or M	59 (16)	314 (84)	373	
	Others	9 (8)	108 (92)	117	
Randomization	Yes	17 (10)	149 (90)	166	0.096
	No	51 (16)	273 (84)	324	
Sample size	< 100	51 (14)	308 (86)	359	0.727
	≥ 100	17 (13)	114 (87)	131	
ECOG/WHO PS	0-1	6 (10)	52 (90)	58	0.407
	Others	62 (15)	370 (85)	432	
Age Upper Limit	Yes	19 (13)	125 (87)	144	0.778
	No	49 (14)	297 (86)	346	
Anti-angiogenesis	Yes	3 (16)	16 (84)	19	0.763
	No	63 (13)	408 (87)	471	

BM: brain metastasis; M: maintenance; ECOG: Eastern Cooperative Oncology Group; WHO: World Health Organization; PS: performance status

ES-SCLC patients with BMs in the real-world setting do not benefit from clinical trials that do not include BM cases. New therapeutic interventions for these patients are unlikely to develop while they remain ignored by researchers. Expanding eligibility criteria to include patients with BMs would provide benefits in several aspects. In a population with a high incidence of BMs such as ES-SCLC, efficacy and safety effects unique to patients with BMs may exist. If the efficacy and safety of an investigational agent is assessed at an early phase, late phase studies may enroll patients to assess central nervous system (CNS) penetration along with related efficacy and safety. A successful example is a phase III trial comparing alectinib *vs.* crizotinib in advanced non-small cell lung cancer where alectinib demonstrated a CNS response rate of 81% in a population with 40% BM at baseline^[12]. ASCO and Friends of Cancer Research strongly recommend investigation in patients with BMs in the setting of prospective trials rather than relying on post-marketing experience^[4]. They state that previously treated and radiographically stable BM cases for at least four weeks should be included in prospective trials of all phases, whereas active/progressive BMs may require a study-to-study approach. A group from the FDA supports their recommendation by suggesting exclusion of only those who are symptomatic or are taking medications with known drug interactions^[13].

Current staging methods of SCLC includes CT scans of chest/abdomen/pelvis, positron emission tomography scan, mediastinal staging procedures via bronchoscopy, and MRI of the brain. Lesions in the CNS have been previously assessed only clinically in early ages, while CT of the head came of use in the 1980s-early 1990s. With the now more recent use of MRI, more asymptomatic BM cases are presumably detected, likely explaining the increase of the conditional group as commented above. However, radiographic screening of BM was mandated only in 32% of studies overall, and 23% of studies published in the 2010s (data not shown). Changes in the management of BMs may also explain the trend over the decades. As stereotactic radiosurgery became available for small CNS lesions, more cases were identified that were previously treated with radiation therapy. In fact, the cases in the conditional group have grown over the decades [Table 2]. They also include asymptomatic cases, those previously treated with radiation or surgery, and those in stable condition without the need for high dose steroids.

We are aware that removing restrictions in eligibility criteria may not necessarily result in rapid increases in enrollment of the previously-excluded population. For instance, a recent trial in extensive-stage SCLC that allowed the presence of BMs accrued a very limited number of patients^[15]. Attitudes of investigators will hopefully change as ASCO/Friends recommendations become more appreciated in the next several years.

We acknowledge limitations in this study. With a retrospective search, there might be studies not covered by our search strategy. Studies not available in English publication were excluded, indicating potential publication bias. The articles in the earlier years had limited space to provide information such as detailed eligibility criteria, whereas recent articles can be more informative with online supplemental datasets. Information regarding eligibility of patients with leptomeningeal metastases were not available in most studies. Few agents with specific concerns on CNS disease such as anti-angiogenesis drugs have been tested in a limited number of trials. These potential biases may need to be considered for the interpretation of this study.

In conclusion, a significant number of ES-SCLC trials continue to exclude patients with BM, although conditional allowance has increased over the decades. Future studies need to further ease eligibility regarding BM according to ASCO/Friends recommendations.

DECLARATIONS

Acknowledgments

We thank the Parkview Research Center for administrative support.

Authors' contributions

Substantial contributions to conception and design of the study and performed data analysis and interpretation: Komiya T

Data acquisition, as well as provided administrative, technical, and material support: Komiya T

Manuscript writing: Komiya T, Chaaya G, Deshotels L, Powell E, Guddati AK

Availability of data and materials

List of clinical trials as primary data source can be found as supplemental information.

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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Meeting Abstracts

Open Access



Meeting abstracts of Colossal Facet Conference - 3rd World Congress on Cancer 2019 “New strategies to prevent, diagnose and treat Cancer based on Precision Medicine”

Prague, Czech Republic; 23-25 Sep 2019; Published: 27 Feb 2020

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Editorial Note

This special issue of *Journal of Cancer Metastasis and Treatment* is dedicated to the proceedings of the 3rd World Congress on Cancer 2019 held in Prague (Czech Republic) during September 23-25 (<http://colossalfacet.com/cancer-conference/>).

The theme of the Conference Cancer 2019 was “New strategies to prevent, diagnose and treat Cancer based on Precision Medicine”. The conference focused on the biomolecular mechanisms of cancer development, on the altered energetic metabolism in cancer cells, on the cancer patient’s metabolic alterations, and on the diagnostic and therapeutic approaches. Major topics included: pathogenetic mechanisms (oncogenes, oncosuppressors, DNA repair, cancer stem cells, epigenetics, inflammation, and immune responses); metabolism (nutrition, fasting, and obesity) and cancer; novel strategies for prevention, diagnosis and therapy (imaging, phytochemicals, and nanotheranostics). The conference gathered academicians and young inspired scientists from all around the world with the aim to strengthen the international cooperation in the fight against cancer. One other major goal of this conference was, in fact, to create an atmosphere of interactions between young and senior scientists to favor novel cooperation that can bring new and more efficacious strategies to understand and fight cancer. The Organizing Scientific Committee was formed by renowned scientists in the field who delivered a keynote Speech. Thomas N Seyfried delivered the honorary lecture. In addition, we had invited lectures, oral communications, and poster presentation from registered attendees from all around the world.

This special issue collects the contribution in the form of original research papers or review articles from all participants at the conference.

Keywords: Autophagy, biomarkers, cachexia, cancer metabolism, cancer therapy, cell death, epigenetics, metastasis, tumor microenvironment, precision medicine



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1. Amino acid depleting enzymes alone or in combinations as a therapeutic strategy for cancer treatment

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Background and aim: Significant differences exist between the metabolism and antioxidant requirements of normal and malignant cells. Tumor cells depend on exogenous nutrients in their microenvironment to fulfill the elevated energy requirements and for maintaining appropriate intracellular antioxidant levels. Deprivation of amino acids results in growth inhibition or death of tumor cells by the modulation of various signaling cascades and in some cases redox balance. We have been evaluating potential therapeutic enzymes that degrade critical amino acids required for tumor growth. These engineered human enzymes include one that degrades either *L*-cysteine and one that degrades methionine.

Experimental procedures: (1) *in vitro* cell culture experiments to evaluate cell survival using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and crystal violet assays; (2) metabolomics analyses of amino acids and metabolites; (3) analyses of oncogenic cell signaling, reactive oxygen species (ROS) levels, and DNA damage as well as cell cycle changes using flow cytometry; (4) *in vivo* allograft and xenograft tumor experiments with various cancer cell lines.

Results: Depletion of extracellular *L*-cys/cystine led to depletion of intracellular *L*-cys, decreased levels of intracellular glutathione (GSH), and increases in intracellular ROS leading to activation of cellular signaling pathways, oxidative DNA damage, and ultimately cancer cell death. Cyst(e)inase, given *i.p.*, significantly reduced serum levels of *L*-cys and significantly inhibited tumor growth *in vivo* of both prostate and pancreatic cancer xenograft and allograft tumor models. Notably, targeting a second antioxidant pathway together with cyst(e)inase (*i.e.*, the thioredoxin pathway) using a thioredoxin reductase inhibitor led to synergistic cancer cell killing and also sensitized tumor cells found to be more resistant to cyst(e)inase alone. These and other studies on the mechanisms associated with the potential anticancer activity of Cyst(e)inase are presented. In addition, we have also studied the potential therapeutic application of a human engineered methionine (*L*-met) degrading enzyme called methionine gamma lyase (hMGL). Both mechanistic studies as well as *in vivo* preclinical therapeutic studies demonstrate significant efficacy against several cancers with hMGL.

Conclusion: Depletion of amino acids such as *L*-cys and *L*-met using human engineered enzymes offer novel approaches for treating cancer either given alone or more likely in combination with other agents.

2. Investigating metabolic cancer vulnerabilities by high-content metabolomic screening

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Background and aim: The important role of cell metabolism in furthering cancer development and growth is increasingly recognized^[1,2]. Recent advances in high-throughput metabolomics technology are leading to

its growing role during the development of new drugs targeting metabolic vulnerabilities in multiple cancer diseases^[3,4]. However, drug discovery is limited by the unsuitability of animal models for high-throughput drug screening. Moreover, animal studies may not adequately predict the clinical efficacy of therapeutics in humans. These limitations motivated researchers to develop new three-dimensional (3D) *in vitro* models to better mimic the *in vivo* tumor microenvironment^[5].

Experimental procedure: Here, we introduce a novel high-content metabolomics screen based on high-resolution direct infusion mass spectrometry (DIMS) technology able to monitor the metabolic response of drug-treated mammalian cells in 3D 96-well format. This rapid and systematic metabolomic method was validated on multiple cancer and normal cells, cultured either in individual or in co-culture cell systems using ¹³C- ¹⁵N labeled tracer analysis.

Results: Novel synergistic combination of drugs were identified utilizing the metabolic profiling obtained using DIMS. These include chemotherapies targeting the metabolic reprogramming of cancer cells, including mitochondrial oxidative phosphorylation and glutaminolysis.

Conclusion: Overall, the rapid data acquisition and improved detection limits of mass spectrometry are paving the way for applications of metabolomics in preclinical screening^[5,6], opening new opportunities in drug discovery and personalized medicine.

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3. Clinical application of artificial intelligence in ovarian cancer

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Among several types of gynecologic cancer, ovarian cancer is the most lethal type. Due to the absence of specific symptoms and effective biomarkers, the survival rate of ovarian cancer is poor. Moreover, platinum resistance is a major obstacle in ovarian cancer treatment. Thus, accurate biomarkers associated with chemoresistance and recurrence of the cancer are necessitated. To establish personalized therapeutic strategies for ovarian cancer patients, a prediction model that precisely predicts patient responses to chemotherapy and diagnosis could be computed and incorporated. Although many prediction models for cancer have been suggested, few models specific for ovarian cancer have been proposed. Thus, we performed integrative analysis incorporating both clinico-pathologic and multi-omics data and developed prediction models for diagnosis and prognosis of ovarian cancer. In addition, we conducted metagenome

analysis of patients with benign tumors and ovarian cancer to construct an early detection model of ovarian cancer. Gene expression data from TCGA database have been analyzed using the deep neural network model to develop a model for predicting platinum-sensitivity in high-grade serous ovarian cancer. Lastly, we integrated clinico-pathologic and multi-omics data to reveal multiple factors associated with ovarian cancer progression.

4. Genetic and epigenetic regulation of therapy resistance in ovarian cancer by long non-coding RNAs

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Background and aim: Ovarian cancer remains the most fatal gynecological cancer in the world, with a five-year survival rate of only 46% for the localized disease and 29% for the distant-stage disease. With the recent analysis of cancer genome, long non-coding RNAs (lncRNAs) are emerging as critical players in the pathobiology of many cancers, thus identifying them as new genomic targets for precision cancer medicine. Therefore, we sought to identify the critical lncRNA(s) involved in ovarian cancer genesis, progression, therapy resistance, and disease recurrence.

Experimental procedure: To identify the lncRNAs critically involved in ovarian cancers, we carried out a global analysis of mRNAs as well as lncRNAs that are differentially expressed in patient-derived ovarian cancer cells, using a series of biased and unbiased transcriptome analyses.

Results: The results indicate 1351 lncRNAs and 1591 mRNAs were significantly dysregulated in patient-derived cancer cells compared to normal fallopian tube-derived epithelial control cells. Co-expression network analysis of coding and noncoding RNAs identified the etiological role for several, thus far, unidentified lncRNAs and mRNAs in ovarian cancer. Further analyses indicated that the lncRNA-regulated gene expression network in ovarian cancer involves both genetic and epigenetic mechanisms.

Conclusion: Our findings with representative lncRNAs indicate that they can serve as a novel diagnostic as well as prognostic biomarkers. In addition, the findings that the silencing of specific lncRNAs inhibits xenograft tumor growth identify them as new therapeutic targets in ovarian cancer.

5. Radiotherapy and immunotherapy promote tumoral lipid oxidation and ferroptosis

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Background and aim: Cancer immunotherapy restores or enhances the effector function of CD8⁺ T cells in the tumor microenvironment. Radiotherapy can indirectly stimulate CD8⁺ T cell function through innate immune signaling. Direct connections between radiotherapy and adaptive CD8⁺ T cell function

remain undefined. Ferroptosis is a recently discovered form of cell death and results from iron-dependent accumulation of lipid peroxides. It is unclear whether, and how, ferroptosis is involved in T cell immunity, cancer immunotherapy, and radiotherapy efficacy.

Experimental procedure: To understand the importance of ferroptosis in immunotherapy and radiotherapy efficacy, we used genetic deletion of key ferroptosis effector genes and pharmacologic agonists and antagonists. Lipid oxidation was quantified using C11-BODIPY. A wide variety of *in vivo* and *ex vivo* analyses was performed in tumor and immune cells.

Results: We show that immunotherapy-activated CD8⁺ T cells and radiotherapy enhance ferroptosis-specific lipid peroxidation in tumor cells, and that increased ferroptosis contributes to the anti-tumor efficacy of immunotherapy and radiotherapy. Mechanistically, interferon gamma (IFN γ) released from CD8⁺ T cells downregulates the expression of SLC7A11, a subunit of the glutamate-cystine antiporter system xc⁻, impairs the uptake of cystine by tumor cells, and, as a consequence, promotes tumor cell lipid peroxidation and ferroptosis.

Conclusion: This work establishes a novel mechanism through which CD8⁺ T cells function. This work expands our understanding of the interactions between immunotherapy and radiotherapy.

6. Polyploidy and the origin of human tumors

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Polyploid giant cancer cells (PGCCs) have long been observed in cancer and were thought originally to be nondividing. Surprisingly, the formation of blastomere by cleavage division after the formation of the zygote, with progressive decrease in cell size and increase in nuclear to cytoplasmic ratio, is the first step in embryogenesis, also shows abundant polyploidy. The evidence from our laboratories demonstrated that the stress-induced PGCCs can divide by endoreplication (endocycle and endomitosis), which leads to increased nuclear to cytoplasmic ratio, in turn leading to dedifferentiation of somatic cells and acquisition of embryonic stemness. Therefore, formation of PGCCs in somatic cells may represent a previously overlooked endogenous embryonic program that can be activated to dedifferentiate somatic cells into stem cells of various potencies for tumor initiation. Based on these data, I propose that human tumors originate from stem cells at a specific developmental hierarchy, which can be achieved by dualistic origin: dedifferentiation of the zygote (sexual) via the blastomeric-mediated cleavage division during normal development or transformation from damaged or aged mature somatic cells via a blastomeric-like embryonic program (asexual) via formation of PGCCs. Initiation of the tumor begins with stem cells that have uncoupled the differentiation from the proliferation program, which results in stem cell maturation arrest. Thus, the birth of a tumor can be viewed as a triad that originates from stem cells via dedifferentiation through a blastomeric or blastomeric-like program, differentiation along Waddington's landscape, and arrested at a specific developmental hierarchy. The significance of polyploid blastomere-like cancer stem cells in cancer therapy is discussed.

7. Ketogenic diet as a cancer treatment: *in vitro* quantification

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Background: The glucose deprivation Restricted Ketogenic diet (KD-R) in combination with Metformin use, is a non-toxic broad-spectrum approach that targets the important metabolic differences between normal and cancer cells. The optimal use of this approach for cancer treatment is investigated using *in vitro* tests.

Method: Tests were carried out at 3-mmol/L blood glucose (BG) to mimic the BG effect of KD-R in combination with Metformin. Two breast and one cervical cancer as well as one non-tumorigenic cell were used.

Results: The different cell lines were affected differently. This suggests that glucose deprivation via KD-R and Metformin will not equally affect different cancers. All cell lines were most adversely affected after three weeks. Cell growth decreased to 32% for the most glucose avid cancer cell line.

Partial recovery occurred after three months. Full cancer extinction can thus not be reached with only KD-R and Metformin. Adjuvant treatments are needed. These treatments should be done when the cancer cells are at their most vulnerable, i.e., three weeks after reaching a BG level of 3 mmol/L.

Future work: Future work will entail adjuvant treatments such as chemotherapy together with KD-R and Metformin. Results should be available before the conference. The focus of our conference presentation is on the latest results.

8. DNA repair and damage response in personalized brain cancer chemotherapy

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Background and aim: The first-line chemotherapeutic for malignant glioma is the DNA methylating agent temozolomide. The mechanism of cell death triggered by the minor DNA lesion, O⁶-methylguanine, induced by the agent is well described. It rests on conversion of the lesion through mismatch repair into DNA double-strand breaks (DSB) that trigger downstream pathways including apoptosis and senescence^[1]. Consequently, corresponding repair pathways are expected to have a great impact on temozolomide resistance, and evidence was provided for the involvement of O⁶-methylguanine DNA methyltransferase (MGMT), mismatch repair, and DSB repair by homologous recombination through BRCA2 and Rad51^[2] as well as XRCC3^[3]. However, only MGMT found the way into the clinic, being used as predictor for therapy outcome^[4].

Experimental procedure: A battery of cell und molecular biological methods was applied, including apoptosis, senescence, and autophagy measurements.

Results: We compared methods of determining the MGMT promoter methylation status, which corresponds to MGMT silencing and therapy, and showed that MS-HRM is superior compared to methylation-specific polymerase chain reaction^[5]. We also show that downstream of O⁶-methylguanine derived DSBs are ATR/ATM triggered pathways that activate apoptosis and senescence. Thus, data are shown demonstrating that the SIAH1-HIPK2-p53ser46 pathway plays a key role in regulating temozolomide-induced apoptosis^[6]. The question of temozolomide threshold doses in activating survival and death pathways is also addressed^[7].

Conclusion: MGMT, mismatch repair and the SIAH1-HIPK2-p53ser46 pathway are key elements in personalized glioblastoma therapy with DNA-alkylating drugs.

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9. Metabolic management of glioblastoma

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Glioblastoma multiforme (GBM) remains among the most aggressive and difficult to manage primary brain tumors in humans. Abnormalities in the number, structure, and function of GBM mitochondria compromise energy metabolism through OxPhos. Glucose and glutamine are recognized as the major fermentable fuels that drive GBM growth through glycolysis and glutaminolysis, respectively. The glutamine antagonist, 6-diazo-5-oxo-L-norleucine (DON), was administered together with a calorically restricted ketogenic diet (KD-R) to treat late-stage orthotopic growth in two syngeneic mouse models of GBM: the highly invasive mesenchymal tumor, VM-M3, and the high-grade stem cell glioma, CT-2A. DON targets glutaminolysis while KD-R reduces glucose and, at the same time, elevates neuroprotective and non-fermentable ketone bodies. The diet/drug therapeutic strategy caused massive tumor cell death or mitotic arrest, while reversing disease symptoms and improving overall survival without toxicity. The therapeutic strategy also reduced edema, hemorrhage, and inflammation associated with rapid tumor growth. Moreover, the KD-R diet facilitated DON delivery to the brain and allowed a lower nontoxic dosage to achieve therapeutic effect. Data from human case reports are also presented. These findings support the importance of glucose and glutamine in driving GBM growth and provide a plausible therapeutic strategy for the non-toxic metabolic management of GBM and any cancer with mitochondrial defects.

10. Novel perspectives on the immune environment of acute myeloid leukemia using multiomyx

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Background and aim: Acute myeloid leukemia (AML) is a clinically and molecularly heterogeneous disorder. Despite its poor prognosis, the treatment of AML remains largely unchanged over the past several decades with high-dose chemotherapy remaining the mainstay of therapy. This has led to interest in exploring novel therapeutic approaches, such as bispecific antibodies, chimeric antigen receptor T cells, tumor vaccines, and immune checkpoint inhibitors.

The bone marrow (BM) constitutes the home niche for leukemic cells. Tumor microenvironment (TME) is defined as the cellular environment in which the tumor exists. This environment is made up of endothelial, stromal, and immune cells and plays a key role in the development, propagation, and survival of cancer cells. The immune microenvironment has been well described in several hematologic malignancies, including Hodgkin lymphoma, acute lymphoblastic leukemia, chronic myeloid leukemia, and chronic lymphocytic leukemia, but less is known about the microenvironment in AML.

We studied the myeloid subsets in bone marrow tissues of normal and AML patients using MultiOmyx technique. We aimed to clarify the clinical significance of these cells in the AML patients.

Experimental procedure: MultiOmyx is an exclusive proprietary multiplex immunofluorescent technology that overcome the challenges for immuno-oncology biomarker profiling. It enables detection and visualization of up to 60 biomarkers on a single formalin-fixed paraffin-embedded (FFPE) slide and co-expression analysis of up to 25 stains on a single cell, which is unattainable with the conventional immunohistochemistry (IHC) technique. Other advantages include: quantitative single cell classification, measures of marker intensity (mean, median, and total), and full spatial context for measuring the distances between cells with different immunophenotypes.

Results: Myeloid subsets present in tumors are heterogeneous and play a crucial role in promoting cancer development and metastasis. Tumor associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) all contribute to an immunologically permissive microenvironment for cancer cells. Based on surface markers expression, MDSC can be further subdivided into granulocytic MDSC (G-MDSC, polymorphonuclear MDSC) and monocytic MDSC (M-MDSC). MDSC have also been shown to express immune checkpoint ligands such as programmed death-ligand 1 (PD-L1) that can suppress T cell responses *in vitro*. There is little information regarding MDSCs in AML. TAMs can be polarized by signals from their environment into two major subsets, called M1 and M2 macrophages. Acute myeloid leukemia blasts have been shown to differentiate monocytes from healthy donors into an M2-like phenotype in transwell coculture assays. In our study, we were able to highlight the immune landscape of AML and compared it with the landscape for normal bone marrow. We observed that both M-MDSC and G-MDSC accumulated within the TME in AML BM samples, with higher frequency of G-MDSCs over M-MDSCs. The data also reveal abundant M2 macrophages present in the TME of the AML samples. The detection of both MDSCs and M2 macrophages in these samples supports the hypothesis that these cells contribute to the establishment of an immunosuppressive TME. Using the MultiOmyx proprietary algorithm, which takes into account the staining patterns, we quantified the counts and density of different tumor-resident myeloid subsets and measured the spatial distance from the different subsets of tumor-resident myeloid cells to CD34+ blasts in AML samples. We highlighted the correlations between the immunosuppressive myeloid cells and the different subsets of T cells including T-regulatory cells in AML clinical biopsy samples. TAMs and MDSCs are emerging as potential biomarkers for diagnosis and prognosis of cancer as well as therapeutic targets of many immunomodulating agents. As demonstrated in this study, MultiOmyx multiplexed panel has the potential to monitor the changes of immunosuppressive myeloid cells in response to immune modulating drugs such as MDSC-targeting drugs (e.g., PDE-5 inhibitors and COX-2 inhibitors), TAM-targeting agents (e.g., anti-CSF1R), and combination therapy in treatment of AML.

11. Radiation oncology updates in treatment of prostate cancer

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I am a radiation oncologist at MD Anderson Cancer Center with a large proportion of my practice dedicated to prostate cancer. I would like to update the audience with the basic algorithms of radiation, hormonal, and systemic therapy for low, intermediate, and high risk. This talk would include information on active surveillance, definitive radiation treatment, brachytherapy as single modality and boost treatment, adjuvant and salvage (post-operative) radiation therapy, and hypofractionation and stereotactic body radiation therapy. I understand the audience is a variety of clinicians in the medical field. I would tailor the talk to provide an overview so all can understand what is available to all prostate cancer patients and the updated studies to support these recommendations.

12. Novel DNA modification in cancer

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Genetic drivers of cancer can be dysregulated through epigenetic modifications of DNA. Although the critical role of DNA 5-methylcytosine (5mC) in the regulation of transcription is recognized, the functions of other non-canonical DNA modifications remain obscure.

We report the identification of novel DNA N6-methyladenine (N6-mA) modifications in human tissues and implicate this epigenetic mark in human disease, specifically the highly malignant brain cancer glioblastoma. Glioblastoma markedly upregulated N6-mA levels, which co-localized with heterochromatic histone modifications, predominantly H3K9me3. N6-mA levels were dynamically regulated by the DNA demethylase ALKBH1, depletion of which led to transcriptional silencing of oncogenic pathways through decreasing chromatin accessibility. Targeting the N6-mA regulator ALKBH1 in patient-derived human glioblastoma models inhibited tumor cell proliferation and extended the survival of tumor-bearing mice, supporting this novel DNA modification as a potential therapeutic target for glioblastoma.

Furthermore, ALKBH1 controls the hypoxia responding genes in glioblastoma. Collectively, our results uncover a novel epigenetic node in cancer through the DNA modification N6-mA. The regulators of this new modification could serve as novel therapeutic targets in cancer therapy.

13. Evaluation of undiagnosed liver masses that do not exhibit typical imaging features, including even Stage C HCC

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Background and aim: Sometimes, despite all blood examinations, transabdominal ultrasound, and computerized tomography with or without magnetic resonance imaging by multidisciplinary approach, we cannot reach a strict diagnosis in patients with liver masses. In this study, we aimed to evaluate undiagnosed liver masses that do not exhibit typical imaging features.

Material and methods: In this study, we retrospectively evaluated 140 patients with undiagnosed liver mass(es) without any typical imaging features. Then, percutaneous liver biopsy by transabdominal ultrasound guiding was performed in 121 patients to obtain a liver specimen. A single gastroenterologist who has much experience with radiologic biopsies performed all biopsies in this study. This study included the years 2011-2013. A single experienced radiologist reevaluated images from the records in 2014.

Results: A pathologist evaluated 121 patients' liver specimens. The distribution of the diagnosis is as follows: 45 patients with metastasis, 24 patients with hepatocellular carcinoma (HCC), 16 patients with nothing, 8 patients with advanced stage chronic liver disease, 5 patients with neuroendocrine tumours (NET), 5 patients with dysplastic nodule or well-differentiated HCC, 4 patients with cholangiocarcinoma, 4 patients with pseudotumor (secondary to infections), 2 patients with steatosis, 2 patients with hemangioma, 1 patient with steatohepatitis, 1 patient with extramedullar hematopoiesis, 1 patient with necrotizing granuloma, 1 patient with biliary cirrhosis (sistozomiazis), 1 patient with cyst hydatid, and 1 patient with mixed tumor (HCC + cholangiocarcinoma). The radiologist reevaluated the radiologic records of 70 patients. The distribution of these patients is as follows: 27 patients with HCC, 11 patients with chronic liver disease findings without any mass, 12 patients with metastasis, 6 patients with cholangiocarcinoma, 3 patients with hemangioma, 5 patients with abscess (1 with fasciola and 1 with cyst hydatid), 2 patients without any liver abnormality, 1 patient with dysplastic nodule, 1 patient with angiomyolipoma, 1 patient with gallbladder tumor, and 1 patient with focal nodular hyperplasia. A further distribution of the 27 patients with HCC is shown in Table 1 according to the Barcelona Clinic Liver Cancer Staging System.

Discussion: Our results show that HCC, even with Stage C, is one of the major causes of the liver masses that do not exhibit typical imaging features. HCC due to none B and none C was also a significant portion in this group of patients. More than half of the patients with HCC had normal serum α -fetoprotein level even in HCC patients with Stage C. As expected, life expectancy was related to the stage of the disease.

14. Therapeutic effects of trehalose liposomes against tumors along with apoptosis

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Background and aim: Trehalose stabilizes membranes and proteins in cells most likely by hydrogen bonding. In this study, inhibitory effects of trehalose liposomes (DMTre) composed of *L*- α -dimyristoylphosphatidylcholine (DMPC) and trehalose micelles (TreC14) on the growth of tumors along with apoptosis were obtained *in vitro* and *in vivo*.

Experimental procedure: DMTre were prepared by the method of sonication of a mixture of DMPC and TreC14 in a buffer solution with no organic solvent^[1,2]. The thickness of fixed aqueous layer (TFAL) of DMTre was evaluated from the zeta potential by an electrophoretic light scattering measurement. The fusion and accumulation of DMTre in tumor cell membrane including a fluorescence probe was observed using confocal laser microscopy. Activation of caspases for tumor cells induced by DMTre was analyzed using a flow cytometer. Assessment of therapeutic effects of DMTre against xenograft mice and orthotopic graft bearing mice model of carcinoma was performed.

Results: Hydrodynamic diameter (d_{hy}) of DMTre composed of 30 mol% DMPC and 70 mol% TreC14 was 100 nm with single and narrow range of size distribution, which can avoid reticular endothelial system *in vivo*. An increase in TFAL values of DMTre was obtained in a dose-dependent manner. DMTre inhibited the growth of breast and lung tumor cells leading to apoptosis with the activation of caspases. The suppression of tumor weight of xenograft mice model of carcinoma treated with DMTre after inoculation with breast tumor cells was obtained along with apoptosis. A remarkable reduction of volume and weight in subcutaneous tumors on subcutaneous lung carcinoma-bearing mice administered with DMTre was obtained.

Conclusion: Anti-tumor activities of DMTre against carcinoma-bearing mice along with apoptosis were obtained. The results of this study could contribute to the development of therapeutic agents for patients with carcinoma in future clinical application.

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15. The effect of interactions between temozolomide and dexamethasone on the profile of 84 selected proteins in glioblastoma multiforme cells

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Background and aim: In patients with glioblastoma multiforme (GBM), standard chemotherapy with temozolomide (TMZ) is always supplemented with dexamethasone (DXM). Even though for years DXM has been applied as a “gold standard” in therapy of vasogenic edema, intracranial pressure, and mass effect, recent controversial results have challenged the widely accepted dogma concerning its using in therapy of GBM. The results of experimental studies emphasize that DXM may increase the aggressiveness of GBM by promoting the proliferation and invasiveness of cancer cells.

The aim of our study conducted on two primary glioblastoma lines obtained from patients and on the commercial line T98G was to assess the effects of TMZ and DXM, as well as their interaction, on the profile of 84 proteins involved in the process of carcinogenesis. The tests were performed using the Proteome Profiler Human XL Oncology Array Kit (R & D) in cells cultured under two oxygen conditions: physiological for tumor hypoxia (2.5% oxygen) or in standard laboratory conditions (20% oxygen) frequently used in *in vitro* studies.

Results: Our results confirm the pro-tumorigenic properties of DXM but they also show that the response of GBM commercial and primary cell lines to DXM given to culture medium with or without TMZ is variable and depends on oxidation of the microenvironment.

Conclusion: It can be concluded that DXM and TMZ administered together or separately may induce different effects which depend on the degree of hypoxia prevailing in the malignant brain tumor.

16. Sensitive detection of metabolic abnormalities in adult T-cell leukemia/lymphoma and induction of specific leukemic cell death using photodynamic therapy

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Adult T-cell leukemia/lymphoma (ATL) is an aggressive T-cell neoplasm caused by human T-cell leukemia virus type I (HTLV-I). Therapeutic interventions have not been associated with satisfactory outcomes. We showed that the porphyrin metabolic pathway preferentially accumulates the endogenous photosensitive metabolite, protoporphyrin IX (PpIX), in ATL, after a short-term culture with 5-aminolevulinic acid (ALA). PpIX accumulated 10-100-fold more in ATL leukemic cells when compared to healthy peripheral blood mononuclear cells (PBMCs). Patient specimens showed dynamic changes in flow cytometry profiles during the onset and progression of ATL. Furthermore, 98.7% of ATL leukemic cell death in the ATL patient specimens could be induced with 10 min of visible light exposure, while 77.5% of normal PBMCs survived. Metabolomics analyses revealed that a specific stage of the metabolic pathway progressively deteriorated with HTLV-I infection and at the onset of ATL. Therefore, this method will be useful for diagnosing and identifying high-risk HTLV-I carriers and high-risk indolent ATL patients who appeared to have developed or are likely to develop the aggressive subtypes with single cell resolution. Photodynamic therapy in the circulatory system may be a potential treatment due to its highly-specific, non-invasive, safe, simultaneous, and repeatedly-treatable modalities.

17. Preoperative localization of breast lesions: analysis of current techniques

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Image-guided preoperative localization of breast lesions is a relatively common procedure. This presentation describes the most common localization options available commercially - wire localization, radioactive seed localization, radiofrequency reflector localization, and magnetic seed localization - and outlines the advantages and disadvantages of each. This information may help radiologists, surgeons, pathologists, and hospital administration as they seek to add value and provide patient-centered care.

18. Innovative technologies for cancer diagnosis and management metal-organic framework encapsulation for biospecimen and biotherapeutic preservation

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Background and aim: Handling, transport, and storage of biospecimens such as blood and urine without refrigeration are extremely challenging. This formidable challenge leads to an inevitable reliance on a “cold chain” for shipping, handling, and storage of biospecimens throughout the world. The cold chain requirement impedes biospecimen procurement from under-served populations and resource-limited settings where refrigeration and electricity are not reliable or even available.

Experimental procedure: Here, we introduce a universal biospecimen preservation approach based on nanoporous material encapsulation for preserving protein biomarkers in biofluids under non-refrigerated storage conditions. We used urinary neutrophil gelatinase-associated lipocalin and plasma CA-125 as the model protein biomarkers and measured their concentrations before and after encapsulation by enzyme-linked immunosorbent assay (ELISA).

Results: We found that encapsulation in a zeolitic imidazolate framework-8 (ZIF-8), a nanoporous material, can preserve protein biomarkers in urine and plasma for weeks at room temperature and 40 °C. The preservation efficacy for ELISA assay was greater than 85%, comparable to freezing liquid samples at -20 °C. The protein biomarkers in the relevant biofluids were first encapsulated within the nanoporous ZIF-8 crystals, then dried on paper substrates via a dry spot sample collection method, and later reconstituted for analysis. This technology also preserves the biologic activity of insulin in liquid form for therapy.

Conclusion: This eco-friendly technology greatly improves biospecimen and biotherapeutic handling in resource-limited settings. The technology may be applicable to vaccine preservation, storage, and transport at ambient temperature. Overall, this environmentally-friendly and energy-efficient approach will alleviate huge financial and environmental burdens associated with “cold chain” facilities and extends biomedical research and treatment benefits to underserved populations from regions/populations currently inaccessible.

19. Microrna-335-5p as a suppressor of metastasis and invasion in gastric cancer

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Background and aim: Gastric cancer is the fifth most common cancer worldwide. It is mainly diagnosed via endoscopic examination, which is unsuitable for screening and patient follow-up. A deeper knowledge of its development and progression can contribute to discovering effective preventive strategies. To understand this complex process, we focus on microRNAs and exosomes and their metastatic and invasive potential on gastric cells.

Experimental procedure: We evaluated the expression of several candidate miRNAs in 38 gastric cancer tissues and 22 plasma samples from gastric cancer patients and compared them to adjacent not-tumor tissues and plasma from symptomatic patients without cancer, respectively. We performed an association analysis of the expression of microRNA-335-5p with clinicopathological features and survival curves. For *in vivo* study, we injected intravenously microRNA-335-5p-loaded exosomes into immunodeficient mice with intraperitoneal tumors.

Results: MicroRNA-335-5p is downregulated in advanced gastric cancer (GC) tissues relative to their paired non-tumor tissues. This downregulation is associated with worse survival rates of patients. We also demonstrated decreased levels of microRNA-335-5p in total plasma and exosomes isolated from plasma samples from GC patients, when compared to symptomatic patients without cancer. In our *in vivo* model of intraperitoneal carcinogenesis, we observed less metastasis but more necrosis in organs of mice with microRNA-335-5p-loaded exosomes and all mice lacked ascites.

Conclusion: MicroRNA-335-5p is downregulated in both types of gastric cancer samples. The difference in expression of this microRNA in plasma of gastric cancer patients versus patients without cancer is so profound that it can be considered as a possible candidate for non-invasive diagnosis of gastric cancer and the *in vivo* results may suggest a therapeutic role for miRNA-335.

20. RANBP9 as potential target in non-small cell lung cancer

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Non-Small Cell Lung Cancer (NSCLC) is by far the number one cause of cancer related death in the Western world. Despite the progress made with targeted therapies and immuno-checkpoint inhibitors, the vast majority of patients still undergo treatment with genotoxic drugs such as platinum-based compounds. Studies testing whether DNA damaging agents sensitize NSCLC tumors to targeted- or immuno-therapies are ongoing. However, only a better understanding of the mechanisms of the DNA damage response can lead to the validation of biomarkers predictive of response to genotoxic agents and the discovery of novel targets.

We found that overexpression of the scaffold protein Ran Binding Protein 9 (RANBP9) is pervasive in NSCLC. Most importantly, patients with higher levels of RANBP9 have a worst treatment outcome (Tessari *et al.*^[1], 2018). Mechanistically, RANBP9 is not only a target (Matsuoka *et al.*^[2], 2007) but also,

surprisingly, an enhancer of the Ataxia telangiectasia mutated (ATM) kinase signaling (Palmieri *et al.*^[3], 2016). Indeed, the depletion of RANBP9 in NSCLC cells abates ATM activation and its downstream targets such as p53. Predictably, RANBP9 KO cells are more sensitive than controls to inhibition of the ataxia and telangiectasia-related kinase, but not ATM. Interestingly, the absence of RANBP9 renders cells more sensitive to drugs inhibiting Poly (ADP-ribose)-Polymerase (PARP) (Tessari *et al.*^[1], 2018).

We present results of our *in vitro* and *in vivo* investigation aimed at revealing the mechanisms responsible for increased sensitivity to specific genotoxic drugs when RANBP9 is absent. For this purpose, we generated human NSCLC cell lines and new mouse models of NSCLC in which endogenous RANBP9 can be specifically ablated in cancer cells or, alternatively, tagged with V5-HA for its unequivocal detection. Tumors of this latter group will enable proteomic studies to identify unknown RANBP9 interactions upon DNA damaging treatment.

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21. One carbon metabolic enzymes play important roles for cancer cells and cancer stem-like cells

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Background and aim: Emerging evidence suggests that cancer stem-like cells (CSCs) are responsible for drug-resistant tumor recurrence^[1-6]. The one-carbon (1C) metabolism incorporates carbons as building blocks of purine and pyrimidine that are used for DNA replication and RNA transcription. In the mitochondria, there are four major enzymes in 1C metabolism. These enzymes are strongly expressed in cancer cells, while it is scarcely expressed in normal cells. We investigated the role of MTHFD2 and MTHFD1L among them in cancer cells and CSCs.

Experimental procedure: We depleted expression of MTHFD2 and MTHFD1L in lung cancer cells and breast cancer cells by using small interfering RNA (siRNAs) or small hairpin RNA (shRNAs). By using these cells, we examined cell proliferation and sphere-forming ability *in vitro* and *in vivo*. We also examined expression levels of stemness markers.

Results: We showed that MTHFD2 and MTHFD1L play important roles for cancer cell proliferation, stem-like properties, and drug resistance. Knockdown of *MTHFD2* led to accumulation of 5-aminoimidazole carboxamide ribonucleotide (AICAR), an intermediate of the purine synthesis pathway, in association with reduced stem-like properties.

Conclusion: MTHFD2- or MTHFD1L-mediated mitochondrial 1C metabolism appears critical for survival of CSCs through consumption of AICAR, leading to depletion of the intracellular pool of AICAR. Because CSCs are dependent on MTHFD2 and MTHFD1L, therapies targeting MTHFD2 may eradicate tumors.

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22. Differential roles of the redox-sensitive transcription factor, Nrf2 in multistage carcinogenesis

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Background and aim: Nuclear factor E2-related factor 2 (Nrf2) is a redox-sensitive transcription factor regulating the expression of a battery of genes encoding antioxidant and carcinogen detoxifying enzymes. In contrast to its tumor suppressive functions in normal cells, Nrf2 facilitates tumor growth and progression through metabolic reprogramming in some cancer cells. Our previous study has demonstrated that 15-deoxy- $\Delta^{12,14}$ -prostaglandin J_2 and 4-hydroxyestradiol induce overactivation of Nrf2 and consequently overexpression of its target protein, heme oxygenase-1 (HO-1), in human breast cancer cells.

Experimental procedure: In this study, we investigated the involvement of Nrf2 in experimentally induced hepatocarcinogenesis by utilizing Nrf2 null mice as well as wild type animals. The liver tumor was induced by intraperitoneal injection of diethylnitrosamine (DEN). The expression of Nrf2 and its target genes and proteins were measured by RT-PCR and Western blot analyses. The cell proliferation was determined by immunohistochemical analysis of Proliferating Cell Nuclear Antigen expression.

Results: Nrf2 expression, nuclear translocation, and transcriptional activity were enhanced in liver tumors. Overactivated Nrf2 was required for hepatoma growth in DEN-induced HCC. Following DEN treatment, Nrf2 genetic disruption reduced expression of pentose phosphate pathway-related enzymes, the depletion of which has been associated with an amelioration of HCC incidence. Nrf2-deficient mice resisted DEN-induced hepatocarcinogenesis.

Conclusion: The cellular stress response or cytoprotective signaling mediated via the Nrf2 is often hijacked by cancer cells. This may facilitate the remodeling of the tumor microenvironment, making it advantageous for the autonomic growth of cancer cells, metastasis, angiogenesis, tolerance to anticancer therapy, and self-renewal activity of stem-like cells. Notably, Nrf2 overactivation upregulates antioxidant gene expression in breast cancer stem cells, which contributes to the manifestation and maintenance of stemness.

23. The importance of sequential mutations in pancreatic tumorigenesis

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Background and aim: Genetically engineered animal models (GEMMs) are established robust platforms for exploring the molecular mechanisms underlying the progression of pancreatic precancerous lesions to invasive PDA (pancreatic ductal adenocarcinoma). For example, using Pdx1-Cre to activate mutant *Kras*^{G12D} allele in the pancreas induces full spectrum of premalignant PanIN (pancreatic intraepithelial neoplasias) lesions that can eventually progress to invasive PDA (reviewed in^[1]). We and others have reported that concomitant inactivation of the tumor suppressors *p16*, *p19*, *p53*, or TGF- β receptor type 2 (*TgfbR2*) can synergize with oncogenic *Kras*^{G12D} in promoting the progression of the non-invasive PanINs to invasive cancer *in vivo*^[1,2]. In contrast, the inactivation of *Smad4* or *Acvr1b* in the context of mutant *Kras*^{G12D} preferentially promotes the development of pancreatic IPMNs (intraductal papillary mucinous neoplasms) but not PanINs^[1,3]. Collectively, these data suggest that the order in which tumor-suppressor genes are inactivated may influence the development of pancreatic tumor subtypes. To further investigate the importance of sequential mutations in pancreatic tumorigenesis, we generated double heterozygous *Smad4*^{fllox/+}; *p16*^{+/-}; *LSL-KRAS G12D*; *Pdx1-Cre* GEMM and asked how spontaneous inactivation of the second allele might impact the development pancreatic precancerous lesions.

Experimental procedure: *Smad4*^{fllox/+}; *p16*^{+/-}; *LSL-KRAS G12D*; *Pdx1-Cre* mice were examined and characterized in comparison to *p16*^{+/-}; *LSL-KRAS G12D*; *Pdx1-Cre* and *Smad4*^{fllox/+}; *p16*^{+/-}; *Pdx1-Cre* GEMMs.

Results: *Smad4*^{fllox/+}; *p16*^{+/-}; *LSL-KRAS G12D*; *Pdx1-Cre* mice shared similar medium survival and tumor progression to *p16*^{+/-}; *LSL-KRAS G12D*; *Pdx1-Cre* mice (PanIN to PDA). Molecular analyses showed that biallelic inactivation only occurred at the *p16* locus in the PanINs and PDA from *Smad4*^{fllox/+}; *p16*^{+/-}; *LSL-KRAS G12D*; *Pdx1-Cre* GEMM.

Conclusion: Our results support the previous observations that the sequential inactivation of tumor-suppressor genes in the context of oncogenic *Kras*^{G12D} can dictate the development of pancreatic precancerous lesions. More importantly, the sequential mutations observed in mice mirror those detected in human patient specimens and thus illustrating that the order of genetic mutations is as critical as the mutated genes themselves in influencing tumor development and progression.

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24. Cyr61 promotes tip cell activity through VEGFR2-Hippo pathway in tumor angiogenesis

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Cyr61 stimulates active angiogenesis in various tumors, although the mechanism is unknown. Here, we report that Cyr61 enhances the activity of tip cells during angiogenesis by regulating VEGFR2-Hippo pathway. Microvessel networks and directional vascular cell migration patterns were deformed in Cyr61-knockdown zebrafish embryos. Moreover, Cyr61 promoted the endothelial sprouting activity in angiogenesis. Cyr61 induced the interaction of integrin $\alpha v \beta 3$ with VEGFR2, which activated downstream MAPK/PI3K signaling pathways, YAP/TAZ, and Rho effector mDia1 to enhance tip cell activity and Cyr61 itself. Integrin $\alpha v \beta 3$ inhibitor repressed tip cell number and sprouting in postnatal retinas from endothelial cell-specific Cyr61 transgenic mice (*VE-Cadherin:Cyr61*), and allograft tumors in Cyr61 transgenic mice also showed hyperactive vascular sprouting. Cancer patients with high Cyr61 expression have poor survival outcomes and positive correlation with *integrin $\alpha v \beta 3$* and high YAP/TAZ. Thus, our data underscore the positive feedback regulation of tip cells by Cyr61 through integrin $\alpha v \beta 3$ /VEGFR2 and YAP/TAZ activity, suggesting a promising therapeutic intervention for pathological angiogenesis.

25. Silibinin targets bone morphogenic protein 2 in its efficacy against ultraviolet B radiation-induced promotion/progression of microscopic basal cell carcinoma formation

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Background and aim: Non-melanoma skin cancers (NMSCs) account for about half of all malignancies diagnosed annually in the United States. Around 80% of NMSCs are basal cell carcinoma (BCC) and 20% are squamous cell carcinoma (SCC). Whereas the efficacy of several chemopreventive agents has been examined and reported against both BCC and SCC, a majority of these studies have focused on the test agent's activity in a long-term setting to determine the number of tumors formed. Notably, the studies evaluating the efficacy of chemopreventive agents during early stage(s) of BCC development are lacking. Accordingly, utilizing the well-established patched (Ptch)+/- mouse model of ultraviolet b (UVB)-induced BCC formation, we excised skin samples from UVB exposed mice prior to tumor formation to study the promotion/progression of BCC and to determine the target(s) of silibinin, a well-known skin cancer (SCC) chemopreventive agent, in BCC tumor growth inhibition.

Experimental procedure: We used a multifactor approach: (1) long-term ultraviolet B radiation-induced mouse skin tumorigenesis in Ptch heterozygous mice focusing on BCC; (2) investigating and quantifying expression of molecular regulators and cyclobutane pyrimidine dimers by immunohistochemistry and/or immunoblotting; and (3) real-time PCR with mouse signal transduction pathway finder PCR array.

Results: At as early as one month, we found that UVB exposure significantly increased the number of mast cells in Ptch+/- mice by about 48% ($P < 0.05$), which was completely inhibited (to control levels) by silibinin topical treatments. In Ptch+/+ mice, which do not develop BCC tumors, we did not observe any increase in mast cells following UVB exposure, suggesting this could be a specific pathway in the development of BCC. To decipher the molecular mechanism of these findings, we performed a PCR profiler array analysis of several genes involved in signal transduction pathways which showed strong differences between Ptch+/+ and Ptch+/- mice that were unexposed, UVB irradiated, and silibinin treated. Most notably, following UVB exposure for one month, in Ptch+/- mice, the expression of Bone Morphogenetic Protein 2 (BMP-2), Hairy/enhancer-of-split related with YRPW motif 1 (Hey1), and Inhibitor of DNA binding 1 (Id1) was significantly upregulated when compared to Ptch+/+ mice. Additional studies focusing on BMP-2 found that silibinin strongly inhibits UVB-induced expression of BMP-2 in Ptch+/- mouse

skin. Consistent with these results, we also found that silibinin strongly attenuates UVB-induced BMP-2 expression and DNA damage in Ptch+/- mouse skin *ex vivo*. Regarding BCC formation, silibinin treatment inhibited UVB-induced microscopic BCC formation in Ptch+/- mice; microscopic tumor number and size were reduced by 73% and 84%, respectively. Together, our results suggest a possible role of BMP-2 in early stages of BCC development and that silibinin plausibly acts through BMP-2 to inhibit microscopic BCC formation.

Conclusion: Our current findings in BCC model, together with previous studies in SCC model, suggest that silibinin could be a multi-target agent capable of being a chemopreventive agent for both types of NMSCs.

26. A novel sulforaphane-regulated gene network in prevention of breast cancer-induced osteolytic bone resorption

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Bone is the most preferred site for colonization of metastatic breast cancer cells for each subtype of the disease. The standard of therapeutic care for breast cancer patients with bone metastasis include bisphosphonates (e.g., zoledronic acid), which have poor oral bioavailability, and a humanized antibody (denosumab). However, these therapies are palliative and a subset of patients still develop new bone lesions and/or experience serious adverse effects. Therefore, a safe and orally bioavailable intervention for prevention/therapy of osteolytic bone resorption is still a clinically unmet need. This study demonstrates prevention of breast cancer-induced bone resorption by a small molecule (sulforaphane, SFN) that is safe clinically and orally bioavailable. *in vitro* osteoclast differentiation was inhibited in a dose-dependent manner upon addition of conditioned media from SFN-treated breast cancer cells representative of different subtypes. Targeted microarray coupled with interrogation of TCGA dataset revealed a novel SFN-regulated gene signature involving cross-regulation of runt-related transcription factor 2 (RUNX2) and nuclear factor- κ B and their downstream effectors. Both RUNX2 and p65/p50 expression were higher in human breast cancer tissues compared to normal mammary tissue. RUNX2 was recruited at the promotor of *NFKB1*. Inhibition of osteoclast differentiation by SFN was augmented by doxycycline-inducible stable knockdown of RUNX2. Oral SFN administration significantly increased the percentage of bone volume/total volume of affected bones in the intracardiac MDA-MB-231-Luc model, indicating *in vivo* suppression of osteolytic bone resorption by SFN. These results indicate that SFN is a novel inhibitor of breast cancer-induced osteolytic bone resorption *in vitro* and *in vivo*. These findings necessitate clinical investigations to determine the effect of SFN administration on osteolytic bone resorption in women with metastatic breast cancer. This study was supported by grant CA225716 awarded by the National Cancer Institute.

27. Targeting of TM4SF5-mediated regulation of metabolic functions to overcome hepatic cancer

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Background and aim: Liver is an organ that can metabolize diverse nutrients and its cancers show arginine auxotroph, which involves arginine delivery from extracellular diet sources and lysosomal protein

degradation products. Among the genes involved in hepatic cancer, transmembrane 4 L six family member 5 (TM4SF5), which is structurally similar to the tetraspanins with 4 transmembrane domains, is shown to be highly expressed in cancerous liver tissues and correlated with many hepatic metabolism genes. However, it is unknown whether and how TM4SF5 is involved in arginine metabolism in livers.

Experimental procedure: We examined whether TM4SF5 expression can be involved in the mTORC1 signaling pathway, since mTORC1 signaling is a central hub of various cellular metabolism processes.

Results: First, we found shuttling of TM4SF5 between plasma membrane and lysosomal (late endosomal) membrane, depending on availability of amino acids. Further, upon resupply of arginine to arginine-starved cells, lysosomal TM4SF5 was associated with mTOR, leading to an increased S6K phosphorylation. The association between TM4SF5 and mammalian target of rapamycin (mTOR) appeared to require the C-terminal regions of TM4SF5 and kinase activity of mTOR. In addition, an endosomal arginine transporter (SLC38A9) and a cytosolic arginine sensor (Castor1) were found to be associated with TM4SF5, indicating TM4SF5 as an arginine sensor on late endosomal (lysosomal) membrane. Interestingly, the association of Castor1 with TM4SF5 was negatively regulated by L-arginine, but concomitantly the association between mTOR and TM4SF5 increased. Furthermore, certain residues in the extracellular loop 2 of TM4SF5 bound to arginine. Thus, association of TM4SF5 with mTOR, SLC38A9, and arginine on lysosomal membrane might allow TM4SF5 to propagate arginine response to mTORC1 by directly sensing arginine in the lysosome as well as to elevate the cytosolic arginine pool for cellular homeostasis.

Conclusion: Therefore, these observations suggest TM4SF5 as an arginine sensor on late endosomal membrane and as a promising therapeutic target candidate for the arginine auxotrophy of hepatic cancers.

28. Targeting cancer stem cells in malignant mesothelioma

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Background and aim: Mesothelioma is an aggressive, treatment resistant, and fatal cancer of the mesothelial lining of the pleural and peritoneal cavities that is initiated by exposure to asbestos or nanotubes. Surgical reduction and chemotherapy are first line treatments, but recurrence of highly aggressive and drug-resistant disease is common. Disease recurrence is associated with expansion of mesothelioma cancer stem cells (MCS cells). Thus, new treatments are needed for this disease that target the cancer stem cell population.

Experimental procedure: We used genetic gene expression and knockdown approaches, signaling studies, xenograft tumor studies, transcriptome analysis, and protein structure studies to characterize the role of the transglutaminase 2 (TG2) cancer stem cell survival factor in enhancing MSC cell survival and function.

Results: We showed that tissue transglutaminase (TG2), a cancer stem cell survival and drug-resistance protein, is highly enriched in human mesothelioma tumors and mesothelioma cancer stem cells (MCS cells) and drives MCS cell spheroid formation, invasion, and migration. TG2 knockdown or TG2 inhibitor treatment reduces MCS cell survival, spheroid formation, Matrigel invasion, migration, and tumor formation. These are important observations as MCS cells comprise a highly aggressive subpopulation of tumor that forms rapidly growing and aggressive tumors. In addition, transcriptome analysis reveals that TG2 loss is associated with reduced levels of mRNA encoding a wide range of cancer stem cell and epithelial-mesenchymal transition proteins, and that TG2 knockdown reduces expression of transcripts and proteins encoding pro-cancer matrix proteins including collagens COL1A2 and COL3A1 that are involved in metastasis. Mesothelin, a mesothelioma cell-specific MCS cell survival protein and attachment factor, is also reduced in TG2 knockdown cells.

Conclusions: These studies indicate that TG2 is highly overexpressed in MCS cells and drives the cancer stem cell phenotype to enhance MCS cell stemness, survival, and invasion, and suggests that TG2 is an important candidate mesothelioma cancer stem cell therapy target.

29. Forward genetics to discover tumor suppressor in colorectal cancer

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The nuclear factor κ B (NF- κ B) plays pivotal roles in inflammatory and immune responses and in cancer. Therefore, understanding its regulation holds great promise for disease therapy. Using validation-based insertional mutagenesis, a powerful technique established by us, we discovered a novel negative regulator of NF- κ B (named NNRN1) in colorectal cancer (CRC). We showed that NNRN1 overexpression downregulated the expression of NF- κ B-dependent genes, many of which are related to cancer. Additionally, compared to the vector control group, overexpression of NNRN1 in HEK293 cells or CRC HT29, DLD1, and HCT116 cells dramatically reduced NF- κ B activity, cellular proliferation, anchorage-independent growth, and migratory ability *in vitro*, and, unsurprisingly, significantly decreased xenograft tumor growth *in vivo*. In contrast, shNNRN1 knockdown cells showed the opposite effect. Furthermore, co-immunoprecipitation (Co-IP) experiment confirmed that NNRN1 may form a complex with the p65 subunit of NF- κ B. Importantly, immunohistochemistry data exhibited much lower NNRN1 expression level in CRC patient tumor tissues compared to normal tissues, indicating that NNRN1 may function as a tumor suppressor in CRC. To conclude, our findings for the first time uncovered the negative regulatory function of NNRN1 in NF- κ B signaling, and present NNRN1 as an innovative therapeutic target in CRC treatment.

30. Immunogenic cell death in myeloid leukemia

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Background and aim: We investigated the effect of pharmacologically active compounds that act as immunoadjuvants able to trigger a cancer stress response and release of damage-associated molecular patterns (DAMPs) in myeloid leukemia^[1]. These processes result in a chemotherapeutic response with

a potent immune-mediating reaction. Several parameters determine whether a compound can act as an immunogenic cell death (ICD) inducer including the nature of the inducer, the premortem stress pathways, the cell death pathways, the intrinsic antigenicity of the cell, and the potency and availability of an immune cell response^[2].

Experimental procedure: We used a multifactor approach: (1) detecting endoplasmic reticulum (ER) stress markers; (2) investigating and quantifying caspase-dependent or independent cell death; (3) measuring the release of danger associated molecular patterns; (4) quantifying phagocytosis of compound-treated cells by both murine and human monocyte-derived macrophages; (5) performing colony formation assays and *in vivo* zebrafish xenografts; and (6) performing vaccination assays with immunocompetent mice.

Results: We identified ICD-inducing capacities of old (coumarinics) and novel (stemphol and cardiac glycoside UNBS1450) inducers of immunogenic cell death together with venetoclax and experimental BH3 mimetics. We detected their capacity to trigger synergistic cell death in myeloid leukemia in an attempt to overcome apoptosis-resistant myeloid leukemia alone or in combination with other chemotherapeutic compounds.

Conclusion: The identification of hallmarks of ICD is important in determining the prognostic biomarkers for new therapeutic approaches and combination treatments^[3]. In myeloid leukemia, combination treatments of ICD-inducing pharmacological agents^[4] with Venetoclax showed positive synergistic effects^[5] allowing to confer immunogenicity to otherwise cytotoxic non-immunogenic treatments.

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31. The role of autophagy in inflammatory cytokines-induced epithelial to mesenchymal transition in cancer

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The peculiar hallmark distinguishing malignant from benign tumors is the ability of the former to invade the extracellular matrix and metastasize to near and distant organs. This process implies an epigenetic change in the expression of genes that leads to a reversible phenotypic change of the cancer cells from epithelial-like to mesenchymal-type known as epithelial-to-mesenchymal transition (EMT). The tumor microenvironment plays a pivotal role in this process, the major players being the pro-inflammatory cytokines IL-6 and IL-8 released by cancer associated fibroblasts, immune cells (M2 macrophages), and cancer cells themselves.

Autophagy, a lysosome-driven catabolic process for degradation of self-constituents, participates in the stress response for maintaining cell homeostasis. It has been shown that autophagy is down-regulated during cell locomotion, while it is induced when cells arrest their migration.

We found that pro-inflammatory cytokines promote cancer cell migration following down-regulation of autophagy in the migratory cells. We also investigated at the molecular level the mechanisms through which the cytokines modulate autophagy.

Our data highlight the role of autophagy in cancer cell EMT and migration, offering opportunities for therapeutic interventions to prevent invasion and metastasis.

32. Vasculogenic mimicry in glioblastoma and melanoma

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Background and aim: Neo-angiogenesis is the most studied mechanism of vascularization in tumors and refers to sprouting of new blood vessels from pre-existing ones and it may be inhibited by natural compounds. Vasculogenic mimicry (VM) is an alternative mechanisms of tumor vascularization providing a means by which some tumors can escape anti-angiogenetic therapy. VM occurs in glioblastoma (GBM) and melanoma, both tumors of neuroepithelial derivation. We investigated the role of *REST/NRSF* gene in the pathophysiology of VM as well as the effects of compounds such as the histone deacetylase inhibitors (HDACis) to interfere with VM.

Experimental procedure: To measure tube formation, cell migration, and invasion, we used: *in vitro* tube formation assay on Matrigel, Boyden chamber migration assay, wound healing assay, invasion test, and real-time migration monitoring. To measure cell viability, we used: MTT and trypan blue exclusion test. To transfect cells, we used: lipofectamine standard protocol.

Results: We analyzed a number of GBM and melanoma cell lines. We found that the expression of *REST* parallels the ability to migrate and to form tubes on Matrigel. Upon genetic or chemical down-regulation of *REST* (via siRNA or dominant negative mutant or HDACi), we observed a decrease in migration ability as well as tube formation. Finally, we found that different histone deacetylase inhibitors impair vasculogenic mimicry from glioblastoma cells.

Conclusions: Our findings show that *REST/NRSF* gene is an important molecular player in the pathophysiology of vasculogenic mimicry in GBM and melanoma and that HDAC inhibitors alone can impair the formation of tubes from GBM cells.

33. Mouse tumor susceptibility alleles identify pathways for intervention in multiple myeloma

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Background and aim: Multiple Myeloma (MM) is a clonal proliferation of neoplastic plasma cells in the bone marrow. Despite recent therapeutic advances, drug resistance and MM progression is common. Mouse plasma cell tumors model these antibody producing neoplasms. Long-term genetic studies utilizing backcross, and congenic strain analyses coupled with positional cloning strategies and functional studies identified *Cdkn2a*, *Mtor*, and *Mndal* as plasmacytoma susceptibility genes. Tumor incidence data in congenic strains carrying resistance alleles of *Cdkn2a* and *Mtor* led us to hypothesize that drug combinations affecting these pathways are likely to have an additive, if not synergistic, effect in inhibiting tumor cell growth.

Experimental procedure: Drug combination [mTOR and histone deacetylases (HDAC) inhibitors] activity and synergy were measured in B cell neoplasms and NCI-60 cell lines. *in vivo* activity was assessed in xenograft experiments. Co-expression network analyses of microarray data from *in vitro* drug treatment delineated the cooperative mTORi/HDACi transcriptional response. Selectivity of the response for genes differentially regulated in MM was determined by GSEA of datasets from healthy controls and MM patients. The combination's potential clinical utility was evaluated by developing a multivariate survival prediction model from the response signature in a MM patient dataset. Functional enrichment and transcription factor activity testing of the response signature delineated the combination's biological activities.

Results: The combination was active and synergistic in 90% of cell lines and controlled *in vivo* tumor growth for 12 weeks. Combination response signature genes were correlated with improved survival and the signature was functionally enriched for cell cycle, apoptosis, antigen presentation, and DNA damage response. The combination is predicted to repress oncogenic factors and activate tumor suppressors (RB1 and CDKN2A).

Conclusion: The traditional and novel systems-level genomic approaches used to assess combination activity, disease specificity, and clinical potential demonstrate the efficacy of combined mTORi/HDACi, and warrant further investigation in clinical trials.

34. tRNA-derived fragment AS-tDR-007333 promotes cell proliferation in NSCLC through interacting with HSPB-1

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Background: tRNA derived-fragments (tRFs) comprise a new class of non-coding small-molecule RNA. Recent studies suggest that tRFs are involved in the development and progress of several cancers. However, the impact of tRFs in non-small cell lung cancer (NSCLC) remains elusive.

Methods: NSCLC-related tRFs were determined by RNA-seq. Expression of tRFs in tumor tissues, plasma, and NSCLC cell lines was analyzed with qRT-PCR. The effect of tRFs on NSCLC malignancy was evaluated *in vitro* by loss- and gain-of-function assays. RNA-seq was conducted to screen for the target genes of tRFs. The mechanism of action of tRFs was explored with RNA pulldown, RNA immunoprecipitation (RIP), and qRT-PCR.

Results: RNA-seq identified seven differentially expressed tRFs between pre-and postoperative plasmas in patients with NSCLC. Among them, the expression of a novel tRF termed AS-tDR-007333 was significantly upregulated in preoperative plasma, NSCLC tissues, and NSCLC cell lines. Overexpression of AS-tDR-007333 promoted NSCLC cell (PC9, HCC827, and A549) proliferation, while knockdown of AS-tDR-007333 inhibited cell growth. RNA-seq showed that up-regulation of AS-tDR-007333 led to the activation of oncogenes such as *MED29*, *AL049829.1*, *SCHIP1*, *SAMD12*, *MRFAP1*, and *SHISA5*. RNA pulldown and RIP analyses revealed that AS-tDR-007333 can bind directly with heat shock protein beta-1 (HSPB-1). Rescue assays demonstrated that HSPB1 was involved in AS-tDR-007333 mediated NSCLC cell proliferation.

Conclusion: Our study reveals an oncogenic role of AS-tDR-007333 in NSCLC, suggesting that it may be a novel target for diagnosis and the treatment of NSCLC.

35. Breast tumor-on-chip

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Background and aim: Breast cancer is the most common invasive cancer among women. There are several chemotherapeutic and radiotherapeutic approaches available but they have certain limitations. Over the past few years, improved understanding of the microenvironment heterogeneity of breast cancer has allowed the development of more effective treatment strategies. However, researchers have still not been able to recapitulate the entire tumor microenvironment to study tumor progression and invasion. In this way, more complex 3D *in vitro* cancer models have been developed. These 3D tumor models still lack the cell-cell and cell-tissue interactions and more balanced interstitial fluidic flow that are present within living systems. Furthermore, mimicking different physiological conditions and collecting samples from tumor microenvironment are also difficult. In this direction, the breast tumor-on-chip model has emerged as an alternative system to study the tumor microenvironment and decipher its role in metastasis. In this work, a microfluidics system was integrated into a 3D breast tumor to bridge the gap between 2D and animal model effectively and evaluate the efficacy of anti-cancerous drugs. These microfluidic systems contain small chambers for cell culture, which enable control over local gradients and the ability to maintain the interstitial fluidic flow of the local breast tumor microenvironment.

Experimental procedure: In this work, the multi-compartment microfluidics platform was generated by designing a specific PDMS chip with three channels that are separated by specific barriers (50 μm). The cancerous and fibroblast cells (cocultures) were suspended with collagen hydrogel and loaded into the central channel and one of the side channels was used to grow the endothelial cells to make this system vascularized. The barriers inside the chips allowed exchanging signaling molecules.

Results: The cancer cells in the presence of fibroblast cells grew well in these microchannels, which was confirmed with live/dead assay. At this stage, we have obtained the preliminary data and are still working in this area.

Conclusion: Integration of microfluidics system into breast tumor will add another toolset that can make a more efficient testing platform for the current therapeutic development pipeline.

36. Giant mediastinal mixed germ cell tumor, a rare case report and review of literature

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Introduction: Germ cell tumors are relatively rare, embryologically-derived from reproductive cells, and usually arise in the gonads. Mediastinal germ cell tumors are estimated to be about 1%-3% of all germ cell tumors and are generally seen in the anterior mediastinum, while metastatic lesions are mostly seen in the posterior mediastinum. The most aggressive germ cell tumor subtypes are choriocarcinoma, embryonal carcinoma, and yolk-sac tumors. Seminomas only rarely spread distantly. The presentations vary, ranging from accidental findings on routine radiography to life-threatening respiratory and cardiovascular compromise, which can also present as gigantic intrathoracic germ cell tumors, as in our case.

Case report: A 30-year-old male patient, not known to have any chronic illness, was referred from TB hospital center due to history of dyspnea, cough, and loss of appetite with weight loss for more than four months and no history of chest pain or hemoptysis. Chest X-ray was performed and showed complete obliteration of the right side of thorax; pleural effusion was suspected, and pleural TB and empyema were diagnosed. He was started on a tuberculosis drugs, antibiotics, and received chest drain with a slightly bloody fluid. Patient did not improve and was referred to our hospital. Computed hospital of chest with contrast revealed a very large mass obliterating the right side of chest, pushing the trachea and mediastinum to the left side with minimal effusion on both sides. Pleural US revealed mass and effusion but no empyema. Differential diagnosis was mediastina mass, adenocarcinoma, thymic carcinoma, lymphomas, fibroma, or fibrosarcoma. US guided transthoracic fine needle biopsy from the right-side mass revealed mixed germ cell tumor. The patient's condition had rapidly deteriorated prior to confirming the diagnosis or starting with treatments and died because of difficult airway breathing due to deviated and compressed airway and possible pneumothorax after transthoracic biopsy.

Conclusion: Germ cell tumors are aggressive and rapidly growing cancers. The literature reports the nature of the extragonadal mediastinal germ cell tumor as appearing as a giant mass occluding the whole lung, compressing the great vessels, and adhering to the chest wall, pericardium, and lung, as in our case. This leads to a worse prognosis. The estimated event-free survival at 10 years after combined treatment is 80.4%. Chemotherapy, debulking, and pneumectomy are the treatments for such cases.

37. MGMT, BRCA1 and MEG3 methylation status in triple-negative breast cancer

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Background and aim: Breast cancer is one of the most common cancers in women worldwide. The most severe type of breast cancer is triple-negative breast cancer (TNBC) due to unfavorable clinical course

and poor prognosis. The development of cancer is often associated with dysregulation of epigenetic mechanisms, including DNA methylation. The aim of our study was to evaluate *MGMT* (O6-methylguanine DNA methyltransferase), *BRCA1* (breast cancer 1), and *MEG3* (maternally expressed 3) methylation in TNBC.

Experimental procedure: In this study, 44 TNBC patients were included. The methylation status of the *MGMT*, *BRCA1*, and *MEG3* promoter regions were analyzed by methylation-specific PCR.

Results: *MGMT*, *BRCA1*, and *MEG3* promoter methylation was found in 70.4%, 61.3%, and 61.3% of TNBC patients, respectively. Moreover, we showed that the frequency of *MGMT* and *BRCA1* methylation is higher in older patients compared to younger patients (P -value for *MGMT* is $P = 0.0194$ and for *BRCA1* is $P = 0.0188$). Additionally, in one of TNBC patient with glandular and squamous histopathological components, it was shown that the promoter status of all analyzed genes changed from methylated to unmethylated after chemotherapy of this patient.

Conclusion: The high frequency of *MGMT*, *BRCA1*, and *MEG3* methylation indicates that epigenetic changes are important mechanisms in breast cancer. Moreover, our results indicate that *MGMT* and *BRCA1* methylation may have greater impact in the development of breast cancer in older patients compared to younger patients.

38. Sphingosine kinase 2 in oral squamous cell carcinoma

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Background and aim: Sphingosine kinase 2 (SK2) is one of the enzymes responsible for producing sphingosine-1-phosphate (S1P)^[1]. Recently, SK2 has been associated with protective autophagy and survival, and regulation of p21 in breast and colon cancer cells^[2,3]. However, the role of SK2 in oral squamous cell carcinoma (OSCC) is still unclear. Thus, our study aimed to investigate the involvement of SK2 in autophagy and proliferation in OSCC cells.

Experimental procedure: HN13 and HN12 (OSCC) cell lines were transduced with short hairpin RNA interference against SK2 and a lentiviral vector containing cDNA for SK2, respectively. Cell cycle analyses were performed by propidium iodide staining and flow cytometry. Western blotting and immunofluorescence assays were adopted to analyze protein levels and cellular distribution.

Results: HN13 cells with SK2 knockdown showed a decrease of pAkt, c-MYC, and LC3 levels (an autophagy marker) while p21 was increased. SK2 knockdown in HN13 cells caused cell arrest in S phase with reduction of the cells in G2/M. SK2 overexpression in HN12 cells led to an increase of pAkt, c-Myc, and LC3 levels.

Conclusion: Our work is the first to demonstrate the role of SK2 in proliferation and autophagy in OSCC cells. Other studies are in progress to understand the molecular mechanism underlying the role of SK2 and its potential as a target.

Financial support: FAPESP (grant: 2016/19103-2; scholarship: 2018/14225-8, CAPES, CNPq- Brazil).

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39. DNA methylation markers for noninvasive detection of early stage colorectal cancer

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Background and aim: Colorectal cancer (CRC) is the most common cancer in Singapore. Earlier detection enhances chances of a cure and facilitates reducing CRC mortality rates. Colonoscopy is currently the gold standard for CRC diagnosis, but a somewhat troublesome and invasive procedure makes its acceptance not high in the general public as a screening tool. Epigenetic silencing of tumor-related genes by promoter methylation is common in CRC, but no biomarker has been proven to be individually of sufficient sensitivity or specificity in routine clinical practice. The aim of this study was to identify tumor-derived methylated genes in the serum of stage IIA CRC and assess their diagnostic potentials for early stage of colorectal cancer.

Experimental procedure: In this prospective study, DNA methylation levels were measured by quantitative methylation-specific PCR. Two genes (*PPP1R3C* and *ADHFE1*) were first investigated in serum samples of an exploratory set of stage IIA CRC case-controls. Methylation results were verified in the sera of a test set comprising 50 stage IIA cases and 50 age- and gender-matched healthy controls. The receiver operating characteristic curve was constructed for assessment of assay performance.

Results: Serum methylation levels of *PPP1R3C* and *ADHFE1* were significantly higher in stage IIA patients as compared to healthy controls (both $P < 0.001$, Mann-Whitney *U* test). Areas under the receiver operating curve (AUCs) using serum methylation levels of *PPP1R3C* and *ADHFE1* were 0.60 [95% confidence interval (CI), 0.48-0.71] and 0.73 (95%CI, 0.62-0.83), respectively. At a specificity of 80%, the assay sensitivities of methylated *PPP1R3C* and *ADHFE1* were 26% and 56%, respectively.

Conclusion: Serum methylation levels of *ADHFE1* might be useful for minimally invasive detection of early stage II colorectal cancer. Validation studies in larger and independent cohorts and identification of additional markers are necessary.

40. Quantification of HER2 protein using multiple reaction monitoring-mass spectrometry in formalin-fixed paraffin-embedded (FFPE) breast cancer tissue specimens

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Human epidermal growth factor receptor 2 (HER2) protein is often overexpressed in breast cancer and is correlated with a worse prognosis; thus, accurate detection of HER2 by using optimum techniques is

crucial to provide appropriate cares for patients. However, no technique is the universal gold standard to detect accurate HER2 status. In this context, we established a multiple reaction monitoring (MRM) assay to quantitate HER2 protein that improves upon existing methods in differentiating between each HER2 status in FFPE tissue specimens. We developed a targeted proteomic assay based on multiple reaction monitoring-mass spectrometry (MRM-MS) and quantified levels of HER2-MRM protein in breast cancer FFPE tissues.

We analyzed a total of 210 breast cancer FFPE tissue specimens, which were comprised of HER2 0 ($n = 30$), HER2 1+ ($n = 30$), HER2 2+FISH- ($n = 61$), HER2 2+FISH+ ($n = 59$), and HER2 3+ ($n = 30$). We applied normalization factors that can represent the tumor size to simplify the overall experimental work-flow and raise the accuracy and precision of the results of HER2 quantification. In this context, the ratio between the quantification data of HER2 peptides by MRM assay and the normalization factor can be a new factor for determining HER2 status.

To select the most suitable normalization factor that can differentiate ambiguous IHC results of HER2 (HER2 2+FISH- vs. HER2 2+FISH+), which cannot be distinguished by IHC, area under the receiver operating curve (AUROC) values were calculated by using each normalized value of the 120 HER2 2+ samples. To determine whether the data generated by MRM matched with the data obtained by IHC and FISH scores, the quantitative data of a HER2 peptide normalized by a Junctional adhesion molecule A (JAM1) peptide with the highest AUROC values were used. The Mann-Whitney U test determined that significant differences were found in all HER2 and FISH groups, and especially the MRM data can distinguish between HER2 2+FISH- and HER2 2+FISH+ ($P < 0.000$), which cannot be differentiated by IHC. In addition, the MRM data distinguished the HER2 positive group that was expected to benefit from trastuzumab therapy from the HER2 negative group ($P < 0.000$).

We developed an experimental work-flow that is simple and clear enough to automate by introducing normalization factors for accurate HER2 status determination through MRM assay. The MRM assay that we developed clearly distinguished the equivocal HER2 status that could not be classified by the conventional method, IHC, as well as the overall HER2 classification. Our developed assay using MRM for determining HER2 status would provide clinicians with valuable diagnostic information and ensure that all patients whose breast cancers express HER2 proteins have the opportunity to receive proper treatment.

41. Amino-functionalized nanoparticles promote toxicity in ovarian cancer cells by impinging on autophagy

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Background: In the last decades, nanotheranostics has obtained great attention for its potential application in the biomedical field by combining multimodal imaging along with selective targeting therapy on the same nanoplatforms^[1]. However, the contributions of metabolic, genetic, or epigenetic features of tumor cells and tumor microenvironment in the cellular response to the nanoparticles have not been fully addressed^[2].

Aim: We investigated the cellular stress response to polystyrene nanoparticles (PS-NPs) functionalized with amino groups in two ovarian cancer cell models differing in the expression, among others, of relevant proteins involved in endocytosis (caveolin-1, CAV-1) and in pro-survival/pro-death pathways (PTEN and TP53).

Results: NH₂-PS-NPs were toxic in both cell lines, leading to primary necrosis that was time- and dose-dependent, yet with different mechanisms of toxicity. In OVCAR3 cells, which are PTEN and TP53 mutated and CAV-1 deficient, autophagy was insufficient to protect the cells from NH₂-PS-NPs toxicity. Autophagy inducers prevented while autophagy gene silencing exacerbated NH₂-PS-NPs-induced cell death. By contrast, in OAW42 cells, which express wild-type PTEN, TP53, and CAV-1, NH₂-PS-NPs strongly impaired autophagosome formation and increased production of the mitochondrial anion superoxide, resulting in ATG4 inactivation. Accordingly, resveratrol, a nutraceutical known to inhibit the formation of anion superoxide, rescued ATG4-mediated autophagy and reduced NH₂-PS-NPs toxicity.

Conclusion: Taken together, our findings point out the relevance of the genetic background of target cells, which determines the type and consequences of the stress response elicited by the NPs. Our data outline the necessity of a better assessment of the genetic/epigenetic and metabolic status of the target cells when designing theranostics for cancer therapy, in full agreement with the principle of personalized medicine.

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42. Glucose-dependent autophagy control of cancer cell migration

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Background and aim: Because of its aggressiveness and its diagnosis at very late stage, Ovarian cancer^[1] remains one of the main leading causes of death among women^[2]. IL-6 is an inflammatory cytokine over-expressed in serum and ascitic liquid of ovarian cancer-affected patients^[3]. One of the hallmarks of cancer is the so-called Warburg effect, which consists of an alteration of glucose metabolism^[4]. The goal was to investigate the mechanisms underlying the involvement of glycolysis and its mechanistic link with autophagy in cancer cell migration.

Experimental procedure: To mimic a pro-inflammatory tumor microenvironment, we treated ovarian cancer cells with IL-6, in absence or presence of glucose. To examine the molecular pathways linking glycolysis and autophagy in cell motility, we employed the metabolically inert glucose analog 2-Deoxy Glucose (2-DG). Additionally, we used resveratrol (RV), a nutraceutical with anti-cancer properties known to interfere with the utilization of glucose.

Results: We found that glucose is necessary for cell migration, with IL-6 promoting glucose uptake and cell motility. On the contrary, inhibiting glucose uptake or its utilization blocks cancer cell migration while up-regulating autophagy.

Conclusion: Our data indicate that the up-regulation of autophagy promoted by glucose deprivation hampers ovarian cancer cell migration.

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43. Trousseau's syndrome in association with lung adenocarcinoma

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Background: Trousseau's syndrome (TS) is a hypercoagulability manifestation of paraneoplastic syndrome, known as a variant of cancer-associated thrombosis and defined as a migratory thrombophlebitis found typically in patients with an underlying malignancy. TS commonly occurs in pancreatic cancer (24%), lung cancer (20%), prostate cancer (13%), and stomach cancer (12%), followed by breast and colon cancer.

Case presentation: Here, we describe the case of a 50-year-old male patient, who is a nonsmoker. During a checkup for work, he was found to have a highly positive Mantoux test (TBT), thus a chest X-ray was ordered. He had a previous chronic history of burning sensation in both feet, responding to analgesic drugs. There was no history of shortness of breath, cough, fever, night sweating, weight loss, loss of appetite, or fatigue. Auscultation of chest X-ray revealed a mass in the left upper lobe of lung. The computed tomography (CT) of chest showed a 5.5 cm × 4.3 cm left lingual superior segment lobulated mass with left hilar and mediastinal lymph node enlargement. In addition, the CT reported bone metastasis in vertebra, confirming the bone scan. Tumor markers were negative. CT guided biopsy for this lesion in the left upper chest was performed and the histopathology result showed poorly differentiated adenocarcinoma. The following molecular studies were negative: epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase, ROS, and PD-1. The patient was referred to the oncology center as a case of lung adenocarcinoma with distant metastasis, stage T4bN2bM1, and started in cisplatin and Alimta chemotherapy. Spiral CT revealed incidental finding of multiple filling defects, indicating segmental pulmonary embolism. Due to leg pain, Doppler of lower limb was also performed, showing deep venous thrombosis in the left limb. Enoxaparin was commenced at full dose. This case report indicated a TS cancer-associated thrombosis. The patient, after receiving the first cycle of chemotherapy, was discharged on enoxaparin, was stable, and returned to his job.

Conclusion: TS, a paraneoplastic manifestation, must be considered in patients with advanced stages of cancer, regardless of the primary site of the cancer. In lung cancer, paraneoplastic syndrome presents more frequently with small cell carcinoma (in 10% of patients), but, regarding TS in the literature, previous cases reported adenocarcinoma was the most prevalent histology associated with thrombosis.

44. Delphinidin chloride and its hydrolytic metabolite gallic acid promote differentiation of regulatory T cells and have an anti-inflammatory effect on the allograft model

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Regulatory T cells (Tregs) control the reactivity of other T cells to prevent excessive inflammatory responses. They also play a role in preventing autoimmune diseases; however, when they are overproduced, they decrease vital immunity, which can lead to invasion of external pathogens. Therefore, it is most important in preventing the development of immune diseases to maintain the homeostasis of these cells. Delphinidin chloride is an anthocyanidin and known to have antioxidant activities. However, its structure is very unstable and easily decomposed. One of these degradation products is gallic acid, which also has antioxidant effects. In this study, we examined the effect of these materials on Tregs in controlling immune response. It was found that these materials further promote differentiation into Tregs, and TGF- β and IL-2 related signals are involved in this process. Furthermore, it was verified that a variety of immunosuppressive proteins were secreted more, and the function of induced Tregs was also increased. Finally, in the allograft model, we could find a decrease in activated T cells when these materials were treated because they increased differentiation into Tregs. Therefore, these two materials are expected to become new candidates for the treatment of diseases caused by excessive activation of immune cells, such as autoimmune diseases.

Practical application: Delphinidin, a kind of anthocyanin rich in pigmented fruits, and its hydrolytic metabolite, gallic acid, are known to have antimicrobial and antioxidant properties. In this experiment, it was shown that delphinidin and gallic acid had an effect of increasing the differentiation of regulatory T cells, and the effect of suppressing the function of memory T cells was also observed. Due to these functions, delphinidin and gallic acid might have the potential to be used as immune suppressive agents in organ transplant and autoimmune disease patients or be a model for food development associated with the immune system.

45. Association between heavy metal cadmium and the warburg effect in breast cancer - preliminary results

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Background and aim: Warburg effect is a cancer hallmark described as reprogramming of energy metabolism, in which cells produce energy mainly due to glycolysis instead of oxidative phosphorylation. The Warburg effect is extremely important for the survival of tumor cells, particularly under hypoxia, but it may also occur under aerobic conditions (hence, it is called aerobic glycolysis). Although Warburg effect

was discovered almost 100 years ago, it is still not known whether it is a cause or a consequence of cancer. Interestingly, there are few studies investigating the association between the known carcinogenic factors and the Warburg effect. The aim of this study was to analyze the association between carcinogenic metal cadmium (Cd) and the Warburg effect in breast cancer.

Experimental procedure: We conducted an observational study on 100 women with breast cancer, from whom fragments of tumor tissue and tumor-adjacent tissue were collected, in order to compare Cd contents and molecular effect of the Warburg effect. In both types of tissue, we determined Cd content and the expression of mRNA of HIF-1 α (the key driver of the Warburg effect) and other proteins associated with the Warburg effect (including glucose transporters, glycolytic enzymes, or kinases regulating glycolysis). In addition, urinary Cd concentration as a marker of environmental exposure was analyzed. To investigate the effect of Cd on the Warburg effect *in vitro*, we analyzed molecular and metabolic markers of the Warburg effect (lactate concentration and pyruvate kinase activity) in MCF-7 cells exposed to non-toxic, environmentally relevant concentrations of Cd for 72 h (short term exposure) and six months (imitation of chronic exposure to Cd).

Results: In the preliminary study of 15 patients, we observed significant positive correlation between urinary Cd concentration and the expression of HIF-1 α , in both tumor ($r = 0.80$, $P < 0.001$) and tumor-adjacent tissues ($r = 0.75$, $P < 0.001$). Cd content in tumor tissue was also significantly correlated with the expression of PDK1 (pyruvate dehydrogenase kinase 1; $r = 0.48$, $P < 0.001$). Preliminary data analysis of MCF-7 cell line showed that HIF-1 α expression was also significantly increased upon Cd exposure (1-20 μ M).

Conclusion: Preliminary results of this study may suggest a possible link between Cd exposure and the molecular effects of the Warburg effect. Complete data analysis, including metabolic markers, will allow formulating final conclusions.

46. The oyster can adapt to a harsh environment in the marine coast: does it mimic cancer cells?

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Background and aim: The tumor physical microenvironment is extreme. Interestingly, the rocky intertidal zone is among the most physically harsh environments on Earth. The oyster *Crassostrea gigas*, living in this habitat, is among the champions of physiological adaptation to extreme environments. Our hypothesis is that environmental adaptation of oyster cells can mimic cancer cells inside the tumor.

Experimental procedure: Oysters were challenged to three extreme environments in the field, at high, medium, and low bathymetric levels. Biochemical analyses were done to identify the environmental responses.

Results: At two times during the experiment, we sampled all the organs of oysters in the field, extracted total proteins from flesh, and performed laboratory analysis, in order to obtain a rapid picture of metabolic

activities linked with extreme environmental responses. The first results demonstrated that challenged oysters in high/medium bathymetry exhibited a low weight gain, increased hexokinase activity, and increased mitochondrial functioning. We also quantified an up-regulation of AMP-activated protein kinase (AMPK) activation, a key energy sensor that controls glucose, lipid, and protein metabolism in *C. gigas*. Interestingly, up-regulation of AMPK was initially reported as a hallmark of cancer cells to support the high-energy demand of highly proliferative cells.

Conclusion: We propose the oyster as a new model for cancer research, to identify mechanisms underlying the ability of cells to adapt to a harsh environment. The oyster is a marine invertebrate that evolved 500 million years ago and we are convinced that it could help us to identify common ancestral pathways for cell adaptation to a harsh environment, for a better understanding of cancer cells functioning inside the tumor.

47. Resveratrol-induced modulation of non-coding RNA in ovarian cancer cells

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Background and aim: Ninety percent of the human genome is transcribed, of which only 2% encodes proteins; the remaining sequences enclose non-coding genes^[1,2]. Non-coding RNAs (ncRNAs) play a crucial role in the regulation of several biological processes and their dysregulation may influence cancer development, functioning as oncogenes or tumor suppressors. ncRNAs generally are divided into two main groups based on size: long non-coding RNAs (lncRNAs) of about 200 nucleotides, acting as positive or negative transcription modulators, and microRNAs (miRNAs) of about 20-22 nucleotides, acting as post-transcriptional silencing molecules^[3,4]. The aim of our work was to investigate whether Resveratrol (RV), a polyphenolic compound with anticancer properties^[5], could modulate ncRNAs in ovarian cancer cell lines.

Experimental procedure: Ovarian cancer cells were treated with RV (100 µM). Total RNA was isolated from the cells and mRNA was amplified and labeled. Labeled specimens were fragmented and hybridized to Human Whole Genome Oligo Microarrays. One hundred nanograms of total RNA were treated following the miRNA microarray protocol. RNA was dephosphorylated, denatured, ligated, and labeled. Samples were hybridized to Human miRNA Microarray. DIANA TOOLS was used to retrieve predicted microRNA targets and Gene Ontology for predicting their involvement in biological processes.

Results: We show that RV modulates non-coding transcripts that impact on cancer cell features.

Conclusion: Our data support the view that RV treatment can be effective in cancer therapy on regulating epigenetic mechanisms involved in cancer development.

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48. The microbiota-derived metabolite butyrate inhibits colorectal cancer cell migration via modulation of autophagy

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Background: Colorectal cancer (CRC) is the third most common cause of cancer deaths worldwide. The etiology of CRC involves host genetic predisposition and environmental factors, among which the diet plays an important role. The proportion of dietary fiber and meat consumed influences the composition of intestinal microbiota, which get energy from ingested food^[1]. One of the main metabolites produced by gut microbiota is the short chain fatty acid butyrate. Butyrate exerts a beneficial role in the maintenance of intestinal epithelium integrity through various mechanisms^[1]. Autophagy is the main cellular process that promotes a balanced macromolecular turnover and guarantees cell homeostasis^[2].

Aim: We studied the anti-migratory and anti-inflammatory properties of butyrate, a probiotic metabolite, in a colorectal cancer cellular model. Furthermore, we investigated the molecular pathways underlying these effects, with a particular focus on autophagy.

Experimental procedure: HCT116 colorectal cancer cells were treated with 5-mM sodium butyrate and 50-ng/mL interleukin-6 (IL-6). To study cell motility, a wound healing scratch assay was performed. Cellular homogenates were employed for protein expression studies through Western blot analysis. Immunofluorescence was performed on fixed cells.

Results: We found that butyrate counteracts colorectal cancer cell migration, even in the presence of IL-6, a well known pro-inflammatory cytokine. This effect is accompanied with a reduced expression of activated STAT3 and Twist1. Furthermore, the probiotic metabolite prevents IL-6-induced expression of N-cadherin, a typical hallmark of epithelial-to-mesenchymal transition. In addition, butyrate strongly accelerates the autophagy flux, alone and in co-presence with IL-6, suggesting autophagy as a putative mechanism responsible for slowing down cell motility.

Conclusion: Taken together, our findings identified anti-cancer properties of butyrate, in particular its ability to counteract IL-6-induced colon cancer cell migration, by upregulating autophagy.

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49. Parasites and cancer

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This PowerPoint presentation is based on our work at Parasitology Center, Inc. (PCI), in Scottsdale, Arizona, USA and covers the diagnosis, pathology, relationships with cancer, and treatment of human

parasitic infections in the United States based on our own patient history and testing. The conceptual thesis and practical observations of extensive damage of parasites to human tissues and the initiation of host defense strategies causing out of control cell divisions leading to metastasis is emphasized. A brief introduction to laboratory procedures, misdiagnoses/mistreatment, and impact on public health, especially cancer, is made. A systematic treatment of protozoan and helminth (worm) parasites follows, emphasizing epidemiology and exposure, symptoms, and gross pathology. Herbal and allopathic remedies including our own anti-parasitic herbal product Freedom/Cleanse/Restore are presented. All topics are illustrated with 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 and treatment of intestinal pathogenic bacteria that usually cause GI symptoms similar to those caused by intestinal parasites.

50. Role of biobanks in cancer research

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Background and aim: Biobanks are an important tool for biomedical research and a pillar of personalized medicine. Biobanks are collections of biological material and the associated data and information stored in an organized system, for a population or a large subset of population (OECD definition)^[1]. During last three decades, the role of biobanks has increased dramatically. They act as sources for a wide range of biomedical research and support the basic principles of personalized medicine. They contribute to prevention, early diagnosis, prognosis, right treatment, therapy monitoring, and optimal approach to a patient^[2]. Cancer was and remains as one of the main causes of mortality and morbidity worldwide. The number of cancers, especially rare cancers, make cancer diseases serious candidates for personalized medicine approaches.

How can biobanks contribute to the process of cancer treatment?

Biobanks as sources of various human biological material from different patients, from different regions, males and females, young and elderly, with other comorbidities, before and after surgery, different social and environmental associations, different ethnic groups, *etc.*, offer a wide range of samples for any type of research. Based on these characteristics, biobanks are a cornerstone for the discovery of new biomarkers, drugs, techniques, applications, and innovations^[3]. On the other hand, data and information connected with samples are another source for modeling, data applications, and artificial intelligence applications, with respect to ethical, legal, and social issues. Personalized medicine principles guarantee the best possible approach to every patient, which means prevention, early diagnosis, treatment, and treatment monitoring. Biobanking is a phenomenon that is intrinsically based on international collaboration, including samples, data, and information exchange, as no one institution can cover all the challenges all over the world by offering biobanking. Biobanking requires a multidisciplinary international strategy.

Conclusion: Biobanks are efficient tools for new biomedical research and a personalized medicine approach to every patient and contribute to support healthcare systems, international collaboration, biomedical research, and innovation.

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51. Cancer chemoprevention with mitochondria-targeted compounds

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We synthesized two mitochondria-targeted compounds, namely mito-honokiol (Mito-HNK) and mito-lonidamine (Mito-LND), that facilitate their mitochondrial accumulation. This dramatically increases their potency and efficacy against highly metastatic lung cancer lines *in vitro*, orthotopic lung tumor xenografts, and brain metastases *in vivo*. Both Mito-HNK and Mito-LND are > 100-fold more potent than their parent compounds in inhibiting cell proliferation and mitochondrial complexes, stimulating reactive oxygen species generation, and oxidizing mitochondrial peroxiredoxin-3. Interestingly, Mito-HNK appears to induce apoptosis via suppressing the phosphorylation of mitoSTAT3, while Mito-LND induces autophagic cell death via inactivating AKT/mTOR/p70S6K signaling. Both Mito-HNK and Mito-LND cause no toxicity in mice, even when administered for eight weeks at > 20 times the effective cancer inhibitory dose. A highly synergistic effect is observed when combining the two compounds and its mechanistic basis is being vigorously pursued. Collectively, these findings show that mitochondrial targeting compounds are a promising preventive/therapeutic approach to mitigate lung cancer development and brain metastasis.

52. The efficacy of ketogenic diet with concomitant intranasal perillyl alcohol as a novel strategy for therapy of recurrent glioblastoma

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Background: It has been hypothesized that persistent ketotic hypoglycemia might represent a potential therapeutic strategy against high-grade gliomas. Perillyl alcohol (POH) is a non-toxic, naturally-occurring, hydroxylated monoterpene that exhibits cytotoxicity against temozolomide-resistant glioma cells, regardless of O6-methylguanine-methyltransferase promoter methylation status. This study aimed to evaluate the toxicity and therapeutic efficacy of intranasal POH administered in combination with a ketogenic diet (KD) program for the treatment of patients with recurrent glioblastoma.

Patients and methods: Thirty-two patients were divided into two groups - KD or standard diet - both associated with intranasal POH ($n = 17$ and $n = 15$, respectively). The nutritional status and anthropometric parameters of patients were measured. Patients who adhered to the KD maintained a strict dietary regimen, while receiving inhalation of POH (55 mg, four times daily) in an uninterrupted administration schedule for three months. Neurological examination and imaging analysis (magnetic resonance imaging) were used to monitor disease progression. Clinical toxicity and overall survival were correlated with tumor size, topography, extent of peritumoral edema, and frequency of seizures. In the KD patient, strict compliance with the KD was confirmed by measuring the levels of ketone bodies in the urine (9/17 patients) three times per week.

Results: After three months of well tolerated treatment, we observed a partial response in 77.8% (7/9 patients), stable disease in 11.1% (1/9), and 11.1% (1/9) presented with progressive disease. Among the patients assigned to the standard diet (control group), the partial response was 25% (2/8 patients), stable disease was 25% (2/8), and progressive disease was 50% (4/8 patients). The patients assigned to the KD group presented with: reduced frequency of seizures, a slight increase in lean muscle mass, reduced serum lipid levels, and decreased low-density lipoprotein cholesterol (LDL-C) levels.

Conclusion: These results are encouraging and suggest that KD associated with intranasal POH may represent a viable option as an adjunct therapy for recurrent GBM.

Review

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The management of hyponatremia in cancer patients: a practical view in Spain

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How to cite this article: Marquina G, Gomez-Hoyos E, Runkle I. The management of hyponatremia in cancer patients: a practical view in Spain. *J Cancer Metastasis Treat* 2020;6:6. <http://dx.doi.org/10.20517/2394-4722.2019.39>

Received: 13 Oct 2019 **First Decision:** 6 Jan 2020 **Revised:** 28 Jan 2020 **Accepted:** 18 Feb 2020 **Published:** 11 Mar 2020

Science Editor: William P. Schieman **Copy Editor:** Jing-Wen Zhang **Production Editor:** Jing Yu

Abstract

Hyponatremia is a common disorder among cancer patients and is associated with a poor prognosis in several malignancies. It is classified by volumic status into hypovolemic, euvoletic, and hypervolemic hyponatremia. Clinical history, physical examination, and blood and urine tests are important for a correct classification and diagnosis of hyponatremia, to assure correct management. Treatment of hyponatremia in cancer patients depends on the etiology of hyponatremia, as well as on the chosen therapy for the tumor. Supportive care is also a factor to be taken into account.

Keywords: Hyponatremia, sodium, cancer, syndrome of inappropriate antidiuretic hormone secretion, antidiuretic hormone, supportive care

INTRODUCTION

Hyponatremia is the most frequent electrolyte disorder found in cancer patients^[1]. It is defined as a serum sodium level (SNa) of less than 135 mmol/L. Hyponatremia is generally classified into mild (130-135 mmol/L), moderate (120-129 mmol/L), and severe (< 120 mmol/L) according to the SNa, and is usually correlated with its symptoms^[2]. However, the velocity of descent should also be taken into account, as severe, acute hyponatremia is also defined as a drop in sodium levels of more than 10 mmol/L in 48 h. The precise prevalence of hyponatremia in patients with cancer has yet to be determined. The



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Table 1. Physical examination approach to classify hyponatremia regarding volume status^[18]

Physical examination	
Orthostatism	Orthostatism can often be found in hypovolemia
Manual ocular pressure	Manual ocular pressure can be low in hypovolemia
Internal jugular venous pressure	Inspection of internal jugular vein. The maximum height of the pulse of this vein reveals the pressure in the right cardiac atrium: A maximum pulse height below the angle of the sternum in a reclining patient indicates hypovolemia; A height between 1 and 3 cm above the sternal angle indicates euvolemia or hypovolemia with cava vein thrombosis or severe pulmonary hypertension; A height over 4 cm indicates elevated right atrial pressure, as is found in congestive heart failure
Edema/increase of liquids in a "third space"	Hypervolemic status usually presents with an increased third space such as edema or ascites

frequency of hyponatremia varies depending on the type of tumor, clinical scenario, and the threshold used for definition of hyponatremia. However, up to 47% of patients in the oncology ward^[3] have been found to present hyponatremia. Furthermore, hyponatremia can precede the diagnosis of malignancy, with incidences between 1% and 40%^[4].

Historically, hyponatremia has been more frequently associated with small cell lung cancer than with other tumors^[5]. However, other publications establish that this hydro-electrolytic alteration can be detected in any cancer patient^[3,6]. Direct cancer-induced hyponatremia could be due to ectopic arginine vasopressin secretion, inducing the Syndrome of Inappropriate Antidiuretic Hormone Secretion (SIADH). Furthermore, there is emerging evidence for expression of sodium-transporting proteins in cancer^[7].

Hyponatremia can also be attributed to other etiologies that are not related *per se* to the cancer. What is more, hyponatremia can be induced by cancer-related complications, as well as the anti-cancer treatment itself^[8] or the side effects of cancer therapy. These include diarrhea, nausea, vomiting, pain, nephrotoxicity, adrenal insufficiency (due to adrenal metastases), *etc.*^[5].

Hyponatremia can be a potential negative prognostic factor in patients diagnosed with solid tumors or hematological malignancies such as lung cancer, breast cancer, lymphoma, and colorectal cancer^[5]. In cirrhosis, hyponatremia is associated with a higher morbidity and mortality. Decompensated cirrhosis in liver cancer patients represents an additional complicating factor^[9].

Hyponatremia detected in in-hospital cancer patients is associated with a longer hospital length of stay and an increased risk of mortality^[4,6,10-13].

The impact of correction of hyponatremia on patient survival has yet to be ascertained. In some patient series, the correction of hyponatremia correlates with an improvement in quality of life and an improved prognosis^[4,14].

THE DIAGNOSTIC APPROACH TO HYPONATREMIA IN CANCER PATIENTS

The diagnostic approach in cancer patients should be the same as for any patient with hyponatremia^[2,15]. The physical examination is fundamental, since it can establish the volume status of the patient [Table 1]; the clinical history, blood and urine tests are also necessary [Table 2] to determine the etiology [Table 3]^[16,17].

The initial step in laboratory evaluation of hyponatremia, after detecting a sodium level below 135 mmol/L, is to assure that hyponatremia is truly present. High glycemic levels, or mannitol infusion can induce translocational hyponatremia. In fact, total blood or serum sodium levels must always be corrected in patients with hyperglycemia. In patients receiving mannitol infusion, a normal plasma osmolality will rule

Table 2. Basic blood and urine tests for the diagnosis of the cause of hyponatremia

Blood test (serum)	Urine test	Gasometer
Protein		
Glucose		
Urea		
Creatinine	Creatinine	
Osmolality	Osmolality	
Sodium	Sodium	Sodium
Potassium	Potassium	
Chlorine	Chlorine	
Cortisol		
TSH		
T4		

TSH: thyroid stimulating hormone; T4: thyroxine

Table 3. Approach to diagnosis

Etiology approach for hyponatremia	
The basic hyponatremia approach is based on clinical history, physical examination, full blood test (as described in Table 2), timing of the onset of hyponatremia, symptoms, and type of hyponatremia [Table 4]	
Urine sodium: essential for the differential diagnosis of hypovolemic hyponatremia.	With renal sodium loss (Urine sodium > 25 mmol/L): diuretics, bicarbonate intake, primary adrenal insufficiency (Addison's disease), isolated hypoaldosteronism, and salt wasting syndrome Without sodium renal loss (Urine sodium < 20 mmol/L): gastrointestinal losses (vomiting and diarrhea), burns, hemorrhage, and pancreatitis
Urine osmolality: essential for the differential diagnosis of euvoletic hyponatremia	≤ 100 mOsm/kg: secretion of the ADH is inhibited, polydipsia with or without low solute intake, water intoxication, and administration of hypotonic fluids > 100 mOsm/kg: Secretion of ADH is not inhibited, ACTH deficit, severe hypothyroidism, pain, postsurgical stress, nausea, vomiting, the syndrome of inappropriate antidiuretic hormone secretion, use of thiazides, <i>etc.</i>

ADH: antidiuretic hormone; ACTH: adrenocorticotrophic hormone

out true hyponatremia. Pseudohyponatremia, induced by high protein or lipid levels, can be excluded by the determination of total blood sodium by gasometer.

The best and most direct way to ascertain whether the patient presents hypovolemic or euvoletic eunatremia is by neck inspection of the highest point of the internal jugular vein pulse [\[Table 1\]^{\[18\]}](#). The evolution of serum creatinine together with SNa is also a good parameter. Serum creatinine usually increases when natremia drops in the hypovolemic patient and usually decreases along with hyponatremia in the euvoletic patient [\[14\]](#).

An appropriate intervention depends on determining the timing of hyponatremia onset, the severity of the neurological symptoms, and the voletic classification [\[Table 4\]](#). This information, together with clinical history and blood and urine tests, is the basis for determining the etiology of hyponatremia [\[Table 3\]](#).

Note that a single patient could experience different voletic episodes (e.g., hypovolemic after having been euvoletic) sequentially [\[19\]](#). That is why clinical examination remains necessary to assure correct management of hyponatremia at any given point of time, and reevaluation of patients is essential.

Hyponatremia in oncology patients is often considered primarily euvoletic, secondary to SIADH [\[20\]](#). However, some studies have found that hypovolemic or hypervolemic hyponatremia is more prevalent in hospitalized cancer patients [\[3,6\]](#).

The clinician should remember that the diagnosis of SIADH is always a diagnosis of exclusion [\[21\]](#), in a euvoletic patient with a urine osmolality higher than 100 mOsm/kg, in the absence of pain, nausea, diuretics, adrenocorticotrophic hormone (ACTH) deficit, diuretic use, or severe hypothyroidism. ACTH

Table 4. Hyponatremia clinical approach

Hyponatremia approach		
Timing of the onset of hyponatremia	Acute: onset less than 48 h earlier Chronic: onset more than 48 h earlier *Hyponatremia should be considered chronic when timing of onset is unknown	
Neurological symptoms	Mild	Impaired capacity for concentration Cognitive deficit Gait disturbances and falls Memory loss Anorexia
	Moderate	Cramps Drowsiness Headache Nausea Vomiting Asthenia Impaired gait and falls Confusion
	Severe	Lethargy Stupor Seizures Coma Respiratory distress Sudden death
Type of hyponatremia: volumetric classification	Hypovolemic Euvolemic Hypervolemic	

*Onset unknown, - in this case it must be considered as chronic

deficit is often overlooked, and all patients who are not receiving pharmacological steroid doses should have cortisol levels determined.

TREATMENT OF HYPONATREMIA IN CANCER PATIENTS

Hyponatremia should be treated to both correct clinical symptoms and permit adequate oncological and nutritional therapy. Furthermore, correction of hyponatremia could potentially influence the cancer patient's quality of life.

A patient who is a candidate for chemotherapy

Severe hyponatremia ($Na < 120$ mmol/L)

The management of cancer patients is exactly the same as is the case for a non-oncological patient. Hypertonic saline solution (3% sodium chloride) should be administered in i.v. infusion or in bolus therapy, regardless of the type or etiology of hyponatremia. The rate of correction will vary if hyponatremia is chronic or acute. In acute hyponatremia (< 48 h), there are no established limits for correction of hyponatremia. In chronic hyponatremia (> 48 h) or when the timing of the onset of hyponatremia is unknown, the goal of correction in the first 24 h should be a Na rise of 4-6 mmol/L, reached during the first 6 h of treatment, to reduce cerebral edema [Figure 1].

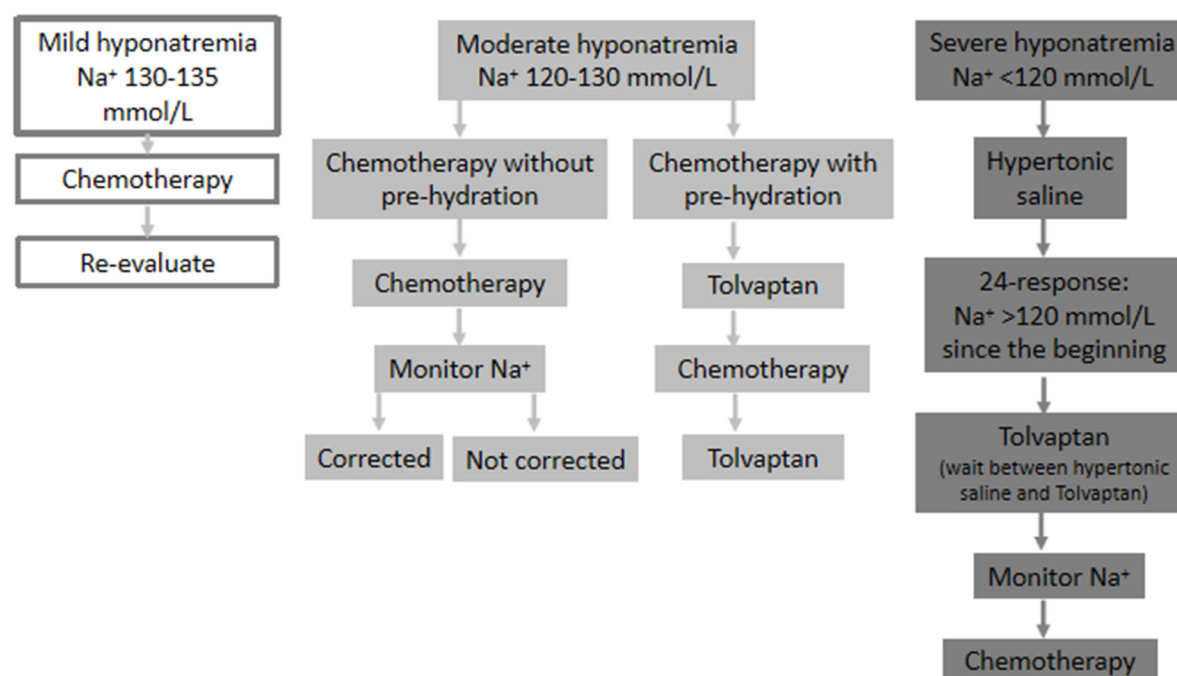
Additional treatments for hyponatremia must be avoided during the first 24 h of correction, except for associated furosemide in patients with heart failure or the addition of potassium chloride in patients with initial hypokalemia^[2,14] [Table 5]^[22].

Hypercorrection of Na should be avoided in patients with chronic hyponatremia (> 48 h from onset) or when the timing of the onset of hyponatremia is unknown. Patients presenting risk factors for the Osmotic Demyelination Syndrome (ODS) (hypokalemia, malnutrition, liver failure, and alcoholism) should not present 24-h Na rises above 8 mmol/L during the first or second 24 h of therapy. Therefore, following Hypertonic saline therapy, Na should be monitored every 6-8 h. If Na re-descends, hypertonic saline can

Table 5. The 24- and 48-h goals for correction of chronic hyponatremia (adapted from^[22])

ODS risk	Minimum 24 h SNa rise (mmol/L)	24 h Goal (mmol/L)	Maximum 24 h SNa rise (mmol/L)	48 h Goal (mmol/L)	Maximum 48 h SNa rise (mmol/L)
Low	4-8	6-8	10-12	6-8	18
High	4-6	6	8	4-6	8/day

ODS: osmotic demyelination syndrome; SNa: serum sodium

**Figure 1.** When the patient is a candidate for chemotherapy (modified from Escobar *et al.*^[29])

be administered anew. If SNa levels have reached the desired goal hours before 24 h have elapsed since the start of treatment, desmopressin (DDAVP) can be associated at doses of 1-2 µg every 6-8 h until the 24 h have elapsed, to prevent SNa levels from continuing to increase. Diuresis should be monitored and DDAVP administered in the case of polyuria.

A marked exception to the use of hypertonic saline is the suspicion of an adrenal crisis. In this case, established protocols for treatment should be applied, with i.v. hypertonic saline only used if the patient's SNa fails to rise adequately^[2,19].

Mild/moderate hypovolemic hyponatremia

Management will depend on the cause of hyponatremia. Diarrhea and vomiting are frequent side effects of chemotherapy. Antiemetics and adequate hydration and salt intake can be enough to correct non-severe hypovolemic hyponatremia, although i.v. isotonic saline can be required.

If it is mild hyponatremia, the patient can proceed with chemotherapy and control serum sodium levels in outpatient clinic.

Mild/moderate hypervolemic or euvolemic hyponatremia

In patients with a diagnosis of SIADH, fluid restriction can be attempted if patients are not candidates for surgery, hyperhydration, or nutritional supplements or support. Furthermore, patients at nutritional risk could limit protein intake when reducing fluids, as the latter are usually needed to eat solids. Fluid

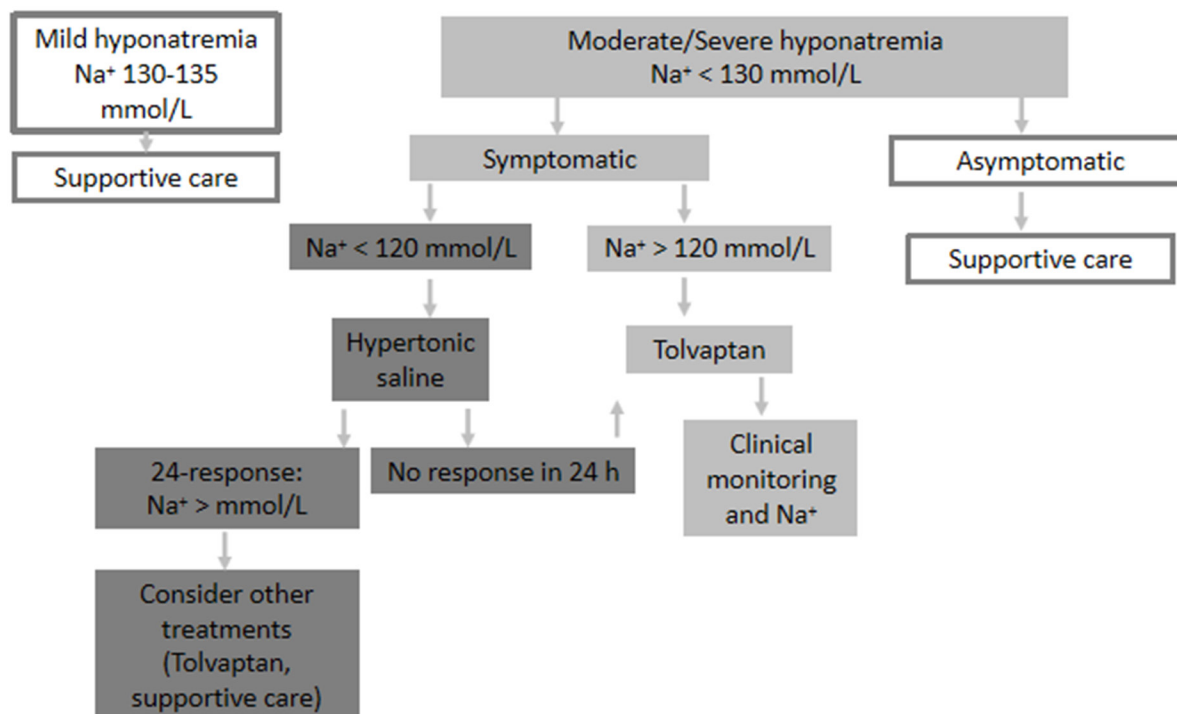


Figure 2. When the patient is not a candidate for chemotherapy (modified from Escobar *et al.*^[29])

restriction refers to all administered liquids, including i.v. medication and semisolids administered in the diet.

Fluid restriction should not be attempted if the patient's biochemistry indicates their kidneys are unable to eliminate free water. To ascertain response, the Furst formula can be applied.

The first approach is fluid restriction and increased salt intake in diet, applying the Furst formula^[23]:
(Urine sodium + Urine K)/Serum sodium

The result of this formula will predict whether fluid restriction will be effective for the treatment of hypervolemic or euvolemic hyponatremia, and the threshold of the liquid restriction: (1) < 0.5: fluid restriction of < 1000 cc/day; (2) 0.5-1: fluid restriction of < 500 cc/day; (3) > 1: fluid restriction ineffective.

In cancer patients with a high prevalence of and risk for malnutrition and the frequent need for dilution of chemotherapy, fluid restriction is often not viable^[14,19].

Evidence in favor of the use of fluid restriction in patients with hypervolemic hyponatremia is scant^[24]. However, its use has been incorporated into therapeutic algorithms of congestive heart failure. If there is no response to fluid restriction, Tolvaptan could be considered^[25].

Patients who are not candidates for chemotherapy

Supportive treatment and treatment of hyponatremia should be prioritized according to the severity of the symptoms, rather than the level of hyponatremia *per se* [Figure 2].

Hyponatremia due to SIADH in cancer patients

Tolvaptan is approved for the treatment of SIADH-induced hyponatremia in adult patients^[26-28].

The Spanish Medical Oncology Society (SEOM) has developed an algorithm which could be useful for the management of hyponatremia secondary to SIADH in cancer patients^[29] based on the prior algorithm developed in Spain for hyponatremia patients^[30].

According to the SEOM algorithm, management will depend on the two scenarios mentioned above: the patient is a candidate *vs.* a non-candidate for chemotherapy.

When the patient is a candidate for chemotherapy [Figure 1]

- (1) Mild hyponatremia (130-135 mmol/L): proceed with chemotherapy. Re-evaluate during the next cycle. Note that there are discrepancies in this point, as Tolvaptan would prevent the exacerbation of hyponatremia and the development of severe hyponatremia following the first cycle of chemotherapy^[19];
- (2) Moderate hyponatremia (120-130 mmol/L): consider Tolvaptan if chemotherapy requires pre-hydration, hyponatremia is progressively worsening, or hyponatremia is symptomatic. As mentioned above for mild hyponatremia, Tolvaptan could prevent worsening hyponatremia;
- (3) Severe hyponatremia (< 120 mmol/L): use the same as treatment as for severe hyponatremia. Once 24-48 h have elapsed following therapy with hypertonic saline solution, Tolvaptan could be started.

When SIADH-induced hyponatremia is caused by the anti-cancer treatment itself (for example, vincristine)^[8], a modification of cancer therapy should be considered. When a change in medication is not feasible, SIADH in these cases should be treated with Tolvaptan.

When the patient is not a candidate for chemotherapy [Figure 2]

As mentioned above, supportive treatment and treatment of hyponatremia should be prioritized according to the severity of the symptoms, rather than the level of hyponatremia *per se*:

- (1) Mild hyponatremia (130-135 mmol/L): focus on supportive care management;
- (2) Very symptomatic moderate/severe hyponatremia (< 130 mmol/L): consider Tolvaptan. If < 120 mmol/L, treat the acute phase with hypertonic sodium solution and, once stabilized, consider Tolvaptan;
- (3) Mild symptomatic moderate/severe hyponatremia (< 130 mmol/L): focus on supportive care management.

DECLARATIONS

Authors' contributions

Wrote and reviewed the manuscript: Marquina G, Gomez-Hoyos E, Runkle I

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

Not applicable.

Consent for publication

Not applicable.

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Original Article

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Combinatorial treatment of curcumin or silibinin with doxorubicin sensitises high-risk neuroblastoma

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How to cite this article: Fonseka P, Gangoda L, Pathan M, Angela DG, Mathivanan S. Combinatorial treatment of curcumin or silibinin with doxorubicin sensitises high-risk neuroblastoma. *J Cancer Metastasis Treat* 2020;6:7.
<http://dx.doi.org/10.20517/2394-4722.2019.024>

Received: 16 Aug 2019 **First Decision:** 20 Nov 2019 **Revised:** 17 Feb 2020 **Accepted:** 2 Mar 2020 **Published:** 13 Mar 2020

Science Editor: Godefridus J. Peters **Copy Editor:** Jing-Wen Zhang **Production Editor:** Jing Yu

Abstract

Aim: Neuroblastoma is a pediatric cancer of the sympathetic nervous system. Using various parameters including stage of the disease, amplification status of N-Myc, DNA index and histopathology, neuroblastoma can be stratified into low- and high-risk groups. Recent advances in treatment have significantly improved the survival rate of low-risk neuroblastoma patients. However, the overall survival rate of high-risk neuroblastoma group, especially N-Myc amplified patients, is poor. Moreover, the survivors of both low- and high-risk neuroblastoma manifest adverse side effects to chemotherapy and thus their quality of life is impaired. Considering all these factors, there is an urgent need to develop therapeutic strategies with natural compounds to improve the survival rate and to reduce the side effects. In this study, we hypothesised that the mesenchymal nature of neuroblastoma cells is a reason, at least in part, for the aggressive and treatment resistant phenotype.

Method: In order to validate our hypothesis, we used publicly available RNA-Seq data, *in vitro* assays and xenograft mouse models.

Results: Using a combinatorial treatment of mesenchymal-to-epithelial inducers (curcumin or silibinin) with doxorubicin significantly increased the cell death in a panel of neuroblastoma cells *in vitro*. Follow up analysis *in vivo*, confirmed the therapeutic benefit of utilising the combination of curcumin with doxorubicin. The combinatorial therapy significantly reduced the tumor burden and increased the survival of mice implanted with high-risk neuroblastoma cells.



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Conclusion: Taken together, this study shows the efficacy of using curcumin in combination with doxorubicin to improve the survival rate and has the potential to enhance the quality of life of neuroblastoma patients.

Keywords: Neuroblastoma, epithelial-to-mesenchymal transition, curcumin, silibinin, combinatorial therapy

INTRODUCTION

Neuroblastoma is the most common extracranial solid tumour that occurs in childhood^[1]. It usually arises from the sympathetic nervous system and originates from the neuroepithelial cells of neural crest tissues^[2]. The clinical behaviour of this complex disease is highly diverse, ranging from a benign tumour mass with no symptoms to a progressive and fatal disease with resistance to current treatments^[2,3]. This aggressive cancer is the most common cancer diagnosed during the first two years of human life^[4]. The most important prognostic factor in neuroblastoma is the stage of the disease while the age of the patients remains an independent prognostic factor. Interestingly, infants less than 12 months with stages beyond 1, have significantly better disease-free survival rates than older children who are diagnosed with the same stage^[5-8]. The histopathologic features of tumours are also classified as favourable or unfavourable depending on the differentiation of neuroblasts and Schwannian stroma content^[9]. In addition, the presence of amplified transcription factor N-Myc in neuroblastoma patients (about 20%) strongly correlates with poor prognosis and tumour dissemination. As amplification of N-Myc has a profound effect on clinical outcome in neuroblastoma patients, N-Myc copy number has been used as a biomarker^[10-12]. The heterogeneity of neuroblastoma has resulted in decrease in the effectiveness of the therapeutic strategies over the past decades. However, tumours with favourable prognostic markers do not require as intense chemotherapy as the tumours with adverse factors. Hence, the Children's Oncology Group has developed a risk group stratification system in order to categorise the treatment strategies^[13]. This system is mainly based on the stage of the disease, amplification status of N-Myc, DNA index and histopathology.

Even though the overall outcome of the neuroblastoma patients has improved recently, the survival rates of the children with high-risk neuroblastoma have not shown a substantive improvement^[14,15]. Hence, there is a need for better therapeutic avenues to treat high-risk neuroblastoma^[1,16,17]. Regardless of the efforts and current developments in treating high-risk patients, most of them relapse due to acquired drug resistance. Moreover, the survivors of high-risk neuroblastoma have manifested adverse effects to current therapeutics, which in turn has impaired their quality of life. Some of the uncovered effects from radiotherapy and surgery are damages to eyes, osteoporosis and various other musculoskeletal abnormalities^[18,19]. Unfortunately, damages to renal tubes, chronic abnormalities in electrolytes, impaired sexual maturation, premature menopause and growth hormone deficiency can also occur due to long-term exposure to chemotherapy^[20-25]. When considering all these factors, there is an urgent need of developing therapeutic strategies with natural compounds that increase the anti-cancer activity with lower side effects to enhance the quality of life of neuroblastoma patients.

Epithelial-to-mesenchymal transition (EMT) is a cellular process where epithelial cells lose their adhesion properties and turn mesenchymal. This highly plastic and dynamic shift towards the mesenchymal state is considered as EMT, wherein the expression of the adhesion proteins are downregulated so as to promote migration and invasion^[26,27]. On the contrary, mesenchymal-epithelial transition (MET) is the reverse of EMT, wherein the expression of adhesion proteins are upregulated and the cells lose the migratory phenotype^[28]. It is well established that EMT regulates cancer metastasis. Recent evidence suggests that EMT also regulates chemotherapeutic drug resistance in several cancer types. Human colorectal cancer (CRC) cell lines KM12L4 and HT29 displayed EMT because of oxaliplatin resistance by translocating β -Catenin to the nucleus^[29]. Hepatocellular carcinoma cells resistant to 5-Fluorouracil (5-FU) also showed

induction of EMT with the upregulation of Twist and the down regulation of E-Cadherin^[30]. Similarly, activation of Snail in CRC cell lines led to increased motility and invasiveness with increased resistance to 5-FU^[31]. Collectively, these data suggest that EMT plays a role in chemoresistance. Neuroblastoma cells, due to their origin, are more mesenchymal and it is highly likely that the inherent resistance to treatment could be partly attributed to EMT. It is unclear whether the utility of MET inducers along with standard chemotherapeutic drugs could increase the sensitivity of the neuroblastoma cells.

Curcumin and silibinin are natural components that have exhibited anti-cancer activities and can induce MET in many adult cancers with less or no toxicity^[32,33]. These natural active ingredients have been implicated in suppressing various growth and pro-invasive signalling pathways as well as inducing cell death in a variety of cancer cells^[34-36]. In non-N-Myc amplified SH-SY-5Y neuroblastoma cells, curcumin was shown to induce cell death *in vitro*^[37]. However, *in vivo* studies that highlight the therapeutic potential of curcumin and silibinin in treating high-risk neuroblastoma cells are currently lacking. In addition, the utility of these natural compounds in combinatorial therapy with doxorubicin *in vivo* has not been examined. In this study, we examined the combinatorial effect of curcumin or silibinin in sensitising neuroblastoma cells to the chemotherapeutic drug doxorubicin both *in vitro* and *in vivo*.

METHODS

RNA-Seq analysis

The RNA-Seq data for NBL and CRC cell lines and tissues were downloaded from published literature^[38]. The mRNA expression (log₂ RPKM values) of genes categorised as epithelial and mesenchymal were plotted using MATLAB. Genes (315) classified as epithelial and mesenchymal were downloaded from published literature^[39]. Statistical analysis to calculate the significance was performed using the Pearson's chi-square test. *P*-values less than 0.05 were considered statistically significant.

Cell culture

The neuroblastoma cell lines SK-N-BE2, SH-SY5Y and SK-N-AS were cultured in 150 cm² tissue culture flasks (BD FalconTM) in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO, Life Technologies) medium supplemented with 10% (v/v) fetal calf serum (GIBCO, Life Technologies) and 100 units/mL of penicillin-streptomycin (GIBCO, Life Technologies). IMR32 neuroblastoma cells were cultured in Minimum Essential Medium (GIBCO, Life Technologies) medium. The cells were incubated at 37 °C with 5% CO₂. SK-N-BE2 (CRL-2271TM) and IMR32 (CCL-127TM) cells were purchased from ATCC®, while SH-SY5Y and SK-N-AS cells were kindly gifted by Dr Julie Atkin.

Whole cell lysate preparation

Neuroblastoma cells were treated with 10 μM curcumin and 100 μM silibinin for 24 and 48 h prior to preparation of cell lysates. Cells were lysed as described previously^[40] using 4 × sodium dodecyl sulfate (SDS) loading dye [2% (w/v) SDS, 125 mM Tris-HCl pH 7.4, 12.5% (v/v) glycerol and 0.02% (w/v) bromophenol blue]. Briefly, loading dye (1.5 mL) was added to culture dishes and evenly spread using the cell lifter (Fisher Biotec). The lysate was then collected in thick wall polyallomer tubes (Beckman Coulter) and centrifuged at 100,000 g for 1 h (TLA 100.2, Beckman). Supernatant was collected and stored at -80 °C for further analysis.

SDS-PAGE and Western blotting

Equal amount of protein samples was prepared in 4 × SDS loading buffer with 100 mM DTT (Astral). Samples were then denatured by heating at 95 °C for 2 min and were run on a NuPAGE® 4%-12% Bis-Tris precast gel (Life Technologies). The gels were run at 150 V for 1 h in NuPAGE® MES SDS Running Buffer (Life Technologies). Proteins were transferred on to nitrocellulose membrane using iBlot dry blotting system

(Invitrogen™) at 20 V for 7 min. The membrane was incubated with blocking solution containing 5% (w/v) skim milk in Tris-buffered saline with 0.05% Tween 20 (TTBS) [100 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.05% (v/v) Tween 20] for 45 min. The membrane was washed three times with TTBS (10 min each) and probed with the relevant primary antibody overnight at 4 °C. The membrane was again washed with TTBS over 30 min. Subsequently, the blot was probed with appropriate IRDye (LI-COR®) or peroxidase (Sigma-Aldrich®) conjugated secondary antibody for 1 h at room temperature. The blot was then washed three times with TTBS over 30 min. For the visualisation of the protein bands probed with IRDye (LI-COR®), ODYSSEY CLx (LI-COR®) machine was used.

Cell death assay

Cells (5×10^3 per well) were seeded in a 24-well plate in 500 µL DMEM medium and allowed to adhere for 48 h at 37 °C in the presence of 5% CO₂. Cells were then treated with or without doxorubicin (1 µM) and incubated for 48 h. For combinational treatment studies, curcumin (10 µM) or silibinin (100 µM) treatment was performed 24 h before the addition of doxorubicin (pre-treatment) as well as on the same day of doxorubicin treatment (combinational). After 48 h of cancer therapeutic drug treatment, cells were scraped and resuspended. Supernatant (300 µL) was then transferred in to a 96-well plate and spun at 300 g for 5 min before discarding the supernatant. The remaining pellet was then resuspended with 200 µL of propidium iodide (PI) buffer [0.1% (w/v) TritonX 100 and 50 µg/mL Propidium iodide (Sigma Life Science®)] and was incubated overnight at 4 °C. For this, 2×10^4 cells were used in the analysis. Results from fluorescence activated cell sorting CANTO II (BD Biosciences) were then analysed using FlowJo (TreeStar).

Establishment of tumour xenografts

SK-N-BE2 cells (5×10^6) were subcutaneously injected to athymic Balb/c nude female mice (8 weeks old). The cells were suspended in Matrigel before the injections. After formation of tumours, the mice were injected intraperitoneally (i.p.) with dimethyl sulfoxide (DMSO) (control), curcumin (40 mg/kg), doxorubicin (5 mg/kg) and combinational treatment of doxorubicin and curcumin three times a week. Tumour size and the weight of the mice were measured daily. The tumour volume was calculated according to the formula $\frac{1}{2}(W^2 \times L)$. According to the Australian code of practice for the care and use of animals for scientific purposes and La Trobe Ethics Committee guidelines (AEC 14-15), mice were sacrificed when the tumour size reached 1500 mm³.

Statistical analysis

Statistical significance of experiments was analysed by student *t*-test and *P* values less than 0.05 were considered to be statistically significant. CI (Combination index) was calculated using Chou-Talalay method^[41]. A violin plot was generated using MATLAB and the *P* value of the violin plot was also calculated using MATLAB^[42].

RESULTS

Neuroblastoma cells exhibit mesenchymal signature

Prior to commencement of the experiments, publicly available RNA-Seq data for neuroblastoma tissues and cells were queried for the expression of EMT genes to examine the hypothesis of whether the neuroblastoma cells are mesenchymal. To validate the expression of epithelial and mesenchymal genes, publicly available RNA-Seq data for neuroblastoma cell lines and tissues were examined. In total, 315 genes implicated in EMT were retrieved from the public literature^[43] and the expression profile of these genes were plotted in a panel of neuroblastoma cell lines and tissues [Figure 1]. Interestingly, agreeing with our hypothesis, neuroblastoma cell lines (*n* = 8) and tissues (*n* = 157) exhibited a high mesenchymal and low epithelial gene expression. These results suggest that neuroblastoma cells are mesenchymal and hence could contribute to the aggressive phenotype.

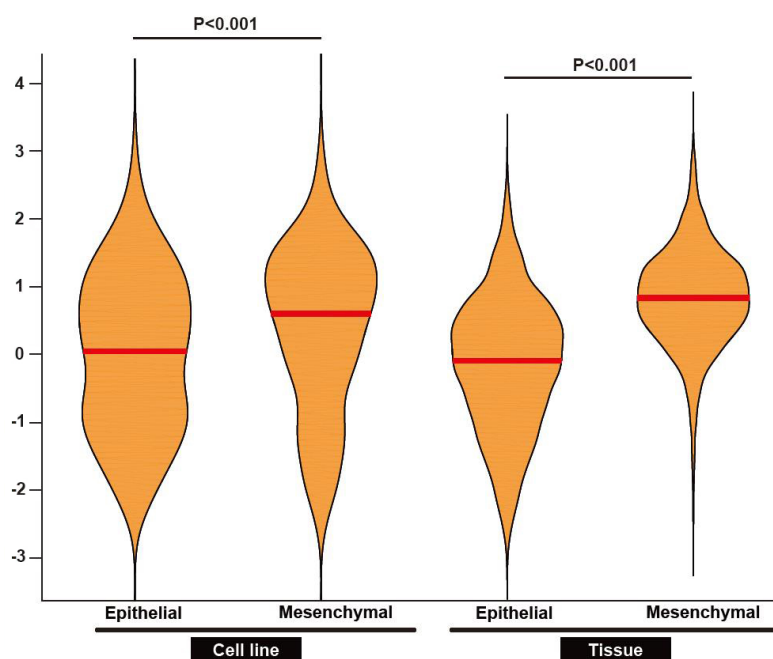


Figure 1. Neuroblastoma cells and tissues have a high mesenchymal gene signature. Violin plot representation of epithelial and mesenchymal gene expression in neuroblastoma cell lines ($n = 8$) and tissues ($n = 157$) is depicted. The red line represents the median. P value was determined by Mann-Whitney test

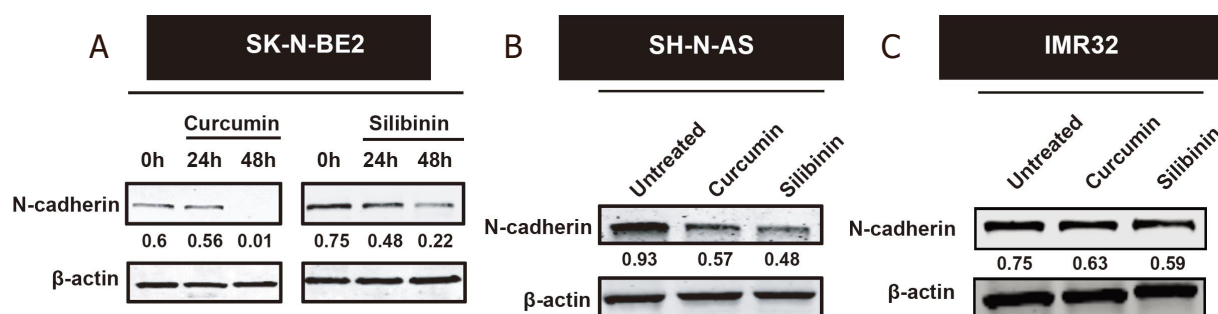


Figure 2. Curcumin and silibinin reduced the expression of the mesenchymal marker N-Cadherin. A: Western blot analysis for N-Cadherin in SK-N-BE2 cells; B: Western blot analysis for N-Cadherin in SH-N-AS cells; C: Western blot analysis for N-Cadherin in IMR32 cells. Drug concentration: 10 μ M curcumin or 100 μ M silibinin. β -actin was used the loading control. The N-cadherin band intensities are normalised to respective band intensities of β -actin

Curcumin and silibinin reduce the expression of mesenchymal marker N-Cadherin

If the mesenchymal nature of neuroblastoma cells account for the treatment resistance, induction of an epithelial phenotype by MET inducers could sensitise the neuroblastoma cells to doxorubicin. To test this hypothesis, MET inducers such as curcumin and silibinin were utilised in this study. It has been previously established that curcumin and silibinin can reverse EMT in certain cancer types^[44,45]. Consistent with the literature, treatment of the cancer cells with curcumin and silibinin reduced the expression of mesenchymal marker N-Cadherin [Figure 2A-C]^[46,47]. N-cadherin is a well-established mesenchymal marker that promotes cell motility and migration^[48]. The reduction of N-Cadherin levels was only observed at 10 μ M for curcumin and 100 μ M for silibinin. Epithelial markers E-Cadherin and Keratin 18 could not be detected in the neuroblastoma cells with or without the MET inducers. Similarly, the expression of another mesenchymal marker, Vimentin, did not change in the neuroblastoma cells treated with or without curcumin or silibinin (data not shown).

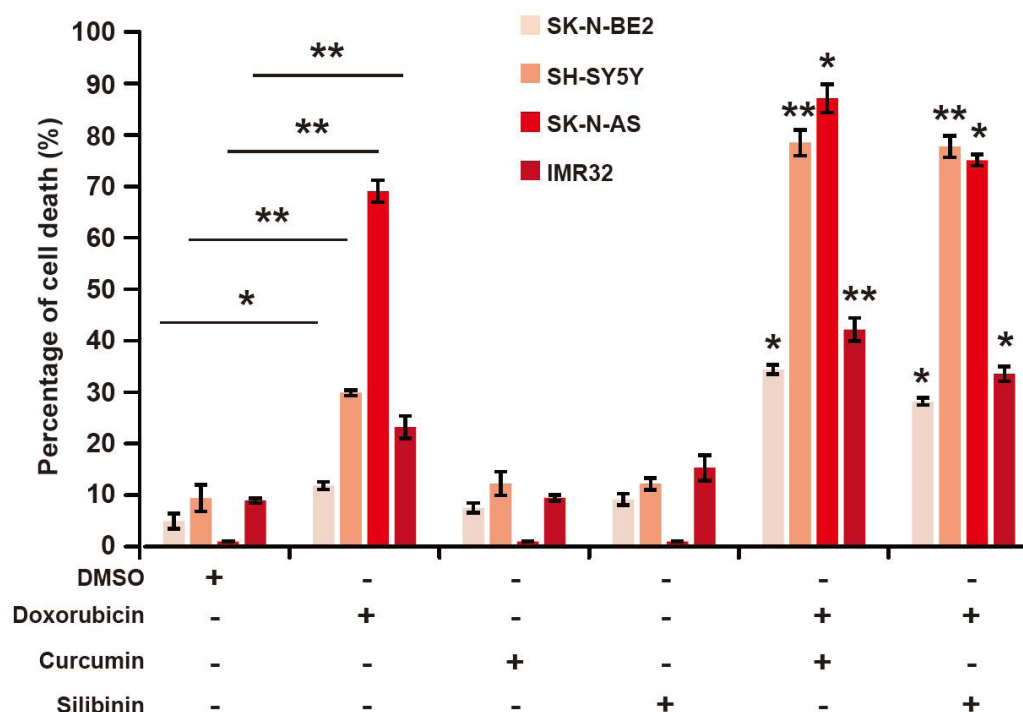


Figure 3. Curcumin and silibinin increases the sensitivity of neuroblastoma cells to doxorubicin. FACS cell death assay on a panel of neuroblastoma cells treated with 10 μ M curcumin or 100 μ M silibinin in the presence of 1 μ M doxorubicin ($n = 3$). Data are presented as mean \pm SEM, * $P < 0.05$; ** $P < 0.01$ as determined by Student's t -test. Significance of the percentage of cell death in combinational treatments was calculated in comparison to respective doxorubicin only treatments. DMSO: dimethyl sulfoxide; FACS: fluorescence activated cell sorting

Combinatorial treatment of curcumin or silibinin with doxorubicin sensitises neuroblastoma cells

Curcumin and silibinin are natural compounds that have many anti-cancer properties including induction of MET^[34-36]. As neuroblastoma cells, especially high-risk ones, are aggressive and resistant to treatment, the combinatorial effect of curcumin or silibinin with doxorubicin was examined to understand their therapeutic potential. A panel of four neuroblastoma cells, namely SH-SY-5Y, SK-N-AS, SK-N-BE2 and IMR32, was selected for the *in vitro* analysis. Among these neuroblastoma cells, SH-SY-5Y and SK-N-AS cells are low-risk and do not contain N-Myc amplification^[49]. On the contrary, SK-N-BE2 and IMR32 cells are high-risk neuroblastoma cell models with N-Myc amplification (> 0 copies)^[49]. Consistent with the literature, treatment of the low-risk neuroblastoma cells (SH-SY-5Y and SK-N-AS) with the chemotherapeutic agent doxorubicin (1 μ M) induced significant cell death [Figure 3]. Whilst the basal cell death for SK-N-AS cells was about 1%, incubation with doxorubicin induced nearly 70% cell death, an increase by 70-fold. Similarly, SH-SY-5Y cells exhibited more than three-fold cell death (30%) upon doxorubicin treatment. However, the high-risk neuroblastoma cells (SK-N-BE2 and IMR32) showed lesser percentage of cell death (11.8% and 23%, respectively) when incubated with the chemotherapeutic agent doxorubicin (1 μ M). This relates to a 2.3- and 2.6-fold increase in cell death in SK-N-BE2 and IMR32 cells, respectively.

Next, the combinatorial effect of curcumin or silibinin with doxorubicin was evaluated in the panel of neuroblastoma cells. As shown in Figure 3, neither curcumin (10 μ M) nor silibinin (100 μ M) induced significant cell death in any of the neuroblastoma cells. The concentration for curcumin and silibinin was chosen as they reduced the expression of N-Cadherin in neuroblastoma cells. When performing the

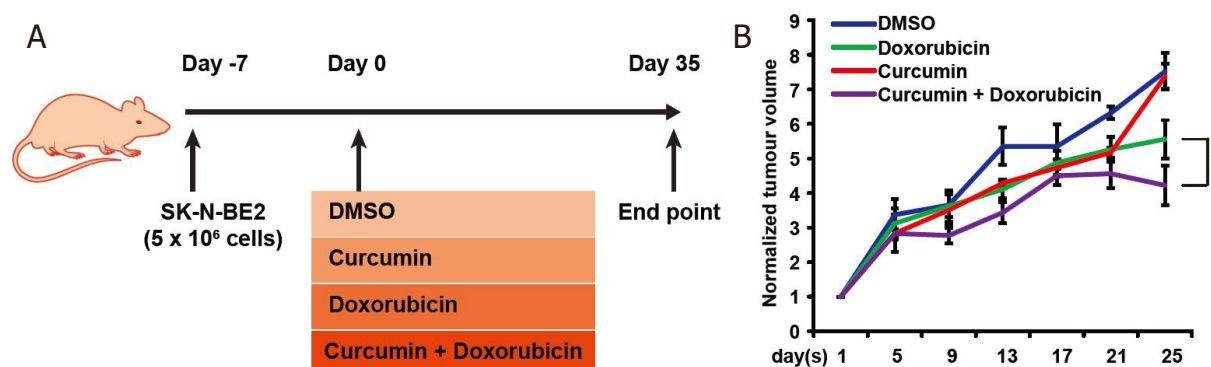


Figure 4. Combinatorial therapy reduces the tumour burden. A: schematic representation of *in vivo* work to examine the efficacy of combinatorial therapy on neuroblastoma tumour burden. B: relative tumour volume across a period in the presence of curcumin and/or doxorubicin ($n = 3$). Data are presented as mean \pm SEM, $*P < 0.05$ as determined by Student's *t*-test. DMSO: dimethyl sulfoxide

combinational treatments, the cells were treated with curcumin and silibinin 24 h prior to doxorubicin treatment to allow the induction of MET. Nevertheless, combination of curcumin with doxorubicin induced significant cell death in both the low- and high-risk neuroblastoma cells. Compared to the doxorubicin alone treatment, a 2.9-, 2.6-, 1.3- and 1.8-fold increase in cell death was observed in SK-N-BE2, SH-SY-5Y, SK-N-AS and IMR32 cells, respectively. Moreover, the Combination index (CI) values obtained for the treatments were < 0.1 , indicating a synergism. The CI values were 0.56 and 0.5 for SK-N-BE2 and SH-SY-5Y cells, respectively. Consistent with these results, combinatorial treatment of silibinin and doxorubicin also induced significant cell death, compared to doxorubicin alone, in the panel of neuroblastoma cells. Taken together, these data suggest that curcumin and silibinin can increase cell death induced by doxorubicin in both low- and high-risk neuroblastoma cells. The data also suggested that curcumin along with doxorubicin was more effective in inducing cell death in the neuroblastoma cells.

Combinational treatment reduced the tumour burden and increased the survival of mice implanted with neuroblastoma

Several studies have examined the effect of curcumin or silibinin on inducing cell death in neuroblastoma cells^[37]. However, the therapeutic potential has not been examined *in vivo*. Furthermore, it is unclear whether a combinatorial treatment that increases cell death in neuroblastoma cells also works *in vivo*. Hence, to validate the effect of combinatorial treatment *in vivo*, nude mice were injected with the high-risk SK-N-BE2 neuroblastoma cells (5×10^6) subcutaneously. N-Myc amplified SK-N-BE2 cells are more proliferative and resistant to doxorubicin treatment [Figure 3] than the other neuroblastoma cells used in this study, hence was chosen as a model cell line. After formation of tumours, the mice were administered (i.p.) with DMSO (control), doxorubicin, curcumin or a combination of doxorubicin and curcumin twice per week [Figure 4A]. Here, curcumin was chosen as the curcumin treatment had more effect on N-cadherin expression level [Figure 2A] and cell death [Figure 3] than silibinin. The tumour volume was monitored daily in the control and treatment groups. Tumour volume was the highest in mice that received DMSO or curcumin alone [Figure 4B]. Consistent with the *in vitro* data and literature, mice that were treated with doxorubicin alone had a significant decrease in the tumour volume. Encouragingly, the tumour volume in mice treated with both doxorubicin and curcumin was significantly smaller compared to those receiving only doxorubicin. There were no visible side effects from any of the treatments and no change in body weight of the mice was observed [Figure 5A]. Importantly, mice that received combinatorial treatment exhibited a higher survival rate [Figure 5B]. Taken together, these results suggest that treatment of curcumin in combination with a chemotherapeutic drug may be a viable strategy to treat neuroblastoma patients, most importantly high-risk neuroblastoma patients with N-Myc amplification.

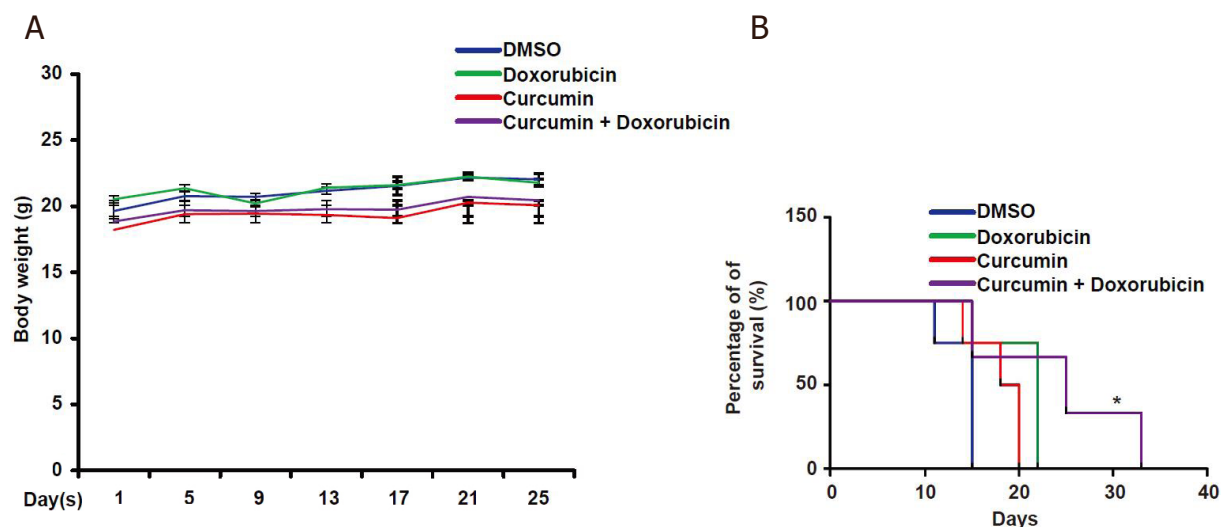


Figure 5. Combinatorial therapy increases the survival of mice implanted with neuroblastoma cells. A: graphical representation of body weight of mice over the period in the presence or absence of curcumin and doxorubicin ($n = 3$). Treatment with curcumin and doxorubicin does not impart visible side effects in mice. B: Kaplan-Meier analysis of the survival rate of mice bearing neuroblastoma tumour undergoing treatment. Combinatorial treatment significantly enhanced the survival of mice (* $P < 0.05$). DMSO: dimethyl sulfoxide

DISCUSSION

Neuroblastoma is the most common extracranial solid tumour in children under the age of five. Depending on risk factors, neuroblastoma can be divided into low- and high-risk^[50]. Even though the survival rates of low-risk neuroblastoma have improved significantly, the survival rates of high-risk neuroblastoma have remained poor^[51]. Among the risk factors, amplification of the oncogene N-Myc is detected in about 20% of neuroblastoma patients and considered high-risk^[52]. Hence, therapeutic strategies to manage high-risk N-Myc amplified neuroblastoma cells is needed.

Curcumin and silibinin have shown anticancer properties by modulating several signalling pathways^[34,36,53-55]. More importantly, it is documented that curcumin and silibinin have the ability to repress proteins that are involved in EMT and metastasis^[45,47,56]. We sought to elucidate the role of curcumin or silibinin in combination with doxorubicin.

First, the results from publicly available RNA-Seq analysis suggest that the mesenchymal-like phenotype exhibited by neuroblastoma cells could be one of the potential reasons for the aggressiveness. In the current study, we targeted the utility of curcumin or silibinin in sensitising neuroblastoma cells to doxorubicin. Combinatorial therapy of curcumin and doxorubicin sensitised the highly aggressive neuroblastoma cells both *in vitro* and *in vivo*. Here, we utilised four neuroblastoma cell lines, among which SK-N-BE2 and IMR32 possess N-Myc amplification and hence are categorised as high-risk aggressive neuroblastoma. N-Myc is not amplified in SH-SY-5Y and SK-N-AS cells, which were used to identify the usage of combinatorial therapy in a wider range of neuroblastoma cells.

Agreeing with the previous literature, curcumin and silibinin reduced the expression of the mesenchymal marker N-Cadherin and induced MET^[37]. However, the combinatorial effect with doxorubicin can also be attributed to other anti-cancer activities of these MET inducers, such as inhibition of p53, pAkt and STAT3 signalling pathway^[34,53,57]. Moreover, recent findings also suggest curcumin and silibinin as agents that can inhibit the cancer stem cells growth^[58,59]. Nevertheless, based on these results, curcumin and silibinin are interesting candidates for combination with standard chemotherapeutic drugs including doxorubicin for

neuroblastoma treatment. As mentioned above, despite aggressive therapy, the overall survival for high-risk neuroblastoma patients is < 50% at five years^[60]. In fact, the survival rate of relapsed stage 4 neuroblastoma patients as per the International Neuroblastoma Risk Group database between 1990 and 2002 is 8%^[61]. Alarming, the survival rate drops below 4% for N-Myc amplified neuroblastoma patients.

Hence, the findings from this study have clear potential therapeutic benefits to increase the survival of the high-risk neuroblastoma patients. Importantly, as curcumin and silibinin have been shown to be safe and well-tolerated in randomised clinical trials^[62-67], they can be readily utilised for neuroblastoma therapy.

DECLARATIONS

Author contributions

Conceived and directed the entire project: Mathivanan S

Performed the experiments: Fonseka P, Angela DG, Gangoda L

Performed bioinformatics analysis: Pathan M

Prepared the figures, drafted and finalized the manuscript with inputs from other authors: Fonseka P, Mathivanan S

Read and approved the manuscript: Fonseka P, Gangoda L, Pathan M, Angela DG, Mathivanan S

Availability of data and materials

Not applicable.

Financial support and sponsorship

Suresh Mathivanan is supported by ANZ Trustees and Ramaciotti Establishment Grant. Suresh Mathivanan is supported by Australian Research Council Future Fellowship (FT180100333) but the project was not funded by the basic science fellowship. Angela Di Giannatale is supported by a Grant from the Italian Ministry of Health (GR-2016-02364088). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Animal study was conducted according to Australian code of practice for the care and use of animals for scientific purposes and La Trobe Ethics Committee guidelines (AEC 14-15).

Consent for publication

Not applicable.

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Original Article

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Development of a Functional Assessment of Chronic Illness Therapy item library and primary symptom list for the assessment of patient-reported adverse events associated with immune checkpoint modulators

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How to cite this article: Webster KA, O'Connor ML, Hansen AR, Kircher S, Jim HSL, Dicker AP, Janda M, Ala-leppilampi K, Bingham CO III, Feliciano J, Henry NL, Steffen McLouth LE, Cella D. Development of a Functional Assessment of Chronic Illness Therapy item library and primary symptom list for the assessment of patient-reported adverse events associated with immune checkpoint modulators. *J Cancer Metastasis Treat* 2020;6:8. <http://dx.doi.org/10.20517/2394-4722.2019.38>

Received: 7 Oct 2019 **Accepted:** 24 Feb 2020 **Published:** 13 Mar 2020

Science Editor: Pravin D. Potdar **Copy Editor:** Jing-Wen Zhang **Production Editor:** Jing Yu

Abstract

Aim: To develop a comprehensive item library of patient-reported, immunotherapy-related adverse events (irAEs) that draws from and expands on the Functional Assessment of Chronic Illness Therapy (FACIT) Measurement System.



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Methods: Literature review and iterative expert input. Based on a literature review of irAEs, we developed a framework of immunotherapy classes and their associated symptoms. Clinical experts then reviewed iterations of symptom summaries and item maps linked to the immunotherapy framework. Experts provided content review and feedback was shared across experts until consensus was reached. The iterative process facilitated creation of a Primary Symptom List associated with immune checkpoint modulators (ICMs), drawn from the larger set of symptoms. Existing FACIT items were mapped to the symptom list, and new items were written as needed to create the item library.

Results: The full item library of irAEs is comprised of 239 items, covering 142 unique symptoms across 75 inflammatory reactions/immune conditions. A subset of 66 items comprises a Primary Symptom List considered most common/relevant to ICM treatment. This includes gastrointestinal, skin, pulmonary, neurologic, musculoskeletal, and multiple miscellaneous and constitutional symptoms.

Conclusion: The FACIT Immunotherapy Item Library is a compilation of 239 self-report items that capture the wide range of AEs experienced by people receiving immune treatments. A subset of 66 items comprises a Primary Symptom List meant for ICM therapy. Use of items selected from this library is encouraged in clinical research and clinical practice evaluation.

Keywords: Immunotherapy, immune checkpoint modulators, quality of life, immune-related adverse events, patient reported outcomes, cancer, oncology

INTRODUCTION

The emergence of immune checkpoint modulators (ICMs) in cancer treatment has produced both optimism and uncertainty among oncologists and patients. Over the last decade, the demonstrated efficacy of cytotoxic lymphocyte antigen-4 (CTLA-4) inhibitors, programmed cell death protein-1 (PD-1) inhibitors, and PD-1 ligand (PD-L1) inhibitors to induce prolonged responses in advanced cancers has been well-documented^[1-3]. Such results were first noted in the treatment of metastatic melanoma, and the United States Food and Drug Administration (FDA) has since approved the use of ICMs for the treatment of non-small cell lung cancer, renal cell carcinoma, Hodgkin lymphoma, urothelial carcinoma, head and neck cancers, and other tumors^[4,5]. A recent meta-analysis of randomized clinical trial data involving the use of anti-PD1/PD-L1 monoclonal antibodies to treat more than 6500 patients confirmed increased overall response rates when compared to usual care, including chemotherapy and targeted therapy^[6]. While the greatest gains were seen in patients with melanoma and those treated in the first-line setting, improved response rates associated with ICMs were seen across all tumor types. However, despite justifiable optimism surrounding ICMs and other biologic therapies, their relative success is tempered by uncertainty as to which patients are likely to benefit, and new challenges in the detection and management of a host of potential short- and long-term adverse events unique to immune-mediated therapy^[7]. Clinicians and patients discussing treatment options for advanced cancer must balance this ambiguity alongside their desire to pursue every possible path toward longer life^[8].

ICM treatment tolerability can complicate treatment decision-making, even for those patients who are promising candidates for ICM therapy. Immune-related adverse events (irAEs), including gastrointestinal, dermatologic, endocrinologic, cardiopulmonary, musculoskeletal, and other autoimmune complications, occur in many patients treated with ICMs^[4,9-15]. One study found that adverse events occur in up to 90% of patients treated with CTLA-4 and 70% of patients treated with anti-PD-1/PD-L1 agents^[13]. In trials using single anti-PD-1/PD-L1 agents, the rates of Grade 3 and 4 toxicities capable of hospitalizing patients and leading to treatment discontinuation ranged from 10% to 20%^[7]. IrAEs are diverse, can impact

almost any organ system, and can be potentially life-threatening (e.g., colitis and pneumonitis). They include the development of inflammatory and autoimmune conditions with unpredictable onset, as well as delayed and late effects that may persist long after treatment ends and require ongoing treatment with immunosuppressive agents in some cases^[5,16].

In the oncology community, recent efforts to catalog and classify ICM-associated toxicities and to set standardized management guidelines are helping treating clinicians understand the complexities of immunotherapy and to better diagnose and manage their patients' symptoms. Importantly, between 2017 and 2018, extensive guidelines for the management of immunotherapy-related toxicities were issued by the National Comprehensive Cancer Network in collaboration with the American Society of Clinical Oncology, the European Society for Medical Oncology, and the Society for Immunotherapy of Cancer Toxicity Management Working Group^[4,9,10]. Nevertheless, the era of immunotherapy is still in its infancy; more data are needed to understand risk factors, clinical phenotypes, and the nature, trajectory, and severity of short- and long-term immunotherapy-related toxicities, as well as the requirements for and responses to other immunosuppressive agents. These data are needed to more accurately inform clinicians and patients about how such toxicities affect the therapeutic risk-benefit ratio associated with ICMs. The availability of a patient-reported, targeted symptom assessment of irAEs for use in both the clinical trial and clinical practice settings would help address this important need.

Patient-reported outcome assessment in immunotherapy

Patient-reported outcome (PRO) data represent a rich and currently underutilized resource that can contribute significantly to our knowledge of patients' experiences with symptoms of cancer, its treatment, and impact on health-related quality of life (HRQOL). PROs are defined as "any report of the status of a patient's (or person's) health condition, health behavior, or experience with healthcare that comes directly from the patient, without interpretation of the patient's response by a clinician or anyone else"^[17]. In clinical trials, PROs enhance understanding of treatment toxicity, HRQOL, and treatment value^[18-22]. In clinical practice, PROs aid early symptom detection, symptom management, and treatment decision making^[23-27].

PRO measurement systems frequently used in oncology include the Functional Assessment of Chronic Illness Therapy Measurement System (FACIT), the European Organisation for Research and Treatment of Cancer (EORTC), and the Patient-Reported Outcomes Measurement Information System (PROMIS)^[28-30]. FACIT, EORTC, and PROMIS systems provide valid and reliable measurement of disease-related physical, functional, social, and emotional concerns and some treatment toxicities. More recently, Basch and colleagues developed the Patient-Reported Outcomes-Common Terminology Criteria for Adverse Events (PRO-CTCAETM) measurement system, a compendium of PRO items uniquely targeted to the assessment of symptomatic treatment-related toxicities in oncology care^[31]. PRO-CTCAE allows for the use of individually selected questions drawn from a pool of over 100 items organized into 14 National Comprehensive Cancer Network (NCCN)-designated toxicity domains, advancing the acceptability of customizable forms. Although these measurement systems have advanced our understanding of treatment toxicity and HRQOL, they were developed prior to the widespread use of ICMs. To date, there are no established PRO measures that assess the full range of common and unique symptoms of AEs related to ICM treatment^[32,33].

We therefore set out to develop an inclusive list and a library of patient-reported items that assess adverse events associated with immunotherapy, intended to be conceptually and structurally similar to the PRO-CTCAE, enabling selective assessment of subsets for specific use. In this paper, we report on the development of a conceptual framework and extensive FACIT Immunotherapy Item Library. We also describe the parallel development of a Primary Symptom List, a representative subset of library items selected by our expert panel via a modified Delphi technique. The FACIT Immunotherapy Item

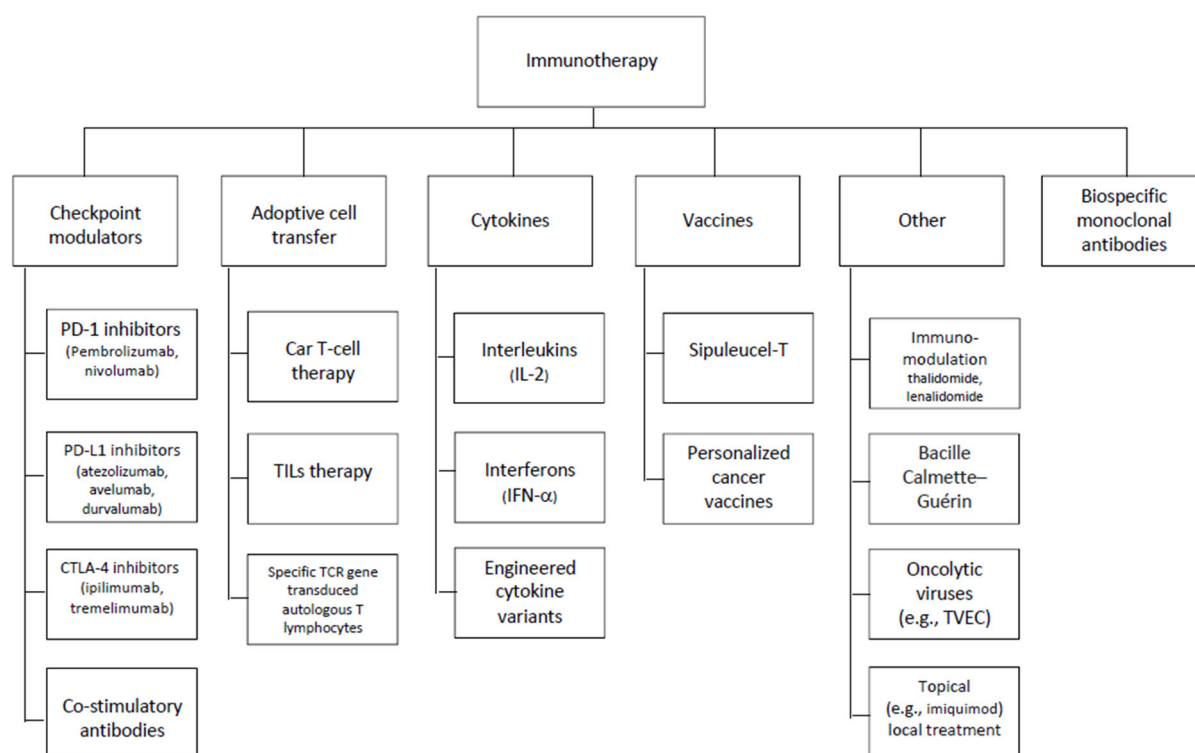


Figure 1. Classes of immunotherapy

Library offers targeted assessment options, enables custom form creation, and has the flexibility needed to accommodate new content in order to stay current with the changing landscape of immunotherapy treatment.

METHODS

A consortium of investigators across several institutions, represented by the author list, convened around a commitment to produce a set of PRO items to assess symptoms associated with immunotherapy. [Figure 1](#) depicts the range of immunotherapies we considered. The array of treatments covered by [Figure 1](#) produced a large number of candidate adverse events to capture, leading us to recognize that a modular approach to assessment would be most practical, perhaps even necessary, for most applications.

Although our ultimate goal is to catalogue immunotherapy toxicities generally (and as depicted in [Figure 1](#)), we began with where the most experience and interest lies, namely the adverse effects associated with immune checkpoint modulators (ICMs; [Figure 1](#), Column 1). We therefore expected this would be an exercise in identifying common toxicity profiles and their associated symptoms, followed by a prioritizing of item content. We reviewed published literature to inform an immunotherapy construct definition and conceptual framework. We mapped identified symptom experiences and impact onto previously developed survey items from the FACIT Measurement System, modifying existing items and drafting new items as necessary. This process produced a working version of an irAE item library. A shorter list of the more common and clinically relevant symptoms was developed based on iterations of symptom review and feedback from an expert panel, and then incorporated into the library. [Figure 2](#) depicts our development process, representing parallel tracks of literature review and expert input.

Expert input

A convenience sample of clinicians and researchers whose expertise aligned with project goals was identified to provide ongoing input and guidance during the ICM Primary Symptom List development.

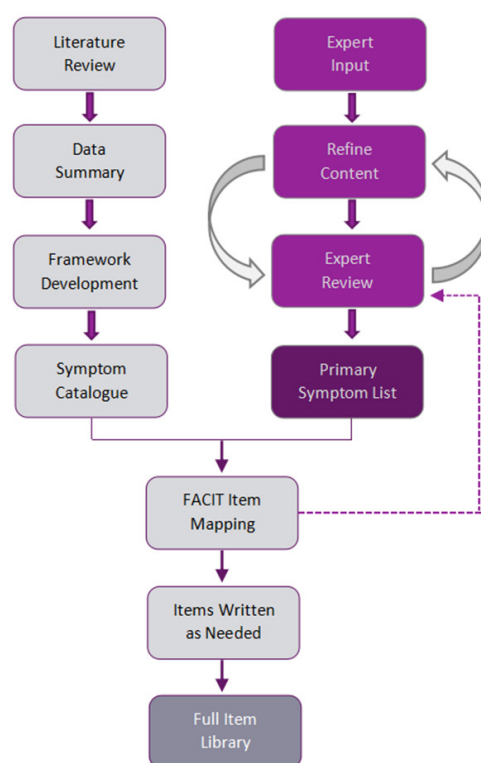


Figure 2. Development process. FACIT: Functional Assessment of Chronic Illness Therapy

Experts with clinical experience treating patients with ICM therapies or researching ICM-related AEs were included in an advisory panel. We used panel input to compile an initial list of commonly seen and/or clinically relevant ICM-related adverse event symptoms. Then, we used iterations of symptom summaries, item mapping, content review, and feedback (informally through e-mail and formally via online surveys) for consensus building and to facilitate selection of final items for the Primary Symptom List. We used a modified Delphi technique for formal online surveying. The modified Delphi technique allows for consensus building through a sequence of two or more rounds of panel member review and feedback until sufficient agreement has been obtained regarding priority content^[34,35].

Literature review

The goals of the literature review were: (1) to select recently issued guidelines for the identification and management of irAEs to help guide the development of a conceptual framework; (2) to identify ICM-related toxicity profiles and their associated organ-system based conditions; and (3) to catalogue and summarize key patient symptoms from the current literature on ICM therapies. As our goal was to capture a representative range of information to inform the conceptual framework and document treatment-related toxicities, we conducted a targeted literature review. Our main data sources were PubMed, Embase, and CINAHL. The search strategy utilized keyword terms associated with ICMs, cancer, and toxicities combined with Boolean operators (“OR” and “AND”) to identify immunotherapy keywords in the titles and abstracts of articles. We also searched select textbooks and reputable websites (e.g., those of the American Society of Clinical Oncology, the National Comprehensive Cancer Network, and the European Society for Medical Oncology) for articles or chapters relating to ICM side effects and their clinical management. Inclusion criteria for relevant articles or chapters included those written in English and published in peer-reviewed journals within the last five years or in similarly recent authoritative medical textbooks. Articles and chapters that fit the inclusion criteria were assigned to a team member for full text review, and relevant content was summarized in an Excel spreadsheet.

Framework development

For framework guidance, we reviewed publications related to recently issued guidelines for the identification and management of irAEs. We selected seminal publications, highlighted key thematic information from each, and organized our framework accordingly. Collectively, this provided a conceptual framing for PRO measurement in the context of a complex matrix of ICM-related adverse events.

Data summaries

From our literature review, we extracted all relevant information regarding ICM-induced inflammatory reactions and immune conditions, as well as their associated toxicity profiles and symptomatology. Data were summarized in Excel spreadsheets, which allowed for detailed descriptions of symptom experience. We added expert-identified symptoms to the composite and highlighted these for Primary Symptom List consideration. Symptoms identified for possible inclusion in the library were reviewed by a team of experts with extensive experience in PRO measure development for relevance, redundancy, and appropriateness as measured by self-report. Symptoms for the Primary Symptom List were iteratively reviewed by our expert panel. Redundant or similar symptoms were collapsed, and symptoms that could not be measured via patient-report were excluded.

Item mapping

Once a comprehensive list of symptomatology had been finalized, we organized immune conditions (inflammatory responses and autoimmune syndromes) and symptom profiles according to our conceptual framework. We then mapped symptoms onto existing items in the FACIT Measurement System via the FACIT Item library (<https://wizard.facit.org>), filling gaps by modifying existing items when possible and writing new items as necessary^[28,36]. The FACIT Measurement System includes over 100 distinct self-report questionnaires that assess a wide variety of disease- and treatment-related symptoms, functional abilities (physical, mental, and social), general perceptions of health and well-being, and other aspects of health-related quality of life. The FACIT item library is a collection of more than 700 unique health-related PRO questions that appear in the FACIT Measurement System. Most FACIT items have demonstrated face and content validity, and were created with direct input from patients and expert clinicians. Many items have also been translated into up to 70 languages using a standardized, rigorous translation methodology^[37,38] and tested for comprehension by native speakers.

For the immunotherapy library, our team identified the most suitable item for symptom assessment, and then included other items related to impact or bother as appropriate. For the Primary Symptom List, when more than one FACIT item was available, our expert panel assisted in selecting the top 1-3 items targeted to symptom assessment in the setting of ICM therapy.

RESULTS

Expert input

Our panel of experts was tasked with identifying a Primary Symptom List of ICM-related adverse events. Experts were MDs ($n = 7$), PhDs ($n = 4$), and a PhD candidate ($n = 1$), with 2-8 years of experience treating cancer patients with ICM therapies and/or researching the incidence, nature, and HRQOL impact of irAEs in the context of collaborative clinical and translational trials.

Experts identified an initial list of 61 commonly seen or clinically relevant irAE symptoms for ICM Primary Symptom List inclusion. We then mapped symptoms onto existing items in the FACIT item bank, which included those that address symptom presence, symptom bother and/or emotional or functional impairment associated with symptom experience (e.g., “I have stiffness or tightness in my joints” and “Joint stiffness limits my usual activities”). Mapped irAE symptoms yielded 153 unique FACIT items.

Table 1. Primary symptom list

I have diarrhea (diarrhoea)	I have pain in my chest
I have to limit my activities because of diarrhea (diarrhoea)*	I feel lightheaded (dizzy)
I must move my bowels frequently to avoid accidents*	My eyesight is blurry
I am constipated	I have trouble with coordination
I am bothered by reflux or heartburn	I am able to maintain my balance*
I have pain in my stomach area	I have pain in my joints
Stomach pain interferes with my daily functioning*	I have stiffness or tightness in my joints
I have swelling in my stomach area	Joint stiffness or tightness limits my usual activities
I feel bloated*	Joint pain limits my usual activities
I have nausea	I have weakness in my arms or legs
I have been vomiting	I am bothered by muscle pains
I have noticed blood in my stool	I am bothered by swelling in certain areas of my body
I have a loss of appetite	I get headaches
I feel fatigued	I am bothered by headaches*
My fatigue keeps me from doing the things I want to do*	I urinate more frequently than usual
I have a lack of energy	I am losing weight
I feel tired*	I am bothered by a change in weight*
My skin (or scalp) itches	I have had fevers
My skin (or scalp) is dry or “flaky”	I am bothered by fevers (episodes of high body temperature)*
I am bothered by dry skin	I have had chills
I am bothered by cracking or peeling of my skin	I am bothered by chills*
I am bothered by blistering of my skin	I feel nervous
I am bothered by vitiligo (white patches on my skin)	I have episodes of heart racing
The skin on my feet hurts	I am bothered by sweating
Pain on the bottom of my feet interferes with my walking	My eyes are dry
I have mouth sores	My eyes feel sandy or gritty
I am bothered by a skin rash	My mouth is dry
The skin on my hands hurts	My mouth and throat are dry*
I am bothered by a change in my skin’s sensitivity to the sun	I am bothered by dry mouth*
I have pain in my hands or feet when I am exposed to cold temperatures	I have pain in my sinus area
I have been short of breath	I am bothered by side effects of treatment
I have been coughing	I am bothered by new allergy-like reactions (e.g., to foods, insects, pollen)
I have been wheezing (whistling sound when I breathe)	I am bothered by short-term treatment reactions that I experience immediately after, or within 24 h of, an infusion (such as chills, dizziness, hives, rashes lasting no more than 24 h)

*Designates a supplemental item

For symptoms where there was no corresponding FACIT item, we adapted a similar, existing item (e.g., replaced “eyes” with “mouth” for item HN3 “My mouth is dry”) or wrote a new item [e.g., “I am bothered by vitiligo (white patches on my skin)”].

We then asked our expert panel to review selected PRO items, and identify the most relevant ones for ICM treatment assessment. We used consensus and input from our internal team of measure developers to refine the list to 47 items covering 32 symptoms, and then sent it back to the panel members for a second iteration of review. During this review our panel helped: (1) identify items that were a misfit or symptoms still missing from the list; (2) confirm that item text targeted desired symptom assessment; and (3) refine item wording for newly written items as needed. Again, we used consensus and internal team input to hone the list to 53 primary and 13 supplemental items, and then sent it back to our panel for one final review to confirm content and identify any lingering item(s) that should be omitted or symptom(s) that should be included.

The final Primary Symptom List includes 66 items assessing 48 symptoms deemed common or clinically relevant to ICM adverse event symptom monitoring. Symptoms include: gastrointestinal (diarrhea, constipation, reflux/heartburn, abdominal pain, abdominal swelling, abdominal bloating, nausea, vomiting,

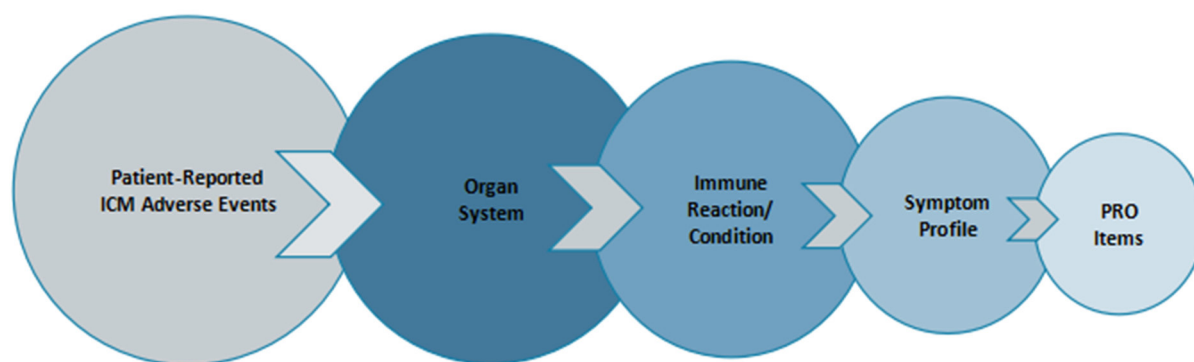


Figure 3. Conceptual framework. PRO: patient-reported outcome; ICM: immune checkpoint modulators

blood in stool, and mouth sores); skin (rash, itch, dryness, cracking/peeling, blistering, vitiligo, sensitivity to sun, and pain); lung (shortness of breath, cough, wheezing, and chest pain); neurologic (coordination and balance); musculoskeletal (joint pain, joint stiffness, swelling, muscle weakness, and muscle pain); and miscellaneous/constitutional (fatigue, headache, weight gain, weight loss, appetite, lightheaded/dizziness, blurry vision, urinary frequency, fevers, chills, nervousness, palpitations, sweating, dry mouth, dry eyes, sandy/gritty eyes, sinus pain, incidence of new allergies, and acute treatment reactions). Of the 66 items, 53 are primary and 13 supplemental [Table 1]. All symptoms are part of the larger FACIT Immunotherapy Item Library.

Conceptual framework development

To inform our conceptual framework, we used recently issued guidelines for the identification and management of immunotherapy-related toxicities from The American Society of Clinical Oncology, in collaboration with the National Comprehensive Cancer Network, the European Society for Medical Oncology, and the Society for Immunotherapy of Cancer Toxicity Management Working Group^[9,10,39]. Collectively, these guidelines identify primary ICM-related immune conditions by organ system, highlighting key symptoms for each, which we then used as the organizing conceptual structure of our immunotherapy library. The content of the item library includes a taxonomy of patient-relevant endpoints according to: organ system > immune reaction/condition > symptom profile > PRO questions [Figure 3].

Literature review

Our literature review yielded 25 articles documenting irAE incidence, management guidelines, toxicity profiles, and associated symptomatology^[4,9,11-15,40-57]. From these, we identified 75 possible inflammatory reactions/immune conditions across 11 organ systems, ranging from very common to rare. Organ systems were categorized as: (1) cutaneous; (2) gastrointestinal; (3) hepatic; (4) lung; (5) endocrine; (6) musculoskeletal; (7) renal; (8) nervous; (9) hematologic; (10) cardiovascular; and (11) ocular. Identified inflammatory reactions/conditions and their symptom profiles were summarized, and all associated symptoms measurable by patient report were retained for PRO item mapping or development [Table 2]. We identified a total of 142 unique symptoms, several of which overlap immune conditions. Symptoms include: gastrointestinal [abdominal bloating, abdominal fullness, abdominal pain, abdominal swelling/distention, pain radiating to the back, blood in stool, diarrhea, frequent bowel movements, constipation, heartburn (burning chest pain), regurgitation, nausea, vomiting, vomiting blood, blood in stool, rectal bleeding, and mouth sores]; skin (rash, rash-itch, rash-blister, rash-pain, rash-burning, skin depigmentation, skin itch, skin dryness, skin cracking/peeling, skin blisters, skin pain, skin thickening, skin thinning, nail pitting, nail ridging, touch sensitivity, sun sensitivity, hair loss, and face reddening); lung (shortness of breath, shortness of breath lying down, cough, cough lying down, wheezing, chest pain, chest pressure, and edema); neurologic (coordination, balance, memory, confusion, disorientation, eyelid droop, facial droop,

Table 2. ICM-related inflammatory reactions/autoimmune conditions and their associated symptoms

Organ system (n = 11)	Inflammatory reaction/condition (n = 75)	Symptoms (n = 142 unique symptoms)
Cutaneous	Rash; pruritus; psoriasis; pitted or ridged nails; vitiligo; Steven's Johnson's syndrome; Lyell's syndrome (toxic epidermal necrosis); alopecia; Sweet syndrome	Rash, rash-pain, rash-blister, rash-itch, skin-itch, skin-dry/cracked, skin-peeling, skin-burning, skin-thickened, nails-pitted, nails-ridged, skin-depigmentation, fever, skin-pain, mouth-blisters, mouth-pain, malaise, hair loss
Gastrointestinal	Colitis; pancreatitis; stomatitis/oral mucositis; lichenoid mucositis; esophagitis; gastritis; celiac disease; reflux; cholangitis; intestinal perforation; cholecystitis; gastric hemorrhage; paralytic bowel obstruction	Diarrhea, abdominal-pain, weight-loss, fever, nausea, vomiting, blood in stool, fatigue, pain radiating to the back, shortness of breath, mouth-sores, mouth-pain, taste changes, swallowing-difficulty, swallowing-pain, chest-pain (burning), heartburn, abdominal-fullness, appetite-loss, abdominal-bloating, regurgitation, skin-itchy, eyes-itchy, mouth-dry, urinary-infrequency, thirst-increased, abdominal-distention, sensitivity-food, vomiting-blood, rectal bleeding, constipation
Hepatic	Hepatitis; acute liver failure	Fatigue, abdominal-pain, joint-pain, jaundice, abdominal-swelling, nausea, vomiting, malaise, disorientation, confusion, sleepiness, dark urine
Renal	Interstitial nephritis; nephrotic syndrome; acute kidney injury	Weight-gain, edema, fever, urine-blood, nausea, vomiting, urinary-frequency, urinary-infrequency, urine-foamy, appetite-loss, shortness of breath, fatigue, confusion, weakness
Lung	Pneumonitis; pleuritis; sarcoidosis/sarcoid-like granulomatosis	Shortness of breath, cough, fatigue, appetite-loss, weight-loss, chest-pain, fever, joint-pain, joint-swelling, lymph swelling, rash, vision-blurred, eyes-pain
Endocrine	Hypophysitis/hypopituitarism; primary hyperthyroidism; primary adrenal insufficiency; hyperglycemia/autoimmune diabetes; diabetic ketoacidosis; primary hypoparathyroidism; thyrotoxicosis/hyperthyroidism; Cushing's syndrome	Headache, fatigue, muscle-weakness, nausea, appetite-loss, weight-loss, vision-blurred, intolerance-cold, intolerance-heat, constipation, skin-dry, weight-gain, muscle-pain/ache, joint-pain, joint-stiffness, joint-swelling, depression, memory impairment, abdominal-pain, nausea, vomiting, diarrhea, lightheadedness/dizziness, thirst-increased, urinary-frequency, appetite-increase, irritability, anxiety, palpitations, bone pain, sweating-excessive, bowel-frequent movements, bruising-easy, face-reddening, skin-thinning, moon face appearance, mood changes
Musculoskeletal	Myalgia; arthralgia; inflammatory arthritis; dermatomyositis; myopathy; inflammatory myositis; polymyalgia rheumatica; Sicca-Sjogren's like syndrome; sarcoidosis-like reactions	Muscle-pain/ache, joint-pain, joint-stiffness, joint-swelling, joint-decreased range of motion, skin-swelling, back pain, skin-rash (violet or red), skin-itch, skin-pain, sensitivity-touch, muscle-weakness, muscle-soreness, fatigue, swallowing-difficulty, shortness of breath, joint-ache, groin-pain, fever (low-grade), malaise, depression, appetite-loss, weight-loss, mouth-dry, face-pain, tooth-decay, mouth-pain, eyes-dry, taste changes, shortness of breath, lymph swelling, cough, wheezing, eyes-pain, eyes-redness, functional interference/eat and sleep interference
Nervous	Neuropathy; Guillain-Barre syndrome; myelopathy; transverse myelitis; meningitis; encephalitis; myasthenic syndrome/myasthenia gravis; facial nerve palsy	Numbness, pain, pain-burning (nerve), muscle-weakness, coordination-difficulty, paralysis, intolerance-heat, sweating-excessive, sweating-decreased ability, dizziness/lightheadedness, tingling, numbness, balance-problems, urine-loss of control, bowel-loss of control, sensitivity-touch, fever, headache, stiff neck, nausea, vomiting, confusion, sleepiness, sensitivity-light, muscle-pain/ache, joint-ache, fatigue, eyelid-drooping, muscle-weakness (that is better with rest), vision-double, swallowing-difficulty, voice changes, drooling, facial droop, jaw pain, taste changes
Hematologic	Hemolytic anemia; aplastic anemia; idiopathic thrombocytopenia purpura; pancytopenia; hemophilia	Fever, weakness, dizziness, confusion, urine-blood, paleness, jaundice, palpitations, bruising-easy, bleeding-pinpoint (petechiae), bleeding-easy, bleeding-gum, bleeding-nose, blood in stool, fatigue, shortness of breath, joint-pain, joint-swelling, lightheadedness/dizziness
Cardiovascular	Myocarditis; vasculitis/temporal arteritis; interstitial lung disease; pericarditis; cardiomyopathy; heart failure; pericardial effusion; Raynaud's phenomenon	Chest pain, fatigue, shortness of breath, edema, palpitations, fever, headache, weight-loss, muscle-pain/ache, muscle-pain, sweating-nocturnal, rash, numbness, weakness, tingling, cough-dry, shortness of breath-lying down, cough-lying down, abdominal-swelling, chest-pressure, lightheadedness/dizziness, wheezing, weight-gain, urinary frequency-nocturnal, chest-fullness, fingers-cold, toes-cold, functional impairment such as reduced exercise intolerance
Ocular	Uveitis/choroiditis; conjunctivitis; scleritis; episcleritis; blepharitis; retinitis; orbital myositis	Eyes-redness, eyes-pain, sensitivity-light, vision-blurred, vision-floaters, vision-decreased, eyes-itchy, eyes-gritty, eyes-crusty, eyes-watery, eyes-pain with eye movement, eyelids-itchy, eyelids-red/swollen, vision-loss, vision-impaired color vision, vision-double, eyelid-drooping

ICM: immune checkpoint modulators

face pain, stiff neck, numbness, tingling, burning pain, back pain, paralysis, sweating-decreased ability, muscle-weakness, sleepiness, drooling, double vision, light sensitivity, jaw pain, voice changes, swallowing difficulty/choking, bladder control, and bowel control); musculoskeletal (joint pain, joint stiffness, joint swelling, muscle pain/soreness, decreased range of motion, functional interference, and bone pain); eyes (eye

pain, eye itch, eye redness, dry eyes, sandy/gritty eyes, crusty eyes, watery eyes, pain with eye movement, eyelid swelling, and eyelid itch); hematologic (bleeding, bleeding easily, bleeding gums, bleeding nose, pinpoint bleeding-petechiae, bruising, bruising easily, and lymph swelling); mood (depressed, nervous/anxious, irritable, and mood changes); and miscellaneous/constitutional (general pain, fatigue, headache, weight loss, weight gain, appetite increase, appetite decrease, lightheaded/dizziness, malaise, weakness, paleness, jaundice, fevers, low-grade fever, chills, sweating excessive/nocturnal, palpitations, dry mouth, taste changes, thirst, sinus pain, blurred vision, decrease vision, loss of vision, vision floaters, impaired color vision, pain radiating to the back, swelling, urinary frequency, urinary infrequency, foamy urine, blood in urine, dark urine, tooth decay, moon face appearance, heat intolerance, cold intolerance, food sensitivity, cold fingers, cold toes, groin pain, new allergies, and acute treatment reactions).

Item mapping

For the FACIT Immunotherapy Item Library, 142 unique symptoms were identified for item mapping, which included 48 from the Primary Symptom List. Once symptom content had been fully determined, the study team met to align symptoms with candidate items from the overall FACIT item library. We identified all items related to a given symptom (e.g., for fatigue, there were 16 items covering fatigue experience and impact), and then selected the one(s) that best captured symptom experience or impact related to ICM therapy. We used descriptions from experts and the literature to corroborate item selection. This process yielded a final total of 239 PRO items retained for immunotherapy item library inclusion, which includes a subset of 66 items from the Primary Symptom List. The full set of immunotherapy items can be found at <https://wizard.facit.org/>.

DISCUSSION

The FACIT Immunotherapy Item Library was developed with input from the literature, clinicians, and researchers and constitutes the first published compendium of PRO items targeting the vast array of irAEs in ICM therapy. Library content is aligned with current practice guidelines and conceptually organized by organ system, inflammatory reaction/condition, and their associated toxicity symptom profiles. PRO items address adverse events associated with immunotherapy generally, and a subset of items for ICM treatments, which include the related functional, social, and/or emotional impact of those symptoms when applicable. Use of items from the widely-used FACIT Measurement System ensures PRO assessment based on well-validated items that have undergone extensive face and content validity, are written at the sixth grade reading level or less, and are available in as many as 70 languages. All items use a standard five-point Likert scale (0 = not at all; 1 = a little bit; 2 = somewhat; 3 = quite a bit; and 4 = very much) and seven-day recall period.

Library format permits for custom form development, as well as expansion based on new or combination therapies and into other immunotherapy classifications. Asking every question in the library would of course be burdensome to a patient in clinical research, especially when other questionnaires are also likely to be desired to measure disease-related symptoms, function, health perceptions, and quality of life. We advocate for the thoughtful, judicious use of subsets of these questions, assembled by a survey builder (FACIT Build-a-PRO; <https://wizard.facit.org/>), to track the most likely or relevant adverse events in a given trial. This practice of custom building has been recommended for use of the PRO-CTCAE^[31] and we endorse that same approach here. Users can draw items from the full library ($n = 239$) or select from a targeted subset of expert-endorsed items (Primary Symptom List; $n = 66$). The Functional Assessment of Cancer Therapy-Immune Checkpoint Modulator (FACT-ICM) is also newly available from Hansen and colleagues - a 25-item toxicity subscale for patients receiving ICM therapy^[58]. The FACT-ICM was developed using a mixed methods approach and is designed to assess HRQOL in the context of ICM irAEs, providing value in research and clinical settings related to symptom assessment and quality of life. Hansen

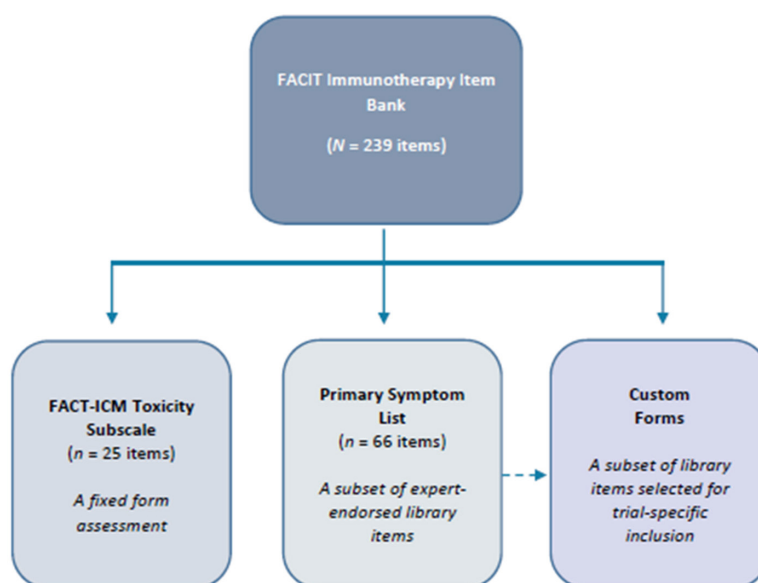


Figure 4. Current FACIT immunotherapy assessment options. FACIT: functional assessment of chronic therapy

and colleagues offer users a fixed-form assessment, developed in accordance with FDA PRO guidelines, with items that are included in the overall item library^[17,58]. A second initiative by Jim and colleagues aims to develop a FACT measure with items reflective of common patient-reported toxicities in lung cancer patients receiving immunotherapy^[59]. They have analyzed PRO and other data from the Addario Lung Cancer Foundation international patient registry to describe patient-reported toxicities and quality of life outside the context of a clinical trial in lung cancer patients treated with immune checkpoint inhibitors. Figure 4 depicts current FACIT immunotherapy adverse event assessment options.

In conclusion, using literature review, expert consensus, and previously developed items from the FACIT Measurement System, we developed an item library that collectively targets full-spectrum PRO assessment of ICM-related adverse events. Library item selection for tailored forms allows for targeted, concise, clinically relevant measurement of symptoms and concerns to monitor when assessing the value of ICM treatment for patients with cancer. Collection of PRO data in the emerging field of immuno-oncology will make it possible to more accurately assess the added adverse event burden associated with new combination regimens and support longitudinal studies to evaluate the long-term effects of both single-agent and combination therapies. At the point of care, PRO data can support shared decision-making between clinicians and patients, and facilitate early detection of, and communication among, the wide range of specialists required to provide comprehensive care for immunotherapy-related adverse events. Direct patient report of immunotherapy-related adverse events may help to identify early symptoms before clinical signs are manifest to the clinician. For example, patients report joint pain and stiffness prior to clinical recognition of myalgia/arthralgia. Earlier detection and recognition of these adverse effects may enable early symptom management and/or treatment modifications that contribute to better quality of life over time.

DECLARATIONS

Acknowledgments

We gratefully acknowledge Dr. Patricia Ganz and Dr. Janet Parsons for their contributions to this project.

Authors' contributions

Contributed to the design, analysis and interpretation of the results: Webster KA, O'Connor ML, Cella D
Contributed to the item review procedures and the writing and editing of the manuscript: Webster KA,

O'Connor ML, Hansen AR, Kircher S, Jim HSL, Dicker AP, Janda M, Ala-leppilampi K, Bingham CO III, Feliciano J, Henry NL, Steffen McLouth LE, Cella D

Availability of data and materials

Not applicable.

Financial support and sponsorship

Laurie E. Steffen McLouth was supported by NCI R25 CA122061 (PI: Avis); Adam P. Dicker received additional support from the National Cancer Institute, the Prostate Cancer Research Program (Dept. of Defense), the American Society of Radiation Oncology, the American Society of Clinical Oncology, NRG Oncology, and the Prostate Cancer Foundation; Josephine Feliciano received additional support from Bristol Myers Squibb and Astra Zeneca.

Conflicts of interest

Heather S.L. Jim is a consultant for RedHill BioPharma Ltd and Janssen Scientific Affairs; Aaron R. Hansen is a consultant for Merck (compensated) and GSK (compensated), and has received grant/research support from (Clinical Trials for institution) from Genentech/Roche, Merck, GSK, Bristol-Myers Squibb, Novartis, Boston Biomedical, Boehringer-Ingelheim, AstraZeneca, Medimmune, Janssen, Karyopharm, and MacroGenics; David Cella is the President and owner of FACIT.org; Kimberly A. Webster is an owner of FACIT.org; Clifton O. Bingham III is a consultant to Bristol Myers Squibb, Regeneron/Sanofi, and Genentech/Roche, and has received grant support from Bristol Myers Squibb; Adam P. Dicker is an advisor for Roche, EMD Serono, Celldex, Janssen, Cybrexa, Self Care Catalysts, Oncohost, ThirdBridge, and Noxopharm (compensated), and to Google LaunchPad Accelerator, Dreamit Ventures, and Evolution Road (uncompensated). He has also provided expert testimony for Intellectual property (Wilson Soncini); Josephine Feliciano is a consultant to Astra Zeneca, Genentech, Eli Lilly, Pfizer, and Merck. Sheetal Kircher, Monica Janda, Laurie E. Steffen McLouth, N. Lynn Henry, Mary O'Connor and Kari Ala-leppilampi declare no relevant interests, financial or otherwise.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Technical Note

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Cytoreductive surgery with hyperthermic thoracoabdominal chemotherapy in stage IV ovarian cancer - a technical description

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How to cite this article: Ray MD, Kuppusamy R, Kumar N, Garg R. Cytoreductive surgery with hyperthermic thoracoabdominal chemotherapy in stage IV ovarian cancer - a technical description. *J Cancer Metastasis Treat* 2020;6:9.
<http://dx.doi.org/10.20517/2394-4722.2020.16>

Received: 18 Feb 2020 **First Decision:** 23 Mar 2020 **Revised:** 26 Mar 2020 **Accepted:** 3 Apr 2020 **Published:** 17 Apr 2020

Science Editor: Pravin D. Potdar **Copy Editor:** Jing-Wen Zhang **Production Editor:** Tian Zhang

Abstract

Systemic chemotherapy for peritoneal disease in ovarian carcinoma is associated with a recurrence rate of more than 75%, and most of the cases are confined to the peritoneal cavity. The propensity of locoregional treatment failure has paved the way for the discovery of cytoreductive surgery with intra-cavitary chemotherapy. Cytoreductive surgery (CRS) is the present-day treatment modality for a variety of peritoneal carcinomatosis including ovarian cancer, and multi-visceral resection is critical for completion of CRS. In cases of diaphragmatic infiltration by tumor deposits, partial resection leads to a diaphragmatic rent, which can be used for the perfusion of chemotherapeutic drugs into the pleural cavity. Disease transmission from the peritoneal to pleural cavity is a poor prognostic factor however. Hence, intrathoracic hyperthermic chemotherapy may be a reasonable treatment option for ovarian carcinoma with malignant pleural effusion or pleural deposits. Hyperthermic intraperitoneal chemotherapy (HIPEC) is added to the treatment plan in cases of complete CRS but this is a technically demanding procedure. Therefore, performing hyperthermic intrathoracic chemotherapy on top of CRS and HIPEC may be even more complicated for such advanced cancers. The technique of combining HIPEC and hyperthermic intrathoracic chemotherapy is also commonly known as hyperthermic thoracoabdominal chemotherapy (HITAC). The perioperative morbidity and mortality may be remarkably high in such scenarios. We describe our CRS technique with HITAC, which was performed in three FIGO stage IVA ovarian carcinoma patients with metastatic pleural effusion after complete CRS. The patients were retrospectively identified from a prospectively maintained database. All had partial diaphragmatic resection followed by HITAC as part of CRS treatment. Surgical techniques



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are outlined along with accompanying intra-operative images. Patient demographics, clinical and follow-up details were also described briefly. No comparative analysis with control patients was done. Adjustments in chemotherapy dose are not mandatory for HITAC. Of three patients, one had intrathoracic recurrence on follow-up; no mortality was recorded HITAC is a complex and potentially harmful procedure whose toxicity profile is still poorly known. Morbidity was not life-threatening and survival was acceptable.

Keywords: Hyperthermic intraperitoneal chemotherapy, hyperthermic intrathoracic chemotherapy, ovarian carcinoma, cytoreduction surgery, peritoneal carcinoma index

INTRODUCTION

Previously, peritoneal disease was considered terminal and systemic chemotherapy was offered for palliative intent while palliative surgery only had a role in symptom relief. With the advent of cytoreductive surgery (CRS) in the early 1990's, it is now the accepted treatment modality for a subset of patients with peritoneal carcinomatosis from pseudomyxoma peritonei, appendiceal adenocarcinoma and mesothelioma, and has also showed promising results in selected patients with ovarian, colorectal and gastric cancer^[1]. The purpose of CRS is to resect all macroscopic disease through peritonectomy and involved viscera followed by intraperitoneal chemotherapy by targeting residual microscopic disease through provision of a high intraperitoneal concentration with lower systemic toxicity^[2]. If CRS is complete, the more provocative procedure hyperthermic intraperitoneal chemotherapy (HIPEC) is performed in the same setting^[2,3]. HIPEC improves both quality of life and survival^[4]. These treatments are based on the concept that when disease is limited to the peritoneal cavity, it is still considered locoregional. The comprehensive CRS approach was described by Dr. Sugarbaker^[3] in 2007. Evidence for hyperthermia is based on accelerated cell death at 41-43 °C in experimental settings^[5]. The additive toxic effects of HIPEC have also been documented in the literature^[6]. Interval CRS and HIPEC resulted in longer recurrence-free and overall survival among FIGO stage III epithelial ovarian cancer than surgery alone, and did not result in excessive side effects^[7]. It also offers a significant survival benefit to patients with recurrent epithelial ovarian cancer, especially in patients with complete CRS^[8]. There is no randomized controlled study or feasibility study demonstrating the efficacy of hyperthermic thoracoabdominal chemotherapy (HITAC) over HIPEC however. Erasmus *et al.*^[9] reported that chemotherapeutic drugs were also absorbed from the pleural cavity like the peritoneal cavity. In the case of HITAC, the intrapleural concentration of chemotherapeutic drugs was persistently high compared to plasma. The current study is focused on the technical aspects and feasibility of HIPEC and HITAC in ovarian cancer patients. It does compare HIPEC alone with HIPEC and HITAC. Cognizant of the beneficial effects of HIPEC in selected patients with ovarian cancer, the same strategy was applied through HITAC in patients with thoracic involvement. This is the first study of its kind in the Indian patient population.

MATERIALS AND METHODS

This is a retrospective study of three prospectively selected patients with ovarian carcinoma and metastatic pleural effusion treated with CRS and HITAC after neoadjuvant chemotherapy. The aim was to describe the technical aspects of the surgery with brief descriptions of the postoperative outcomes and treatment-related morbidities on follow up.

CRS technical aspects

For CRS, a midline laparotomy extending from the xiphoid process to symphysis pubis was performed to provide greater exposure of the abdomen. Bilateral pelvic and retroperitoneal lymph node dissection with total omentectomy were done routinely as a part of CRS in ovarian cancer apart from total hysterectomy and salpingo-oophorectomy. Regarding peritonectomy, we do not routinely practice total peritonectomy

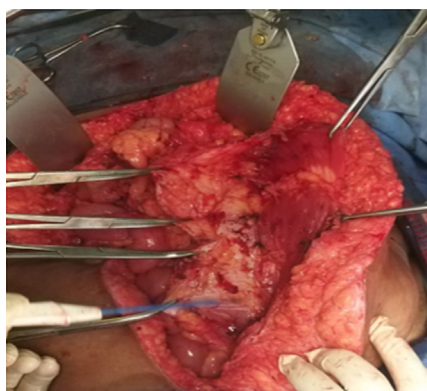


Figure 1. Peritonectomy using multiple artery forceps for retraction

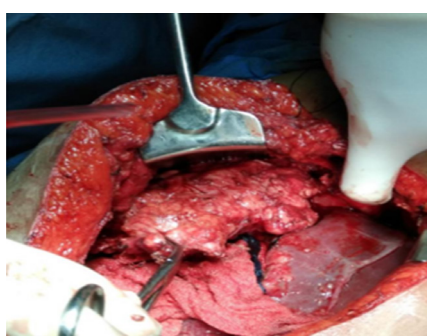


Figure 2. Diaphragmatic peritonectomy with full-thickness resection of diaphragmatic deposits

in all cases. Selective peritonectomy was performed in the region(s) macroscopically affected by the tumor. Total peritonectomy was performed in two cases in the present study, which had gross peritoneal disease. Total peritoneal stripping in continuity is a technically demanding procedure, hence in the current study, we followed the split technique, which involves stripping and removal of the entire peritoneum in five parts - right subdiaphragmatic peritoneum along with Glisson's capsule, left sub-diaphragmatic peritoneum, right and left parietal wall/paracolic gutter peritoneum and pelvic peritoneum. Peritonectomy was performed by holding and lifting the peritoneal edges with multiple artery forceps [Figure 1].

Surgical dissection was performed using monopolar diathermy with a sharp tip and diathermy settings at 30 coagulation spray mode although many surgeons prefer ball tip diathermy in pure cut mode. Additional visceral organ resection (colectomy, colo-proctectomy, splenectomy, gastrectomy, appendicectomy, cholecystectomy, liver resection and small bowel resection) may be performed, depending upon involvement. Whenever bowel resection is required, we prefer resection-anastomosis before HIPEC. Bowel edema, erythema and other hyperthermic chemotherapy-induced changes due to HIPEC at the edges of the bowel wall may become a constant threat for anastomotic leaks. For diaphragmatic peritonectomy, access and exposure of the diaphragmatic peritoneum were of utmost importance. Adequate exposure was obtained with the Omni-Tract surgical retractor and the liver was completely mobilized, except at the area of the hepatic veins and the suprahepatic inferior vena cava. For full-thickness large solid deposits involving the diaphragm, we performed full thickness diaphragmatic resection in two cases of the present study. In another case, partial resection of the hemidiaphragm was created for HITAC. Full-thickness resection of the diaphragm and the subsequent diaphragmatic rent created are shown in Figure 2. Pleural nodules were excised through the same rent in one case. The other two cases had only pleural effusion with no pre-operative or intra-operative evidence of metastatic pleural deposits. Total parietal pleurectomy was

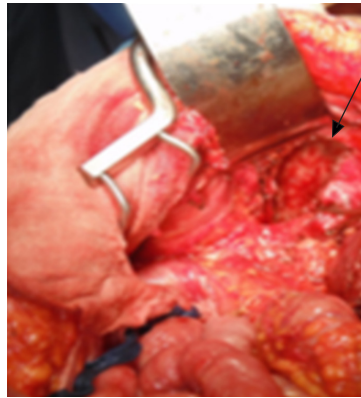


Figure 3. Diaphragmatic rent after partial diaphragmatic excision (black arrow)

not performed. We aspirated the pleural effusion in each case and dissected a few pleural deposits with electro-diathermy in one case. Thorough pleural lavage with chemotherapeutic perfusate into the pleural cavity was performed during HIPEC.

The technique of diaphragmatic resection

For diaphragmatic resection, one must be aware of the anatomy of the diaphragm in relation to the phrenic nerve. The phrenic nerve originates mainly from the 4th cervical nerve, but also receives contributions from the 5th and 3rd cervical nerves (C3-C5).

The right phrenic nerve enters the diaphragm through the central tendon or inferior vena cava opening. On the right side, it courses relatively more medially throughout its thoracic course to various structures like the right brachiocephalic vein, SVC and pericardium over the right atrium. The inferior vena cava lies medially and reaches under the surface of the diaphragm by passing through the inferior vena cava foramen in the central tendon.

The left phrenic nerve pierces the superior surface of the muscular part of the diaphragm, just to the left border of the heart.

Both nerves divide or trifurcate at, or just above the diaphragm. The branches travel together into the diaphragmatic musculature, while small sensory branches supply the peritoneum over the central part of the diaphragm. The larger motor branches separate within the diaphragm into four major nerves trunks - sternal, anterolateral, postero-lateral and crural. The nerve trunks travel partly within the diaphragmatic muscle and innervate the inferior surface covered by peritoneum. Therefore, the diaphragmatic incision has to be made circumferentially to avoid the main phrenic nerve trunks.

In the present study, we made an incision in the above-mentioned manner and excised the tumor deposits.

The patient's head end was lowered during HITAC procedures, so that the chemotherapeutic fluid can gain easy access to the thoracic cavity by free flow from the abdominal cavity.

Reconstruction of the diaphragm after HITAC procedure

In two cases, we had to incise approximately one-fourth of the diaphragm [Figure 3]. The diaphragmatic defects were repaired primarily with polypropylene 1-0 suture. In another case, the peritoneal defect was almost 50% and required reconstruction with polypropylene mesh, fixed with polypropylene 1-0 suture. In all three cases, we placed the ICD in the triangle of safety.



Figure 4. Demonstrating the creation of coliseum for performing hyperthermic intraperitoneal chemotherapy



Figure 5. Showing the coliseum (semi-open technique) and hyperthermic intraperitoneal chemotherapy tubes with adhesive sheet

HITAC: technical aspects

HIPEC was performed using the open Coliseum technique [Figure 4] as described by Sugarbaker^[3]. We used the Omni-Tract surgical retractor for exposing the abdominal cavity. Skin edges were suspended using interrupted polyester sutures fixed to the horizontal arms of the retractor to create an open space in the abdominal cavity, as depicted in Figure 4. An avascular plane of about 2-3 cm was routinely created between the anterior rectus sheath and subcutaneous tissue so that the rectus sheath and muscle would be immersed in the HIPEC perfusate.

An adhesive plastic sheet was incorporated to prevent spillage of the chemotherapy solution and heat loss [Figure 5]. A slit was made in the plastic cover to allow manual access to the abdomen and pelvis during HITAC. This helped in cases of blocked tubings during HIPEC and was also used to ensure uniform distribution of the perfusate and heat by constant, intermittent manipulation of the perfusate. A specially designed wooden spatula was used for stirring the heated chemotherapy solution to ensure uniform distribution as well as heat. Elbow-length gloves were worn for manual stirring and debris removal from the HIPEC tubing ports.

In cases of ovarian carcinoma, cisplatin was used as a chemotherapeutic drug in HITAC. Apart from routine drug dosage calculation using body surface area, another method of estimation uses the

approximate volume of the peritoneal cavity in litres. Dosage was calculated at 50 mg of cisplatin per litre. The chemotherapeutic solution was prepared by a resident doctor/specialized staff nurse using aseptic technique and full body personal protective equipment in a separate room adjacent to the surgical suite. The desired temperature from the inflow tube was kept in the range between 42 °C to 44 °C and out-flow was maintained at 41 °C to 43 °C. Per the literature, microscopic as well as tumour deposits up to 2.5 mm are destroyed by the synergistic effects of hyperthermia (42 °C) and chemotherapy^[5]. To achieve the desired temperature in the abdominal cavity, the temperature in the heat exchanger of the machine was regularly titrated depending on the recorded temperature. During combined HIPEC and HITAC for 60 min with close intra-operative monitoring of core body temperature, hemostasis was ensured before starting. We also constantly monitored the flow rate and nature of fluid content in the tubings. The inflow tube was kept in the pelvis away from critical areas and the great vessels. The average perfusate volume was 2.30 L, depending upon the capacity of the abdominal cavity. We used a triple-layered filtration mask, tightly fit to the face. It was also desirable to have a surgical smoke evacuator work continuously under the plastic sheet during perfusion and we used this in a number of cases. After completion of the procedure, the perfusate was aspirated, hemostasis ensured, and lung expansion confirmed before closure of the pleural defect. We did not perform peritoneal saline irrigation after completion of HITAC although some surgeons prefer to do so after clearing out the chemotherapeutic solution at the end of the procedure. Ipsilateral single intercostal drainage tube would then be placed. Two soft abdominal drains, one each in the sub-hepatic region and pelvis, were routinely placed in all cases. Additionally, we also placed a suction drain in the subcutaneous cavity after mobilizing the rectus sheath as mentioned earlier and this was removed on day 3 routinely unless drainage was more than 100 mL in the last 24 h.

Intraoperative monitoring

The role of the anesthesiologist is crucial during CRS with HITAC because of the extensive resection and long duration of procedures. The addition of hyperthermia in HITAC presents further challenges for the clinicians so a team approach is paramount. The main concern is related to the various physiological changes that can occur during CRS with HITACe, where hyperthermia and the use of chemotherapeutic agents concurrently may affect body systems. These concerns relate mainly to major fluid shifts, respiratory, hemodynamic, renal, hepatic, hematological and metabolic changes along with electrolyte, fluid and thermal imbalances. The maintenance of normal physiology remains the main goal. Ventilatory strategies are also of a major concern, not only because of abdominal surgery but also from exposure of the thorax to chemotherapy drugs and hence, the need for single lung ventilation.

Preoperative assessment and optimization are thus required for optimal outcomes. A thorough history and examination is key and includes routine assessment along with evaluation of prior drug therapy including chemotherapy, analgesics or drugs for associated comorbidities. Preoperative rehabilitation is also emerging as an important management tool because of its various beneficial aspects in enhanced recovery after surgery.

Appropriate monitoring is essential for patients undergoing CRS and HITAC. Apart from routine conventional intra-operative monitoring (electrocardiogram, non-invasive blood pressure monitoring, pulse oximeter, capnography, temperature), certain additional monitoring strategies are required for such interventions. For airway management, the conventional endotracheal tube is used routinely with oropharyngeal core body temperature probe monitoring. However, single lung ventilation is desirable in cases of pleural deposit excision. Anesthesia induction is usually done using propofol, fentanyl and atracurium and maintained with atracurium, fentanyl and inhalational agents like sevoflurane or desflurane in oxygen and air mixture. Based on the extent of the abdominal mass and the patient's clinical condition, ventilator strategies may require further planning.

Table 1. Showing the demographic and clinical details with follow-up status

	Patient SI. No 1	Patient SI. No 2	Patient SI. No 3
Age (years)	45	46	29
Date of registration	08-05-2016	04-06-2018	27-06-2017
Prior surgery	Staging laparotomy	None	None
FIGO stage	IVA	IVA	IVA
NACT (cycles/regimen)	6#TP, 6#Gem + CDDP, 1#TP	12#TP	3#TP
Date of surgery	20-05-2019	08-11-2018	12-03-2018
Types of CRS	Secondary CRS	Interval CRS	Interval CRS
CRS procedure	Disease limited peritonectomy + omental cake excision + terminal ileum and limited right colon resection anastomosis	TAH + BSO + B/L PLND + RPLND + total omentectomy + pouch of douglas and liver deposit excision	TAH + BSO + B/L PLND + RPLND + total omentectomy + right diaphragmatic stripping + selective peritonectomy
CRS duration (min)	370	410	330
Blood loss (mL)	1150	600	450
PCI	23/39	2/39	15/39
HITAC drug	Mitomycin	Cisplatin	Cisplatin
Drug dosage (mg)	30	100	100
Duration (min)	60	45	60
Temperature (°C)	42	42	42
Perfusate	Normal saline	Normal saline	Normal saline
Perfusate volume (L)	2.5	3.0	2.5
CC score	1	0	0
Comorbidity	Cl. Dindo II	Cl. Dindo III	Cl. Dindo II
Adj chemotherapy	3#TP	6#TP + Bev	3#TP
Follow up status	Alive & disease-free	Alive with disease	Alive & disease-free

SI. No: serial number; FIGO: International Federation of Gynecology and Obstetrics; NACT: neoadjuvant chemotherapy; TP: Paclitaxel/Carboplatin; CDDP: Cisplatin; TAH: total abdominal hysterectomy; BSO: bilateral salpingo-oophorectomy; B/L PLND: bilateral pelvic lymph node dissection; PAND: paraaortic node dissection; CRS: cytoreduction surgery; PCI: peritoneal carcinomatous index; HITAC: hyperthermic thoracoabdominal chemotherapy; CC score: completeness of cytoreduction score; Cl. Dindo: Clavien Dindo score; Adj: adjuvant; Bev: Bevacizumab

Goal-directed fluid therapy is desirable for fluid management. Monitoring for fluid management is routinely done using urine output measurement and non-invasive methods such as cardiac output monitors for assessing the fluid status to guide management. Multimodal analgesia is required for optimal outcomes. The use of thoracic epidural analgesia with local anesthetic and opioids appears to be acceptable in the current study. Coagulopathy needs to be identified and corrected as necessary. The use of point of care tools for assessing coagulopathy remains promising. Postoperative monitoring is crucial as these patients continue to have various physiological changes for days in the postoperative period. The patient should be monitored closely for fluid balance, hemodynamic fluctuations, renal impairment, coagulopathy and electrolyte imbalances. Such patients also need DVT prophylaxis in the postoperative period using pharmacological and/or mechanical measures.

RESULTS

CRS with HIPEC and HITAC were performed in three patients with ovarian carcinoma and peritoneal carcinomatosis after neoadjuvant chemotherapy.

Patients were 29-46 years with a mean age of 40 years. All patients resided in urban localities and were of middle class socioeconomic status.

Patient serial number “1” is a 45-year-old female with known hypothyroidism. She underwent staging laparotomy at another institution for ovarian malignancy in December 2015 and histopathology was FIGO stage IB. The patient did not undergo any adjuvant chemotherapy. Six months later, she self-referred to our tertiary centre in May 2016 with symptoms of cough, breathlessness and abdominal distension and was diagnosed as FIGO stage IVA, recurrent ovarian carcinoma. The patient underwent chemotherapy with

6 cycles of TP (Paclitaxel/Carboplatin), 6 cycles of Gem/CDDP (Gemcitabine/Cisplatin) and 1 cycle of TP (Paclitaxel/Carboplatin). Post-chemotherapy, the patient had partial response and she proceeded with secondary CRS and HITAC as mentioned in Table 1. In the early postoperative period, the patient did not develop significant surgical morbidity (Clavien Dindo grade III/IV). Following three cycles of adjuvant chemotherapy, the patient remains disease-free with the last follow-up on 12-02-2020.

Patient serial number “2” is a 46-year-old female with no known comorbidities nor significant family history. She initially presented to another institution with symptoms of abdominal pain, constipation, fever and weight loss. She was diagnosed with Koch's abdomen and received tuberculosis treatment for 1 year. However, she had persistent and worsening of symptoms, so a right-sided intercostal drainage tube was placed for a right pleural effusion. Image-guided pleural biopsy was performed and histopathology was suggestive of poorly differentiated carcinoma which was immunopositive for CK7+, ER+ and focal CA125+. The patient then self-referred to our hospital. After thorough work-up, she was planned for weekly TP neoadjuvant chemotherapy followed by surgical reassessment before proceeding with interval CRS with HITAC. Details of the surgical procedure were mentioned earlier and are based on pre- and intra-operative clinical findings. Post-operative histopathology was consistent with FIGO stage IVA. In the early postoperative period, the patient developed a recurrent right pleural effusion which necessitated another right intercostal drainage tube. After delayed clinical recovery, six cycles of adjuvant TP and bevacizumab were administered to the patient. The patient is currently alive with persistent disease at the last follow-up on 13-03-2020 and is still on bevacizumab based chemotherapy.

Patient serial number “3” is a 29-year-old female with no known comorbidities, significant family history nor past medical or surgical history. She presented with a dry cough and shortness of breath for 10 months. The patient was worked-up and diagnosed with ovarian carcinoma with right sided malignant pleural effusion (FIGO IVA). Multidisciplinary tumor board discussion advised for TP based neoadjuvant chemotherapy followed by CRS and intraperitoneal/intrathoracic chemotherapy. Post 3 cycles TP, the patient underwent interval CRS with HITAC. The procedure details are mentioned in Table 1 and the postoperative period was uneventful. Final histopathology reported the same FIGO stage disease because of similar tumor deposits on the pleura. Three cycles of adjuvant TP regimen were administered to the patient. At the last follow-up on 19-09-2019, the patient was alive and disease-free.

CONCLUSION

CRS with HITAC is a complex and evolving procedure. These are viable treatment options for cases of ovarian carcinoma with peritoneal carcinomatosis and pleural disease in the post neoadjuvant chemotherapy setting. Macroscopic disease can be removed with CRS and the remaining microscopic disease can be dealt with through HITAC to reduce thoracic recurrences. In this study, there were no life-threatening surgical morbidities. No mortality was recorded till the last follow-up. Following the technique described in this study, CRS with HITAC can be safely performed and replicated easily without additional morbidity or need for extra resources for HITAC. However, multicenter studies with larger numbers of patients and longer follow-up is warranted to establish reproducibility and acceptance of the procedure.

DECLARATIONS

Acknowledgments

We like to acknowledge our head of department Professor (Dr.) S V S Deo, DR BRA-IRCH, AIIMS, New Delhi for constant support and inspiration. The acknowledgment extends to all faculty and senior residents of the Department of Surgical Oncology and Department of Onco-anesthesia and Palliative Care for intraoperative and postoperative patient care.

Authors' contributions

Substantial contributions to the conception or design of the manuscript, writing manuscript, revising it critically for important intellectual content and to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the manuscript: Ray MD

Data collection, data analysis, data curation, drafting the work, manuscript writing, editing, conceptualisation, technical and material support, study supervision, revising it critically for important intellectual content and proofreading with final approval of the manuscript version to be published: Kumar N

Data collection, data analysis, drafting the work, manuscript writing, drawing pictorial depiction and formatting, editing, provided technical, and material support: Kuppusamy R

Manuscript writing and final approval of manuscript version to be published: Garg R

Have read and approved the manuscript: Ray MD, Kuppusamy R, Kumar N, Garg R

Availability of data and materials

The data (operative pictures, further case details, follow up data) to support the findings of this study are available on request from the corresponding authors.

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 study has been performed following the "Declaration of Helsinki" and approved by the "institutional ethics committee, AIIMS, New Delhi, India" vide reference no: IEC-592/03.11.2017, AA-3/29. Informed consent to participate in the study has been obtained from participants.

Consent for publication

Not applicable. No identifying images or other personal or clinical details of participants are presented in the manuscript that compromise anonymity.

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Review

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Unlikely role of glycolytic enzyme α -enolase in cancer metastasis and its potential as a prognostic biomarker

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How to cite this article: Schofield L, Lincz LF, Skelding KA. Unlikely role of glycolytic enzyme α -enolase in cancer metastasis and its potential as a prognostic biomarker. *J Cancer Metastasis Treat* 2020;6:10. <http://dx.doi.org/10.20517/2394-4722.2019.43>

Received: 5 Dec 2019 **First Decision:** 4 Mar 2020 **Revised:** 5 Mar 2020 **Accepted:** 24 Mar 2020 **Published:** 17 Apr 2020

Science Editor: Stephen J. Ralph **Copy Editor:** Jing-Wen Zhang **Production Editor:** Jing Yu

Abstract

Reliance on glycolysis for energy production is considered a hallmark of cancer and the glycolytic enzyme α -enolase is overexpressed in a range of cancer types. However, recent studies have revealed that α -enolase is involved in a variety of unrelated physiological processes and can be found in multiple unexpected cellular locations. This review focuses on the unlikely role of α -enolase as an extracellular plasminogen-binding receptor localised to the plasma membrane. Conversion of plasminogen to plasmin on the surface of cancer cells enhances their ability to invade through stroma by activating collagenases and degrading fibrin as well as extracellular matrix proteins. Increased expression of α -enolase is associated with increased migration and invasion of cancer cells, and decreased metastasis-free survival in patients with several cancer types, including non-small cell lung, pancreatic, breast and colorectal cancers. Due to its overexpression in a range of cancer types and multi-functional roles in key areas of tumour metabolism and metastasis, α -enolase may be useful as a universal cancer prognostic biomarker or therapeutic target.

Keywords: Alpha-enolase, ENO1, metastasis, migration, invasion, proliferation, plasminogen-binding receptor



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INTRODUCTION

All cancer cells have a high energy demand due to their increased rate of proliferation^[1]. Increased glycolysis is considered a hallmark of cancer^[2] and investigating glycolytic enzymes may yield new therapeutic approaches for cancer treatment. Enolases are key glycolytic enzymes^[3], and increased expression of one isoform, α -enolase, has been identified in several cancer types^[4-22]. This review provides an overview of the expression of α -enolase and key functions it controls in cancer cells, with a focus on the potential role of α -enolase as a cancer prognostic biomarker or therapeutic target.

ENOLASE IS A GLYCOLYTIC ENZYME THAT HAS THREE ISOFORMS

Enolases (EC 4.2.1.11) are metalloenzymes that catalyse the dehydration of 2-phospho-*D*-glycerate to phosphoenolpyruvate in the glycolysis pathway [Figure 1], and catalyse the hydration of phosphoenolpyruvate to 2-phospho-*D*-glycerate in the reverse anabolic pathway during gluconeogenesis^[3]. In mammals, the three genes *ENO1*, *ENO2*, *ENO3* encode three isoforms, with expression being regulated in a tissue-specific manner. Alpha-enolase (*ENO1*) is ubiquitously expressed, whereas γ -enolase (*ENO2*) is primarily expressed in neurons and neuroendocrine tissues, and β -enolase (*ENO3*) in muscle tissues^[23]. Active enolase consists of a dimer in which two subunits face each other in an antiparallel formation^[24], and requires two non-covalently bound magnesium ions as cofactors for enzyme activity^[25].

ALPHA-ENOLASE IS A MULTI-FUNCTIONAL PROTEIN

Although many glycolytic enzymes are considered to be housekeeping proteins, α -enolase expression can vary dramatically depending on the stress, metabolic, or pathological state of the cell. A retrospective proteomic meta-analysis identified that α -enolase was the most differentially expressed protein in humans and rodents irrespective of tissue type and pathological condition^[26]. Disrupted expression and/or activity of α -enolase has been reported in several pathologies with distinct aetiologies, including Alzheimer's disease, systemic sclerosis, rheumatoid arthritis, bacterial infections and hepatic fibrosis^[27-38].

Apart from its role in the glycolytic pathway, recent studies have revealed that α -enolase is a multi-functional protein that controls a variety of cellular processes, including proliferation, survival, migration and invasion. Additionally, using an alternative transcription start codon, the *ENO1* gene can produce a 37 kDa protein, c-myc promoter-binding protein (MBP-1). MBP-1 localises to the nucleus, where it acts as a transcription repressor by binding to the c-myc P2 promoter^[39], helping regulate and maintain the function of the glycolysis pathway.

ALPHA-ENOLASE EXPRESSION IS ALTERED IN TUMOURS AND VARIES WITH CANCER TYPE

The overexpression of α -enolase is associated with tumour development via a process known as aerobic glycolysis or the Warburg effect. The Warburg effect has been hypothesised to be an adaptation mechanism in cancer cells to support the biosynthetic requirements of rapid proliferation. Alpha-enolase expression has been shown to be altered at the mRNA and/or protein level in a range of tumours [Table 1], and generally upregulated in most, including acute myeloid leukaemia (AML), glioma, melanoma, lymphoma, and colorectal, endometrial, gastric, head and neck, liver, ovarian and pancreatic cancer^[4-22].

ALPHA-ENOLASE CAN SHUTTLE BETWEEN CELLULAR COMPARTMENTS

Alpha-enolase can be localised to the cytoplasm and plasma membrane, as well as secreted in exosomes, and its location varies with cancer type. For example, in pancreatic, breast and lung tumours, α -enolase is localised to the plasma membrane^[22,44,45], whereas in melanoma, mesothelioma, non-small cell lung, colorectal and prostate cancer α -enolase is also secreted and found in exosomes^[43,46-49]. Alpha-enolase can

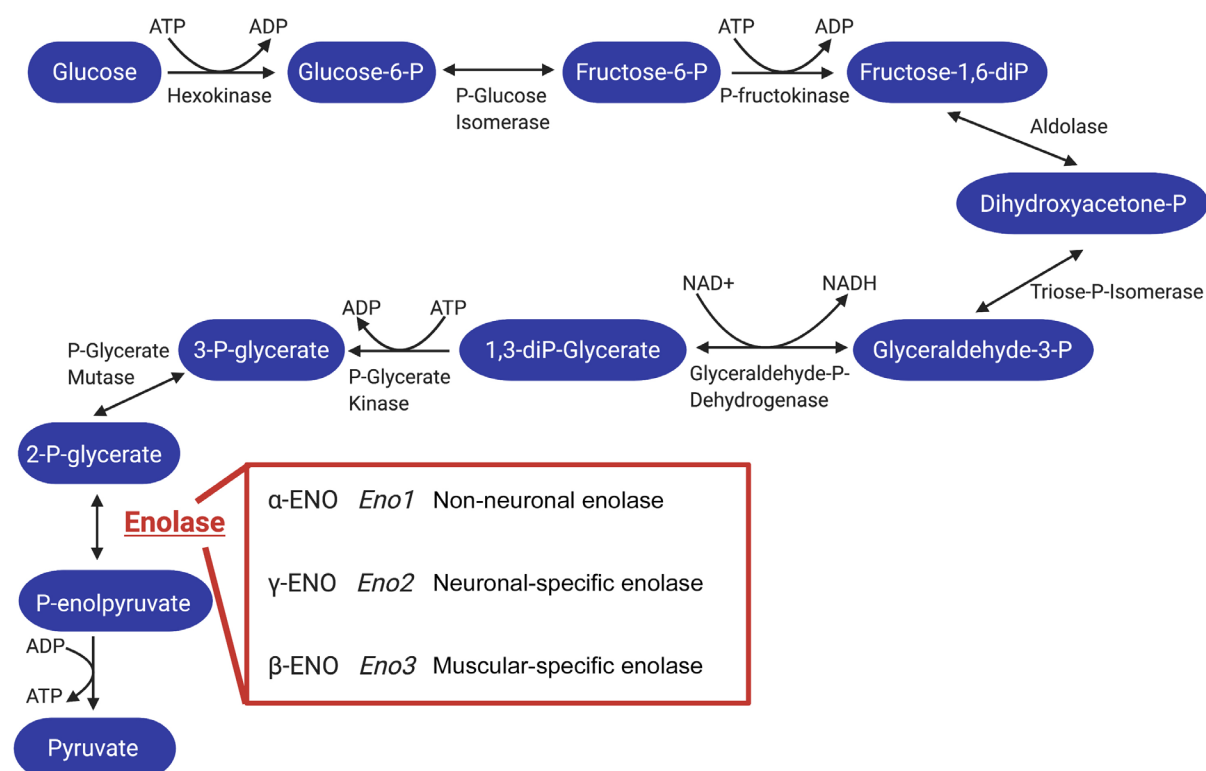


Figure 1. The glycolysis pathway. Enolases catalyse the dehydration of 2-phospho-D-glycerate (2-P-glycerate) to phosphoenolpyruvate (P-enolpyruvate) in the glycolysis pathway

shuttle between compartments, performing different functions when in different subcellular locations, such as surface membrane plasminogen binding, controlling the overall metabolic state of the cell, stress-related or acting as a heat-shock protein, RNA transport, mitochondrial membrane stability, and cell cycle control^[50-55].

INCREASED ALPHA-ENOLASE EXPRESSION ENHANCES CELL PROLIFERATION IN A VARIETY OF CANCERS

In most solid tumours, the Warburg effect causes an increase in total glycolysis under both hypoxic and normoxic conditions [Figure 2]. Enhanced cell proliferation leads to increased anabolic needs, and cancer cells remodel metabolic processes by diverting nutrients to anabolic pathways to satisfy increased cellular energy demands^[56]. Therefore, the Warburg effect may provide cancer cells with an advantage when competing with non-cancerous tissues for nutrients. This suggests that increased α-enolase expression will contribute to enhanced proliferation commonly observed in cancer cells.

Indeed, upregulated α-enolase expression has been shown to regulate cell proliferation in various solid tumours *in vitro*^[11,13,57-61], and to increase tumour growth in a HCT116 colorectal cancer xenograft model *in vivo*^[11] [Table 2]. Conversely, silencing of α-enolase in glioma, pancreatic, lung, endometrial, colorectal and breast cancer cells was found to induce cell cycle arrest and senescence, and also to reduce tumour volume in CFPAC-1 pancreatic, MDA-MB-231 breast and U-87MG glioma xenograft models *in vivo*^[6,11,12,62,63]. Furthermore, α-enolase is also implicated in the control of apoptosis and sensitivity to chemotherapeutic agents, as silencing of *ENO1* in cancer cells induced apoptosis and increased sensitivity to cisplatin and 5-fluorouracil *in vitro*^[13,62,64]. Unexpectedly, cells respond to α-enolase silencing by inducing catabolic adaptations that lead to restoration of pyruvate, acetyl-CoA bulk and oxidative phosphorylation,

Table 1. The expression of α -enolase is altered in cancer

Cancer	Sample type	Detection method	Alteration (frequency of alteration)	Ref.
AML	Peripheral blood and bone marrow from AML patients ($n = 41$) and healthy patients ($n = 20$)	Microarray	Increased	[4]
Breast cancer	Breast cancer tissue with adjacent matched normal tissue ($n = 24$)	Western blot	Increased (100%)	[9]
	Breast cancer and normal tissue ($n = 244$)	Quantitative real-time PCR	Decreased	[10]
Cervical cancer	Squamous cell carcinoma ($n = 33$) and normal cervical tissue ($n = 17$)	2D-DIGE and MALDI-TOF mass spectrometry	Increased	[40]
Colorectal cancer	SW620 cell line (lymph node metastasis) compared to SW480 (primary lesion)	2D-DIGE	Increased	[41]
	Colorectal cancer ($n = 48$) and adjacent matched normal tissue ($n = 16$)	IHC and PCR	Increased (56%)	[11]
Endometrial carcinoma	Endometrial cancer ($n = 100$), endometrial atypical hyperplasia ($n = 22$) and normal endometrium tissues ($n = 20$)	IHC	Increased in endometrial cancer (52%) and atypical hyperplasia (31.8%) compared to normal	[12]
Gastric cancer	Gastric cancer and adjacent normal tissue ($n = 94$)	IHC	Increased	[13]
	Primary gastric cancer tissue ($n = 107$)	IHC	Increased (48%)	[14]
Glioma	Primary glioblastoma tissue ($n = 24$)	Quantitative real-time PCR	Increased (68%)	[5]
	Glioma ($n = 136$) and normal brain ($n = 15$) tissue	Quantitative real-time PCR and IHC	Increased (69%)	[6]
Head and neck cancer	Head and neck cancer and adjacent normal tissue ($n = 44$)	Real-time PCR	Increased (50%)	[15]
HCC	HCC ($n = 374$ and $n = 1309$), adjacent matched tissue ($n = 50$), and normal tissue ($n = 1442$) from TCGA and GEO data source; tissue microarray (93 HCC and 87 normal liver tissues)	Microarray and IHC	Increased	[16]
Lymphoma	Pretherapeutic tumour biopsies from peripheral T-cell lymphoma not otherwise classified ($n = 87$)	IHC	Increased	[8]
Melanoma	A375, MeWo, MEL-HO, Colo-800, Colo-853 melanoma cell lines and a normal melanocyte cell line	2D-DIGE and nano-HPLC-chip ion trap mass spectrometry	Increased	[7]
NSLC	Primary NSCLC tissue and matched normal lung ($n = 26$) from RNA and primary NSCLC tissue ($n = 55$) and normal lung tissue ($n = 17$)	Real-time PCR and IHC	Increased	[17]
	Primary NSCLC and adjacent matched normal tissue ($n = 46$)	Western blotting	Decreased (26%)	[42]
	Primary NSCLS tissue ($n = 36$)	PCR	Increased (16%)	[18]
Ovarian cancer	Ovarian cancer ($n = 4$) and ovarian tissue from endometriosis ($n = 1$)	2D-DIGE and LC-MS/MS	Increased	[19]
Pancreatic cancer	Pancreatic cancer and adjacent normal tissue ($n = 31$)	IHC	Increased	[20]
	Pancreatic cancer ($n = 100$) and adjacent normal tissue ($n = 80$)	IHC	Increased (48%)	[21]
	Primary pancreatic and adjacent normal tissue ($n = 3$)	Western blot	Increased	[22]
Prostate cancer	Exosomes from prostate cancer cell lines	Western blot and mass spectrometry	Decreased	[43]

2D-DIGE: two-dimensional differential in gel electrophoresis; HCC: hepatocellular carcinoma; HPLC: high performance liquid chromatography; IHC: immunohistochemistry; LC-MS/MS: liquid chromatography tandem mass spectrometry; MALDI-TOF: matrix assisted laser desorption/ionization time of flight; NSCLC: non-small cell lung cancer; PCR: polymerase chain reaction; AML: acute myeloid leukaemia; TCGA: the cancer genome atlas; GEO: gene expression omnibus

and exhibit an increased expression of proteins involved in both oxidative stress- and sirtuin-induced autophagy^[62]. Taken together, these studies demonstrate that α -enolase is an important regulator of tumour cell metabolism, proliferation and survival, which by definition make it a perfect target for anticancer therapy.

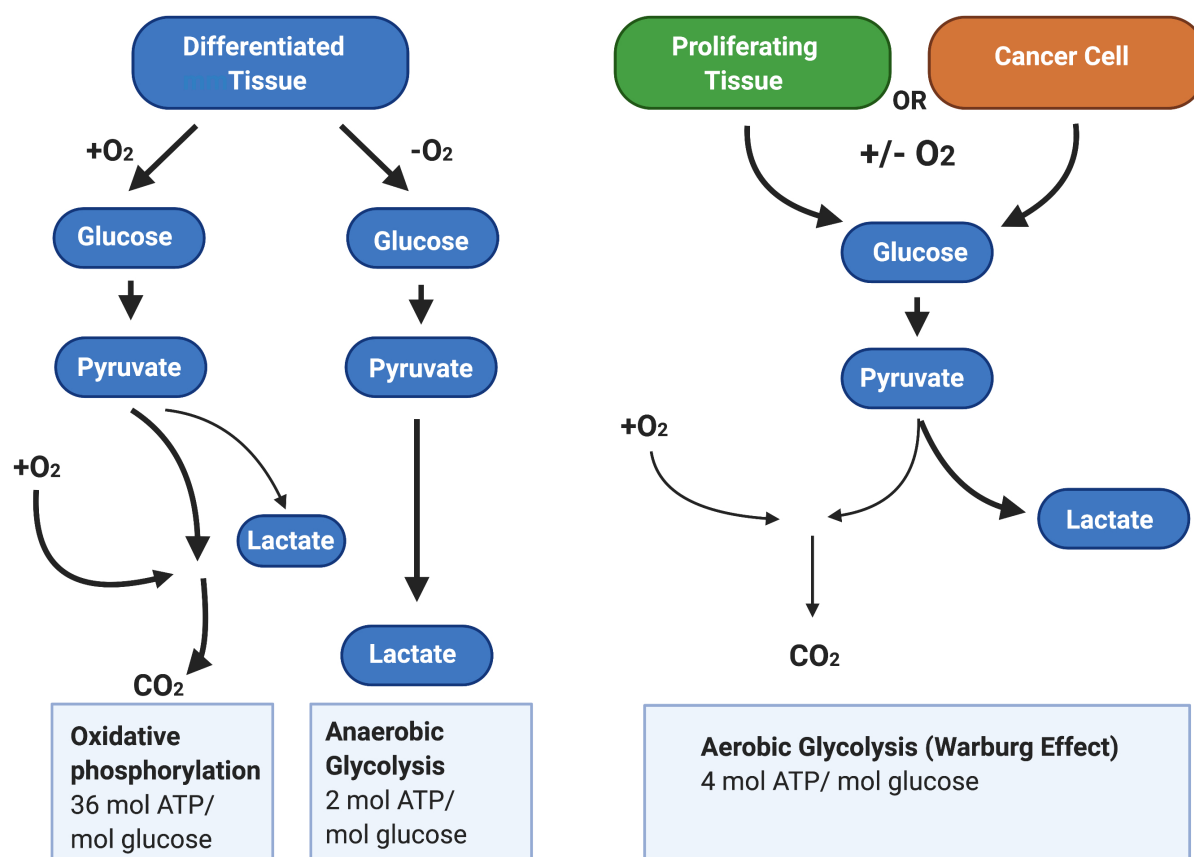


Figure 2. The Warburg Effect in cancer cells. In the presence of oxygen, differentiated tissues first metabolise glucose to pyruvate via glycolysis and then oxidise the majority of the pyruvate to carbon dioxide via oxidative phosphorylation. In situations where oxygen is low, cells redirect pyruvate generated by glycolysis away from oxidative phosphorylation by generating lactate via anaerobic glycolysis. By contrast, cancer cells convert most glucose to pyruvate regardless of whether oxygen is present. This allows cancer cells to meet the increased cellular energy demands

ALPHA-ENOLASE IS A SURFACE PLASMINOGEN-BINDING RECEPTOR

In addition to a role in cell proliferation and survival, α -enolase located on the plasma membrane acts as a plasminogen-binding receptor^[55] [Figure 3]. Plasminogen is a zymogen, which is converted to plasmin in the presence of the activators tissue plasminogen activator or urokinase-type plasminogen activator (uPA)^[65]. This cell surface interaction concentrates protease activity in the tissue surrounding the cell, protecting plasmin from inactivation by circulating $\alpha 2$ -antiplasmin^[66]. Plasmin activates collagenases and degrades fibrin and other matrix proteins, resulting in cell migration and invasion into tissue, ultimately underpinning cancer metastasis and relapse.

Role in invasion and migration

Overexpression of α -enolase has been shown to increase the migration and invasion of hepatocellular carcinoma, colorectal and gastric cancer cells *in vitro*^[11,58,60,61,67] and to enhance colorectal cancer metastasis *in vivo*^[11], demonstrating that it is an important driver of metastasis in multiple cancer types [Table 3]. Conversely, knockdown or pharmacological inhibition of α -enolase decreased the migration and invasion of glioma, colorectal, pancreatic and endometrial carcinoma *in vitro*^[6,12,63,68,69], and decreased tumorigenesis and metastasis of endometrial carcinoma *in vivo*^[12]. Furthermore, binding of recombinant α -enolase to the surface of prostate cancer cells was shown to promote cell migration via its plasminogen receptor activity^[70]. By contrast, anti- α -enolase monoclonal antibodies inhibited plasminogen-dependent invasion of human pancreatic cancer cells *in vitro* and metastasis formation *in vivo*^[71] and also lung cancer cell invasion *in vitro* and growth *in vivo*^[72] [Table 3].

Table 2. Alpha-enolase controls cancer cell proliferation and survival

Cancer	Experimental model	Effect of modulation of ENO1 expression	Ref.
Bladder cancer	Overexpression and knockdown in T253 and 5637 cells	Knockdown decreased cell proliferation and colony formation. Overexpression increased cell proliferation and colony formation	[59]
Breast cancer	Downregulation in MDA-MB-231 cells	Downregulation decreased cell proliferation and survival <i>in vitro</i> and reduced tumour growth <i>in vivo</i>	[62]
Colorectal cancer	Overexpression and knockdown in HCT116 cells	Overexpression promoted cell proliferation and tumour growth <i>in vivo</i> ; Decreased expression decreased cell proliferation and tumour growth <i>in vivo</i>	[11]
Endometrial carcinoma	Knockdown in HEC-1B and Ishikawa	Decreased expression reduced cell proliferation <i>in vitro</i> and tumourigenesis <i>in vivo</i>	[12]
Gastric cancer	Knockdown in MGC-803 and MKN45 cells	Knockdown led to cell cycle arrest at the G ₁ phase and promoted apoptosis, and repressed the rate of cell proliferation and colony formation	[13]
	Knockdown in MKN45 cells	Knockdown decreased cell proliferation, induced apoptosis and increased sensitivity to chemotherapeutics	[64]
	Knockdown in AGS cells and overexpression in SGC7901 cells	Knockdown decreased proliferation and colony formation, whereas overexpression increased cell proliferation and colony formation	[60]
	Overexpression in AGS cells	Overexpression increased cell proliferation and colony formation	[61]
Glioma	Knockdown in U-87MG cells	Knockdown suppressed cell proliferation and colony formation <i>in vitro</i> and tumour growth <i>in vivo</i>	[6]
HCC	Knockdown in HCC cells	Knockdown inhibited cell growth	[58]
Lung cancer	Knockdown in NCI-H441 cells	Knockdown decreased cell proliferation and survival	[62]
Pancreatic cancer	Knockdown in CFPAC-1 cells	Downregulation decreased cell proliferation and survival <i>in vitro</i> and reduced tumour growth <i>in vivo</i>	[62]
Retinoblastoma	Knockdown in Y79 cells and overexpression in Meri-RB1 cells	Knockdown led to cell cycle arrest at the G ₁ phase, decreased cell proliferation and increased apoptosis. Overexpression increased cell proliferation.	[57]

HCC: hepatocellular carcinoma

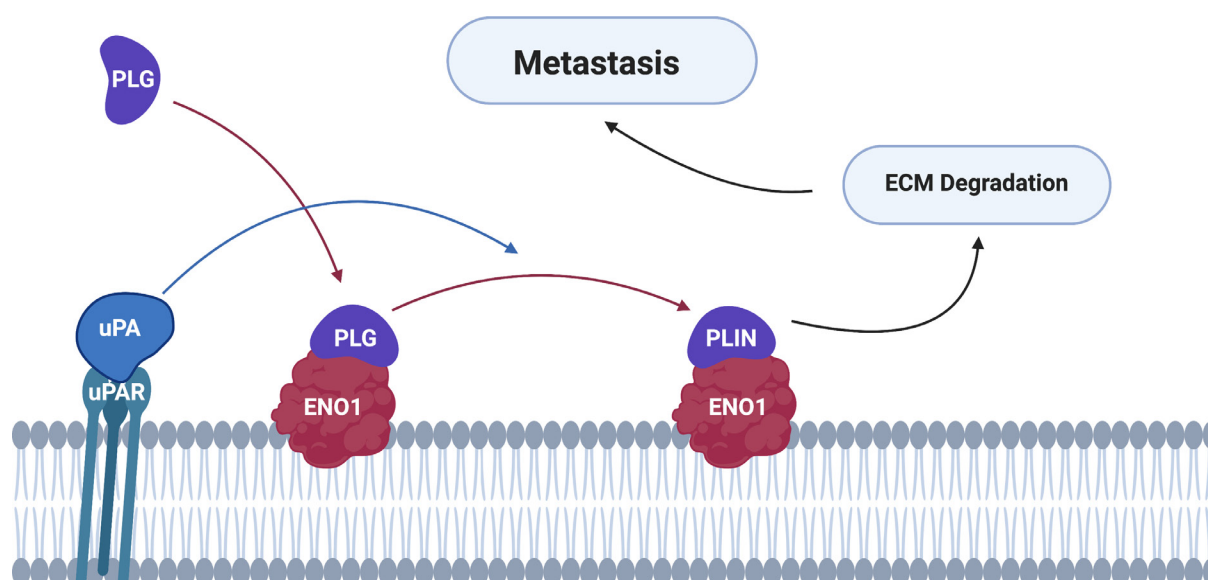
**Figure 3.** Alpha-enolase acts as a surface plasminogen-binding receptor to mediate cancer cell invasion and metastasis formation. PLG binds to its receptors and is subsequently converted to PLIN by plasminogen activators (e.g., uPA). Cell surface-associated plasmin facilitates degradation of the ECM, allowing tumour cells to invade and metastasise into other tissues. PLG: plasminogen; PLIN: plasmin; ECM: extracellular matrix; uPA: urokinase-type plasminogen activator

Table 3. Alpha-enolase is a potential regulator of metastasis

Cancer	Experimental model	Effect of modulation of ENO1 expression	Ref.
Colorectal cancer	Overexpression and knockdown in HCT116 cells	Overexpression increased migration and invasion both <i>in vitro</i> and <i>in vivo</i> ; decreased expression decreased migration and invasion both <i>in vitro</i> and <i>in vivo</i>	[11]
	Treatment of HCT116 cells with CS5931 (peptide inhibitor)	Treatment with CS5931 decreased cell migration and invasion	[69]
Endometrial carcinoma	Knockdown in HEC-1B and Ishikawa	Decreased expression reduced migration and invasion <i>in vitro</i> and metastasis <i>in vivo</i>	[12]
Gastric cancer	Knockdown in AGS cells and overexpression in SGC7901 cells	Knockdown decreased migration, and overexpression increased migration	[60]
	Overexpression in AGS cells	Overexpression increased migration	[61]
Glioma	Knockdown in U251-MG-WBP2 cells	Knockdown decreased migration	[63]
	Knockdown in U-87MG cells	Knockdown suppressed migration and invasion	[6]
HCC	Knockdown in HCC cells	Knockdown inhibited migration	[58]
	Overexpression in HepG-2 cells	Increased migration and invasion	[67]
Lung cancer	Anti-human ENO1 antibody and knockdown in A549 cells	Downregulation and adoptive transfer of anti-human ENO1 antibody decreased invasion <i>in vitro</i> and <i>in vivo</i>	[72]
Pancreatic cancer	Knockdown in CFPAC-1 cells	Knockdown decreased migration and invasion, and reduced adhesion to fibronectin and collagen and increased adhesion to vitronectin	[68]
	Anti-human ENO1 antibody	Decreased invasion <i>in vitro</i> and metastasis <i>in vivo</i>	[71]

HCC: hepatocellular carcinoma

ALPHA-ENOLASE IS A TUMOUR-ASSOCIATED ANTIGEN

Externalisation of α -enolase by cancer cells exposes it to the immune system as a tumour-associated antigen that has been found to induce autoantibody production in cancer patients, including those with acute and chronic leukaemias, melanoma, and lung, breast, gastric and pancreatic cancers^[22,45,73-79]. In pancreatic cancer, T cells activated by α -enolase-pulsed dendritic cells lysed pancreatic cancer cells, but not normal human keratinocytes *in vitro* and inhibited CF-PAC-1 tumour growth *in vivo*^[22]. In oral squamous cell carcinoma, an HLA-DR8-restricted human α -enolase peptide was recognised by CD4⁺ T cells and produced a cytotoxic response against OSC-20 cells^[80]. Additionally, vaccination with *ENO1* in Kras^{G12D}/Cre and Kras^{G12D}/Trp53^{R172H} mice prior to development of pancreatic carcinoma delayed tumour growth and increased survival^[81]. Taken together, these studies suggest that immune responses directed against α -enolase may be immunostimulatory and ultimately beneficial to patients.

ALPHA-ENOLASE IS A PROGNOSTIC FACTOR FOR MULTIPLE CANCER TYPES

In addition to being overexpressed in many cancers, α -enolase has been identified as a putative prognostic biomarker in a range of tumour types [Table 4]. Whilst *ENO1* expression was not associated with tumour stage in colorectal cancers, it was significantly correlated with tumour size and presence of distant metastases^[11]. Alpha-enolase expression was positively correlated with lymph node status in endometrial and gastric cancer patients^[12,13,60], and increased α -enolase expression in endometrial, gastric, lung, lymphoma and hepatocellular cancer patients was associated with worse overall survival^[8,12,13,16,73,82]. Furthermore, increased α -enolase expression was correlated with worse distant metastasis-free survival in breast cancer patients^[9], worse disease-free survival in hepatocellular carcinoma and chordoma^[16,83] and worse progression-free survival in lung cancer patients^[73]. By contrast, downregulation of α -enolase is a predictor of poor prognosis in clear cell renal cell carcinoma (ccRCC)^[84], demonstrating that α -enolase may control different cellular functions in ccRCC when compared to other cancers.

Autoantibodies generated against α -enolase in its capacity as a tumour-associated antigen represent an additional type of prognostic biomarker that may be assayed in serum. The presence of autoantibodies against α -enolase correlated with longer disease-free survival and overall survival in pancreatic and lung cancer patients^[45,85-87] [Table 4]. Furthermore, compared with healthy individuals, α -enolase antibodies

Table 4. α -Enolase is a prognostic biomarker for a range of cancer types

Cancer	Sample type	Patient outcome	Ref.
Breast cancer	Kaplan-Meier Plotter database ($n = 5143$ breast cancer patients)	Increased mRNA expression correlated with worse DMFS	[9]
Chordoma	Cervical or sacral spine chordomas ($n = 39$)	Increased protein expression was associated with worse disease-free survival	[83]
CLL	Sera from CLL patients ($n = 86$)	Presence of anti- α -enolase antibodies was predictive of a shorter time to first treatment	[90]
Colorectal cancer	Colorectal tumour tissues ($n = 41$)	Protein expression correlated with tumour size and distant metastasis	[11]
Endometrial cancer	Endometrial cancer tissue ($n = 100$)	Protein expression correlated with lymph node status and depth of myometrial invasion; patients with high expression had worse OS	[12]
Gastric cancer	Gastric cancer tissue ($n = 76$)	Protein expression correlated with lymph node metastasis and TNM stage	[60]
	TCGA dataset ($n = 410$ gastric cancer patients); Gastric cancer tissue ($n = 94$)	Protein expression correlated with high TNM stage and metastasis; Increased mRNA was associated with poor OS	[13]
HCC	TCGA dataset ($n = 374$ HCC tissues); meta-analysis of 12 cohorts in GEO database	Increased mRNA was associated with poor OS and disease-free survival; Protein expression correlated with high TNM stage and was negatively correlated with OS	[16]
	Sera from HCC patients ($n = 61$)	Anti- α -enolase antibodies were lower in patients without microvascular invasion compared to those with microvascular invasion	[91]
Lung cancer	Kaplan-Meier Plotter database ($n = 348$ lung cancer patients); Lung adenocarcinoma tissue ($n = 37$)	Increased mRNA and protein was associated with poor OS; Increased expression was associated with bone metastasis incidence	[82]
	Malignant pleural effusion samples ($n = 54$)	High protein was associated with poor OS and PFS	[73]
	Plasma from non-small lung carcinoma patients ($n = 85$)	Patients with a higher increase in anti- α -enolase had a lower hazard ratio and better PFS	[85]
	Sera from patients with lung cancer ($n = 72$), benign lung diseases ($n = 69$), and healthy individuals ($n = 70$)	Autoantibodies were higher in lung cancer sera compared with sera from normal and benign lung disease patients; Autoantibodies were higher in stage I/II than in stage III/IV	[89]
Lymphoma	Peripheral T-cell lymphoma not otherwise classified tissue ($n = 87$)	Increased protein correlated with worse OS	[8]
Pancreatic cancer	Sera from pancreatic ductal adenocarcinoma patients ($n = 120$)	Presence of auto-antibodies correlated with a better clinical outcome	[86]
	Sera and PBMCs from pancreatic ductal adenocarcinoma patients ($n = 15$)	Patients with > 20% peripheral α -enolase-specific T cells or anti- α -enolase antibodies showed a better OS	[87]
ccRCC	Primary ccRCC tissue ($n = 360$) and TCGA dataset ($n = 428$)	Negative correlation between protein expression, tumour stage and grade. Patients with higher mRNA had lower hazard ratio of recurrence and longer OS	[84]

ccRCC: clear cell renal cell carcinoma; CLL: chronic lymphoblastic leukaemia; DMFS: distant metastasis-free survival; GEO: gene expression omnibus; HCC: hepatocellular carcinoma; OS: overall survival; PBMC: peripheral blood mononuclear cell; PFS: progression-free survival; TCGA: the cancer genome atlas; TNM: tumour node metastasis

are decreased in stage IV lung and breast cancers^[88], and are lower in stage III/IV than in stage I/II lung cancer patients^[89]. By contrast, the presence of anti- α -enolase antibodies in sera from chronic lymphocytic leukaemia (CLL) patients is predictive of a shorter time to first treatment^[90], indicating that the presence of α -enolase antibodies are indicative of a disrupted immune system in CLL. Taken together, these studies suggest that autoantibodies against α -enolase are a good prognostic factor in pancreatic, lung and breast cancers, and provide further evidence that targeting α -enolase may be beneficial in solid tumours.

ENOLASE INHIBITORS ARE POTENTIAL ANTICANCER AGENTS

Due to its important cancer-related roles, enolase is one of several glycolytic enzymes being examined as a potential anticancer therapeutic target. Polyamine sulphonamide analogues have proven particularly effective at inhibiting α -enolase activity. Two such compounds have been further developed and shown to

be cytotoxic to KG-1 (AML) cells and to the AML leukaemic stem cell fraction, with minimal effects on normal healthy stem cells^[92]. This report highlights that α -enolase is an actionable therapeutic target that may be useful in the treatment of cancer, particularly AML.

CONCLUSION

Alpha-enolase plays a supportive role in cancer progression and has been implicated in three of the hallmarks of cancer: cellular energetics and metabolism; cell proliferation; and invasion and migration. In cancer cells, α -enolase is overexpressed and localised on the surface, where it acts as a key promotor of metastasis, driving invasion through plasminogen activation and extracellular matrix degradation. In several cancer types, patients develop an immune response against α -enolase, and anti- α -enolase antibodies can be detected in their sera. Increased expression of α -enolase mRNA, proteins or autoantibodies are associated with decreased metastasis-free survival in several cancer types, including non-small cell lung, pancreatic, breast and colorectal cancers. Future examination of the expression and function of α -enolase in cancers may ultimately result in α -enolase becoming a therapeutic target and prognostic biomarker for a range of cancer types.

DECLARATIONS

Authors' contributions

Contributed to the drafting and editing of this manuscript: Schofield L, Lincz LF, Skelding KA

Availability of data and materials

Not applicable.

Financial support and sponsorship

This work was funded by grants from the Calvary Mater Newcastle and Hunter Medical Research Institute.

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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Perspective

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Letting go of the physical exam: embracing telehealth solutions to oncology

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How to cite this article: Overman MJ. Letting go of the physical exam: embracing telehealth solutions to oncology. *J Cancer Metastasis Treat* 2020;6:11. <http://dx.doi.org/10.20517/2394-4722.2020.06>

Received: 17 Jan 2020 **First Decision:** 27 Mar 2020 **Revised:** 12 Apr 2020 **Accepted:** 21 Apr 2020 **Published:** 28 Apr 2020

Science Editor: Pravin D. Potdar **Copy Editor:** Jing-Wen Zhang **Production Editor:** Tian Zhang

Despite the diminishing value of the physical exam in the management of many chronic diseases, it still represents a core component of clinical office visits^[1]. In part, this reflects the reimbursement landscape that continues to support its use at every clinical encounter. However, as healthcare pivots towards a new focus on value-based care, it is imperative that we move beyond the confines of the in-person clinical encounter and embrace the tremendous potential for telehealth solutions to healthcare delivery.

The management of chronic diseases frequently relies on the subjective component of care represented by symptom management or the provision of information. In addition, in many chronic diseases, new diagnoses are made based on objective measures of laboratory or imaging testing. This is particularly true in medical oncology, where symptoms generally reflect well-defined toxicity profiles from anti-cancer systemic therapy or the space-occupying effects of metastatic disease, which in almost all settings would be diagnosed via imaging testing. In a medical oncology clinic, the two most common visit types are by patients undergoing active systemic anti-cancer therapy or surveillance for potential cancer recurrence.

In the first setting, medical oncology providers spend their time managing various symptoms such as nausea, fatigue, depression, anxiety, diarrhea, or pain. In addition, at almost all of these visits, laboratory studies are conducted to provide adequacy of blood counts for treatment and chemistries to assess dehydration. The critical aspects of these visits are subjective symptom assessment, laboratory review, education related to symptom management, and the prescription of symptomatic medications and anti-cancer therapy. The physical exam however, frequently represents time that is not allocated for symptom management education. Without the need to see each patient in person, patients could have laboratory testing, vital signs testing, and telehealth visits better integrated into their schedules. In fact, telehealth



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experiences in other internal medicine subspecialties have demonstrated a reduction in driving time and missed work^[2]. As well, systemic anti-cancer therapy represents a recurring event in patients' lives so optimization of their time should be our focus.

In the second context of surveillance, the physical exam is not needed when recent diagnostic imaging such as computed tomographic scans have just been conducted. In addition, when tumor markers are normal and a patient does not have new symptoms, a physical exam is of questionable value.

In essence, by tailoring in person clinic visits towards when there is highest need, we can help to integrate our delivery of care into the lives of our patients better. In medical oncology where patients' lives are often measured in months or years, this improved integration would reflect a higher quality of life away from the medical system for our patients. This change in approach would also align with where many medical oncologists feel we need to be spending our time: educating our patients about symptom management and their cancer journey. Such time spent on cancer prognosis, end-of-life experience, clinical-trial understanding, symptom prevention and management should reduce the downstream utilization of high cost emergent care facilities and hospitalizations during and at the end of life. The ability to tailor visits to the needs of patients would enable in person visits to be reserved and dedicated to the critical discussions of end-of-life care planning, prognostication discussions or restaging discussions.

Making such changes to a medical oncology clinic to be fully integrated with telehealth delivery will not be easy as technological, liability, financial, and logistic scheduling challenges will exist^[3]. In addition, the physical examination still has a symbolic meaning to patients as an acknowledgment of their complaints and an expected part of a physician office visit^[4]. Studies have also demonstrated that the physical examination is a way for physicians to build empathetic relationships with their patients^[5]. These concerns will need to be addressed and discussed with patients as telehealth moves forward. Could open acknowledgment of the missing aspect of touch and examination be a mechanism to attenuate this loss?

As we move towards a vision where we optimally integrate our practice with the lives of our patients, we must first agree to move away from our historical reliance on the physical examination. It is time taken not only to optimize a patients' time at the end of life, but also to optimize their time throughout the entire cancer care journey.

DECLARATIONS

Authors' contributions

The author contributed solely to the article.

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

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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Editorial

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Radiotherapy of brain metastases from lung cancer: evidences and areas of research

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How to cite this article: Kepka L. Radiotherapy of brain metastases from lung cancer: evidences and areas of research. *J Cancer Metastasis Treat* 2020;6:12. <http://dx.doi.org/10.20517/2394-4722.2020.35>

Received: 21 Apr 2020 **Accepted:** 21 Apr 2020 **Published:** 11 May 2020

Science Editor: Lucio Miele **Copy Editor:** Jing-Wen Zhang **Production Editor:** Jing Yu

Historically, the standard treatment for brain metastases (BM) from lung cancer involved neurosurgical resection and radiotherapy. Chemotherapy was applied, to a lesser extent, mainly for small-cell lung cancer (SCLC). However, with the ever-changing landscape of lung cancer therapy, the approach to the treatment of BM from lung cancer is also evolving. Generally, there has been a tendency to avoid whole-brain radiotherapy (WBRT) and to use more focused forms, i.e., stereotactic radiosurgery (SRS). Currently, in patients with WHO performance status 0-2 having up to 4 BM, local therapy (surgery or SRS) without WBRT is recommended^[1]. The rationale for such an approach is based on evidence that the omission of WBRT minimizes neurocognitive toxicity and there is no difference in overall survival between local therapy (SRS or surgery) only and WBRT^[2-4]. Nevertheless, a substantial proportion of lung cancer patients have multiple BM and for them, WBRT remains the primary treatment modality, unless their general performance status is very poor. For such patients with poor performance status, WBRT has no benefit over the use of steroids alone^[5,6]. The prognosis of patients with BM from lung cancer is considered poor with a median overall survival of about 4 months with WBRT^[7,8]. However, it was shown that WBRT in NSCLC patients with BM having EGFR mutations and ALK rearrangement had improved overall survival^[9,10]. Novel therapies such as targeted agents for BM in lung cancer with driver mutations and immune checkpoint inhibitors have greater intracranial efficacy compared to conventional chemotherapy. In turn, this also promoted research towards combining these novel agents with SRS or WBRT. While the tumor's molecular status may have an impact on the decision to delay WBRT or SRS in subgroups of patients, there is insufficient data to make more definitive recommendations currently.



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The optimal management of patients with BM from lung cancer should consider both patient (performance status, age, comorbidities, cognitive function, patient's preferences) and tumor (number/volume of BM, location, molecular subtype, extracranial disease control and systemic treatment available) related factors. There is always a trade-off between better intracranial control of disease offered by WBRT and better preservation of cognition with SRS but at the price of poorer intracranial control. This requires a detailed discussion with the patient and shared decision-making is recommended in such cases. Numerous strategies have been employed to preserve neurocognition after WBRT such as the use of neuroprotective medication and/or hippocampal avoidance (HA). Recently, the results of a randomized phase III trial comparing WBRT (30 Gy in 10 fractions)/memantine/HA to WBRT/memantine in 518 patients with multiple BM referred for WBRT were published. It was reported that WBRT with HA preserves cognitive function better after treatment compared to WBRT without HA, with no difference in intracranial control and overall survival. Thus HA should be a standard approach in all good performance status patients with BM undergoing WBRT with no metastases in the HA zone^[11].

This special issue of the *Journal of Cancer Metastasis and Treatment* aims to summarize the current knowledge on the treatment of BM from lung cancer with radiation by presenting the available evidence and discussing new perspectives and areas of research. The review articles by Kirakli and Yilmaz^[12] and Gutiérrez-Valencia *et al.*^[13] highlight the latest developments and evidence available for the use of radiotherapy in BM, and question the role of adjuvant WBRT after surgery or SRS. The issue of extending the use of SRS for more than 3-4 BM is also discussed, as we do not have evidence that directly compares such an approach to WBRT.

The planning and delivery of radiotherapy to multiple BM is a complex issue. Dumane *et al.*^[14] present their experience with the use of knowledge-based planning, which is a new approach to treatment planning and this may lead to a fully automated planning process. Knowledge-based planning utilizes dose distributions from prior treatment plans to build a model that can predict the same for new patients. The authors then demonstrated that such an approach might be used efficiently for the complex planning involved in the use of volumetric modulated arc therapy to treat multiple BM using a single isocenter.

A comprehensive review by Mudra *et al.*^[15] provides the evidence regarding the use of novel systemic agents in combination with SRS for the management of BM from lung cancer. Limited evidence is available however, on the use of systemic therapy (targeted therapy, immune checkpoint inhibitors) as firstline treatment with the omission of radiation and the authors warn that such an approach should be used with caution and only for very selected patients. On the other hand, the sequence of use of systemic agents and radiation remains uncertain and is still under investigation.

In SCLC, about 40%-50% of patients will develop BM during the course of their disease. Owing to the distinct clinical characteristics of BM from SCLC, these patients are usually excluded from prospective trials on the value of WBRT and local ablative treatments such as surgery or SRS. Instead, WBRT in combination with chemotherapy has long been a standard approach in the setting of SCLC. However, data on the neurocognitive toxicity of WBRT, occurrence of BM after earlier use of prophylactic cranial irradiation, as well as the increasing availability of stereotactic radiotherapy technologies, have all led to the increasing use of SRS and omission of WBRT in SCLC. Various issues arising from the use of radiation for BM from SCLC in numerous clinical scenarios are discussed^[16].

Finally, this issue also contains a systematic review on the clinical effectiveness of neuroprotective agents given during WBRT: memantine, methylphenidate and donepezil. Some evidence to support the use of memantine to delay cognitive decline in patients undergoing brain irradiation was demonstrated, although at 24 weeks this did not reach statistical significance ($P = 0.059$). Despite fairly large usage of memantine

during WBRT, more data is needed to confirm whether there is truly a long-standing neuroprotective effect of this drug for patients undergoing WBRT. Results for the two other agents were inconclusive^[17].

The Guest Editor and Contributors for this special issue of *Journal of Cancer Metastasis and Treatment* hope that clinicians will find these articles interesting and useful for their daily practice.

DECLARATIONS

Authors' contributions

The author contributed solely to the article.

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

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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Multi-layered control of PD-L1 expression in Epstein-Barr virus-associated gastric cancer

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How to cite this article: Miliotis CN, Slack FJ. Multi-layered control of PD-L1 expression in Epstein-Barr virus-associated gastric cancer. *J Cancer Metastasis Treat* 2020;6:13. <http://dx.doi.org/10.20517/2394-4722.2020.12>

Received: 1 Feb 2020 **First Decision:** 7 Apr 2020 **Revised:** 23 Apr 2020 **Accepted:** 12 May 2020 **Published:** 23 May 2020

Science Editor: Pravin D. Potdar **Copy Editor:** Jing-Wen Zhang **Production Editor:** Jing Yu

Abstract

Gastric cancer (GC) is the fifth most common cancer worldwide. In approximately 10% of GC cases, cancer cells show ubiquitous and monoclonal Epstein-Barr virus (EBV) infection. A significant feature of EBV-associated GC (EBVaGC) is high lymphocytic infiltration and high expression of immune checkpoint proteins, including programmed death-ligand 1 (PD-L1). This highlights EBVaGC as a strong candidate for immune checkpoint blockade therapy. Indeed, several recent studies have shown that EBV positivity in GC correlates with positive response to programmed cell death protein 1 (PD-1)/PD-L1 blockade therapy. Understanding the mechanisms that control PD-L1 expression in EBVaGC can indicate new predictive biomarkers for immunotherapy, as well as therapeutic targets for combination therapy. Various mechanisms have been implicated in PD-L1 expression regulation, including structural variations, post-transcriptional control, oncogenic activation of intrinsic signaling pathways, and increased sensitivity to extrinsic signals. This review provides the most recent updates on the multi-layered control of PD-L1 expression in EBVaGC.

Keywords: Epstein-Barr virus, gastric cancer, immunotherapy, programmed death-ligand 1

INTRODUCTION

Gastric cancer (GC) is the fifth most common and third most deadly cancer worldwide^[1]. Due to the lack of early symptoms, patients are usually diagnosed with locally advanced or metastatic cancer, when



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Table 1. Immunological and molecular characteristics of GC molecular subtypes

Molecular subtype	EBV-positive	MSI	CIN	GS
Frequency (TCGA STAD) % ^[4]	9	22	50	20
TIL abundance ^[5,6]	High	High	Low	Low
PD-L1 expression ^[4-6]	High	High	Low	Low
Somatic mutations ^[4]	Standard rate of mutations (non-hypermethylated) Common <i>PI3KCA</i> , <i>ARID1A</i> mutations	Hypermethylated Recurrent mutations in <i>TP53</i> , <i>KRAS</i> , <i>ARID1A</i> , <i>PIK3CA</i> , <i>ERBB3</i> , <i>PTEN</i> and <i>HLA-B</i>	Non-hypermethylated Common <i>TP53</i> mutations	Non-hypermethylated Common <i>RHOA</i> , <i>CDH1</i> mutations
Other molecular characteristics ^[4]	Hypermethylation (EBV-CIMP, frequent <i>CDKN2A</i> silencing) Amplification in 9p24.1 chromosomal region (<i>PD-L1/PD-L2/JAK2</i>)	Hypermethylation (MSI-associated gastric CIMP, frequent <i>MLH1</i> silencing)	Gene amplifications, frequent in TKRs (<i>EGFR</i> , <i>VEGFA</i>) and deletions	

*“High” indicates that the corresponding molecular subtype is typically associated with high levels of TIL or PD-L1, respectively. “Low” indicates that the corresponding molecular subtype is not typically associated with high levels of TIL or PD-L1, respectively. MSI: microsatellite instability; CIN: chromosomal instability; GS: genomically stable; TCGA: the cancer genome atlas; STAD: stomach adenocarcinoma; TILs: tumor infiltrating lymphocytes; PD-L1: programmed death-ligand 1; PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; ARID1A: AT-rich interaction domain 1A; TP53: tumor protein p53; KRAS: Kirsten rat sarcoma viral oncogene homolog; ERBB3: Erb-B2 receptor tyrosine kinase 3; PTEN: phosphatase and tensin homolog; HLA-B: human leukocyte antigen B; RHOA: ras homolog family member A; CDH1: cadherin 1; CIMP: CpG island methylator phenotype; CDKN2A: cyclin dependent kinase inhibitor 2A; JAK2: Janus kinase 2; MLH1: MutL homolog 1; TKR: tyrosine kinase receptor; EGFR: epidermal growth factor receptor; VEGFA: vascular endothelial growth factor A; GC: gastric cancer

conventional lines of therapy are less effective. The 5-year survival rate for GC in the United States varies by stage and ranges from 68% in patients with localized cancer to 5% in patients with distant metastases^[2].

The majority of GCs (90%-95%) are adenocarcinomas^[3]. The Cancer Genome Atlas (TCGA) recently undertook a comprehensive molecular characterization of hundreds of gastric adenocarcinomas and proposed classification of GC into four molecular subtypes: Epstein-Barr virus (EBV)-positive, microsatellite instability (MSI), chromosomal instability (CIN), and genomically stable (GS) subgroups^[4]. EBV-positive tumors are characterized by DNA hypermethylation, frequent phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) mutations, and programmed cell death ligand 1 (PD-L1)/programmed cell death ligand 2 (PD-L2)/Janus kinase 2 (JAK2) overexpression; MSI tumors have high mutation and DNA methylation rates; CIN tumors contain chromosomal alterations affecting mainly tyrosine kinase receptors; and finally, GS tumors are chromosomally stable and have a high frequency of cadherin 1 (*CDH1*) and Ras homolog family member A (*RHOA*) mutations [Table 1]^[4]. This review will focus on EBV-positive tumors.

EBV is a double-stranded DNA virus that belongs to the herpesvirus family. An estimated 90% of the human population show signs of previous infection with EBV^[7]. The virus is usually transmitted orally through saliva. Primary infection is most commonly asymptomatic, but it can lead to acute mononucleosis in a subset of the population, primarily adolescents and young adults^[8]. Following primary infection, the virus establishes a lifelong latent infection in the host. EBV can remain latent in both lymphocytes and epithelial cells, where it expresses only a subset of its genes. Depending on which viral genes are expressed, latent EBV infection is typically classified into four latency programs, known as latency 0, I, II, and III [Table 2]^[7].

In 1964, EBV was the first human virus to be associated with cancer, when it was discovered in Burkitt's Lymphoma^[9,10]. Since then, EBV has also been linked to other lymphomas, including Hodgkin lymphoma, diffuse large B-cell lymphoma (DLBCL), and Natural Killer/T-cell lymphomas (NK-T lymphomas)^[11]. In addition, EBV has been associated with certain epithelial cancers, notably Nasopharyngeal Carcinoma (NPC) and GC. In both lymphoid and epithelial cancers, EBV persists in a latent state in infected cells^[11]. However, different EBV-associated cancers demonstrate different viral gene expression patterns [Table 2].

Table 2. Viral genes expressed in each latency program

Latency program	EBV gene expression pattern	Cancer*
0	No viral proteins expressed	
I	EBER 1 & 2, BART miRNAs, EBNA1 <i>Latency I</i> +/- LMP2A	BL GC
II	<i>Latency I</i> + LMP1, LMP2A, LMP2B	NPC, NK-T lymphomas, HL
III	<i>Latency II</i> + EBNA2, EBNA3s, EBNA-LP, BHRF1 miRNAs	PTDL, AIDS-related lymphomas (IB-DLBCL)

*This column indicates some cancers that are typically associated with each latency program. Within each cancer type (e.g., BL and DLBCL) there might be subtypes that show different patterns of latent expression. EBER: Epstein-Barr encoding region; BART: BamHI A rightward transcripts; miRNA: microRNA; BHRF1: BamHI fragment H rightward open reading frame 1; EBNA1: EBV nuclear antigen; LMP: latent membrane protein; EBNA-LP: EBNA leader protein; BL: Burkitt's lymphoma; GC: gastric cancer; NPC: nasopharyngeal carcinoma; DLBCL: diffuse large B-cell lymphoma; NK-T lymphomas: natural killer/T-cell lymphomas; HL: Hodgkin lymphoma; PTDL: post-transplant lymphoproliferative disorder; IB-DLBCL: immunoblastic DLBCL; EBV: Epstein-Barr virus

In EBV-associated GC (EBVaGC), the virus expresses all latency I products, meaning EBV nuclear antigen 1 (EBNA1), Epstein-Barr encoding region small noncoding RNAs, BamHI A rightward transcripts (BARTs), and BART miRNAs, while around 50% of the cases also show Latent Membrane Protein 2A (LMP2A) expression, which is typically associated with latency II^[12]. LMP1, another latency II protein, is typically not detected in EBVaGC samples^[12-14]. There is strong geographical variation in the prevalence of EBVaGC but overall around 10% of gastric adenocarcinomas worldwide are classified as EBV-positive^[15].

In EBVaGC, the EBV genome is mainly maintained as a nuclear episome and cancer cells within the tumor show ubiquitous and monoclonal EBV infection^[12,16]. The monoclonality of EBV infection suggests the clonal selection of virus-infected cells in early stages of cancer development. The role of EBV in gastric carcinogenesis is still under investigation, but EBV infection is thought to contribute to GC progression or maintenance, both directly through the activity of viral proteins or RNAs and indirectly through the induction of somatic alterations in the host genome and epigenome^[17].

PD-L1 EXPRESSION IN EBVAGC

Multiple studies have shown that EBVaGC is commonly characterized by high lymphocytic infiltration in the tumor microenvironment, coupled with overexpression of immune-related genes, including *PD-L1* (also known as *CD274*)^[4,5,18]. *PD-L1* is a glycoprotein, expressed by both cancer cells and stromal immune cells in the tumor, that engages the programmed cell death 1 (PD-1) receptor expressed on the surface of infiltrating cytotoxic T cells (CTLs)^[19]. The interaction between *PD-L1* and PD-1 leads to the inhibition of the tumor-infiltrating CTLs, preventing them from attacking and eliminating tumor cells. *PD-L1* is only one of multiple immune checkpoint genes that are known to be upregulated in EBV-positive compared to EBV-negative cancers. *PD-L2*, Lymphocyte activation gene-3 (*LAG3*), T cell immunoglobulin and mucin domain (*Tim-3*), Cluster of differentiation 80 (*CD80*), Cluster of differentiation 86 (*CD86*), and Indoleamine 2, 3-dioxygenase 1 (*IDO1*) are also upregulated, but *PD-L1* has received particular interest because the PD-1/*PD-L1* axis is the target of some recent breakthrough cancer therapies^[20]. Monoclonal antibodies that block the interaction between *PD-L1* and PD-1, thus restoring the ability of the immune system to surveil and attack the tumor, have shown promising results as therapeutic agents against multiple cancers, including non-small cell lung cancer and melanoma^[19]. Recently, the Food and Drug Administration (FDA) approved pembrolizumab, a mAb targeting PD-1, as a third-line therapy for advanced gastric tumors that are positive for *PD-L1* expression based on immunohistochemical (IHC) staining^[21,22].

The clinical efficacy and adverse effects of PD-1/*PD-L1* therapy vary tremendously among patients. High expression of *PD-L1* in the tumor has been implicated as a significant predictive biomarker for positive response to PD-1/*PD-L1* therapy^[23]. However, several clinical studies have demonstrated that some tumors with high *PD-L1* expression do not respond to PD-1/*PD-L1* therapy, while some tumors with moderate or

low PD-L1 expression do show beneficial responses. This discrepancy could be attributed in part to the fact that direct determination of PD-L1 expression in the tumor, which is typically performed by IHC assays, has been proven to be difficult and inconsistent^[24,25]. Understanding the molecular mechanisms that control PD-L1 expression in cancer may give rise to more accurate biomarkers for positive response to PD-1/PD-L1 therapy. In addition, regulators of PD-L1 expression could serve as targets for potential combination therapies.

In a phase II clinical trial, Kim *et al.*^[6] identified EBV-positivity and high mutational load as reliable and independent biomarkers for the clinical efficacy of pembrolizumab in GC patients and recommended considering pembrolizumab as an early therapeutic option for EBVaGC. MSI-high (MSI-H) tumors are characterized by high rates of somatic mutations, resulting in increased presentation of neoantigens and thus stimulation of anti-tumor immunity. As is the case in EBV-positive GCs, MSI-H tumors have high levels of tumor-infiltrating lymphocytes (TILs)^[26]. The FDA has already approved the use of front-line pembrolizumab monotherapy in advanced MSI-H solid tumors of any origin, including stomach^[27].

EBVaGC appears to employ some common and some unique mechanisms for PD-L1 regulation. This review presents the most recent findings on PD-L1 regulation in EBVaGC and discusses some of the discrepancies in the literature, parallels with other EBV-associated cancers, and questions to be addressed in the future [Figure 1].

EVOLUTIONARY PRESSURE FOR PD-L1 OVEREXPRESSION

EBVaGC shows high levels of TILs and thus is under strong evolutionary pressure for the development of immune evasion strategies^[4]. One such strategy is PD-L1 overexpression, which is evident in both cancer and stromal immune cells. It is speculated that the high immune activity in the EBVaGC microenvironment reflects the strong immunogenicity of EBV in the body. Up to 5% of the circulating CD8⁺ T cells in EBV-infected individuals are believed to be reactive to lytic or latent EBV antigens^[28]. Indeed, several groups have detected expression of a subset of lytic genes in EBVaGC^[29-31]. According to Borozan *et al.*^[29], the lytic expression pattern detected in EBVaGC, which includes subsets of both early and late lytic proteins, does not indicate lytic, or abortive lytic, replication. However, the presentation of lytic viral antigens by infected cancer cells might be driving further immune activation. Camargo *et al.*^[32] detected higher levels of circulating antibodies targeting both latent and lytic proteins in patients with EBV-positive compared to EBV-negative GCs, even though virtually all patients in the study were seropositive for antibodies against EBNA and the viral capsid. This further suggests that lytic proteins are expressed in EBVaGC and potentially contribute to the activation of the host immune response.

SOMATIC STRUCTURAL VARIATIONS

Somatic genomic alterations include short variations, such as single nucleotide substitutions and short insertions/deletions (indels), as well as long variations, also known as structural variations (SVs)^[33]. SVs affect large chromosomal regions (longer than 50 base-pairs) and include amplifications, deletions, inversions, and translocations^[33]. Somatic changes occur continuously in the life of an individual and are usually repaired, but their accumulation over time can contribute to carcinogenesis^[33]. EBVaGC only appears in a small percentage of EBV-infected individuals and typically long after primary EBV infection suggesting that somatic genomic changes are likely required for cancer development^[34].

Gene amplification

Recent high-throughput sequencing studies have revealed different genomic alterations that lead to PD-L1 overexpression in EBVaGC. TCGA performed a somatic copy-number aberrations analysis in gastric adenocarcinoma samples and identified a frequent (15% of EBVaGC cases) somatic focal amplification at the chromosomal region 9p24.1, which includes the genes *PD-L1*, *PD-L2*, and *JAK2*^[4]. This amplification

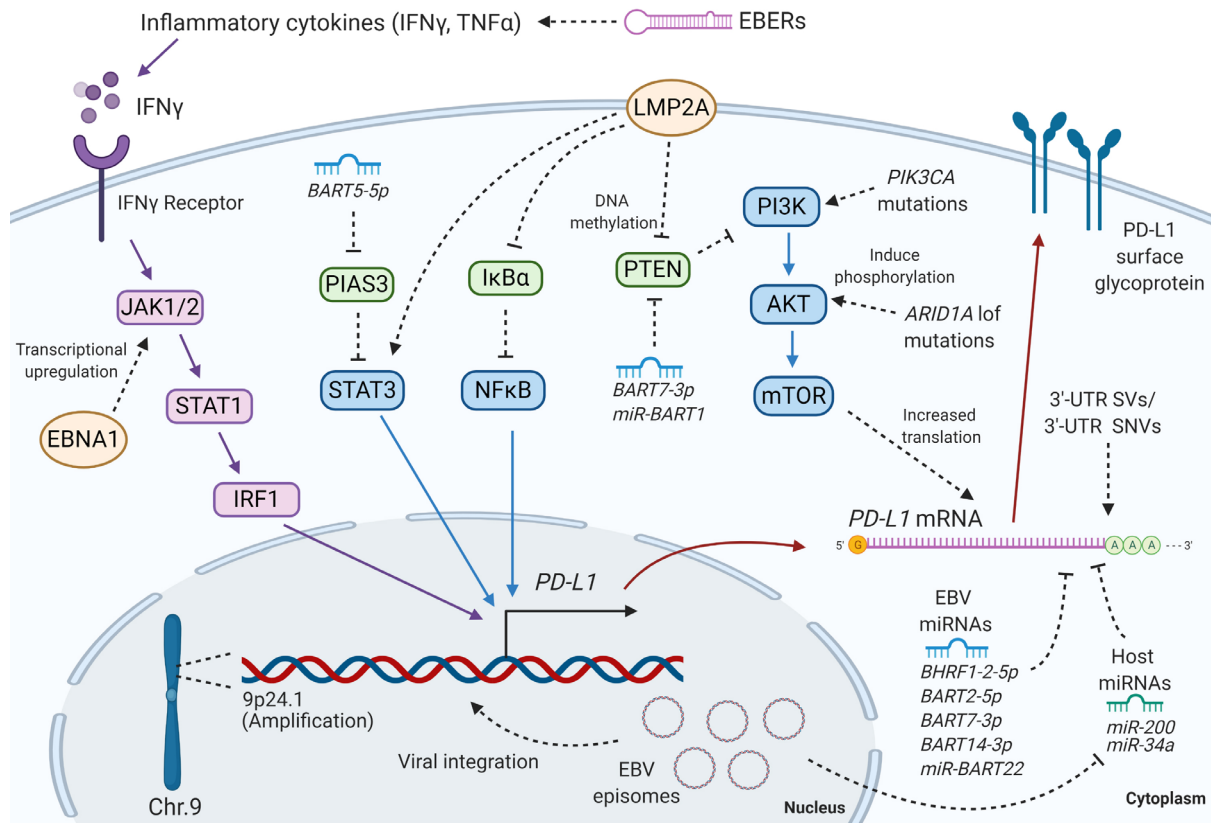


Figure 1. The mechanisms of PD-L1 regulation in EBVaGC discussed in this review. EBV: Epstein-Barr virus; EBVaGC: EBV-associated gastric cancer; PD-L1: programmed cell death ligand 1; IFN γ : interferon gamma; TNF α : tumor necrosis factor alpha; EBER: Epstein-Barr encoding region; JAK: Janus kinase; STAT: signal transducers and activators of transcription; IRF: interferon regulatory factor 1; EBNA1: EBV nuclear antigen 1; BART: BamHI A rightward transcripts; PIAS: protein inhibitor of activated STAT 3; I κ B α : nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; NF κ B: nuclear factor kappa-light-chain-enhancer of activated B cells; LMP2A: latent membrane protein 2A; PTEN: phosphatase and tensin homolog; PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PI3K: phosphoinositide 3-kinase; AKT: protein kinase B; ARID1A: AT-rich interaction domain 1A; mTOR: mammalian target of rapamycin; 3'-UTR: 3' untranslated region; SV: structural variation; SNV: single nucleotide variation; BHRF1: BamHI fragment H rightward open reading frame 1. Created with BioRender.com

was significantly more common in EBV-positive than EBV-negative tumors. Importantly, they showed that the 9p24.1 amplification correlated positively with *PD-L1* mRNA levels in the tumor^[4]. Saito *et al.*^[35] detected 9p24.1 amplification events at a similar rate in a different EBVaGC cohort and demonstrated a direct association between *PD-L1* copy number and protein expression. Using fluorescence in-situ hybridization and IHC staining, they showed that *PD-L1* genomic amplifications were specifically detected in cancer cells that showed high PD-L1 protein expression. Similar amplification patterns have been detected in other cancers, including hepatocellular carcinomas (HCC) and DLBCL^[23,36].

Integration of EBV to the host genome

In EBVaGC, the EBV genome is mostly maintained in the nucleus as a circular episome^[16]. Some studies have also reported viral integration events, even though they are considered rare^[4,37,38]. TCGA identified the presence of RNA sequencing (RNAseq) reads corresponding to a fusion between the host *PLGRKT* gene and the EBV gene *BHLF1* in one EBVaGC sample (FP-7998-01)^[4]. This is notable given that the *PLGRKT* lies within the 9p24.1 chromosomal region and is adjacent to the *PD-L1* locus. It was later shown in the same sample, that the amplified region containing the integrated EBV genome (Copy Number = 4) has a breakpoint in the *PD-L1* 3' untranslated region (3'-UTR)^[37]. Xu *et al.*^[38] performed a genome-wide EBV integration analysis in multiple malignancies and identified that EBV integrates primarily in unstable

chromosomal regions of the host genome. They identified EBV integration events in 25% (10/39) of the GC samples analyzed, with some of the integration breakpoints mapping close to known tumor suppressor genes^[38]. None of their samples had integrated virus close to *PD-L1*, but studies with bigger EBVaGC sample sizes are necessary to identify the frequency and significance of viral integration in or close to the *PD-L1* locus. Viral integration in the host genome has been associated with increased PD-L1 expression in other virus-associated cancers. Cao *et al.*^[20] identified integrated HPV genomes in the *PD-L1* or *PD-L2* loci in three cases from the TCGA Head and Neck Squamous Cell Carcinoma cohort and showed that these integration events correlated with elevated PD-L1 and PD-L2 expression.

3'-UTR structural variations

SVs in the *PD-L1* 3'-UTR have also been associated with increased PD-L1 expression in EBVaGC^[34,37]. Kataoka *et al.*^[37] analyzed RNAseq data from all TCGA cancer types and searched for 3'-UTR disruptions in *PD-L1*. The authors identified *PD-L1* 3'-UTR truncations in 31/10,210 cancer cases and showed that they correlated with high PD-L1 expression. The highest frequency of 3'-UTR truncations was found in DLBCL (4/48) and GC (9/415), with a third of the GC samples (3/9) being EBV-positive. Therefore, around 10% of EBVaGC samples in TCGA were found to have *PD-L1* 3'-UTR SVs^[34,37]. In a follow-up study, Kataoka *et al.*^[34] analyzed samples from multiple EBV-associated lymphomas and found that *PD-L1* 3'-UTR SVs were significantly more common in EBV-positive compared to EBV-negative lymphomas. They report that *PD-L1* 3'-UTR genomic truncations in cell lines and mouse models promote PD-L1 overexpression and immune evasion, consistent with the patient data^[37].

POST-TRANSCRIPTIONAL REGULATION

The 3'-UTR contains sequences or structural regions, called regulatory elements, that are important for the post-transcriptional regulation of a gene. These regulatory elements control binding to miRNAs and RNA-binding proteins (RBP), which influence mRNA stability, translation rate, and localization^[39]. miRNAs are short non-coding RNAs that silence gene expression by binding to complementary sequences in the 3'-UTR of target mRNAs. miRNA-mRNA binding usually triggers mRNA degradation or blocks translation. The fact that 3'-UTR shortening has such a profound effect on PD-L1 expression in multiple cancers indicates that PD-L1 is under tight post-transcriptional control^[37].

3'-UTR short variations

Mutations in the 3'-UTR have the capacity to remove existing or create new binding sites for miRNAs and RBPs. Some germline and somatic mutations in the 3'-UTR of *PD-L1* have been shown to correlate with PD-L1 expression in gastric and other cancers^[40-44]. Wu *et al.*^[43] analyzed 728 GC samples and found that the AA and AG genotypes in rs2297136, a germline single nucleotide polymorphism (SNP) located in the 3'-UTR of *PD-L1*, were associated with lower PD-L1 protein levels. They reported that the miRNAs miR-324-5p and miR-362 are predicted to bind to that region of the *PD-L1* 3'-UTR, but no validation experiments were pursued. Wang *et al.*^[44] polymerase chain reaction (PCR)-amplified and sequenced the 3'-UTR of *PD-L1* in hundreds of GC and matched normal samples and identified a frequent guanine-to-cytosine somatic mutation that correlated with increased PD-L1 protein expression. It was shown that this mutation maps to a seed-binding region for miR-570 and it was proven experimentally that it increases PD-L1 expression by disrupting miR-570 binding^[44]. To date, most studies looking at *PD-L1* 3'-UTR mutations have been low-throughput, with small sample sizes or targeted on specific SNP locations. There has not been a comprehensive study looking at the frequency and effect of all possible somatic and germline variants in the *PD-L1* 3'-UTR in EBVaGC or other EBV-associated cancers. The fact that SVs in the *PD-L1* 3'-UTR appear to occur more frequently in EBV-positive than EBV-negative cancers raises the question of whether short variants in the 3'-UTR could be an alternative or parallel mechanism for PD-L1 overexpression. Large-scale variant calling studies in gastric and other cancers, including the TCGA somatic mutation data, have mostly relied on whole exome sequencing data and exclude 3'-UTR sequences. This has created a gap in our understanding of 3'-UTR variations in cancer in general.

Host miRNAs and RBPs

Multiple miRNAs have been implicated in the control of PD-L1 expression in GC^[45]. Some miRNAs, such as miR-152 and miR-200, target the 3'-UTR of PD-L1 directly. Other miRNAs, such as miR-19a and miR-19b, affect PD-L1 levels indirectly, by targeting signaling pathways or transcription factors that regulate PD-L1 expression^[45-48].

EBVaGC has been reported to exhibit a distinct host miRNA expression profile from other GC subtypes^[4,49,50]. Differentially expressed miRNAs include ones that have been independently shown to target PD-L1, with the most notable example being miR-200^[48]. miR-200 is a family of miRNAs found in two distinct genomic clusters and consists of miR-200a, miR-200b, miR-429, miR-200c, and miR-141^[51]. miR-200a, miR-200b, and miR-429 form a cluster on chromosome 1, while miR-200c and miR-141 form a cluster on chromosome 12. miR-200b, miR-200c and miR-429 share the same seed sequence, while miR-200a and miR-141 have a seed sequence that differs from the others by one nucleotide. The *PD-L1* 3'-UTR contains one binding site for each seed-sharing functional cluster of miR-200 miRNAs and all miR-200a, b, and c have been shown to directly silence PD-L1 expression^[48].

The miR-200 family has been shown to be downregulated in EBV-positive compared to EBV-negative GC samples, as well as in EBV-negative GC cells following infection with recombinant EBV (rEBV) *in vitro*^[52,53]. Whether or how EBV latent proteins downregulate miR-200 remains unclear. Shinozaki *et al.*^[53] reported that overexpression of any of *EBNA1*, *LMP2A*, or *BARF0* (a *BART* transcript) in EBV-negative GC cells (MKN74 cell line) leads to miR-200a/b transcriptional repression, while overexpression of EBERs downregulates the mature miRNAs post-transcriptionally. The authors concluded that EBV latent proteins and RNAs act synergistically to downregulate miR-200. This is in contrast with a study by Marquitz *et al.*^[52] on a different EBV-negative GC cell line, AGS. They reported that rEBV-infected AGS (AGS-EBV) cells showed consistent downregulation in some tumor suppressor miRNAs, including miR-200, when compared to the parental uninfected cells. However, *EBNA1* or *LMP1* overexpression in AGS did not affect the expression levels of miR-200 and most of the other EBV-downregulated miRNAs^[52]. They speculate that EBV-mediated cellular miRNA downregulation might not be mediated by the latent viral proteins, but by viral miRNAs, through their effect on host transcription factors, or by EBERs^[52]. Another possible mechanism of host miRNA downregulation in EBV-infected cells is methylation of their promoter sequences. EBV infection in GC cells has been shown to promote extensive DNA methylation, partly through the activity of the latent protein LMP2A, which induces DNA methyltransferases (DNMTs)^[54,55]. Indeed, the miR-200c promoter contains CpG islands that have been shown to be methylated by DNMT3a in GC^[56].

Research from our group described an EBV gene/host miRNA/PD-L1 regulation axis in B cell lymphomas, where the EBV protein EBNA2 induces PD-L1 expression by transcriptionally repressing miR-34a, a miRNA that downregulates PD-L1^[57]. EBNA2 is a latency III-associated protein and thus is not expressed in GC. However, miR-34a has been shown to be downregulated in GC overall and to be transcriptionally repressed by EBNA1 in EBVaGC^[58,59].

The *PD-L1* 3'-UTR contains multiple adenosine-uridine (AU)-rich elements (AREs) that are known to serve as binding sites for different RBPs^[60]. In GC, the RBP tristetraprolin (TTP) has been shown to bind to AREs in the *PD-L1* 3'-UTR and to promote mRNA destabilization, leading to reduced PD-L1 expression^[62]. In addition, in NPC, the EBV latent protein LMP1 reduces TTP expression through extracellular-signal-regulated kinase 1/2 (ERK1/2) activation^[63]. Nonetheless, there has not been an association between EBV infection and TTP expression in GC.

Viral miRNAs

EBV expresses 25 precursor miRNAs (pre-miRNAs) and 44 mature miRNAs. Three pre-miRNAs are derived from the BamHI fragment H rightward open reading frame 1 (BHRF1) region of the viral genome,

while the rest are derived from the BART region^[64]. BART miRNAs are thought to play an important role in EBVaGC and are highly abundant, accounting for up to 15-20% of the total miRNA pool in some EBVaGC cell lines^[65]. Some studies have demonstrated that xenografts of EBV-infected GC cell lines, including AGS-EBV and SNU719 cells, in immunocompromised mice show up to 10- or 100-fold overexpression of BART miRNAs compared to their parental cell lines^[66,67]. This suggests that BART miRNAs might be particularly important for cancer progression *in vivo*. BART miRNAs are known to play a range of roles in GC, targeting host cell apoptosis, cell cycle, and metastasis^[68,69]. Their role in immune evasion is less well studied in GC, although multiple immune-related functions have been attributed to BART miRNAs in other EBV-associated cancers^[64].

In contrast to BART miRNAs, which are expressed in all EBV latent stages, BHRF1 miRNAs are thought to be expressed only in latency III and lytic replication. BHRF1 miRNAs are commonly reported as barely detectable in GC tissues and cell lines^[4,54,62]. However, Treece *et al.*^[49] analyzed miRNA expression in FFPE tissues from 78 cases of gastric adenocarcinoma, including 20 EBVaGCs, and reported that BHRF1-2-5p was significantly overexpressed in EBV-infected vs. EBV-negative cancers, although to a lower extent than BART miRNAs. Marquitz *et al.*^[52] also detected low expression of BHRF1 miRNAs in sequencing from EBV-infected GC cells (AGS-EBV). Both groups attributed the detection of BHRF1 miRNAs to possible low levels of viral replication. Given that several studies have reported the expression of a subset of lytic transcripts in EBVaGC, even in the absence of lytic replication, the level and importance of BHRF1 miRNA expression remain to be determined. In an *in vitro* model of EBV-driven B-cell differentiation, BHRF1-2-5p was found to downregulate PD-L1^[70]. The same study identified potential binding sites for some BART miRNAs in the *PD-L1* 3'-UTR, including BART19-3p, but overexpression of the miRNA did not appear to influence PD-L1 expression. Four BART miRNAs, BART2-5p, BART7-3p, BART14-3p, miR-BART22 were found to interact with *PD-L1* mRNA in a high-throughput photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) study in lymphoblastoid cell lines, but no validation experiments have been performed^[71,72]. A recent study reported that miR-BART5-5p, which shares seed homology with the host miRNAs miR-18a-5p and miR-18b-5p, leads to signal transducers and activators of transcription 3 (STAT3)-dependent transcriptional PD-L1 upregulation by targeting the STAT3 inhibitor Protein Inhibitor Of Activated STAT 3 (PIAS3)^[73].

INTRINSIC SIGNALING

PD-L1 expression can be dysregulated by oncogenic activation of signaling pathways like the JAK/STAT, phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR), Mitogen-activated protein kinase/ERK kinase (MEK)/ERK, and Jun/Activator protein 1 (AP-1) pathways. These pathways act independently or synergistically to control PD-L1 expression, at the transcriptional, post-transcriptional, and post-translational stage^[74-76]. Their importance in PD-L1 regulation tends to vary among different cancer types. The constitutive activation of an intrinsic signaling pathway in cancer is usually the result of mutations or SVs in genes of key components or regulators of the pathway. In the case of virus-associated cancers, viral proteins can also induce constitutive signaling in infected cells.

Signaling activation by host gene mutations

A frequently mutated gene in EBVaGC cancer is *PIK3CA*, which encodes a catalytic component of the PI3K kinase^[4,77]. The most common variants in *PIK3CA* are associated with increased PI3K signaling activity^[77]. The PI3K pathway is thought to regulate PD-L1 expression in a tissue-specific manner. Loss of Phosphatase and tensin homolog (PTEN) leads to activation of the PI3K pathway and induction of PD-L1 expression in gliomas and colorectal cancer^[78,79]. In gliomas, Parsa *et al.*^[79] showed that the PI3K/Akt/mTOR pathway increases *PD-L1* mRNA translation through polysomal recruitment. In the case of GC, there is conflicting evidence for the importance of the PI3K pathway in PD-L1 regulation. Supporting the importance of PI3K signaling in promoting PD-L1 expression, Kim *et al.*^[80] showed that the PI3K inhibitor

LY294002 reduced PD-L1 expression in three GC cell lines. In addition, Menyhárt *et al.*^[81] performed hierarchical clustering to determine the mutations that could best stratify TCGA GC patients based on PD-L1 expression. They showed that the mutation status of *PIK3CA* served as the best root node for the stratification, while mutations in other immune-related genes like *MEF2C*, *SLC11A1*, and *KIF15* could help further refine it, suggesting potential interactions between these genes for the control of PD-L1 expression^[81]. However, Mimura *et al.*^[82] performed experiments with a panel of GC cell lines and determined that the PI3K inhibitor wortmannin did not affect PD-L1 expression. Besides, Seo *et al.*^[77] analyzed 112 EBV-positive GC samples and reported that *PIK3CA* mutations did not show any correlation with PD-L1 expression or TIL abundance. Therefore, the importance of *PIK3CA* mutations and PI3K signaling in the control of PD-L1 expression in GC and EBVaGC is still unclear, and may be dependent on interactions with other mutations and signaling pathways.

Another gene that is commonly mutated in EBVaGC is AT-Rich Interaction Domain 1A (*ARID1A*), which encodes an important component of the SWItch/Sucrose Non-Fermentable (SWI/SNF) chromatin remodeling complex^[4]. Mutations in *ARID1A* are usually loss-of-function mutations. Kim *et al.*^[80] analyzed 273 GC samples and showed that ARID1A protein loss correlated with PD-L1 positivity (as defined by IHC staining), independently of EBV or MSI status. They also showed that *ARID1A* knockdown *in vitro* directly leads to PD-L1 overexpression, through the activation of the PI3K/Akt pathway. To account for the variability in PD-L1 expression among *ARID1A*-mutated tumors, the authors looked for additional mutations that could be acting synergistically with *ARID1A* mutations. They found *KRAS* mutations in the three *ARID1A*-mutated MSI-H tumors with the highest PD-L1 expression, while two other samples that harbored *KRAS* but not *ARID1A* mutations did not show elevated PD-L1 levels. These data further suggest that PD-L1 expression is controlled by multiple oncogenic signaling pathways acting in coordination.

Signaling activation by viral latent proteins

The EBV latent membrane proteins (LMPs) are potent oncoproteins and are known to activate oncogenic signaling cascades in EBV-associated malignancies. In epithelial cancers, LMP2A has been found to regulate the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), PI3K/Akt, MEK/ERK, and transforming growth factor beta (TGFβ) pathways^[83-86]. In GC specifically, LMP2A has been reported to lead to constitutive NFκB activation by inhibiting the expression of the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα), which negatively regulates NFκB activity^[83,84]. The mechanism of IκBα downregulation appears to be unrelated to promoter methylation and remains to be determined. Independent studies have shown that NFκB promotes PD-L1 transcription in GC^[87-89], but no direct association has been made between LMP2A, NFκB, and PD-L1. As mentioned above, LMP2A is also thought to be important for promoting the DNA hypermethylated state observed in EBVaGC. Hino *et al.*^[90] showed that LMP2A induces phosphorylation and activation of STAT3, which in turn leads to overexpression of DNMT1, thus changing the DNA methylation landscape of the cell. They showed that one of the targets of DNMT1 is *PTEN*, which encodes a negative regulator of the PI3K pathway. The hypermethylation of the *PTEN* promoter reduces PTEN expression and leads to overactivation of the PI3K pathway^[90]. Interestingly, PTEN is also known to be directly targeted by miR-BART1 and BART7-3p in NPC^[91,92]. Moon *et al.*^[93] reported that the small interfering RNA (siRNA)-mediated knockdown of LMP2A did not affect PD-L1 expression in SNU719, an EBVaGC cell line. However, more studies with additional methods of manipulating LMP2A expression and activity are required to elucidate its role in PD-L1 regulation.

INTERFERON GAMMA-INDUCIBLE PD-L1 EXPRESSION

Multiple studies have reported that GC patients with higher levels of CD8⁺ TILs also have higher PD-L1 expression^[94-96]. This suggests that PD-L1 overexpression in EBVaGC is at least partly a result of the evolutionary pressure from the adaptive immune response acting against the cancer. When CD8⁺ T cells are

activated, such as through the recognition of neoantigens or viral antigens presented by cancer cells, they produce inflammatory cytokines like tumor necrosis factor alpha (TNF α) and interferon gamma (IFN γ)^[97,98]. IFN γ has been shown to induce PD-L1 expression in various cancers^[99]. In GC, PD-L1 induction by IFN γ appears to occur mainly through the JAK/STAT/Interferon regulatory factor 1 (IRF1) signaling axis^[82,100-102]. The extent to which different cancer cells are responsive to IFN γ and the downstream effects of IFN γ exposure vary among cancer types and molecular subgroups within a cancer type.

In TCGA and other GC cohorts, when EBVaGC samples are compared to EBV-negative ones, they demonstrate elevated IFN γ signatures, indicated by higher expression of IFN γ , JAK/STAT signaling components, and several IFN γ -induced genes^[4,103]. In addition, *in vitro* studies have shown that EBVaGC cell lines induce PD-L1 expression in response to IFN γ to a much higher extent than EBV-negative GC cell lines^[93,104]. PD-L1 induction in response to IFN γ has also been shown to be significantly elevated in other EBV-associated epithelial malignancies, such as NPC^[105]. In NPC, the viral protein LMP1 acts synergistically with IFN γ to induce PD-L1 expression through the activation of the JAK3/STAT3, NF κ B, and AP-1 signaling pathways. In GC, EBNA1 has been shown to promote IFN γ -induced PD-L1 overexpression. Moon *et al.*^[93] showed that *EBNA1* knockdown in SNU719, an EBVaGC cell line, resulted in the transcriptional downregulation of JAK2. *EBNA1* knockdown also resulted in a small but significant reduction in constitutive and IFN γ -induced PD-L1 levels. However, ectopic EBNA1 expression in the EBV-negative GC cell line AGS did not affect constitutive or IFN γ -induced PD-L1 expression. In contrast, Su *et al.*^[106] reported that AGS-EBV cells are more sensitive to IFN γ /TNF α treatment, showing higher downstream PD-L1 upregulation, than the uninfected parental cell line. This suggests that other viral factors, in addition to EBNA1, might be necessary for increasing IFN γ -induced PD-L1 expression in EBV-infected GC cells. Further studies are required to determine how EBNA1 and other viral or host factors promote increased IFN γ sensitivity and PD-L1 expression in EBVaGC.

Nakayama *et al.*^[107] analyzed 43 EBVaGC samples and showed that the number of EBV genomes per cancer cell (EBV copy number) correlates positively with PD-L1 expression. Similarly, Strong *et al.*^[31] performed cellular gene expression analysis on 12 EBV-positive and 20 EBV-negative TCGA GC samples and showed that, following hierarchical clustering, the 4 EBV-positive samples that had a much higher EBV coverage depth (in RNAseq data) than the rest of the EBV-positive samples formed a well-defined gene expression cluster. When they compared expression between “high” and “low” EBV GC samples, a large proportion of the genes that were upregulated in the “high” EBVaGC group were immune-related, including IFN γ , STAT1, IRF1, and multiple IFN γ -induced genes^[31]. As the authors state, EBERs, which have been shown to induce IFN γ and TNF α production in peripheral blood mononuclear cells *in vitro*, could be contributing to the elevated IFN γ signature found in “high” compared to “low” EBV GC samples^[108].

CONCLUSION

Even though GC is declining in the United States, it still has one of the lowest 5-year survival rates of any cancer type^[2]. This highlights the need for new therapeutic strategies, especially for metastatic cases that have the poorest prognosis and account for most of the new diagnoses every year. The molecular heterogeneity of GC correlates with the response rate to different therapies, indicating that different approaches should be considered for different molecular subgroups. The FDA recently approved pembrolizumab as second-line therapy for patients with advanced MSI-H tumors of any type, including GC^[109]. In the last few years, several molecular and clinical studies present EBVaGC as another subgroup of GC that could benefit from early-line treatment with immune checkpoint inhibitors^[6,107].

In EBVaGC, high immune activation in the tumor microenvironment likely acts as a driving force for the selection of immune escape mechanisms such as PD-L1 overexpression. Different mechanisms act independently or synergistically to induce PD-L1 expression. These include somatic genomic modifications,

oncogenic activation of intrinsic signaling pathways, increased sensitivity to PD-L1 inducing signals from the tumor microenvironment, and post-transcriptional control mechanisms. Overall, the regulation of PD-L1 expression in EBVaGC is poorly understood and further studies are necessary to explain how EBV and host factors contribute to it. Insight into the important genetic and epigenetic factors that control PD-L1 expression in EBVaGC and other cancers could reveal new biomarkers for positive response to immunotherapy, as well as novel therapeutic targets.

DECLARATIONS

Acknowledgments

We especially appreciate Dr. Maria Mavrikaki, Allison Baker, Tanvi Saxena, and Jason Nomburg for their comments on this manuscript.

Authors' contributions

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Availability of data and materials

Not applicable.

Financial support and sponsorship

Slack FJ acknowledges the NCI Outstanding Investigator Award (R35CA232105) and the support of the Ludwig Institute at Harvard and the V Foundation.

Conflicts of interest

Slack FJ discloses financial interests and SAB roles with Mira DX and MiRNA Therapeutics. The other author declares no competing financial interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Opportunities and challenges in developing tissue-agnostic anti-cancer drugs

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How to cite this article: Li IW, Krishnamurthy N, Wei G, Li G. Opportunities and challenges in developing tissue-agnostic anti-cancer drugs. *J Cancer Metastasis Treat* 2020;6:14. <http://dx.doi.org/10.20517/2394-4722.2020.27>

Received: 31 Mar 2020 **First Decision:** 29 Apr 2020 **Revised:** 2 May 2020 **Accepted:** 18 May 2020 **Published:** 27 May 2020

Science Editor: Godefridus J. Peters **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

The rapid advances in the understanding of oncogenic process and the maturation of affordable precision diagnostic tools have enabled the development of targeted therapeutic agents, such as those targeting BCR-ABL, epithelial growth factor receptor L858R, EML4-anaplastic lymphoma kinase, and BRAF V600E, to treat cancers that harbor specific molecular alterations. Traditionally, each targeted drug has been developed for a particular tumor type where such alteration is most frequently found. Recently, the widespread adoption of next generation sequencing has led to an increase in the identification of rare and ultra-rare alterations, and, in some cases, the same rare alterations are found across multiple tumor types. The rarity of these alterations makes clinical trials traditionally designed for specific tumor types infeasible. As a result, tissue-agnostic trials have been developed to study the efficacy of these treatments and increase patient access. This review summarizes current successful cases of tissue-agnostic development, such as drugs targeting tropomyosin receptor kinase fusions, and proposes the next wave of potential tissue-agnostic targets, including fusions of *ROS1*, anaplastic lymphoma kinase, fibroblast growth factor receptor, and rearranged during transfection. In addition, the advantages and the challenges of such approach are discussed in the context of clinical development and approval.

Keywords: Tissue agnostic, basket trial, tropomyosin receptor kinase, anaplastic lymphoma kinase, *ROS1*, fibroblast growth factor receptor, rearranged during transfection



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INTRODUCTION

The past two decades have witnessed a significant paradigm shift in cancer treatment practices based on increased understanding of oncogenic process. Molecular alterations in specific targets, usually kinases, can result in constitutive activation of the targets and their downstream signaling activities, leading to unchecked cellular proliferation, resistance to cell death, promotion of angiogenesis, and evasion of immune surveillance, all of which are hallmarks of cancer^[1]. Matching a patient's cancer with a therapeutic agent designed to specifically address the underlying molecular alteration has become the cornerstone of precision oncology^[2]. Alongside the rapid advancement in cancer biology, the technical revolution of molecular diagnostic platforms, particularly high-throughput next generation sequencing (NGS), has made comprehensive profiling of tumor tissue and liquid biopsy samples feasible and affordable, not only for scientific interrogation of cancer genome, transcriptome, and epigenome for target discovery and mechanistic characterization, but also for patient selection and stratification in the clinical setting^[3,4].

Several large-scale cancer sequencing efforts involving thousands of patient samples have not only confirmed relatively frequent molecular alterations such as mutations in Kirsten rat sarcoma gene (*KRas*), tumor protein p53 (TP53), and epithelial growth factor receptor (*EGFR*), but also revealed, in many cases for the first time, low- and ultra-low frequency mutations that otherwise had been difficult to detect without high-throughput deep sequencing in large number of samples. For instance, in a study by Armenia and colleagues^[5], whole exome sequencing data from 1,013 cases of prostate cancer (680 primary and 333 metastatic tumors) and matched germline were assembled and uniformly analyzed. The study identified a total of 97 potential oncogenic genes, about 70 of which had not been previously implicated in the disease. The majority of these newly identified mutated genes were found in less than 5% of the 1,013 cases. In statistical terms, this is known as a “long-tail” distribution; in other words, some genes are mutated in comparatively many cases, but many genes with oncogenic mutations are only found in few cases. This “long tail” distribution also suggests that additional discovery of rarely mutated oncogenic drivers is likely to continue along with the dramatic increase in the number of tumors sequenced. A similar “long tail” distribution has also been observed in other tumor types, such as lung adenocarcinoma^[6], head and neck^[7], and breast^[8]. Arguably, this “long tail” phenomenon exists in most, if not all, tumors. Interestingly, some of the same “long tail” genes are found across many distinct tumor types, suggesting common underlying mechanism of tumorigenesis^[9,10].

The United States Food and Drug Administration (FDA) and other regulatory agencies generally approve anti-cancer drugs on the basis of efficacy and safety data obtained from clinical trials with patients of a particular tumor type. An example of this “one target, one tumor type” is the FDA's 2001 landmark approval of imatinib, a kinase inhibitor of Abelson tyrosine kinase (c-ABL), for the use in treating BCR-ABL positive, chronic myeloid leukemia (CML), which heralded a new era in approval of drugs for single indications with characteristic gene alterations^[11]. A decade later, crizotinib, a small molecule tyrosine kinase inhibitor (TKI) of mesenchymal-epithelial transition factor (c-MET), anaplastic lymphoma kinase (ALK), c-ros oncogene 1 (ROS1), and recepteur d'Origine Nantais (RON), received accelerated approval for the treatment of patients with locally advanced or metastatic non-small cell lung cancer (NSCLC) with EML4-ALK fusion. The approval was based on two single-arm trials demonstrating objective response rates (ORRs) of 50% and 61% and median response durations of 42 and 48 weeks^[12].

Even with the life-changing success of the “one target, one tumor type” approach, it is important to remember that cancer is a complex disease. On the one hand, tumors that originate from the same tissue or organ can be segmented into multiple subtypes, each of which can be defined by differentiating molecular, pathological, and etiological features^[13-15]. On the other hand, some distinct and seemingly unrelated tumors of different histology can be traced back to a common dominant genetic defect that can be exploited for therapeutic intervention by the same targeted agent, regardless of the histological tumor type

or anatomical site of origin^[16]. Recently, drugs that target these common features, for example microsatellite instability-high (MSI-H)/mismatch repair deficient (dMMR)^[17] and tropomyosin receptor kinase (TRK) fusions^[18], across multiple tumor types have been approved by the FDA as the first wave of tissue-agnostic therapies (one target, all/many tumor types).

This review summarizes the current status of the tissue-agnostic approach and proposes additional molecular alterations, with the emphasis on oncogenic fusions, that are potential targets for drug discovery and development.

FIRST TISSUE-AGNOSTIC APPROVALS

The FDA and its international counterparts traditionally approve cancer drugs on the basis of clinical studies in patients of a particular tumor type. Even for biomarker-driven approvals such as erlotinib and crizotinib, these drugs have generally been approved for a specific tumor type that harbors the target of interest.

In 2017, however, a significant paradigm shift took place, when the FDA granted accelerated approval of pembrolizumab, an anti-programmed cell death protein 1 (PD-1) therapy, in adult and pediatric patients with locally advanced or metastatic solid tumors of any tumor type (hence, tissue agnostic) that are dMMR or MSI-H, who have progressed after prior treatment, and who have no satisfactory alternative treatment options^[17]. This approval was based on collective data from several clinical trials. In the Phase II study code-named KEYNOTE-016 in patients ($n = 58$) with progressive metastatic carcinoma, high somatic mutation burden was associated with significant prolonged progression-free survival (PFS). In addition, two separate studies (KEYNOTE-158, $n = 19$; KEYNOTE-164, $n = 61$) specifically enrolled solid tumor patients with MSI-H or dMMR. Additional data from KEYNOTE-12 ($n = 6$) and KEYNOTE-28 ($n = 5$) were included in the dataset after retrospective analysis of MSI and MMR status. By tumor type, among 149 patients with MSI-H and/or dMMR cancers across the five trials, the majority ($n = 90$) had colorectal cancer (CRC), while 14 other distinct tumor types accounted for the remaining 59 patients. Collectively, the ORR was 39.6%, which included 11 complete responses (CR) and 48 partial responses (PR). The response rate for patients with colorectal cancer and those with other cancers were similar.

About a year later in November 2018, the second tissue-agnostic cancer therapy, larotrectinib, won accelerated approval by the FDA for the treatment of adult and pediatric patients with solid tumors that have a neurotrophic receptor tyrosine kinase (*NTRK*) gene fusion without a known acquired resistance mutation, that are either metastatic or where surgical resection is likely to result in severe morbidity, and who have no satisfactory alternative treatments or whose cancer has progressed following treatment^[19]. It is the second tissue-agnostic FDA approval for the treatment of cancer, and the first small molecule TKI that gained the tissue-agnostic status. The approval was based on clinical outcome in 55 patients with unresectable or metastatic, *NTRK*-fusion-positive solid tumors from three multicenter, open-label, single-arm clinical trials: LOXO-TRK-14001 (NCT02122913), SCOUT (NCT02637687), and NAVIGATE (NCT02576431). The identification of positive *NTRK* gene fusion status was prospectively determined in local laboratories using NGS or fluorescence in situ hybridization (FISH). The ORR was 75%, including 22% CR and 53% PR across 12 cancer types, with the most common being salivary gland tumors (22%), soft tissue sarcoma (20%), infantile fibrosarcoma (13%), and thyroid cancer (9%), as well as lung, melanoma, gastrointestinal stromal tumor (GIST), and colon cancer.

Soon after, the *TRK/ROS1* inhibitor, entrectinib, was also granted accelerated approval by the FDA for the treatment of adults and pediatric patients 12 years of age and older with solid tumors that have a *NTRK* gene fusion without a known acquired resistance mutation, are metastatic or where surgical resection is likely to result in severe morbidity, and have progressed following treatment or have no satisfactory

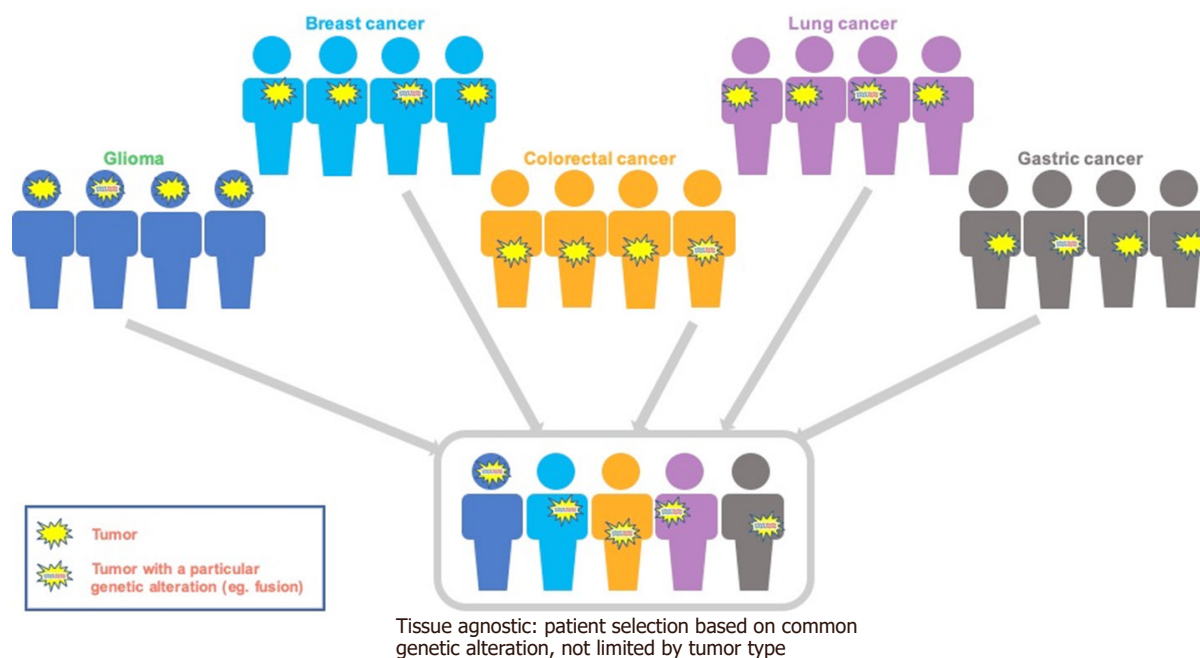


Figure 1. Tissue-agnostic approach offers biomarker-informed treatment strategy regardless of histological origin of the tumor

standard therapy. The approval was based on clinical outcome in 54 adult patients across three multicenter, single-arm, clinical trials: ALKA, STARTRK-1 (NCT02097810), and STARTRK-2 (NCT02568267). The identification of positive NTRK gene fusion status was determined in local laboratories or a central laboratory using nucleic acid-based tests prior to enrollment. Among 54 adult patients, the ORR was 57%, with 7.4% of patients achieving CR. The most common cancers were sarcoma, NSCLC, mammary analog secretory carcinoma, breast, thyroid, and colorectal. Compared to larotrectinib, the patient populations of entrectinib leaned more heavily on adult patients with more prior lines of therapies^[20]. Importantly, entrectinib also showed meaningful responses in brain cancer patients and those whose tumors metastasized to the brain^[21].

The approvals of these tissue-agnostic therapies represented a new paradigm in cancer treatment and validated the notion that, under certain circumstances, the biomarker in essence, rather than the tissue origin, would define the disease [Figure 1].

It is important to point out that the latest tissue-agnostic approvals are both small molecule receptor tyrosine kinase (RTK) inhibitors that treat oncogenic fusions in rare tumors. There are a number of novel compounds in development for other oncogenic fusion genes^[22], and it is highly likely that next tissue-agnostic approval will be from one of these experimental agents.

ONCOGENIC GENE FUSIONS

In the past several decades, cancer epidemiological and molecular studies have identified a variety of genetic alterations including point mutations, chromosomal rearrangements and translocations, gene amplification, and overexpression that are believed to play a driver role in various cancer histologies^[10]. Many of these changes lead to constitutive activation of the oncoprotein and downstream signaling pathways, resulting in uncontrolled cell proliferation, survival, and migration, which are hallmarks of cancer^[23].

Oncogenic gene fusions are somatic genetic alteration caused by interchromosomal translocation, intrachromosomal translocation, insertion, deletion, tandem duplication, inversion, chromothripsis^[23], and read-through^[24]. The first identified cancer-causing fusion gene is BCR-ABL gene, product of a reciprocal interchromosomal translocation between the q arms of chromosomes 9 and 22 that occurs in more than 96% of patients with CML^[25]. The first fusion gene in epithelial solid tumors, rearranged during transfection (RET)-CCDC6, was found in papillary thyroid carcinoma more than 30 years ago^[26]. Since then, many gene fusions have been discovered, facilitated by large scale sequencing efforts such as those championed by The Cancer Genome Atlas (TCGA), International Collaboration for Clinical Genomics (ICCG), International Cancer Genome Consortium (ICGC), and numerous other institutional studies. With the technological advancement in detection methods, the identity of gene rearrangement partners, the spectrum of tumor histologies where the gene rearrangements have been found, and their overall prevalence have significantly expanded in the past few years.

For instance, a recent study by Gao *et al.*^[27] interrogated 9,624 samples belonging to 33 cancer types in the TCGA collection and identified 25,664 distinct fusion events. Importantly, among all fusions involving receptor and non-receptor kinases, 1,275 cases contain an intact kinase domain, many of which are believed to be the sole onco-driver in a particular tumor biopsy. Many of these fusion events lead to constitutive activation of the kinase activity and downstream signaling pathways including mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) cascades, which enables cells to hyper-proliferate and evade apoptosis^[28-30] [Figure 2]. The mechanisms of activation include overexpression of the kinase as a result of the activity of the promoter of the fusion partner, constitutive ligand-independent dimerization of the fusion kinase proteins, and release of kinase auto-inhibitory mechanism. Since kinases are generally druggable targets, studies such as this provided the rationale for developing small molecule targeted therapies to treat fusion-driven hematological and solid tumors^[31-34].

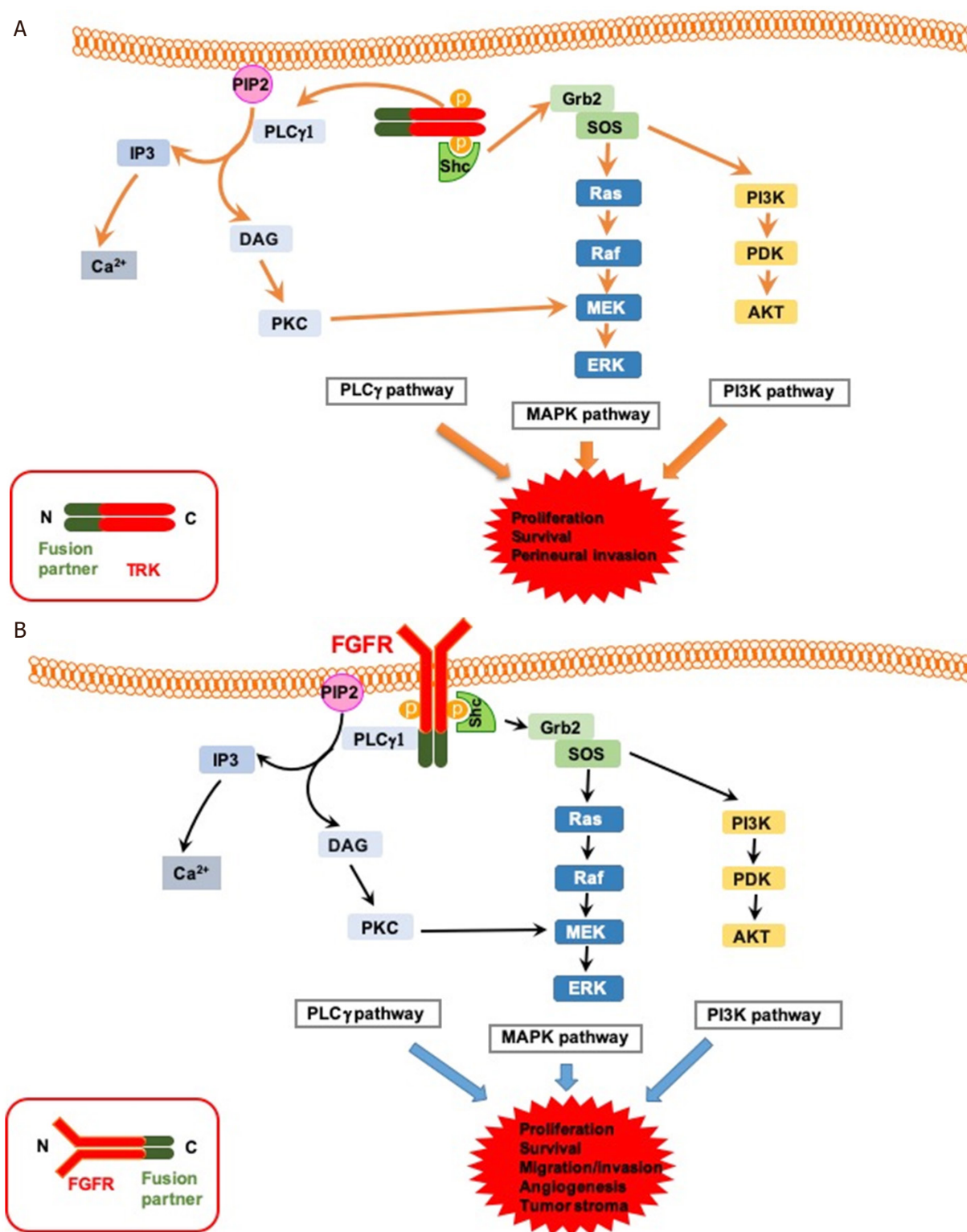
THE LONG-TAIL PHENOMENON AND TISSUE-AGNOSTIC DEVELOPMENT

Although conceivably, specific drugs can be developed to address these distinct fusion proteins in each of the tumor types involved individually, in reality, with the exception of a few cases, such as ALK and ROS1 fusions in NSCLC^[35] and fibroblast growth factor receptor (FGFR) fusions in cholangiocarcinoma^[36], the majority of the fusions occur at low frequencies^[37]. The low and ultra-low frequency alterations sometimes are called the “long tail”^[38]. As discussed above, the rarity of the fusions and the resulting small patient pool make the development of a particular targeted drug for a single tumor type impractical.

One potential solution to address this challenge lies in the observation that a number of recurring gene fusions, such as those formed by ALK, ROS1, FGFR, NTRK, and RET, have been identified in multiple cancer histologies. For example, ALK fusions are found in anaplastic large cell lymphoma^[39], NSCLC^[40], papillary thyroid cancer^[41], colorectal cancer^[42], renal cell cancer^[43], and esophageal cancer^[44], as well as in spitzoid tumors^[45]. Similar to ALK fusions, FGFR fusions have been reported in a wide range of tumors such as cholangiocarcinoma, breast cancer, prostate cancer, NSCLC, gastric adenocarcinoma, colorectal adenocarcinoma, and glioblastoma, with a large number of distinct fusion partners^[46]. The long-tail phenomena (rare and ultra-rare patient populations) and recurring fusions across multiple tumor types necessitate biomarker-driven cross-tumor type clinical trials, to enroll a sufficient number of patients for efficacy and safety assessment and to offer patients with a rare actionable mutation access to an experimental therapy.

TRK

The tyrosine kinase receptors TRKA, TRKB, and TRKC, are encoded by neurotrophic tropomyosin receptor kinase (*NTRK*) genes *NTRK1*, *NTRK2*, and *NTRK3*, respectively. Their ligands are neurotrophins, a family



of nerve growth factors including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins^[47]. Neurotrophin binding to TRK receptors results in receptor autophosphorylation

and activation of downstream signaling cascades. Physiologically, the TRK family members play a key role in normal central and peripheral neuronal cell development and differentiation. *NTRK* gene mutations, overexpression, splice variants, and fusions/rearrangements have been found in a number of human cancer types^[48].

The first *NTRK* gene fusion, *TPM3-NTRK1*, was identified in a colon carcinoma biopsy through a transformation assay^[49]. Subsequently, *TPM3-NTRK1* has been identified in additional CRC tumors^[50], sarcoma^[51], lipofibromatosis-like neural tumors^[52], spitzoid melanoma^[53], invasive mucinous adenocarcinoma of the lung^[54], and papillary thyroid carcinoma^[55]. With the use of advanced molecular diagnostic techniques, additional *NTRK1* fusion partners have been identified in various tumor types, with 5' fusion partners including LMNA, SQSTM1, BCAN, CD74, IRF2BP2, MDM4, MPRIP, and others^[56].

Similarly, oncogenic fusions have also been identified with *NTRK2* and *NTRK3* genes, as well as across a variety of cancer histologies. For example, *NTRK2* forms fusions with partners such as *AGBL4*, *NACC2*, *QKI*, and *VCL*, which were identified in non-brainstem high-grade glioma, soft tissue liposarcoma, head and neck squamous cell carcinoma, pilocytic astrocytoma, ganglioglioma, and diffuse intrinsic pontine glioma. *NTRK3* fusion partners include *ETV6*, *BTBD1*, and *EML4*, which were identified in diffuse intrinsic pontine glioma, congenital fibrosarcoma, papillary thyroid carcinoma, mammary analog secretory carcinoma (MASC) of the thyroid gland, secretory breast cancer, and inflammatory myofibroblastic tumor^[48,57-60], as well as in hematological malignancies such as acute myeloid leukemia, Philadelphia-like acute lymphoblastic leukemia, and chronic eosinophilic leukemia^[61-63].

NTRK gene fusions have also been reported in pediatric solid tumors. For instance, oncogenic gene fusions involving the *NTRK3* kinase domain have been identified in congenital fibrosarcoma and pediatric mesoblastic nephroma and acute leukemias^[57]. A survey of 2 pediatric cancer databases, St. Jude pediatric cancer database (PeCan; total $n = 1,604$) and the University of Michigan database (Peds-MiOncoSeq; total $n = 91$) resulted in the identification of three gene rearranged-cancers, one each involving *NTRK1*, *NTRK2*, and *NTRK3* in a sarcoma, a low-grade glioma, and a B-cell acute lymphoblastic leukemia, respectively. In addition, the following tumor types, which are largely confined to the pediatric patient population, are also known to harbor *NTRK* gene fusions: congenital or infantile fibrosarcoma, secretory (juvenile) breast cancer, mesoblastic nephroma, and intrinsic pontine gliomas^[64].

Fusion of 3' *NTRK* gene sequences encoding the tyrosine kinase domain to various 5' partner sequences via intra- or inter-chromosomal rearrangement results in an oncogenic chimera protein that can ligand-independently homodimerize, autophosphorylate, and constitutively activate downstream signaling pathways, such as MAPK, PI3K/protein kinase B (AKT), and phospholipase C (PLC)- γ , which can result in hyperproliferation and cell survival in tumors expressing these proteins. The growth of cancer cells thus becomes dependent on or "addicted" to this abnormal kinase signaling^[65].

Although oncogenic *NTRK* gene fusions are observed across a large number of adult and pediatric solid and hematological tumors, they are rare events in most common cancers (e.g., frequency of $< 0.1\%$ in NSCLC or CRC). Although much higher frequencies of *NTRK* fusions are present in certain tumor types such as MASC, these cancers are ultra-rare, collectively representing less than 1% of all malignancies. As a result, the overall population of *NTRK*-fusion-positive patients is very small^[66].

The rarity of the molecularly defined patients and the vastly diverse histologies of the patients clearly called for an innovative, tissue-agnostic approach. Fortunately, data generated in preclinical studies provided rationale to perform tissue-agnostic clinical trials in multiple molecularly defined cancers^[56]: (1) regardless of the fusion partner or the tissue of origin, the *NTRK* gene fusions result in a constitutively active kinase

and provide the driving force for transformation and tumor progression; and (2) regardless of the identity of the fusion partners, TRK inhibitors such as entrectinib exhibit similar anti-tumor potency in cell lines harboring *NTRK1*, *NTRK2*, or *NTRK3* fusion genes (i.e., *TPM3-NTRK1*, *LMNA-NTRK1*, *SQSTM1-NTRK1*, *BCAN-NTRK1*, *MPRIIP-NTRK1*, *AFAP1-NTRK2*, *VCL-NTRK2*, and *ETV6-NTRK3*), and in *NTRK*-fusion-positive xenograft models derived from various tumor types. For instance, tumor growth inhibition was observed in cancer cell line-derived xenograft models of CRC harboring *TPM3-NTRK1* fusion, AML harboring *ETV6-NTRK3* fusion, and NSCLC harboring *MPRIIP-NTRK1* fusion, as well as in patient-derived xenograft (PDX) models of metastatic CRC harboring *LMNA-NTRK1* fusion, head and neck cancer harboring *ETV6-NTRK3* fusion, and sarcoma harboring *TPM3-NTRK1* fusion^[67].

As discussed above, the preclinical observations have been clinically validated in several clinical trials that led to the regulatory approvals of larotrectinib and entrectinib as the first two small molecule anti-cancer drugs that carry a tissue-agnostic label.

ROS1

ROS1 belongs to the insulin-receptor superfamily of receptor tyrosine kinases and plays a role in relaying growth signals from the environment outside the cell into the cell's nucleus. It is an orphan receptor tyrosine kinase with no known binding ligand. Genetic changes in *ROS1*, such as gene rearrangements, mutations, or copy number increases, create oncogenes that can lead to cancer^[68]. *ROS1* gene rearrangements create fusion proteins with constitutively active kinase domains that activate downstream signaling pathways leading to oncogenic properties in cells, including uncontrolled proliferation and resistance to cell death with prolonged tumor cell survival. These pathways include Ras-ERK for cellular proliferation and the Janus kinase/signal transducer and activator of transcription (JAK/STAT) and PI3K/AKT pathways, which regulate cell survival (anti-apoptosis) and proliferation. *ROS1* fusion proteins may also activate the mammalian target of the rapamycin pathway, which is critical for the regulation of protein translation. Cancers that have these pathways activated tend to be more aggressive, with invasion and metastasis leading to poor patient survival^[69].

In NSCLC patients, *ROS1* fusion protein is found in approximately 1%-2.5% of patients^[70,71]. *ROS1* gene rearrangements have also been detected in a variety of other cancers, including glioblastoma multiforme^[72,73]; biliary tract carcinoma (3.9%)^[74]; ovarian cancer, gastric adenocarcinoma (0.61%)^[75]; CRC (0.85%)^[76]; inflammatory myofibroblastic tumor, angiosarcoma, and epithelioid hemangioendothelioma^[69,70,75,77]; and Spitz nevus (benign) (25.3%), atypical Spitz tumors (6.2%), and spitzoid melanomas (9.1%)^[45].

Thus far, more than two dozen *N*-terminal fusion partners have been identified^[78]. All the fusion proteins retain the *ROS1* kinase domain, but rarely its transmembrane domain^[79]. The most common *ROS1* fusion partner is *CD74*^[80]. Other commonly observed *ROS1* fusion partners include *SDC4*, *SLC34A2*, *LRIG3*, *EZR*, and *TPM3*^[77,78]. A survey of cBioPortal for Cancer Genomics (<https://www.cbioportal.org>) and The Cancer Genome Atlas (TCGA) generated the following breakdown of *ROS1* fusion partners: 38% *CD74*, 12% *EZR*, 12% *SLC34A2*, 9% *SDC4*, 6% *CEP85L*, 6% *GOPC*, and rare cases of *CLTC*, *GOLGB1*, *SLC4A4*, *TFG*, *TMEM181*, and *TPM3*. More than half of the partners have dimerization domains that are retained in the fusion, presumably leading to constitutive *ROS1* tyrosine kinase activation. Additional mechanism of activation of the *ROS1* fusion proteins may include removal of the auto-inhibitory domain from the full-length *ROS1* as the result of the fusing event^[69]. Recent survey of responses to crizotinib in 106 NSCLC patients with *ROS1* fusions of various fusion partners (49.1% *CD74*, 17% *EZR*, 14.2% *SDC4* and 4.7% *TPM3*) showed no significant difference among patients with various types of *ROS1* fusion partners in overall survival (OS) and progression-free survival (PFS)^[81].

Clinically, multiple *ROS1* inhibitors have been approved for *ROS1*-fusion-positive NSCLC. Although the clinical efficacy of *ROS1* inhibitors has not been systemically established, several preclinical studies have

shown support of potential broad efficacy across tumor types that are driven by *ROS* fusion. For instance, Davare *et al.*^[73] showed that *CEP85L-ROS1* and *GOPC-ROS1* are transforming oncogenes in cells of astrocytic lineage, and they are sensitive to pharmacologic inhibition with several *ROS1* inhibitors *in vitro*. Furthermore, systemic therapy with a BBB-penetrant *ROS1* inhibitor, lorlatinib, significantly prolonged survival in an intracranially xenografted, *ROS1*-fusion-positive glioblastoma tumor model. In a separate study^[82], *ROS1* inhibitors were able to inhibit FIG-*ROS*-driven cholangiocarcinoma *in vitro* and *in vivo*. These data provide the rational support to a potential tissue-agnostic approach for treating *ROS1*-fusion-positive cancers.

ALK

ALK belongs to the insulin-receptor superfamily and aberrant ALK fusion proteins lead to self-activation and constitutive activity within cancer cells via activation of signal transduction pathways and intracellular kinases that drive uncontrolled tumor cell growth, metabolism, and survival^[83]. In addition to anaplastic lymphomas (ALCL), ALK oncogenes are found in a number of cancers such as NSCLC, diffuse large B-cell lymphoma, neuroblastomas, colorectal cancer, inflammatory myofibroblastic tumors (IMT), esophageal/gastric cancers, and renal cell cancers^[35,83]. The currently available ALK inhibitor drugs, crizotinib, ceritinib, alectinib, and brigatinib, have demonstrated clinical benefit in NSCLC^[84]. In a recent report based on the CREATE Study in eight European countries, Schoffski *et al.*^[85] showed that, in ALK-fusion-positive IMT, crizotinib treatment resulted in 50% ORR (6 responders out of 12 patients) with nine-month median duration of response and 73% one-year PFS. On the contrary, the ORR was 14% (one out of seven) in ALK-negative IMT. In addition to the CREATE study, similar or higher ORRs were observed in ALK-fusion-positive patients enrolled in studies COG and PROFILE 1013. These two studies also demonstrated ORRs of 53%-88% in ALCL.

FGFR

The FGFR protein family consists of four highly conserved transmembrane receptor tyrosine kinases (FGFR1-4). Receptor activation by the fibroblast growth factor (FGF) ligands or oncogenic alterations leads to intracellular signaling to promote cell proliferation, differentiation, morphogenesis and patterning, angiogenesis, and survival^[86]. The FGFR signaling pathway is aberrantly activated in multiple types of human cancers through various molecular alterations including point mutations, gene amplification and overexpression, and chromosomal rearrangements/translocations. Many of these changes lead to constitutive receptor activation and upregulation of the downstream signaling pathways, leading to uncontrolled cell proliferation, survival, and migration, which are hallmarks of cancer. Both the overall frequency of FGFR alterations and the relative distribution of the types of alterations vary by cancer type^[46,87,88].

FGFR fusions are the result of gene rearrangements and have been detected in different types of human cancers^[46,89] [Table 1]. Particularly, *FGFR2* fusions with different partners, such as *BICC1*, *TACC3*, *CCDC6*, and *AHCYL1*, have been detected in approximately 10%-20% of intrahepatic cholangiocarcinomas^[90-92]. Lower frequencies of *FGFR1-3* fusions have also been detected in breast cancer, bladder cancer, glioblastoma, head and neck squamous cell carcinoma, low-grade glioma, lung adenocarcinoma, lung squamous cell carcinoma, ovarian cancer, prostate adenocarcinoma, and thyroid carcinoma^[46,87,88,93]. *FGFR* fusion partners generally contain dimerization or oligomerization domains that lead to ligand-independent constitutive activation of the receptor and downstream RAS-MAPK and JAK-STAT signaling pathways^[88], resulting in uncontrolled cell proliferation, survival, and migration, which are hallmarks of cancer. In solid tumors, these *FGFR* fusions are typically formed by fusing near full-length *FGFR* with intact kinase domain at the N-terminus and various fusion partners at the C-terminus (Type II fusions), suggesting that these may serve as potential therapeutic targets via kinase inhibition. However, it is also possible for the fusion to

Table 1. FGFR fusions and fusion partners in solid tumors

Cancer Type	5'-gene	3'-gene
Bladder	<i>FGFR1</i> <i>FGFR3</i>	<i>NTM</i> <i>TACC3, TNIP2, JAKMIP1, BAIAP2L1</i>
Breast	<i>FGFR1</i> <i>RHOT1</i> <i>NSD3</i> <i>ERLIN2</i> <i>FGFR2</i>	<i>ADAM18</i> <i>FGFR1</i> <i>FGFR1</i> <i>FGFR1</i> <i>CCDC6, AFF3, CASP7, NCALD, WHSC1</i>
Cervical cancer	<i>FGFR3</i>	<i>TACC3</i>
Cholangiocarcinoma	<i>FGFR2</i>	<i>AFF4, AHCYL1, BICC1, CCDC6, VCL, CLIP1, POC1B, CELF2, CREB5, DNAJC12, HOOK1, KCTD1, KIAA1217, KIAA1598, MGEA5, NOL4, OPTN, PARK2, PCMI, PPHLN1, RASAL2, SLMAP2, SORBS1, STK26, STK3, TACC3, TBC1D1, TFEC, TRA2B, UBQLN1, WAC, ZMYM4</i>
Colorectal cancer	<i>FGFR2</i>	<i>NPM1, COL14A1</i>
Gastric cancer	<i>FGFR2</i>	<i>C10orf68, PDHX, TACC2</i>
Glioblastoma	<i>FGFR3</i>	<i>TACC3</i>
Head and neck squamous cell carcinoma	<i>FGFR3</i>	<i>TACC3, TPRG1</i>
Lung squamous cell carcinoma	<i>BAG4</i> <i>FGFR2</i> <i>CCAR2</i> <i>FGFR3</i>	<i>FGFR1</i> <i>CCAR2, CIT, KIAA1967</i> <i>FGFR2</i> <i>TACC3</i>
Mesothelioma	<i>FGFR2</i>	<i>CASC15</i>
Ovarian cancer	<i>FGFR2</i>	<i>USP10</i>
Prostate adenocarcinoma	<i>FGFR2</i> <i>FGFR3</i>	<i>KLK2, PPAPDC1A, SLC45A3</i> <i>AES</i>
Renal cell carcinoma	<i>FGFR3</i>	<i>TACC3</i>
Thyroid cancer	<i>FGFR2</i> <i>VCL</i>	<i>OFD1</i> <i>FGFR2</i>

FGFR: Fibroblast growth factor receptor

occur so that the *FGFR* gene remains intact on the 3' end of the gene (Type I fusions) allowing the fusion partner to be present on the 5' end^[88,94]; these fusions are mostly found in hematological malignancies^[95].

Given the significance of constitutive *FGFR* signaling in tumorigenesis and progression, small molecule inhibitors targeting this pathway have been developed and their anti-tumor activities are currently being evaluated in clinical trials^[88,96,97]. For instance, recent results from several clinical trials in *FGFR2*-fusion-positive cholangiocarcinoma have demonstrated meaningful clinical efficacy, which supports potential approvals as second line therapy for the treatment of advanced cholangiocarcinoma with *FGFR2* fusions. For example, in a Phase II study of infigratinib in 71 cholangiocarcinoma patients with *FGFR2* fusions, ORR 31% and SD 58% were observed, with median PFS and OS of 6.8 and 12.5 months, respectively^[98]. Similar results were reported based on an interim update from the Phase II study of pemigatinib in *FGFR2*-fusion-positive cholangiocarcinoma patients^[99]. Erdatinib, recently approved for advanced or metastatic urothelial carcinoma with susceptible *FGFR3* mutations, has demonstrated efficacy in therapeutic trials for cholangiocarcinoma patients with *FGFR2* fusions, although these trials contained fewer patients^[100]. Additionally, a covalent pan-*FGFR* inhibitor, futibatinib, has shown limited efficacy in cholangiocarcinoma patients previously treated with a different *FGFR* inhibitor, suggesting a potential utility for later line therapy when drug sequencing is needed^[101].

In addition to cholangiocarcinoma, there is evidence, albeit very limited, that *FGFR* inhibitors work in other solid tumors with *FGFR1*, -2, or -3 fusions^[97,102-104]. Based on these data there is a rationale for performing tumor-agnostic clinical trials in molecularly defined cancers to maximally benefit patients with serious and life-threatening diseases.

RET

RET encodes a single-pass transmembrane receptor tyrosine kinase important for normal cellular proliferation, development, and maintenance. It has four cadherin-like repeats at the *N*-terminal

extracellular domain, a cysteine-rich region, a transmembrane domain, and C-terminal cytoplasmic tyrosine kinase domain^[105]. Under normal conditions, wild-type *RET* is activated through binding of glial cell line-derived neurotrophic factor (GDNF) family of ligands^[106] and a co-receptor, and it functions through the modulation of downstream signaling including RAS-MAPK, PI3K-AKT, and phospholipase C γ (PLC γ) pathways^[107,108]. During development, *RET* protein plays an important role in the development of the enteric nervous system^[109] and homeostasis of neural, neuroendocrine, hematopoietic, and male germ tissues^[106,110].

In certain cancers, aberrant, ligand-independent *RET* activation is associated with gain of function *RET* mutations or gene rearrangements (fusions). The first known *RET* fusion was an in-frame fusion of *CCDC6-RET* identified in a patient with papillary thyroid carcinoma^[111]. Subsequently, *RET* fusions were reported in 13%-43% of papillary thyroid carcinomas^[112], and multiple fusion partners have been reported. *RET* fusions mostly occur in irradiation-induced papillary thyroid carcinoma^[112].

The first group of *RET* fusions in lung cancer, *KIF5B-RET*, was reported in 2012^[113] with estimated incidence rates between 1.3% and 6% of lung adenocarcinomas tested. The *KIF5B-RET* fusion contains *RET* kinase domain fused with a coiled-coil domain from *KIF5B*, which mediates homodimerization and ligand-independently activates the oncogenic pathways by autophosphorylation. It is also believed that the fusion event eliminates the auto-inhibitory domain of *RET*^[107,108]. Later studies with much larger samples put the fusion rate at 1%-2%^[114,115]. Interestingly, a review of 936 patients with surgically resected NSCLC suggested that *RET*-fusion-positive patients tended to be associated with younger age, never-smoker status, early lymph node metastases, poor differentiation, and a solid-predominant subtype^[116]. Not surprisingly, and similar to other oncogenic drivers, *RET* fusions are largely mutually exclusive from other known oncogenic alterations^[117].

Besides coiled-coil domain-containing protein 6 (*CCDC6*) and *KIF5B*, other partners of *RET* include nuclear receptor coactivator 4 (*NCOA4*), the tripartite motif-containing 33 (*TRIM33*), myosin VC gene (*MYO5C*), EPH receptor A5 gene (*EPHA5*), CAP-Gly domain containing linker protein family member one gene (*CLIP1*), ELKS/RAB6-interacting/CAST family member one gene (*ERC1*), phosphatidylinositol binding clathrin assembly protein gene (*PICALM*), FERM domain containing 4A gene (*FRMD4A*) *RUN*, *RYVE* domain containing two gene (*RUFY2*), tripartite motif containing 24 gene (*TRIM24*), tripartite motif containing 27, and many others. All of these fusion counterparts have a dimerization domain that induces ligand-independent activation of the *RET* kinase^[118-120].

In addition to papillary thyroid cancer and lung adenocarcinoma, *RET* fusions have been identified in other solid tumors, including colorectal cancer (CRC)^[121,122], breast cancer^[121], and Spitz tumor^[45,123] [Table 2].

Given the importance of *RET* fusions in cancer biology and preclinical support of targeting *RET* as a potential intervention agent for cancer^[124,125], multiple selective small molecule kinase inhibitors, such as *RXDX-105*^[124], *BLU-667*^[126], and *LOXO-292*^[127], have entered clinical development. In the most recent update^[128], *LOXO-292* demonstrated a 68% ORR in *RET*-fusion-positive NSCLC patients who had previously received chemotherapy. Additionally, *BLU-667*^[129] achieved 60% response in second line *RET*-fusion-positive NSCLC and 63% response in *RET*-altered medullary thyroid cancer (MTC) who had previously been treated with Caprelsa or Cabometyx.

CHALLENGES

Six years ago, Lacombe et al.^[130] in their opinion article “The dream and reality of histology agnostic cancer clinical trials”, while appreciating the need and advantages of tissue-agnostic trials, expressed great uncertainty whether a true tissue-agnostic approach was feasible and approvable. Six years later, however,

Table 2. RET fusions and fusion partners in solid tumors other than lung cancer

Tumor histology	RET fusion detection rate (%)	Fusion partners
PTC	6% (Kondo et al. ^[112] 2006, Stransky et al. ^[121] 2014)	<i>AKAP13, FKBP15, HOOK3, PCM1, PRKAR1A, SPECC1L, TBL1XR1, TRIM24, TRIM27, CCDC6, ERC1, KIF5B, NCOA4, GOLGA5, KTN1, RFG9</i>
CRC	0.2%-0.4% (Stransky et al. ^[121] 2014, Le Rolle et al. ^[122] 2015)	<i>CCDC6, NCOA4</i>
BC	0.1% (Stransky et al. ^[121] 2014)	<i>ERC1</i>
Spitz tumors	3% (Wiesner et al. ^[145] 2014)	<i>GOLGA5, KIF5B</i>

PTC: Papillary thyroid cancer; CRC: colorectal cancer; BC: breast cancer; RET: rearranged during transfection

with three tissue-agnostic cancer drugs approved and more than a dozen in various stages of development, the tissue-agnostic approach is becoming a viable route for demonstrating efficacy of a targeted agent in multitude of tumor types with shared molecular aberration or target as the common denominator. This approach is especially attractive for those cancers with rare or ultra-rare patient populations. At the same time, it is important to acknowledge that there are still many challenges and limitations in this emerging area of research and development.

The first challenge is to determine, at the target and biology levels, whether same aberrations in different histologies have similar biological, functional, and pathological significance. The preclinical data and clinical experience in targeting *NTRK* fusions clearly confirmed that *NTRK* fusions are the single dominant oncogenic driver in fusion positive cancers, independent of tissue origin of the cancer^[131]. Therefore, *NTRK* fusions represent an ideal tissue-agnostic target. On the other hand, one of the prominent failures during early days of tissue-agnostic exploration involved BRAF targeting in different tumors including melanoma, thyroid carcinoma, and colorectal cancer^[132]. Whereas vemurafenib was efficacious in BRAF V600E melanoma^[133] and thyroid carcinomas^[134], it failed to halt colorectal cancer with the same BRAF mutation^[135], partly due to a tissue-specific feedback activation of EGFR pathway in CRC patients^[136]. This exemplifies the role of histological context plays in certain cancers that influence the drug-target response. It is unclear what level of influence the tissue context has on the oncogenic fusions. Will the oncogenic fusions of *ALK*, *ROS1*, *FGFR*, and *RET* behave similarly to *NTRK* fusions upon treatment? For example, it has been shown that different *ROS1* fusions exhibited different subcellular localizations^[79], which could lead to varied levels of activation and pathway involvement. Whether differential subcellular localization is a more general feature regulating oncogenesis across different oncoprotein fusions remains unclear. Therefore, extensive translational research efforts need to be an integral part of these trials to guide patient selection strategy.

The clinical development path for tissue-agnostic indication can be challenging. For instance, how is the sample size determined for each of the tumor types? What are the common endpoints, considering each tumor type is likely to have distinct natural history, standard of care option(s) and treatment algorithm (line of therapy), reference response rates and duration of response, and survival end points? Particularly, response assessment criteria would require cross-tumor harmonization, since these can differ depending on tumor type. There is no standard design of basket trials, especially for the very rare and ultra-rare patient populations. For instance, larotrectinib was conditionally approved based on a 55-patient trial that spanned 12 distinct tumor types, some of which were represented by just one patient. Will this happen to a future trial and still get approved? It is obvious that in these trials the statistical analyses are different from well-established practices and innovative approach will be needed to support drug development decisions. Operationally, basket trials require well-coordinated effort from different specialists and their teams of the respective departments, which are typically organized by organ site. This holds particularly true for the collection and processing of the patients' biological material for molecular diagnostics.

The challenges in the regulatory processes should not be ignored either. Regulatory agencies in different countries and geographic regions, such as US/North America, European Union, Japan, and other Asia-Pacific countries, may have different degrees of acceptance of the tissue-agnostic approach. It is encouraging that recently the regulatory authorities in the US (larotrectinib and entrectinib), EU (larotrectinib), and Japan (entrectinib) all gave the green light to tissue-agnostic indication for *NTRK* fusions. However, the comfort level of these agencies may be different if the next agent does not have the splendid response rates larotrectinib and entrectinib exhibited, and the future approvals are likely to be reviewed on case-by-case basis and no established playbook exists.

CONCLUSION

With the rapid advances in cancer genomics, drug design and precision diagnostics, the field of oncology drug development has entered an era when both traditional tissue-restricted and innovative tissue-agnostic approaches provide precedented approval paths. Despite the challenges, we anticipate that tissue-agnostic approvals will continue to grow and expand the therapeutic options for cancer patients in need.

DECLARATIONS

Authors' contributions

Researched and gathered the material and data: Li IW

Reviewed the manuscript: Li IW, Wei G, Li G

Drafted the manuscript: Krishnamurthy N, Wei G, Li G

Proposed the concept: Li G

Availability of data and materials

Not applicable.

Financial support and sponsorship

Li IW and Krishnamurthy N were supported by the Emperor Science Awards, an initiative of Stand Up To Cancer (SU2C), made possible by support from Genentech, Bristol-Myers Squibb Company, and Novartis.

Conflicts of interest

Wei G and Li G are employees of QED Therapeutics and equity owners.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Technical Note

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Evaluating different routes of extracellular vesicle administration for cranial therapies

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How to cite this article: Ioannides P, Giedzinski E, Limoli CL. Evaluating different routes of extracellular vesicle administration for cranial therapies. *J Cancer Metastasis Treat* 2020;6:15. <http://dx.doi.org/10.20517/2394-4722.2020.22>

Received: 9 Mar 2020 **First Decision:** 13 Apr 2020 **Revised:** 1 Jun 2020 **Accepted:** 5 Jun 2020 **Published:** 18 Jun 2020

Science Editor: Robert J. Griffin **Copy Editor:** Cai-Hong Wang **Production Editor:** Tian Zhang

Abstract

Aim: Human stem cell-derived extracellular vesicles (EV) provide many advantages over cell-based therapies for the treatment of functionally compromised tissue beds and organ sites. Here we aimed to highlight multiple administration routes for the potential treatment of various forms of brain injury.

Methods: Human neural stem cell-derived EV were isolated from conditioned media and administered via three distinct routes: intrahippocampal transplantation, retro-orbital vein injection, and intranasal. EV were administered after which brains were evaluated to determine the capability of EV to translocate into normal tissue.

Results: Data showed no significant differences in the amount of EV able to translocate across the brain, indicating the functional equivalence of each administration route to effectively deliver EV to the brain parenchyma.

Conclusion: Findings show that both systemic administration routes (retro-orbital vein or intranasal delivery) afforded effective penetrance and perfusion of EV throughout the brain in a minimally invasive manner, and point to a translationally tractable option for treating certain neurological disorders including those resulting from cranial irradiation procedures.

Keywords: Extracellular vesicles, cranial therapy, cognitive dysfunction

INTRODUCTION

Extracellular vesicles (EV) are secreted by nearly every mammalian cell type and contain a wealth of bioactive cargo capable of modulating target cell function through a variety of paracrine signaling



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mechanisms^[1]. Depending on such factors as cellular origin, cargo contents, membrane composition, and target cell indications, interactions of EV with damaged, diseased, or otherwise compromised tissue beds can promote functional recovery^[1,2]. As membrane-bound vesicles, EV are typically divided into two groups based on size and mode of formation. Microvesicles (MV) tend to be larger (100 nm to 1 µm), and are directly assembled from cellular contents and released by outward budding of the cell membrane^[3]. Exosomes are smaller (30-100 nm) intraluminal vesicles within endosome-derived multivesicular bodies that then fuse with and release from the plasma membrane^[4]. For the resolution of radiation injury, no clear evidence has demonstrated a therapeutic advantage of human stem cell-derived MV over exosomes or vice versa, so the EV-based treatments used in this study include the full-size range of vesicles secreted into the conditioned medium by the proliferating human neural stem cells.

Compared to stem cell therapies, the ability of EV to stimulate regenerative healing while eliminating risks of teratoma/tumor formation and confounding complications associated with immune suppression, indicate their potential translational utility. While regenerative approaches for implementing stem cell treatments in the context of radiation injury hold tremendous potential^[5], EV circumvents certain stem cell-based caveats due to their low immunogenicity, long-circulating half-life and ability to cross the blood-brain barrier^[2,6,7]. Recent work from our laboratory has demonstrated the functional equivalence of EV and human stem cells following intra-cranial delivery to the irradiated hippocampus^[8,9]. These studies demonstrated that both therapies mitigated radiation-induced cognitive dysfunction while preserving host neuronal morphology and attenuating neuroinflammation^[8,9]. EV-based therapies have also been used to reduce indications of neuroinflammation or cognitive dysfunction associated with chemobrain^[10], other neurodegenerative conditions^[11,12], and physical injury^[13-15], indicating their widespread tolerance and broad efficacy in the brain.

Migration of EV through the extracellular space or circulation provides the routes whereby EV can interact with target cells, presumably through interactions between transmembrane proteins on the EV and specific receptors on the surface of the target cell. Recipient cells internalize EV via either fusion with the plasma membrane or more commonly by endocytosis^[16]. This then initiates the functional transfer of critical bioactive cargo containing lipids, proteins, organelles, and an assortment of nucleic acids including microRNA (miRNA). The ability of EV to target and functionally interact within the radiation-injured tissue bed provides a heretofore unexplored area for resolving a wide range of dose-limiting normal tissue toxicities associated with the radiotherapeutic management of cancer. For these reasons we embarked on a targeted technical study to evaluate whether other non-surgical administration routes could deliver hNSC-derived EV to the parenchyma of the brain.

METHODS

Stem cell culture, isolation, and labeling of EV

Growth, culturing and maintenance of human neural stem cells (hNSC, H9-derived ENstem-A, Millipore) was approved by the Institutional Human Stem Cell Research Oversight (HSCRO, #2007-5629) and Institutional Biosafety Committees. The growth of hNSC and harvest of conditioned medium from hNSC has been described previously^[9,10]. All cultures were tested and found to be negative for mycoplasma with MycoAlert Mycoplasma Detection Kit (Lonza, Cat# LT07-118; Basel, Switzerland).

For *in vivo* tracking, EV were labeled with PKH26 (Sigma-Aldrich, Cat# PKH26PCL; St. Louis, MO) the day before transplantation. The EV were then resuspended in Diluent C and incubated with Dye Solution for 2 min with intermittent mixing as per the manufacturer's protocol. The dye was quenched with 1% bovine serum albumin in water, and EV were isolated through ultracentrifugation and washed as described^[17].

Administration of EV

All animal experimentation described in this study was per the guidelines provided by the NIH and approved by all Institutional Animal Care and Use Committees (IACUC #AUP-18-032). Male wild type mice (C57Bl6/J, Jackson) were maintained in standard housing conditions, respectively ($20^{\circ}\text{C} \pm 1^{\circ}\text{C}$; $70\% \pm 10\%$ humidity; 12 h:12 h light and dark cycle) and had free access to standard rodent chow and water. Four-month-old wild-type C57Bl/6J male mice were divided into the following three groups receiving EV: Intracranial (IC), retro-orbital (RO), and intranasal (IN). For each route of administration, a total of 6.70×10^6 EV were delivered in a total volume of 8 μL (4 sites), 50 μL and 20 μL for IC, RO and IN respectively.

IC delivery: Mice were anesthetized (4%) and maintained on 2% (v/v) isoflurane/oxygen for the stereotaxic implantation of EV. Surgical injections were performed using a 32G microsyringe at an injection rate of 0.25 $\mu\text{L}/\text{min}$. Each hippocampus received 2 distinct injections of EV per hemisphere in an injection volume of 2 μL (EV in sterile hibernation buffer) per site for a total of 4 injections (8 μL) per animal. Stereotactic coordinates from the bregma were anterior-posterior (AP): -1.94, mediolateral (ML): -1.25, dorsal-ventral (DV): -1.50 for the first site and AP: -2.60, ML: -2.0, and DV: -1.5 for the second site.

RO delivery: Mice were anesthetized with 4% isoflurane and gentle pressure was applied using the fingers against the skin that is ventral and dorsal to the eye. A dermal syringe with a 29G needle, bevel down, was inserted gently to the retro-orbital vein by the corner of the eye. The 50 μL EV injection was applied in a slow, smooth fashion. The needle was carefully removed and the mouse was monitored until fully recovered (within 3-5 min) from anesthesia.

IN delivery: Mice were lightly anesthetized (2% isoflurane) and prepared for IN administration of EV^[18]. Manual restraint was used to hold the mouse in a supine position with the head elevated. The end of a 20 μL micropipette was placed at the external nares, and then the 20 μL solution was poured in slowly by the nasal tip. Mice were held for about 10 s before returning to the holding cage to recover.

Intracranial imaging

Two days post-EV treatment, mice were deeply anesthetized using isoflurane and euthanized via intracardiac perfusion using 4% paraformaldehyde (Sigma) in 100 mmol/L phosphate-buffered saline (PBS; pH 7.4, Gibco). Brains were cryoprotected using a sucrose gradient (10%-30%) and sectioned coronally into 30 μm thick sections using a cryostat (Microm, Thermo Scientific, US). For each endpoint 4 representative coronal brain sections from each experimental group were selected at approximately 15 section intervals to encompass the rostrocaudal axis from the middle of hippocampus including regions of the prefrontal cortex (PFC), the subventricular zone (SVZ) and the dentate gyrus (DG), which were then stored in PBS. Tissues ($n = 54$) were DAPI nuclear counterstained mounted onto slides and sealed in slow fade/antifade mounting medium (Life Technologies). Confocal analyses were carried out using multiple Z-stacks taken at 5-mm intervals using a confocal laser scanning microscope (Nikon Eclipse TE2000-U, EZ-C2 interface). Individual Z-sections were then analyzed using Nikon Elements software (version 3.0). Images were deconvoluted using AutoQuant X3 and surface analysis was performed with Imaris (v8.5, BitPlane, Inc., Switzerland).

Statistical analysis

Statistical analyses were carried out using GraphPad Prism (v6) software. One-way analyses of variance (ANOVA) were used to assess significance between the control, irradiated, and irradiated group receiving EV. When overall group effects were found to be statistically significant, a Bonferroni's multiple comparisons test was used to compare the 9 Gy with individual experimental groups. Data in the text are presented as means \pm SEM, and all analyses considered a value of $P \leq 0.05$ to be statistically significant.

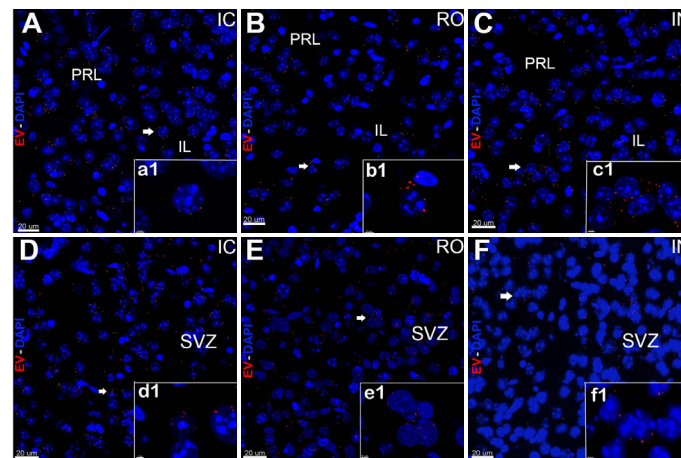


Figure 1. *In vivo* tracking of extracellular vesicles (EV) in the prefrontal cortex and subventricular zone after intracranial (IC), retro-orbital (RO), or intranasal (IN) injections. hNSC-derived-EV labeled with fluorescent dye were transplanted using stereotaxic IC (A, D), RO (B, E), or IN (C, F) injections. The brain tissues were fixed at 48 h post-surgery and sections imaged using confocal microscopy. Confocal Z-stacks were collected at 60 × magnification and qualitatively demonstrate that injected EV (red, DAPI nuclear counterstain, blue) migrated to the pre-limbic (PRL) and infra (IL) limbic structures of the PFC (A-C) and the SVZ (D-F). Magnified images (a1-f1) demonstrate localization of EV in close vicinity of the cell bodies after IC, RO, and IN administration. Scale bars: 20 μm (A-F) and 3 μm (a1-f1)

RESULTS

Extensive migration of EV in the host brain via distinct administration routes

Past data has shown that intracranial grafting of hNSC-derived EV affords significant neurological improvements in the irradiated brain^[9]. In that work, cranial irradiation was associated with significant behavioral deficits that were ameliorated by EV treatments. The neurological benefits of hNSC-derived EV grafted into the hippocampus prompted efforts to determine whether alternative (and non-surgical) routes of administration would suffice for the delivery of EV to the parenchyma of the brain. EV derived from a single batch were administered to mice via IC, RO, and IN routes, after which distinct brain regions (PFC, SVZ, DG) were imaged 2 days following treatments to assess brain penetrance of EV delivered through each route.

Compared to IC grafting, data demonstrated that systemic delivery routes provided comparable doses of EV to the brain [Figures 1 and 2]. Intracranial grafting of EV [Figure 1A and D], showed equivalent levels when compared to the brains in which EV were injected RO [Figure 1B and E] or delivered IN [Figure 1C and F] in the PFC [Figure 1A-C] or the SVC [Figure 1D-F]. Similar observations were obtained from comparisons of EV content for the hippocampal DG following each administration route [Figure 2]. Quantification of EV fluorescence between the different administration routes and/or subregions of the brain did not reveal consistent trends indicating that one delivery route was more or less efficacious than another [Figure 3A]. Similar findings were obtained when the number of fluorescent EV puncta were quantified throughout the same brain regions [Figure 3B]. Delivery of EV via IC, RO, and IN routes were all found to penetrate the different subregions of the brain at roughly equivalent levels, where differences found between either method of quantification did not reach statistical significance [Figure 3]. These findings corroborate our past data, where comparable distributions of EV were found between ipsi- and contra-lateral sites when delivered via unilateral IC route^[19]. Current data indicate that qualitatively similar yields and widespread distribution of EV can be obtained throughout the brain using various administration routes.

DISCUSSION

While certain applications of EV-based therapies have begun, their potential for the resolution of radiation-induced normal tissue toxicities remains relatively unexplored. Our past work demonstrating

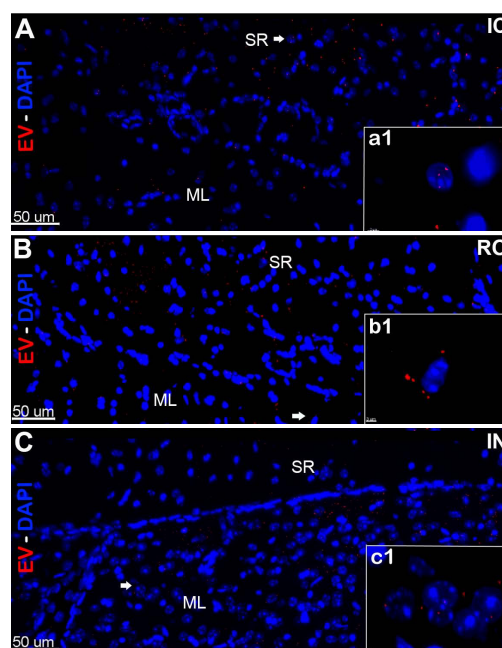


Figure 2. *In vivo* tracking of extracellular vesicles (EV) in the hippocampus after intracranial (IC), retro-orbital (RO), or intranasal (IN) injections. hNSC-derived EV labeled with fluorescent dye were transplanted using stereotaxic IC (A), RO (B), or IN (C) injections. Brain tissues were fixed at 48 h post-treatment, sections were imaged using confocal microscopy and Z-stacks were collected at 60 magnification. Fluorescently-labeled EV (red; DAPI nuclear counter-stain, blue) were located and migrated through the CA1 stratum radiatum (SR) and granule cell molecular layers (ML) in the host hippocampus. Magnification (a1-c1) demonstrates the close vicinity of EV around the cell bodies after IC, RO, and IN administration. Scale bars: 50 μm (A-C) and 3 μm (a1-c1)

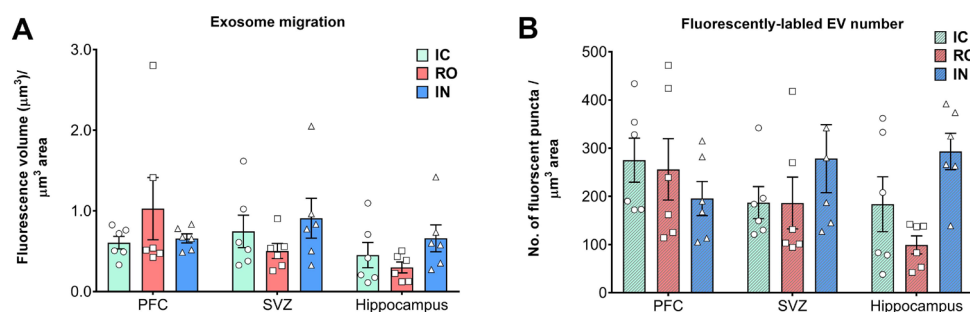


Figure 3. Quantification of extracellular vesicles (EV) throughout the brain. The volume of EV fluorescence intensity (A) or the yield of fluorescent EV puncta plotted (B) as a function of administration route reveal the relatively equal and widespread distribution of EV throughout the prefrontal cortex (PFC), subventricular zone (SVZ), and hippocampus. Differences between the yields of EV quantified between administration route and brain subregion were not found to be statistically different

the neuroprotective benefits of cranially grafted EV, when substituted for stem cells, into the irradiated brain laid the foundation for much of the current work. The ability of EV to ameliorate radiation-induced cognitive dysfunction is noteworthy if not remarkable, especially given that a single treatment via cranial graft was successful in reducing serious and multifaceted normal tissue complications associated with the radiotherapeutic management of brain cancer. Importantly, we have now demonstrated the feasibility of delivering EV through non-surgical routes, thereby providing a more tractable and appealing alternative for translating EV therapies to the clinic.

Our current study was designed to advance potential therapeutic applications of EV, by demonstrating the practical feasibility of delivering EV through multiple routes. While EV surface markers and the content will greatly dictate *in vivo* targeting and efficacy, specifics related to disease, insult, and/or injury will largely

dictate whether a targeted or systemic administration will provide the most optimal treatment strategy. Furthermore, to avoid any possible confounding impact on concurrent cancer treatments, we envision that such treatments would transpire after the cessation of radiotherapy and/or chemotherapy. This proposition is also supported by recent evidence demonstrating that irradiation can significantly alter the protein, lipid, and miRNA cargo of EV derived from cancer and normal cells and circulating EV found within the plasma^[20-22].

In any case, this targeted study demonstrates the potential feasibility of administering EV through systemic routes, thereby avoiding the requirement for more invasive surgical procedures. The equivalence found between administration routes for delivering EV to various subregions of the brain is provocative but not without caveats. Inherent uncertainties are associated with comparisons of these data, as the different EV administration routes selected lead to variable (and unavoidable) sample dilution *in vivo*. Importantly, while the net amount of EV between each treatment was held constant, each administration route necessitated different volumes for proper biological distribution. For instance, IC injections cannot accommodate volumes over 2 µL/site and systemic injections (RO at 50 µL, IN at 25 µL) require larger boluses to facilitate more homogeneous delivery. Notwithstanding, further work is still required to more rigorously validate whether systemic administration of EV affords functionally equivalent neuroprotection to the otherwise compromised or irradiated CNS.

So where does the field of EV therapy stand for the treatment of radiation and other normal tissue toxicities? Future studies should seek to define optimal cellular sources of EV to delineate the mechanism of action, to identify bioactive cargo, and to pinpoint efficacious EV dosing regimens. While current data points to several possible options for delivering EV to the brain, in humans, intravenous routes are likely to provide the best combination of widespread availability and feasibility for repeated treatment regimens. Clearly, a more systematic and complete characterization of EV surface markers and the content will be required to translate these approaches to the clinic and be necessary to evaluate other potential risks. While the lack of teratoma formation and reduced immunogenic response inherent to EV therapies are clear benefits, certain safety issues remain to be thoroughly addressed, especially in the area of cancer treatments. Further work must determine whether such approaches activate “cold” or latent cancers or alter the growth of recurrent malignancies when administered after the cessation of specific cancer treatments. Despite the caveats associated with any burgeoning therapy, EV provide a potentially attractive therapeutic avenue for resolving normal tissue toxicities associated with radiotherapy, injury, disease, and aging. Studies here provide the proof of principle highlighting the tremendous potential of EV-based therapy and underscore that such pursuits are warranted.

DECLARATIONS

Author contributions

Performed experiments, analyzed data, wrote paper: Ioannides P

Performed experiments, analyzed data: Giedzinski E

Designed experiments, analyzed data, wrote paper: Limoli CL

Availability of data and materials

Data can be made available upon request. Materials can be provided pending availability.

Financial support and sponsorship

This work was supported by the NINDS grant (R01 NS074388) to Limoli CL.

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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Deubiquitination in prostate cancer progression: role of USP22

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How to cite this article: Nag N, Dutta S. Deubiquitination in prostate cancer progression: role of USP22. *J Cancer Metastasis Treat* 2020;6:16. <http://dx.doi.org/10.20517/2394-4722.2020.23>

Received: 24 Mar 2020 **First Decision:** 14 Apr 2020 **Revised:** 7 May 2020 **Accepted:** 2 Jun 2020 **Published:** 18 Jun 2020

Science Editor: Zhou Wang **Copy Editor:** Cai-Hong Wang **Production Editor:** Tian Zhang

Abstract

Prostate cancer (PCa) is the leading cause of cancer death in men. With more therapeutic modalities available, the overall survival in PCa has increased significantly in recent years. Patients with relapses after advanced second-generation anti-androgen therapy however, often show poor disease prognosis. This group of patients often die from cancer-related complications. Multiple approaches have been taken to understand disease recurrence and to correlate the gene expression profile. In one such study, an 11-gene signature was identified to be associated with PCa recurrence and poor survival. Amongst them, a specific deubiquitinase called ubiquitin-specific peptidase 22 (USP22) was selectively and progressively overexpressed with PCa progression. Subsequently, it was shown to regulate androgen receptors and Myc, the two most important regulators of PCa progression. Furthermore, USP22 has been shown to be associated with the development of therapy resistant PCa. Inhibiting USP22 was also found to be therapeutically advantageous, especially in clinically challenging and advanced PCa. This review provides an update of USP22 related functions and challenges associated with PCa research and explains why targeting this axis is beneficial for PCa relapse cases.

Keywords: USP22, prostate cancer, SAGA, Deubiquitin

INTRODUCTION

Epidemiologically, prostate cancer (PCa) is the most common cancer in men and second most common cancer related death worldwide^[1]. Over the past few years, treatment modalities have improved, albeit modestly, the overall survival of PCa patients. The fate of advanced PCa remains the same however, and androgen deprivation therapy (ADT) is the standard of care in such cases. PCa eventually recurs within



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6 months to 2 years in the form of highly aggressive castration resistant prostate cancer (CRPC)^[2-5]. While the treatment of CRPC with second generation ADT such as Abiraterone or Enzalutamide is promising, eventually, the cancer progresses to metastatic disease [called metastatic CRPC (mCRPC)], especially in the bone^[6]. Chemotherapy with docetaxel is the first choice for treatment with mCRPC. Unfortunately, mCRPC patients have died due to complications related to metastatic transformation of PCa^[7-11]. Interestingly, these mCRPC often lose the androgen-receptor dependency and are associated with the loss of tumor suppressor proteins such as tumor protein p53 (TP53) and retinoblastoma 1 (RB1)^[12,13]. Genome-wide sequencing analysis has found some of the unique variations in the chromosomal sequence but no such driver mutation/s in PCa has been ascertained to correlate with cancer progression^[14-16]. Moreover, not all primary PCa cases progress to CRPC. Therefore, to understand the indolent vs. aggressive nature of PCa, gene-expression analysis is highly important. To correlate CRPC progression and to stratify the therapeutic regimen, high throughput sequencing analysis of various stages of PCa to correlate genetic expression profiles with the therapy-resistant state has been attempted. The expression of a 11-gene signature in primary prostate tumors was shown to correlate with therapeutic failure in PCa patients^[17]. Further characterization has shown that this 11-gene signature is a powerful predictor of distant metastasis and poor survival. Amongst these eleven genes, a specific deubiquitinases, named USP22, has been over-expressed following PCa progression. Further evidence indicates the importance of USP22 in a multi-faced pathway, which often correlates with a poor prognosis of PCa independently^[18-20].

CLASSIFICATION OF DEUBIQUITINATING ENZYMES

The protein ubiquitin (Ub) plays an important role in tissue homeostasis. Ub modification is a reversible phenomenon that is coordinated by the deubiquitination pathway^[21-24]. Deubiquitinating enzymes (DUBs) belong to either cysteine proteases [such as ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), *etc.*] or metalloproteases [Jab1/Mov34/Mpr1 (JAMM)], which are important for maintaining normal physiological homeostasis^[21,25]. Approximately 100 DUBs are encoded in the human genome. DUBs are involved in various physiological processes including the processing of Ub precursors, reversal of ubiquitination and removal of poly-ubiquitin chains^[20,26]. Therefore, DUBs regulate a series of cellular processes and functions including proteolysis, apoptosis, cell cycle progression, gene expression, DNA repair, maintenance of telomeric length, spermatogenesis, and so on^[27-30]. One such conserved ubiquitin-specific protease is USP22 and it has been well characterized, relating to various physiological and pathological processes^[19,31].

UBIQUITIN-SPECIFIC PEPTIDASE 22

The ubiquitin-specific peptidase 22 (USP22) belongs to the USPs family of DUBs and is highly conserved from yeast to vertebrates. In yeast, the USP22 homologue known as Ubp8, is complexed with Sgf73, Sgf11 and Sus1 to form the deubiquitylase module (DUBm) of the SAGA (Spt-Ada-Gcn5 Acetyl transferase) complex. The SAGA complex has a multi-disciplinary role in gene-expression and RNA-transport. Like yeast Ubp8, USP22 also forms a DUBm complex with the human orthologue ATXN7L3, ENY2 and ATXN7 and functions as a DUB unit of the human SAGA complex^[32,33].

ROLE OF USP22 IN CELLULAR PROCESSES

As a part of the SAGA complex, transcriptional activation by deubiquitination of lysine-123 of histone-H2B is enhanced^[34,35]. Later it was identified that histone-H2A ubiquitination can be processed by USP22. Ubiquitination of H2A by the polycomb group of proteins is related to transcriptional repression. However, whether deubiquitination of H2Aub (monoubiquitinated histone) by USP22 reverses the phenotype is not yet clearly established^[36].

Other than histones, USP22 also regulates the ubiquitination status of a large number of non-histone proteins. One of the most important functions of USP22 is to regulate telomeric length. Telomeric repeat

binding factor 1 (TRF1) functions as a negative regulator of telomere length by inhibiting the access of telomerase to the telomeric region of chromosomes. Poly-ADP-ribosylation of TRF1 by Fbx4 releases it from the telomere, which in turn gets ubiquitinated and degraded by the proteasomal pathway. On the contrary, as a part of the SAGA complex, USP22, by deubiquitinating TRF1, restores its protein level and thereby maintains telomeric length. Depleting USP22 decreases TRF1 levels and enhances cell death by genotoxic insults^[27]. The deubiquitination activity of USP22 is also important for the stability of Sirtuin 1 (SIRT1)^[37]. By deacetylating, SIRT1 negatively regulates the transcriptional activity of p53 and thereby, p53 dependent apoptosis^[38]. Deubiquitination also stabilizes another important transcription factor called c-Myc by the similar SIRT1 mediated pathway. In short, by deubiquitinating a number of transcriptional regulators such as Hes1, NFAT, COX-2, SNF1, *etc.*, USP22 maintains their homeostatic functions within the cell^[39-42]. A compensatory mechanism also involves SAGA, c-Myc and SIRT1. The enhanced stability of c-Myc through a USP22 dependent manner increases its transcriptional activity, which in turn, increases SIRT1 expression. However, the increase in SIRT1 levels enhances its deacetylation activity, which in turn, deacetylates USP22 and other SAGA components, thereby decreasing the enzymatic activity of USP22^[43]. Interestingly, not all deubiquitinations altered protein stability; it also changes the molecular function of the protein. Deubiquitinating lysine-63 of FBP1 enhances its recruitment to the chromosome^[44]. The role of USP22 in B cells is also important for its functionality. Complete ablation of USP22 in primary B cells was found to impair the classical non-homologous end joining and thereby, affects both V(D)J recombination and class switch recombination for the development of various IgG and IgE subtypes^[45].

Little is known about USP22 regulation however. Reports indicate that USP22 transcription is regulated by Sp1 and the PKA/CREB dependent pathway^[46,47]. USP22 is also regulated and stabilized by phosphorylation. Phosphorylation of USP22 at T147 and S237 by CDK1 increases the deubiquitination status of cyclin B1 in a cell cycle dependent manner. USP22 mediated deubiquitination of Cyclin B1 promotes its nuclear accumulation and cell cycle progression^[48]. USP22 is ubiquitously expressed in human subjects as well as in mice. In mice, USP22 expression was detected as early as in E4.5. Loss of both USP22 alleles results in an embryonic lethality starting at E10.5 and no live embryos were recovered after E14.5. Embryonic expression patterns indicate that the potential functions of USP22 relate to the development of extra-embryonic tissues and the loss of function of embryonic USP22 fails to establish vascular interactions with the maternal circulatory system, which leads to immense hypoxic stress induced lethality. Loss of USP22 is also associated with impairments in transforming growth factor β , vascular endothelial growth factor receptor-2 and platelet derived growth factor signaling axes in endothelial cells, and pericytes have been shown to be implicated with detrimental effects on cell survival, differentiation and vessel formation. However, the heterozygous loss of USP22 in mice is still viable but with retardation of growth and brain development^[38,45]. USP22 expression is also important for embryonic stem cell (ESC) differentiation into the embryonic body where Sox2 expression needs to be suppressed. Studies have reported that USP22 functions as a transcriptional repressor by occupying and deubiquitinating H2B at the Sox2-promoter region during the differentiation of ESC into the embryonic body. USP22 expression is also important for regulating neural stem/progenitor cell maintenance through the Notch signaling pathway^[18]. Deubiquitination by USP22 stabilizes the expression of Hes1 protein that negatively modulates neuronal differentiation. On the contrary, depletion of USP22 delays Hes1 oscillation and thereby, induces neuronal differentiation from neuronal progenitor stem cells^[39]. Overall, USP22 functions in multiple pathways to maintain cellular homeostasis and physiological functions of cells.

USP22 EXPRESSION IS FREQUENTLY ALTERED IN CANCER

Altered expression of USP22 was first detected in microarray studies from patient tissue cohorts where the expression of 11-gene signatures in stem like cells correlates with poor prognosis of the cancer^[17]. Over the years, upregulation of USP22 has been validated in several cancers such as breast, colorectal, pancreatic, lung, ovarian, bladder, lymphoma, glioma, mesothelioma, neuroblastoma, *etc.*^[17,31,49-53]. USP22

mainly functions as a part of the SAGA complex and depletion of USP22 alters the expression of a variety of transcriptional regulators that ultimately affect the cellular conserved pathway or cell metabolism^[54]. On the contrary, overexpression of USP22 often stabilizes the transcriptional effector proteins that directly or indirectly influence gene expression. Higher expression of USP22 is associated with increased risk of cancer recurrence and poor disease-free survival^[19]. USP22 expression also correlates with cell cycle progression. In fact, depletion of USP22 has been shown to be associated with cell cycle arrest, mainly at the G0/G1 phase^[18,48,55,56]. Moreover, the depletion of USP22 was shown to decrease *in vivo* tumor growth^[19,55].

The oncogenic role of USP22 in cancer stem cells (CSC) has been identified as a poor prognostic factor in multiple cancer models. Mechanistic studies indicate that by deubiquitination, USP22 has been associated with the stabilization of a variety of its downstream proteins that are important for the development and maintenance of CSC including BMI1. It has also been shown that the increased stability of BMI1 induces CSC populations by inducing the expression of stemness associated genes such as CD133, SOX2, OCT4 and NANOG and thereby, favor the progression of gastric cancer^[57]. The role of USP22 and BMI1 in glioma associated stem cells has also been reported. Under hypoxic conditions, USP22 stabilizes BMI1 to induce CSC formation for cancer progression in glioma models^[58].

In non-small cell lung cancer (NSCLC), the upregulation of USP22 was reported to be associated with advanced stage or recurrent NSCLC and considered as a poor prognostic marker for overall survival^[59]. Knockdown of USP22 in an *in vivo* model was shown to decrease tumor angiogenesis, impair non-homologous DNA damage repair pathways and significantly improve the therapeutic efficacy of cisplatin. USP22 upregulation affects a broad range of pathways in NSCLC to maintain tumor aggressiveness. Cisplatin-resistant lung adenocarcinoma cells were shown to be associated with upregulation of USP22. According to that model, USP22 enhances DNA damage repair and cisplatin resistance by deubiquitinating histone H2A, which in turn facilitates the phosphorylation of histone H2AX. In addition, USP22 was shown to decrease the acetylation of Ku70 by stabilizing SIRT11 via deubiquitination. Ku70 acetylation dissociates the Bax-Ku70 interaction and thereby, induces apoptosis by favoring mitochondrial translocation of Bax. However, upregulation of USP22 in lung adenocarcinoma inhibits Bax-mediated apoptosis in cisplatin-resistant cells^[52]. Upregulation of USP22 was also shown to be associated with chemotherapy-resistant pancreatic cancer cell survival by enhancing autophagic activity^[60]. In breast and colorectal cancer, upregulation of USP22 was reported to be associated with decreased therapeutic efficacy of the HSP90 inhibitor ganetespib. Depletion of USP22 in an *in vivo* model of colorectal cancer was shown to increase the therapeutic potentiation of ganetespib^[61].

In gastric cancer, the co-expression of USP22 and BMI1 was shown to be associated with shorter disease-free survival and a poor prognosis for overall survival^[62]. This was similarly reported in colon cancers. The upregulated expression of USP22 was significantly correlated with both a decrease in relapse-free survival and overall survival. An *in vitro* study showed that the upregulation of USP22 mediated the enhanced expression of BMI1 and Cyclin D2, and was responsible for increased cell proliferation and the metastatic behavior of colon cancer cells^[63]. In hepatocellular carcinoma, the enhanced expression of USP22 was shown to be an independent factor for a poor prognosis with tumor progression^[64]. The enhanced stability of c-Myc following USP22 mediated deubiquitination was reported to be associated with breast cancer cell proliferation and metastatic activity^[43]. The upregulation of USP22 was also reported to be associated with a poor prognosis in papillary thyroid carcinoma^[65] and glioma^[66]. In retinoblastoma, the depletion of USP22 has been shown to induce cancer cell apoptosis by suppressing the TERT/P53 signal pathway^[67].

In the majority of cancers, USP22 functions like an oncogene. Tumor suppressive functions however, were also reported in certain cancer models such as acute myeloid leukemia (AML) and colorectal cancer. Recently, in an *in vivo* model, it was shown that the deletion of USP22 from *Mx1-Cre* mice carrying

Kras^{G12D/+} was associated with shorter survival compared to *Kras*^{G12D/+} mice. Further studies indicate that mice that received myeloid progenitor cells carrying USP22 deletion and mutated *Kras*^{G12D/+} had an AML phenotype. As a mechanism, USP22 was shown to positively regulate protein expression of the transcription factor PU.1, which is important for myeloid and B-lymphoid cell development. Depletion of USP22 directly affected myeloid specific gene expression in *Kras*^{G12D/+} mutated mice, which further led to the development of AML^[68]. Contradictory functions of USP22 in the development of colorectal cancer have been reported. One such study showed that intestine specific USP22 deletion impaired the tumor phenotype associated with *Apc* mutation and positively correlated with the intestinal tumor burden and decreased survival. Mechanistically, the loss of USP22 resulted in increased mTOR activity and has been linked to the tumorigenic properties of colorectal carcinoma^[69].

Over-expression of USP22 is observed in aggressive PCa and has been associated with its oncogenic function. In the following section, we will concentrate mainly on the role of USP22 in the development of CRPC and treatment-resistant PCa.

USP22 AND PROSTATE CANCER

During PCa progression, increase in copy numbers as well as enhanced expression of androgen receptor (AR) (along with its splice variant formation) often led to aggressive therapy resistant phenotypes^[70,71]. Therefore, targeting AR is the most favorable choice to limit PCa progression. Over the years, improvement in AR targeted therapy has increased overall survival to some extent, however, recent clinical studies indicate that individuals are becoming resistant to second generation anti-androgen therapy. Therefore, understanding therapy resistance pathways may provide better or alternative solutions to target PCa. Since the 11-gene signature was shown to predict PCa recurrence and therapy resistance, the contribution of individual genes and 5-year PCa survival was analyzed in mCRPC cases. High expression of Ki-67, BUB1, KNTC1 and USP22 showed significant association with poor 5-year survival^[18]. Further, it was shown that the concerted expression of USP22, AR and Myc in PCa cells predicted the worst prognosis of the disease. USP22 plays an important role in AR protein stability and recruitment to AR-binding regions to drive AR driven cancer cell proliferation and tumor growth in CRPC cells. Later, it was shown that USP22 is equally important for protein stability of AR-variants. The upregulation of USP22 also promotes AR/Myc driven gene expression independent of androgens in the CRPC cell line, implicating that USP22 has a tremendous impact on genes that are regulated by AR and Myc in CRPC cells. This might be important to the phenomenon of anti-androgen therapy resistance^[18]. Interestingly, analysis of patient data with mCRPC validates that point (<https://www.cbioportal.org/>)^[72]. Analysis of the coordinated expression of USP22 and AR between abiraterone/enzalutamide (2nd generation anti-androgen therapy) in naïve vs. exposed groups revealed that USP22/AR expression is upregulated in patients who have progressed to mCRPC under treatment conditions. However, in the treatment naïve group, such correlation was not ascertained [Figure 1]. Patients who are resistant to abiraterone/enzalutamide therapy often develop neuroendocrine-like PCa. Further analysis of patient data (GSE126078) indicates that in pathologically validated neuroendocrine PCa, USP22 expression is significantly higher compared to metastatic sites, which did not develop the neuroendocrine phenotype [Figure 2]. In general, bone is the preferred metastatic site for PCa. However, neuroendocrine PCas often develop visceral metastasis. USP22 expression in visceral metastatic sites (<https://www.cbioportal.org/>)^[72] was significantly higher compared to bone [Figure 3]. Therefore, further validation of the earlier observations and selective upregulation of USP22 are associated with therapy resistance and progression of the disease. This group of patients need an alternative form of treatment and the early detection and stratification of these patients will be beneficial.

Increased expression of USP22 was also observed with progression of primary PCa. Analysis of the Oncomine database showed that USP22 expression increases with increased Gleason score [Figures 4 and 5]^[73,74], indicating that during progression of PCa, USP22 expression can be a predictive factor for advanced

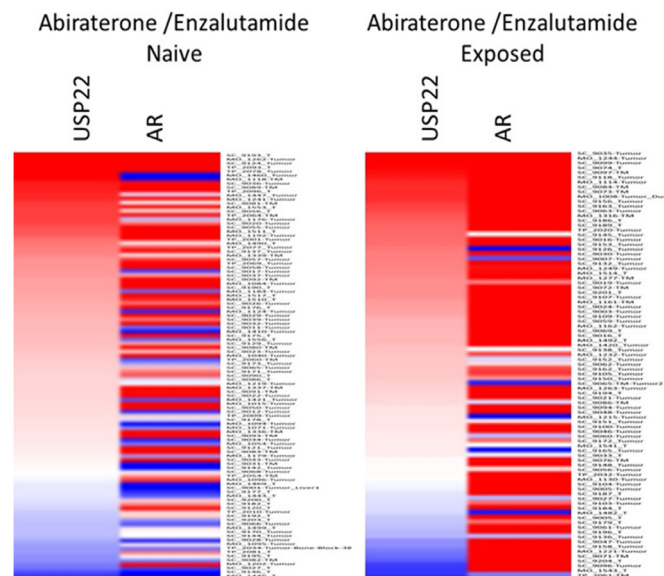


Figure 1. Analysis of ubiquitin-specific peptidase 22 (USP22) and androgen receptor (AR) expression from metastatic biopsy samples deposited in https://github.com/cBioPortal/datahub/tree/master/public/prad_su2c_2019

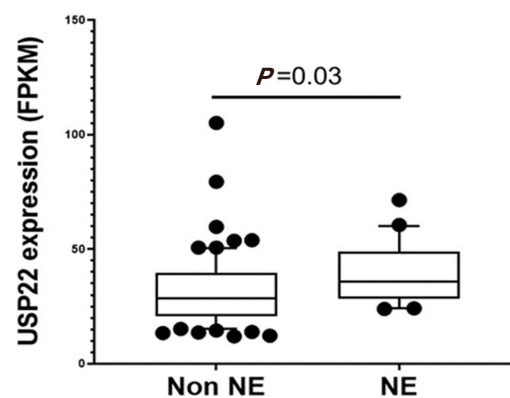


Figure 2. Ubiquitin-specific peptidase 22 (USP22) expression between neuroendocrine (NE) vs. patients who did not develop neuroendocrine PCa (Non NE) using the GSE126078 database

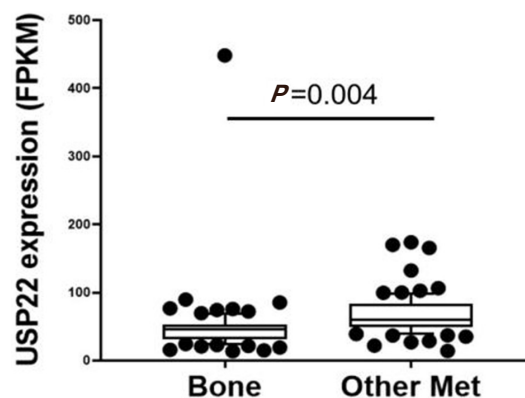


Figure 3. Ubiquitin-specific peptidase 22 (USP22) expression compared between bone and visceral metastatic sites (Other met) using the expression data deposited in cbiportal (https://www.cbiportal.org/study/summary?id=prad_su2c_2019, SU2C/PCF Dream Team, PNAS 2019)

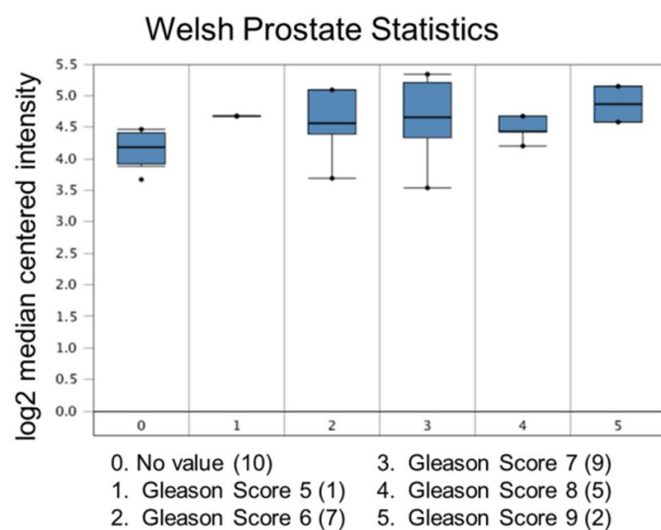


Figure 4. Ubiquitin-specific peptidase 22 expression across the Gleason Score. Number of patients are in parenthesis

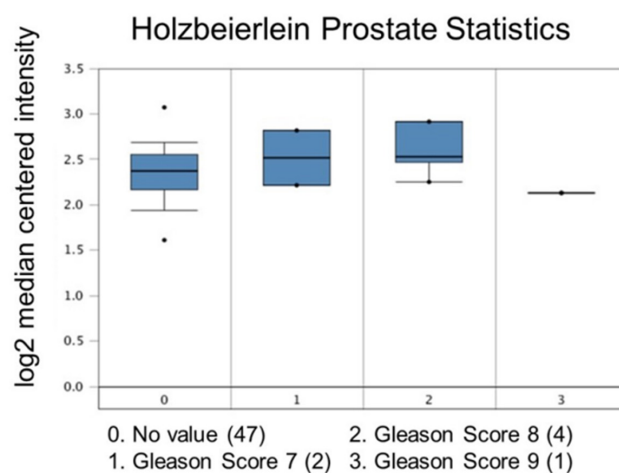


Figure 5. Ubiquitin-specific peptidase 22 expression across the Gleason Score. Number of patients are in parenthesis

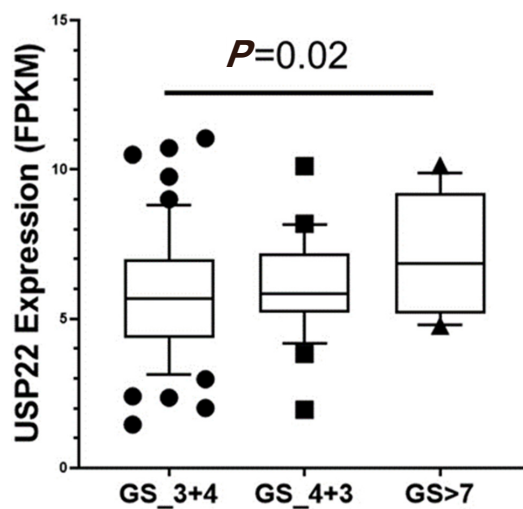


Figure 6. Ubiquitin-specific peptidase 22 expression across the Gleason Score (GS) using the database GSE54460

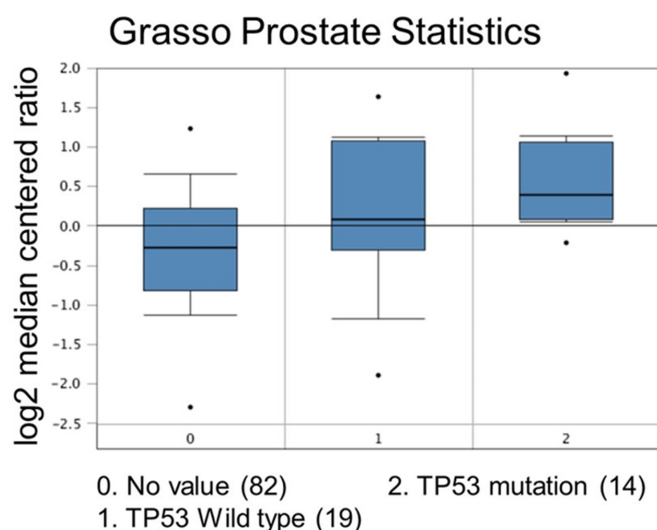


Figure 7. Comparison study for ubiquitin-specific peptidase 22 expression with TP53 mutation status. Number of patients are in parenthesis

disease. Further analysis of GSE54460 expression data supports increased USP22 expression with higher grades of PCa [Figure 6]. Moreover, advanced PCa patients often have functionally inactive TP53. Oncomine analysis of the Grasso cohort indicates that TP53 mutation is associated with increased expression of USP22 [Figure 7]^[75]. Therefore, primary PCa patients who have higher expression of USP22 with functionally inactive TP53 might be candidates for alternative therapeutic approaches. Various clinical trials are currently ongoing with upfront administration of chemotherapy such as cabazitaxel for patients who have started to develop disease progression in the early stage. In future, analysis of such cohorts will address whether such therapy can be beneficial for those who have showed early upregulation of USP22 expression.

To mimic that hyperactivation/overexpression state, the role of USP22 functions in PCa progression has recently been redefined in a genetically modified mouse model. According to the model, prostate specific upregulation of USP22 is associated with a hyperproliferative phenotype, an indication of aberrant cell proliferation. Moreover, studies have also showed that overexpression of USP22 is important for cellular survival following a genotoxic insult by DNA-damaging agents. In line with their finding, the authors have identified the nucleotide excision repair pathway protein XPC as a substrate for USP22, which modulate XPC polyubiquitination status following the DNA-damage response and thereby, efficiently recruited it into the damage foci. Interestingly, the depletion of USP22 in PCa cells affects efficient DNA repair and therefore, presents a therapeutic challenge^[19].

Although USP22 was identified almost 15 years earlier as an important oncogenic driver for therapy resistant prostate cancer, not much work has been carried out to understand its importance in the development of mCRPC. As part of the SAGA complex, how inappropriate stoichiometric upregulation of USP22 in PCa drives AR/Myc mediated gene-expression remains unresolved. Also, whether USP22 plays an independent role in PCa progression is not well understood.

OTHER USPS IN PROSTATE CANCERS

Importantly, other ubiquitin specific proteases or USPs have long been recognized in the progression of advanced PCa.

USP2a (also known as USP2) has been associated with PCa development. More than 50% of cases with PCa have USP2a overexpression. Increase in USP2a selectively deubiquitinates and stabilizes MDM2, which

is important for the proteasomal degradation of p53 in PCa cells. p53 is the negative regulator of Myc in many cases. USP2a mediated enhanced stability of MDM2 abrogates p53 accumulation and its tumor suppressive functions. Therefore, the inhibition of p53 mediated transactivation of transcriptional activity indirectly stabilizes Myc accumulation in cells and thereby, enhances the development of aggressive PCa transformation. The deubiquitination activity of USP2a was also found to stabilize the anti-apoptotic gene fatty acid synthase and thereby induce cells to develop neoplastic transformation. The depletion of USP2a has also been shown to abrogate such cellular transformation^[76,77].

USP7 has been associated with PCa and plays a negative role for PTEN nuclear localization. PTEN is generally regarded as a protein phosphatase that dephosphorylates the phosphatidylinositol (3,4,5)-triphosphate to inhibit AKT signaling. However, PTEN's role in the nuclear DNA repair system associated with tumor suppressive functions has been well recognized. Following mono-ubiquitination, PTEN moves into the nucleus and participates in the repair processes. In PCa, over-expression of USP7 expels this ubiquitinated-PTEN to the cytosol and activates the cells towards transformation. Interestingly, in the presence of androgen, USP7 was identified as a co-regulator of AR. Studies also suggest that USP7 mediated AR-deubiquitination enhance the AR-transcriptional ability that promotes cell proliferation and PCa aggressiveness. Moreover, single nucleotide polymorphisms that affect USP7 function has been associated with the development of intermediate risk PCas^[78,79].

USP19 silencing directly affects the growth of several prostate cancer cell lines, suggesting a putative role in carcinogenesis^[80]. USP19 deubiquitinates and stabilizes KPC1, an E3 ligase for p27. Interestingly, the effects of decreased nuclear levels of p27, resulting in a poor prognosis, have already been described in prostate cancer^[81]. USP19 regulates the levels of p27, although p27 is not a USP19 substrate. Reports indicate that the disruption of USP19 inhibits a series of PCa cell proliferation by arresting cells in the G1 to S phase transition through stabilization of the cyclin-dependent kinase inhibitor p27^[80]. Increased stability of AR by USP12, USP14 and USP26 has been linked to the development of aggressive PCa^[82-84]. Recent reports indicate that the overexpression of USP33 in PCa confers docetaxel resistance by inhibiting JNK activation and apoptosis^[85].

In the context of PCa, most USPs are overexpressed; however, USP9x was found to be down regulated in advanced PCa and was associated with higher Gleason scores. This downregulation increases the local invasiveness of PCa cells, possibly through the ERK activation pathway^[86].

Among all the DUBs, available data suggest that USP22 functions often overlapped with other reported USPs in the context of progression and development of therapy resistant PCas. Therefore, USP22 targeted therapy or broad-spectrum inhibitors that can abrogate the functions of a group of USPs may be a better therapeutic agent in PCa.

TARGETING USP22 IN ADVANCED PROSTATE CANCER TREATMENT

Recent studies have suggested that USP22 is emerging as a potential oncogenic driver in relation to PCa. As a member of the cysteine protease family, its catalytic domains are somewhat conserved amongst family members. Therefore, the development of inhibitors specifically against one such member is challenging. Efforts have been made to develop small molecule inhibitors against the allosteric sites of USP22. However, till now, no such specific inhibitor has been validated to target USP22. Recently, Pirarubicin (4'-O-tetrahydropyranyl doxorubicin, THP), an anthracycline (analogue of another chemotherapeutic agent known as doxorubicin), has been shown to inhibit USP22 expression in a condition-specific manner^[87]. Reports indicated that protein kinase A (PKA), protein kinase B or mitogen activated kinase-mediated phosphorylation of CREB-1 bind and activate the USP22 promoter for its synthesis. The addition of THP abrogates PKA activity and decreases CREB-1 phosphorylation, thereby inhibiting USP22 expression and

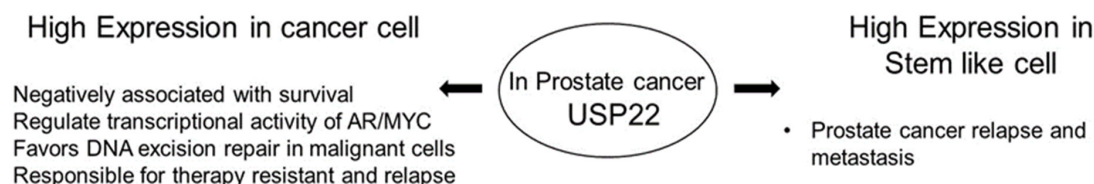


Figure 8. Summary of ubiquitin-specific peptidase 22 (USP22) role in prostate cancer

USP22 mediated tumorigenic activity. Betulinic acid (BA), a small molecule isolated from white birch trees, has been shown to inhibit an array of DUBs. BA was also showed to reduce AR protein stability and selectively kills PCa cells. Another multi-DUB inhibitor WP1130 has been shown to selectively kill PCa cells. Treatment with WP1130 also reduces AR expression in CRPC cells. Therefore, BA and WP1130 have the potential to enhance the therapeutic efficacy of CRPC cells and the published literature suggests that the combination of these inhibitors with enzalutamide increases the therapeutic window for the treatment of advanced PCa patients^[88]. With such growing knowledge, scientists have tried to develop exosite inhibitors against the various DUBs. One such inhibitor, P5091, is highly selective against USP7 and has been shown to induce apoptotic cell death in therapy resistant multiple myeloma cells^[89]. However, its selectivity and specificity as an agent in PCa remains unknown.

CONCLUSION

In summary, the oncogenic role of upregulated USP22 in the progression and development of treatment resistance of PCas has been observed [Figure 8]^[19]. Accumulated evidence indicates that USP22 possibly functions independent of the SAGA complex in the progression of PCas. Increased acetylation and enhanced activity of GCN5 has been reported to be associated with advanced PCa. However, there is a lack of studies to ascertain any relationship between upregulated USP22 and other members of the SAGA complex in the development of aggressive PCas. Moreover, in advanced PCas, the coordinated function of upregulated Myc and USP22 indicates the lack of feedback regulation by hyperactivated Myc. Therefore, to develop better targeted therapeutic approaches, a comprehensive understanding about the functional interactions among the various sub-units of SAGA and their relationships with AR/Myc is important. Moreover, the differential functions of USP22 in the normal prostate, aggressive disease and disease progression are not fully understood. Thus, defining the role of USP22 will be beneficial for the development of future therapeutic modalities.

DECLARATIONS

Authors' contributions

Wrote the paper jointly and SD analyze the data: Nag N, Dutta S

Availability of data and materials

Not applicable.

Financial support and sponsorship

This work was supported by grants (1R21CA241234-01) to Dutta S.

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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Case Report

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Cyclophosphamide, fluorouracil and low-dose interleukin-2 and salvage combination chemotherapy in advanced cutaneous squamous cell carcinoma

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How to cite this article: Lo Re G, Doretto P, Lo Re F, Matrone F, Ermacora A, Marus W, Pizzichetta MA, Sulfaro S. Cyclophosphamide, fluorouracil and low-dose interleukin-2 and salvage combination chemotherapy in advanced cutaneous squamous cell carcinoma. *J Cancer Metastasis Treat* 2020;6:17. <http://dx.doi.org/10.20517/2394-4722.2020.11>

Received: 31 Jan 2020 **First Decision:** 28 Apr 2020 **Revised:** 1 May 2020 **Accepted:** 26 May 2020 **Published:** 24 Jun 2020

Science Editor: Pravin D. Potdar **Copy Editor:** Cai-Hong Wang **Production Editor:** Tian Zhang

Abstract

A 70-year-old female with metastatic cutaneous squamous cell carcinoma (cSCC) and low-grade non-Hodgkin's lymphoma, not amenable to cisplatin combination therapy, was treated with cyclophosphamide (Cyc)-fluorouracil (FU)-interleukin-2 (IL-2) in light of high tumor immunogenicity and the potential activity of this regimen. Cyc 300 mg/m² and FU 500 mg/m² intravenously on day 1 and IL-2 4.5 MIU/day on days 3-6 and 17-20 subcutaneously every 4 weeks; Carboplatin (C) AUC 2 and paclitaxel (P) 85 mg/m² on days 1, 8 and 15 ± capecitabine (Cape) every 4 weeks. After partial remission (PR) of lung metastases and local control with two cycles of first therapy followed by PR with five cycles of CP ± Cape, right mastectomy was performed with evidence of viable tumor. Subsequently, the patient underwent 3 cycles of chlorambucil and is alive after 13 months of follow-up. Safety and activity of chemo-immunotherapy and salvage treatment can be achieved in cSCC.

Keywords: Cutaneous squamous cell carcinoma, cyclophosphamide, fluorouracil, interleukin-2, regulatory T cells, myeloid-derived suppressor cells



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INTRODUCTION

In 2018, cutaneous squamous cell carcinoma (cSCC) was reported to be the 5th most common type of cancer with 5.8% and 0.7% rates of incidence and mortality respectively^[1]. It shows racial and gender differences with greater incidence in white than black subjects and in men than women. The incidence increases with age, with an average age around 60 years^[2]. Tumor aggressiveness is associated with histological type. In fact, well-differentiated histologic subtypes such as keratoacanthoma and verrucous carcinoma are associated with low metastatic potential and do not seem related to human papillomavirus (HPV) infection^[3]. On the contrary, there are histological variants characterized by greater tumor aggressiveness, metastatic potential and poor prognosis and are represented by desmoplastic^[4] and adenosquamous^[5] cSCC. However, the absence of negative features such as epithelial dysplasia and stromal invasion in verrucous carcinoma can determine differential diagnostic difficulties with other benign entities^[6]. The most important prognostic factors are tumor diameter > 2.0 cm^[7], tumor depth (< 2 mm vs. > 2 mm)^[8] and perineural involvement^[9], which are highly associated with local recurrence, nodal metastases and disease-specific death. Sun exposure, age, fair skin, and immunosuppression are the main risk factors. Immunosuppression, associated with organ transplantation^[10] or other lymphoproliferative^[11] or solid tumors^[12], negatively affects the behavior of the disease and probably also the responsiveness to treatments. Surgery and radiotherapy are the main modalities of treatment once the diagnosis has been made or after loco-regional recurrence with good results in terms of relapse-free survival, which is influenced by the state of immunosurveillance^[13]. The treatment of metastatic disease up to now has been by chemotherapy^[14-19], and it should be noted that cisplatin (DDP)^[14] and fluorouracil (FU)^[15] are the most active chemotherapeutic agents. Regarding anti-epidermal growth factor receptor (EGFR) therapy, cetuximab^[14] seems to be more active than panitumumab^[20] with complete response (CR) of 68% and 12.5%, overall response rate (ORR) of 78% and 31% and progression-free survival of 25 and 8 months, respectively.

In comparison, ORR of patients treated with DDP is 45% comprising 22% CR with a median disease-free survival of 14.6 months^[14]. One of the mechanisms of DDP resistance could be linked to MiR-3619-5p downregulation, responsible for cell proliferation^[19]. To overcome drug resistance, considering the synergistic action between different types of chemotherapeutic agents such as DDP and fluoropyrimidines [e.g., FU^[16] or capecitabine (Cape)]^[17], DDP and taxanes^[21], and taxanes and fluoropyrimidines^[22], there is a rationale for a combination of these drugs. Recently, we have witnessed an explosion in research on new immunotherapeutic agents that have come into clinical practice both in solid and hematological tumors. In 2018, the US FDA approved cemiplimab-rwlc, a human anti-programmed death 1 (PD-1) monoclonal antibody, which blocks the interaction of PD-1 with programmed death ligand-1 (PD-L1) and represents the first and sole treatment specifically approved and available for advanced cSCC. The approval of cemiplimab-rwlc was based on a combined analysis of data from a phase II trial (EMPOWER-CSCC-1 Study 1540) and a phase I trial with two advanced cSCC expansion cohorts (Study 1423). In 108 patients with metastatic or locally advanced disease, there was a 47.2% objective response, and G_{≥3} SAE was observed in 29% of cases^[14]. Regarding other anti-PD1 agents such as nivolumab^[24-28] and pembrolizumab^[29-35], there are positive reports with small series [Table 1].

Regarding the negative effect of immunosuppressive cells such as regulatory T lymphocytes (Tregs) and myelo-derived suppressor cells (MDSCs) on resistance to treatment with clinically unfavorable outcome and in light of the possible inhibitory interference of cyclophosphamide (Cyc) and FU on this cell population, the aim of the study was to evaluate an innovative chemo-immunotherapy modality including interleukin-2 (IL-2) in the treatment of cSCC.

Our patient with low performance status had advanced cSCC originating from the right breast and concomitant non-Hodgkin lymphoma (NHL), and was therefore not amenable to combination

Table 1. Anti-PD-1 agents in cSCC

Agent	Study	Drug dose	No. PTS	RR (CR)	PFS (mo)	Toxicity G \geq 3 SAE
Cemiplimab ^[23]	1,540		59	41 (7)	(n.r.) MDR > 6 mo 57%	29%
Cetuximab Nivolumab ^[24]	case	n.d.	1	CR	12+	n.d
Ipilimumab Nivolumab ^[25]	case	n.d.	1	Path CR	5	Allograft rejection
Nivolumab ^[26]	case	3 mg/kg/2 weeks	3	PR 2, SD 1	12+	--
Nivolumab ^[27]	case	3 mg/kg/2 weeks	3	PR 1, SD 2	5.5-7+	--
Nivolumab ^[28]	case	3 mg/kg/2 weeks	1	PR	4.5	--
Pembrolizumab ^[29]	cases	2 mg/kg/3 weeks	1	PR	5+	--
Pembrolizumab ^[30]	cases	2 mg/kg/3 weeks	5	CR 1, PR SD 1, PD 1	3-21	Severe weakness (2)
Pembrolizumab ^[27]	case	2 mg/kg/3 weeks	2	PR 1, SD 1	4+, 7+	--
Pembrolizumab ^[31]	case	2 mg/kg/3 weeks	2	PR 2	--	--
Pembrolizumab ^[32]	case	2 mg/kg/3 weeks	1	PR	11+	--
Pembrolizumab ^[33]	phase 2	200 mg IV/3 weeks	10	40%	n.r.	hepatitis and pneumonitis
Pembrolizumab ^[34]	case	2 mg/kg/3 weeks	1	PR	n.r.	--
Pembrolizumab ^[35]	case	2 mg/kg/3 weeks	1	CR	24+	--

PTS: patients; No.: number; CR: complete remission; PR: partial remission; SD: stable disease; RR: response rate; Path CR: pathological CR; PFS: progression-free survival; MDR: median duration of response; n.d.: not done; n.r.: not reported; SAE: serious adverse event

chemotherapy including DDP. Taking into consideration the high immunogenicity of cSCC, even if burdened by the immunosuppressive effect of NHL, this depletion strategy on Tregs and MDSCs by Cyc and FU could allow effective immune stimulation by IL-2. An alternative treatment with carboplatin, paclitaxel \pm Cape was foreseen in case of intolerance or ineffectiveness of the therapy to reach palliative mastectomy.

CASE REPORT

Clinical history and response

A 70-year-old female patient, a teacher by profession and of the Caucasian race, underwent hysterectomy for fibromatosis in 1995. In December 2017, she went to the emergency room because of the presence of exophytic vegetation 10 cm in diameter and localized in the right hemithorax. The lesion had appeared a year before and showed recent bleeding. After biopsy resection, the pathological diagnosis of a cSCC with lymph node metastasis (pT2L1V1N2) was made. A simultaneous marginal low-grade NHL (stage IV) was diagnosed. The patient was treated with radiotherapy on the right chest wall, 50 Gy/20 fractions, which were completed in August 2018. In September 2018, mammography detected local recurrence of a 35-mm nodule with polylobed contours in the right breast. The lesion was confirmed by ultrasound, which detected retroareolar ductal ectasia with dense intraductal content and satellite node of 0.6 cm in the upper internal quadrant of the breast. No significant focal lesions in the left breast were evident. Multiple bilateral axillary lymph nodes were detected. A needle biopsy of the right breast lesion was performed and confirmed the pathological diagnosis of poorly differentiated cSCC, polypoid, ulcerated, initially infiltrating the hypodermis. Thereafter, the immunohistochemistry for programmed death ligand-1 (PDL-1) and microsatellite instability were negative. Considering the new efficacy of anti-PD-1, at present not yet available, the ineligibility for DDP-containing regimen due to weight loss and poor performance status and the chance of low efficacy of alternative chemotherapy, after health authorization of chemo-immunotherapeutic regimen, from November 2018 to January 2019, she was treated with Cyc-FU-IL-2. The treatment was well tolerated, and the only reported problem was flu-like symptoms, which were controlled with paracetamol. On physical examination after two cycles of therapy, the patient showed initial local response [Figure 1A-C, Figure 3A and B] and size reduction of lung metastasis on CT scan [Figure 3D and E]. However, after a short-lasting response [Figure 1C] due to local progression [Figure 1D] from February to March 2019, the patient was treated with weekly low-dose carboplatin (C) AUC 2 and paclitaxel (P) 85 mg/m² (CP) on days 1, 8 and 15 every 4 weeks with initial objective response [Figure 2A] followed by progression [Figure 2B]. Thereafter, from March to June 2019, Cape 1000 mg/day for 14 days was combined with CP for 3 cycles. From response evaluation



Figure 1. Clinical presentation and course of breast neoplastic lesion during chemo-immunotherapy with cyclophosphamide, fluorouracil and interleukin-2. A: (November 2018): presence of polylobed and ulcerated neoplastic mass of 7 cm × 7 cm and overlying vegetative lesion of 4 cm located on the right breast; B: (December 2018): 2 weeks, after chemo-immunotherapy reduction in size and consistency of the neoplastic mass of 5 cm × 7 cm and of polylobed overlying vegetative lesion of 3 cm located in the right breast; C: (December 2018): 5 weeks after chemo-immunotherapy, further reduction in size and thickness of nodules in the right breast; D: (January 2019): increase in size of the polylobed and ulcerated neoplastic mass of 8 cm × 8 cm with nodules above greater than 4.5 cm located in the right breast after chemo-immunotherapy

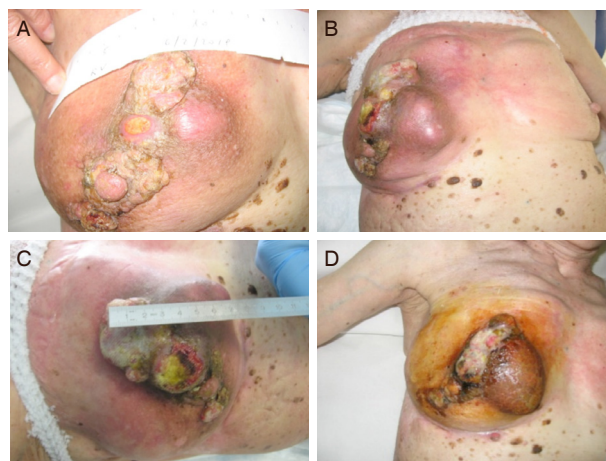


Figure 2. Clinical presentation and course of breast neoplastic lesion during chemotherapy with carboplatin, paclitaxel ± capecitabine. A: reduction in size and thickness of the nodules with ulcerated area and fibrous material in the right breast after chemotherapy (February 2019); B: progression in size and thickness of nodules with ulcerated area and fibrous material within the neoplastic mass in the right breast after chemotherapy (March 2019); C: initial reduction in thickness of the polylobed nodules with ulcerated and necrotic areas and fibrin-hemorrhagic material above the neoplastic mass in the right breast (April 2019); D: reduction in size of the polylobed and ulcerated neoplastic mass of 6 cm × 6 cm located in the right breast after chemotherapy (June 2019)

by physical examination and radiological back-up, there was a reduction in size and thickness of the skin lesion in the right breast [Figure 2C and D, Figure 3C] with good tolerance and a further reduction of lung metastasis [Figure 3F]. After 5 cycles of chemotherapy, in June 2019, the patient was submitted to right simple radical mastectomy and the pathological diagnosis was poorly differentiated cSCC G3 according to WHO. Nipple, margins of resection and muscle level were free from tumor. However, after three months in September 2019, progression of disease was detected in multiple lymph nodes sites, on the chest wall along with the appearance of new pleural and lung metastatic lesions (not shown). Considering the progression and supposed negative impact of NHL, she then underwent three cycles of therapy with chlorambucil, aiming to improve the immunosuppressive role of lympho-proliferative disease. The patient is alive and in a rather good shape after 13 months from the beginning of systemic therapy.

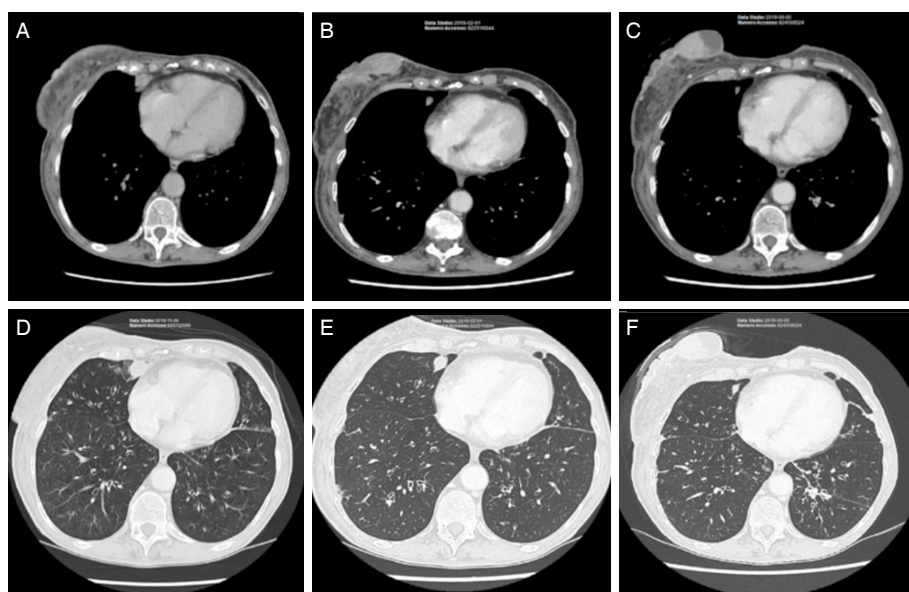


Figure 3. CT scan presentation and clinical course of local and metastatic lesions during chemo-immunotherapy with cyclophosphamide, fluorouracil and interleukin-2 and salvage carboplatin, paclitaxel \pm capecitabine regimen. A: neoplastic masses of 19 mm \times 13 mm to the infero-lateral quadrant and 40 mm \times 35 mm to the medial quadrant of the right breast. Metastatic lesion of 11 mm \times 25 mm to the middle lung lobe anteriorly (November 2018); B: dimensional increase of the heterogeneous lesion localized in the right central breast of 63 mm \times 34 mm, with current ulcerative aspects of the overlying skin after chemo-immunotherapy (February 2019); C: partial reduction of density due to necrosis and size of the polylobed and ulcerated neoplastic mass of 7 cm located in the right breast after salvage chemotherapy (June 2019); D: metastatic lesion of 11 mm \times 25 mm to the middle lung lobe anteriorly (November 2018); E: reduction in size of metastatic lesion of 11 mm \times 7 mm to the middle lung lobe anteriorly after chemo-immunotherapy (February 2019); F: further reduction in size of metastatic lesion of 10 mm \times 6 mm to the middle lung lobe anteriorly after salvage chemotherapy (June 2019)

Treatment protocol

The chemo-immunotherapy combination included intravenous Cyc 300 mg/m² and FU 500 mg/m² on day 1 and subcutaneous low-dose IL-2 4.5 MIU/day on days 3-6 and 17-20 every 4 weeks. A premedication with metoclopramide and paracetamol was planned. The cycle was repeated every 4 weeks for three cycles. If an objective response (CR) or PR or disease stabilization was documented upon clinical and radiological back-up every two months, in the absence of serious toxicities or refusal of treatment, the therapy was continued for another three cycles. Blood count, creatinine, alanine aminotransferase (ALT), gamma glutamyl transpeptidase (γ -GT), bilirubin, calcium, lactic dehydrogenase (LD), alkaline phosphatase, peripheral blood lymphocyte immunophenotype CD3⁺, CD4⁺, CD8⁺, CD19⁺, CD16⁺, HLA-DR⁺/CD3⁺/CD8⁺ and Treg (CD3⁺/CD4⁺/CD25⁺/CD127⁺) were determined before every cycle, and blood count, creatinine, ALT, bilirubin and blood lymphocyte immunophenotype (CD3⁺, CD4⁺, CD8⁺, CD19⁺, CD16⁺), HLA-DR⁺/CD3⁺/CD8⁺ and Treg (CD3⁺/CD4⁺/CD25⁺/CD127⁺) on days 3 and 17 of each cycle.

Salvage therapy: carboplatin (C) AUC 2 and paclitaxel (P) 85 mg/m² (CP) day 1, 8 and 15 every 4 weeks. In the presence of further disease progression, the addition of Cape 1000 mg/day for 14 days to CP was expected. A premedication with ondansetron during treatment was employed.

Blood count, creatinine, ALT, γ -GT, bilirubin, calcium, LD, alkaline phosphatase were determined before every cycle and blood count, creatinine, ALT, bilirubin on days 1 and 8. Radiological response was determined every 3 months.

DISCUSSION

Advanced cSCC is an orphan disease and the main treatment is represented by radiotherapy, anti-EGFR antibodies and chemotherapy. Unfortunately, these treatments do not offer long-lasting results with a

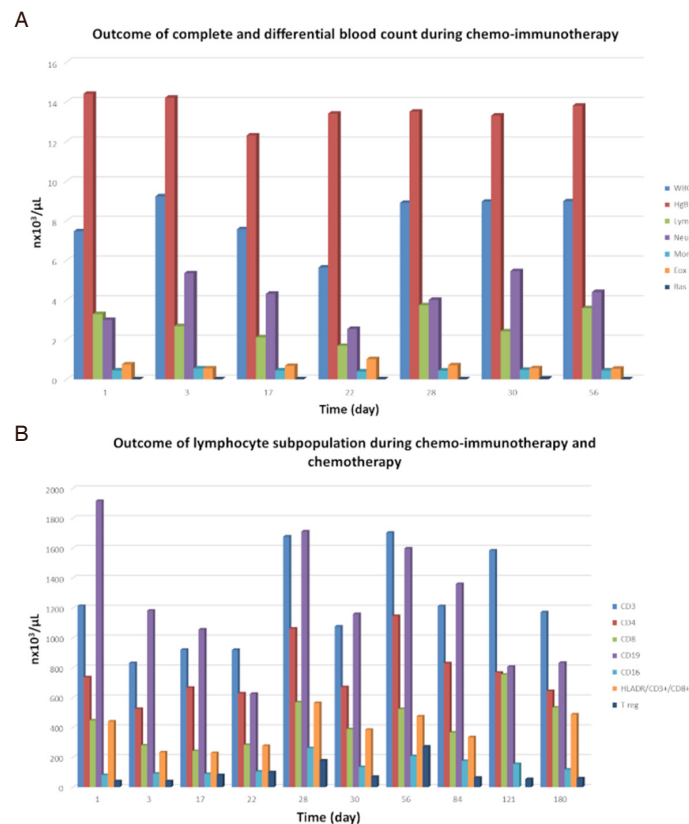


Figure 4. Outcome of complete and differential blood count and immunophenotyping during chemo-immunotherapy and salvage chemotherapy. A: The blood count showed an initial increase in white blood cells (WBC), neutrophilic granulocytes (N) and lymphocytes (Lym) followed by their decrease during chemo-immunotherapy; B: An undulating trend of CD3⁺, CD4⁺, CD8⁺, CD19⁺, CD16⁺, HLA-DR⁺ and Tregs and a transient decrease in Treg count were observed after chemo-immunotherapy. Subsequently, after an increase in Treg count, there was a decrease during chemotherapy. CD3⁺: cluster of differentiation 3 T cell; CD4⁺: CD4⁺ (helper) T cell; CD8⁺: CD8⁺ (cytotoxic) T cells; CD19⁺: cluster of differentiation 19 B-lymphocyte; CD16⁺: type I transmembrane receptor mediating antibody-dependent cellular cytotoxicity (ADCC) by NK cells; HLA-DR⁺: histocompatibility class II allele T cell; Treg: regulatory T cells CD4(+) CD25(+)Foxp3(+)

range of 8 to 25 months. The disease becomes more resistant especially when it is associated with a state of immunosuppression resulting from post-transplant therapy or neoplastic disease such as lymphomas. This scenario becomes permissive to the immunosuppression exercised above all by Tregs and MDSCs, as well as by tumor-associated macrophages. cSCC shows a high tumor mutation burden (TMB), a condition that makes immunotherapy effectiveness highly possible. Recently, cemiplimab-rwlc, an anti PD-1 checkpoint agent was approved by the FDA for the treatment of cSCC. Regarding other immunotherapeutic agents such as IL-2, which has been shown to be effective in metastatic renal cell carcinoma and cutaneous melanoma, it has not been tested in this disease in human subjects. However, in the animal model, subcutaneous perilesional administration of IL-2 resulted in a high remission rate and long-lasting response, which was significantly satisfactory when administering high doses instead of low ones^[36]. IL-2 is a 15.5 kDa cytokine secreted predominantly by CD4⁺, CD8⁺ T cells, natural killer cells, and activated dendritic cells^[37]. IL-2 can stimulate cells expressing both a high affinity for the trimeric receptor α , β , γ chains or a low affinity dimeric receptor α , γ chains for IL-2. IL-2 can stimulate cell growth in CD8⁺ cells and differentiation of memory lymphocytes, and maintain and expand the CD41⁺ Tregs, reducing the risk of uncontrolled immune activity and autoimmunity^[38]. Furthermore, it has a differentiating effect on CD4 T cells, and its action can be stimulatory or inhibitory in the different T helper subtypes^[39]. The immunosuppressive effect seems to be exerted also by MDSCs. It can occur indirectly through the increase in Tregs and for the expression of indoleamine 2, 3-dioxygenase (IDO) on MDSCs^[40] and through the production of TGF- β and retinoic acid^[41]. Similarly the overexpression of IDO by the dendritic cells

with consequent depletion of tryptophan determines immunosuppression through their blocking of the maturation and induction of T cell apoptosis^[42].

Considering the key immunosuppressive role played by these cells, with Tregs and MDSCs being the most studied, and their negative relationship with tumor stage, prognosis, and resistance to treatment^[43], preliminary experience with Cyc and FU, active on both types of suppressive cells, combined with IL-2 was reported in heavily pre-treated solid tumors, with interesting results both from a clinical and laboratory point of view^[44].

Our patient with advanced cSCC showed for the first time how a chemo-immunotherapy regimen including IL-2 was able to produce a fleeting response even on the primary and more-lasting tumor response on the metastatic lesion. Furthermore, the blood count and immunophenotype showed an initial increase in white blood cells, neutrophilic granulocytes and lymphocytes followed by their decrease [Figure 4A] and a transient decrease in the Treg count during chemo-immunotherapy [Figure 4B], respectively. In addition, a subsequent decrease in Tregs was observed during salvage chemotherapy [Figure 4B]. This transient effect could be explained by the concomitant presence of lymphomas and Treg stimulation by IL-2 with detrimental effect on the immune system with consequent unfavorable response to chemo-immunotherapy. A more favorable outcome could be hypothesized in the presence of adequate immunosurveillance especially in a high TMB tumor such as cSCC. A confirmation of the poor efficacy of chemotherapy along with the combination of carboplatin and paclitaxel employed after chemo-immunotherapy failure, despite the theoretical synergism and additive antitumor activity for increase of carboplatin-DNA adduct formation^[45], has been reported. Noteworthy is the ability of a fluoropyrimidine (Cape) to reverse resistance to the previous carboplatin combination therapy through the upregulation of thymidine phosphorylase activity by paclitaxel and subsequent Cape activation^[22,46], the decrease of Treg count and tumor response.

In conclusion advanced spinocellular carcinoma of the skin remains a pathology with severe treatment difficulties due to primary resistance, worsened by a state of immunosuppression resulting from organ transplantation or other tumors. It is desirable to improve our knowledge of the resistance mechanisms and to investigate prospectively innovative therapeutic strategies to improve the therapeutic index and the control of the disease.

DECLARATIONS

Acknowledgments

We warmly thank Dr. Federica G. Ravizza for editing. Finally, we thank the doctors and nurses who supported us in this study.

Authors' contributions

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Used in attributes: Lo Re G, Doretto P, Lo Re F, Matrone F, Ermacora A, Marus W, Pizzichetta MA, Sulfaro S

Availability of data and materials

The source of the data is PUBMED and proceeding ASCO.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Informed consent to treatment was accepted and signed by the patient after ethical approval by the competent facility.

Consent for publication

Patient consent for publication.

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Perspective

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RANBP9 as potential therapeutic target in non-small cell lung cancer

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How to cite this article: Tessari A, Soliman SHA, Orlacchio A, Capece M, Amann JM, Visone R, Carbone DP, Palmieri D, Coppola V. RANBP9 as potential therapeutic target in non-small cell lung cancer. *J Cancer Metastasis Treat* 2020;6:18.
<http://dx.doi.org/10.20517/2394-4722.2020.32>

Received: 7 Apr 2020 **First Decision:** 6 May 2020 **Revised:** 17 May 2020 **Accepted:** 4 Jun 2020 **Published:** 24 Jun 2020

Science Editor: Ciro Isidoro **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Non-small cell lung cancer (NSCLC) remains the leading cause of cancer-related deaths in the Western world. Despite progress made with targeted therapies and immune checkpoint inhibitors, the vast majority of patients have to undergo chemotherapy with platinum-based drugs. To increase efficacy and reduce potential side effects, a more comprehensive understanding of the mechanisms of the DNA damage response (DDR) is required. We have shown that overexpressby live cell imaging (Incuyion of the scaffold protein RAN binding protein 9 (RANBP9) is pervasive in NSCLC. More importantly, patients with higher levels of RANBP9 exhibit a worse outcome from treatment with platinum-based drugs. Mechanistically, RANBP9 exists as a target and an enabler of the ataxia telangiectasia mutated (ATM) kinase signaling. Indeed, the depletion of RANBP9 in NSCLC cells abates ATM activation and its downstream targets such as pby live cell imaging (Incuy53 signaling. RANBP9 knockout cells are more sensitive than controls to the inhibition of the ataxia and telangiectasia-related (ATR) kinase but not to ATM inhibition. The absence of RANBP9 renders cells more sensitive to drugs inhibiting the Poly(ADP-ribose)-Polymerase (PARP) resulting in a "BRCAness-like" phenotype. In summary, as a result of increased sensitivity to DNA damaging drugs conferred by its ablation *in vitro* and *in vivo*, RANBP9 may be considered as a potential target for the treatment of NSCLC. This article aims to report the results from past and ongoing investigations



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focused on the role of RANBP9 in the response to DNA damage, particularly in the context of NSCLC. This review concludes with future directions and speculative remarks which will need to be addressed in the coming years.

Keywords: RANBP9, RANBP10, Scorpins, DNA damage, DNA repair, DNA damage response, CTLH complex, cisplatin, non-small cell lung cancer, PARP, BRCAness-like phenotype

OUTLINE

As an introduction, we will briefly mention the state-of-the-art strategies in the treatment of NSCLC, as well as the main molecular features of RANBP9. Moving on, we will then succinctly discuss RANBP9 in cancer generally, before focusing on the role of RANBP9 specifically in the cellular response to DNA damage of NSCLC cells.

INTRODUCTION

Non-small cell lung cancer

Non-small cell lung cancer (NSCLC) remains the leading cause of cancer-related deaths in the developed world^[1-3]. The standard of care for NSCLC during the last decades has been the use of combination chemotherapy, including that of platinum-based drugs^[4,5]. Significant progress has been made in the treatment of this devastating disease through the development of targeted therapies for tumors expressing oncogenic driver alterations (examples include that of *EGFR* mutations and *ALK* rearrangements)^[6-8]. Nevertheless, the vast majority of NSCLC patients treated with targeted therapy commonly exhibit *de novo* or *acquired* resistance^[7].

Treatment with immune checkpoint inhibitors (ICIs) has been transformational in the management of NSCLC. These drugs have now set new standards to the point that ICIs evolved to become first-line treatment^[9-14]. However, for unknown reasons, an estimated half of the NSCLC patients are refractory to this new modality and the majority become resistant after an initial response^[15-18].

From a clinical and basic science perspective, the relationship between tumor mutational burden and response to ICIs is of great interest^[19]. It is known that tumors with high mutational burden tend to be immunologically “hot”, displaying favorable responses to treatment^[20]. Therefore, the specific mechanisms of the DNA damage response (DDR) causing the tumor to be more vulnerable to ICIs represent an active area of research and investigation. Clinical trials testing whether the use of low doses of DNA damaging agents may sensitize advanced NSCLC to targeted or immuno-therapies are ongoing^[21-23]. Therapeutic regimens include both platinum-based drugs and ICIs for advanced NSCLC, which have shown superior results compared to the use of chemotherapy alone^[21,24]. Upon first line or after failure of targeted- and/or immune therapies, the vast majority of patients will undergo treatment with platinum-based cytotoxic drugs. Hence, seeking out new modalities of DDR which can be used as biomarkers to better stratify patients, or as new therapeutic targets represents a valuable clinical and experimental goal^[25,26].

Molecular features of RANBP9

The RAN Binding Protein 9 (RANBP9; a.k.a. RANBPM) is a scaffold protein consisting of 5 regions/domains known to mediate protein-protein interactions^[27] [Figure 1A]. RANBP9 is highly conserved throughout evolution, suggesting that they maintain critical biological functions^[28,29]. The perturbation of its expression has shown that RANBP9 modulates the stability, turnover, and consequently signaling, of a number of proteins involved in crucial biological processes/signaling pathways^[30]. It is thought that RANBP9 exhibits these effects as a component of a ubiquitously expressed multi-subunit structure known

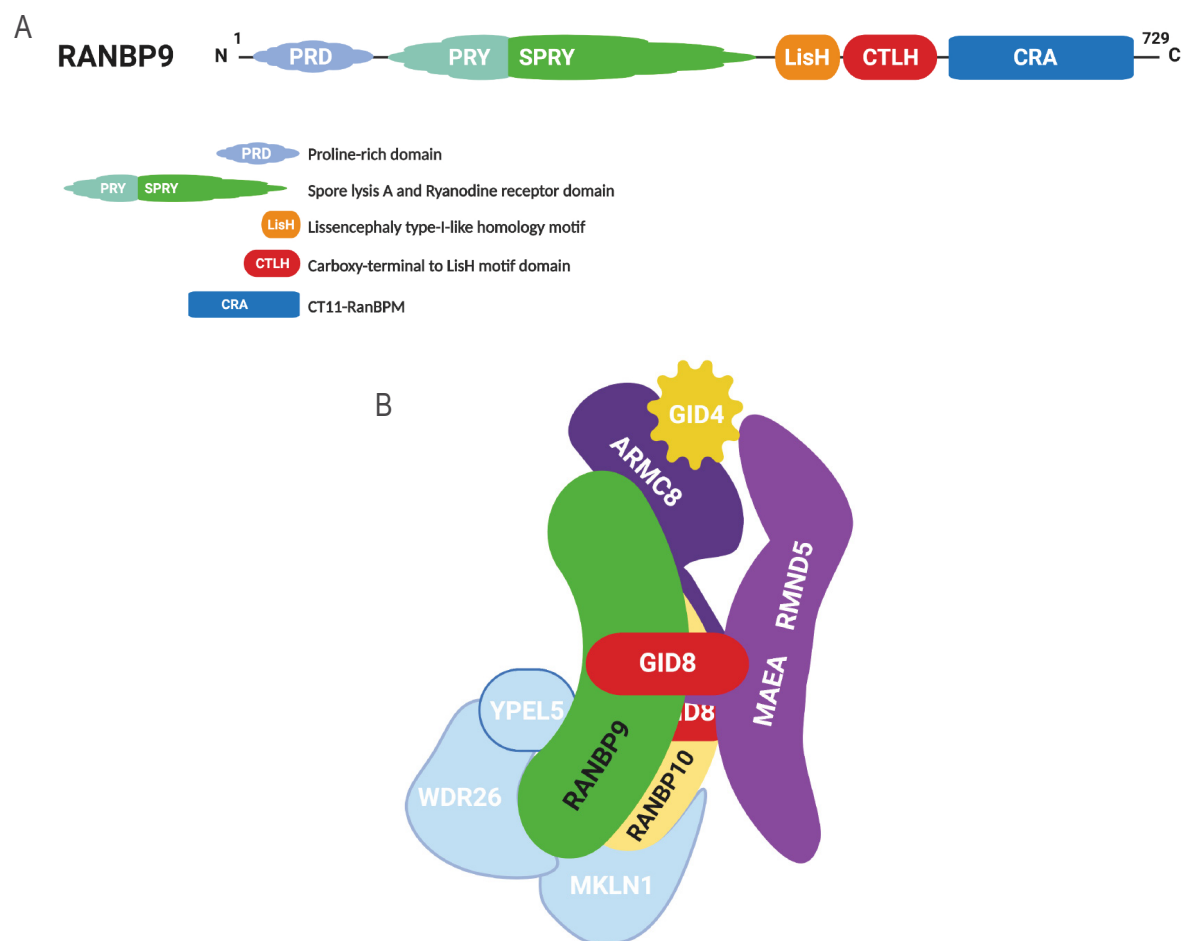


Figure 1. RANBP9 and the CTLH complex. A: RANBP9 is 729 amino acid protein that bears 5 regions/domains that are known to be instrumental for protein-protein interactions; B: the CTLH complex is an evolutionarily conserved E3 ligase multi-subunit structure equivalent of the GID complex in yeast. In its known configuration, the CTLH complex is a heterodecameric structure with a “core” made of a GID8 dimer, RANBP9, and ARMC8 (based on Liu *et al.*^[33]). Due to the similarities with RANBP9, it is likely that RANBP10 is also a core component. GID4 is a “peripheral” component recognized to act as substrate receptor. Other peripheral CTLH members are MKLN1, WDR26, and YPEL5, whose functions and placement within the structure are not well defined. For more detailed info about the CTLH complex and its members in cancer please refer to Huffman *et al.*^[34]. RANBP9: RAN binding protein 9; CTLH: C-terminal to LisH domain

as the C-terminal to LisH domain (CTLH) complex, the nomenclature of which is derived from one of the common protein-protein interaction domains shared by most of its members [Figure 1B]^[31-33]. In its reported configuration, the CTLH complex is heterodecameric, functioning as an unconventional E3 Ligase^[31,32]. While RANBP9 and GID8 in conjunction make up the scaffold, two other CTLH members known as MAEA and RMND5, provide the enzymatic activity^[31,32]. Currently, despite limited knowledge the E3 complex as a whole may serve as a key role in cancer biology^[34].

RANBP9 and cancer

With regard to human disease, RANBP9 has been initially studied for its potential involvement in abnormal brain and gonadal development, as well as in Alzheimer’s disease^[35-39]. Nevertheless, after it has become clear that RANBP9 is linked to critical cancer-causative pathways and to hallmarks of cancer in general, its investigative interest has shifted towards tumor-related paradigms. RANBP9 has been shown to demonstrate tumor-suppressive effects *in vitro*. For example, when acutely over-expressed, RANBP9 is proapoptotic in nature^[40,41]. Further, it increases stability of other accepted tumor suppressors such as p73

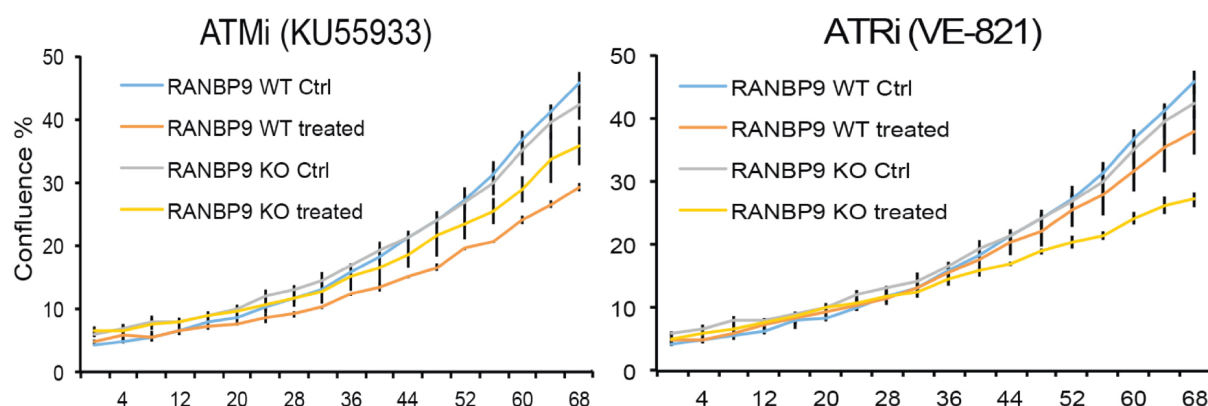


Figure 2. The absence of RANBP9 in NSCLC cells results in increased sensitivity to ATR inhibition. A549 RANBP9 WT controls and A549 RANBP9 KO clones were treated with the ATM inhibitor KU5933 (10 μ mol/L; left panel) and the ATR inhibitor VE-821 (10 μ mol/L; right panel). Growth was monitored automatically by live cell imaging (IncucyteTM) and cell confluence was quantitated in three replica plates. RANBP9: RAN binding protein 9; NSCLC: non-small cell lung cancer; ATR: ataxia and telangiectasia-related; KO: knockout; ATM: ataxia telangiectasia mutated

or mammalian Lethal Giant Larvae-1^[42,43]. Reduced expression of RANBP9 has been associated with distant metastasis and chemo-resistance in gastric cancer^[44]. The silencing of RANBP9 in colorectal cancer cells also resulted in an increase of cell growth *in vitro* and tumorigenesis *in vivo*^[45]. However, a straightforward cancer suppressive role is clearly inconsistent with the ability of RANBP9 to enhance Receptor Tyrosine Kinases or MAPK signaling^[46-49].

While it remains unclear whether RANBP9's tumor suppressive effects is observed *in vitro*, most studies agree that RANBP9 is over-expressed in a variety of highly prevalent tumor types including that of NSCLC^[44,45,50-52].

RANBP9 in NSCLC

RANBP9 and the entire CTLH complex are constitutively expressed and respond to different modalities of cellular stress^[31,32,53]. In order to elucidate the underlying biological mechanisms which could be relevant to the treatment of NSCLC, we focus on studying RANBP9 in the DNA damage response in NSCLC. Our specific interests arose from the observation that γ H2AX staining lingered for longer periods of time in RANBP9 knockout (KO) mice testes^[35,54]. In addition, RANBP9 had been listed as putative target of the ATM kinase in a seminal study by Matsuoka *et al.*^[55]. In this regard, we previously demonstrated that ATM phosphorylates RANBP9 on at least three Serine residues following DNA damage in NSCLC cells. We also showed that the absence of RANBP9 blunts the effect of ATM signaling^[56]. This observation prompted us to test the sensitivity of NSCLC cells to DNA damaging agent such as ionizing radiation (IR) and Cisplatin (CDDP), which are frequently used on patients. Our results show that RANBP9 KO NSCLC cells exhibit increased sensitivity to both IR and CDDP^[56].

The significance of our findings was highlighted in a retrospective study where we observed that negative correlation existed between the levels of RANBP9 protein expression and response to platinum-based treatment in NSCLC patients^[50]. As part of the same study, we also determined that the absence of RANBP9 in NSCLC cells caused an increase in the sensitivity to Poly-ADP ribose phosphorylase (PARP)-inhibitors. Recently, our findings were substantiated indirectly by an innovative high throughput CRISPR screening, where the combined ablation of MAEA, UBE2H, and WDR26 (all recognized members of the CTLH complex) was found to be associated with an augmented sensitivity to PARP inhibitors^[57]. Hence, we speculate that the lack of RANBP9 alone or in combination with other members of the CTLH complex may result in a "BRCAness-like" phenotype in NSCLC^[58]. This could be clinically important in light of the active

pursuit of biomarkers with “BRCAness-like” status in malignancies other than breast and ovarian cancer, including that of NSCLC^[59,60].

Herein, we show that RANBP9 KO NSCLC cells have increased sensitivity to ATR inhibitors while not having the same response to ATM inhibitors [Figure 2]. In essence, we hypothesize that RANBP9 levels of expression may be predictive of patient response to specific DNA damaging agents in the clinics. Nevertheless, a prospective study will be necessary to prove this hypothesis^[50].

As a whole, RANBP9 is highly expressed in NSCLC compared to normal adjacent tissue^[50]. However, we have found that the levels of protein expression may not necessarily correlate with the cellular transcription levels. We found this lack of correlation in commonly used NSCLC cell lines as well as in a limited number of freshly extracted NSCLC patient samples^[50]. This observation needs to be further substantiated and confirmed in other studies. However, it is not unusual for proteins which are part of macromolecular complexes to modulate other proteins’ stability^[61,62]. In addition, RANBP9 is involved in the response to stress and it is conceivable that protein levels are not always regulated by mRNA expression^[61,62]. If confirmed, the lack of correlation between RANBP9 protein and mRNA amounts will have profound implications. In fact, it indicates that only the study of protein levels can provide a reliable assessment of the expression of RANBP9.

Taken together, RANBP9 is highly expressed in NSCLC cells as compared to normal lung tissue^[50,51]. However, this does not preclude the untested possibility that RANBP9 may possess a tumor suppressive function during the initial phases of NSCLC tumorigenesis due to its role in promoting genomic stability. This hypothesis needs to be tested in relevant preclinical models. However, it is conceivable that RANBP9 opposes initiation but may later become advantageous for tumor progression, which is similar to TGF- β related signaling pathways^[63,64].

ONGOING INVESTIGATIONS

Studying the role of RANBP9 in the context of cellular response to stress and DNA damage is a major focus of our group. We are currently exploring three specific aspects of RANBP9 biology in response to genotoxic stress. The first is the close association of RANBP9 with the “guardian of the genome” known as p53^[65]. The second relates to the mechanisms underlying the augmented sensitivity to DNA damaging drugs caused by the lack of RANBP9. Finally, we also consider the potential partial functional redundancy of RANBP10. Due to the presence of high homology, this second Scorpion (Spry-Containing Ran binding ProteIN) and paralog cannot be ignored, and it is likely a major confounding factor in establishing the importance of RANBP9^[66,67].

RANBP9 and p53 in the DDR

As a consequence of impairment of ATM signaling in the absence of RANBP9, we have reported that phosphorylation of p53 on Serine 15 is severely compromised, affecting the total expression of p53 as shown in Figure 3^[50,56]. The relationship between RANBP9 with p53 is likely more complex than anticipated and worthwhile to be further investigated. Although an interaction has been previously described between RANBP9 and a specific isoform of p73 by co-IP and colocalization, three groups including ourselves have failed to demonstrate a physical interaction between RANBP9 and p53 by co-IP^[42,68] (and Coppola, unpublished results). Nevertheless, a high throughput study reported a co-IP between RANBP9 and the p53 R273H mutant that needs to be further validated^[69]. In summary, it appears that the effects of the absence of RANBP9 on p53 total and phosphorylation levels upon DNA damage are indirect. How the absence of RANBP9 negatively affects ATM-kinase remains to be clarified, but the blunted ATM activity could potentially explain the decrease in p53 levels. On the other hand, an impaired ATM signaling may be only one of the possible mechanisms through which RANBP9 affects p53 abundance and activity.

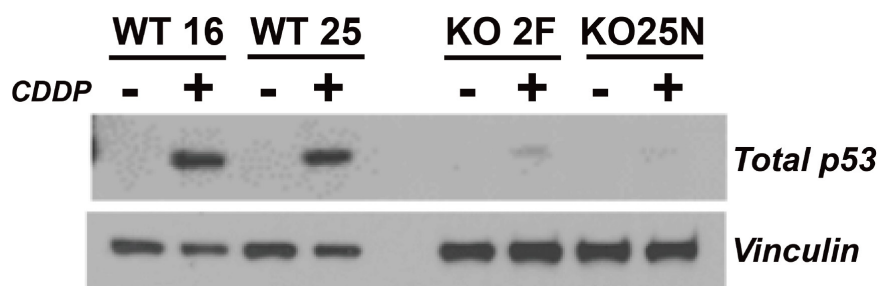


Figure 3. The absence of RANBP9 causes a marked reduction of p53 levels in NSCLC cells subject to genotoxic stress. Two independent A549 RANBP9 WT controls and RANBP9 KO clones were exposed to 10 $\mu\text{mol/L}$ CDDP for 24 h. WB shows that RANBP9 KO cells have a severe reduction of total p53 levels. This is likely due to the blunted p53 phosphorylation of Serine 15, which is a target of the ATM kinase (as shown in Palmieri *et al.*^[56], 2016 and Tessari *et al.*^[50], 2018). RANBP9: RAN binding protein 9; NSCLC: non-small cell lung cancer; KO: knockout

Considering that p53 is degraded by MDM2^[70], an unexplored explanation for our findings is that a potential functional link exists between RANBP9 and MDM2. Alternatively, RANBP9 has been reported to co-localize with Tip60 (a.k.a. KAT5)^[71]. Tip60 mediates histone dynamics in conjunction with PARP1^[72,73]. Although the acetylation by Tip60 of ATM has recently been put into question^[74], it is undisputed that Tip60 acetylates and stabilizes p53^[75]. It is therefore possible that the absence of RANBP9 further impinges on the stabilization of p53 operated by Tip60 [Figure 4]. Tip60 acetylates p53 on K120, which is crucial for p53-dependent apoptosis^[76].

RANBP9 was also reported to have interaction with the homeodomain-interacting protein kinase 2 (HIPK2)^[77], causing phosphorylation of p53 on Serine 46 in situations where the DNA damage is beyond repair. Similarly to the K120 acetylation, the phosphorylation of this residue is particularly important in deciding the fate of the cell upon DNA damage and would be in line with the suggested role for RANBP9 in mediating apoptosis^[40,78]. To complicate the jigsaw regarding the relationship of RANBP9 with ATM, p53, and HIPK2, the latter kinase was degraded via a p53-controlled pathway during recovery from sub-lethal DNA damage^[79].

Altogether, RANBP9 appears to be intricately linked to p53 on multiple levels following DNA damage. Therefore, RANBP9 levels play an important role in fine-tuning the activity of the guardian of the genome [Figure 4].

The absence of RANBP9 confers sensitivity to DNA damage

From a clinical point of view, the absence of RANBP9 renders cells more sensitive to DNA damaging agents such as IR and CDDP, but also to inhibitors of ATR or PARP. This warrants a pre-clinical investigation of RANBP9 as a potential target of therapy which may serve to ameliorate the cancer cell response and resistance to these drugs. However, it is also worthwhile to systematically test RANBP9 KO cells for drug sensitivity and attempt to find additional vulnerabilities caused by the absence of this protein. In fact, RANBP9 is associated with pathways other than the DDR and consequently its absence might result in fatal damage to cancer cells.

RANBP10 may partially compensate for the absence of RANBP9

To date, the study of RANBP9 has largely ignored the existence of the highly homologous RANBP10, which shares four out of five protein-protein interaction domains^[47]. The genetic deletion of these two proteins consequently results in two very different phenotypes^[35,36,80]. However, considering the similarity in protein sequences and genomic organization, they appear to have evolved as duplication of the ancestral yeast Gid1^[28,29]. We proposed that these proteins may have partially overlapping functions and RANBP10 has been found to be post-translationally modified following DNA damage^[66,81-83].

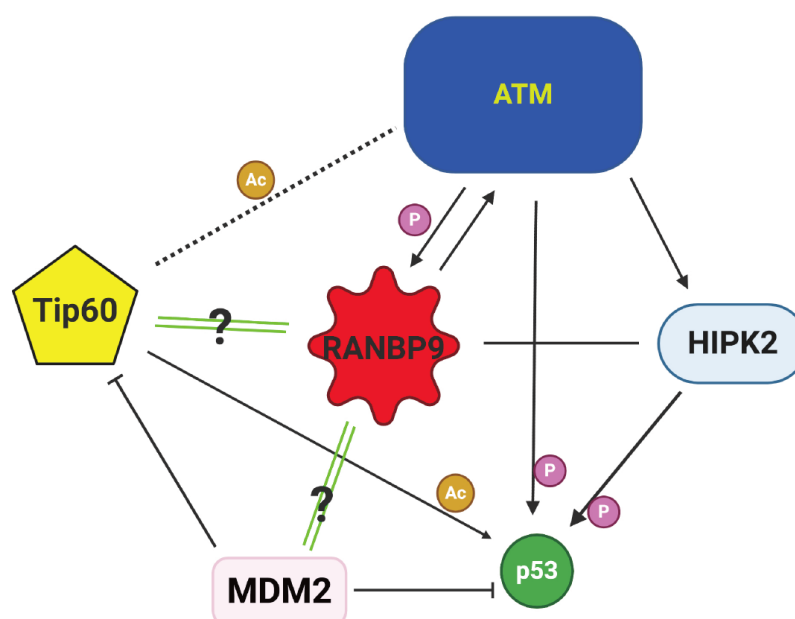


Figure 4. Schematics of the ATM-p53-Tip60-MDM2-HIPK2-RANBP9 potential connection in NSCLC cells subject to genotoxic stress. RANBP9 appears to be at the center of an intricate network determining cell fate during DDR. Green double lines with question marks indicate active areas of investigation. RANBP9: RAN binding protein 9; ATM: ataxia telangiectasia mutated; NSCLC: non-small cell lung cancer; HIPK2: homeodomain-interacting protein kinase 2

From an experimental perspective, it is conceivable that the similarities between the two Scorpins hinders a clear identification of RANBP9 and nullifies the functional effects of RANBP9 deletion. To test this hypothesis, it will be necessary to perform experiments by deleting both RANBP9 and RANBP10 simultaneously.

Finally, a partial redundancy between the two Scorpins would also explain in part why these genes alone are rarely linked with susceptibility to DNA damaging agents.

Using CRISPR/Cas9, we have recently engineered a novel murine model in which RANBP9 is tagged at the C-terminus with both V5 and HA (RANBP9 with double Tag = RanBP9-TT). This model has been validated through the use of immunohistochemistry and by coimmunoprecipitation, showing that the expression and interactions of the tagged protein faithfully recapitulates those of the wild type RANBP9. This new murine strain will be instrumental in obtaining data *in vivo* about the RANBP9-immunoprecipitated proteome, without the risk of using antibodies that may recognize RANBP10^[84].

CONCLUSIVE REMARKS AND FUTURE PERSPECTIVES

It is clear that treatment of cancer with modalities based on the administration of a single therapeutic agent is rarely successful, whilst combined therapies are more efficacious in the clinical setting. Rationalized drug combinations in anticipation for precision medicine should be developed based on the mechanisms which allow cancer cells to resist and thrive. In particular, RANBP9 has been linked to cell proliferation, cell death, cell adhesion and migration. It has been shown to interact with Receptor Tyrosine Kinases at the membrane, intracellular messengers, and nuclear transcription factors^[30,85]. In light of these multiple links with critical signaling pathways and critical biological processes, RANBP9 potentially provides a target to block important mechanisms of cell resistance to therapy. Therefore, our group and others have proposed RANBP9 as potential target for cancer therapy^[50,56,85]. However, this protein is ubiquitously expressed and additional work will be required to take this translational concept to the clinics.

Firstly, we have not fully elucidated the molecular role of RANBP9 in the DDR. The nuclear accumulation within hours after genotoxic stress suggests the participation of RANBP9 in the resolution of the damage. Apart from being constitutively expressed in the CTLH E3 ligase complex, RANBP9 has also been physically linked to the proteasome^[86]. Therefore, it is conceivable that it mediates the turnover of proteins that are directly involved in the repair of DNA that need to be disposed of according to the tightly concerted choreography of the DDR^[87,88].

With regards to the subnuclear localization of RANBP9 during the DDR, a question which remains is whether RANBP9 is physically present at sites of damage. Novel tools such as the cell and mouse lines with the endogenous RANBP9 tagged may be instrumental in answering this *in-vivo* question.

In addition, Tip60 modulates the acetylation of DNA following cellular damage^[89,90]. Whether the absence of RANBP9 results in differences in histone acetylation should be a major topic of investigation.

For future clinical management and stratification of patients, a thorough investigation on the outcomes of treatment in the absence of RANBP9 should be conducted using cells containing different mutational status of p53^[91]. With regard to this, the p53 pathway is the main regulator of cell metabolism^[92-94]. Recently, RANBP9 has been shown to impinge on crucial metabolic nodes such as AMPK and MTOR signaling including processes such as autophagy^[53]. Therefore, the metabolic consequences as a result of the absence of RANBP9 should be investigated to ascertain whether drugs targeting specific metabolic pathways should be used in combination with DNA damaging agents.

In summary, RANBP9 is highly expressed in NSCLC, participating in critical signaling pathways. Therefore, targeting this specific protein may significantly weaken the ability of tumor cells to survive and proliferate when treated with DNA damaging or other types of drugs. Similar consideration may be made with other malignancies in which RANBP9 has been found to be highly expressed.

DECLARATIONS

Authors' contributions

Performed experiments: Tessari A, Soliman SHA, Orlacchio A, Capece M, Palmieri D

Elaborated data, prepared figures: Tessari A, Soliman SHA, Palmieri D

Read and edited the manuscript: Amann JM, Visone R, Carbone DP, Palmieri D

Conceived research, wrote manuscript, prepared figures: Tessari A, Palmieri D, Coppola V

All authors approved the manuscript.

Availability of data and materials

Not applicable.

Financial support and sponsorship

This work was supported in part by funding provided by the Ohio State University Comprehensive Cancer Center (P30 CA016058).

Conflicts of interest

The authors declare that they have 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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Magnetic nanotechnologies for early cancer diagnostics with liquid biopsies: a review

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How to cite this article: Su D, Wu K, Saha R, Liu J, Wang JP. Magnetic nanotechnologies for early cancer diagnostics with liquid biopsies: a review. *J Cancer Metastasis Treat* 2020;6:19. <http://dx.doi.org/10.20517/2394-4722.2020.48>

Received: 19 May 2020 **First Decision:** 20 May 2020 **Revised:** 11 Jun 2020 **Accepted:** 17 Jun 2020 **Published:** 10 Jul 2020

Academic Editor: Wei Zhang **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Liquid biopsy has become an emerging technology in the detection of cancer related biomarkers as well as the continuous monitoring of cancer treatment. There has been extensive research on the applications of magnetic nanotechnologies in liquid biopsies from the separation of target analytes to the detection of cancer biomarkers. Magnetic separation plays an important role in increasing both the efficiency and sensitivity of the liquid biopsy process. The detection of cancer biomarkers through magnetic nanosensors also possesses many advantages such as low background noise, high sensitivity, short assay time, and the ability to detect multiple biomarkers at the same time. This review focuses on the recent advances of magnetic nanotechnologies in liquid biopsies for cancer detection and its future potential in comparison with other technologies.

Keywords: Liquid biopsy, magnetic, cancer, nanotechnology, biosensor

INTRODUCTION

Liquid biopsy is a new diagnostic concept that has received much attention in the past few years. It was originally defined as “the test on the blood sample of the patient to study the circulating tumor cells or cell free DNA derived from the tumor cells in the blood” by the National Cancer Institute^[1]. Compared to the conventional invasive biopsy that contains cells or tissues from lesion, liquid biopsy is a noninvasive technique whose target analytes include circulating tumor cells (CTCs), circulating tumor DNA (ctDNA)



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and RNA (ctRNA), and exosomes^[2-4]. To date, liquid biopsy has been performed in various biological fluids including peripheral blood, urine, ascites, pleural effusion, *etc.* for early diagnosis, screening, prognosis assessment, detection of minimal residual disease, and the design of personalized treatment for cancer therapy^[3,5]. The target biomarkers are firstly separated and enriched via a variety of separation technologies, followed by the determination of the biomarker concentration using different biosensor platforms. Many novel nanotechnologies have been developed in the liquid biopsy field. Loeian *et al.*^[6] fabricated a nanotube-CTC chip with the ability to selectively capture CTCs in the blood sample. It was shown that this nanotube-based device can successfully capture CTCs from the peripheral blood of breast cancer patients with a range of 0.5-28 CTCs per mL^[6]. Yu *et al.*^[7] developed a fluorescent probe that can release the outmost antibodies after binding with CTCs, making it possible to release and recycle CTCs in future liquid biopsy processes^[7]. Besides the separation and detection of CTCs, a polymerase chain reaction (PCR) approach was proposed for the detection of ctDNA by using a quencher-free fluorescent probe DNA and graphene oxides. This method can detect as low as 49 pg of epidermal growth factor receptor (EGFR) exon 19 detection DNA with a detection limit of 0.1%^[8]. A comprehensive review on the current liquid biopsy technologies is contributed by Tang *et al.*^[9].

Of all the proposed technologies for liquid biopsy, magnetic nanotechnologies stand out for the ease in cell manipulation under magnetic field during biomarker separation^[10-12], as well as low background noise and high theoretical sensitivity during biomarker detection due to the fact that most of the biological environment is non-magnetic. However, as with other types of biosensors, the sensitivity of the magnetic biosensors also depends on the system setup, the surface biofunctionalization processes, and the intrinsic sensitivity of the sensing segment. In this review, magnetic separation technologies using high-moment magnetic nanoparticles (MNPs) as tags are introduced. Microfluidic channels are reviewed in the following section regarding their crucial role in both magnetic separation and magnetic sensing. Finally, two types of magnetic nanotechnologies for liquid biopsy are introduced: magnetoresistance (MR) sensors as a surface-based liquid biopsy technology and nuclear magnetic resonance (NMR) as a volume-based liquid biopsy technology.

MAGNETIC SEPARATION

The majority of cell-based liquid biopsy research has been focused on the detection of CTCs since it is the major cause of death and can be detected by non-invasive techniques from patients' blood samples^[13]. However, due to the low abundance of CTCs, cell sorting or cell separation is required prior to the detection process. To date, various devices have been developed to separate the CTCs from other undesired background substances. Cell size-based separation is a label-free technique that sorts the target cells out according to their unique properties such as size and stiffness^[14,15]. Alternatively, specific binding-based separation captures the target cells on patterned surfaces through chemical/immunoassays. While cell size-based devices exhibit high throughput and label-free characteristics, the specificity of the capture is often sacrificed due to the variation of the size and stiffness of the CTCs. On the other hand, techniques based on binding cells to certain surfaces show higher specificity, but suffer from low throughput and difficulties in cell recycling^[12,16-18]. Magnetic separation falls into the category of specific binding-based technologies. However, instead of binding the target cells to surfaces, MNPs are employed to mark the target cells, which are captured onto magnetized surfaces subsequently. Unlike the aforementioned techniques, the captured cells can be released easily by removing the magnetic field. In this section, high-moment MNPs are introduced first as an important approach to increase the capture efficiency. Optimization of the capture process from both surface functionalization and device configuration aspects is also summarized.

High-moment magnetic nanoparticles

MNPs have been widely used as biomarkers for biosensing and could also be used to mark the CTCs for liquid biopsy. By labeling CTCs using MNPs, these CTCs can be separated by applying an external

magnetic field. The migration velocity of a MNP under the application of a homogeneous magnetic field, i.e. the magnetophoresis process, largely depends on the magnetic moments of the MNPs. Bruus *et al.*^[18] theoretically calculated the magnetophoretic force acting on a spherical particle in non-homogeneous field. It is given by equation:

$$F_{MAP} = 2\pi\mu_o f_{cm} a^3 \nabla [H_{ext}(r_o)^2] \quad (1)$$

where ∇H_{ext} is the gradient of the external magnetic field, the diameter of the particle is represented by a , and f_{CM} is the Clausius-Mossotti factor of magnetization (CM). f_{CM} is represented as: $(\mu - \mu_o)/(\mu + 2\mu_o)$. Here, μ and μ_o are the magnetic permeabilities of the spherical particle and vacuum, respectively.

Iron oxide MNPs, such as γ -Fe₂O₃ and Fe₃O₄, are widely used in the biosensing area due to their good stability and biocompatibility. However, the saturation magnetization of iron oxide MNPs is around three times smaller than that of high-moment MNPs such as Fe, FeCo, Fe₁₆N₂, *etc.*^[19,20]. These high-moment MNPs can provide much higher magnetic forces and, as a result, enhance the capture efficiency and reduce the dose of MNPs needed for liquid biopsy. Many research groups have reported the synthesis of high-moment MNPs. Generally, two approaches are used to synthesis high-moment MNPs: the bottom-up approach and the top-down approach. For the bottom-up approach, MNPs are formed from atoms that nucleate and grow into nanoparticles. For example, a gas-phase condensation (GPC) method is used to obtain these atoms either by thermal evaporation or sputtering. By cooling down these atoms, nucleation starts and then the nuclei grow into nanoparticles of various shapes. High-moment MNPs such as Fe, and FeCo have been successfully synthesized using this GPC method^[21,22]. In addition, high-moment MNPs with biocompatible shells such as SiO₂, Ag, and Au can also be synthesized by the GPC method. FeCo MNPs are also reported by using a wet chemistry method^[23], which also belongs to the bottom-up approach. In contrast, for the top-down approach, MNPs are made from raw bulk materials that are broken down to small nanoparticles, such as the ball milling method. Chakka *et al.*^[24] successfully prepared Fe, Co, and FeCo MNPs by using a surfactant-assisted ball milling method, and the size of these MNPs can be smaller than 10 nm. These high-moment MNPs are promising candidates for liquid biopsy.

Magnetic separation in liquid biopsy

Besides the search for novel materials and synthesis techniques to develop high-moment MNPs, surface functionalization strategies also play an important role in the cell-particle interactions, which can greatly impact the capture efficiency and specificity. The most commonly used technique to conjugate MNPs to the target cells is by functionalizing antibodies on MNP surfaces that can bind specifically to the antigens on the target cells^[25,26]. However, extra surface coating is required to further enhance both the efficiency and the specificity of the cell capture process. A good example would be the biomimetic cell-membrane-camouflaged nanoparticles. Rao *et al.*^[27] coated platelet (PLT)-leukocyte (WBC) hybrid membranes followed by the modification of antibodies [Figure 1]. They showed that the PLT membranes can recognize and communicate with CTCs, thus enhancing the binding efficiency. On the other hand, the WBC coatings can reduce the interactions between the MNPs and the white blood cells in the background. Combining the characteristics of both coatings, the hybrid membrane-coated immunomagnetic beads exhibited an improvement of capture efficiency from 66.68% (commercial product) to 91.77% with an increase in cell purity, too.

In addition to the optimization of MNPs, much attention has been paid to developing novel cell separation devices. In magnetic separation, the device performance largely depends on the strength and spatial distribution of the magnetic field. Traditional magnetic separation utilizes permanent magnets that are in the size range of several millimeters to centimeters, which suffers from small magnetic field gradient, low density of magnetic traps, and poor control over the magnetic field profile. To increase the capture

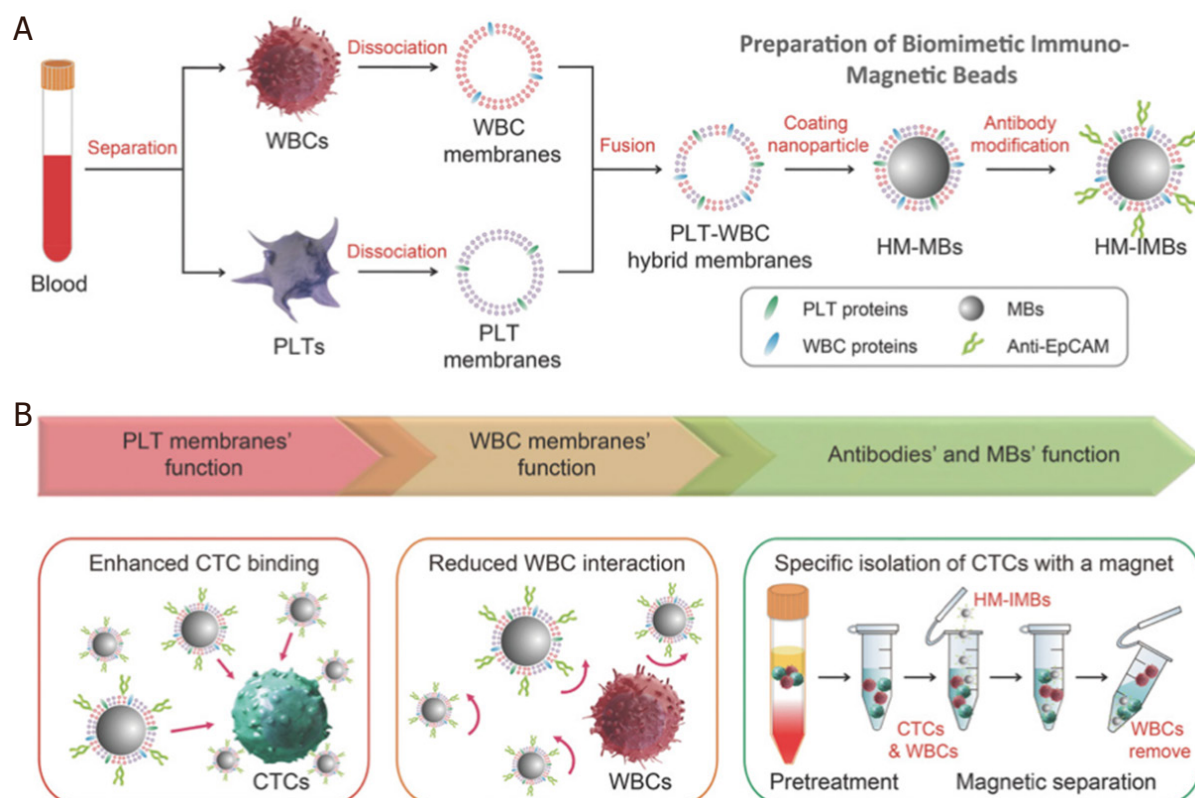


Figure 1. Schematic of the preparation process for the hybrid membrane-coated immunomagnetic particles (A) and the function of PLT and WBC membranes (B)^[27] (reprinted with permission from John Wiley and Sons). PLT: platelet; WBC: leukocyte

efficiency, especially for rare cell separation, magnetic structures with micro- or nanometer sizes are proposed. Chen *et al.*^[25] fabricated micromagnet arrays with the inkjet printing technology. In this work, the MNPs were firstly mixed with the printing ink and then printed with predefined pattern on the glass slide. After the evaporation of the liquid printing ink by heating, the MNPs self-assembled into a micromagnet structure. A permanent magnet was placed under the printed micromagnets to generate the magnetic field for long-range capture and magnetize the micromagnets for short-range trapping of the CTCs. It was found that the printed micromagnets led to a 26% increase in the capture efficiency. Besides the inkjet-printed magnets, a microfabricated magnetic sifter was proposed by Earhart *et al.*^[28]. A silicon wafer was patterned with hexagonal arrays of 40- μm holes with permalloy coatings. The blood sample was firstly labeled with magnetic tags and then pumped through the hexagonal holes under an external magnetic field. Due to the high magnetic gradient at the edge of the holes, the magnetically labeled target cells were captured while the unlabeled cells passed through the holes. A capture efficiency of 90% was reported for high epithelial cell adhesion molecule (EpCAM) expressing cells.

MICROFLUIDIC CHANNELS

The integration with microfluidic channels has become a “common sense” during the process of designing novel liquid biopsy platforms to facilitate highly efficient cell separation, to reduce the biomaterial consumption, to minimize the detection time, and to increase the density of functional devices. In this section, the fabrication process of microfluidic channels is reviewed, followed by their application in magnetic separation. Microfluidic channels can also be integrated with magnetic sensors during liquid biopsy. However, since microfluidic channels are usually not the core of discussion in most magnetic sensing applications, they are reviewed together with magnetic sensors in Section 4.

Fabrication of microfluidic channels

Poly(dimethylsiloxane) (PDMS) is the most commonly employed material in the fabrication of the microfluidic channels since it is low-cost, chemically inert under most circumstances, nontoxic, and compatible with lithography processes^[29]. Conventional 2D microfluidic channels can be made by a soft lithography process. A master mold is firstly fabricated by photolithography, followed by the curing of PDMS on it. Due to the low surface free energy and elasticity, the cured PDMS can replicate the shape of the master mold and be released without causing any damage. Both reversible and irreversible sealing can be realized between PDMS and the channel substrate^[30]. Reversible sealing can be achieved either by applying a pressure greater than 5 psi or through adhesive tapes, while irreversible sealing can be formed by plasma treatment of the contact surface^[31]. Despite the straight-forward fabrication processes, many challenges exist for the PDMS microfluidic channels based on soft lithography, such as flow profile problems due to leakage and uneven pressure, low fabrication efficiency, and poor flexibility due to the need of fabricating the master molds.

There has been much progress in developing both novel materials and fabrication technologies for the microfluidic channels^[32,33]. The ability to manufacture 3D structures and the additive nature of the process have made 3D printing a promising candidate in the fabrication of microfluidic channels. A polyjet-based 3D printed fluidic device was developed by Gross *et al.*^[34]. The device design was completed with the aid of computer-aided software and then converted to a STL file prior to printing. A rigid and transparent channel was developed with precise control of the channel dimension (1 mm × 0.8 mm × 2 mm). Besides 3D printing, other techniques have also been explored to fabricate 3D microfluidic channels. Song *et al.*^[35] proposed a metal wire removal process, where a thin soldering wire with a 3D circular shape was employed. After pouring PDMS onto the metal wire and curing it, the metal wire was melted out via heating. With this simple process, circular, helix-shaped, and double helix-shaped microfluidic channels can be easily prepared. In another work, a direct laser writing approach was used to prepare microfluidic channels embedded in fused silica with an aspect ratio over 1000^[36]. Direct laser writing usually results in high surface roughness. In this work, wet etching and glass drawing process were conducted after the laser writing, which significantly reduced the surface roughness from 50.3 nm to 0.29 nm.

Microfluidic channels in magnetic separation

The design of microfluidic channels play an important role in optimizing the efficiency of magnetic separation. Inglis *et al.*^[37] fabricated a device with ferromagnetic Ni strips underneath the separation chamber at angle of 10° to the bottom of the continuous flow [Figure 2A]. When an external magnetic field is applied, the flux lines are concentrated on the Ni strips. Magnetically labeled cells [in this case, the white blood cells (WBCs)] deviate away from the flow direction and move along the direction of the strips. In this way, they achieved separation of WBCs from red blood cells (RBCs). In another technique, Hans and Frazier applied a uniform magnetic field normal to the ferromagnetic Ni wires which was fabricated along the length of the microfluidic channel^[38]. They magnetically deformed the ferromagnetic wire using high magnetic field gradient, which generates the magnetic field gradient. By playing around with the direction of the magnetic field, they got the fabricated device to work in “diamagnetic capture mode” and “paramagnetic capture mode” that very efficiently separated the diamagnetic WBCs, cells and tissues, and the paramagnetic deoxy-hemoglobin RBCs.

Afshar *et al.*^[39] designed a system to meet the requirements of specific bioassays to study the on-chip agglutination assays for the detection of rare analytes by particle coupling as doublets. They integrated the PDMS microfluidic channels with soft magnetic microtips of varied shapes and sizes to provide the magnetic field gradient for the particles. The magnetic tips serve as the field concentrators and are positioned in the near vicinity of the microfluidic channel to generate even higher magnetic actuation forces. This device was experimentally tested to achieve three goals with two sets of superparamagnetic

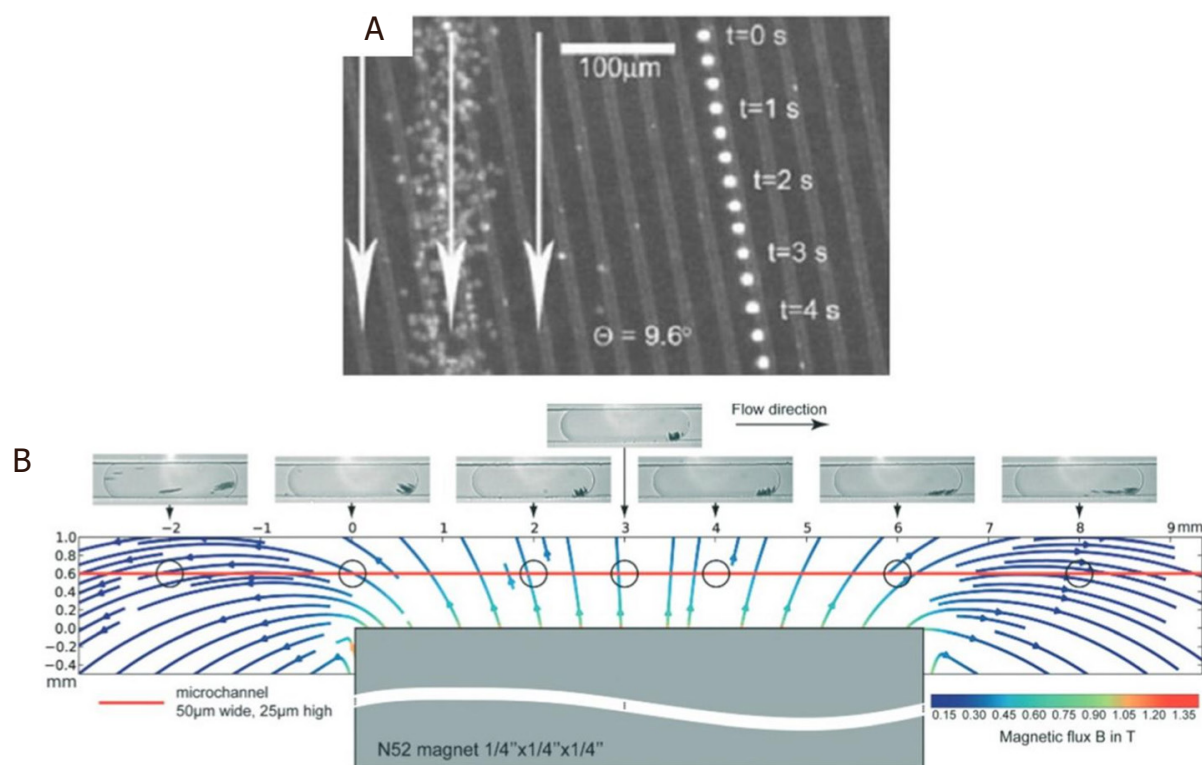


Figure 2. Schematic design of a Ni wire that can be operated in diamagnetic and paramagnetic mode for the separation of diamagnetic WBCs, cells, and tissues from paramagnetic deoxy-hemoglobin RBCs (A)^[37] (reprinted with permission from AIP Publishing); Schematic overview of the droplet based magnetic bead separation from a microfluidic channel (B)^[40] (reprinted with permission from Royal Society of Chemistry). WBC: leukocyte; RBC: red blood cell

particles of sizes $1\mu\text{m}$ and $2.8\mu\text{m}$: (1) dosing and immobilization of desired number of magnetic beads; (2) targeted release of the beads in a highly confined particle stream; and (3) continuous magnetophoretic size separation in-flow with high resolution.

Brouzes *et al.*^[40] reported a droplet-based microfluidic method to separate desired molecules in a rapid and, most importantly, continuous fashion. They accomplished this by at first marginalizing functionalized superparamagnetic beads within the droplet using magnetic field and then splitting the same droplet with one containing the majority of magnetic beads and the other containing the minority part. They quantitatively and qualitatively analyzed the factors which affect the marginalization and the splitting of the droplet. Furthermore, they studied how the marginalization affects the droplet velocity. Figure 2B shows the MNP distribution and orientation as a function of position with respect to the magnet. Most of the MNPs aggregate towards the center of the magnet. However, the aggregation is not exactly at the center of the magnet because of the internal magnetic field flow lines. Finally, they correctly assessed that this droplet-based technique is well-suited for applications in single cell genomics and proteomics. As a foresight, they claimed that this method could also be used to separate mRNA bound to poly-dT functionalized MNPs from single cell lysates to prepare cDNA cell microarrays.

Weddemann *et al.*^[41] reported theoretical calculations supported by experiments for the separation of several particles based on size using the combined hydrodynamic and magnetophoretic forces. Jung *et al.*^[42] designed another such device with slanted ridge arrays in a microfluidic channel. This kind of configuration was reported to have a larger magnetophoretic force of $7.68\mu\text{N}$ in comparison to 0.35pN from the traditional devices. With this design of microfluidic channels, $91.68\% \pm 2.18\%$ of the *E. coli* bacterial cells labeled with MNPs were separated from undiluted whole blood sample at a rate of 0.6 mL/h . This is a

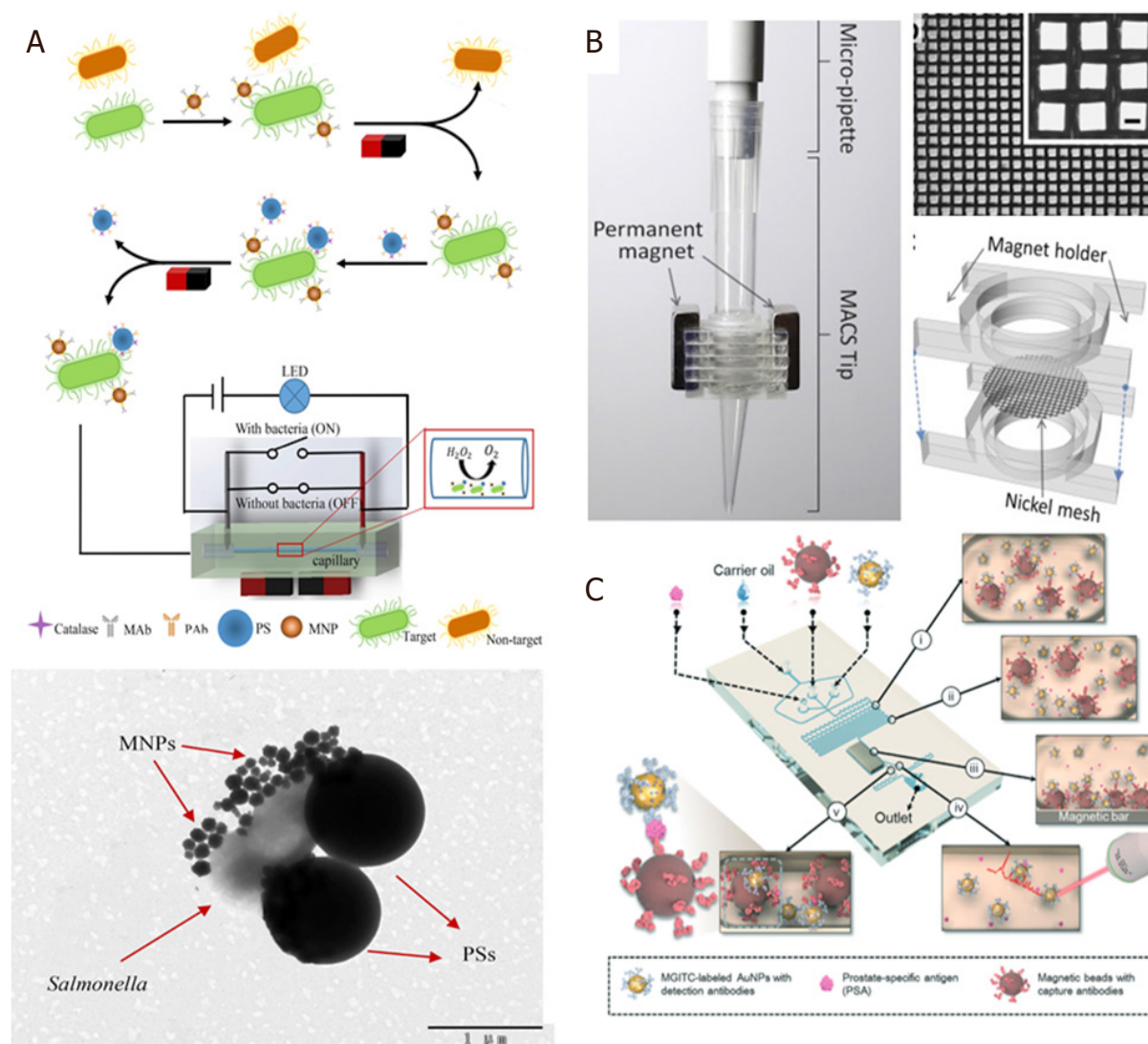


Figure 3. Schematic set-up for the microfluidic channel based immunomagnetic separation including enzyme catalysis and electrical signal-off for detection of *Salmonella* (A)^[43] (reprinted with permission from Elsevier); MACS tip for immunomagnetic separation consisting of rectangular Ni meshes and permanent neodymium magnets on both sides of the Ni mesh (B)^[46] (reprinted with permission from Elsevier); schematic microfluidic channel design for the wash-free magnetic immunoassay for PSA using SERS droplet-based sensor for readout (C)^[47] (reprinted with permission from Royal Society of Chemistry). MACS: magnetic activated cell sorting; PSA: prostate specific antigen; SERS: surface enhanced Raman scattering

significant improvement when compared to conventional devices where only $23.98\% \pm 6.59\%$ of *E. coli* could be separated.

Of the other works on using microfluidics for magnetic separation, Hou et al.^[43] reported the detection of *Salmonella* from spiked milk using microfluidic signal off strategy to monitor changes in electrical signal. This strategy helped in amplification of biological signals and the limit of detection (LOD) of the *Salmonella* was reported to be 33 CFU/mL with an assay time of 2 h. Although it was not a rapid procedure, the LOD seems promising. The schematic setup for their detection of *Salmonella* is shown in Figure 3A. The mixture models for bio-magnetic separation using microfluidics were demonstrated by Khashan et al.^[44]. Wu et al.^[45] reported size selective separation of magnetic microspheres using microfluidic channels. Their experimental results were corroborated with numerical simulations. Oh et al.^[46] modified the tip of a micropipette with Ni wire meshes and demonstrated magnetic activated cell sorting (MACS) [Figure 3B].

This facilitated high throughput and gradient magnetic separation by simple pipetting procedure, thereby leading to high rate of separation of bacteria from whole blood in addition to successful bacterial culture and analysis of the sorted bacteria without off-tip processing. Gao *et al.*^[47] reported a novel wash-free magnetic immunoassay technique for prostate specific antigen (PSA) that employs a surface enhanced Raman scattering-based microdroplet sensor (SERS) for readout [Figure 3C]. A wash-free approach was demonstrated to detect PSA antigens with a reported LOD of 0.1 ng/mL.

MR SENSORS

MR biosensors have been studied for the past 30 years as a sensitive surface-based detection approach. During the detection process, the target analytes are captured by MNPs, which can subsequently bind to the sensor surface through the corresponding antibodies or complementary DNAs functionalized on the surface, resulting in a capture antibody-antigen-detection antibody-MNP complex or a probe DNA-target DNA-MNP complex. The surface functionalization technologies for both antibody-antigen as well as DNA-based magnetic assays are comprehensively^[48]. Under an external magnetic field, the MNP tags can generate stray fields, which will result in the resistance change of the MR sensors. Since the sensor signal is proportional to the number of MNPs in proximity to its surface, higher analyte concentrations will result in higher sensor signals. MR sensors possess multiple advantages as compared to other biosensing techniques. Detection based on magnetic field results in low background noise as most biological samples are paramagnetic, diamagnetic, or nonmagnetic. The sensor signals are also less affected by the chemical environment of the sample, such as pH and temperature^[49]. With the development of nanofabrication technologies, MR sensors can also be integrated into high-density chips, which makes it possible to realize multiplexed detection as well as the development of point-of-care (POC) detection with minimized device size^[50,51]. In this section, magnetoresistance effect are firstly introduced from a fundamental viewpoint, followed by the surface functionalization strategies and some examples of MR sensors' application in liquid biopsy. The integration of MR sensors with POC devices is also reviewed.

Magnetoresistance

Three different MR effects have been applied to the field of biosensors. Anisotropic magnetoresistance (AMR) refers to the phenomenon where the electrical conductivity of a spontaneously magnetized materials depends on the relative orientation of the electrical current and the magnetization. Since it was first discovered in Ni and Fe in 1857, the physical origin of the AMR effect has been extensively studied and was found to originate from the anisotropic scattering of electrons due to the spin-orbital coupling^[52], which was firstly described in the two-current model^[53]. Although AMR is one of the earliest discoveries in the family of MR effects, the AMR ratio in most material systems are relatively low, which limited the signal from AMR devices until the discovery of the giant magnetoresistance (GMR) effect^[54,55]. Nevertheless, AMR sensors still possess many advantages such as high sensitivity to the angle between magnetic field and the current direction (application in angular sensors), lower cost, and simple material system.

As opposed to the simple ferromagnetic films in AMR devices, GMR and tunneling magnetoresistance (TMR) exist in multilayer stacks^[56,57]. In a stack with alternating ferromagnetic layers and spacers, the magnetization orientation in the ferromagnetic layers can be altered by an external magnetic field. The electrical resistance of the structure increases when the magnetizations are parallel in adjacent ferromagnetic layers and decreases when the magnetizations are antiparallel. GMR and TMR occur when the spacer is a conductive metal layer and an insulating layer, respectively. TMR and GMR stacks possess much higher MR ratio than AMR sensors, which makes them better candidates for the sensing applications where the sensor signal and sensitivity are the top priorities^[58,59]. Compared to GMR sensors, TMR sensors generally exhibit higher MR ratio, which results in higher sensor signals as well as sensitivity. However, TMR sensors also suffer from poor linearity and larger noise. Furthermore, the complexity in TMR device fabrication as well as the need of top electrodes also induces difficulties in the design of TMR biosensors. Both GMR and TMR sensors' application in liquid biopsies are reviewed in Section 4.2.

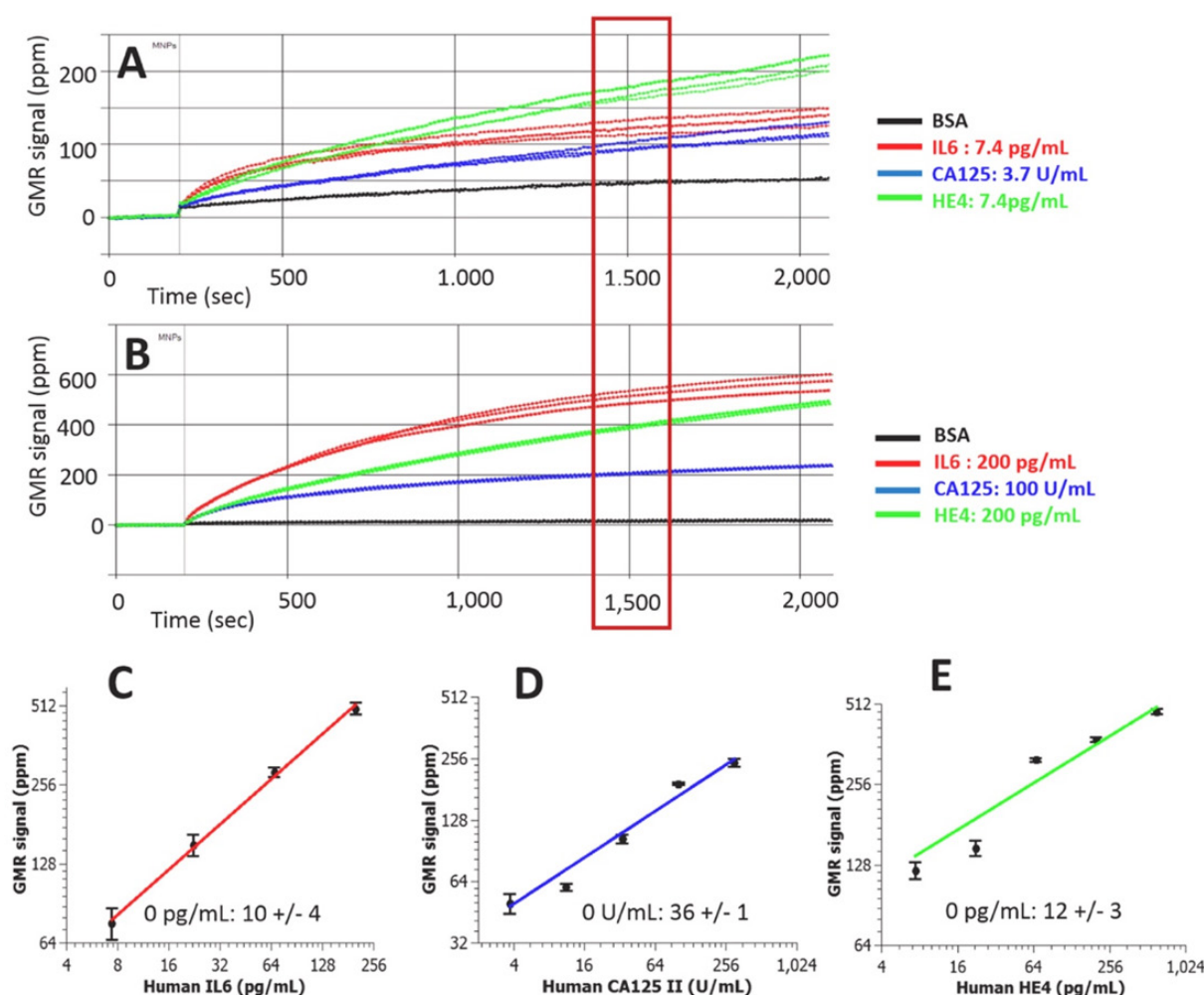


Figure 4. Real-time GMR sensor for multiplexed detection of the biomarkers (A,B); Calibration curves of multiplexed IL6, CA125 II, and Human HE4 assays (C-E)^[61] (reprinted with permission from Elsevier). GMR: giant magnetoresistance; IL6: interleukin 6; CA125: cancer antigen 125; HE4: epididymis protein 4

MR sensors for liquid biopsy

Immunoassay-based liquid biopsy

The elevated or reduced concentration of protein biomarkers in blood, saliva, or tissue can serve as indicators of cancer status^[60]. Since single biomarker detection can lead to false positive or false negative cases, multiplexed detection systems are required to achieve specific and sensitive cancer diagnosis, which can be achieved in MR sensors. During multiplexed detection, densely packed MR sensors are fabricated on a single chip with simultaneous signal readout. The sensors can be divided into different groups which are functionalized with different capture antibodies that are specific to various biomarkers. Klein *et al.*^[61] developed a multiplexed GMR sensing system that can detect three ovarian cancer biomarkers at the same time. The detection limit for cancer antigen 125 (CA125 II), human epididymis protein 4 (HE4), and interleukin 6 (IL6) are 3.7 U/mL, 7.4 pg/mL, and 7.4 pg/mL, respectively [Figure 4]. Another GMR sensing system reported by Gao *et al.*^[62] shows the capability of detecting 12 tumor biomarkers at the same time in only 15 min. It was shown that the analytical sensitivity was either comparable or better than the commercially available single-analyte immunoassays. Besides the aforementioned work, various kinds of biomarkers have been validated in the GMR sensor system such as urinary endoglin for prostate cancer^[63], eotaxin, and tumor necrosis factor alpha^[64]. Recently, Zhu *et al.*^[65] proposed to replace the traditional MNPs with 2D MoS₂-Fe₃O₄ nanostructures (MOFE). With the addition of layered MoS₂ materials, the loading

density of MNPs are increased, resulting in higher sensor signal. With the integration of the MOFE probes, the GMR system was capable of detecting as few as 100 exomes.

Besides GMR sensing platforms, magnetic tunneling junction (MTJ) sensors have also been demonstrated as a proof-of-concept for the detection of liver cancer biomarkers^[66]. The CoFeB/MgO/CoFeB MTJ exhibited a MR ratio of 122% and a sensitivity of 0.95%/Oe at room temperature. Three concentrations of alpha-fetoprotein were successfully detected. However, there has not been any further research in multiplexed cancer biomarker detection with MTJ sensors to the authors' best knowledge. The lack of experimental results for MTJ-based liquid biopsies can be attributed to the following reasons: (1) the complex stack structures of MTJs can result in large variations of performance between different MTJ devices, making it hard to generate calibration curves for different biomarkers; (2) the top electrodes of the MTJs tend to increase the distance between the sensor and the magnetic tags, leading to a decrease in sensor signal; and (3) it is hard to realize linear transfer curves in MTJ sensors, which further complicates the process of correlating sensor signal to the analyte concentrations.

DNA-based liquid biopsy

Besides antibody-antigen-based liquid biopsy, DNA is another important analyte for cancer detection. The ability of DNA detection has been demonstrated for both MTJ and GMR sensors. A proof-of-concept detection of DNA was achieved in a MTJ sensor with a Al₂O₃ barrier^[67]. Later, a quantitative detection of DNA was achieved by integrating 64 MgO MTJs on a single chip. However, there was not much work on MTJ-based DNA detection related to cancer detection. On the contrary, GMR sensors have been extensively studied as potential candidates for DNA-based liquid biopsies. Nesvet *et al.*^[68] reported the integration of methylation specific PCR to melting curve analysis on GMR sensors to enhance the sensitivity for methylation detection. By measuring the difference of melting point between the DNA probes that targeted on methylated or unmethylated cytosine-guanine dinucleotides (CpG) sites, the system was able to detect the methylated DNA with an analytical LOD down to 0.1%. It was also shown by the same group that this melting curve approach can simultaneously profile five mutation and four methylation sites in human melanoma cell lines^[69]. The detection of cell-free (cfDNA) was demonstrated by Dias *et al.*^[70]. The capture DNA probes were firstly spotted manually on the GMR chip, followed by the integration of a microfluidic channel. The magnetically labeled target DNA fragments (ALU115 and ALU247) was then introduced to the sensor surface through the microfluidic channel. A detection limit of picomolar range was achieved upon optimization.

POC devices

Ever since the discovery of MR sensors as a potential biosensor, MR-based POC devices have had a very exciting past showing a very promising future. Nevertheless, this topic has been subjected to extensive reviews^[48,71,72]. There have been two decades of research on improving the sensitivity of the GMR sensor, which was inevitably followed by untiring attempts from several groups worldwide to develop GMR-based POC devices that have proved extremely successful in rapid detection of multivariate pathogens. A research group from Stanford University has developed a device, named Eigen Lifescience, along with a customized App, all fit in the size of a smartphone. This smartphone-shaped device operating on the basis of GMR-based biosensor has been reported to be capable of diagnosing several harmful pathogens such as Hepatitis B virus^[73] and detecting DNA hypermethylation from melanoma cancer cells^[68] as well as prostate cancer antigen^[74], protein signatures in mouse lymphoma^[75], HIV and leukocytosis^[76], RNA sequencing^[77], and lung cancer^[78]. A research group from the University of Minnesota has developed another handheld device for GMR-based biosensing that has been commercialized. The device, better known as Z-Lab [Figure 5], has been reported to diagnose several diseases such as ovarian cancer^[61] and H1N1 influenza virus^[50], which has also been very recently reported to be detected wash-free by the same device^[51]. Drew A. Hall's group from the University of California, San Diego has worked hard to improve the electronic circuitry

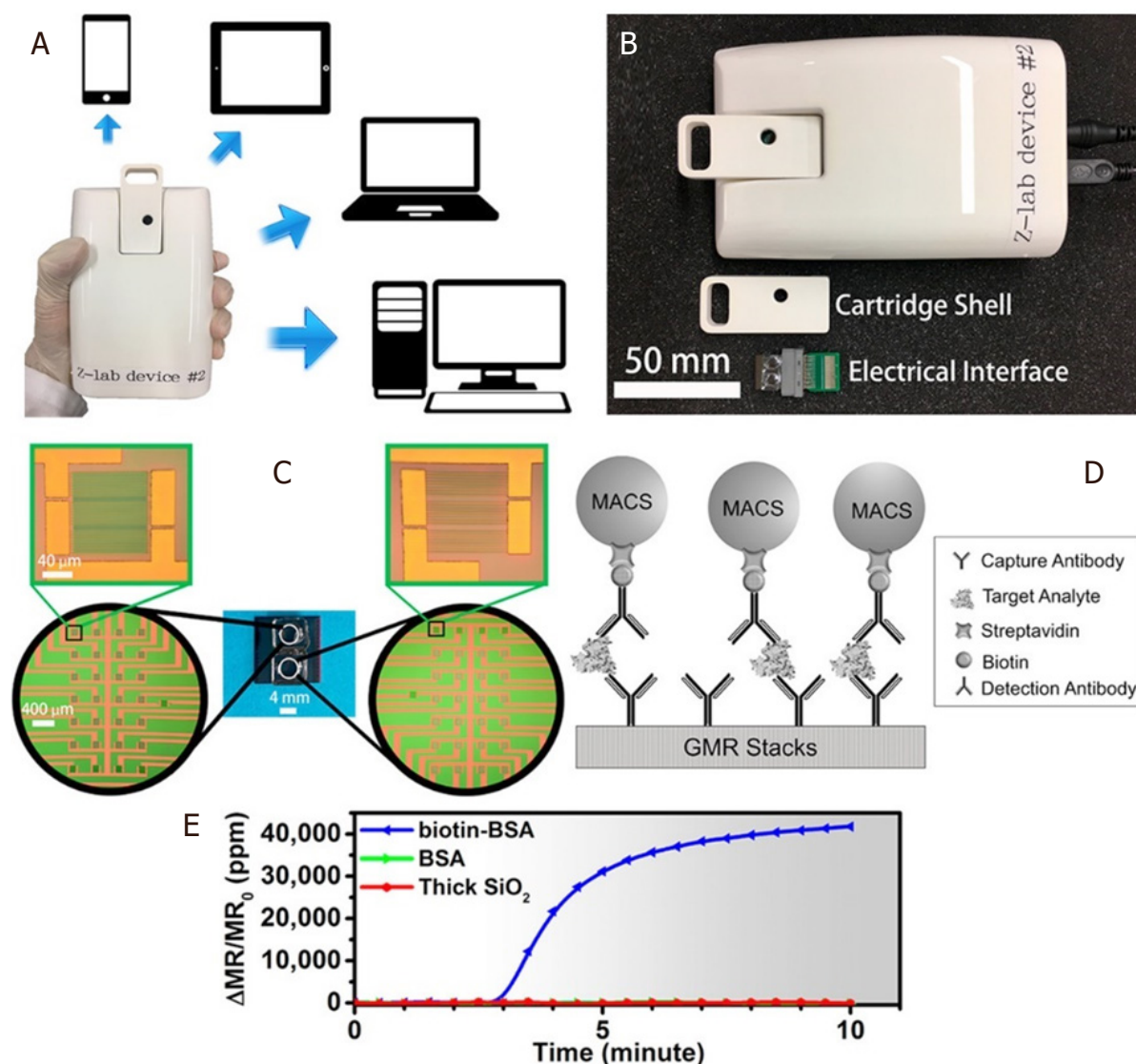


Figure 5. The Z-lab device can be connected to multiple types of devices such as cell phones, tablets, laptops, and desktop computers (A); the Z-Lab platform consists of three parts: a plastic cartridge, an electrical interface connecting the electrodes from GMR chip to the circuit board, and a handheld device (B); the layout of the GMR sensors (C); schematic illustration of the magnetic immunoassays on the sensor surface (D); real-time binding curve readout from Z-lab device (E)^[50] (reprinted with permission from American Chemical Society). GMR: giant magnetoresistance

of the GMR-CMOS interface in addition to noise removal circuit for such handheld device, thereby immensely contributing to the sensitivity of the handheld device^[79,80].

NMR

Origin of NMR signal

Elements that contain an odd number of protons and/or neutrons, such as ^1H , ^{15}N , ^{17}O , ^{31}P , etc., show intrinsic nonzero nuclear spins that give rise to small magnetic moments which can be observed by the NMR spectroscopy. Under a strong constant magnetic field B_0 , the magnetic moments are in equilibrium states, i.e., aligned either with or against the field denoted as M_0 in Figure 6A^[81,82]. A perturbation of the nuclear spins from equilibrium will occur when a weak oscillating magnetic field [referred to as radio frequency (rf) pulse] is applied whose frequency closely matches the nuclei's intrinsic Larmor frequency.

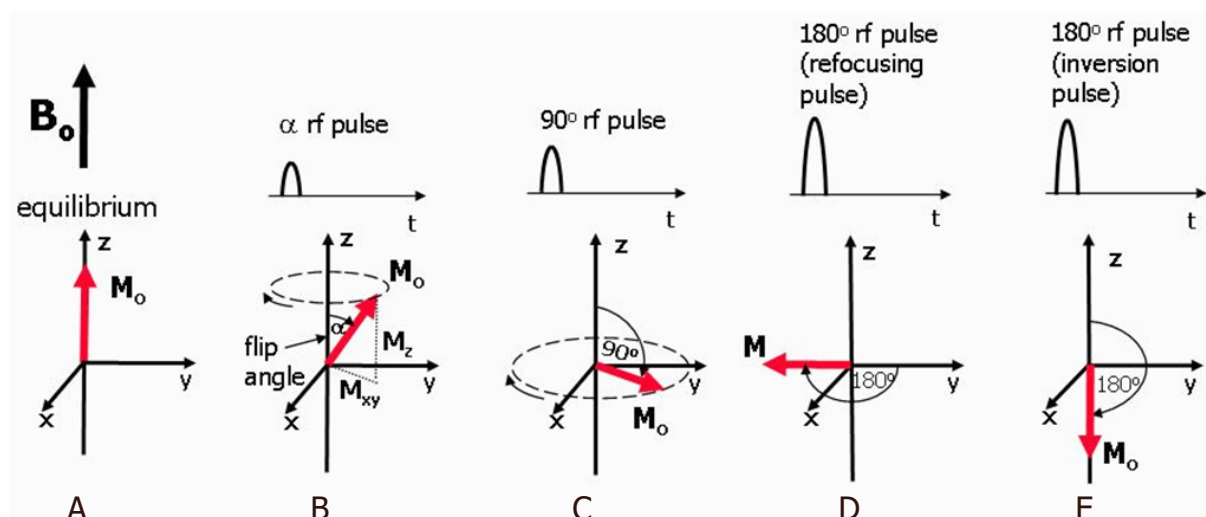


Figure 6. Equilibrium state. M_0 is aligned along the z-axis (A); when a rf pulse is applied, M_0 makes an angle with the z-axis and rotates around the axis in the direction of the curved arrow. The magnetization can be split into two components, M_z and M_{xy} . The rotating M_{xy} component generates the detectable NMR signal (B); the maximum detectable signal amplitude after a single rf pulse occurs when M_0 lies entirely in the plane of the x- and y-axes as this gives the largest M_{xy} component. This pulse has a 90° flip angle and is referred to as a 90° rf pulse or saturation pulse (C); a 180° rf refocusing pulse is usually applied while there is transverse magnetization already rotating in the xy-plane and is used to instantaneously flip the transverse component of magnetization through 180° about an axis also rotating in the xy-plane (D); a 180° inversion pulse is usually applied at equilibrium and is used to rotate the net magnetization through 180° from the positive to the negative z-axis. This is also known as a magnetization preparation pulse (E)^[81]

The Larmor frequency is proportional to the strength of the magnetic field and is also known as the resonant frequency as protons only absorb energy at this characteristic frequency. The time-domain signal responses by the total magnetization of nuclear spins are analyzed in NMR spectroscopy. As shown in Figure 6B, the net magnetization M_0 is flipped by an angle of α , and it can be split into two components: M_x (longitudinal component) and M_{xy} (transverse component). Figure 6C shows a 90° rf pulse that delivers energy to rotate the M_0 through 90° . This 90° rf pulse produces the largest possible transverse magnetization and NMR signal. The 180° rf pulses depicted in Figure 6D and E are used to prepare the net magnetization M_0 before the application of an excitation pulse.

NMR metrics: T1, T2, and T2*

Immediately after the rf pulse, the net magnetization relaxes back to its original state, along the z-axis. This relaxation process can be split into two distinct relaxation processes that relate to M_z and M_{xy} . The longitudinal relaxation, also known as T1 relaxation, is the recovery of z-component M_x along the z-axis to its original value at equilibrium. The transverse relaxation, also known as T2 relaxation, is the decay of xy-component M_{xy} . Figure 7 shows the longitudinal relaxation process as well as the T1 time constants after a 90° rf pulse^[82]. T1 is the time at which the M_x component has recovered to 63% of its value at equilibrium.

For the transverse magnetization components, the phase angles spread out, there is a loss of coherence, and the magnetic moments are no longer together, as shown in Figure 8. The NMR signal detected from receiver coil is seen as an oscillating magnetic field that gradually decays. T2 is the time at which the magnetization has decayed to 37% of its initial value, which is caused by the interactions between neighboring proton spins. The local magnetic field non-uniformities cause the dephasing and result in varied Larmor frequencies for protons at different locations. This local field inhomogeneity results in the observed actual decay and T2* time constant. In the following sections, we review MNPs as contrast agents in NMR-based applications, where the MNPs generate local dipolar fields that cause local field inhomogeneity and, thus, tune the T2* time.

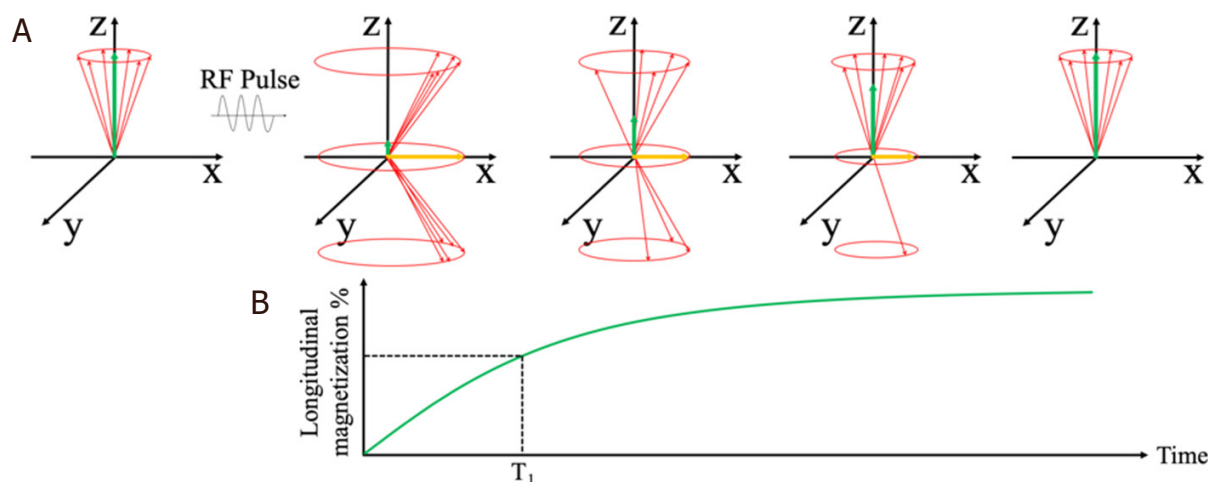


Figure 7. Schematic view of longitudinal magnetization components after the application of a 90° rf pulse. The longitudinal component M_z recovers to equilibrium value through an exponential process (A); Longitudinal relaxation time T_1 at which the M_z recovers to 63% of its equilibrium value (B)^[82] (reprinted with permission from IOP Publishing, Ltd)

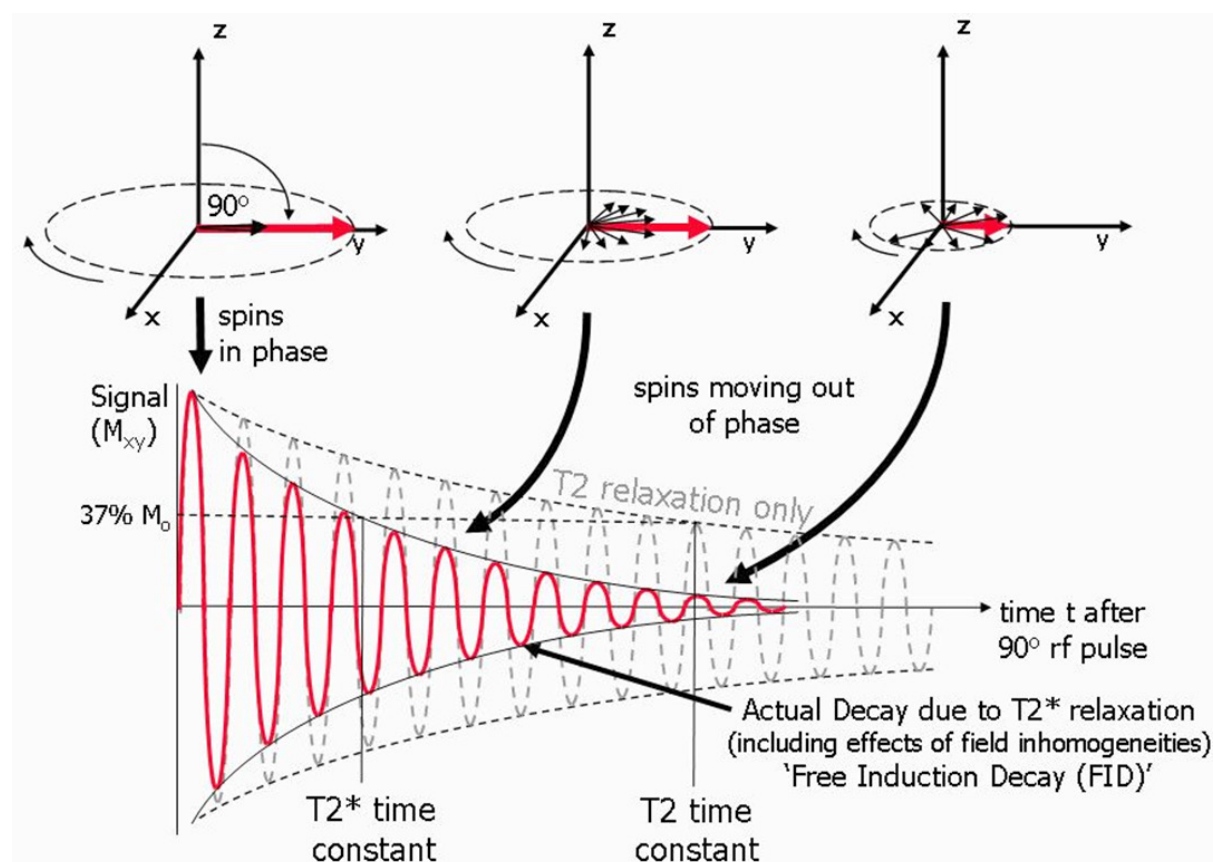


Figure 8. Schematic view of transverse magnetization components after the application of a 90° rf pulse. The transverse component M_{xy} decays to 37% of its initial value. The observed T_2^* relaxation time as a result of local field non-uniformities^[81]

MNPs as contrast agents in NMR-based applications

In NMR-based applications, T_1 , T_2 , and T_2^* are used as metrics. Nowadays, NMR has been widely used for determining the structures of organic compounds and biomolecules as well as in magnetic resonance

imaging (MRI)^[83-87]. Since the biological samples show negligible magnetic susceptibility, MNPs are introduced as contrast agents for *in vitro* detection of biomolecules and cells based on NMR in recent years^[88-91]. For most NMR-based applications, the primary NMR signals come from hydrogen nuclei contained within water or lipid molecules, due to: (1) intrinsic nonzero nuclear spins; and (2) natural abundance in the form of water and lipid molecules.

The foregoing sections describe MNP-based bioassays using technologies such as magnetoresistive and Hall sensors that directly measure the magnetic fields from MNP-labeled biological compounds. However, for MNP enhanced NMR bioassays, NMR spectroscopy exploits MNPs as proximity sensors that modulate the spin-spin relaxation time of water molecules adjacent to the MNPs, where the MNPs generate local dipolar fields that modulate the proton relaxation rates of billions of neighboring water molecules; thus, the analytical signals are directly generated from the whole sample volume. These MNPs are contrast agents in NMR to improve sensitivity in turbid samples with reduced sample preparation. In this section, we review the NMR-based diagnostics using MNPs for a wide range of applications including DNA, RNA, proteins, small molecules, tumor cells, etc.

NMR-based cancer diagnostics

MNPs along with NMR has been applied for many *in vitro* liquid biopsies including the detection of *Listeria monocytogenes*, *Salmonella*, and *Cronobacter sakazakii* from food^[92,93] as well as the microcystin-LR (MC-LR) from water^[94]. In this section, we review the applications in the area of cancer detection.

Lee's group reported a DMR (diagnostic magnetic resonance) system based on NMR technology and MNP contrast agents. Conceptually, the DMR system consists of a microNMR (μ NMR) chip containing microcoils, a microfluidic network for sample handling, on-board NMR electronics, and a small permanent magnet, as shown in Figure 9B-D. The microfluidic network in Figure 9C can effectively mix MNPs and biofluid sample (thus speeding up the specific binding to target analytes) by generating chaotic advection through the meandering channels. The NMR circuits in Figure 9E are designed to perform T₂ and T₁ measurements. The detection of MNP-labeled tumor cells is achieved by exploiting the T₂-shortening effect of MNPs in NMR measurements, where the MNPs generate local dipolar fields with strong spatial dependence to destroy the coherence in the T₂ relaxation of neighboring water protons. These MNP-labeled cells consequently show faster decay of NMR signal, or shorter T₂ relaxation time than non-targeted cells, as shown in Figure 9A.

Using this DMR system, Ghazani et al.^[95] reported the detection of circulating tumor cells (CTCs) directly from whole blood sample without primary purification. The detection is achieved by the combined and simultaneous sensing of four cancer biomarkers: EpCAM, HER-2, EGFR, and MUC-1. The monoclonal antibodies against EpCAM, HER-2, EGFR, and MUC-1 are conjugated with (E)-cyclooct-4-enyl 2,5-dioxopyrrolidin-1-yl carbonate (TCO-NHS), while the MNPs are tetrazine (Tz) modified. Each whole blood sample (7 mL) is lysed and cell pellets resuspended in PBS buffer. Then, TCO-modified antibodies are added and incubated for 15 min. Samples are washed twice and tetrazine-modified MNPs are added and incubated for 15 min. After another two cycles of washing steps, the mixture is suspended in 20 μ L of PBS for μ NMR measurements. The total labeling and incubation procedure takes approximately 30 min, as shown in Figure 10A. They reported the NMR measurements on 58 patients with confirmed epithelial malignancies. As shown in Figure 10B, these blood samples from 58 patients were profiled for four cancer biomarkers: EpCAM, MUC-1, HER-2, and EGFR. Although a notable fraction of the samples shows negative results for each biomarker, the combined biomarker analysis is able to correctly identify 99.2% of samples as malignant. As a comparison, Khosravi et al.^[96,97] reported a nanotube-antibody micro-array chip for the detection of breast cancer cells from 0.85 mL spiked blood. A ~90% sensitivity and a 90% specificity in capture of 1000 SKBR3 breast cancer cells in blood using anti-Her2-functionalized devices are achieved.

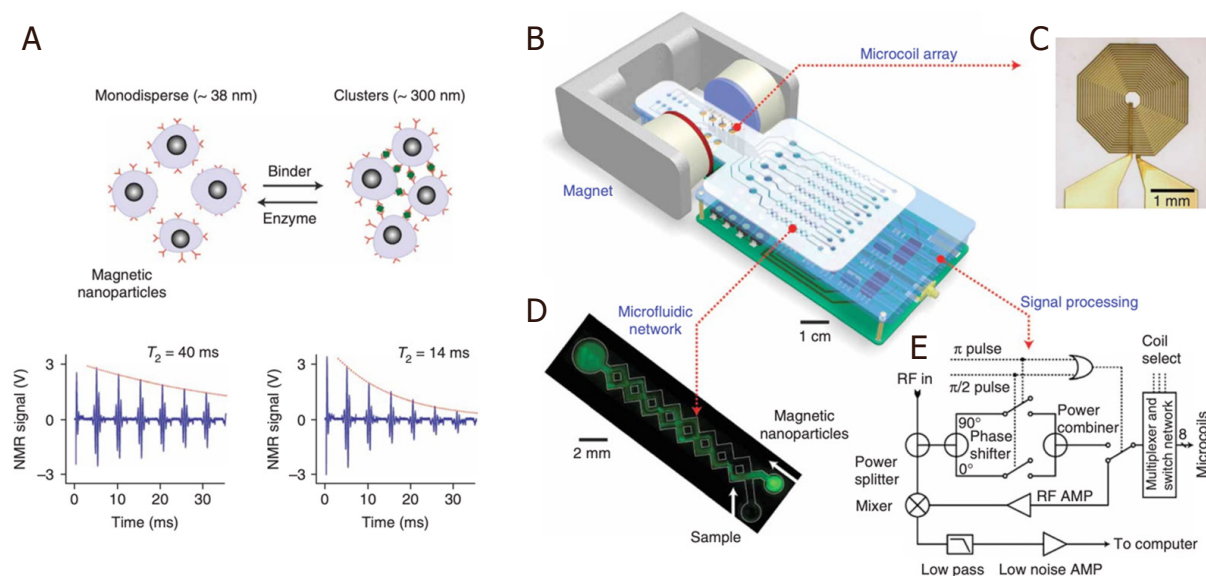


Figure 9. Principle of MNP-enhanced NMR bioassay. When monodispersed MNPs cluster upon binding to targets, the self-assembled clusters become more efficient at dephasing nuclear spins of many surrounding water protons, leading to a decrease in T_2 relaxation time. The bottom panel shows an example of the proximity assay measured by the DMR system. Avidin was added to a solution of biotinylated magnetic nanoparticles, causing T_2 to decrease from 40 to 14 ms (A); schematic diagram of the DMR system (B); the microcoil generates rf pulses (C); the microfluidic network (D); schematic of the NMR electronics (E)^[89] (reprinted with permission from Springer Nature)

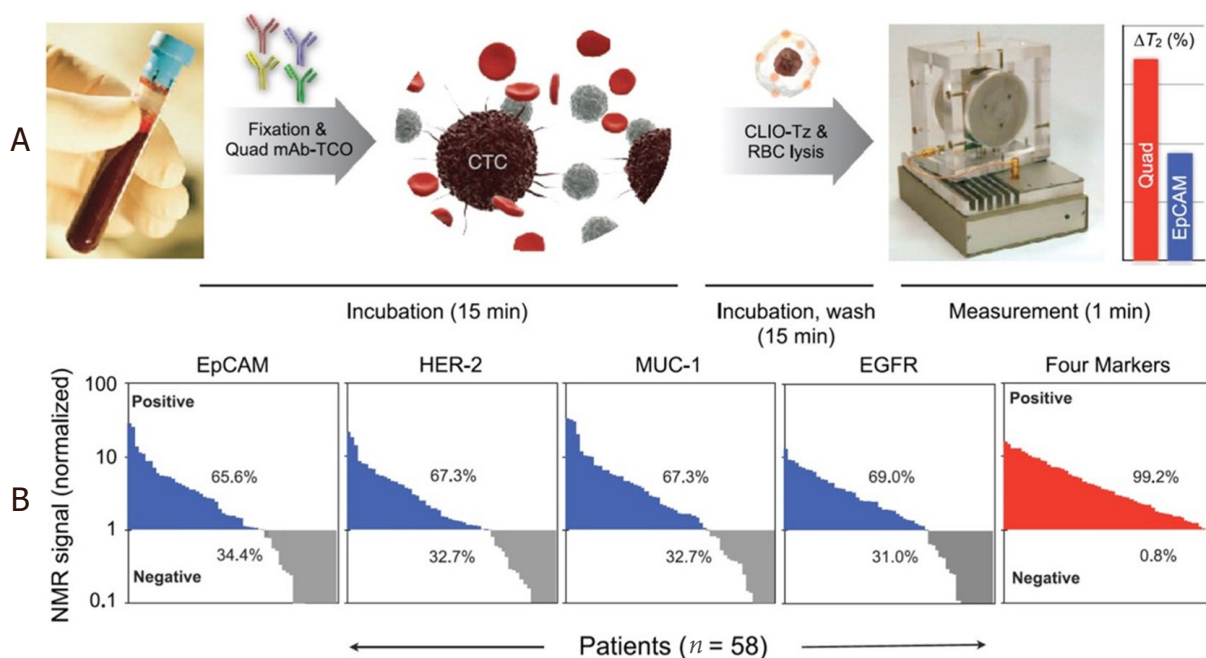


Figure 10. Schematic of the μ NMR system. TCO-labeled antibodies are added to whole blood. Red blood cells are then lysed and the cells spun down before reaction with tetrazine-modified MNPs. The process of labeling antibodies and targeting nanoparticles requires less than 30 min. Biomarker measurements are then taken using the μ NMR device (A); Biomarker expression, as assessed by μ NMR, showed that significant percentages of cancer patients ($n = 58$) were negative for EpCAM (34.4%), HER-2 (32.7%), MUC-1 (32.7%), and EGFR (31.0%). Combining these markers, however, enabled identification of nearly all cancer patients (99.2%) (B)^[95]. Reprinted with permission from Elsevier. μ NMR: micro nuclear magnetic resonance; EpCAM: epithelial cell adhesion molecule; HER-2: human epidermal growth factor receptor 2; MUC-1: mucin 1; EGFR: epidermal growth factor receptor

Later, in the same group, Loeian *et al.*^[6] reported applying the nanotube chip for capturing CTCs from peripheral blood samples of breast cancer patients (stages 1-4). The CTCs (based on CK8/18, HER2, and EGFR) were successfully captured from 7/7 breast cancer patient samples and no CTCs were captured from healthy controls ($n = 2$). Salahandish *et al.*^[98] reported an electrochemical biosensor for detecting as low as 2 cells/mL SK-BR3 breast cancer cells with a fast response time of 30 min. This electrochemical biosensor demonstrated an efficiency of > 90% for capturing cancer cells in whole blood sample without sample preparation and cell staining. Other platforms that detect CTCs from non-clinical samples have also been reported but are not covered in this review. For example, Tian *et al.*^[99] reported an enzyme-free ELISA for HER2 detection from serum samples utilizing copper oxide nanoparticles as signal amplification probes.

Haun *et al.*^[100] clinically tested on suspected lesions in 50 patients and validated in an independent cohort of another 20 patients using this DMR system. For each patient, a one- to two-pass fine-needle aspirate from a suspected abdominal malignancy was obtained, followed by a series of routine core biopsies for conventional standard-of-care analysis. Each fine-needle aspirate sample was washed with 1-2 mL of buffered saline and processed for μ NMR measurements of 11 markers. These markers included nine well-established cancer-related markers (EpCAM (epithelial cell adhesion molecule), MUC-1 (mucin 1, cell surface associated), HER2, EGFR (epidermal growth factor receptor), B7-H3, CK18, Ki-67, p53, and vimentin), a count of CD45-positive cells, and total cell density. Their results show the μ NMR-based measurements are comparable with the accepted gold standards such as enzyme-linked immunosorbent assay (ELISA), fluorescence-activated cell sorting (FACS), and immunohistochemistry (IHC). The correlation coefficients between μ NMR and ELISA, FACS, and IHC measurements for expression of EGFR are 0.99, 0.98, and 0.93, respectively. It was shown that EGFR and HER2 had a good correlation (coefficient = 0.6), whereas EpCAM and HER2 had a poor correlation (coefficient = 0.1). The comparison of different detection technologies for liquid biopsy is shown in Table 1.

CONCLUSION

Liquid biopsy is an emerging research field with great promise in serving as a noninvasive technology for cancer diagnosis and cancer therapy. Magnetic nanotechnologies play an import role in both biomarker separation and biomarker detection. MNPs can bound to the target biomarkers through immunoassays and facilitate the separation of the biomarkers through the magnetophoresis effect. MNPs with high magnetic moment have been synthesized together with biocompatible surface coatings that can accommodate the bounding between MNPs and target biomarkers. Besides synthesizing novel MNPs, the research on magnetic separation also focuses on the optimization of the magnetic field configuration as well as the integration with other biomarker concentration technologies to achieve high biomarker capture efficiency, high specificity, and ease of integration with biosensing platforms. To realize precise fluidic control, minimum biological sample consumption, and better performance in biomarker separation and biosensing, microfluidic channels have become a key part in the designing of liquid biopsy platforms. Apart from the traditional mold casting techniques, novel approaches such as 3D printing and laser writing have been proposed to fabricate microfluidic channels with more complexed structures and higher resolutions.

Biosensors based on magnetic nanotechnologies exhibit low background noise and are less influenced by the biological and chemical environment during detection, since most of the biological samples are paramagnetic. Magnetoresistance sensors including GMR and TMR sensors detect the signal from the MNPs that are brought into proximity of the sensor surface via immunoassays or DNA-based assays. Various biomarkers have been demonstrated for liquid biopsy applications. Point-of-care devices based on MR sensors have been developed by several groups, which could pave the way for bedside liquid biopsies with high sensitivity, high portability, and short detection time. In addition to MR sensors, NMR bioassay platforms along with MNP contrast agents exploit the magnetic resonance technology for the detection of cancer cells. Samples containing MNP-labeled cells show faster relaxation in NMR signals due to the

Table 1. Comparison of different platforms for liquid biopsies

Technology	Target analytes	Substrate	Sensitivity	Assay time	Ref.
GMR	CA125 II, HE4, IL6	PBS	3.7 U/mL, 7.4 pg/mL, 7.4 pg/mL	15 min	[61]
GMR	Endoglin	Urine	83 fM	10 min	[63]
GMR	hcG	Serum	1 pM	3 min	[64]
GMR	exosome	PBS	100 counts	30 min	[65]
GMR	Methylated DNA	Denaturation buffer	0.1%	30 min	[68]
GMR	cfDNA(AU115, AU247)	NA	pM range	80 min	[70]
TMR	AFP	PBS	2 µg/mL	NA	[66]
TMR	Commercial ssDNA	PBS	5 nmol/L	NA	[67]
NMR	Circulating tumor cells (CTCs)	Whole blood	~3 individual CTCs per sample (1-10 mL blood)	30 min	[95]
NMR	Nine cancer-related markers: EpCAM, MUC-1 (cell surface associated), HER2, EGFR, B7-H3, CK18, Ki-67, p53, and vimentin	Whole blood	See Table 2 ^[95]	60 min	[100]

GMR: giant magnetoresistance; TMR: tunneling magnetoresistance; NMR: nuclear magnetic resonance; EpCAM: epithelial cell adhesion molecule; MUC-1: mucin 1; EGFR: epidermal growth factor receptor

local magnetic field inhomogeneity caused by MNPs. This type of NMR signal detection is based on magnetic interactions (interactions between neighboring proton spins) and, thus, requires minimal sample purification steps, which, as a result, reduces cells loss and simplifies the assay procedures. In the past few years, the NMR platform has been considerably advanced and developed into a sensitive and robust detection tool for a wide range of biomarker/cell detections. Magnetic nanotechnology, with its application in both biomarker separation and detection, has demonstrated great potential for the development of liquid biopsy platforms to achieve sensitive, efficient, and portable cancer diagnosis and cancer therapy.

DECLARATIONS

Authors' contributions

Abstract, sections 1, 2.2, 3.1, 4.1, 4.2, 6 was contributed by Su D.

Section 5 was contributed by Wu K.

Sections 3.2 and 4.3 was contributed by Saha R.

Section 2.1 was contributed by Liu J.

Supervised the completion of this review: Wang JP

Availability of data and materials

Not applicable.

Financial support and sponsorship

This study was financially supported by the Institute of Engineering in Medicine of the University of Minnesota through FY18 IEM Seed Grant Funding Program and the Doctoral Dissertation Fellowship.

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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Commentary

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Could zinc dipicolinate be used to “smuggle” zinc into prostate cancer cells?

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How to cite this article: McCarty MF, Iloki-Assanga S, Lujan LL. Could zinc dipicolinate be used to “smuggle” zinc into prostate cancer cells? *J Cancer Metastasis Treat* 2020;6:20. <http://dx.doi.org/10.20517/2394-4722.2020.47>

Received: 17 May 2020 **First Decision:** 24 Jun 2020 **Revised:** 24 Jun 2020 **Accepted:** 6 Jul 2020 **Published:** 19 Jul 2020

Academic Editor: Rafat A. Siddiqui **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Although prostate epithelium concentrates zinc for the purpose of promoting citrate secretion, it loses its capacity to import zinc while undergoing malignant transformation. This exclusion of zinc may be necessary for the viability of prostate cancer, as measures which increase the intracellular zinc content of prostate cancers lead to cell death, oxidative stress, and a marked reduction in ATP, suggestive of mitochondrial damage. The anti-fungal drug clioquinol, which can act as a zinc ionophore, can markedly slow the growth of human prostate cancer in nude mice, and has been proposed as a clinical therapy for prostate cancer. However, clioquinol is currently only available as a topical agent, as it was linked to subacute myelo-optic neuropathy with oral use. A more practical option for promoting zinc transport may be offered by the nutraceutical zinc dipicolinate, a stable chelate in which four coordination positions of zinc are occupied by two molecules of the tryptophan metabolite picolinic acid. Zinc dipicolinate is a highly effective supplemental source of zinc that has been shown to be more potent than soluble zinc salts for alleviating the symptoms of acrodermatitis enteropathica, a genetic zinc deficiency disorder reflecting homozygous loss of functional ZIP4 zinc importers in enterocytes. This suggests that the zinc dipicolinate complex is sufficiently stable and lipophilic to transfer zinc across cellular membranes. If so, it may have potential for “smuggling” zinc into prostate cancer cells. Hence, cell culture and rodent studies to evaluate the impact of zinc dipicolinate on human prostate cancer are warranted.

Keywords: Prostate cancer, zinc, clioquinol, picolinic acid, ZIP4



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VIABILITY OF PROSTATE CANCER MAY REQUIRE EXCLUSION OF ZINC

Prostate epithelium is characterized by high intracellular levels of zinc, particularly within the mitochondria^[1]. This intra-mitochondrial zinc is believed to promote the proper function of prostate epithelium by inhibiting aconitase activity, thereby causing an accumulation of citrate in the Krebs cycle^[2,3]. Much of this citrate is exported into the seminal fluid, where it serves as an energy substrate for spermatozoa.

However, malignantly transformed prostate epithelium is far lower in intracellular zinc, reflected greatly diminished expression or activity of transporter proteins - ZIP1, ZIP2, and ZIP3 - that import zinc^[4-8]. This loss of intracellular zinc appears to be essential to the viability of the transformed cells, as measures which restore high intracellular zinc levels - exposure to high extracellular zinc, or treatment with zinc ionophores such as pyrithione or clioquinol - slows their proliferation and up-regulates cell death^[8-11]. *In vivo*, continual intravenous infusion of zinc, injection of zinc acetate directly into tumors, or parenteral administration of the zinc ionophore clioquinol has notably slowed the growth of human prostate cancers in nude mice^[12-14]. In particular, administration of clioquinol was associated with an 85% growth retardation of a ZIP-1-deficient human prostate cancer^[14].

In a range of human prostate cancer cells lines, increasing intracellular zinc with zinc pyrithione led to necrotic cell death associated with plummeting ATP levels, oxidative stress, and activation of ERK and PKC^[10]. The antioxidants N-acetylcysteine (NAC) and trolox protected against cell death in this system; NAC, but not trolox, likewise blunted the decline in ATP. Since prostate epithelium tends to concentrate zinc in mitochondria, it would be of interest to know whether excessive zinc uptake by mitochondria mediates the oxidative stress and reduction in ATP seen after prostate cancer cells are exposed to zinc pyrithione. In addition to inhibiting aconitase activity, zinc is also capable of inhibiting complex III of the respiratory chain, with a K_i of about 100 nmol/L^[15-18].

Could malignant transformation of prostate epithelium somehow sensitize their mitochondria to the toxic impact of excessive zinc? The mitochondria of cancer cells are prone to structural abnormalities - possibly reflecting mutations in mitochondrial or nuclear DNA - which increase their propensity to produce superoxide^[19,20]. Defects of the mitochondrial respiratory chain or of ATP synthase activity that moderately boost mitochondrial superoxide generation can be expected to promote cellular proliferation, angiogenesis, and mutagenesis; hence, they may act as tumor promoters, in which case these defects would be selected for^[20-23]. The exceptionally high mitochondrial zinc levels of prostate epithelium presumably reflect increased expression or activity not only of ZIP1, but also of one or more zinc transporters -possibly ZnT2 - which import zinc into the mitochondrial inner matrix^[24]. In mammary epithelial cells, ZnT2 transports zinc into mitochondria, and over-expression of this protein lowers cellular ATP levels and oxygen consumption, and promotes apoptosis; oxidant production was not measured in this study^[24].

If this increased intramitochondrial transport of zinc is maintained in transformed prostatic epithelial cells, then high mitochondrial zinc levels might interact with the mitochondrial abnormalities typical of cancer to induce severe dysfunction: excessive production of superoxide, decreased production of ATP, and further mitochondrial structural damage. This sequence of events could evidently be prevented by down-regulation of ZIP1 - which is what in fact is observed in transformed prostate epithelium.

In light of the utility of parenteral clioquinol for controlling growth of a prostate cancer in nude mice, it has been suggested that oral clioquinol could have potential as a therapeutic alternative for prostate cancer control. While it might indeed be the case that some sufficiently modest dose of clioquinol could prove useful in this regard, past clinical experience with oral administration of clioquinol as a fungicide or as a treatment for acrodermatitis enteropathica has been complicated by its association with subacute myelo-

optic neuropathy, characterized by peripheral neuropathy and blindness^[25,26]. Ten thousand patients in Japan were afflicted with this syndrome until oral use of clioquinol was discontinued in Japan. Hence, clioquinol is now available solely for topical use. The zinc-clioquinol chelate has been shown to lead to rapid mitochondrial damage and loss of mitochondrial membrane potential in a melanoma-derived cell line, possibly explaining the clinical toxicity of clioquinol^[27].

ZINC DIPICOLINATE MAY ACT AS A ZINC TRANSPORTER

However, an alternative strategy for boosting the intracellular zinc levels of clinical prostate cancer may be at hand. Zinc dipicolinate is a readily-available nutraceutical, originally patented by the U.S. Department of Agriculture, in which zinc is chelated by two molecules of the natural tryptophan metabolite picolinic acid; 4 coordination positions of zinc are occupied by picolinic acid in this complex. There is reason to suspect that, at least at neutral pH, zinc dipicolinate is sufficiently stable to carry zinc across bilipid layers. When children with acrodermatitis enteropathica (AE) were treated with either zinc dipicolinate or zinc sulfate, the dose of zinc required to prevent exacerbations of this disorder was found to be one-third as high with zinc dipicolinate, as opposed to zinc sulfate^[28]. AE is a hereditary zinc deficiency syndrome in which those afflicted are heterozygous for loss of function of ZIP4, the chief zinc importer expressed by the apical membranes of enterocytes^[29,30]. The superior utility of zinc dipicolinate in this syndrome, as opposed to forms of zinc that ionize readily (such as zinc sulfate), seems likely to reflect the ability of the zinc dipicolinate chelate to carry zinc across enterocyte membranes in the absence of zinc transporter proteins. Furthermore, in healthy human subjects, when zinc was administered at 50 mg daily as either zinc dipicolinate, zinc citrate, or zinc gluconate, zinc dipicolinate was shown to have a significantly greater impact on zinc levels in erythrocytes, hair, and urine^[31]. When nursing rat mothers were fed zinc as either dipicolinate or acetate, the zinc content of the kidney or liver of nursing pups was higher after the dipicolinate supplement^[32].

If zinc dipicolinate is sufficiently stable and lipophilic to “smuggle” zinc into enterocytes lacking ZIP4, might it not also be able transport zinc into prostate cancer cells lacking ZIP1 activity? This possibility could be readily tested in prostate cancer cell cultures and, if preliminary results are promising, in nude mice xenografted with human prostate cancer. The possibility that zinc dipicolinate supplementation might also have potential for prevention of prostate cancer might also be envisioned, as reduction in intracellular zinc is believed to arise at an early stage of prostate cancer evolution^[33].

While therapies which boost intracellular zinc in prostate cancer might at best be expected to slow prostate cancer progression, the fact that such therapy might boost oxidative stress and lower ATP levels in prostate cancer cells raises the possibility that preceding zinc therapy might render prostate cancer more sensitive to hyperthermia and/or high-dose intravenous ascorbate^[34]. The selective susceptibility of cancer cells to high extracellular levels of ascorbate - which generate a high flux of hydrogen peroxide into the these cells - may reflect increased cancer production of superoxide, which can interact with hydrogen peroxide in a transition metal-catalyzed reaction to generate deadly hydroxyl radicals^[34,35]. And the lethality of whole body-tolerable hyperthermia (42 °C) to cancer cells may be potentiated by hydrogen peroxide; conversely, overexpression of mitochondrial superoxide dismutase protects a prostate cancer cell line from 43 °C hyperthermia^[36-39]. Mitochondrial superoxide production by zinc-treated cancer cells might be potentiated by concurrent treatment with dichloroacetate, which can increase the availability of pyruvate to mitochondria by inhibiting pyruvate dehydrogenase kinase; the latter is highly active in many cancers owing to up-regulated hypoxia-inducible factor-1 activity^[34,40,41].

DECLARATIONS

Authors' contributions

Conceived and wrote the first draft: McCarty MF

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Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there is no conflict of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Mouse tumor susceptibility genes identify drug combinations for multiple myeloma

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How to cite this article: Zhang S, DuBois W, Zhang K, Simmons JK, Hughitt VK, Gorjifard S, Gaikwad S, Peat TJ, Mock BA. Mouse tumor susceptibility genes identify drug combinations for multiple myeloma. *J Cancer Metastasis Treat* 2020;6:21. <http://dx.doi.org/10.20517/2394-4722.2020.40>

Received: 5 Mar 2020 **First Decision:** 17 Jun 2020 **Revised:** 3 July 2020 **Accepted:** 7 Jul 2020 **Published:** 26 Jul 2020

Academic Editor: Ciro Isidoro **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Long-term genetic studies utilizing backcross and congenic strain analyses coupled with positional cloning strategies and functional studies identified *Cdkn2a*, *Mtor*, and *Mndal* as mouse plasmacytoma susceptibility/resistance genes. Tumor incidence data in congenic strains carrying the resistance alleles of *Cdkn2a* and *Mtor* led us to hypothesize that drug combinations affecting these pathways are likely to have an additive, if not synergistic effect in inhibiting tumor cell growth. Traditional and novel systems-level genomic approaches were used to assess combination activity, disease specificity, and clinical potential of a drug combination involving rapamycin/everolimus, an *Mtor* inhibitor, with entinostat, an histone deacetylase inhibitor. The combination synergistically repressed oncogenic *MYC* and activated the *Cdkn2a* tumor suppressor. The identification of *MYC* as a primary upstream regulator led to the identification of small molecule binders of the G-quadruplex structure that forms in the NHEIII region of the *MYC* promoter. These studies highlight the importance of identifying drug combinations which simultaneously upregulate tumor suppressors and downregulate oncogenes.

Keywords: Complex genetic trait, plasma cell tumor, multiple myeloma, entinostat, rapamycin, drug combinations, *Cdkn2a*, *Mtor*, *Mndal*, *MYC*



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INTRODUCTION

The majority of human cancers arise in response to exposure to environmental factors and carcinogenic agents that may lead to somatic mutations. Signatures of these mutational processes are often evident in the sequences of cancer genomes^[1]. Genetic and epigenetic factors also play an important role in determining which exposed individuals will develop tumors. Most tumor susceptibility models in humans and experimental animals have focused on the inherited abnormality of a single gene such as germline mutations of *Rb* or *p53*. These particular single locus lesions are predisposed to tumor formation because they harbor strong “altered function” alleles. However, it is estimated that such strong germline alleles may only account for approximately 2%-14% of human cancers which implies that another paradigm is required to explain the other 86%-98%^[2]. The individuals in whom these latter cancers arise must either lack a germline genetic component, or tumor development in these individuals represents an inherited trait that may depend on several genes or epigenetic modifiers, in concert with environmental stressors, thus presenting cancer as a complex genetic trait.

Genome wide association studies of cancer development provide a systematic approach to identifying genes that may influence cancer risk^[3]. Genome-wide linkage studies in genetically uniform strains of mice can provide a window into the more complex genetics associated with human cancers and may be used to model certain patient subpopulations. Thus in 1993, we chose to look at the inheritance of mouse plasmacytoma (PCT) susceptibility alleles associated with genetic variants segregating in immunocompetent backcross mice between *BALB/c* and *DBA/2* strains of mice^[4].

EXPERIMENTAL SYSTEM: IDENTIFICATION OF MOUSE TUMOR SUSCEPTIBILITY PATHWAYS TO TARGET

Human multiple myeloma (MM) is a clonal proliferative of neoplastic plasma cells in the bone marrow. Mouse plasma cell tumors model certain aspects of these antibody producing neoplasms. Plasmacytomagenesis in *BALB/cAn* mice is a complex genetic trait with 40%-60% penetrance in non-specific pathogen free mice^[5]. Through our genome-wide mapping studies utilizing genetic crosses with *DBA/2* mice (0% tumor incidence), together with the development and use of a series of *C.D2* congenic strains, coupled with representational difference analysis and positional cloning, we determined that *Cdkn2a* (*p16*), *Mtor*, and *Mndal* contribute to PCT susceptibility and resistance [Figures 1 and 2]^[4-11]. *Pctr1-2* are localized in non-contiguous, non-overlapping segments of mouse Chr 4, and *Pctm*, a modifier of PCT, on Chr 1. The two *Pctr* loci on Chr 4 are susceptibility loci in *BALB* mice while in *DBA* mice, they are resistance loci as evidenced by backcross and congenic strain analyses. The genes identified for *Pctr1* and *Pctr2* are, *Cdkn2a* (*p16*) and *Mtor*, respectively. The *BALB* alleles of both *p16* and *Mtor* encode efficiency and hypomorphic alleles whose functional activities are much less active than the respective *DBA* alleles. In contrast to *Pctr1* and *Pctr2*, the *Pctm* locus on Chr 1 encodes a resistance allele in *BALB* and a susceptibility allele in *DBA*. In fact, the candidate, *Mndal*, for the *Pctm* locus is deleted in *DBA* mice, but is present and functionally active in *BALB* mice^[12].

Compound allelic variation in both coding and promoter sequences, found in *Cdkn2a* [*p16* exon 2: G232A in ANK repeat domain, RREB cis regulatory element (CRE)] and *Mtor* (exon 11: R628C in HEAT repeat domain; MZF1 CRE)^[6,11,16,17], contribute to the complex genetics associated with PCT susceptibility in *BALB/c* mice^[4,9]. Hypomorphic activity of the promoter and coding regions of the *BALB* alleles of both *p16* and *Mtor* is associated with tumorigenesis after exposure to pristane, suggesting that both *Cdkn2a* (*p16*) and *Mtor* can act as tumor suppressors in PCT development in response to stress and in an allele-dependent manner^[11,15-17].

BALB/c mice are susceptible because they harbor several tumor susceptibility loci that act in concert to produce the susceptible phenotype [Figure 2]. We hypothesize the combination of these relatively subtle

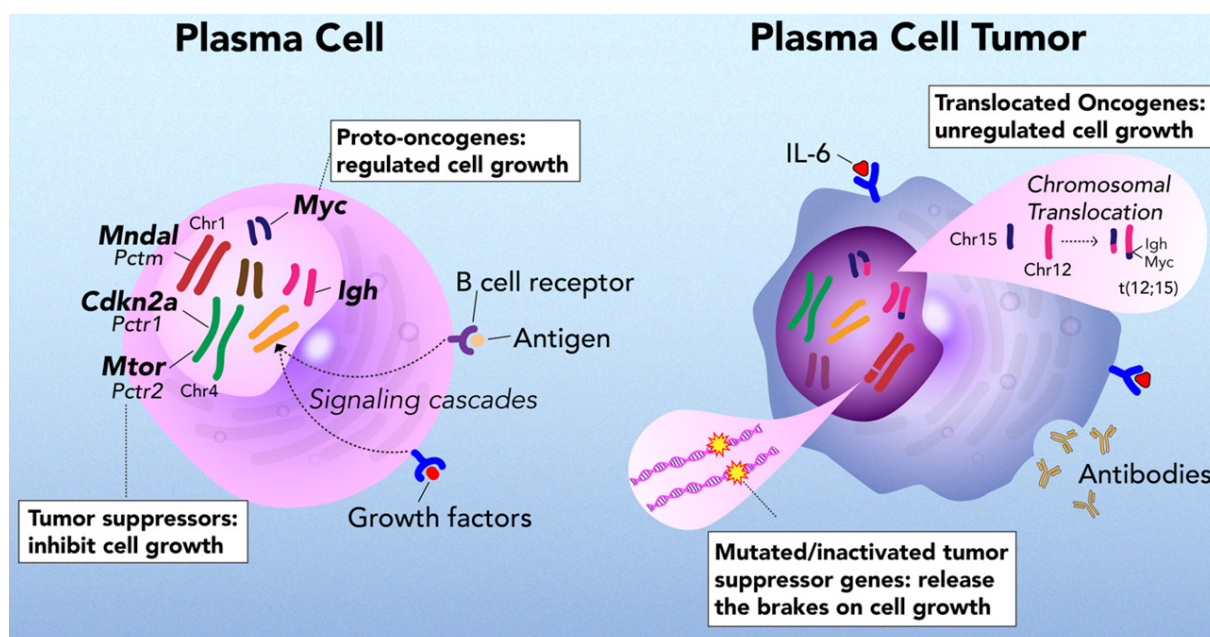


Figure 1. *MYC* regulates cell growth in plasma cells and are dysregulated by translocation in plasma cell tumors^[13,14]. During plasma cell tumor development, *Cdkn2a* (*p16*) and *Mndal* (interferon inducible gene) expression is low and *Mtor* expression is increased^[6,11,12,15]

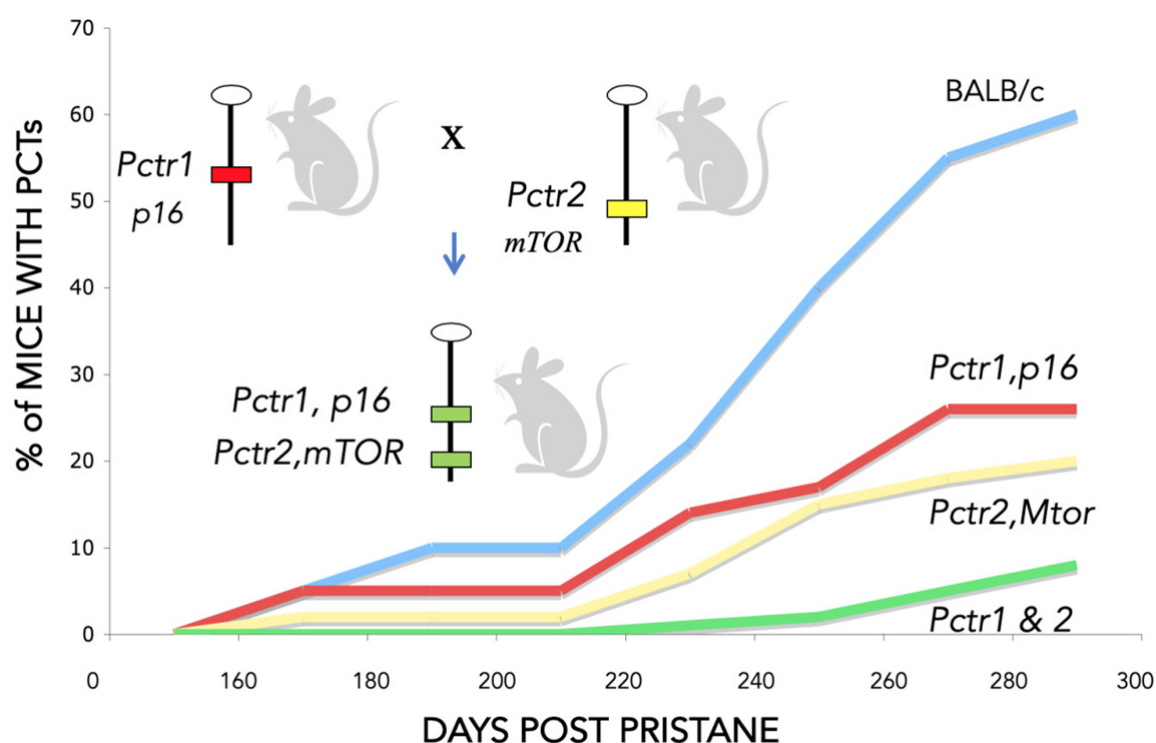


Figure 2. *BALB/c* congenic strains of mice carrying two (*p16* and *Mtor*) *DBA/2* plasmacytoma resistance alleles are more resistant to tumor formation than congenics carrying only one of the *Pctr* alleles

allelic defects tip the balance toward both uncontrolled cell growth and a lack of appropriately timed cell death and removal from the cell cycle.

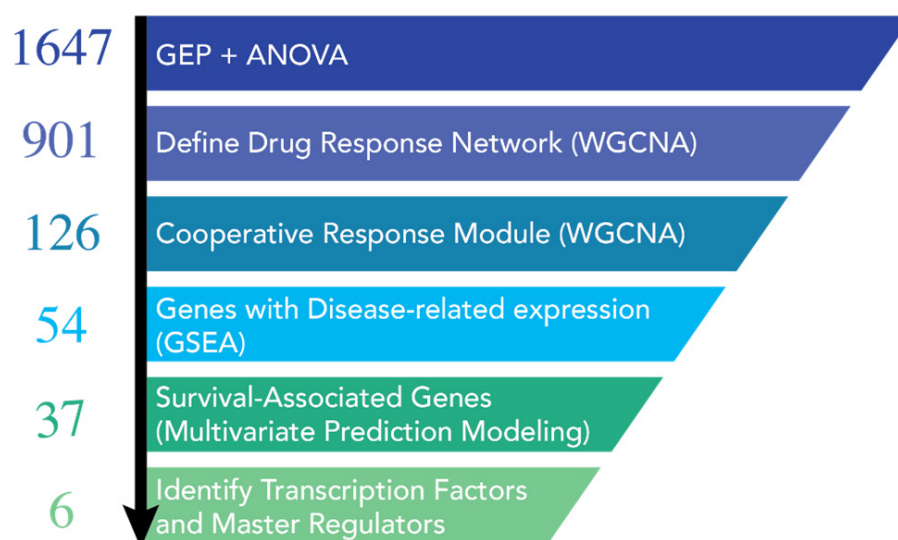


Figure 3. Graphical summary of the systems workflow used to dissect the mechanism of action for the mTOR inhibitor (mTORi) and HDAC inhibitor (HDACi) drug combination^[19]. Initial ANOVA analysis from our gene expression profiling data started with 1647 differentially expressed genes. Weighted gene co-expression network analyses determined that there were 901 genes in the entire drug response network. Of these 901 genes, 126 genes could be assigned to the drug combination network. These genes were then evaluated for enrichment in myeloma vs. normal samples from the same patient (GEO databases) and by multivariate prediction modeling to assess their association with patient survival. 37 disease-specific genes were chosen for further analyses. When the data for the 37 genes (PatentUS2014357660-A1) was evaluated by IPA, 6 master regulators, including *MYC*, *Rb*, and *Cdkn2a* were identified. ANOVA: analysis of variance; GEO: gene expression omnibus; IPA: ingenuity pathway analysis

BALB/c congenic strains of mice carrying two tumor resistance alleles (*Pctr1* and *Pctr2*) are more resistant than mice carrying only one of the resistance alleles [Figure 2]. This led us to hypothesize that drug combinations targeting these pathways are likely to have a cooperative effect in inhibiting tumor cell growth. The *p16/Rb* and *Mtor/PI3K* pathways are frequently dysregulated in both mouse plasma cell tumors and in human multiple myeloma^[18-20].

SYNERGISTIC DRUG COMBINATION PHENOCOPIES RESISTANCE ALLELES

The activity of combining *Mtor* and histone deacetylase (HDAC) inhibitors, rapamycin and entinostat respectively, chosen to target the mouse tumor susceptibility pathways (*p16/Rb* and *Mtor/PI3K*) was found to be synergistic in limiting the growth of a number of B lineage tumor cell lines, including mouse plasma cell tumors, and the human B cell neoplasms, mantle cell lymphoma, and multiple myeloma^[20]. We found that combining rapamycin and entinostat elicited responses distinct from a simple combination or the additive effects of the two drugs^[19]. As such, we developed a rational, unbiased approach to uncover mechanisms of drug synergy for this combination.

Systems approach

We evaluated the synergistic activity of combining *Mtor* and HDAC inhibitors at the organismal, cellular, and molecular levels with a cross-disciplinary “systems pharmacology” approach [Figure 3]^[19]. While the future impact of these specific *Mtor*/HDAC findings is intrinsically linked to the outcome of clinical investigations, there is broader potential for further application and development of our approach. The integration of patient datasets in the identification of a core synergistic response signature offers particular opportunities for the development of companion diagnostics to aid in the clinical development of these combinations. Gene expression-based signatures of cooperative drug responses may prove beneficial for pre-treatment stratification of patients most likely to benefit from a particular drug combination, or as an early response biomarker specific for the combination response and intrinsically linked to expression

patterns correlated with improved prognosis. Our approach in this study was enabled by the availability of high-quality, publicly available tumor gene expression datasets from large cohorts of myeloma patients that included either extensive survival annotation or comparisons of healthy vs. tumor tissues (GSE4581 and GSE6477)^[21-25]. The schema depicted below illustrates the approach that we employed to understand the mechanism of drug synergy between the HDAC inhibitor, entinostat and an *Mtor* inhibitor, everolimus^[19].

The upstream predictors identified as “activated” from the drug combination by ingenuity pathway analysis (IPA) from the 37 patient-survival associated genes included *Cdkn2a* (*p16/p19*), *p53*, and *Rb*. *MYC*, *E2F*, and *TBX2* were predicted as “inhibited” by the combination. The combination worked cooperatively to lower *MYC* protein stability, partially through FBXW7-mediated degradation^[19]. The combination also worked to increase the activity of the *Rb1/Cdkn2a* tumor suppressor pathways^[20]. The drug combination enhanced the overall survival rate of tumor-bearing *BALB-bclxl* transgenic mice and lowered *MYC* protein levels in tumors of these immunocompetent mice. Our studies in the NCI-60 cell line panel found that most tumors, regardless of their tissue of origin, responded synergistically to the mTORi/HDACi combination. In early molecular classification schemes of multiple myeloma patients based on heirarchical clustering of gene expression in myeloma samples, seven clusters were identified as proliferation (PR), low bone, multiple myeloma SET domain (MMSET), hyperdiploid, cyclin D1 (CD-1), cyclin D2 (CD-2) and avian musculoaponeurotic fibrosarcoma (MAF)^[21]. Using gene expression data from samples within these same subgroups, we determined a gene score for our 37 drug-responsive genes to predict how many patients would be expected to benefit from combination treatment. Roughly 50% in most subgroups were predicted to benefit; there were two exceptions: all patients in the PR group and only 17% in the CD-2 group were predicted to benefit from the drug combination based on their expression scores for the 37 gene signature^[19]. This is of course, hypothetical and would need to be tested in a clinical trial. In addition, the drug combination did not have a direct effect on gene expression of genes involved in determining Zhan et al.^[21]'s proliferation index. Cells with mutations in *MYC* residues required for its degradation did not respond to the drug combination^[19].

TARGETING MYC TRANSCRIPTION AND DEGRADATION

Our systems analyses led us to explore a more direct approach to targeting *MYC*. We screened a small molecule microarray library for binders of the G-quadruplex located in the NHEIII region of the *MYC* promoter, and identified a benzofuran-containing molecule, D089, that could stabilize the G4 structure and inhibit *MYC* transcription^[26]. We demonstrated that D089 inhibited *MYC* with greater affinity than other G4-containing genes (e.g., *RAS*, *VEGF*, *BCL2*, and *Rb1*). The small molecule was relatively potent in inhibiting multiple myeloma cell proliferation but was ineffective in tumor cell lines that had deleted the portion of the *MYC* promoter containing the G4 sequence^[26]. In subsequent studies, we analyzed a series of analogs to find one, DC-34, that was more potent in its activity against myeloma cells. In these studies, we were able to define an nuclear magnetic resonance structure of the small molecule bound to the G4 structure which should allow structure-guided design of even more potent compounds^[27]. The discovery of a drug that directly targets *MYC* has been elusive, and thus far there are no approved drugs for this indication. The development of a more direct approach for inhibiting *MYC* activity seems warranted given the overall importance of *MYC* to a wide range of tumor types^[28].

Our early work involving retroviral induction studies of mouse PCTs suggest that inhibition of *MYC* alone may not be curative^[29]. Early induction studies with retroviral vectors clearly showed that overexpression of *MYC* alone could not induce PCTs; but when *MYC* was paired with *RAS* or *RAF*, high incidences of plasma cell tumors could be induced even in PCT-resistant strains of mice^[29]. These data are in agreement with recent studies indicating that *MYC* mutations are acquired secondary genetic events in myeloma progression^[30]. A key aspect of the mTORi/HDACi combination is its ability to decrease *MYC* protein stability; however, in some myeloma cell lines, we have observed a “compensatory” *MYC* mRNA increase

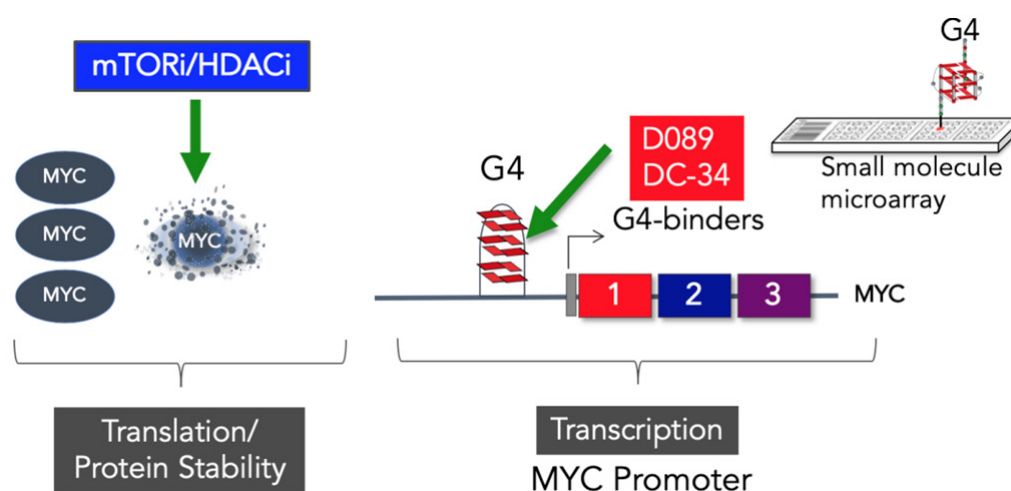


Figure 4. Drug combinations involving mTOR and HDAC inhibitors have a cooperative effect leading to *MYC* protein degradation. Small molecules targeting the G-quadruplex structure in the *MYC* promoter inhibit *MYC* transcription. HDAC: histone deacetylase; mTOR: mechanistic target of rapamycin

with combination treatment, although the steady-state protein level is decreased^[19,20]. Thus, developing a combinational approach to *MYC* inhibition by inhibiting both transcription and post-translational activity [Figure 4] might be more effective in providing a longer treatment window. Our drug combination studies also highlighted the importance of not only inhibiting *MYC*, but also up-regulating *Rb1/Cdkn2a* pathways, again suggesting that a *MYC* G4 stabilizer may not be effective as a single agent^[19,20]. Combining a *MYC* inhibitor with agents that can upregulate the *RB1/CDKN2A* pathways, such as CDK or HDAC inhibitors or other chromatin modifiers, may ultimately be more effective^[31,32].

CONCLUSION

Since the initial sequencing of the human genome in 2001 and the myeloma genome in 2011, there has been a tremendous growth in the generation and availability of high-throughput MM omics datasets^[33-35]. As a result of this, our knowledge and understanding of genetic underpinnings of MM tumor evolution has seen a similar expansion^[36-38]. Tumor heterogeneity, both across different patients and between individual subclones within the same patient, has been shown to play an important role in MM disease progression, prognosis, and response to therapeutic treatments^[36,39-41].

In a recent study by Maura *et al.*^[42] serial whole genome sequencing (WGS) of 30 MM patients was collected and used to determine the chronological order of key driver events that occur during myeloma tumor evolution. In most patients, early driver events such as hyperdiploidy (including the characteristic trisomies of odd chromosomes), immunoglobulin translocation, and chromothripsis tended to precede whole genome duplication, chromoplexy, and point mutation events. In addition to these general patterns of driver event timings, Maura *et al.*^[42] also found several examples of co-occurring or mutually exclusive events such as a co-occurrence between t(11;14) and t(14;16) chromosomal translocations and a mutually exclusive pattern of TRAF3 deletions with these same translocations. By combining the data from the 30 patients with an additional 804 patients from the MMRF CoMMpass trial^[43], Maura *et al.*^[42] were similarly able to detect important driver somatic mutations in MM, including well-known driver genes such as *KRAS*, *NRAS*, and *DIS3*, as well as novel putative driver mutations in genes encoding histone linkers (HIST1H1B, HIST1H1D, HIST1H1E, and HIST1H2BK), and mutations in or near genes involved in nucleosome binding.

Approximately 35%-40% of MM patients have IgH translocations (Chr 14), juxtaposed to an assortment of partners [MMSET (NSD2), FGFR2, MAF, CD-1 and D3 on other chromosomes (4, 6, 8, 11, 16 and

20)]^[44,45]. In contrast, about 80% of mouse PCTs carry translocations of the IgH locus on mouse Chr 12 juxtaposed to the *MYC* locus on Chr 15^[13]. Many myeloma mouse genetically engineered models have focused on the dysregulation (overexpression or knock-out) of a particular gene or pathway, most notably, the dysregulation of *MYC* or *BCL2*^[46,47], as well as the earlier spontaneous 5T models that have M spikes and develop bone lesions^[48]. Adoptive B cell transfer mouse models also provide a novel approach to study MM pathogenesis^[49]. Vlummens *et al.*^[50] comprehensively reviewed numerous murine models, ranging from xenografts to immunocompetent spontaneous and transgenic models, for studying both the etiology and pathogenesis of MM. More recently, Rajagopalan *et al.*^[51] generated a *Nras*^{LSL Q61R/+} mouse which takes advantage of crossing *Vk*MYC* mice to mice harboring a Q61R *NRAS* mutation (as found in WGS studies of myeloma)^[35,42,46]. This rapid model also develops both bone lesions and M spikes.

In contrast, the focus of our studies has been on genetically inherited alleles of genes in immunocompetent strains of mice that predispose the mice to peritoneal plasmacytoma development. In the past several years, more than 17 risk loci for multiple myeloma susceptibility have now been mapped to unique regions of the human genome^[52-57]. The one gene in common with our studies is the *Cdkn2a* locus; it is a tumor susceptibility gene in both mouse plasma cell tumors by genetic linkage studies^[4,6,15] and in genome-wide association studies (GWAS) in human multiple myeloma^[52]. Much progress has been made in understanding the omics of myeloma through GWAS^[52-57], eQTL^[58], and WGS^[35,42] studies of myeloma patient samples. These studies have helped to identify new targets for intervention of myeloma disease progression and form the basis for developing companion diagnostics for drug treatments.

In our studies, we have viewed cancer treatment through the lens of cancer as a complex genetic trait by using pristane-induced mouse PCT as the model^[4]. A goal in the molecular identification of these susceptibility/resistance genes has been to uncover the signaling pathways that are involved in promoting or controlling B cell neoplasia and to understand how these pathways may act in concert to contribute to or limit tumor progression. Tumor incidence data in congenic strains of mice, constructed to harbor different combinations of resistance alleles^[5,9], led to the hypothesis that drug combinations affecting these pathways are likely to have at least an additive, if not synergistic effect in inhibiting tumor cell growth. We investigated experimental therapeutic approaches to target myeloma; that led to the twin goals of upregulating tumor suppressor activities and downregulating oncogenic processes simultaneously.

Our initial preclinical studies focused on *Mtor* inhibition, through targeting *Mtor* kinase activity, coupled with HDAC inhibition, which inhibits histone deacetylation. HDAC inhibitors can target a broad spectrum of genes involved in chromatin modification, including those that regulate the *Rb1* and *p16* pathways. Our mechanistic analysis of the successful targeting of these two pathways, which induced synergistic anti-tumor activity in susceptible tumors, identified *MYC* as an important upstream driver regulated by the combined pathways through their cooperative effects on *MYC* protein degradation. Drug combinations targeting the two signaling pathways (*Cyclin D/CDK/Cdkn2a/Rb* and *PI3K/AKT/Mtor*) identified by our genetic analysis of PCT susceptibility, were indeed synergistic in their activity, not only for myeloma, but also a variety of tumor types as shown in their broad synergistic activity in the panel of NCI-60 cell lines. In fact, the only NCI-60 cell line for which this combination was antagonistic had a mutation in *FBXW7* which is involved in *MYC* protein degradation^[19].

MYC is often overexpressed and/or dysregulated in cancer, including mouse PCT, as well as human myeloma and Burkitt's lymphoma^[30,59,60]. *MYC* has often been considered an undruggable target, yet many researchers are pursuing a number of avenues to downregulate *MYC*, including drug combinations such as the one described above to target post-translational steps, such as protein stability. In addition, *MYC*'s transcription factor activity requires dimerization with its binding partner MAX (*MYC*-associated factor x), and many efforts have focused on interrupting this complex to downregulate its transcription factor

activity^[61]. Furthermore, *JQ1*, a BET (bromodomain and extra-terminal domain) inhibitor, can also inhibit *MYC* transcription, as well as other pathways^[62]. We and others have focused on interfering with *MYC* gene transcription by an alternative inhibitory mechanism involving small molecules that stabilize complex DNA structures (G-quadruplexes) which form transiently in the *MYC* promoter^[26,27,63,64].

Our studies to identify cooperative targets of mTORi/HDACi inhibition have: (1) provided a basic approach for broader application to identify potential biomarkers of drug combinations utilizing weighted gene coexpression network analyses combined with gene set enrichment analyses of survival annotated patient datasets; (2) identified upstream regulators/drivers of drug responses leading to a mechanistic understanding of how the combination is acting (upregulation of tumor suppressive pathways (*Rb1* and *p16*) and downregulation of oncogenic pathways (*MYC* and *E2F1*), leading to *MYC* degradation; and (3) demonstrated that cell lines carrying mutations in *FBXW7* or surrounding *MYC* residues Thr58,Ser62, involved in *MYC* degradation are not likely to respond to the combination of rapamycin/everolimus and entinostat. While *MYC* is known to be deregulated in a majority of cancers, its direct drug targeting has been elusive. We hope that our work to identify and develop a new class of compounds targeting *MYC* transcription will lead to new pharmacological agents for *MYC* inhibition. Our studies suggest that it would be clinically useful if these inhibitors were coupled with drugs that simultaneously upregulate tumor suppressors. Our studies are designed to provide the pre-clinical rationale and evidence of synergistic mechanisms required to advance candidate combinations for preclinical assessment in patient-derived cells and eventually in clinical study.

DECLARATIONS

Acknowledgments

We wish to thank our many collaborators and lab members for their contributions to the primary research referenced in this review: Douglas Lowy, Aleksandra Michalowski, Jyoti Patel, Patrick Sullivan, John S. Schneekloth, Jr., David Calabrese, Michael Kuehl, Ola Landgren, Robert Hawley, Anaisa Quintanilla-Arteaga, Patricia Wiley, Nana Gayabah-Kessie, Kenneth Felsenstein, Elena Leon, Zaw Phy, Daniel Connors, Ben Gamache, Nick Watson, Max Eiden, Matt Steinsaltz, Maudeline Etienne, Joy Gary, Edward Ramsey, Marianne Krall, Cecilia Padlan, Danielle Holiday, Jenefer Dosik, Margaret Pruitt, Dena Tran, Siegfried Janz, Alexander Kovalchuk, Elizabeth Mushinski, Mari Hirano, Rania Salem, Jing Huang, and R. Mark Simpson. We also thank Richard Nordan, Michael Potter, J. Frederic Mushinski, and Valery Bliskovsky (in memoriam) for their many contributions to these studies.

Authors' contributions

Made conceptual contributions to the manuscript: Zhang S, DuBois W, Zhang K, Simmons JK, Hughitt VK, Gorjifard S, Gaikwad S, Peat TJ, Mock BA

Wrote the manuscript: Simmons JK, Hughitt VK, Gorjifard S, Gaikwad S, Peat TJ, Mock BA

Designed several of the figures: Gorjifard S

Availability of data and materials

Not applicable.

Financial support and sponsorship

This work was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research and the MMRF (Multiple Myeloma Research Foundation).

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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Cross-talk between microRNAs, long non-coding RNAs and p21^{Cip1} in glioma: diagnostic, prognostic and therapeutic roles

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How to cite this article: Santoni G, Morelli MB, Nabissi M, Maggi F, Marinelli O, Santoni M, Amantini C. Cross-talk between microRNAs, long non-coding RNAs and p21^{Cip1} in glioma: diagnostic, prognostic and therapeutic roles. *J Cancer Metastasis Treat* 2020;6:22. <http://dx.doi.org/10.20517/2394-4722.2020.49>

Received: 22 May 2020 **First Decision:** 10 Jun 2020 **Revised:** 23 Jun 2020 **Accepted:** 2 Jul 2020 **Published:** 30 Jul 2020

Academic Editor: Giuseppe Lombardi **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Glioblastoma multiforme is considered one of the most common malignant primary intracranial tumors. Despite treatment with a combination of surgery, chemotherapy and radiotherapy, patients with glioblastoma multiforme have poor prognosis. It has been widely accepted that the occurrence, progression, and even recurrence of glioblastoma multiforme strictly depends on the presence of glioma cancer stem cells. The presence of glioma stem cells reduces the efficacy of standard therapies, thus increasing the imperative to identify new targets and therapeutic strategies in glioblastoma patients. In this regard, the p21^{Cip1} pathway has been found to play an important role in the maintenance of the glioma stem cells. It has been shown that this pathway regulates cancer stem cell pool by preventing hyperproliferation and exhaustion. MicroRNAs, endogenous small non-coding RNAs, and long non-coding RNAs, regulate post-transcription gene expression. These are not only altered in glioma, but also in other cancer types, and are involved in tumor development and progression. Notably, they have also been shown to modulate the expression of proteins in the p21^{Cip1} signaling pathway. This review highlights the extent and complexity of cross-talk between microRNAs, long non-coding RNAs and the p21^{Cip1} pathway, and demonstrates how such interplay orchestrates the regulation of protein expression and functions in glioma and glioma stem cells.

Keywords: Glioma, microRNA, long non-coding RNA, p21^{Cip1}, glioma stem cells



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INTRODUCTION

According to the World Health Organization (WHO) classification, Glioblastoma multiforme (GBM) is a grade IV malignant glial tumor which displays astrocytic differentiation. It is considered one of the most common malignant primary intracranial tumors^[1]. Despite treatment with a combination of surgery, chemotherapy and radiotherapy, GBM patients have poor prognosis^[2]. In 1940, the terms primary glioblastoma (pGBM) and secondary glioblastoma (sGBM) were first used by Scherer to distinguish between rapidly progressing *de novo* tumors and tumors derived from a pre-existing astrocytoma^[3]. According to Scherer^[3], pGBM and sGBM display the same histomorphological hallmarks, while differing in biological properties such as type of evolution, clinical manifestation and progression. The differences among primary and secondary GBM arise at the genotypic and epigenetic levels. It is currently widely accepted that the occurrence, progression and even recurrence of both pGBM and sGBM, depend on the accumulation of mutations in neural stem cells, which undergo transformation into glioma cancer stem cells (GSCs). GSCs are characterized by self-renewal and asymmetric cell division, thus allowing the production of proliferating progenitor cells with stem cell features and differentiated cancer cells^[4]. Importantly, GSCs are more resistant to radio- and chemotherapy respect to the proliferative progenitors present in the tumor. The p21^{Cip1} pathway plays an important role in the maintenance of the GSC pool because it induces a quiescent state that prevents hyperproliferation and exhaustion. p21^{Cip1} is involved in cell cycle arrest which occurs in response to DNA damage. It stimulates DNA repair, thus reducing genetic instability. Thus, p21^{Cip1} is fundamental in maintaining the GSC pool and its genomic integrity^[5].

As the presence of GSCs reduces the efficacy of standard therapy, there is an increased imperative to identify new targets and therapeutic strategies in GBM patients.

P21^{Cip1}: P53-DEPENDENT AND INDEPENDENT REGULATION

The proteins, p21^{Cip1}, p27^{KIP1} and p57^{KIP2}, belong to the Cip/Kip family of cyclin dependent kinase inhibitors (CKIs) and are found in mammals. Although p21^{Cip1} and p27^{KIP1} were initially classified as tumor suppressor proteins due to their involvement in the p53-dependent cell cycle arrest, it is now evident that their roles are more complex. Dysregulation of Cip/Kip protein may provoke specific defects in its tumor suppressor activity, while its oncogenic functions may be preserved, thus supporting cancer development. p21^{Cip1} acts as a tumor suppressor through binding and inhibiting CDK/cyclin complexes and the proliferating cell nuclear antigen (PCNA), thus blocking cell proliferation^[6]. However, it has been shown that p21^{Cip1} also displays additional functions such as stem cell pool preservation, regulation of cell migration and apoptosis^[7,8]. While some cancer types do not express p21^{Cip1}, its over-expression or cytoplasmic localization has been associated with poor prognosis in several malignant tumors^[8] [Figure 1].

p21, a potent cyclin-dependent kinase (CDK) inhibitor, also known as p21^{Cip1}/Waf1/CDKN1A, is a small protein consisting of 165 amino acids. p21 is able to arrest the cell cycle in the G1/S and G2/M phases by inhibiting CDK4, 6/cyclin-D and CDK2/cyclin-E, thus controlling E2F activity^[8,9]. The principal transcription regulator of p21^{Cip1} is p53. In fact, DNA damage and oxidative stress enhance p53 activity and trigger p21^{Cip1} expression^[10]. In addition, under the influence of certain factors, the p21^{Cip1}-dependent cell cycle arrest can be also regulated in a p53-independent manner. As such, p21^{Cip1} can be transactivated by the BRCA1 variants in a p53-dependent and independent manner^[11]. Additionally, transforming growth factor β (TGF β) and mitogen activated protein kinase control p21^{Cip1} expression by phosphorylation of SMAD1 and SMAD3, while c-Myc over-expression abrogates TGF β -mediated p21^{Cip1}/SMAD binding^[12]. The E3 ubiquitin ligase, Makorin Ring Finger Protein-1 [a protein involved in cell cycle regulation] reduces p21^{Cip1} levels by p53 ubiquitination and direct p21^{Cip1} polyubiquitination^[13]. Contrarily, Double homeobox4, which enhances p21^{Cip1} promoter activity via the transcription factor Sp1, increases p21^{Cip1} expression levels^[14]. In addition, promoter activity and p21^{Cip1} transcription can be stimulated in a p53-independent

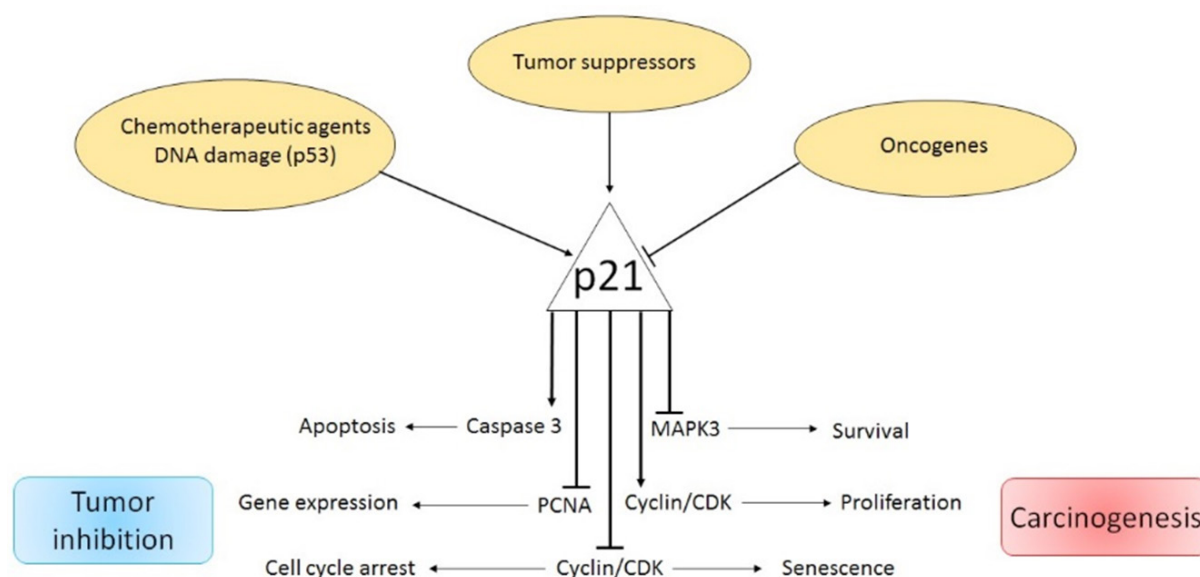


Figure 1. Dual role of p21^{Cip1} pathway in cancer

pathway via over-expression of $\beta 1$ -integrin receptors, and engagement of the transcription factor Sp1 and the P300 co-activator p21^{Cip1}^[15]. Down-regulation of p21^{Cip1} mRNA expression can also be induced by c-Myc through interactions with the initiator binding zinc finger transcription factor, MIZ-1^[16]. Similarly, the promyelotic zinc finger can bind to the p21^{Cip1} promoter, thus blocking the Sp1/p21^{Cip1} promoter interaction and reducing p21^{Cip1} expression^[17]. The liver kinase B1 is a serine/threonine kinase that activates AMPK and regulates cell growth and apoptosis by triggering a p53-dependent p21^{Cip1} increase^[18,19]. Enhanced p21^{Cip1} levels, induced by increased p53 and Sp1/DNA binding activity, have been also found in human fibroblasts during replicative senescence^[20-22]. Moreover, the alkylating agent Temozolomide (TMZ) induces G2-M cell cycle arrest and senescence through the Mre11-Rad50-Nbs1 (MRN) complex and activation of the ATR/CHK1 axis. TMZ-induced senescence requires a functional p53, a functional NF- κ B pathway, as well as a sustained p21^{Cip1} induction. Upon TMZ exposure, as a consequence of the E2F1/DP1 complex disruption, a strong repression of the mismatch repair proteins MSH2, MSH6, EXO1 and RAD51 was observed^[23].

The Wnt signaling pathway is a critical regulator of cancer stem cell (CSC) features and has been associated with poor prognosis. Its downregulation is responsible for diminished numbers of CD133⁺CD44⁺ CSCs [in this regard, recent studies on colon cancer have demonstrated that the knock-down of zeste homologue 2 (EZH2), a key component of the Polycomb-Repressive Complex 2, is involved in maintaining the transcriptional repressive state of cycle arrest and apoptosis of CSC-like cells^[24]. EZH2 expression positively correlates with levels of Wnt/ β -catenin pathway target genes. EZH2 is essential for the maintenance of CSC-like cell properties] [Figure 2]^[25]. Several findings demonstrate that EZH2 knockdown inactivates the Wnt/ β -catenin pathway by increasing p21^{Cip1} expression, resulting in G1/S-phase arrest^[25]. EZH2 knockdown in CD133⁺/CD44⁺ SW480 cells has been shown to induce the increase of p21^{Cip1} with consequent reduction of β -catenin, vimentin and c-Myc expression. Moreover, the inhibition of EZH2 via a specific inhibitor promotes enhancement of p21^{Cip1} expression and inactivation of the Wnt/ β -catenin pathway^[25].

Interestingly, the role of EZH2 in chemotherapy drug resistance has also been investigated. A decrease in EZH2 expression was demonstrated to promote apoptosis, cell cycle arrest at the G1/S phase, and reduce expression of multi-drug resistant proteins in TMZ-resistant GBM cells^[26]. GSC maintenance involves the cell-division cycle protein 20 (CDC20), an activator of the E3 ubiquitination ligase complex. Silencing

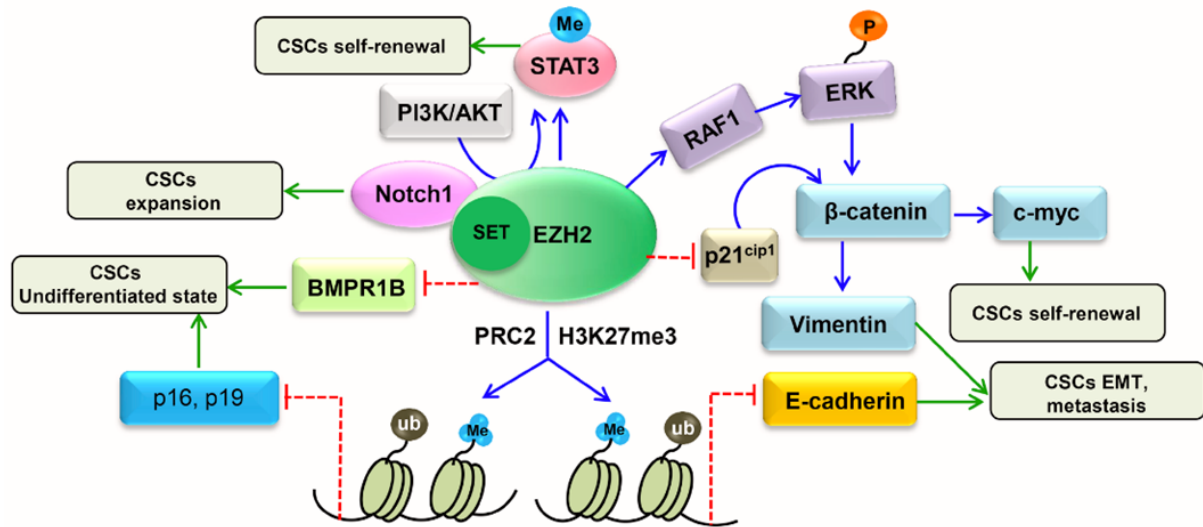


Figure 2. EZH2/WNT/p21^{Cip1} pathway in cancer stem cells^[24]

CDC20 expression attenuates GSC proliferation, self-renewal and *in vivo* tumor growth by inducing apoptosis and cell cycle inhibition. CDC20 maintains the GSC phenotype through p21^{Cip1} degradation. CDC20 inhibition stabilizes p21^{Cip1} and represses Survivin, CDC25C and c-Myc survival gene expression^[27].

In addition, the roles of p21^{Cip1} and p27^{KIP1} in GSCs treated with camptothecin [CPT, a specific inhibitor of the DNA topoisomerase I that induces DNA double-strand breaks in GSCs carrying the homozygous CDKN2A/ARF deletion] have recently been investigated^[28]. The results showed that both p21^{Cip1} and p27^{KIP1} proteins block the cell cycle in both unstressed conditions and in response to genotoxic stress. Cip/Kips expression was upregulated after CPT treatment, with a peculiarly nuclear localization. CKIs protect cancer cells against CPT damage by stopping cell cycle progression. Notably, cells become more susceptible to DNA damage in the absence of p21^{Cip1} and p27^{KIP1} proteins, leading to impaired cell cycle blockage under genotoxic stress. Overall, the existing literature demonstrates that p21^{Cip1} and p27^{KIP1} may act both as tumor suppressors by reducing cell proliferation, and as oncogenes, by increasing cellular resistance in response to DNA damage. A deeper understanding of Cip/Kip functions may be relevant and useful to improve understanding on the mechanisms underlying the acquisition of chemoresistance in cancer^[28].

Finally, p21^{Cip1} is one of the major regulators of cell cycle and has been previously linked to apoptosis resistance in glioma cells^[29]. O(6)-Methylguanine-DNA methyltransferase (MGMT) plays a major role in the resistance to alkylating agents in gliomas^[30]. Hapbold *et al.*^[31] demonstrated that in TMZ-resistant glioma cell line, constitutively expressing MGMT, but not in glioma cell lines negative for MGMT, a strong up-regulation of MGMT levels and elevated p21^{Cip1} mRNA levels and slower cell cycle progression^[31]; however, silencing p21^{Cip1} silencing in resistant vs. normal glioma cells does not evidenced major changes in cell cycle distribution. Mostofa *et al.*^[32] clarified the role for p21^{Cip1} in TMZ-resistance in glioma cells-the p21^{Cip1} protein sequesters PCNA by binding to it during the cell cycle, thus attenuating DNA replication. In human GBM, MGMT harbors a PCNA-interacting protein motif (PIP box), thus associating with PCNA and in turn, p21^{Cip1}. PCNA is strictly controlled by p21^{Cip1}, a strong binder and sequester of the former. Alkylating DNA damage induces MGMT and PCNA colocalization, and this occurs mainly in glioma cells deficient in p21^{Cip1}, as p21^{Cip1} expression directly correlates with higher MGMT mRNA and protein levels, higher MGMT activity and greater resistance to alkylating agents. p21^{Cip1} strongly disrupts MGMT-PCNA complexes within glioma cells. MGMT proteins are downregulated at mid to late S-phase and specifically degraded by the PCNA-dependent ubiquitin-ligase, CRL4^{Cdt2}.

Table 1. miRs targeting p21^{Cip1} in glioma

miRs	Targets	Cell functions	Ref.
miR-7	ND	Pathological grade ↑	[50]
miR-10b	Bim/AP-2/p16/p21 ^{Cip1}	Proliferation, cycle arrest ↓	[34]
miR-15-16	E2F1/Rb	Proliferation ↓	[35]
miR-17-92	CTGF	Apoptosis ↑, proliferation ↓	[37]
miR-34a	YY1	Migration ↓	[42]
miR-92b	TGF/SMAD3	Viability, proliferation ↑	[38]
miR-93	p21 ^{Cip1}	Chemoresistance to Temozolomide ↑	[40]
miR-128	Bmi-1	Proliferation ↓	[43]
miR-139	ND	Pathological grade ↑	[50]
miR-146b-5p	HuR/-catenin	Stemness, radioresistance ↑	[51]
miR-149	Casp-2	Cell survival ↑	[41]
miR-184	KT/NF-κB	Proliferation, invasion ↑	[44]
miR-193b	SMAD3	Proliferation ↑	[39]
miR-223	NFIA	Gliogenesis ↓	[45]
miR-329	E2F1	Cell growth ↓	[46]
miR-454	PDK1	Proliferation ↓	[48]
miR-656	BMPRI1A	Proliferation, migration/invasion neurosphere formation ↓	[47]
miR6798-3p	Ars2	Proliferation, tumorigenesis ↑	[49]

↑: increase; ↓: decrease; Bim: Bcl-2-like protein 11; AP-2: activating enhancer-binding protein 2 gamma; E2F: transcription factor E2F1; CTGF: connective tissue growth factor; PPAR: peroxisome proliferator-activated receptors; YY1: Yin yan 1; TGF: transforming growth factor; SMAD3: mothers against decapentaplegic homolog 3; HuR: Human antigen R; Casp-2: caspase 2; AKT: Serine/threonine protein kinase; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; NFIA: nuclear factor I A; PDK1: pyruvate dehydrogenase kinase 1; BMPRI1A: bone morphogenetic protein receptor type 1; Ars2: arsenate resistance protein

As the p21^{Cip1} protein harbors binding domains for CDKs and PCNA, it serves as an upregulator of MGMT expression, enhancing its transcription and expression, thus providing glioma resistance to alkylating anticancer drugs.

P21^{CIP1} AND MIRNA IN GLIOMA

MicroRNAs (miRs) are endogenous small non-coding RNAs which regulate gene expression post-transcriptionally. They are commonly deregulated in different types of cancer, including gliomas. In cancer cells, miR regulate cell viability, proliferation, differentiation, migration, invasion, apoptosis, chemoresistance and radioresistance. They can function as oncogenes by promoting cell growth or as anti-oncogenes by suppressing tumor growth^[33].

Recent reports indicate a strict relationship between the p21^{Cip1} signaling pathway and miRs [Table 1]. The expression of miRs is significantly upregulated in gliomas as compared to that of adjacent non-tumor tissues or normal brain tissue. The expression of miR-10b, -17-92, -92b, -93, -149, -193b, 6798-3p was elevated in glioma tissues as compared to normal controls and significantly increased with tumor grade progression. It has been demonstrated that survival of GBM patients expressing high levels of miR-10 is lower in comparison with patients with low miR-10 levels indicating that miR-10 may contribute to glioma growth *in vivo*^[34]. miR-10b inhibition enhances the expression of direct miR-10b targets, namely BCL2L11/Bim, TFAP2C/AP-2γ, CDKN1A/p21, and CDKN2A/p16, thereby reducing glioma cell growth by cell-cycle arrest and apoptosis. In cells expressing high levels of p21^{Cip1}, miR-10 represses E2F1-mediated transcription, leading to down-regulation of miR-15/16 and E2F1 target genes, thus delaying progression through to S-phase of the cell cycle. Consequently, miR-15/16 activities are reduced through the repression of many of its targets such as FBXW7, the ubiquitin ligase that destabilizes Cyclin E. However, miR-10b inhibition induces a weaker E2F1 response in GBM cells that express low levels of p21^{Cip1}^[35]. Using the clustered regularly interspaced short palindromic repeats-cas9 system, it has also been demonstrated that ablation of the miR-10b gene, through alterations in the expression of several targets including p21^{Cip1},

strongly reduces glioma cell viability in both *in vitro* and *in vivo* experimental models^[36]. This suggests miR-10b gene editing to be a promising therapeutic approach for permanent elimination of essential regulators of tumor survival.

In addition, the miR-17-92 locus has been found to be amplified in GBM specimens. Inhibition of miR-17-92 activities enhances apoptosis and reduces cell proliferation of GBM spheroids. miR-17-92 inhibition has also been associated with increased expression of CTGF, a direct target of miR-17-92 in GBM spheroids, as well as CDKN1A, E2F1, PTEN^[37].

Several target of miRs are represented by members of the TGF/SMAD pathways. In GBM tissues overexpressing miR-92b, the expression of SMAD3 was found to be reduced when compared with that of normal brain tissues. miR-92b directly affects SMAD3 expression by targeting the 3'-untranslated region. Silencing miR-92b inhibits GBM viability through upregulation of the TGF/SMAD3/p21^{Cip1} signaling pathway. Furthermore, *in vivo* treatment with miR-92b inhibitors was shown to reduce tumor growth, demonstrating the ability of miR-92b to act as an oncogene by promoting GBM cell proliferation. Therefore, miR-92b may be a future potential target for the development of miR-based therapies^[38]. miR-193b has also been shown to be upregulated in glioma patients, while its overexpression has been associated with poor prognosis. Downregulation of miR-193b correlates with decreased cell growth. SMAD3 is a direct target of miR-193b, and downregulation of SMAD3 attenuates miR-193b suppression of glioma proliferation. Thus, miR-193b regulates cell growth through the TGF- β pathway by modulating SMAD3^[39].

Upregulation of miR-93 has also been demonstrated to be associated with advanced malignancy as it promotes the proliferation, migration and invasion of glioma cells. miR-93 regulates the cell cycle by controlling the p21^{Cip1}, p27^{KIP1}, p53 and cyclin D1 expression. Since p21^{Cip1} is a direct target of miR-93, p21^{Cip1} knockout attenuates the suppressive effects of miR-93 on cell cycle progression and colony formation. In addition, the chemosensitization of GBM cells to temozolomide is markedly increased when miR-93 is inhibited^[40]. Overexpression of miR-149 in gliomas augments pro-survival activity, inhibits apoptosis and induces xenografted tumor growth *in vivo*. Given that caspase-2 is a functional target of miR-149, its expression is inversely associated with miR-149 *in vitro*. miR-149 promotes cell survival in U87 and A172 glioma cell lines and targets caspase-2, through p53 and p21^{Cip1} inactivation^[41].

Several miRs have been found to be down-regulated in glioma patients, thus functioning as tumor suppressor genes (e.g., miR-34a, -128, -184, -223 -329 and -656). miR-34a maps to chromosome 1p36.23, a region often deleted in GBM. For this reason, the expression of miR-34a is lower in GBM samples as compared with that of normal brain tissue. In glioma patients, an miR-34a deletion is accompanied by the amplification of epidermal growth factor receptor (EGFR). Notably, mean survival time is shorter in GBM patients with EGFR amplification and miR-34a deletion^[42]. Moreover, enforced expression of miR-34a in GBM cells decreases migration and levels of cyclin-A1, -B1, -D1, and -D3, as well as cyclin-dependent kinases, while increasing the expression of cyclin kinase inhibitor proteins such as p21 or p27^{KIP1}. In *in vivo* xenograft mouse model, the injection of U251 cells overexpressing miR-34a induced the development of smaller tumors compared with tumors derived from wild-type U251 cells. Since miR-34a targets Yin Yang-1, a transcription factor that stimulates the expression of EGFR, the expression of EGFR is reduced in cells overexpressing miR-34a. miR-34a act as a tumor suppressor by inhibiting the growth of GBM cells *in vitro* and *in vivo*^[42].

miR-128 expression reduced glioma cell proliferation *in vitro* and *in vivo* in a glioma xenograft growth model. It reduced Bmi-1 oncogene expression, by direct regulation of the Bmi-1 mRNA 3'-untranslated region, through a miR-128 binding site. Relative to normal brain tissues, Bmi-1 is upregulated and miR-128 is down-regulated in glioma samples. Bmi-1 induces the silencing of several genes through epigenetic

chromatin modifications. It is responsible for decreasing histone methylation [H3K27me(3)] and Akt phosphorylation, while upregulating p21^{Cip1} levels. Bmi-1 also promotes self-renewal of stem cells, while miR-128 [by down-regulating Bmi-1 levels] inhibits self-renewal of glioma^[43]. miR-184 levels are reduced in aggressive human tumor cells. Thus, a potential therapeutic strategy which targets miR-184 may prove useful in reducing cancer aggressiveness. miR-184 inhibits the proliferation and invasion of the U87MG cell line. It arrests the cell cycle and prevents adhesion by upregulating the expression of p53 and p21^{Cip1}, increasing caspase-3/8 activity, suppressing SND1, MMP-2/9, CD44 expression, and the activity of the AKT/NF-κB pathway^[44]. Another miR involved in glioma development is miR-223. It represses nuclear factor I-A (NFIA) expression, a key regulator of gliogenesis. miR-223 and NFIA expression negatively correlate in human GBM tumors. The miR-223/NFIA axis suppresses tumorigenesis in human glioma cells. The NFIA factor directly represses p21^{Cip1}, and is required for tumorigenesis in a mouse neural stem cell model of glioma^[45].

miR-329, which is located on 14q32.31, is down-regulated in glioma and it is able to target E2F1. Its overexpression blocks G1/S transition in LN18 and T98G cell lines and suppresses cell proliferation as well as colony formation. It also decreases the phosphorylation of Akt and cyclin D1, inducing p21^{Cip1} upregulation and suppression of cell growth through the inhibition of the E2F1-mediated Akt pathway^[46].

miR-656 was found to be downregulated in glioma. In human glioma tissues, expression of BMPR1A [a miR-656 target] is negatively correlated with miR-656 levels. miR-656 suppresses glioma cell proliferation, neurosphere formation, migration and invasion with or without exogenous BMP-2^[47]. Knockdown of BMPR1A diminished the antiproliferative effect of miR-656 *in vitro*. Moreover, the canonical BMP/SMAD pathways were shown to be inhibited by miR-656 overexpression. Several molecules, including cyclin B, cyclin D1, matrix metalloproteinase-9, p21^{Cip1} and p27^{KIP1} are involved in miR-656 functions within glioma cells. Ectopic expression of miR-656 reduced tumor size and prolonged the survival of mice, regardless of whether treatment with BMP-2 was administered^[47].

Some miRs, even if down-regulated in gliomas, function as tumor promoters. For example, an important role has been described for miR-454, which is down-regulated in GBM primary tumors and cell lines. Overexpression of miR-454 in GBM cells resulted in the arrest at the G0/G1 phase, resulting in the inhibition of cell proliferation. 3-phosphoinositide-dependent protein kinase-1 (PDK1), one of miR-454 targets, was found to increase miR-454 levels, decrease PDK1 expression, down-regulate Cyclin D1 and upregulate p-Rb and p21^{Cip1}^[48].

In addition, arsenic resistance protein 2 (Ars2), a component of the nuclear RNA cap-binding complex, that is involved in miR biogenesis, proliferation and tumorigenicity, was found to be overexpressed in glioma cell lines^[49]. It is also associated with poorer overall survival in GBM patients^[49]. In fact, not only several miRs have been found to be associated with pathological grade, tumor development and stemness in glioma^[50,51], but also it has been demonstrated that overexpression of Ars2 stimulates glioma cell proliferation and colony formation and colony formation. Knockdown of Ars2 reduces miR-6798-3p expression, causing p53 and p21^{Cip1} upregulation which leads to apoptosis. By using an orthotopic GBM xenograft model, knockdown of Ars2 was found to reduce tumor growth and to extend the survival time of tumor-bearing mice^[49].

ROLE OF LONG NON-CODING AND LONG INTERGENIC NON-CODING RNAs IN GLIOMA AND GLIOMA STEM CELLS

Long noncoding RNAs (LncRNAs) represent a class of long transcribed noncoding RNAs (ncRNA) molecules, which are longer than 200 nucleotides. They do not code for proteins and are involved in tumor

Table 2. Long non-coding RNA and long intergenic non-coding RNA interacting with p21^{Cip1} pathway in glioma

Lnc/LincRNA	Mechanism/s	Cell functions	Ref.
SNHG3	Epigenetic p21/KLE2 repression	Proliferation ↑ apoptosis ↓	[60]
RP11-732M18.3	Binding to 14.3.3 b/a	Cell growth ↑	[57]
SNHG16	Caspase 3/9 reduction	Proliferation and apoptosis ↑	[59]
SNHG20	p21 ^{Cip1} silencing	Proliferation ↑	[71]
PTENP1	SHG44 reduction	Proliferation and metastasis ↓	[62]
FAH83H-AS1	Epigenetic p21 ^{Cip1} silencing	Poor prognosis ↑	[70]
SNH6	ND	Proliferation ↓	[61]
LOC441208	Binding to b-catenin	Cellgrowth ↑	[58]
p21 ^{Cip1}	Reduced HIF-1 autophagy	Radiosensitivity ↑	[69]
p21 ^{Cip1}	HuR upregulation	Stemness and radioresistance ↓	[51]
p21 ^{Cip1}	Binding to hnRNP-K	Apoptosis ↑	[64]
ASEN	Binding to UPF1	Senescence ↓	[72]
p21 ^{Cip1}	CRF/CRFR1	Proliferation and invasion ↑	[73]

↑: increase; ↓: decrease; KLE2: condensin-2 complex subunit kle-2; HIF-1: hypoxic inducible factor 1; HuR: Hu-antigen R; hnRNP-K: heterogeneous nuclear ribonucleoprotein K; UPF1: up-frameshift suppressor 1 homolog; CRF: corticotropin-releasing factor; CRFR1: CRF receptor 1

development and progression. Long intergenic noncoding RNAs (LincRNAs) are long RNA transcripts which control cell differentiation and maintenance of cell identity. All of the above have recently been found to be altered in various cancer types [Table 2]^[52]. LncRNAs have been recognized as regulators involved in different steps of the tumorigenic process. In gliomas, the functions of most LncRNAs are not well known, and the mechanisms controlling the proliferation, invasion, angiogenesis, radiosensitivity or radioresistance, and GBM stemness remain poorly defined^[53]. While many LncRNAs have been identified, only a few have been functionally described in gliomas. Moreover, CSC regulation by LncRNA following radiotherapy and the relationship between LncRNA and tumor spreading and radioresistance have been reported^[54].

Recent studies have evidenced that LncRNAs control the transcription of genes involved in DNA Damage Response (DDR), the latter of which is strictly associated with radiosensitivity and repair capacity^[55,56]. The majority of LncRNAs are overexpressed in tumor cells, hence their inhibition might represent a new therapeutic target for glioma treatment.

Among LncRNA, RP11-732M18.3 is highly overexpressed in glioma cells and functions as an oncogene by interacting with 14-3-3β/α to promote glioma growth^[57].

Its overexpression has been associated with the proliferation of glioma cells and tumor growth both *in vitro* and *in vivo*. LncRNA RP11-732M18.3 stimulates the G1/S cell cycle transition and cell proliferation. It has been found that interaction of LncRNA RP11-732M18.3 with 14-3-3β/α increases the degradation of the p21^{Cip1} protein. In fact, by promoting the recruitment of ubiquitin-conjugating enzyme E2 E1 (UBE2E1) to 14-3-3β/α and with the binding of 14-3-3β/α with UBE2E1, LncRNA RP11-732M18.3 stimulates the degradation of p21^{Cip1}^[57]. Similarly, LOC441204-LncRNA, has been shown to be upregulated in glioma cell lines. It promotes tumor cell growth by stabilizing the β-catenin pathway. LOC441204 can bind to β-catenin and prevent its degradation, thus resulting in downstream p21^{Cip1} repression and cdk4 activation that in turn enhances glioma cell proliferation. The LOC441204 knockdown suppressed tumor cell proliferation of glioma cell lines^[58].

The expression of the small nucleolar RNA host gene 16, SNHG16-LncRNA was upregulated in gliomas. Knockdown of SNHG16 is associated with high p21^{Cip1} expression, poor proliferation and increased apoptosis. Lnc-SNHG16 was shown to inhibit p21^{Cip1} expression and caspase 3/9 activation, while increasing cyclinD1/B1 expression^[59]. The SNHG3 upregulation in glioma promoted cell proliferation,

accelerated cell cycle progression and repressed apoptosis. SNHG3 facilitated the malignant progression of glioma through epigenetically repressing KLF2 and p21^{Cip1} via recruitment of the enhancer of zeste homolog 2 to the promoter of KLF2 and p21^{Cip1}[60]. Similarly, overexpression of SNHG6 promotes the malignant phenotype, while loss-of-function revealed that the silencing of SNHG6 inhibits glioma cell growth in a p21^{Cip1}-dependent manner[61].

The lncRNA PTEN pseudogene-1 (PTENP1) has been described to be involved in the development and progression of several cancers. However, little is known about the molecular mechanism by which lncRNA PTENP1 affects the development and progression of gliomas. Overexpression of PTENP1 suppressed SHG44 expression as well as U251 cell proliferation, invasion and migration by inducing p21^{Cip1} expression while inhibiting p38 signaling[62].

Among lncRNA, the lncRNA-p21^{Cip1} is an intergenic lncRNA which resides on the chromosome 17, upstream of the p21^{Cip1} gene. It was identified ten years ago as being one of the eleven lncRNAs that are increased in expression in response to p-53 induced DNA damage[63].

lncRNA-p21^{Cip1} is the downstream target of p53 and controls the cell cycle, damage of DNA and its repair process[63]. The lncRNA-p21^{Cip1} suppresses the p53-dependent transcription activity and affects p53-repressed genes. It is required to trigger p53-dependent apoptosis of DNA damaged cells through physical association with the ribonucleoprotein K (hnRNP-K)[64].

lncRNA-p21^{Cip1} also regulates the G1/S checkpoint of the cell cycle by modulating the expression of its neighboring gene p21^{Cip1}[65] and mediating radiation-induced apoptosis[66,67]. lncRNA-p21^{Cip1} is a potent suppressor of GSC and works by triggering apoptosis through the NOXA pro-apoptotic gene[68]. It negatively regulates β -catenin activity in GSCs. Downregulation of lncRNA-p21^{Cip1} in GSCs results in upregulation of Hu antigen R (HuR), and this is caused by miR-146b-5p down-regulation. Moreover, knockout of lncRNA-p21^{Cip1} or HuR was shown to increase GSC β -catenin expression, stemness and GSCs radioresistance[51]. Previous findings also showed that lncRNA-p21^{Cip1} is a hypoxia-responsive lncRNA able to regulate the cell cycle and apoptosis. It has also been shown to be responsible for the Warburg effect in cervical cancer. Although hypoxia was found to increase lncRNA-p21^{Cip1} expression in U251MG glioma cell line, its functions in hypoxic glioma remain unknown. Knockdown of lncRNA-p21 induced G2/M phase arrest, promoted apoptosis, decreased cell proliferation and motility, and also reduced autophagy through the HIF-1/Akt/mTOR/P70S6K pathway[69]. These findings are suggestive of a mechanism by which lncRNA-p21^{Cip1} enhances the radiosensitivity of hypoxic tumor cells.

The antisense FAM83H RNA1 (FAM83H-AS1) is known to be upregulated in glioma. Silencing of FAM83H-AS1 suppressed glioma proliferation and apoptosis. FAM83H-AS1 inhibits CDKN1A expression by recruiting EZH2 to the promoter of CDKN1A, thereby influencing cell cycle and proliferation[70].

lncRNAs also regulate cellular senescence. Thus, lncRNA-ASEN, which is expressed in prematurely senescent cells, has been shown to repress cellular senescence by reducing p21^{Cip1} expression, both during and post-transcription. The complex formed by the interaction between lnc-ASEN and UPF1 can suppress p21^{Cip1} transcription through the recruitment of Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2) to the p21 locus. This prevents binding of the transcriptional activator p53 to the p21 promoter through histone modification[64].

CLINICAL ROLE OF MIRNA TARGETING P21^{CIP1} AND LNCRNA-P21^{CIP1} IN GLIOMAS

The identification of glioma-related biomarkers is an urgent necessity as it would enable the development of new therapeutic approaches, while improving the ability to predict responsiveness to chemo- and

radiotherapy. In this regard, several miRs including miR-10b^[34] and miR-92b^[38] have been suggested as targets for the development of miRNA-based therapies. Inhibition of miR-93 enhanced the chemosensitization of glioma cells to TMZ treatment^[40], while miR-17-92 and its target CTGF have been suggested for use in differentiation-promoting therapy in the treatment of GSC^[37]. Conversely, the upregulation of miR-193b^[33] and miR-93 are associated with advanced malignancy^[40] and represent negative prognostic factors. Ars-2, which reduces miR-6798 expression, was associated with poor overall survival in glioma patients^[49].

Finally, Shen *et al.*^[41] have suggest that the miR effect in cancer may also depend on the p53 status of tumor cells. In glioma cell lines harboring wild-type p53, miR-149 showed pro-survival function, as it targeted caspase-2 via the inactivation of p53/p21^{Cip1} pathways, while functioning as a tumor suppressor in proliferation and invasion of glioma cell lines with p53 mutations. miR-149 also inhibited proliferation and migration of glioma cell lines with p53 loss, suggesting its distinct biological function within both p53 wild-type and p53 mutated glioma cells.

LncRNAs have recently emerged as crucial players in the p21^{Cip1} complex signaling network as they control glioma development and progression^[71-73], the activation of GSCs, as well as radioresistance. LncRNAs aberrantly expressed in CSCs in different cancer types such as epatocarcinoma, are active participants in the major signaling pathways governing DDR, DNA repair, apoptosis and epithelial-mesenchymal transition^[54,74]. It has been also reported that the deregulation of LncRNAs plays a role in cancer recurrence and prognosis^[75]. With increasing knowledge on the expression profile of LncRNAs in cancers, their potential roles as biomarkers in diagnosis and prognosis have been highlighted. Expression levels of LncRNAs are closely associated with the real tumor status. Additionally, LncRNAs are more sensitive and specific as compared to the other conventional markers. Furthermore, given that LncRNAs are released in body fluids, this suggests their potential to be utilized in a clinical setting as non-invasive biomarkers^[75].

In gliomas, several dysregulated LncRNAs are involved in proliferation, radioresistance, metastasis and cancer stem cell properties. A strong correlation between high LncRNA expression and clinical parameters has been reported for LncRNA-SNHG20 in glioma patients. High LncRNA-SNHG20 has been associated with larger tumor size, larger extent of resection, more advanced grade (WHO classification), poorer survival status and a higher incidence of recurrence. High SNHG20 levels are predictive of poor prognosis and represent an independent potential prognostic biomarker for glioma patients^[71]. Similarly, overexpression of SNHG3, SNHG6 and FAH83H-AS1, which promote the malignant phenotype, represent a negative prognostic factors^[60,61,70].

Given the up-regulated expression of LncRNAs in glioma patients, and the role in cancer development and progression, LncRNA inhibition (e.g., RPH, SNHG3, SNHG16, LOC441204)^[57-60] would represent a novel diagnostic and therapeutic strategy in gliomas. Moreover, as LincRNA-p21^{Cip1} enhances the radiosensitivity of hypoxic tumor cells, it represents a valuable target for radiation therapy in glioma patients^[69]. Furthermore, in GSCs, the LncRNA-mediated effects also depend on the epigenetic regulation of genes, particularly via recruitment of the Polycomb repressor complex and by acting as competing endogenous RNAs for miRNAs targeting genes involved in stemness and radioresistance, mainly of the WNT/B-catenin and p21^{Cip1} pathways^[76].

Finally, several miRs such as miR26a^[77] and LncRNAs such as Lnc-TALC^[78] have been found to promote MGMT expression and TMZ resistance in gliomas. However, at present no miRs or LncRNAs targeting p21^{Cip1} have been employed to modulate alkylating agent resistance in gliomas.

CONCLUSION

The functions of the vast majority of miRNAs, LncRNAs and LincRNAs encoded by mammalian genome remain largely unknown. However, recent studies indicate that these transcripts play vital roles not only

in cellular physiology, but also in cancer pathology. In this regard, alternations in their regulation have been found to be implicated in the development and progression of glioma^[53,79,80]. Complex cross-talk and interplay between miRNAs, Lnc/LincRNAs and p21^{Cip1} orchestrate the regulation of protein expression and functions in cancer cells. Our understanding of the functional roles and molecular mechanisms of miR and LncRNAs in glioma and GSC is still nascent. Several findings indicate that miRs targeting p21^{Cip1} and more recently LncRNAs signatures correlate with glioma malignancy grade, histological differentiation and prognosis^[47,51,70]. Further in-depth investigation into miRs, LncRNAs and LincRNAs, their complex mechanisms of regulation, as well as their involvement in glioma genesis, metastatic spread and therapeutic resistance will be necessary in order to develop novel and effective diagnostic and therapeutic strategies against primary and secondary GBM tumors.

DECLARATIONS

Authors' contributions

Supervised the work and wrote the manuscript: Santoni G

Contributed to the preparation of the subchapters: Morelli MB, Amantini C

Contributed to the preparation of figures and tables: Maggi F, Marinelli O

Provided critical revision of the manuscript: Nabissi M

Collaborated in the Drafting of the introduction and conclusion: Santoni M

Availability of data and materials

Not applicable.

Financial support and sponsorship

This work was supported by PRIN 2017 and Fondazione Umberto Veronesi (Post-doctoral Fellowship 2019 to Morelli MB).

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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Lobe-specific modulation of B16MET melanoma lung metastases by nephrlin peptide

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How to cite this article: Mascarenhas DD, Ravikumar P, Amento EP. Lobe-specific modulation of B16MET melanoma lung metastases by nephrlin peptide. *J Cancer Metastasis Treat* 2020;6:23. <http://dx.doi.org/10.20517/2394-4722.2020.52>

Received: 17 May 2020 **First Decision:** 24 Jun 2020 **Revised:** 24 Jun 2020 **Accepted:** 06 Jul 2020 **Published:** 30 Jul 2020

Academic Editor: Rafat A. Siddiqui **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Aim: Nephrlin peptide modulates systemic immune responses to trauma in contexts characterized by simultaneous inflammation and immunosuppression. This study explores the possibility that nephrlin peptide may modulate lung metastasis, which also occurs in an environment of concurrent inflammation and immunosuppression.

Methods: B16MET melanoma cells were injected via the tail vein of mice and the development of lung metastases was recorded in animals treated with nephrlin peptide or vehicle by subcutaneous bolus injection daily for three weeks. In a separate experiment, nephrlin was administered by subcutaneous bolus injection for seven days to study the biodistribution of peptide and possible changes to plasma cytokine levels.

Results: Nephrlin significantly suppressed B16MET lung metastases. Suppression was more effective in deep lobes with the poorest access to circulation: accessory > inferior > middle > superior. In a separate biodistribution study in mice, nephrlin showed similar biodistribution levels in kidney, liver, brain, and left lung, but significantly higher accumulation in the lobes of the right lung in a gradient that matched its effectiveness in suppressing metastases (accessory > inferior > middle). The latter environments were also characterized by significantly higher local concentrations of succinate, a proxy for lower levels of oxygenation.

Conclusion: Nephrlin accumulates preferentially in the deep lobes of the right lung in mice and inhibits B16MET right lung metastases in a lobe-specific manner.



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Keywords: Nephrlin, melanoma, metastases, deep lung, lobes, anoxia, oxidative stress, immune modulation

INTRODUCTION

The peptide nephrlin is an inhibitor of Rictor complex derived by fusing a 19 amino acid segment of Protor with the metal-binding domain of human insulin-like growth factor binding protein-3 (IGFBP3)-a 21 amino acid pro-homeostatic sequence that targets and preferentially enters stressed cells^[1-2].

Severe burn trauma is associated with secondary systemic effects including hyper inflammation, hypercatabolism, sepsis, organ failure, loss of glycemic control, delayed wound healing, and cognitive deficits^[3-8]. We have demonstrated the efficacy of nephrlin peptide in combating many of these pleiotropic effects in burn trauma as well as in several rodent models of metabolic and xenobiotic stress^[9-14]. When nephrlin peptide is injected subcutaneously into rodents, its Protor sequence is believed to reduce oxidative stress in target tissues by modulation of Rac1 phosphorylation and, more speculatively, its IGFBP3 sequence may mediate homeostatic immunomodulatory functions in addition to its described transporter functions^[12]. Nephrlin treatment following burn injury reverses epigenetic and signaling changes in kidney tissue that lead to the activation of Rac1, and lowers elevations in markers of systemic oxidative stress such as urinary 8-isoprostane and plasma OHDG^[10-12]. Besides, an analysis of gene expression in the CNS after burn injury showed that nephrlin beneficially modulated the expression of genes associated with astrocytosis, oxidative stress, and immunosuppression^[13]. Trauma is characterized by the co-existence of pro-inflammatory and immunosuppression phenomena, strikingly reminiscent of what has long been described for metastatic niche formation^[15,16]. The role of oxidative stress and pro-fibrotic cytokines has been amply documented as instrumental in the sculpting of the latter environment(s). We asked whether nephrlin might modulate the latter environment, as we have shown that it has beneficial effects on the former.

Previous studies have shown that the lobes of the rodent right lung differ in their access to the arterial and lymphatic circulation, susceptibility to anoxia, and local concentrations of pro-inflammatory factors such as lung elastase^[17]. We were interested in examining the biodistribution and effectiveness of nephrlin peptide in the lobes of the rodent lung, to gain a better understanding of how nephrlin accesses deep lung environments. This variable is relevant in critical care settings, particularly in the context of major comorbidities of sepsis and burn trauma, such as ARDS^[18,19]. Nephrlin has shown beneficial effects in rodent models of sepsis and burn trauma, as noted above.

Finally, lung metastasis is accelerated by profibrotic and pro-metastatic factors such as CCL12 and TIMP-1^[20,21]. We examined the possible effects of nephrlin on the circulating levels of these factors.

METHODS

Reagents

Nephrlin peptide (1) and FITC-labelled tracer peptide, the IGFBP3-derived portion of the nephrlin peptide sequence (fitc-KKGFYKKKQCRPSKGRKRGFCW) were synthesized by Lifetein LLC (Hillsborough, NJ). Antibodies for ELISAs were purchased from Abcam (Cambridge, MA), and chemicals from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Murine models

Animals were housed in clean cages on a 12 h light/dark cycle with access to standard chow and water *ad libitum*. Animals were allowed to acclimate for one week before the experiment. All animal procedures were performed in adherence to the National Institute of Health's Guide for Care and Use of Laboratory

Animals and approved by the Institutional Animal Care and Use Committee (IACUC) of the Molecular Medicine Research Institute. At the end of the study, period animals were euthanized by decapitation as approved by MMRI IACUC guidelines, the NIH's Office of Laboratory Animal Welfare (OLAW), and AVMA recommendations.

For the lung metastasis study, 7-week old female *B6D2F1* mice (Charles River; 18-22 gram body weight) received implantation via tail vein of 1×10^5 B16MET-e100 tumor cells per animal. Beginning on Day 2, animals received either saline or nephrlin peptide (1.2 mg/kg) daily via subcutaneous bolus injection, for 3 weeks ($n = 6$ per group). At the end of the treatment period, animals were sacrificed and lung metastases in each lobe of the right lung were counted.

For biodistribution studies, 10-week old male *C57BL6* mice (Jackson Laboratories; 30 grams body weight) were used. Animals received either saline or nephrlin peptide (4 mg/kg) + FITC-labelled peptide tracer (1:100 molar ratio to nephrlin) via subcutaneous bolus injection once daily for seven days ($n = 3$ per group). The concentration of tracer peptide administered is too low to generate measurable biological effects, of itself. At the end of the treatment period, animals were sacrificed and left lung, left kidney, brain (left hemisphere), and liver (left half) were immediately stored in formalin for further histological analysis. Brain (right hemisphere), right kidney, liver (right half), plasma, and each lobe of the right lung were immediately frozen at -80°C for further analysis.

Plasma cytokine/chemokine analysis

Analysis of plasma was performed using a mouse cytokine 44-plex immunoassay (Custom Plex Discovery Assay, Eve Technologies, Calgary, AB). Statistically significant differences (Student's *t*-test, $P < 0.05$) between saline-treated and nephrlin-treated animals in the biodistribution experiment were found in the plasma levels of two of the 44 analytes tested.

Tissue extracts

Frozen tissue extracts were prepared using the CellLyticMT extraction buffer (Sigma-Aldrich, St. Louis, MO) as specified by the manufacturer. Extracts were stored at -20°C before assay by ELISA (for nephrlin peptide, using in-house polyclonal antibody #2501) or for succinate assay using a kit purchased from Megazyme, Inc (Chicago, IL).

Statistical analysis

Data are presented as means \pm SD unless otherwise indicated. Probability values (P values) were computed using Student's *t*-test and expressed relative to the saline-treated group.

RESULTS

Biodistribution of nephrlin

Figure 1 shows tissue sections of the brain, kidney, left lung, and liver from mice injected with either saline or nephrlin (4 mg/kg) + 1:100 FITC-tracer peptide (see methods). A fairly even distribution of peptide across tissues is visible in the slides, and corroborated by the biodistribution of nephrlin peptide in the brain (0.81 μg nephrlin/mg total tissue protein), kidney (0.97) and liver (0.74), as measured by ELISA. The concentration of nephrlin in the left lung was not measured, as that tissue was fixed in formalin. The concentration of nephrlin and tracer peptide in the right lung was measured. Table 1 shows the distribution of nephrlin in the four lobes of the right lung. There is a gradient of accumulation of peptide ($\mu\text{g}/\text{mg}$ total tissue protein) in the deep lobes: accessory (5.07) > inferior (3.45) > middle (2.84) > superior (2.12). This gradient is reminiscent of that previously shown for lung elastase in rodent lung^[17]. We also measured the concentration of succinic acid in tissue extracts of each lobe of the right lung. Interestingly,

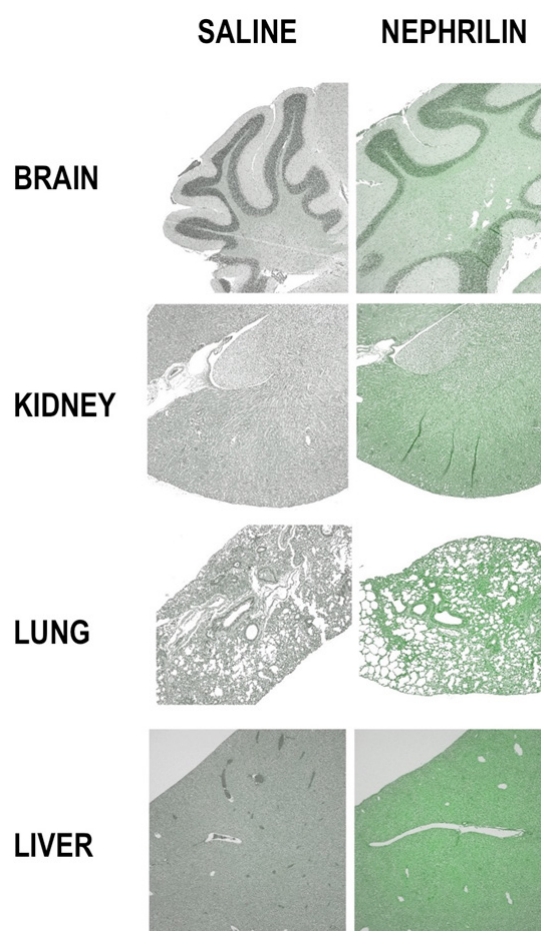


Figure 1. Biodistribution of tracer peptide in major organs. See text and Methods for a description of the tracer peptide and histological methods

Table 1. Biodistribution of nephrilin in right lung lobes^[1]

Treatment Group	Superior	Middle	Inferior	Accessory
Saline-treated	0.11 ± 0.32	0.19 ± 0.22	0.10 ± 0.12	0.10 ± 0.41
Nephrlin-treated	0.12 ± 0.37	0.84 ± 0.58	1.48 ± 0.57 ^{*,#}	3.07 ± 0.90 ^{*,#}

^[1]Tissue nephrlin measured by ELISA (μg nephrlin/mg tissue protein); ^{*}*P* < 0.05 vs. saline-treated group; [#]*P* < 0.05 vs. superior lobe

the concentration of succinic acid was 22% higher (*P* < 0.001) in the accessory, inferior and middle lobes, when compared to the superior lobe, suggesting lower oxygenation levels in those tissues.

Plasma cytokines

Of the 44 cytokine/chemokine analytes measured in plasma, only two differed significantly between the mice treated with nephrlin versus those treated with saline: CCL12 (MCP-5) and TIMP-1 were both significantly lower in the nephrlin group [Figure 2]. Both CCL12 and TIMP-1 are believed to play a pro-fibrotic role^[20,21] and fibrosis is canonically described as a feature of the tumor environment^[22].

Lung metastases

Table 2 shows B16MET melanoma lung metastases in the saline and nephrlin treated groups on Day 21. The average number of metastases decreases along with the superior > middle > inferior > accessory gradient, consistent with relative access to the circulatory system. However, the percent reduction in

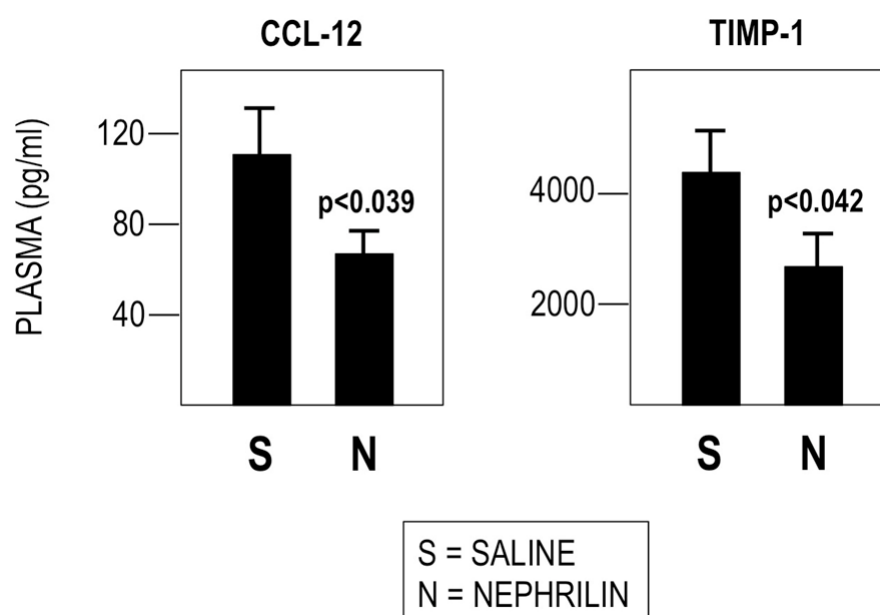


Figure 2. Plasma analytes in mice treated with saline or nephrlin for seven days

Table 2. B16MET melanoma metastases in lung lobes at 3 weeks post-injection^[2]

Treatment Group	Superior	Middle	Inferior	Accessory
Saline-treated	17.83 ± 3.43	15.83 ± 3.43	14.83 ± 2.64	6.17 ± 1.72
Nephrlin-treated	17.33 ± 3.33	11.83 ± 2.56*	10.00 ± 4.29*	3.33 ± 1.63*

^[2]All lobes combined: saline gp 54.67 ± 7.09, nephrlin gp 42.50 ± 7.66, $P = 0.017$; * $P < 0.05$ vs. saline-treated group

metastases in the nephrlin-treated group runs in the reverse direction of that gradient, from a 46% reduction in the accessory lobe to a 3% reduction in the superior lobe. Overall, nephrlin reduces B16MET lung metastases by 22% in all lobes combined ($P = 0.017$).

Figure 3 shows a strong and direct relationship between the accumulation of nephrlin in the various lobes and the reduction in metastases in those tissues ($r^2 = 0.98$).

DISCUSSION

Nephrlin's efficacy in reversing the systemic effects of sepsis and burn trauma^[9-11] appears to involve pro-homeostatic modulation of the underlying immune dysfunction. In this study, we measured the distribution of nephrlin peptide across lung tissues after administration via subcutaneous bolus injection. Surprisingly, substantial preference of peptide accumulation was observed in lobes of the lung that are distal to the circulation, an environment in which we also showed a higher accumulation of succinate, a proxy for anoxia^[23]. In previously published work, a similar gradient was shown for concentrations of lung elastase, fragments of which have been implicated in the inflammatory response within these tissues^[17].

Nephrlin is effective at reducing B16MET metastases to the lung in this murine model. The effectiveness of the peptide is directly proportional to the peptide's preferential accumulation in tissues of the deep lung. The potential clinical significance of this pattern of accumulation should be mentioned here: tissues of the deep lung have been associated with morbidity and mortality of respiratory distress syndromes and pneumonia, often comorbid with kidney dysfunction or sepsis, accounting for much of the deadliest burden in ICUs^[24,25]. Furthermore, these deep tissues are associated with lower oxygen tensions, a feature associated with metastatic niche^[26].

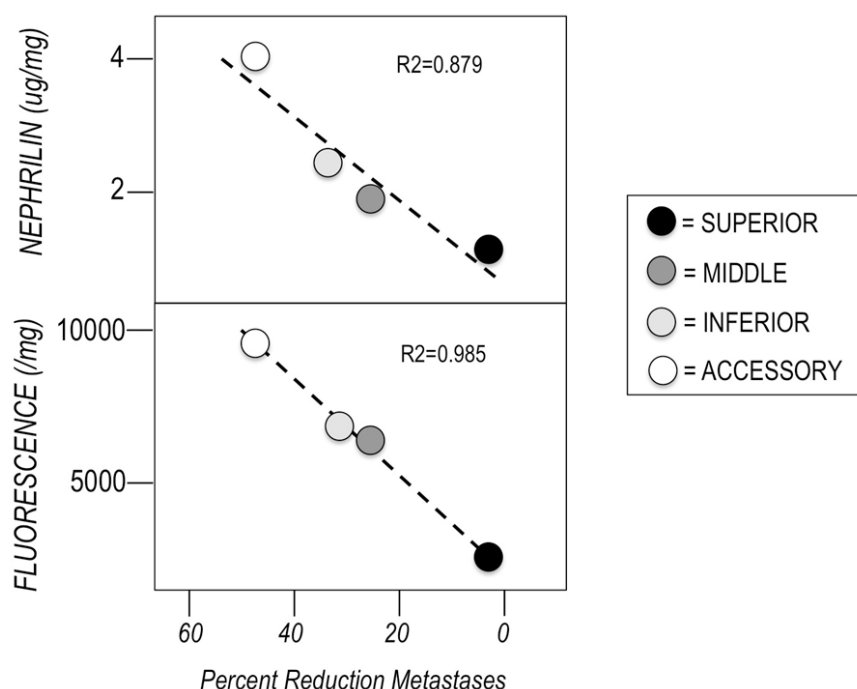


Figure 3. Biodistribution of nephrlin/tracer peptide and inhibition of metastases observed in lung lobes

Our observation that nephrlin treated animals have significantly lower plasma levels of the pro-fibrotic factors CCL12 and TIMP-1 is particularly intriguing. Fibrosis and metastasis have been linked in previous studies^[22]. Whether animals marked at the genetic loci for CCL12 and TIMP-1 might show lowered altered levels of susceptibility to the metastatic spread of melanoma is worthy of exploration.

In the context of metastatic melanoma, the ability to address the formation of a metastatic niche in the deep lung is an intriguing subject for future study. But our results also raise other questions: Can the unusual lung tissue targeting seen with nephrlin peptide in this study presage its use as a vehicle for deep-lung targeted treatments in general? Given the known efficacy of nephrlin peptides in models relevant to other aspects of critical care such as sepsis, burn trauma, and kidney function, the potential usefulness of this peptide for targeting treatments in ARDS is intriguing.

DECLARATIONS

Authors' contributions

Contributed to experimental work, experimental design and data discussion: Mascarenhas DD, Ravikumar P
Contributed to funding the research, discussing the data and manuscript writing: Mascarenhas DD, Amento EP

Availability of data and materials

For additional data requests, please contact the corresponding author.

Financial support and sponsorship

General funds from all participating institutions contributed to the funding of this study.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

All animal procedures were performed in adherence to the National Institute of Health's Guide for Care and

Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (IACUC) of the Molecular Medicine Research Institute.

Consent for publication

Not applicable.

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Original Article

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Burden of chronic diseases among sarcoma survivors treated with anthracycline chemotherapy: results from an observational study

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How to cite this article: Baker LH, Boonstra PS, Reinke DK, Peregrine Antalis EJ, Zebrack BJ, Weinberg RL. Burden of chronic diseases among sarcoma survivors treated with anthracycline chemotherapy: results from an observational study. *J Cancer Metastasis Treat* 2020;6:24. <http://dx.doi.org/10.20517/2394-4722.2020.36>

Received: 21 Apr 2020 **First Decision:** 5 Jun 2020 **Revised:** 12 Jun 2020 **Accepted:** 8 Jul 2020 **Published:** 7 Aug 2020

Academic Editor: Ian Judson **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Aim: Cardiovascular disease is a leading cause of mortality among long-term cancer survivors treated with large total doses of doxorubicin. An increase in coronary artery disease (CAD) among childhood cancer survivors by age 45 has been observed and is driven by primarily anthracycline chemotherapy and to a lesser extent chest radiation that includes the heart in the radiation field. The risk factors and associated chronic diseases (hypertension, *etc.*) are well known for CAD and can be often prevented or treated, thus reducing the risk of CAD in these patients. We piloted a risk-based survivorship clinic in an academic medical center to characterize the distribution of risk factors for CAD and improve the quality of life in a population of sarcoma survivors treated with doxorubicin.

Methods: We followed a prospective cohort of sixty-one survivors of bone and soft tissue sarcoma treated with doxorubicin chemotherapy ($> 400 \text{ mg/m}^2$) and at least 2 years post-therapy attending the sarcoma survivorship clinic. We collected clinical, demographic data, and patient reported outcomes via PROMIS questionnaires annually.

Results: We demonstrated a high burden of chronic diseases in this population. Among six chronic conditions that are known risk factors for CAD (hypertension, diabetes, obesity, chronic inflammation, kidney disease and



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dyslipidemia), more than one-fourth (26%, 16/61) of patients had three or more of these risk factors at baseline visit, and 49% (30/61) had two or more.

Conclusion: The results of this pilot study support the presence of modifiable CAD risk factors in this population of sarcoma survivors. Evidence-based guidelines for high-risk survivors of rare cancers are needed.

Keywords: Coronary artery disease, cardiovascular disease, sarcoma, anthracycline, cancer survivor

INTRODUCTION

In 1973, the Southwest Oncology Group (SWOG) reported a phase II trial of 472 patients accrued in 17 months that demonstrated the clinical efficacy of an exciting new cytotoxic chemotherapy, doxorubicin in treating patients with advanced breast cancer, Hodgkin's lymphoma, and multiple types of sarcomas^[1]. SWOG further reported findings of cardiac toxicity manifested as irreversible heart failure^[2]. In a subsequent follow-up publication, the impact of cumulative dose on long-term effects, most notably cardiac toxicity, leading to dose-limiting treatment schedules was described^[3]. The first attempt to diminish this cardiomyopathy was also reported by SWOG in a randomized clinical trial reporting the benefit and toxicity of doxorubicin administered as a bolus injection vs a continuous infusion over 96 h^[4]. The infusion schedule was associated with less toxicity. The importance of anthracycline chemotherapy in pediatric and adult populations was underscored, but was rapidly followed by the recognition that the long-term effects were unknown, especially in pediatric populations. The launch of the landmark Childhood Cancer Survivor Study in 1994 provided a powerful tool to account for the long-term effects of anthracycline chemotherapy on the first cohort of pediatric patients, those treated between 1970 and 1986^[5]. Following the example of this important study, the focus has been on retrospective analysis and surveillance of the long-term effects of treatment.

Anthracycline chemotherapy and chest radiation make cardiovascular disease (CVD) a leading cause of morbidity and mortality among childhood cancer survivors^[6-9]. Cardiomyopathy secondary to cumulative doxorubicin exposure has been a concern among oncologists, because it is often irreversible and fatal^[10]. Over the years we have learned to prevent this cardiomyopathy by capping the dose and/or adding dexrazoxane^[11]. More recently, the Childhood Cancer Survivor Study suggested that whereas the incidence of cardiomyopathy decreases since the time of treatment, the incidence of coronary artery disease (CAD) increases^[12,13]. Cumulative incidence of CAD now significantly exceeds the cardiomyopathy by age 45^[13]. CAD is also associated with doxorubicin exposure, presumably by altering the innate immune pathway and initiating CAD^[14]. CAD is associated with several modifiable risk factors and chronic diseases including hypertension, dyslipidemia, tobacco use, metabolic syndrome, and diabetes. These chronic illnesses are most often managed by family physicians and primary internists.

Effective management of these risk factors reduces the incidence of CAD^[7]. Exposure to anthracycline chemotherapy and radiotherapy are recognized as CVD risk factors on par with hypertension and diabetes, but are not included in cardiovascular risk prediction tools, nor is high-sensitivity C-reactive protein (hsCRP), which Ridker and colleagues have identified as a key linkage of inflammation induced by anthracycline and CAD, leaving individual practitioners to determine risk^[15-18]. While the American College of Cardiology/American Heart Association (ACC/AHA) guidelines on the management of cholesterol and the guidelines for primary prevention of CVD recommend using the Pooled Cohort Equation to estimate cardiovascular risk in the general population^[19,20], this and other risk calculators do not account for anthracycline chemotherapy or chest radiation, key drivers of CAD risk in survivors of cancer, especially in younger patients. Most risk-scoring systems do not include patients at or younger than age 40, which

is concerning as this is precisely the population of cancer survivors that demonstrates manifestation of CAD. Chow *et al.*^[12] developed a prediction model to account for treatment-related risk factors and age at diagnosis based on 5-year cancer survival, but did not include hypertension, dyslipidemia, and diabetes. Conventional risk factors (obesity, hypertension, dyslipidemia, and diabetes) have been characterized by some as non-cancer related issues^[21]. However, we now have evidence that obesity, hypertension, diabetes and lipid abnormalities can and do result from cancer therapies. The adolescent who has a life-long disability from an amputation-sparing knee reconstruction should expect decreased mobility and an increased risk of chronic pain, depression, and obesity as a consequence of cancer diagnosis and care. In the absence of guidelines for the timing and frequency of surveillance of chronic conditions and management of CAD risk factors, oncology providers are left with inadequate knowledge about (what may be perceived as) non-oncology care, and primary care providers are left with inadequate knowledge about cancer-specific follow-up care^[22].

Adult survivors of childhood cancers, especially those treated with anthracycline chemotherapies, are at a much higher risk of developing chronic conditions than other cancer populations^[5,12,13], making the years post-treatment a crucial window of opportunity to diagnose and manage chronic health conditions. Survivors of osteosarcoma and Ewing sarcoma have a 39-fold greater risk of acquiring severe, life-threatening, and even fatal chronic disease(s) than their siblings^[5]. However, adherence to long-term follow-up among adolescent and young adult survivors of childhood cancers sharply declines after treatment ends, with primary care filling the healthcare gap for survivors of childhood cancers^[23]. A 2014 survey of general internists, published in the *Annals of Internal Medicine*, queried their comfort level and preferences for caring for survivors of childhood cancers^[24]. A sizeable minority of respondents reported being “somewhat comfortable” or “comfortable” caring for Hodgkin’s lymphoma, acute lymphoblastic leukemia, and osteosarcoma patients (36.9, 27.0 and 25.0%, respectively), presumably due to the rarity and treatment complexities of these diseases. A similar survey of 2,520 family physicians in the United States and Canada confirmed that physicians were equally uncomfortable caring for survivors of Hodgkin’s lymphoma, acute lymphoblastic leukemia, and osteosarcoma^[25]. This study further revealed that 81% of respondents had cared for two or fewer survivors of childhood cancer in the preceding five years! It is reasonable to posit that the majority of primary care physicians, including both pediatricians and internists, will never see a patient with osteosarcoma in their practice. Knowledge of rare cancers accumulates with the experience of the oncologist, and long-term care for survivors is no different. Thus, a model of care tailored to the needs of these patients is necessary to prevent and manage chronic late effects.

To move the study of survivorship care forward we sought not to replicate the model of surveillance of the long-term effects of anthracycline chemotherapy, but to actively intervene and treat those long-term survivors for dyslipidemia, hypertension, obesity, diabetes, anxiety and depression. This model was created in recognition that the patients being monitored for recurrence were developing chronic conditions which were not being addressed in part due to lack of knowledge from primary care physicians or a paucity of care, such as in the mental health care field. Recognizing that a “one size fits all” approach will not adequately serve the heterogeneous population of cancer survivors, we piloted a survivorship clinic for survivors of bone and soft tissue sarcomas led by a medical oncologist^[26]. This prospective cohort study was conducted to identify and treat risk factors for CAD among adult survivors of sarcoma, including those diagnosed as children, adolescents and young adults, and adults, as sarcomas occur at all ages. Risk factors for CAD are well described and often modifiable^[14,19]. We share preliminary data from the fifth year of this prospective cohort study confirming a significant burden of chronic diseases in this population.

METHODS

Study population

To be eligible for enrollment in the survivorship clinic, survivors had to be 18 years or older at time of first visit, be at least two years past active chemotherapy (adjuvant or neoadjuvant doxorubicin), and be willing

to return for an annual visit. The first enrollment was in October 2014 and the most recent was in May 2019. Survivors could be either self- or provider-referred and did not have to have received treatment at the host institution. Our study was approved by our institution's Institutional Review Board (HUM00095825), and informed consent was obtained from all patients. Patients who did not consent to participate in the prospective cohort study were not included in this analysis but are still seen in the clinic.

Risk-based survivorship clinic

In the absence of existing clinical guidelines for the care of this population of high-risk survivors of sarcoma, consultation was sought from nephrology, cardiology, endocrinology, lipidology, psycho-oncology, physical medicine, and nutrition in development of the clinical protocol with a focus on laboratory surveillance for the chronic diseases. Biometric and laboratory data were collected to encompass the conventional cardiovascular risk factors of hypertension and elevated total cholesterol, as established by the Framingham Heart Study^[27], obesity and high-sensitivity C-reactive protein hsCRP as found in the Reynolds Risk Score^[28], and diabetes as advocated by the ACA/AHA^[29]. The Multi-Ethnic Study of Atherosclerosis risk calculator includes coronary artery calcium scores, which will be collected going forward^[29]. Importantly, while the Pooled Cohort Equation can calculate a lifetime risk score for persons aged 20-59 years, no risk assessment is intended to accurately predict risk in individuals under age 30 and certainly not with a history of anthracycline chemotherapy and/or chest radiation.

At the enrollment visit and annually thereafter, patients received a comprehensive health examination with a focus on cardiovascular illness, patient-centered surveillance based on past therapy, family history, genetic predispositions, lifestyle behaviors, and comorbid health conditions. Patients were assessed for musculoskeletal dysfunction, metabolic syndrome, diabetes mellitus, hypertension, cardiac diseases, anxiety/fear disorder, depression, renal insufficiency, and obesity. Treatment history, demographic, family history, biometric, and patient risk assessment data were also collected at each visit. The patient's risk assessment was reviewed with the nurse practitioner, who engaged the patients in education about their risks and counseled them on lifestyle changes to reduce their risk of chronic illness, such as exercise, weight reduction, nutrition modification, and mindfulness. Surveillance for disease recurrence was personalized to the primary sarcoma. Clinic visits averaged 60+ min of face-to-face interaction with the clinician. The nurse practitioner prepared the initial draft of the survivorship care plan after each annual visit, which was shared with the patients and their providers. The survivorship care plan included laboratory test results, imaging studies, treatment history, a cancer screening schedule, medication and vaccination review, and management plan. About 1.5-2.5 h were spent on pre-visit patient care and after-visit documentation and in the creation of the survivorship care plan.

Data collection

Patients annually completed a health questionnaire that included family history, fertility, behavior, patient risk assessment, and patient-reported outcome measures, the results of which would be reported in a subsequent paper. We used the Patient-Reported Outcomes Measurement Information System (PROMIS), developed by the National Institute of Health (NIH) to collect PROs using PROMIS Anxiety Short Form 8a v1.0, Depression Short Form 8a v1.0, Sleep Disturbance Short Form 6a v1.0, Pain Interference Ca Bank v1.1, Physical Function Ca Bank v1.1, and Global Health Scale v1.2. Patients completed the health questionnaire via an electronic link prior to each clinic visit to support clinical care, and the data were collected and managed using Research Electronic Data Capture (REDCap) tools hosted at University of Michigan, a HIPAA-compliant web-based data capture application and database^[30]. Biometric data and imaging were included as standard of care. Laboratory and clinical data were extracted from the patient's electronic medical record and entered into the REDCap database.

Table 1. Summary of patient characteristics at first clinic visit (baseline)

Demographic characteristics	Median (IQR) prevalence (n)	Range	Missing
Age (at diagnosis)	24.0 (14.0, 48.0)	(2.0, 67.0)	0
Age (at enrollment)	42.0 (30.0, 60.0)	(18.0, 82.0)	0
Age \geq 40 (at enrollment)	54% (33/61)		0
Years DX to enrollment	15.0 (10.0, 20.0)	(4.0, 42.0)	0
Female sex	59% (36/61)		0
BMI (kg/m ²)	26.9 (24.1, 30.8)	(15.9, 46.5)	1
Smoked, ever smoked	30% (17/56)		2
Alcohol, current use	57% (33/58)		0
Drugs, current use	2% (1/54)		4
Exercise, currently exercise	53% (31/58)		0
Treatment history			
Chemotherapy	100% (61/61)		
Radiation to primary site	49% (30/61)		0
Surgery	89% (54/61)		0
Chemotherapy and radiation	51% (31/61)		0
Clinical lab values			
ALT (IU/L)	21.0 (16.0, 34.5)	(9.0, 65.0)	9
AST (IU/L)	23.0 (20.0, 29.0)	(14.0, 46.0)	9
LDL (mg/L)	85.0 (75.0, 102.5)	(31.0, 175.0)	3
HDL (mg/L)	56.0 (47.0, 73.0)	(29.0, 102.0)	2
Total cholesterol (mg/dL)	169.0 (156.0, 193.5)	(98.0, 266.0)	2
Triglycerides (mg/dL)	119.0 (74.0, 160.0)	(36.0, 269.0)	2
Glucose (mg/dL)	93.0 (83.5, 100.0)	(61.0, 154.0)	2
hsCRP (mg/L)	2.1(0.6, 6.9)	(0.2, 20.0)	4
Creatinine (mL/min)	91.0 (73.0, 112.8)	(41.0, 179.0)	3
Protein/creatinine ratio (mg/g)	77.0 (61.5, 99.0)	(2.6, 161.0)	9
SBP (mmHg)	119.0 (108.0, 135.0)	(85.0, 182.0)	0
DBP (mmHg)	67.0 (60.0, 76.0)	(42.0, 97.0)	0

BMI: body mass index; ALT: alanine aminotransferase; AST: aspartate aminotransferase; LDL: low-density lipoprotein cholesterol; HDL: high-density lipoprotein cholesterol; hsCRP: high-sensitivity C-reactive protein; SBP: systolic blood pressure; DBP: diastolic blood pressure

Statistical analysis

Clinical and laboratory measurements from each annual visit were compared to reference ranges to determine whether the measurement would be considered “abnormal”. The distributions and co-occurrences of abnormal hsCRP (≥ 2.0 mg/L), high body mass index (BMI > 30 kg/m²), and previously diagnosed risk factors (specifically, prior clinical diagnosis of type II diabetes, cardiovascular disease, hypertension, renal insufficiency, or high cholesterol) were graphically summarized. Statistics were stratified by age at enrollment: less than 40 years old (“18-39”) or at least 40 years old (“ ≥ 40 ”). No formal statistical inferences were conducted, due to the exploratory nature of the study and the limited sample size.

RESULTS

A total of 61 patients had an enrollment (“baseline”) visit between October 2014 and May 2019. Among these, 43, 24, 12 and 3 patients had a total of one, two, three, or four subsequent annual follow up visits, respectively. Sixteen patients are no longer followed due to: sarcoma recurrence ($n = 2$); a new primary cancer (1); patient withdrawal due to geographic relocation, time barriers, or loss of insurance (8); physician withdrawal (1); other loss to follow-up (3); or death (1). Among 45 actively enrolled patients, 6 enrolled within 12 months of the current data snapshot, meaning that the maximum possible number of patients with at least one annual follow-up visit was 49 (43 recorded plus 6 potential).

Baseline patient characteristics, including clinical and laboratory data, are summarized in [Table 1](#). The median age at first sarcoma diagnosis was 24 years (range 2-67), and the median age at baseline clinic visit

Table 2. Patient frequency of abnormal readings at baseline, 12 months, and 24 months after enrollment

Variable	Normal Range	Abnormal (%), Baseline	Abnormal (%), 12M	Abnormal (%), 24M
BMI (kg/m ²)	≤ 30	28% (17/60)	18% (6/34)	27% (6/22)
ALT (IU/L)	≤ 30	35% (18/52)	23% (7/31)	14% (3/21)
AST (IU/L)	≤ 35	10% (5/52)	6% (2/31)	5% (1/21)
LDL (mg/L)	≤ 135	9% (5/58)	6% (2/33)	10% (2/21)
HDL (mg/L)	≥ 40	15% (9/59)	3% (1/33)	10% (2/21)
Total cholesterol (mg/dL)	≤ 200	19% (11/59)	21% (7/33)	19% (4/21)
Triglycerides (mg/dL)	≤ 200	15% (9/59)	6% (2/33)	29% (6/21)
hsCRP (mg/L)	≤ 2	51% (29/57)	61% (20/33)	57% (12/21)
Glucose (mg/dL)	≤ 100	20% (12/59)	18% (6/33)	52% (11/21)
Creatinine (mL/min)	≥ 88	45% (26/58)	41% (14/34)	43% (9/21)
SBP (mmHg)	≤ 130	28% (17/61)	32% (11/34)	45% (10/22)
DBP (mmHg)	≤ 80	13% (8/61)	9% (3/34)	14% (3/22)

BMI: body mass index; ALT: alanine aminotransferase; AST: aspartate aminotransferase; LDL: low-density lipoprotein cholesterol; HDL: high-density lipoprotein cholesterol; hsCRP: high-sensitivity C-reactive protein; SBP: systolic blood pressure; DBP: diastolic blood pressure

was 42 years (range 18-82). Twenty-eight patients (46%) were between the ages of 18 and 39 at the time of the first clinic visit and 64% ($n = 39$) were diagnosed at age 39 years or younger. All patients received doxorubicin adjuvant chemotherapy (by definition of our study population), 89% had cancer surgery, and 49% received external radiation (adjuvant, neoadjuvant). The median number of years between diagnosis and baseline visit was 15 years (range 4-42).

Laboratory measurements taken at each visit (baseline, one year, and two years) that fell outside the corresponding reference range and were therefore considered abnormal are summarized in Table 2. Excluding missing values, the measurements with the largest percentage of abnormal values were hsCRP, with 51% (29/57) having a measurement of 2.0 mg/L or greater at baseline, and creatinine clearance, with 45% (25/55) having a measurement below 88 mL/min. Among the patients with a 1-year (12-month) or 2-year (24-month) visit, the percentages of abnormal hsCRP measurements increased slightly to 61 and 57%, respectively, and the percentages of abnormal creatinine clearance measurements increased slightly to 48 and 47%, respectively.

Figures 1 and 2 plot the baseline distribution of risk factors for major cardiovascular events, stratified by age group. From Figure 1, 16 (26%) patients had at least three risk factors, and 30 (49%) had at least two. Nineteen patients aged 40 or older (58%) had at least two CVD risk factors identified, and 12 (36%) had three or more risk factors. In 9 out of these 12 patients, one of the contributing risk factors was hsCRP \geq 2.0 mg/L. Among patients aged 18-39, 11 (39%) had at least two CVD risk factors identified, and four (14%) patients had three or more risk factors. In all four of these patients, one of the contributing risk factors was hsCRP \geq 2.0 mg/L. Figure 2 indicates that the prevalence of high BMI, high total cholesterol, and type 2 diabetes was comparable between patients aged 18-39 and \geq 40. Among patients aged 18-39, hypertension was present in 4 (14%) patients. The biomarker hsCRP, indicative of inflammation and a recognized CAD risk enhancer^[20], was recorded in 26 patients aged 18-39; in 10 of these patients (38%), it was measured at levels > 2 mg/dL, and in 6 patients (23%), it was measured at levels > 10 mg/dL, presented in Figure 3.

DISCUSSION

Retrospective cohort studies of childhood cancer survivors have shown that exposure to anthracycline chemotherapy and external radiation that included the heart in the field at a young age are compounded by conventional risk factors, such as obesity, hypertension, and lipid disorders^[5,7]. A retrospective study of adult cancer survivors similarly identified poorer overall survival for patients who subsequently developed CVD as well as a relationship between therapeutic exposure and the development of CVD^[31]. Our cohort

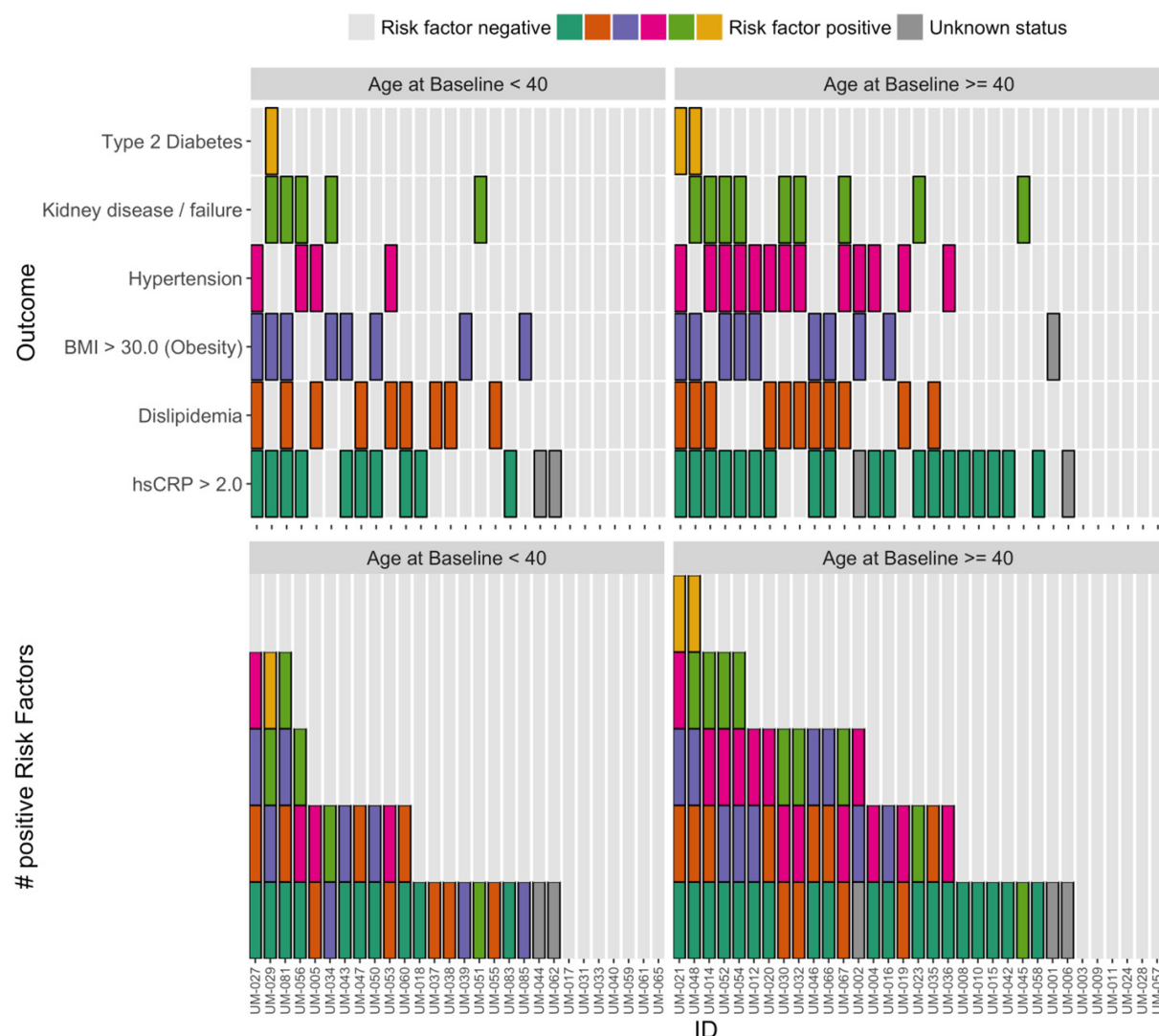


Figure 1. Types (top panel) and numbers (bottom panel) of risk factors identified prior to enrollment by patient, grouped by age at baseline. Note: except for body mass index (BMI) and high-sensitivity C-reactive protein (hsCRP), these are distinct from, and derived using different data than, the baseline laboratory measurements

of sarcoma survivors treated with moderate dosing of anthracyclines confirms the presence of multiple CAD risk factors, beyond what would be expected in a healthy population, particularly so for patients younger than 40. The presence of these chronic conditions demonstrates the need for active prevention and management of late effects, in addition to monitoring for relapse. This clinic was piloted solely for survivors of bone and soft tissue sarcomas treated with anthracycline chemotherapies.

Patients are educated about their risks of developing chronic conditions as a consequence of their sarcoma treatment. Our patients are counseled on behavioral and lifestyle approaches to reduce these risks, and referred to needed medical services such as psycho-oncology when available or a social worker, nutritional counseling, and physical medicine to decrease pain and improve mobility (especially for those who have had limb salvage surgery). Patients who present with a systolic blood pressure > 130 are urged to obtain a home blood pressure cuff and taught to take measurements in accordance with best practices (twice in the morning). Patients are encouraged to report their blood pressure readings electronically with the provider to accurately assess their blood pressure in acknowledgement that some patients experience transient

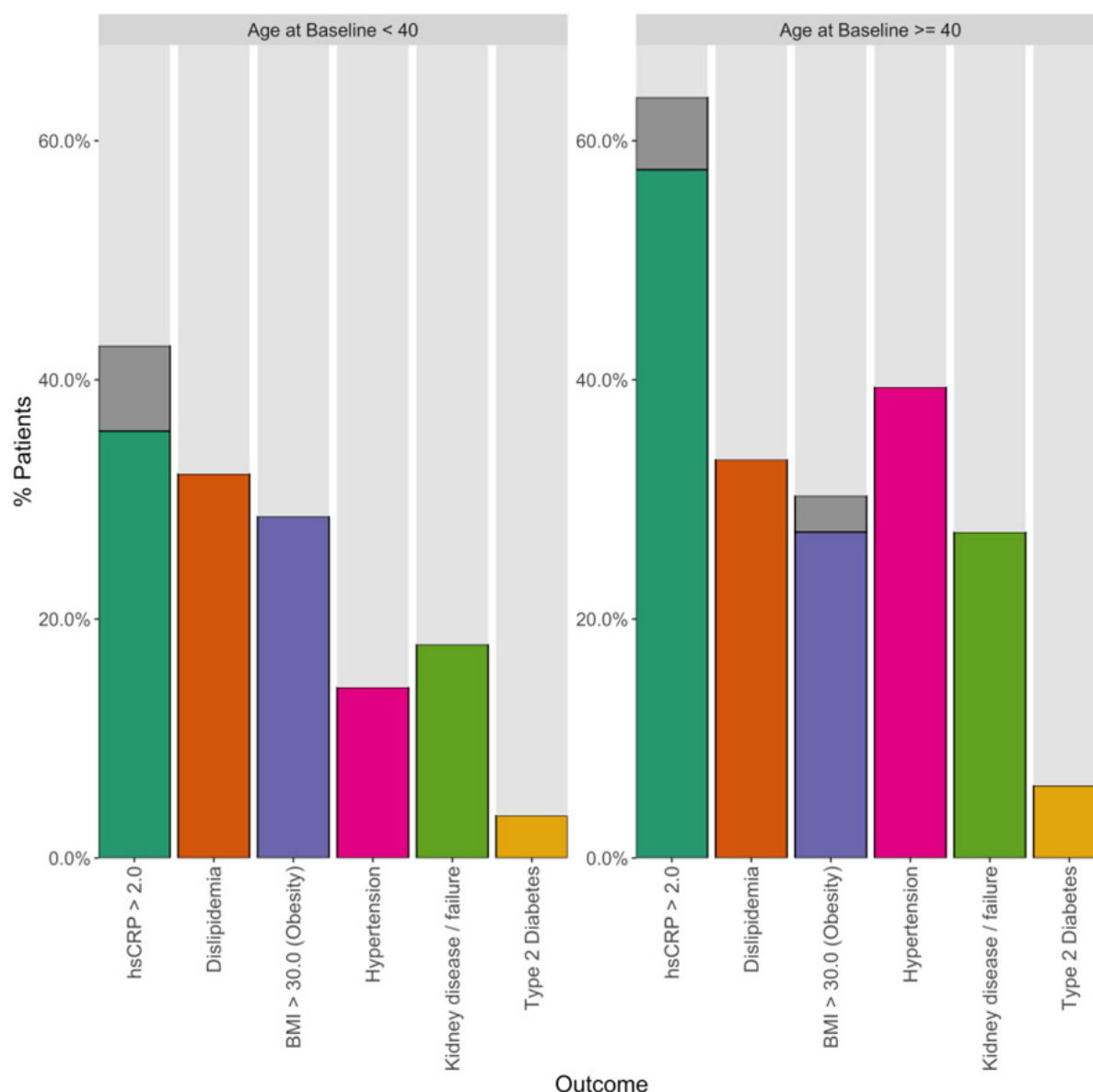


Figure 2. Percentages of individual risk factors identified at enrollment across all patients, stratified by age at baseline. Note: except for body mass index (BMI) and high-sensitivity C-reactive protein (hsCRP), these are distinct from, and derived using different data than, the baseline laboratory measurements

elevations in oncology settings. Going forward, patients will have received a coronary artery calcium score to monitor for coronary artery calcification in lieu of chest X-ray alone. Surprisingly, in our series, three patients aged 18-24 were found to have visible coronary artery calcifications on routine chest computed tomography as part of their malignancy surveillance. We have learned from cardiologists that CAD is at least in part an inflammatory process. We routinely measure hsCRP and more than half of our patients had maintained an elevation of hsCRP; Isolated elevations are most often the consequence of infection. Experimental evidence demonstrates inflammatory changes soon after doxorubicin administration^[32]. All patients with abnormal renal function are cautioned about the use of contrast agents for imaging and ibuprofen for pain management.

All patients are given a survivorship care plan and instructed to share their care plan with their other providers. Staffed by a medical oncologist and nurse practitioner, considerable time is dedicated to

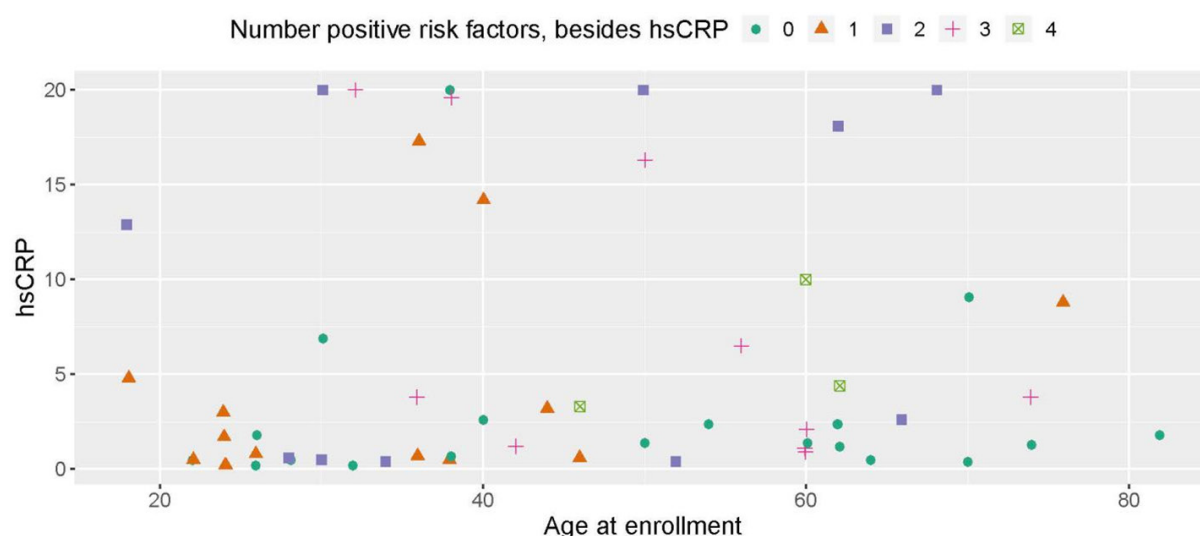


Figure 3. Distribution of high-sensitivity C-reactive protein (hsCRP) measurement by age and positive risk factors

extended clinic visits, pre-visit patient care and post-visit documentation and creation of the survivorship care plan. First visit preparation, face-to-face evaluation, and discussion routinely extend beyond 90 minutes, though visit duration decreases with subsequent visits. We monitor the patient for increased awareness of risk factors and are pleased to see improvement in many patients.

We suggest that patients with bone sarcoma and soft tissue sarcomas exposed to anthracycline chemotherapy are best followed within a medical oncology setting. Medical oncologists have the expert knowledge of sarcoma and sufficient familiarity of survivorship literature as well as the skills in the principles and practice of internal medicine. For example, owing to the widespread use of tyrosine kinase inhibitors oncologists have had to learn again the medical management of hypertension. Management of these complex patients requires knowledge of the patient's treatment history and associated complications, management of the suite of chronic conditions that develop over time, and a foundational relationship between the provider and the patient. It may be necessary to share these responsibilities with a willing primary internist experienced in aggressive management of lipids, hypertension, diabetes, and obesity with clear pathways of care coordination and communication. We have learned at our institution that those trained in Med-Peds are most often receptive to a shared care model.

Benefits to these survivor patients not only can be measured in quality of life but also lifespan. Prevention of catastrophic chronic medical conditions with active management strategies clearly benefits society as well. The cost in time and resources in providing this care is challenging in the current climate of minimizing return visits within oncology, reducing visit times, the utilization of extenders to conduct follow-up care, and transiting patients to primary care for management of their chronic diseases. Medical educators should be encouraged to modify medical or Med-Peds curricula to accommodate this growing societal need as the population of cancer survivors continues to grow. Experts predict that there will be more than 20 million cancer survivors by 2025. The cost of these practices to the patients as observed in our clinic is that patients are not made aware of their risks of developing chronic diseases, are unaware that they should be monitored for chronic diseases following treatment, especially at a young age, and are not having their chronic diseases managed by anyone. The frequency of risk factors in younger survivors of our clinic suggests that there is a window of opportunity to have a great impact on quality of life and longevity among patients already burdened by the experience of cancer at a young age.

We have demonstrated a burden of chronic diseases in a subset of this young population. Evidence-based guidelines are necessary to strengthen the care of sarcoma survivors but not currently offered by the National Comprehensive Cancer Network or any other body. The development of guidelines to care for sarcoma survivors has not been supported by the National Institute of Health in medical oncology. However, NIH support is increasing in pediatric oncology and so is significant support of survivorship issues in more common cancers such as breast, colon, and prostate. We recommend the continuation and expansion of this prospective study to identify and characterize risk factors and to establish guidelines for sarcoma survivors and others treated with anthracyclines. Our cohort provides rich data characteristic of a prospective study. Oncology-based survivorship care for survivors treated with anthracycline chemotherapy necessitates addressing systemic barriers within the current health system. Recognizing that some of the data are not consistent with other data presented on adolescent and young adult survivors only underscores the relevance of disease and treatment specificity when caring for cancer survivors.

Time and resources are needed to expand this model of care to cancer survivors treated with anthracycline chemotherapy. However, the cost to the growing population of cancer survivors will arguably be higher still in terms of the chronic disease burden and overall loss in quality of life. As the survivorship population grows, so will the healthcare burden of unchecked development of chronic diseases within this heterogeneous population. Our obligation to the patients does not end with the pronouncement of cure but continues to shepherd the patient to a healthy future.

DECLARATIONS

Authors' contributions

Contributed to the writing of this manuscript: Baker LH, Boonstra PS, Reinke DK, Peregrine Antalis EJ, Zebrack BJ, Weinberg RL

Made substantial contributions to the conception, design, conduct, interpretation of the results: Baker LH, Reinke DK

Performed data analysis and interpretation of results: Boonstra PS

Made contributions to the interpretation of results: Zebrack BJ, Weinberg RL

Performed data acquisition, interpretation of results, and administrative, technical, and material support as well: Peregrine Antalis EJ

Availability of data and materials

Not applicable.

Financial support and sponsorship

This research was funded by The Hope Foundation and the Robert and Heather Urich Foundation.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

The study was approved by Institutional Review Board (HUM00095825) and consent was obtained from patients included in the study.

Consent for publication

The consent for publication was obtained from patients included in the study.

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Original Article

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Early detection of non-small cell lung cancer in liquid biopsies by ultrasensitive protease activity analysis

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How to cite this article: Udukala DN, Wendel SO, Wang H, Yapa AS, Covarrubias-Zambrano O, Janik K, Gadbury G, Troyer DL, Bossmann SH. Early detection of non-small cell lung cancer in liquid biopsies by ultrasensitive protease activity analysis. *J Cancer Metastasis Treat* 2020;6:25. <http://dx.doi.org/10.20517/2394-4722.2020.45>

Received: 12 May 2020 **First Decision:** 23 Jun 2020 **Revised:** 3 Jul 2020 **Accepted:** 14 Jul 2020 **Published:** 7 Aug 2020

Academic Editor: Wei Zhang **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Aim: A significant fraction of mortalities from non-small cell lung cancer could be prevented, if the cancer would be diagnosed earlier. Nanobiosensors for the ultrasensitive detection of active proteases in serum were designed to detect a significant protease activity signature of non-small cell lung cancer (stage I and higher).

Methods: We determined the activity of nine protease biomarkers in the sera of non-small cell lung cancer patients and compared them with the protease activities of a control group of healthy human subjects using optical nanobiosensors. They consist of a central Fe/Fe₃O₄ core/shell nanoparticle with an attached Fluorescence resonance energy transfer-pair [tetrakis-carboxyphenyl porphyrin (TCPP) and cyanine 5.5]. TCPP is attached to the central nanoparticle via a protease-cleavable tether, whereas cyanine 5.5 is tethered permanently to the dopamine-layer surrounding the nanoparticle.

Results: Based on the activity pattern of urokinase plasminogen activator, matrix metalloproteinases 1, 2, 3, 7, 9, and 13, and cathepsins B and L as well, non-small cell lung cancer could be detected at stage I by means of a liquid biopsy.



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Conclusion: This feasibility study, comprising 33 non-small cell lung cancer patients and 20 apparently healthy subjects, clearly demonstrated the feasibility of minimally invasive early diagnosis of non-small cell lung cancer, starting with stage I.

Keywords: Non-small cell lung cancer, lung cancer diagnosis, protease activity, liquid biopsies, iron/iron oxide nanoparticles, significance table, multivariate model

INTRODUCTION

Lung cancer is the second most common cancer in men and women. For 2020, a total of 228,820 new cases are estimated. For both genders, lung cancer is on top of the cancer mortality ranking in the US, and 135,720 cancer mortalities are anticipated this year^[1]. Approximately 13% of all lung cancers are small cell lung cancers (SCLC), and 84% are non-small cell lung cancers (NSCLC). Here, we focus on the detection of NSCLC by means of optical nanobiosensors capable of determining the activity of signature proteases in serum. In [Figure 1](#), the 5-year survival of NSCLC patients as a function of cancer stage at the time of lung cancer diagnosis is shown. The staging system used for NSCLC in this report is the American Joint Committee on Cancer (AJCC) TNM system, which is based on the size and extent of the main tumor (T), the spread to nearby lymph nodes (N), and the metastasis to distant sites (M)^[2]. It is clearly discernible that the 5-year survival rate significantly decreases if the cancer is diagnosed late. This implies that early detection of cancer saves lives. On the basis of the 5-year survival statistics of NSCLC, we conservatively estimate that about 30 percent of mortalities could be prevented if NSCLC were routinely detected at stage 1 (combined T1a, T1b, and T1c) instead at stages 2 or 3, when it is currently diagnosed^[3]. The optical nanobiosensors developed in the Bossmann group feature sub-femtomolar limits of detection, thereby permitting the diagnosis of NSCLC by means of a liquid biopsy utilizing the serum of cancer patients^[4-7].

Liquid biopsies

In 2000, Veridex introduced the first commercially available liquid biopsy assay, the CELLSEARCH® CTCtest^[8]. In 2016, the Food and Drug Administration (FDA) approved the cobas® epidermal growth factor receptor (EGFR) Mutation Test for determination of the EGFR gene mutation in blood from lung cancer patients^[9]. Commercially available liquid biopsies consist in the detection of circulating tumor cells (CTCs), tumor-derived exosomes, circulating cell-free DNA (cfDNA), microRNA (miRNA), signaling proteins and metabolic enzymes (proteases and kinases)^[10]. Virtually all companies in this field, among them Personal Genome Diagnostics^[11], Genomic Health^[12], Myriad Genetics^[13], Guardant Health^[14] and Pathway Genomics^[15] rely on PCR to detect genetic mutations, and various RNAs that are overexpressed in tumors^[11-15]. CancerSEEK evaluated the plasma levels of 8 cancer-indicating proteins and the presence of mutations in 2,001 genomic positions for detecting 8 solid tumor types, among them pancreatic cancer with an overall percentage of cancer detection of 62% at > 99% specificity^[16]. DETECT-A (detecting cancers earlier through elective mutation-based blood collection and testing) was able to detect 26 cancers by means of a liquid biopsy (nine lung cancers, six ovarian cancers and two colorectal cancers). Seventeen (65%) of the 26 cancers were localized or regional, including five patients with stage I tumors^[17]. The GRAIL technology looks at differences in DNA methylation between cancerous and healthy cells. It is capable of detecting > 50 different cancers from one liquid biopsy and has a very low false positive rate. Regardless of the cancer detection technology used, it is of vital importance to detect cancers at AJCC stage I or earlier to maximize cancer survival^[17]. GRAIL is promising early detection capability, because changes in DNA methylation occur early in cancer^[18,19]. As for all approaches, including the optical nanobiosensors for protease activity profiling discussed here, future comparative clinical trials will permit a true comparison of genomic vs. other methods of early cancer detection.

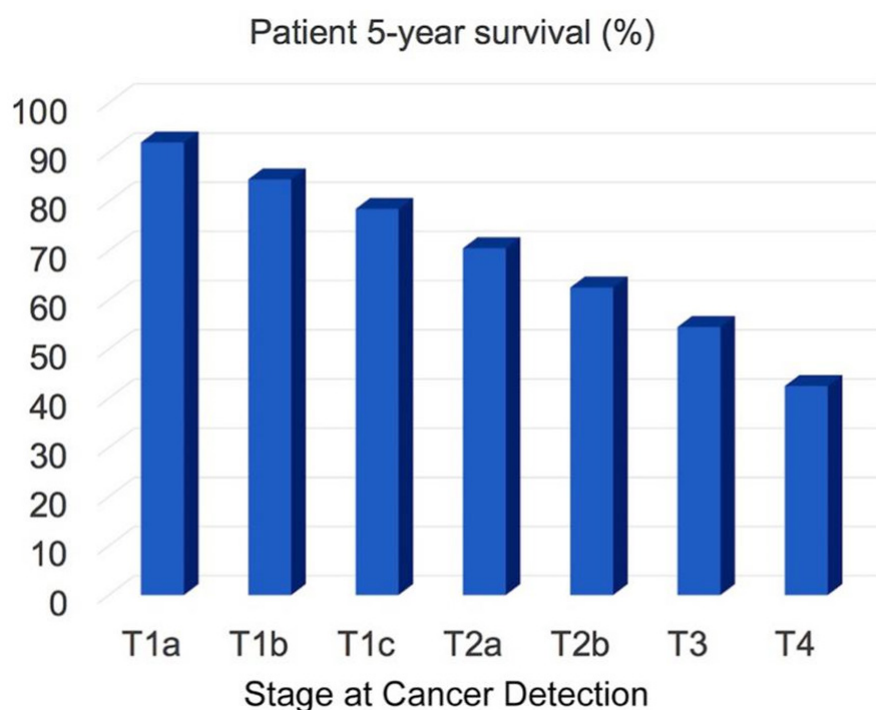


Figure 1. Patient 5-year survival (in percent) as a function of cancer stage at the time of cancer diagnosis. Data from references^[1-3]

Optical nanobiosensors for protease detection

It is established that virtually all solid tumors are characterized by dysfunctional protease expression patterns^[20-22]. These deviations from the proteasome of healthy cells have been successfully used by the authors for the early detection of breast^[5] and pancreatic^[7] cancer. In cancer, numerous proteases, such as matrix metalloproteinases (MMPs)^[23], cathepsins^[24-26], and urokinase plasminogen activator (uPA)^[20,22,25] are either over- or underexpressed, when compared to healthy cells. This enables the detection of solid tumors in liquid biopsies through the simultaneous detection of several proteases in serum^[5,7].

The Bossmann group has continuously developed their patented technology for ultra-sensitive protease detection since 2007^[27-29]. These fluorescence-based optical nanobiosensors are composed of water-dispersible dopamine-coated Fe/Fe₃O₄ core/shell nanoparticles and an attached Fluorescence resonance energy transfer pair^[30] [tetrakis (4-carboxyphenyl) porphyrin (TCPP) and cyanine 5.5]. Both the central nanoparticle and cyanine 5.5 are able to quench photoexcited TCPP, which is attached via a protease-cleavable consensus sequence^[4]. The consensus sequences have been optimized to show only minimal cross-sensitivity between the proteases used^[5] [Figure 2].

Selection of protease biomarkers

About two percent of the human genome encodes proteases, 553 in total^[31]. For detecting NSCLC, we selected the following proteases: MMPs 1, 2, 3, 7, 9 and 13, uPA and cathepsins B and L. This selection is identical to that in a previous study, in which breast cancer at stage 1 could be detected^[5]. Evidently, we have ascertained that this selected group of proteases is indeed implicated in NSCLC^[20,22,23,25,26]. The advantage of using the same panel of proteases for detecting breast cancer and non-small cell lung cancer is that we can compare the resulting “significance tables” and begin to find answers to the question whether protease expression pattern of different types of solid tumors are similar or significantly different. In Table 1, the consensus sequences for the nine proteases used are summarized. Note that the cross-reactivity of these

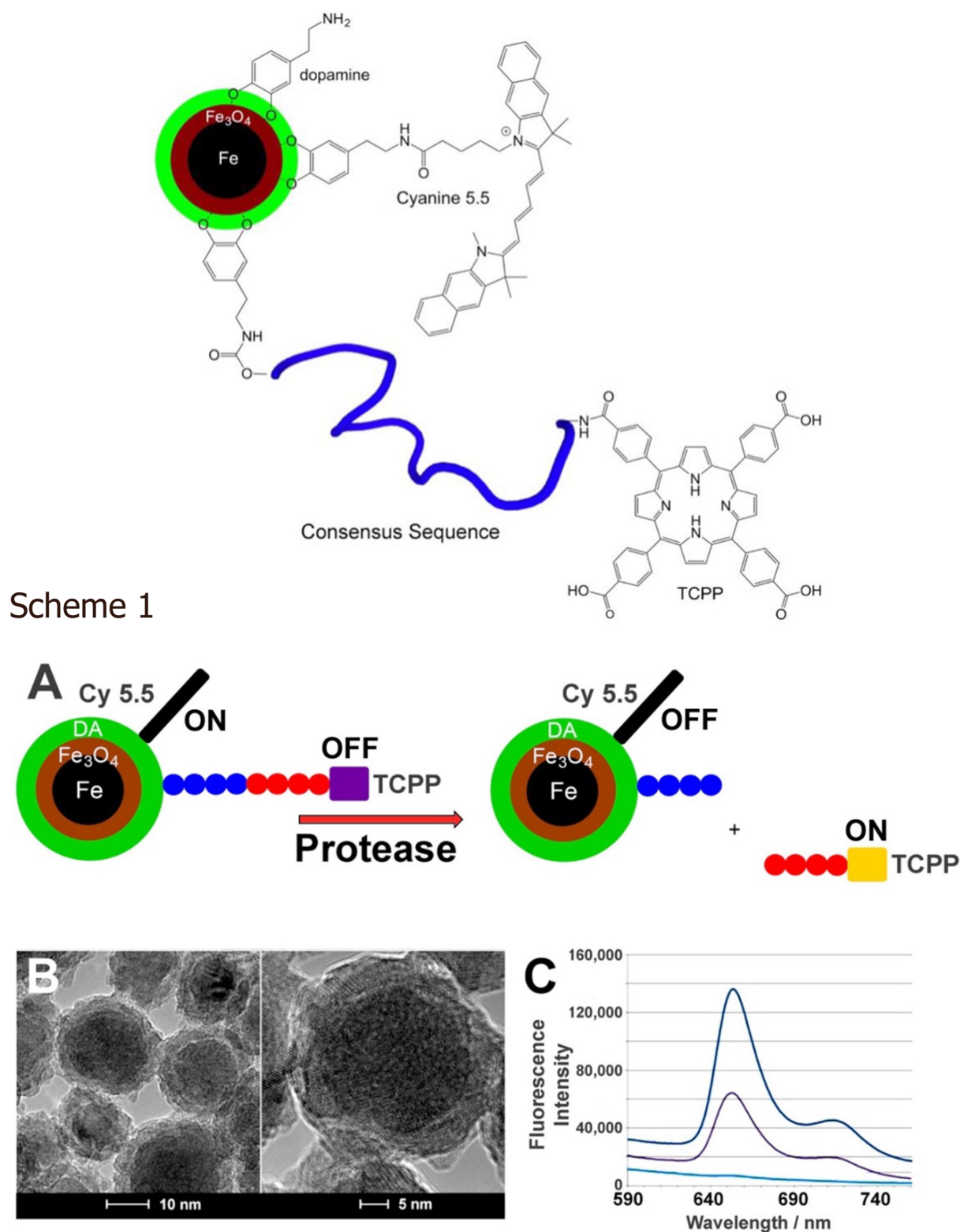


Figure 2. Fe/Fe₃O₄-based nanobiosensor for matrix metalloproteinase 1 detection. TCPP: tetrakis-carboxyphenyl-porphyrin. **Scheme 1.** A: Design principles of a nanobiosensor for protease detection. Consensus sequence: GAGVPMS-MRGGAG (MMP1); The average distance between fluorophore TCPP (tetrakis-carboxyphenyl-porphyrin) and Fe/Fe₃O₄ nanoparticle and FRET-acceptor cyanine 5.5 increases upon cleavage of the oligopeptide tether by a suitable protease, leading to an increase in fluorescence; B: TEM and HRTEM of dopamine-coated Fe/Fe₃O₄ core/shell nanoparticles; C: Typical emission spectra occurring from the nanosensor for MMP 13 after 1 h of incubation at 37 °C (λ_{exc} = 421 nm). low: buffer; middle: nanosensor; high: nanosensor after incubation with MMP13^[4].

Table 1. Consensus sequences in single-letter code for nine proteases that have been identified as cancer biomarkers. Essential amino acids of the consensus sequences are in bold^[32]

Protease	Consensus Sequence	Number of Reported Cleavages in MEROPS ^[32]
MMP1	GAGVPMS-MRGGAG	83
MMP2	GAGIPVS-LRSGAG	3,417
MMP3	GAGRPFS-MIMGAG	2,465
MMP7	GAGVPLS-LTMGAG	196
MMP9	GAGVPLS-LYSGAG	370
MMP13	GAGPQGLA-GQRGIVAG	147
uPA	GAGSGR-SAG	19
Cathepsin B	GAGSLLKSR-MVPNFNAG	632
Cathepsin L	GAGSGVVIA-TVIVITAG	2,938

MMP: matrix metalloproteinase; uPA: urokinase plasminogen activator

consensus sequences is reported in reference^[5]. With the exception of MMP9 and MMP2, which show about 20%, only minor cross reactivities (< 5 rel. %) were observed.

METHODS

Serum samples

We obtained 53 de-identified human serum samples (at -80 °C) from Dr. Tracy Chapman, Southeastern Nebraska Cancer Center (SNCC). All subjects involved in this study were Caucasian. Twenty were apparently healthy volunteers (ages 26 to 68), and 33 were NSCLC patients {9 patients were diagnosed with stage 1 [T1a (3), T1b (2) and T1c (4)], 12 patients were stage 2 [T2a (5) and T3a (7)], and 12 patients were stage 3 (T3)}. The NSCLC patients ranged from 35 to 70 in age. In a previous study, we established that the protease expression pattern of healthy and (breast) cancer patients was not significantly affected by gender. The underlying paradigm of this study was that also in lung cancer, there are no gender differences in protease expression patterns of cancer and healthy human subjects.

Statistical analysis

Bar graphs and box plots to illustrate data ranges and *P*-values^[33] were created using the R software environment for statistical computing^[34,35]. To determine possible differences between cancer patients and healthy control groups for each protease nanobiosensor, *P*-values were derived by using an unpaired and two-sided Student *t*-test with Welch modification. *P*-value (calculated probability) is the probability of finding the observed results if the null hypothesis of a study question is true. The null hypothesis assumes that there is no difference between a selected property of a group of study subjects vs. a control group^[36].

Nanobiosensor synthesis

Detailed descriptions of the syntheses of nanobiosensors and the required components (dopamine Fe/Fe₃O₄ nanoparticles, oligopeptides, TCPP, and cyanine 5.5) have been reported in our previous studies^[15,16,18,19]. Briefly, the nanobiosensors were assembled from dopamine coated Fe/Fe₃O₄ nanoparticles, cyanine 5.5, and peptide sequences that were linked to TCPP on resin^[15]. Thirty-five TCPP and 50 cyanine 5.5 dye molecules were attached on average to each Fe/Fe₃O₄ core/shell nanoparticle, on the basis of statistical modelling^[37,38]. To achieve this synthesis, a solution was prepared by completely dissolving 64 mg of TCPP-linked peptide sequence, 37 mg cyanine 5.5, 45 mg EDC and 45 mg DMAP in 30 mL of anhydrous DMF. In a separate vial, 450 mg of dopamine-coated Fe/Fe₃O₄ nanoparticles were dispersed in 10 mL of anhydrous DMF by sonicating for 20 min. Both solutions were then mixed, sonicated for 10 min, and incubated overnight on a shaker at room temperature. After overnight incubation, the resulting nanobiosensor was collected via centrifugation (5 min at 10,000 rpm), washed with DMF to remove excess dye and unbound components, followed by five washes with cold ether (-10 °C). After each washing step, the nanobiosensor was collected

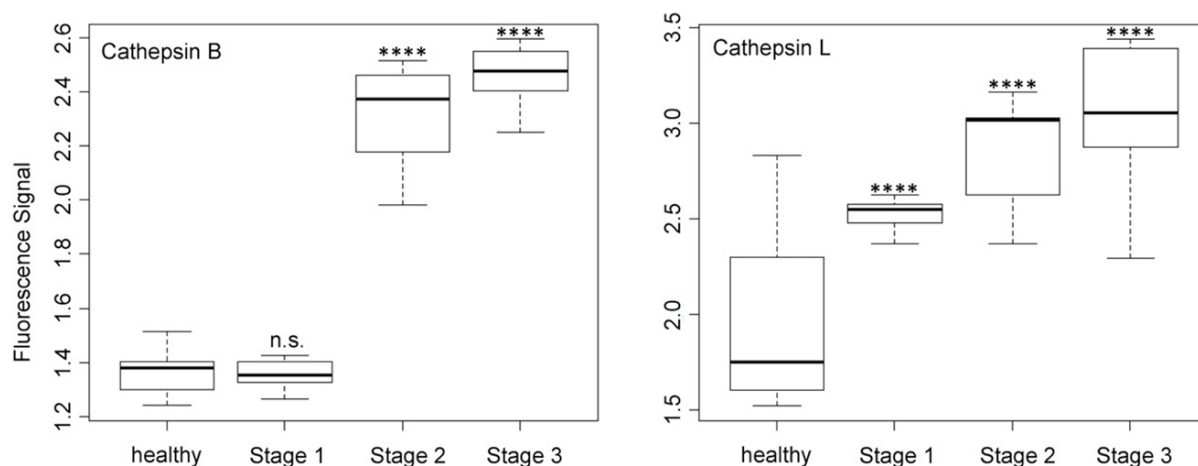


Figure 3. Box plots (indicating the observed data range) for cathepsins B and L. The groups are apparently healthy control group ($n = 20$), non-small cell lung cancer stage 1 ($n = 9$), non-small cell lung cancer stage 2 ($n = 12$), and non-small cell lung cancer stage 3 ($n = 12$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; n.s.: not significant. All biospecimens were obtained from the South Eastern Nebraska Cancer Center. Non-small cell lung cancers had been staged according to the American Joint Committee on Cancer TNM staging system^[2]

via centrifugation. The nanobiosensors were then collected and dried with argon. The nanobiosensors could be stored for more than 1 year at -20°C under argon.

Standard procedure of preparing protease assays

For each protease, 3.0 mg of nanoplatfom were dispersed in 3.0 mL of PBS (phosphate-buffered saline, Aldrich). The suspension was sonicated for 10 min, and 3.0 mL of PBS-dextran (10 mg dextran in 1.0 mL of PBS) were mixed with 75 μL of the nanoplatfom suspension (3.0 mg in 3.0 mL of PBS, see above) and 30 μL of serum. The suspensions were incubated at 37°C for 60 min, followed by the recording of a fluorescence spectrum at 25°C using a Fluoromax2 spectrometer ($\lambda_{\text{em}} = 421 \text{ nm}$, $\lambda_{\text{ex}} = 620\text{--}680 \text{ nm}$).

RESULTS

The major advantages of the fluorescent $\text{Fe}/\text{Fe}_3\text{O}_4$ core/shell nanoparticle-based nanobiosensors are their sub-femtomolar limits of detection, their large ranges extending over at least eight orders of magnitude, and their reliably small experimental errors of ± 3 percent. The calibration of the optical nanobiosensors with commercially available proteases, as well as a modest serum effect on their calibration curves are discussed in detail in an earlier study^[5]. We also established that the statistical analysis of the protease activity in the serum of cancer patients vs. a healthy control group of volunteers can be performed by using the integrated fluorescence signal of the nanobiosensors^[5]. Our cancer detection method by means of liquid biopsies relies on the paradigm that the extensive protease network of human cells is dysfunctional in cancer^[22]. Therefore, unique protease signatures can be established for virtually any solid tumor. We furthermore hypothesized that tumors derived from different cell types should exhibit different protease activity patterns and, therefore, unique protease signatures.

The activities of nine selected proteases (cathepsins B and L, uPA, and MMP1, 2, 3, 7, 9, and 13) in the serum of 33 NSCLC patients and 20 healthy volunteers were determined following the procedure described in the experimental section. In short, each nanobiosensor designed for measuring the activity of one protease and a small serum sample (30 μL) were incubated in dextran-containing PBS buffer for 1 h at 37°C . The dextran was added to prevent the coagulation of the nanobiosensor suspension during the time required for protease sensing. After 1 h, the resulting fluorescence of the suspension was measured, which indicates the protease activity in serum. A series of boxplots and bar graphs [Figures 3-7] show the data

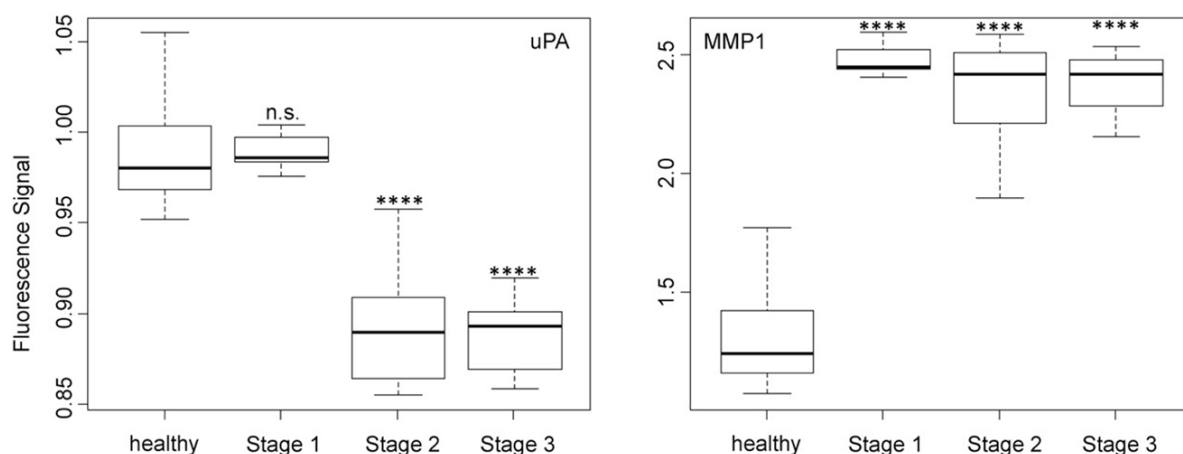


Figure 4. Box plots (indicating the observed data range) for urokinase plasminogen activator and matrix metalloproteinase 1. The groups are apparently healthy control group ($n = 20$), non-small cell lung cancer stage 1 ($n = 9$), non-small cell lung cancer stage 2 ($n = 12$), and non-small cell lung cancer stage 3 ($n = 12$). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; n.s.: not significant. All biospecimens were obtained from the South Eastern Nebraska Cancer Center. Non-small cell lung cancers had been staged according to the American Joint Committee on Cancer TNM staging system^[2]

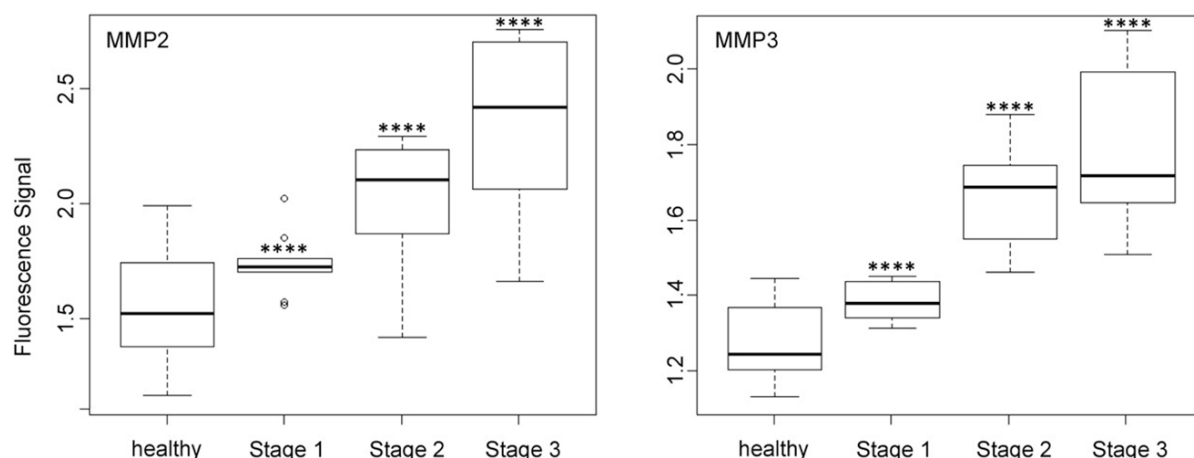


Figure 5. Box plots (indicating the observed data range) for matrix metalloproteinase MMP2 and MMP3. The groups are apparently healthy control group ($n = 20$), non-small cell lung cancer stage 1 ($n = 9$), non-small cell lung cancer stage 2 ($n = 12$), and non-small cell lung cancer stage 3 ($n = 12$). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. All biospecimens were obtained from the South Eastern Nebraska Cancer Center. Non-small cell lung cancers had been staged according to the American Joint Committee on Cancer TNM staging system^[2]

range for each protease that correlates to each cancer stage, as well as the protease activity range of healthy patients.

As shown in Figure 3, cathepsin B was expressed equally by the control group and the group of stage 1 NSCLC patients, whereas the distinct upregulation of cathepsin B activity was observed for stage 2 and 3 patients. Although cathepsin B was unsuited for detecting (sub)stage 1 NSCLC, it provides data for cancer staging via liquid biopsy. In opposite to cathepsin B, cathepsin L showed a steady increase in activity with increasing stage of NSCLC. This behavior is well suited for staging and also permits the detection of stage 1.

The activity of uPA [Figure 4], which is an originally misnamed protease, depended on the NSCLC staging in a very similar manner as cathepsin B. It is noteworthy that the optical nanobiosensors for uPA and MMP9 decreased with increasing protease activities, whereas all other protease nanobiosensors increased

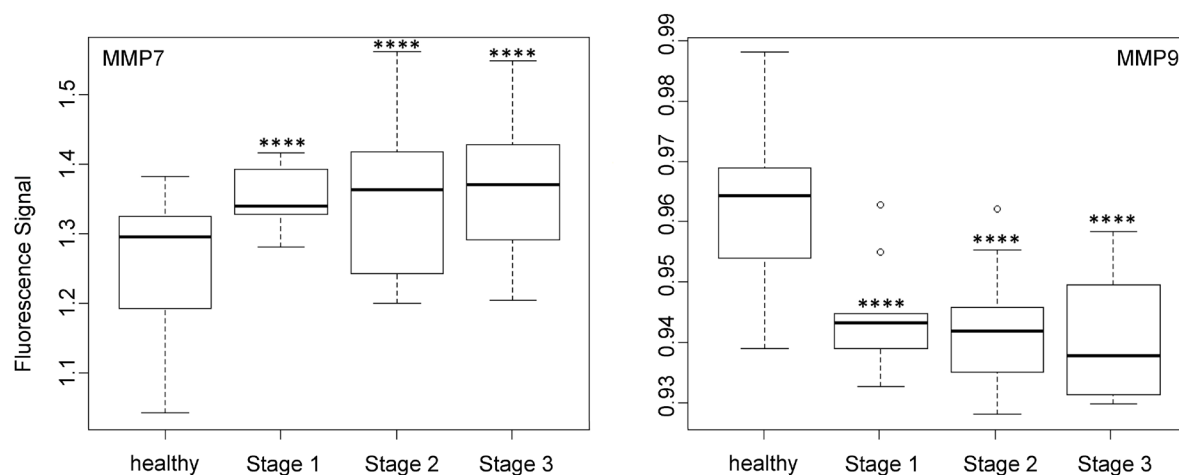


Figure 6. Box plots (indicating the observed data range) for matrix metalloproteinase MMP7 and MMP9. The groups are apparently healthy control group ($n = 20$), non-small cell lung cancer stage 1 ($n = 9$), non-small cell lung cancer stage 2 ($n = 12$), and non-small cell lung cancer stage 3 ($n = 12$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. All biospecimens were obtained from the South Eastern Nebraska Cancer Center. Non-small cell lung cancers had been staged according to the American Joint Committee on Cancer TNM staging system^[2]

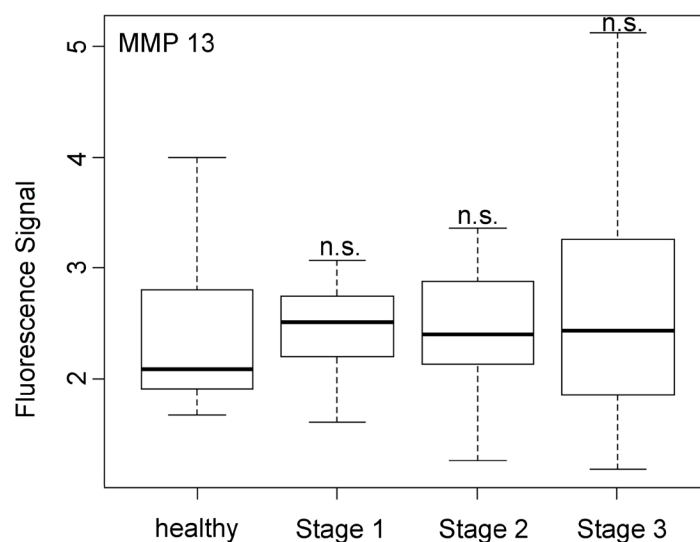


Figure 7. Box plots (indicating the observed data range) for matrix metalloproteinase 13. The groups are apparently healthy control group ($n = 20$), non-small cell lung cancer stage 1 ($n = 9$), non-small cell lung cancer stage 2 ($n = 12$), and non-small cell lung cancer stage 3 ($n = 12$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; n.s.: not significant. All biospecimens were obtained from the South Eastern Nebraska Cancer Center. Non-small cell lung cancers had been staged according to the American Joint Committee on Cancer TNM staging system^[2]

in fluorescence. Similar to cathepsin B, uPA was well suited for NSCLC staging but not for early cancer detection. A very different type of dependence of nanobiosensor fluorescence on NSCLC staging was observed for MMP1 activity, which was significantly increased starting at stage 1! However, it did not change significantly when progressing to stages 2 and 3. This finding makes MMP1 our best biomarker for very early diagnosis of NSCLC.

In contrast to MMP1 and very similar to cathepsin L, the protease activities of MMP2, MMP3, MMP7, and MMP9 in the serum of NSCLC patients increased steadily with escalating cancer stage [Figures 5 and 6].

However, it should be noted that for all three MMPs, their expression levels in the sera of NSCLC patients at stage 1 and the healthy control group were statistically different.

In opposite to all other protease activities studied here in the serum of NSCLC patients, MMP13 was found to be unsuited for both early cancer diagnosis and NSCLC staging. As shown in [Figure 7](#), MMP13 activity was basically the same for healthy subjects and NSCLC patients of all three investigated stages. This finding was unexpected, because MMP13 is known to be involved in endothelial-mesenchymal transition^[39].

DISCUSSION

The sensitivity of the Fe/Fe₃O₄ nanoparticle-based fluorescence nanobiosensors permits the accurate measurement^[5] of the activities of nine signature proteases in serum samples (30 µL) obtained from NSCLC patients. This technology permits the rapid and inexpensive detection of NSCLC at stage 1 by means of a simple liquid biopsy. We estimate costs of approx. \$20 for measuring the activity of the 8 required proteases in serum. Six proteases permit the detection of NSCLC at stage 1. MMP1 is the best candidate for the detection of NSCLC, due to the large increase in activity of 3.16×10^{-9} mol/L of stage 1 patients compared to 1.35×10^{-15} mol/L the control group. Principally, a protease-based liquid biopsy for NSCLS has the potential of significantly reducing lung cancer mortality, because lung cancer treatment would be more successful when the cancer would be detected at stage 1 instead of stages 2 and 3, which is usually the case at present day. It should be noted that this technology works for the detection of virtually all solid tumors, of which many feature distinct protease signatures. When comparing the protease signatures of breast cancer^[5] and NSCLC, there are similarities and differences: (1) for all investigated proteases, increased activities are detected for consecutively higher cancer stages, compared to the control group of healthy subjects; (2) Cathepsin B is more significant for breast cancer than for NSCLC; (3) on the other hand, MMP7 is far more sensitive in detecting NSCLC than breast cancer; and (4) MMP13 is an unsuitable biomarker for cancer detection in either case. Based on this comparison, it is our conclusion that the similarities between the protease expression pattern of NSCLS and breast cancer far outweigh the differences. Therefore, the panel of proteases should be expanded to detect characteristic differences, which could be used for the identification of the type (and the stage) of solid tumor by means of a liquid biopsy. The latter should be offered in regular intervals to all members of cancer risk groups.

The “Significance [Table 2](#)” summarizes our findings. It shows 95% confidence intervals and *P*-values^[33] calculated for the comparison of the members of individual NSCLC stages with the group of healthy volunteers. *P*-values < 0.05 are considered significant and shown in green. Cathepsin L, and MMP1, 2, 3, 7, and 9 permit the detection of NSCLC at stage 1. The statistically non-significant *P*-values are marked in red.

In [Table 3](#), the average activities for all nine investigated proteases are summarized. The calibration curves in the presence of human serum that are discussed in reference^[5] were run again in parallel to the protease measurements described here. They were used to calculate the protease activities for NSCLC patients and the control group of healthy volunteers. To date, most of the protease measurements in cancer research and clinical diagnosis are performed by means of immunoassays^[10]. Whereas the latter measure the total concentration of protease, our optical nanobiosensors determine only the fraction of active proteases. As discussed earlier, a complex protease network exists in human biology that has the ability to form activation cascades^[20,31]. Furthermore, protease zymogens can act as signaling peptides, depending on their glycosylation pattern^[40]. Comparing the concentrations of active and inactive proteases in cancer and numerous other diseases may offer an unprecedented insight into the human proteasome and also provide diagnostic opportunities.

Table 2. Significance table: protease expression pattern of non-small cell lung cancer at stages 1, 2, and 3

		Healthy ¹	Stage 1	Stage 2 ³	Stage 3 ³
Cathepsin B	Signal	1.36	1.36 ²	2.31	2.42
	95%CI	0.03	0.03 ²	0.10	0.12
	P-value		4.32E-01 ²	1.37E-09	9.09E-11
Cathepsin L	Signal	1.92	2.53 ³	2.87	3.05
	95%CI	0.18	0.05 ³	0.16	0.20
	P-value		1.08E-06 ³	6.17E-09	5.85E-09
uPA	Signal	0.99	0.99 ²	0.89	0.89
	95%CI	0.01	0.01	0.02	0.01
	P-value		2.10E-01 ²	3.81E-08	3.95E-13
MMP1	Signal	1.31	2.45 ³	2.34	2.38
	95%CI	0.10	0.09 ³	0.13	0.07
	P-value		7.15E-15 ³	3.00E-12	2.20E-16
MMP2	Signal	1.55	1.74 ³	2.00	2.36
	95%CI	0.10	0.09 ³	0.17	0.22
	P-value		5.91E-03 ³	1.23E-04	3.51E-06
MMP3	Signal	1.28	1.39 ³	1.67	1.79
	95%CI	0.05	0.03 ³	0.08	0.11
	P-value		3.80E-04 ³	3.71E-08	2.61E-07
MMP7	Signal	1	1.35 ³	1.35	1.37
	95%CI	0.04	0.03 ³	0.06	0.06
	P-value		7.40E-04 ³	1.59E-02	4.20E-03
MMP9	Signal	0.96	0.94 ³	0.94	0.94
	95%CI	0.01	0.01 ³	0.01	0.01
	P-value		1.50E-04 ³	8.93E-06	4.43E-06
MMP13	Signal	2.40	2.43 ²	2.44 ²	2.69 ²
	95%CI	0.30	0.34 ²	0.33 ²	0.64 ²
	P-value		4.59E-01 ²	4.30E-01 ²	2.19E-01 ²

¹Healthy control group; ²differences in integrated fluorescence signals between cancer and control groups were not significant; ³integrated fluorescence signal of cancer patients is significantly larger than of control group. Confidence intervals were calculated for each sample using alpha (0.05), the standard deviation and the sample count (n). MMP: matrix metalloproteinase; uPA: urokinase plasminogen activator

Table 3. Activities of cathepsins, MMPs and uPA in serum as a function of stage

Protease	H ¹	S1	S2 ³	S3 ³
Cathepsin B	3.32E-16	3.26E-16 ²	4.91E-14	9.29E-14
Cathepsin L	3.22E-15	2.74E-14 ³	6.82E-14	1.39E-13
uPA	1.12E-15	7.17E-16 ²	2.36E-12	4.25E-12
MMP1	1.35E-15	3.16E-09 ³	7.89E-10	1.37E-09
MMP2	7.04E-15	1.08E-13 ³	1.27E-13	3.70E-13
MMP3	3.56E-14	8.43E-14 ³	2.14E-13	7.65E-13
MMP7	2.98E-15	6.82E-15 ³	6.75E-15	8.12E-15
MMP9	4.39E-13	1.93E-11 ³	3.70E-11	2.38E-11
MMP13	3.01E-15	3.22E-15 ²	3.37E-15 ²	6.75E-15 ²

¹average protease activities in the serum of the group of healthy volunteers; ²differences in integrated fluorescence signals between cancer and control groups were not significant; ³integrated fluorescence signal of cancer patients is significantly higher compared to control group. MMP: matrix metalloproteinase; uPA: urokinase plasminogen activator

A combination of MMP1 and cathepsin B was chosen for a multivariate model. MMP1 achieved a good separation between the healthy group and stage 1, while cathepsin B increased the separation between the later stages, a quality that MMP1 lacked. The parameter's values were linearized (log) and this model's coefficients were highly significant. The R²-value improved to 0.91. The graph shows how the data groupings shift upward (MMP1 influence) and to the right (cathepsin B influence) with increasing cancer stages [Figure 8]. This would allow for a precise detection of stage 1 NSCLC.

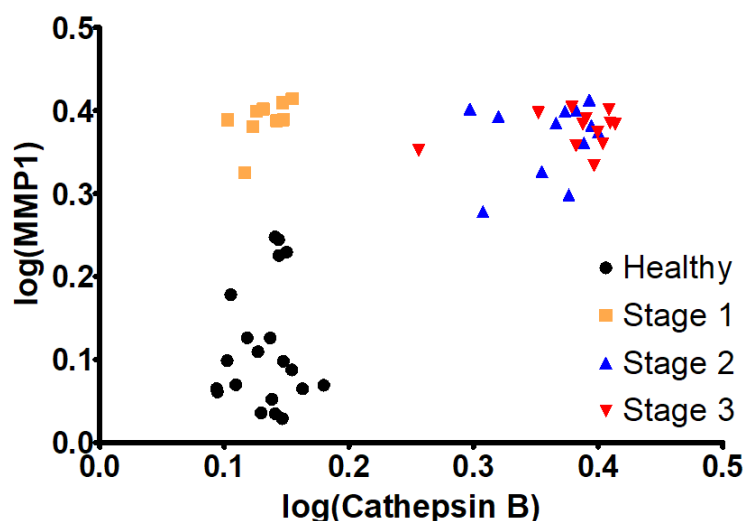


Figure 8. Multivariate model: statistical analysis: P -value of matrix metalloproteinase (MMP)1 slope: 4.34×10^{-13} MMP1 slope: 0.90623; P -value of cathepsin B slope: 9.11×10^{-15} and cathepsin B slope: 1.13514; R^2 value: 0.9181

DECLARATIONS

Acknowledgments

The authors thank Dr. Tracy Chapman, Southeastern Nebraska Cancer Center (SNCC) for the biospecimens used in this study.

Authors' contributions

Synthesis and characterization of the optical nanobiosensors: Udukala DN, Wendel SO, Wang H, Yapa AS

Calibration of the optical nanobiosensors: Udukala DN

Liquid biopsies: Udukala DN, Covarrubias-Zambrano O

Data analysis: Udukala DN, Janik K

Statistical data analysis: Wendel SO, Covarrubias-Zambrano O, Gadbury G

Design of the study reported here: Troyer DL, Bossmann SH

Writing of the manuscript: Covarrubias-Zambrano O, Janik K, Bossmann SH

Availability of data and materials

The raw data will be made available upon request; please contact Dr. Stefan H. Bossmann.

Financial support and sponsorship

National Science Foundation, USA, grant (CBET 1804416).

Conflicts of interest

All authors declared that there are no conflicts of interest

Ethical approval and consent to participate

The biospecimens were collected by Dr. Tracy Chapman, Southeastern Nebraska Cancer Center (SNCC). We received de-identified patient information.

Consent for publication

The deidentified serum samples were purchased from the South Eastern Nebraska Cancer. The activities at Kansas State were classified as “non research” with respect to the biospecimens used.

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Review

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Perspective on dietary isothiocyanates in the prevention, development and treatment of cancer

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How to cite this article: Yeger H, Mokhtari RB. Perspective on dietary isothiocyanates in the prevention, development and treatment of cancer. *J Cancer Metastasis Treat* 2020;6:26. <http://dx.doi.org/10.20517/2394-4722.2020.61>

Received: 16 Jun 2020 **First Decision:** 10 Jul 2020 **Revised:** 16 Jul 2020 **Accepted:** 21 Jul 2020 **Published:** 7 Aug 2020

Academic Editor: Sanjay Gupta **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Epidemiological evidence has highlighted the association of specific diets and a lower incidence of cancer. Foremost, the Mediterranean diet provides high levels of polyphenolics and a high consumption of healthier fats, e.g., as from olive oil. In the Mediterranean region the consumption of vegetables is elevated providing a class of compounds, the isothiocyanates (ITCs) as found in the cabbage family. The ITCs have raised great interest for their health benefits over the past few decades. Some of the key ITC compounds, sulforaphane, phenethylisothiocyanate and benzyl isothiocyanate, have been studied *in vitro* and *in vivo* and the data support their promise for cancer chemoprevention, as anti-tumor agents, and for chemoprotection of normal tissues and organs. Along with other polyphenolic compounds in the diet, in general, they also possess key anti-inflammatory properties thus satisfying the criteria for compounds that could intervene in cancer initiation and progression. In this review we provide a larger overview of the advantages of including ITCs in the diet as food or as supplements and speculate on what could constitute a valuable therapeutic strategy for improving and sustaining good health and countering cancer disease in humans.

Keywords: Mediterranean diet, isothiocyanates, chemoprevention, chemoprotection, anti-tumor, hormesis, mitohormesis, anti-inflammatory



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INTRODUCTION

The association between diet and cancer incidence have generated great interest over many years when evidence was obtained that specific regional plant based diets portended significantly lower numbers of specific cancers^[1] despite confounding factors that should have predicted an incidence closer to other regions. One such association receiving increased attention both for normal health and a lower incidence of cancer is the association with the Mediterranean diet (MedDiet)^[2-4]. The MedDiet, also lower in calories, saturated fats, and higher in fiber, also has strong benefits for reduction in cardiovascular disease and generally, inflammatory conditions^[3,5]. A dissection of the diets in this region highlights the intake of polyphenols and in particular the family of cruciferous vegetables (the *Brassica* cabbage related family) consumed. The Brassica family contains high amounts of a family of precursors and derivatives of isothiocyanates (ITCs). Populations in the region bordering the Mediterranean also consume large amounts of extra virgin olive oil (EVOO) with a high content of unsaturated fatty acids^[4]. Interestingly, although a high amount of oleic acid appears to promote cancer growth, the other components in EVOO (hydroxytyrosol, oleuropein, pinorelinol, apigenin, squalene, and maslinic acid) counter the effects of oleic acid and are indeed protective^[4]. Many other studies support the protective effect of olive oil on gut and the development of colorectal cancer^[6,7]. Our older studies demonstrated that squalene protects bone marrow progenitors against cancer cytotoxic cisplatin, and that apigenin (along with other flavonoids) can significantly inhibit proliferation of neuroblastoma tumor cells^[8]. Recent studies indicate that the high content of squalene epoxidase in cancers (in the cholesterol oxidation pathway that metabolizes cytotoxic squalene) and is associated with worse overall survival^[9]. Thus, although squalene is cytotoxic, its anti-inflammatory activity, and the complex of factors in olive oil together operate positively. Thus protection against cancer rests on a complex of dietary factors in the context of human lifestyle habits and genetic contributions.

In this review we present a broader window on the ITC based family of compounds, including our research on sulforaphane (SFN), and make a case for the value of such phytochemicals in cancer prevention and treatment, as well as for other important physiological benefits that together support why ITC based, and in general phytochemical rich diets, working in concert with other healthful components like olive oil can make a real difference to the overall health of people. The review will cover most of the key issues in this class of ITC compounds and end with insights into potential therapeutic applications.

CANCER RISK

Cancer remains a major risk to health and longevity. Although, according to the National Cancer Institute (USA)^[10,11] the decrease in cancer associated deaths have been decreasing in an encouraging manner, the statistics indicate that cancer still constitutes a significant burden (only second to cardiovascular disease) on health and survival. For example, in 2018, approximately 1,735,350 new cases of cancer were estimated to be diagnosed in the United States, and 609,640 people estimated to die from the disease. By 2020, 1,806,590 new cancer cases and 606,520 cancer deaths have been projected. Relative to older data the drop in death rate is trend setting and reflects earlier detection and better treatments. The most common cancers (listed in descending order according to estimated new cases in 2018) are breast cancer, lung and bronchus cancer, prostate cancer, colon and rectum cancer, melanoma of the skin, bladder cancer, non-Hodgkin lymphoma, kidney and renal pelvis cancer, endometrial cancer, leukemia, pancreatic cancer, thyroid cancer, and liver cancer. From the data the number of new cases of cancer (cancer incidence) is 439.2 per 100,000 men and women per year (based on 2011-2015 cases). The number of cancer deaths (cancer mortality) is 163.5 per 100,000 men and women per year (based on 2011-2015 deaths). Cancer mortality is higher among men than women (196.8 per 100,000 men vs. 139.6 per 100,000 women, respectively). When comparing groups based on race/ethnicity and sex, cancer mortality is highest in African American men (239.9 per 100,000) and lowest in Asian/Pacific Islander women (88.3 per 100,000). Estimated national expenditures for cancer

care in the United States in 2017 were \$147.3 billion. In future years, costs are likely to increase as the population ages and cancer prevalence increases. Costs are also likely to increase as new, and often more expensive, treatments are adopted as standards of care.

From our perspective, if we then compare with the Canadian cancer statistics^[12] we note comparable figures. It is estimated that 1 in 2 Canadians will be diagnosed with cancer in their lifetime, and that 220,400 new cases would be diagnosed in 2019 (113,000 in males and 107,400 in females). In 2020^[13], it is projected that 225,800 Canadians will receive a diagnosis of cancer and 83,300 will die of cancer. These estimates are higher than previously reported for 2019, which are expected given the growing and aging population. In contrast, the decline in age-standardized incidence rates (ASIR) overall shows progress is being made for several leading cancers. For example, the ASIRs for some of the commonly diagnosed cancers are declining, reflecting success in cancer-control activities around primary prevention (e.g., tobacco for lung cancer), as well as changes in detection practices (prostate cancer) and possibly screening (colorectal). In addition, there have been dramatic declines in mortality for these cancers, reflecting not only changes in incidence but also improvements in early detection and treatments. In contrast, the lack of progress with pancreatic cancer and the continued increase in the number of cases and deaths expected each year for this and other cancer type shows further efforts in cancer control are urgently required.

Globally speaking, the World Health Organization (IARC, WHO Cancer Facts Sheet, Global Cancer Observatory, March 2019^[14]) reported data from 2018 as excerpted for this review: Cancer is the second leading cause of death globally, and is responsible for an estimated 9.6 million deaths in 2018. Globally, about 1 in 6 deaths are due to cancer and approximately 70% of these occur in low- and middle-income countries. Around one third of deaths from cancer are due to the 5 leading behavioural and dietary risks: high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco use, and alcohol use. Note that the first two indicator risks relate directly to the values inherent in the Mediterranean diet.

As indicated previously the incidence of cancer in a number of favourable areas in the world tends to decrease with better phytochemical enriched diets suggesting that a lot more has to be done in determining how dietary factors can help to bring the incidence sharply down over time and aided by critical changes in lifestyle habits and physiological burdens. Therefore it is vital to try to understand not only how to treat cancer, but importantly how to prevent cancer (ideally early in life) even in light of the driving forces of genetic susceptibility and epigenetics that can play important roles in subverting normal gene expression^[15]. Finally, it also appears clear that low- and middle-income populations may not have access to sufficient vegetables and fruits in their diets to meet the threshold amount needed for chemoprevention and chemoprotection. In fact, low-income populations because of limited resources do not purchase the healthier fruits and vegetables they would need, thereby significantly limiting the intake of specific nutrients that could effectively prevent cancer, or as we see, also capable of limiting infections, additional contributing vectors. The MedDiet, very much aligned with other diets that favor greater ingestion of vegetables and fruits, may be circumvented by the trend in the lower economic strata towards poorer typical western diets that favor starches and meats.

On this point the MedDiet has been described with four sustainable benefits^[16,17] in terms of nutrition and health, environment, economy, and society and culture. Fresán *et al.*^[18] present a detailed analysis comparing the western diet, MedDiet and provegetarian diet in terms of sustainability, health benefits and cost. The MedDiet had the greatest health benefits but was considered more costly than the others. In a recent review Truzzi *et al.*^[19] examined the broader benefits of sustainability, resilience and health of the MedDiet and indicate that this diet promotes social interaction, protects biodiversity, and ensures conservation and development of traditional activities with a definite emphasis on disease prevention. Given these advantages, for the MedDiet to be transferable to other countries will require a multitude of

changes in dietary habits^[20]. In order to translate the MedDiet to a multiethnic population, for example in Australia, there is a need for a consistent definition of the MedDiet to ensure the key elements are captured and translated so that the translated MedDiet model retains authenticity^[21]. Translating the MedDiet to non-Mediterranean populations with different cultural food habits and cultural customs has been discussed as rather challenging^[22].

On the other hand, there are other diets that are also more plant based and can be equally effective and advantageous like the Jiangnan diet in China^[23] and the Japanese traditional (Washoku) diet characterized by the high consumption of fish and soybean products and low consumption of animal fat and meat. This diet uses the umami taste to enhance palatability^[24]. As vegetarian and vegan diets appear to be rising in popularity one might envision a positive reversal in dietary trends going forward. However, as changing dietary habits tends to be difficult to adapt in many ways we stress the necessity to identify key dietary derived compounds that could be easily and cheaply integrated as inexpensive supplements into the most basic of diets.

THE DIETARY CRUCIFEROUS FAMILY OF ITCs

The cruciferous family of plants, that provide ITCs, includes the brassica species (cabbage family) garden cress, watercress, radish, white mustard and papaya. This class of compounds has been investigated for anti-carcinogenic, chemoprotectant, chemopreventive, and anti-cancer activities, *in vitro* and in animal models, over the past several decades. A number of excellent detailed reviews and perspectives have been published^[25-30]. Key members of the ITC family found in the diet, SFN, phenethylisothiocyanate (PEITC), and benzyl isothiocyanate (BITC), show antitumor efficacy, perhaps selectively, against a variety of different cancers^[31] and thus have received the greatest attention. These authors have reviewed the molecular mechanisms for individual ITCs and emphasized roles for nuclear factor (erythroid-derived 2)-like 2, (Nrf2) and nuclear factor-kappa beta modulation. In addition, another common and widely studied ITC, allylisothiocyanate (AITC) also possesses anti-inflammatory and antioxidant properties that can improve oxidative stress in pathological processes^[32]. Singh *et al.*^[30] reviewed the key features of the ITC, erucin (4-methylthio butyl isothiocyanate; 4-MTB-ITC) closely related to SFN, and studied in rodent models of cancer, showing mechanisms of chemopreventive and anticancer effects including apoptosis and cell-cycle arrest, and also enhancing the phase II metabolizing enzymes, suppressing phase I metabolizing enzymes, with enhancement of Nrf-2 that leads to enhanced antioxidant activity.

The known basic structured compound of the ITC family, SFN, derived through digestion or other means, has been well studied and demonstrated by many groups to possess chemopreventive and ant-tumor activities^[25,29,32-36]. The association with cancer chemoprevention has also been established in animal models and minimally yet in humans^[27]. Metabolic phenotypes can have a modulatory role^[3]. For example, the glutathione-S-transferase T1 phenotype in particular appears to be relevant in how humans physiologically respond to ITCs. Not surprisingly, this speaks to the concept of metabolizer phenotypes for many metabolites (and drugs) in the human population^[37]. Nevertheless, dissecting the mechanism of action of the different ITCs has become an important area of investigation and their negative effects on several key cancer survival and growth pathways have been documented showing commonalities^[25]. SFN itself has been identified as a prime example of a compound that triggers the drug detoxification Nrf2-Keap1 (Kelch-like ECH-associated protein 1) pathway in cells^[38,39]. As a result there is also the caveat that the SFN induced Nrf2 pathway could protect tumor cells against cytotoxic chemotherapeutics^[39]. On the other hand, there are several examples of drug potentiation published. Addition of SFN to taxanes enhanced the anti-tumor efficacy against triple negative breast cancer^[40]. SFN can synergize with cisplatin to suppress human ovarian cancer^[41], and as a component with other anti-tumor phytochemicals^[42]. Studies from our lab have noted the potent anticancer effects of SFN for both pediatric and adult cancers^[35,43,44]. Our approach for therapeutic targeting of several cancers (bronchial carcinoids, bladder cancer, and neuroblastoma) was

to exploit the anti-tumor properties of SFN in combination with targeting of carbonic anhydrase (CA) with acetazolamide, a pan CA inhibitor to perturb the pH regulation necessary for metastatic progression. Results *in vitro* and in xenograft models showed a potent anti-tumor inhibition of growth and survival. Whether this combination could similarly inhibit other cancers has yet to be explored. Our studies also raised the possibility that the Nrf2-Keap1 pathway might actually be functionally deficient in certain cancers thereby negating the presumed cancer chemoprotection effect^[35]. Whether this happens to be the more general case in cancer patients still has to be determined but the low plasma concentrations exhibited by phytochemicals may actually limit or negate tumor cell chemoprotection. An additional interesting aspect revolves around whether Nrf2 activating compounds like SFN could target senescent cancer cells, a potential nidus for metastasis^[45]. Malavolta *et al.*^[45] studied 15 Nrf2 activating compounds including ITCs in the context of adjuvant therapy since these could display senolytic activity.

The growing evidence for ITCs as potent anticancer agents has placed greater recognition on SFN (e.g., being prevalent in broccoli and cabbage, and highly consumed) but it invites speculation that the MedDiet might be advantageous since a number of potent anticancer agents could act cooperatively in all phases of the neoplastic process. It should be noted that a complexity of ITCs are ingested in the MedDiet or other phytochemical diets. The question in terms of cancer prevention is whether dietary factors such as these are introduced in sufficient amounts during development of the fetus, as sourced from the mother, or only appear later in life in the diet, to be maximally beneficial. Phytochemicals with epigenetic modifier properties, such as ITCs, could help to counter environmental toxins exposed to the developing fetus and prenatally^[46]. There is indeed the caveat that this ITC family of dietary plants, for various reasons (e.g., bitterness, pungency), is often voiced as not favored by or agreeing with children and even adults due to digestive disturbances^[47]. However, it should be recognized that other, less known members of the cruciferous family are indeed popular and are regularly consumed at higher amounts, for example, in diets in Asia (in cooked form)^[48]. Thus one would need to determine how specific ITCs could be otherwise administered to achieve the greatest therapeutic benefit.

The essential information on ITCs can be viewed as follows. The three key ITCs, SFN, PEITC, and BITC, are generated from glucosinolates (b-thioglucoside N-hydroxysulfates) by the enzyme myrosinase that is located on the external surface of plant cells that becomes activated and is exposed to the substrates after mastication and cooking or insect damage. ITCs are particularly active via thiocarbamoylation^[49]. Many phytochemicals exhibit both anti-oxidant and pro-oxidant activities dependent on dose and thus phytochemicals via reactive oxygen species (ROS) induction and in combination with chemotherapeutics compromises tumor cell survival or can be chemopreventive^[50]. In addition, ITCs like PEITC can tie up glutathione (GSH) thereby raising ROS levels that can compromise tumor cell survival and in particular cancer stem cells (CSCs)^[51]. Liskova *et al.*^[52] having gleaned the literature emphasize the significant anti-cancer effects of dietary phytochemicals on CSCs in a wide range of cancer types via influencing multiple signaling mechanisms. Targeting of CSCs speaks to the ability to suppress tumor progression from first initiation through malignant progression towards metastasis. Elimination of CSCs or tumor initiating cells would therefore constitute the ideal targets in chemoprevention and anti-tumor targeting during progression. There are documented studies showing targeting of tumor stem cells with good evidence^[38,40,43,53]. Furthermore, considering all the hallmark features of cancer originally elaborated by Hanahan and Weinberg^[54] and now reviewed and expanded on by Girotti *et al.*^[55], who describe how galectins can affect tumor progression, it is obvious that ITCs can have a significant impact on all of these, including galectins^[56]. Thus it is not surprising that there is a building literature on the cancer chemoprevention and anti-tumor progression by ITCs.

ITCs are metabolized in the liver by the mercapturic acid pathway and thereafter eliminated in the urine being detected after consumption of glucosinolates. From the perspective of elimination it is interesting to

note that ITCs are promising chemopreventives for bladder cancer^[57]. In other words, routes of metabolism and elimination may be key sites for their biological activity in certain cancers. Aside from these 3 main natural compounds, more than 20 natural and synthetic ITCs have been studied for their anti-carcinogenic potential and properties^[25,58,59]. In clinical trials conducted or in progress on a variety of cancers, ITCs are showing positive outcomes on different biological parameters^[58]. Importantly the efficacy has varied considerably dependent upon the test system, target tissues, type of carcinogen involved, the particular ITC, dose, and dosing regimen. In a limited series of experiments in humans, activation of a carcinogen was blocked supporting the idea of chemoprevention. High performance liquid chromatography has afforded a reliable means of measuring ITC levels and kinetics.

From the perspective that ITCs might be consumed during cancer therapy, and in trying to understand normal tissue/organ chemoprotection, the following biochemical pathway has received great attention. The ITCs are potent inducers of the phase 2 enzymes, the Nrf2-Keap1 pathway mentioned above, involved in detoxification of carcinogens, mutagens and a larger variety of toxins constituting the protective system in cells, both normal and cancerous^[39]. However, there is thinking that this complexly regulated pathway^[60], being upregulated by ITCs, may counter chemotherapeutic efficacy compromising the therapeutic outcome. On the other hand, the evidence that ITCs can potentiate the efficacy of chemotherapeutics^[40], their rapid metabolism and elimination may actually interfere with the Nrf2 pathway based detoxification scenario.

It is well recognized and known, that tumor cells can invoke other mechanisms of drug resistance of greater impact. Also consider that ITCs have reliable documented broad effects on cell cycle progression, proliferation, apoptosis, and multiple cell survival signaling pathways supporting interference in tumor progression in many cancers and in a dose dependent manner. Concomitantly, the induction of phase 2 enzymes by ITCs may also play a significant role in protecting normal tissues against cytotoxics^[39].

Epigenetics play a significant role in cancer. Relevant to epigenetic regulation, ITCs are weaker but still effective inhibitors of histone deacetylases, histone deacetylases^[35], and although changes in histone acetylation may be minimal, some of their potency may involve modulation of cell activity epigenetically^[61]. In fact, combinations of SFN (and likely other ITCs) with other phytochemicals (e.g., genistein) could operate with enhanced epigenetic activity^[62,63] and via mechanisms involving inhibition of non-coding RNAs (e.g., miRNAs)^[64]. Since epigenetic therapy inhibits metastases by disruption of the metastatic niche, i.e., the tumor microenvironment^[65], ITCs may be advantageous at multiple steps in the malignant process. In skin, evidence exists that ITCs can normalize epigenetic marks altered during tumorigenesis while inhibiting melanoma growth and survival^[66]. PEITC also modulates epigenetic writers and erasers to restrict tumor development^[58]. Regulation of the epigenetic machinery may be a major function of ITCs in chemoprevention and perhaps chemoprotection of normal tissues. Whether the mechanism involves favorably rebalancing miRNAs is still to be determined.

Interestingly, ITCs target mitochondria and the electron transport chain to provoke cancer cell-selective death programming. ITCs incur mitochondrial disruption of electron transport generation to trigger apoptosis as chemopreventives^[67]. The mechanism here for SFN is thought to be targeting critical mitochondrial cysteine residues in complex III but also complex I-II by SFN; it should be noted that ITCs (with the highly reactive R-N=C=S moiety) in general are reactive with accessible cysteine residues in proteins, thus possibly able to inactivate multiple key signaling proteins favoring cancer growth and survival^[68]. As a primary example, SFN suppresses the growth of the very aggressive glioblastoma tumor cells, the stem-cell like spheroids (the CD133 fraction) and xenografts through multiple signaling pathways, with a lessor effect on normal brain cells^[69]. This study also demonstrated it can cross the blood-brain barrier and so may have versatility in enhancing other therapeutics. Despite this ability our own xenograft studies did not show any toxicity to the mice and obviously higher consumption of cruciferous

vegetables have not revealed any cytotoxicity likely due to the more rapid elimination and limiting plasma concentrations of such compounds.

On the other hand, along a cautionary line, the clinical value of ITCs as chemopreventives and anti-cancer agents has been tempered by evidence that immune cell functions (T cell and NK cells) may be compromised even at low doses (i.e., reducing proliferation of peripheral blood mononuclear cells)^[27]. The real question is whether ITCs access these cell compartments *in vivo* during ingestion and whether rapid conjugation (e.g., by GSH) yields derivatives that are less active against immune cells but are still potent chemopreventives. Since ITCs and metabolites are present in urine and plasma in the nanogram range and rapidly eliminated^[70] it is conceivable that this very low plasma level alone counters any deleterious effects, but still permits chemoprevention and anti-tumor effects in a selective uptake and concentration dependent manner. Certainly, as *in vitro* experiments are not confounded in this way, does this indicate that a deleterious effect is yet possible on the immune system? Noteworthy is that ingestion of large amounts of ITCs has not been found to compromise human health likely due to extensive elimination keeping plasma concentrations low^[58]. In this context, even low concentrations may in fact be physiologically active since hormetic effects (see below) and routes of metabolism and tissue accumulation may determine the ultimate efficacy.

It is important to put such observations in the context of diets where cruciferous plants are consumed on a regular basis without any obvious negative outcomes. In fact, epidemiological observations vis-à-vis the MedDiet generally suggests the opposite. Furthermore, plant based diets comprise a very large array of different phytochemicals that can act additively, synergistically or antagonistically or can protect against negative effects^[59]. As examples of synergistic effects between ITCs and other polyphenolics in terms of anti-inflammatory efficacy a number of papers have been published^[71,72]. ITCs in combination with anti-oxidants such as selenium and polyphenolics such as genistein can operate at an epigenetic level^[73,74]. The ITCs AITC and SFN are synergistic for chemoprevention of non-small cell lung carcinoma^[75]. More examples exist, however this area of research is still not mainstream as many scientists have not yet appreciated the larger therapeutic efficacy of the vast complexity of functional dietary factors.

Overall, epidemiological observations favor the beneficial side of ingesting cruciferous plants along with many other unrelated positively influential dietary components (e.g., as found in olive oil). Jaman and Sayeed^[76] reviewed evidence for ellagic acid, sulforaphane, and ursolic acid as possible therapy for breast cancer, and although suggestive positive overall, they raised questions about compounds that could negatively affect both tumor cells and normal cells. As mentioned before, dose is a critical parameter, and as plasma concentrations of phytochemicals tend to be self limiting *in vivo* and phytochemicals often rapidly inactivated or eliminated^[58,70], one needs to consider that most phytochemicals would likely behave within the parameters and kinetics of hormesis^[77].

HORMETIC EFFECTS MAY GOVERN THE EFFICACY OF PHYTOCHEMICALS AND LIMIT TOXICITY

Hormesis, the low dose stress modulatory effect, describes how phytochemicals (including many other agents) are bimodal in their function often acting positively at a significantly lower dose and acting negatively at higher doses^[78-81]. There is the idea that early life stressors can lead to resilience in later life^[78], and if thought of in the context of dietary factors then early exposure to chemopreventive compounds might condition the body for greater resilience or make it refractory to oncogenic stimuli^[80]. At another cellular level, acknowledging that mitochondria are absolutely essential for life, mitohormesis (an adaptive stress response) defines a biological response where reduction in mitochondrial stress can lead to significant increments in health and vitality proscribing what is felt to be the anti-aging paradigm^[3,82]. Mediators of

mitohormesis such as FGF21 and GDF15 have been described vis-à-vis increased lifespan^[82]. It is also proposed that altered mitohormesis primes a subpopulation of cancer cells to upregulate mitochondrial stress response leading to an adaptive metastatic phenotype^[83]. Therefore, theoretically, phytochemicals that have benefits on normal organ function via support of healthy mitochondria may counter cancer altering mitohormesis.

Thus the working concept here, capitalizing on the precepts of hormesis, is that for phytochemicals to be effective as chemopreventives at the earliest stages and continuously as guardians against tumorigenesis, combinations must be sought that work within the low dose range permitting adaptive responses and not toxicological responses^[77]. This would then provide the protection against biological stresses affecting normal homeostasis of tissues and cells and that can intervene in tumorigenesis, whether genetic or epigenetic. To paraphrase the summary statements in the review article on stress response by Zelenka *et al.*^[84] they indicate the following. Upregulation of the cellular stress response pathways are governed by exercise, natural compounds, or drugs that protect the body against carcinogenesis. However, the same stress response pathways may protect cancer cells against metabolic and oxidative stress associated with metastatic dissemination and anticancer therapy. In cancer prevention upregulating stress responses of the body are desirable, but anticancer drugs that downregulate stress response in tumor cells have just emerged. In targeting of the stress response, less specific multi-targeting approaches are often more successful than those targeting a single molecular target. It is noteworthy in stress response research that the dose of the stressor and the time to adaptation dramatically influence the outcome of each study.

Of relevance here is the paradox of hormesis with the low-dose beneficial effects of stressors, viewing nearly all phytochemicals as stressors, but having an intrinsic high dose toxic effect. Thus combinations of phytochemicals, if well selected, when operating at low doses could maintain a precious homeostatic state supporting normal functions and the anti-neoplastic resistant state in all tissues and organs. Biological systems are innately adaptive to stressors and respond ideally if phytochemicals are delivered with optimum duration and exposure.

INFLAMMATION AS A CRITICAL TARGET OF THE CANCER PROCESS

There is consensus amongst most scientists and physicians that inflammation is a critical component of many pathological processes. For example, Tsoupras *et al.*^[85] analyzing the driving force behind cardiovascular disease identified systemic inflammation and not cholesterol as the culprit. Especially in cancer disease progression the inflammatory microenvironment can drive the malignant phenotype^[86]. In fact, any perturbation in normal homeostasis is announced to the immune regulatory system in the body and invites inspection and reaction from a variety of immune cells orchestrated to attempt resolution. In this way it is well recognized that local or systemic inflammation can play a major role in how the cancerous process rolls out^[86]. In the oncogenic microenvironment, as a perturbation of normal tissue homeostasis, inflammatory events figure early on in the pathologic process and exacerbate the damage by humoral factors and through recruitment of immune cells^[86]. This is where the anti-inflammatory activity of phytochemicals and especially ITCs has relevance.

The ITCs and other phytochemicals could have a major chemoprevention impact on the development of cancer precursor lesions, whether as occurring in embryonal derived cancers, for example, Wilms tumor^[87,88] or on *in situ* precursor lesions well documented in adult cancers^[74]. Certainly, the incidence of precursor lesions is significantly higher than the progression to frank cancer^[89]. The derivation of pre-malignant lesions can be understood in the context of the theoretical bases of the two hit hypothesis, initiation and promotion, and the current day elaboration in genetic and epigenetic terms^[62,54]. Furthermore, we now have a much greater understanding of tumor progression towards metastasis, whether incurred locally or via circulating tumor cells^[13,90]; all should be amenable to dietary phytochemicals. Therefore

chemoprevention has a true functional basis; however, thus far scientists have not been able to propose a universal working strategy for implementation of cancer chemoprevention.

In addition, the tumor cell (precursor state or subsequently) microenvironment plays a significant role in how the malignant process evolves and where and how one central player, the cancer associated fibroblasts (CAFs), play a major role^[91]. Thus in this microenvironment of a developing cancer and thereafter during tumor progression (with increasing epigenetic aberrations) towards a metastatic phenotype, immune surveillance is involved and immune responses are elicited and manipulated^[92]. There is a growing understanding that CAFs play a key role in immunosurveillance in the tumor microenvironment. In parallel, and even earlier, innate immune responses elicited from the surrounding normal cells would cause release of inflammatory mediators necessary for the full-blown immune response^[93]. However, in the resulting complex of immune cells both inhibitors and checkpoints are produced and can suppress innate immunity. One can therefore surmise that endogenous anti-inflammatory mediators or exogenously provided as phytochemicals may help modulate the immune response in a positive fashion to thwart malignant progression and permit rapid return to homeostasis.

In this regard advice has yet to emerge on an appropriate diet that could accurately provide the necessary dietary phytochemicals that might prove most effective in blocking cancer initiation and progression and are intrinsically anti-inflammatory^[94-97]. Many phytochemicals possess these properties and a routine intake of the right combinations of phytochemicals that would include the ITCs, well recognized for their anti-inflammatory activity, might fit the bill. In addition, and for example, as a potential combination phytochemical, curcumin stands out in this regard^[98]. Combinations of phytochemicals, selected appropriately, would have a greater inhibitory impact on inflammation, preventively or during progressive states to limit or inhibit the evolution of a cancer and also thereby the damage to normal tissues^[94,99]. This has been the working hypothesis favoring the MedDiet. Of concern, as voiced by Tsoupras *et al.*^[85] is that, if inflammation fails to resolve, due to persistence of a triggering event or unsuccessful block, then chronic inflammation may exacerbate the damaging outcome. This is where a continuous exposure to anti-inflammatory phytochemicals could rebalance the inflammatory state. In tumor progression, tumor cells are able to recruit immune cells to promote tumor progression to a higher state of malignancy and metastasis^[13]. Thus it will be necessary to select phytochemicals, and also other natural compounds, that are not only potent anti-inflammatories but also anti-metastatic. In addition, combinations of anti-inflammatory phytochemicals would likely act additively or synergistically to enhance the positive outcome. In terms of biomarkers that could identify cancer prevention potential it has been noted that a pro-coagulative state is related to not only increased risk of cardiovascular diseases but also certain adult cancers. Grafetstätter *et al.*^[100] assessed lower alcohol and meat consumption population studies that highlight thrombomodulin and thrombopoietin as two markers that quantitatively associate with chronic disease development. Thus other biomarkers should be sought that can predict ongoing biological processes underlying cancer development.

THERAPEUTIC STRATEGY

The cruciferous family of dietary plants provides ITCs, and historically such plants have been used for medicinals, cooking, and in massage oils from their origins in different members of the cruciferous family. In addition to these attributes, as an example, benzyl ITC, has shown bacteriostatic, bacteriocidal and anthelmintic and vermifuge properties at low concentrations. Uppal *et al.*^[101] discuss this but raised the caveat of poor bioavailability for a number of intrinsic chemical properties^[68]. Uppal *et al.*^[101] then showed that within a complex of chitosan nanoparticles as carriers there is significantly improved efficacy. In fact, bio-nanocarriers have proven highly advantageous for efficient delivery of other promising phytochemical therapeutics such as curcumin^[102]. Efforts are being made for improving the bioavailability of ITCs^[103].

Keeping hormetic effects in mind, and the fact that such compounds can accumulate in cells and tissues, would bioavailability still remain a true physiological limitation?

As with many phytochemicals ingested daily over a lifetime, maintenance of a lower plasma concentration and eliminating excess, may be of real benefit since ITCs are also beneficial for normal organ functions as fully evident^[60]. What is of concern is whether humans would maintain dietary practices that could provide the necessary amounts of phytochemicals, like the ITCs, to serve for chemoprevention and chemoprotection. Furthermore, as the evidence for additive and synergistic positive effects amongst a variety of phytochemicals is compelling^[70], one would hope that ideal combinations of phytochemicals could be uncovered to provide supplements that could be included in products acceptable to the general populations. One could envision incorporating such combinations in everyday desirable foods sought by most.

Finally, keeping an open mind, one might envision combining ITCs with other natural related compounds, e.g., curcumin, showing potent anti-tumor and anti-inflammatory activity as well as having positive physiological effects that could provide a better chemoprotective environment. In addition, in light of the metabolic reprogramming of the cancer microenvironment^[104] the widely used drug for treatment of type 2 diabetes (T2D), metformin, a biguanide, derived almost a century ago from parent compound galengin found in French lilac, has shown anti-tumor effects perturbing glucose metabolism in tumor cells^[105]. When considering that hormesis also includes mitohormesis perhaps adding metformin (or a phytochemical equivalent) to the ITCs regimen could prove more effect in targeting tumor evolution or progression in the earliest stages. This of course is only speculative but it does invite supporting further research on this subject. Another factor is that if ITCs from food have limiting bioavailability/absorption could this be balanced out by the beneficial kinetics of hormesis? Since many phytochemicals operate with the kinetics of hormesis, in order to keep the dosage within the positive optimal low dose range, it may be necessary to obtain necessary pharmacokinetic information from acute, intermittent and chronic administration (i.e., ingestion). One might surmise that this strategy would not constitute a burden on lifestyle as many people have their morning coffee (or tea, both with touted health benefits) with little thought to daily frequency. Many folks are also cognizant of the antioxidant value of phytochemicals, however it should also be recognized that phytochemicals operate as anti-cancer agents through multiple mechanisms^[106].

It would bode well for all global communities to re-examine their diets and realign them more effectively with a more uniform country-wide distribution of foods that could attain the goal of improving health in concert with increased longevity. As discussed before many regions of the world can identify diets that constitute wholly or partially the basic components and endorse appropriate supplementations in a cost effective form containing the ITCs, polyphenolics, and healthy fats found in vegetables and fruits. Therefore, the MedDiet may emerge as a medical prescription able to win against unhealthy eating habits from other areas of the globe^[107]. A major global challenge is overriding the wave of obesity in many cultures^[108] with links to the growing rise in T2D^[109]. Therefore it is reasonable to raise the progressive idea of supplementing reasonably balanced diets with the beneficial compounds found in the MedDiet, when bulk produced to lower costs, and deriving the optimal concentrations. Adding an optimal dose of chemoprevention/chemoprotection to the morning coffee or tea, if pre-formulated and not altering taste, etc., would not raise any concerns if proven beneficial. Addressing this aspect Yagishita *et al.*^[110] reviewed the current state of knowledge regarding the different aspects of formulations, bioavailability and efficacy and what might be needed to implement a public health paradigm for global application. Quirante-Moya *et al.*^[111] also question whether bioactive compounds from Brassica species are being studied in the right way given the lack of solid clinical trial data thus far. Moreover, ITC containing plant species are present in cold and hot climates thus permitting global acquisition and evaluation.

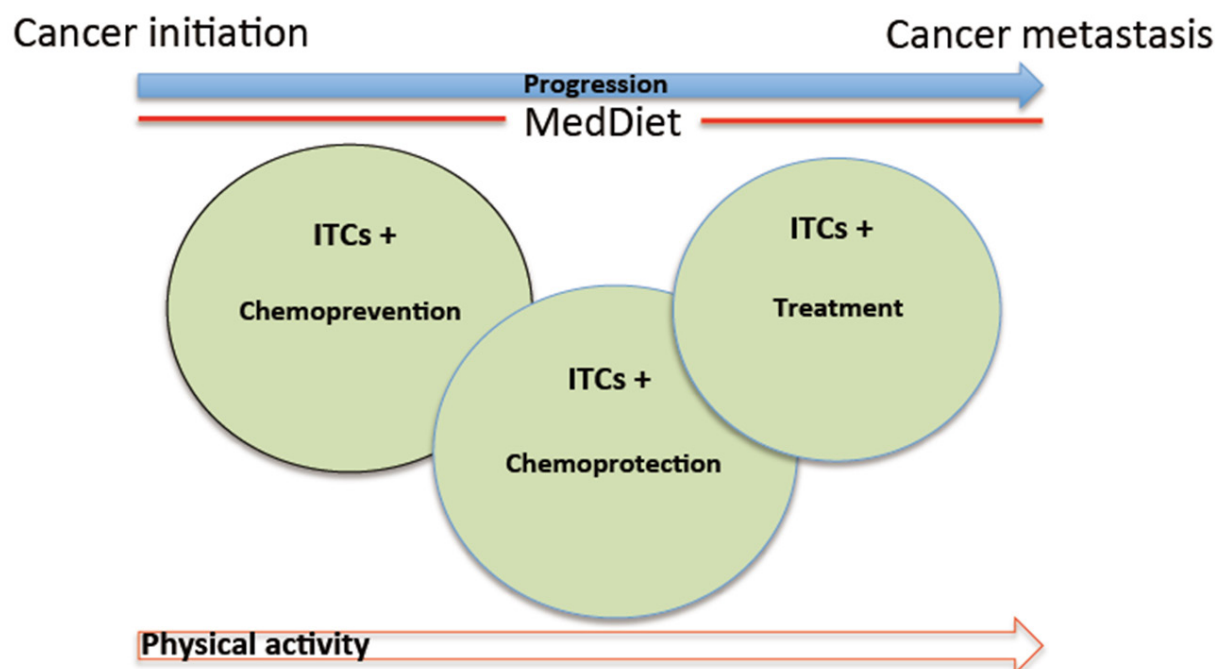


Figure 1. Schema depicts how diets, like the Mediterranean diet (MedDiet), as part of a healthy lifestyle can contribute sustainably and positively to the health of humans, the chemoprevention of cancer, inhibition of tumor progression and metastasis, and for the treatment of cancers. Concomitantly the therapeutic components in the MedDiet can also chemoprotect normal tissues and organs. All these effects are mediated by the isothiocyanates (ITCs), other phytochemicals, and oils present in this diet. ITCs have also shown potentiation of other chemotherapeutic agents, thus expanding their versatility. Maintaining good physical activity running in parallel with a chemoprotective diet can also play a significant role in ameliorating the adverse effects of T2D, obesity and cardiac disease which can exacerbate the deleterious effects of cancer

Thus it is time we view chemoprotection, chemoprevention and hormesis governed homeostasis, as a potential advantage of ITCs and other phytochemicals, and a necessary strategy for well being and a healthier, longer life^[3,58]. From this viewpoint a lot more can be done to bring anti-cancer and health promoting phytochemicals into global diets.

CONCLUSION

Here we have tried to present a new supportive strategy for preventing the onset and the treatment of cancer if attempting to intervene optimally, and if possible, for effectively targeting the entire tumorigenic process especially malignant progression. There are numerous phytochemicals with potential for cancer prevention including ITCs^[26]. Concomitantly we indicate that inclusion of an optimal balance of phytochemicals could also protect normal organs. How these phytochemicals would be selected and cognizant of their interactions has been extensively discussed^[112]; however it will require more studies to determine how ITCs interact with other phytochemicals and if there are effects on bioavailability and function. As a side benefit to the inclusion of ITCs the anti-microbial activity of ITCs^[113] would add an important further element towards achievement of a healthier lifespan.

Undoubtedly, the ultimate goals of preventing and curing cancer are perhaps very lofty at present. At the very least, as stated by others, cancer could be relegated to the status of a non life-threatening disease kept suppressed and sub-clinical. It may turn out, given the complexity of determining the ideal chemopreventive and anti-tumor diets or treatments, that ITCs in combination with other phytochemicals might be capable of controlling cancer with this objective in mind. As a last thought on the subject, a recent article by Pedersen in *The Scientist*^[114] presented a convincing review of evidence for exercise in prevention

and significantly retarding malignant progression of cancer while Musci *et al.*^[115] voiced the same message for maintenance of skeletal muscle and healthspan extension via exercise induced balanced mitohormesis. Given that ITCs could positively influence mitochondrial functions, bolstering mitohormesis, and that cancer cells and normal cells differ sufficiently in mitochondrial functions, ITCs and other phytochemicals could show the desirable selectivity.

Figure 1 schema presents a common sense strategy illustrating that leading a physically active lifestyle and consuming a phytochemical rich MedDiet, where ITCs figure prominently, may be the best defense against cancer and concomitantly for maintaining the health of all organ systems.

DECLARATIONS

Authors' contributions

Contributed equally to the literature search and the writing of this manuscript: Yeger H, Mokhtari RB
First developed the concept and the Figure Schema: Yeger H

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

Not applicable.

Consent for publication

Not applicable.

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Review

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Targeting the JAK/STAT pathway in solid tumors

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How to cite this article: Qureshy Z, Johnson DE, Grandis JR. Targeting the JAK/STAT pathway in solid tumors. *J Cancer Metastasis Treat* 2020;6:27. <http://dx.doi.org/10.20517/2394-4722.2020.58>

Received: 10 Jun 2020 **First Decision:** 15 Jun 2020 **Revised:** 17 Jul 2020 **Accepted:** 27 Jul 2020 **Published:** 21 Aug 2020

Academic Editor: William P. Schiemann **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Aberrant activation of signal transducer and activator of transcription (STAT) proteins is associated with the development and progression of solid tumors. However, as transcription factors, these proteins are difficult to target directly. In this review, we summarize the role of targeting Janus kinases (JAKs), upstream activators of STATs, as a strategy for decreasing STAT activation in solid tumors. Preclinical studies in solid tumor cell line models show that JAK inhibitors decrease STAT activation, cell proliferation, and cell survival; in *in vivo* models, they also inhibit tumor growth. JAK inhibitors, particularly the JAK1/2 inhibitor ruxolitinib, sensitize cell lines and murine models to chemotherapy, immunotherapy, and oncolytic viral therapy. Ten JAK inhibitors have been or are actively being tested in clinical trials as monotherapy or in combination with other agents in patients with solid tumors; two of these inhibitors are already Food and Drug Administration (FDA) approved for the treatment of myeloproliferative disorders and rheumatoid arthritis, making them attractive agents for use in patients with solid tumors as they are known to be well-tolerated. Four JAK inhibitors (two of which are FDA approved for other indications) have exhibited promising anti-cancer effects in preclinical studies; however, clinical studies specifically assessing their activity against the JAK/STAT pathway in solid tumors have not yet been conducted. In summary, JAK inhibition is a viable option for targeting the JAK/STAT pathway in solid tumors and merits further testing in clinical trials.

Keywords: Clinical trials, Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, JAK inhibitors, solid tumors, STAT hyperactivation



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INTRODUCTION

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway is implicated in the development and progression of many cancers^[1,2]. Hyperactivation of STAT transcription factors, has been reported in both hematologic malignancies and solid tumors, including cancers of the breast, lung, liver, head and neck, and stomach, among others^[3-8]. For many of these cancers, increased activation of the JAK/STAT signaling pathway is associated with a worse prognosis, including increased recurrence and reduced overall survival^[1,9,10]. Given the strong association between JAK/STAT hyperactivity and the development and prognosis of multiple cancers, STATs and their upstream activators, JAKs, are being extensively explored as targets for cancer therapy^[1,11-13].

Certain hematologic malignancies such as myeloproliferative neoplasms are associated with specific JAK mutations that serve as predictive biomarkers for JAK-targeted therapy^[14]. The majority of cases of polycythemia vera, essential thrombocythemia, and myelofibrosis are characterized by an activating valine to phenylalanine mutation in JAK2 (JAK2 V617F) that drives the development of these neoplasms^[15]. Clinical trials studying the impact of ruxolitinib, a selective JAK1/2 inhibitor, on polycythemia vera and myelofibrosis demonstrated significant improvement in patient outcomes, leading to Food and Drug Administration (FDA) approval and widespread use of this agent for these diseases^[16-22]. However, mutations in the JAK/STAT pathway are rare in solid tumors, and the role of JAK and/or STAT inhibitors for the treatment of solid tumors is incompletely understood. In this review, we describe the rationale for targeting the JAK/STAT pathway in solid tumors and summarize preclinical studies and clinical trials to date that evaluate the impact of agents targeting this pathway.

JAK/STAT SIGNALING

Ligands, particularly cytokines and growth factors, provide the initial stimulus for activating the JAK/STAT pathway^[23]. Cytokines bind to their corresponding transmembrane receptor subunits, resulting in multimerization with other subunits and close physical interactions of receptor-associated JAKs^[24]. The JAK family of tyrosine kinases consists of JAK1, JAK2, JAK3, and TYK2^[25]. Once the receptor-associated JAKs are placed in close proximity, they become activated via trans-phosphorylation^[24]. Activated JAKs phosphorylate tyrosine residues on the cytoplasmic region of the cytokine receptor to provide docking sites for the Src Homology 2 (SH2) domain of STAT proteins. The binding of a member of the STAT family of proteins (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6) to the phosphorylated receptor intracellular domain results in JAK-mediated tyrosine phosphorylation and activation of the STAT protein [Figure 1]^[26]. In the case of receptors with intrinsic tyrosine kinase activity (e.g., epidermal growth factor receptor, EGFR), ligand binding results in receptor autophosphorylation of tyrosine residues which serve as the docking sites for STATs, and the bound STATs are directly phosphorylated/activated by the receptor tyrosine kinase. Activated STATs dimerize and translocate into the nucleus where they serve as transcription factors, inducing the expression of genes that regulate cellular proliferation, survival, and invasion, as well as the host immune response^[14,24,27,28].

The JAK/STAT signaling pathway is modulated by several negative regulators^[29]. Members of the suppressors of cytokine signaling (SOCS) family of proteins, such as SOCS1 and SOCS3, are inhibitory against JAKs, while cytokine-inducible SH2-containing protein (CIS) blocks STAT binding sites on receptor proteins^[29,30]. Another family of inhibitory molecules, the protein inhibitor of activated STAT (PIAS) proteins, inhibit the binding of STATs to response elements in target genes^[29,31]. Protein tyrosine phosphatase receptors (PTPRs), specifically PTPRT, PTPRD, and PTPRK, have been shown to dephosphorylate STAT3, resulting in STAT3 inactivation; a handful of non-receptor PTPs harbor a similar function^[32-37]. Increased activity of JAKs and STATs coupled with decreased activity of negative regulators can lead to an upregulation of pro-proliferative, anti-apoptotic, and immunosuppressive proteins, potentially driving oncogenesis.

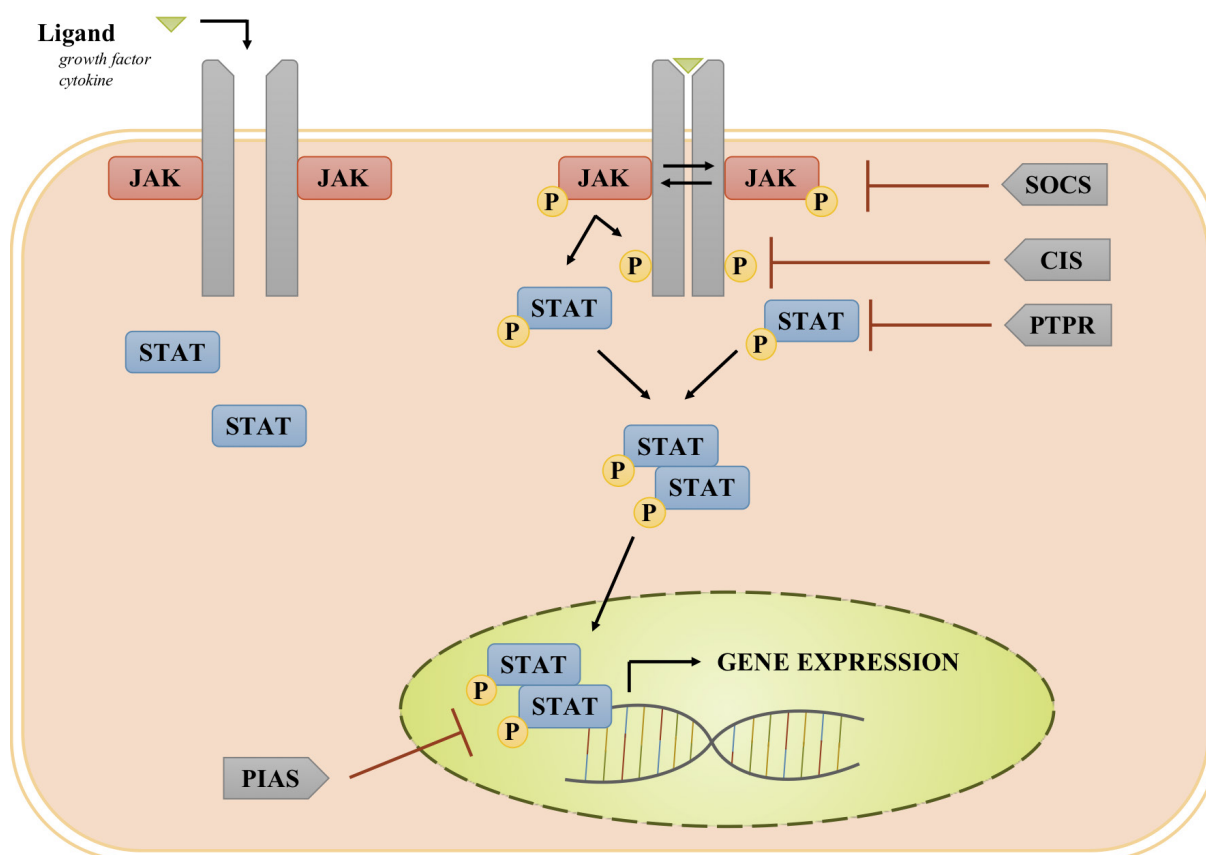


Figure 1. JAK/STAT pathway involving receptors lacking intrinsic tyrosine kinase activity: Upon ligand binding, transmembrane cytokine receptors multimerize, bringing receptor-associated JAKs into close physical proximity. Once activated via transphosphorylation, JAKs phosphorylate the cytoplasmic domain of the receptor to provide a docking site for STAT. The bound STATs are then phosphorylated and activated by JAKs. Activated STATs dimerize and translocate into the nucleus where act as transcription factors. Suppressors of cytokine signaling (SOCS) family of proteins inhibit JAK activation; cytokine-inducible SH2-containing protein (CIS) blocks the STAT docking site on the receptor; Protein inhibitor of activated STAT (PIAS) proteins inhibit STAT binding to promoter regions of target genes; and protein tyrosine phosphatase receptors (PTPRs) dephosphorylate STATs. JAKs: Janus kinases; STAT: signal transducer and activator of transcription

DYSREGULATION OF THE JAK/STAT PATHWAY IN SOLID TUMORS

Hyperactivation of STAT3

Hyperactivation of STATs, particularly STAT3, has been implicated in many cancers. Upstream JAK2 V617F mutations in myeloproliferative diseases and STAT3 mutations in T-cell large granular lymphocytic leukemia provide mechanisms for STAT3 hyperactivity in hematological malignancies^[15,38,39]. JAK1 mutations have been identified in hepatocellular carcinoma (HCC) patient tumors; patient-derived xenografts with JAK1 S703I mutations had elevated levels of phosphorylated STAT3 and STAT5^[40,41]. However, for most cases of solid tumors, activating mutations in this pathway have not been identified^[42].

In most solid tumors associated with hyperactivation of STAT3, disease development and progression has been attributed to either increased cytokine signaling or inhibition of negative regulators of the JAK/STAT pathway^[42,43]. In head and neck cancers (HNC), aberrant activation of STAT3, often through elevated IL-6 levels in the tumor microenvironment, is associated with increased tumor cell proliferation, survival, and metastasis, as well as immunosuppression of tumor-infiltrating immune cells^[44-46]. As in HNC, gastric cancer cell lines exhibit IL-6-mediated STAT3 activation, which leads to increased cell survival and epithelial to mesenchymal transition *in vitro*^[47,48]. Gastric cancer tumors were also found to have increased levels of phosphorylated STAT3 compared to healthy tissue^[49]. In non-small cell lung cancer (NSCLC),

secretion of oncostatin-M (OSM), a member of the IL-6 cytokine family, by cancer-associated fibroblasts increases STAT3 activity through activation of JAK1 and is a possible mechanism of resistance to targeted therapy such as EGFR and MEK inhibitors^[50]. STAT3 hyperactivity seen in pancreatic cancers has been associated with increased IL-22-induced STAT3 signaling and SOCS3 suppression, leading to increased invasion, migration, and angiogenesis^[51-53]. PTPRT, another negative regulator of the JAK/STAT pathway, is silenced via promoter hypermethylation in many cases of HNC and provides a likely mechanism for STAT3 hyperactivation in this cancer^[54]. Loss-of-function mutations in PTPRD have also been implicated^[55]. Hyperactivation of STAT3 has been reported in many other solid tumor malignancies, including breast cancer, HCC, and ovarian cancer, among others^[56-58].

Hyperactivation of other STATs

While less common, hyperactivation of other members of the STAT protein family has been shown in some solid tumors. STAT1 drives aromatase inhibitor resistance in breast cancer, and is highly expressed in estrogen receptor-positive, tamoxifen-resistant breast cancer cell lines, indicating it may be a promising target in this malignancy^[59]. STAT2 is not only highly expressed in ovarian cancer compared to normal ovarian tissue, but is also associated with metastasis and poor overall survival^[60]. STAT2 is also associated with poor overall survival in NSCLC^[61]. Hyperactivity of STAT5 is associated with enhanced cell viability, tumor growth, and recurrence in prostate cancers^[62,63]. In colorectal cancer cell lines, elevated levels of activated STAT6 are correlated with metastasis and decreased apoptosis^[64].

Collectively, there is ample evidence showing that increased JAK/STAT signaling is associated with increased cell proliferation, cell survival, immune evasion, recurrence, and drug resistance in solid tumors; this pathway therefore represents a promising target for therapeutic intervention.

JAK INHIBITORS

While hyperactivation of STATs, primarily STAT3, has been linked to the development and progression of solid tumors, STATs, similar to other transcription factors, have proven difficult to target directly. Therefore, upstream activators of STATs, such as JAKs, have been studied in preclinical and clinical settings as potential therapeutic targets. Several JAK inhibitors have been studied in solid tumors. Figure 2 depicts JAK inhibitors that are: (1) FDA approved and have been tested clinically in solid tumors [Figure 2A]; (2) not FDA approved, but have been tested clinically in solid tumors [Figure 2B]; and (3) have only been tested in solid tumor preclinical models. One multitarget agent (lestaurtinib) has been tested clinically for its activity against other targets [Figure 2C]. To date, there are 10 JAK inhibitors (two of which are FDA approved for other indications) that have been or are currently being investigated across 45 clinical trials in patients with solid tumors (excluding trials that have been withdrawn or in which JAK inhibitor was standard of care in studies investigating other agents) [Table 1]. Some compounds, a few of which are also FDA approved for other indications, have to date only been studied in solid tumor preclinical models.

JAK inhibitors investigated in clinical trials

Ruxolitinib

The JAK1/2-selective inhibitor ruxolitinib is FDA approved for the treatment of polycythemia vera, myelofibrosis, and graft versus host disease, and it has been shown to decrease STAT3 activation in preclinical models of several solid tumors^[18,22,65]. Ruxolitinib inhibited STAT3 activation and decreased cell growth in breast cancer^[66,67], NSCLC^[68], HNC^[69], esophageal cancer^[70], bladder cancer^[71], HCC^[72], cervical cancer^[73], and colorectal cancer^[74,75] cell lines. In pancreatic cancer cells, ruxolitinib treatment was also shown to decrease expression of pro-angiogenic genes and impede epithelial-to-mesenchymal transition^[76,77]. In *in vivo* xenograft models of neuroblastoma^[78,79], HCC (in which there was a JAK1 S703I mutation)^[40], and KRAS-mutated lung adenocarcinoma^[80], among others, ruxolitinib treatment

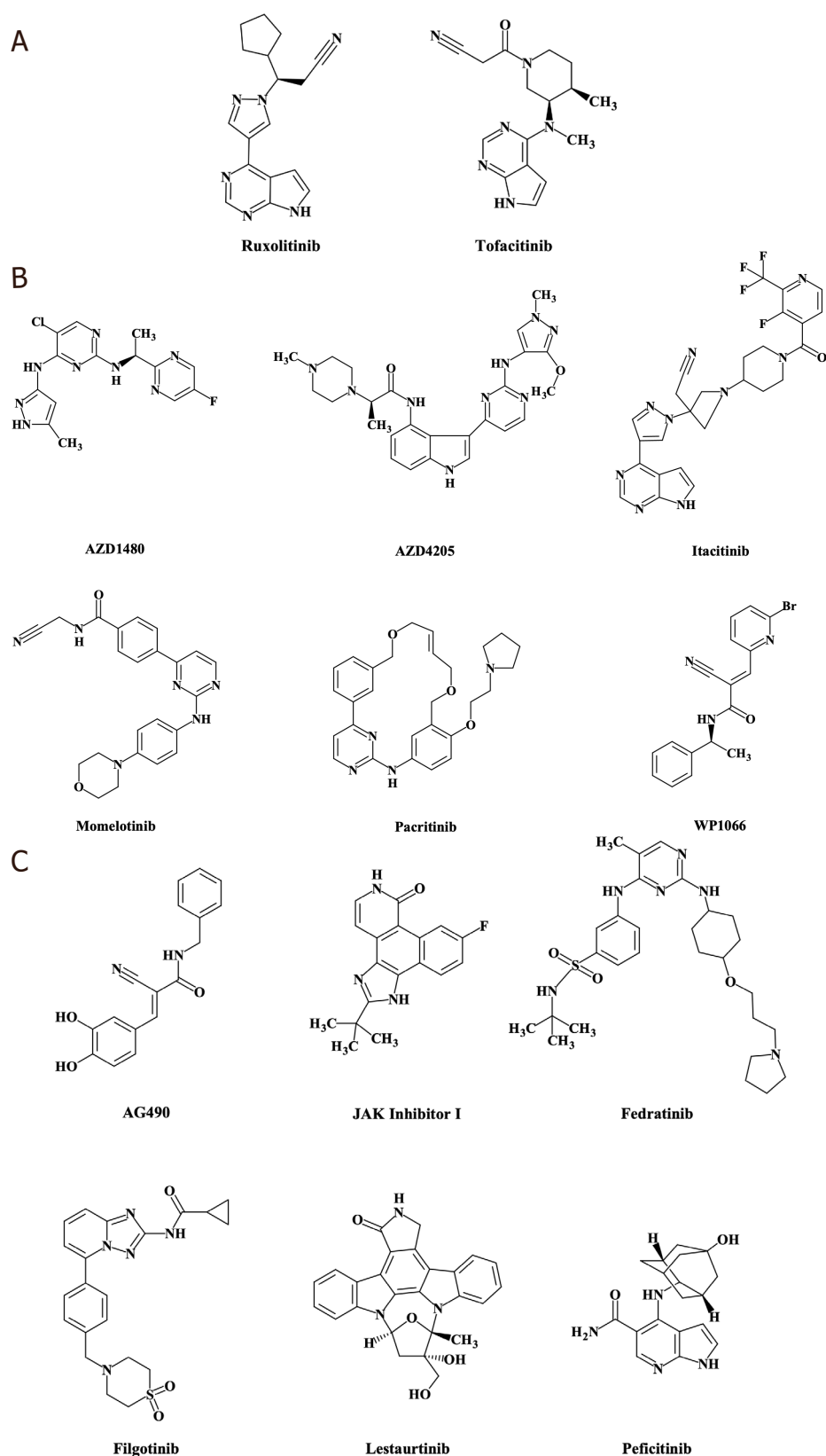


Figure 2. JAK inhibitors: chemical structures for JAK inhibitors described in this review were created using MarvinSketch software downloaded from ChemAxon (Budapest, Hungary) [175,171-183]. Food and Drug Administration (FDA)-approved JAK inhibitors that have been tested clinically in solid tumors (A); JAK inhibitors that are not FDA approved but have been tested clinically in solid tumors (B); JAK inhibitors that have only been tested in solid tumor preclinical models, and lestaurtinib, which has been tested clinically in solid tumors for its activity against other targets (C). Structures for INCB047986 and INCB052793 are not publicly available and therefore are not shown in this figure. *FDA approved for another indication; JAKs: Janus kinases

Table 1. JAK inhibitors tested clinically in solid tumors

Inhibitor	Direct Target(s)	NCT#	Type(s) of solid tumor	Status	Outcome
Ruxolitinib*	JAK1/2	NCT01423604	PDAC	Completed	Improved overall survival in subgroup of patients with inflammation ^[89]
		NCT02117479	PDAC	Terminated	No overall survival benefit ^[90]
		NCT02119663	PDAC	Terminated	No overall survival benefit ^[90]
		NCT02120417	BC	Terminated	Favorable HRQoL, no overall survival benefit ^[91]
		NCT01562873	BC	Terminated	No tumor response ^[92]
		NCT01594216	BC	Completed	None published
		NCT02119676	CRC	Terminated	No overall survival benefit ^[94]
		NCT02119650	NSCLC	Terminated	Unable to interpret efficacy ^[95]
		NCT02145637	NSCLC	Completed	23.3% PR, 70.0% SD ^[96]
		NCT02155465	Lung adenocarcinoma	Completed	Lack of efficacy ^[97]
		NCT01822756	Advanced solid tumors	Terminated	Unable to interpret efficacy ^[98]
		NCT00638378	PC	Terminated	Lack of clinical response
		NCT02955940	PDAC, CRC, BC, NSCLC	Active	
		NCT03153982	HNC	Active	
		NCT02928978	Premalignant breast disease	Active	
		NCT03514069	High-grade gliomas	Active	
		NCT04303403	CRC, PDAC	Active	
		NCT03012230	BC	Active	
		NCT02876302	IBC	Active	
		NCT02041429	IBC	Active	
		NCT02066532	BC	Active	
		NCT02713386	OC, fallopian tube cancer, peritoneal cancer	Active	
Tofacitinib*	JAK1	NCT02788201	UC	Completed	None published
		NCT04034238	Epithelioid mesothelioma, cholangiocarcinoma, PDAC	Active	
AZD1480	JAK1/2	NCT01112397	Advanced solid tumors, not specified	Terminated	pSTAT3 inhibition in granulocytes, neurotoxicity in patients ^[110]
		NCT01219543	HCC, NSCLC, GC	Terminated	None published
AZD4205	JAK1	NCT03450330	NSCLC	Completed	None published
INCB047986	JAK1	NCT01929941	PDAC, BC, non-specified advanced solid tumors	Terminated	None published
INCB052793	JAK1	NCT02265510	Non-specified advanced solid tumors	Terminated	Lack of efficacy
Itacitinib	JAK1	NCT01858883	Variety (84% PDAC)	Completed	Lack of efficacy in JANUS 1 and JANUS 2 trials ^[114]
		NCT04358185	HCC	Active	
		NCT03425006	NSCLC	Active	
		NCT02917993	NSCLC	Active	
		NCT02646748	CRC, endometrial cancer, HNC, lung cancer, BC, PDAC, RCC, UC	Active	
		NCT03670069	Soft tissue sarcoma	Suspended	
		NCT02257619	NSCLC	Terminated	None published
		NCT02559492	Non-specified advanced solid tumors	Terminated	None published
Mometinib	JAK1/2 TBK1	NCT02101021	PDAC	Terminated	No overall survival benefit ^[122]
		NCT02258607	NSCLC	Terminated	No overall survival benefit ^[123]
		NCT02206763	NSCLC	Terminated	Neutropenia ^[124]
		NCT02244489	PDAC	Terminated	None published
Pacritinib	JAK2	NCT02277093	CRC	Terminated	Lack of clinical response
		NCT02342353	NSCLC	Terminated	None published
WP1066	JAK2	NCT04334863	Medulloblastoma, brain metastases	Active	
		NCT01904123	Glioma, brain metastases	Active	

This table summarizes active, completed, or terminated clinical trials registered in ClinicalTrials.gov of JAK inhibitors in solid tumors. Outcomes of the studies were reported in published articles describing the trials or in the study descriptions at ClinicalTrials.gov.

*Agents FDA-approved for other indication. JAK: Janus kinase; PDAC: pancreatic ductal adenocarcinoma; BC: breast cancer; HRQoL: health-related quality of life; CRC: colorectal cancer; NSCLC: non-small cell lung cancer; PR: partial response; SD: stable disease; PC: prostate cancer; HNC: head and neck cancer; IBC: inflammatory breast cancer; OC: ovarian cancer; UC: urothelial cancer; pSTAT3: phosphorylated signal transducer and activator of transcription 3; HCC: hepatocellular carcinoma; GC: gastric cancer; RCC: renal cell carcinoma; TBK1: TANK-binding kinase 1

significantly inhibited tumor growth. Ruxolitinib treatment was associated with an increase in CD8⁺ T cells in pancreatic cancer xenograft models and a decrease in myeloid-derived suppressor cells in KRAS-mutated lung adenocarcinoma models, indicating an impact on immune activity^[52,80].

Ruxolitinib has also been shown to overcome drug resistance and increase sensitivity to several chemotherapeutic or targeted agents. In preclinical *in vitro* and *in vivo* models of cisplatin-resistant NSCLC, with increased JAK2 and STAT3 activation levels, the addition of ruxolitinib to cisplatin decreased STAT3 activation and cell growth, enhanced apoptosis, and inhibited tumor growth^[81]. In myxoid liposarcoma cancer stem cells, which can be resistant to chemotherapy due to upregulated JAK/STAT signaling, ruxolitinib treatment inhibited phosphorylation of STAT3 and cell viability, overcoming chemotherapy resistance^[82]. Ruxolitinib in combination with antibodies against cytokines such as IL-6 (tocilizumab) improved survival in mice bearing ovarian cancer tumors. Ruxolitinib in combination with paclitaxel reduced cell proliferation and colony formation in ovarian cancer cell lines and inhibited tumor growth in *in vivo* models^[83,84]. Ruxolitinib has been shown to improve sensitivity to oncolytic viral therapy in HNC^[85], pancreatic cancer^[86], glioblastoma multiforme (GBM)^[87], and NSCLC^[88]. Collectively, the safety profile of ruxolitinib in conjunction with promising preclinical findings in a variety of tumor models make ruxolitinib an attractive therapeutic agent against solid tumors.

Several clinical trials have studied the impact of ruxolitinib in patients with solid tumors. In a Phase II study of ruxolitinib and capecitabine in patients with pancreatic cancer who failed to respond to gemcitabine, known as the RECAP trial, there was improved survival among a subgroup of patients with inflammation, defined by a C-reactive protein (CRP) greater than the population median of 13 mg/L (NCT01423604)^[89]. Given these initial promising results, ruxolitinib was administered to patients with pancreatic cancer and an elevated CRP in two Phase III trials, JANUS 1 (NCT02117479) and JANUS 2 (NCT02119663). In both trials, patients were randomized to be treated with either ruxolitinib and capecitabine or placebo and capecitabine. However, these studies were terminated as there was no increase in overall or progression-free survival observed in the group receiving ruxolitinib compared with placebo^[90]. The combination of ruxolitinib and capecitabine in breast cancer patients with elevated CRP was also investigated in a Phase II clinical trial (NCT02120417). While patients receiving ruxolitinib and capecitabine had a more favorable health-related quality of life outcome, this study was terminated because there was no improvement in overall survival compared to the group receiving placebo and capecitabine^[91]. A Phase II trial of ruxolitinib in triple-negative breast cancer confirmed inhibition of STAT3 activation in patient tumor samples; however, no clinical response was observed, as evaluated by the RECIST criteria, and the study was terminated (NCT01562873)^[92,93]. The most recently completed clinical trial (results not reported or published) included a Phase II study testing ruxolitinib in combination with exemestane in patients with estrogen receptor-positive breast cancer (NCT01594216). The addition of ruxolitinib to regorafenib in a Phase II trial in patients with colorectal cancer did not show a difference in overall survival or progression-free survival as compared to placebo and regorafenib; therefore, this study was terminated early (NCT02119676)^[94]. Ruxolitinib was also tested in patients with lung cancers. A Phase II trial of ruxolitinib (or placebo), pemetrexed, and cisplatin in patients with stage IIIb/IV or recurrent NSCLC demonstrated that this combination was well-tolerated; the study was terminated without achieving an efficacy endpoint (NCT02119650)^[95]. Partial responses were seen in 31% of patients who received ruxolitinib and in 35% of patients who received placebo. A Phase Ib study of ruxolitinib combined with afatinib, an inhibitor of mutant EGFR, in patients with NSCLC showed that this regimen was both well-tolerated and displayed activity against this malignancy, as 23.3% displayed a partial response and 80% had stable disease (NCT02145637)^[96]. In a Phase I/II study, ruxolitinib combined with the EGFR inhibitor erlotinib in lung adenocarcinoma was shown to be well-tolerated but ineffective (NCT02155465)^[97]. A Phase Ib study of ruxolitinib with gemcitabine or nab-paclitaxel in solid tumors showed that this combination was well-tolerated (NCT01822756). However, efficacy could not be evaluated due to early termination of

the trial after results from JANUS 1 showed no benefit of ruxolitinib and capecitabine compared to placebo and capecitabine^[98]. In a Phase II trial of ruxolitinib in metastatic prostate cancer, there was no significant clinical response and the trial was terminated (NCT00638378). There is currently a rollover study that is providing continued access to ruxolitinib for patients with pancreatic, colorectal, lung, and breast cancers enrolled in previous trials (NCT02955940).

Several ongoing early-stage clinical trials are investigating ruxolitinib as monotherapy. There are two current window-of-opportunity trials: one testing neoadjuvant ruxolitinib in HNC (NCT03153982) and one examining ruxolitinib in premalignant breast disease (NCT02928978). Some trials are also investigating ruxolitinib in combination with other agents. Among these are a Phase I study testing ruxolitinib in combination with temozolomide in patients with high-grade gliomas (NCT03514069), a Phase Ib study of ruxolitinib and trametinib (MEK inhibitor) in colon and pancreatic cancers with RAS mutations (NCT04303403), a Phase I study testing ruxolitinib with pembrolizumab (PD-L1 inhibitor) in triple-negative breast cancer (NCT03012230), two Phase II studies investigating ruxolitinib with chemotherapy in inflammatory breast cancer (NCT02876302, NCT02041429), a Phase I/II trial evaluating ruxolitinib with trastuzumab (HER2 inhibitor) in HER2+ breast cancer (NCT02066532), and a Phase I/II study of ruxolitinib with paclitaxel and carboplatin in ovarian, fallopian tube, and peritoneal cancers (NCT02713386). Ruxolitinib is one of 75 approved agents being tested in a trial that uses the Co-eXpression Extrapolation (COXEN) model to identify biomarkers and to predict which drugs would provide the most benefit to patients with urothelial cancer (NCT02788201).

Tofacitinib

Tofacitinib is a JAK1/3 inhibitor that is FDA approved for treatment of rheumatoid arthritis and ulcerative colitis^[99-102]. Tofacitinib treatment of breast cancer cells prevented activation and nuclear localization of STAT3^[103]. In prostate cancer preclinical models, tofacitinib decreased STAT5 activation and epithelial-to-mesenchymal transition^[104]. This JAK inhibitor is currently being tested in patients with solid tumors (mainly pancreatic adenocarcinoma and cholangiocarcinoma) in a Phase I trial (NCT04034238).

AZD1480

AZD1480 is a selective ATP-competitive JAK1/2 inhibitor that showed promising activity against many solid tumor preclinical models. AZD1480 treatment of cell lines and murine models, including but not limited to GBM^[105], breast cancer^[106,107], HNC^[108], and ovarian cancer^[109], inhibited STAT3 activation, cell viability, and tumor growth. Despite these encouraging preclinical findings, neurotoxicity was observed in a Phase I clinical trial of AZD1480 in solid tumors and halted the development of this agent, leading to the termination of this trial (NCT01112397) and a parallel Phase I study in patients with HCC, NSCLC, and gastric cancer (NCT01219543)^[110].

AZD4205

AZD4205 is a selective JAK1 inhibitor^[111]. In a preclinical NSCLC *in vivo* model, AZD4205 treatment inhibited tumor growth and STAT3 activation; these findings were more significant when AZD4205 was administered in combination with the EGFR inhibitor osimertinib. A Phase I/II clinical trial investigating AZD4205 combined with osimertinib was initiated in patients with NSCLC (NCT03450330).

INCB047986 and INCB052793

INCB047986 and INCB052793 are selective inhibitors of JAK1. INCB047986 was studied in a Phase I clinical trial in breast and pancreatic cancers, among other solid tumors, but the trial was terminated early (NCT01929941). INCB052793 has been studied in multiple myeloma (MM) preclinical models, but there are no reports using this agent in solid tumors. In combination with other anti-MM agents, INCB052793 decreased cell viability and inhibited tumor growth^[112]. A Phase I/II trial was initiated investigating this agent in solid tumors but was terminated due to lack of efficacy (NCT02265510).

Itacitinib

Preclinical studies of the JAK1 inhibitor itacitinib have mostly been conducted in preclinical models of hematological malignancies. In conjunction with INCB054329, an inhibitor of bromodomain and extra-terminal motif proteins, itacitinib inhibited STAT3 activation and tumor growth in MM cell lines and murine models^[113]. Given this effect of JAK1 inhibition on STAT3 activity, clinical studies with this agent were initiated in solid tumors. In a Phase Ib/II study of itacitinib in combination with nab-paclitaxel and gemcitabine in solid tumors (84% of which had pancreatic cancer), 24% of patients responded (all partial response) (NCT01858883). The therapeutic combination was well-tolerated after dose reduction of itacitinib; however, this study was terminated due to another Phase III clinical trial reporting no impact of the JAK1/2 inhibitor ruxolitinib on overall survival in pancreatic cancer^[114]. Other ongoing clinical trials are studying itacitinib in patients with HCC (NCT04358185), NSCLC (NCT03425006 and NCT02917993), and a variety of advanced solid tumors (NCT02646748). A Phase II study in soft tissue sarcoma is currently suspended (NCT03670069); a Phase II study in NSCLC (NCT02257619) and a Phase Ib study in other solid tumors (NCT02559492) were terminated, with no published findings.

Momelotinib

Momelotinib is a JAK1/2 inhibitor that also has activity against TANK-binding kinase 1 (TBK1)^[115,116]. Several preclinical studies in solid tumor models have investigated the impact of momelotinib on the JAK/STAT pathway. Momelotinib has been shown to increase sensitivity of ovarian cancer to chemotherapy in *in vitro* and *in vivo* preclinical models^[117,118]. In combination with paclitaxel, momelotinib inhibited tumor growth, suppressed STAT3 activation, reduced expression of the stem cell marker OCT4, significantly increased the time to recurrence, and decreased tumor burden^[117,118]. Similarly, in GBM preclinical models, momelotinib in combination with temozolomide inhibited STAT3 activation, decreased cell growth, increased apoptosis, and inhibited tumor growth compared to temozolomide monotherapy^[119]. In colorectal cancer cells, momelotinib inhibited STAT5 activation, decreased cell growth, and increased cell death^[120]. These promising preclinical results across several types of solid tumors support further investigation of momelotinib as a therapeutic agent.

Clinical use of momelotinib has been studied extensively in myeloproliferative diseases: in myelofibrosis, treatment with this agent was associated with a reduction in splenic volume that was non-inferior to ruxolitinib^[121]. Its impact in solid tumors is under active clinical investigation. In a Phase I dose-escalation study in patients with untreated metastatic pancreatic cancer, momelotinib in combination with gemcitabine and nab-paclitaxel was well-tolerated; however, limited efficacy and no apparent association between efficacy and increasing dose led to the termination of this trial prior to the initiation of planned Phase III studies (NCT02101021)^[122]. A Phase Ib study of momelotinib combined with trametinib in KRAS-mutated NSCLC showed no improvement in response compared with historic data with trametinib monotherapy (NCT02258607)^[123]. In a Phase Ib study of momelotinib in combination with erlotinib in EGFR-mutated, metastatic NSCLC, patients experienced neutropenia as an adverse effect of this drug combination, and the trial was halted (NCT02206763)^[124]. Another Phase Ib clinical trial of momelotinib with chemotherapeutic agents, capecitabine and oxaliplatin, in pancreatic adenocarcinoma was terminated (NCT02244489).

Pacritinib

Pacritinib is a selective JAK2 inhibitor currently being studied in a Phase III clinical trial for treatment of myelofibrosis (NCT02055781)^[125,126]. In GBM cell lines, pacritinib, alone or in combination with afatinib, inhibited STAT3 activation, cell viability, and spheroid formation^[127-129]. Pacritinib plus afatinib was also shown to decrease tumor burden in mice with GBM tumors^[129]. Similar to momelotinib, pacritinib reduced resistance to temozolomide in GBM *in vivo* models^[127,128]. Pacritinib has been shown to inhibit liver fibrosis and thus may be effective in preventing HCC^[130]. A Phase II trial of pacritinib in refractory colorectal

cancers is ongoing (NCT02277093)^[131]. Pacritinib was also studied in combination with erlotinib in a Phase I/II trial in NSCLC, which was terminated (NCT02342353).

WP1066

WP1066 inhibits JAK2 phosphorylation and causes JAK2 degradation; it is an analog of the JAK2 inhibitor AG490, an agent which was widely tested in preclinical modes of solid tumors^[132,133]. Preclinical studies have shown that WP1066 exhibits anti-cancer activity including inhibition of cell proliferation and survival, and/or inhibition of tumor growth in solid tumors including, but not limited to, bladder cancer, renal cell carcinoma (in which it was shown to inhibit angiogenesis), HNC, GBM, and NSCLC^[134-139]. This agent also inhibited migration and invasion in bladder cancer, hepatocellular carcinoma, and GBM cell lines^[134,139,140]. WP1066 treatment overcame STAT3-mediated cisplatin resistance in oral squamous cell carcinoma and ovarian cancer cell lines, and doxorubicin resistance in breast cancer cell lines^[141-143]. Two current Phase I trials investigating the safety and efficacy of WP1066 are being conducted in pediatric medulloblastomas (NCT04334863) and adult malignant gliomas or brain metastases (NCT01904123).

JAK inhibitors with preclinical evidence supporting activity against solid tumors

While several JAK inhibitors have not yet been tested in patients with solid tumors, they have shown promising anti-cancer effects in preclinical models. Agents such as AG490, the compound from which WP1066 was derived, and JAK inhibitor I have been widely tested in preclinical *in vitro* and *in vivo* models. AG490 inhibited STAT3 activation and exhibited anti-cancer effects such as inhibition of cell growth and induction of apoptosis via targeting of JAK2 in preclinical models of breast cancer^[4], gastric cancer^[48], pancreatic cancer^[144], and gallbladder cancer^[145], among others. JAK inhibitor I is a JAK1/2/3 inhibitor that decreased cell proliferation in breast cancer cells^[146], increased apoptosis in esophageal squamous cell carcinoma cancer stem cells^[147], inhibited STAT3 phosphorylation in HCC cells^[148], and, in combination with cisplatin, decreased PD-L1 expression in prostate cancer cells^[149]. In addition to these agents, there are a handful of inhibitors that have either already been FDA approved or are being tested currently in clinical trials for other indications and have also shown promising findings in solid tumor preclinical models. The following inhibitors, therefore, are potential candidates for clinical testing and use in patients with solid tumors.

Fedratinib

Fedratinib is an orally bioavailable, small molecule, JAK2 inhibitor that is FDA approved for the treatment of myelofibrosis^[150-153]. NSCLC cells have been shown to be sensitive to fedratinib; sensitivity was shown to be correlated with elevated JAK2 expression^[154]. Two studies showed that fedratinib in combination with erlotinib (EGFR tyrosine kinase inhibitor) decreased STAT3 activation and increased apoptosis in erlotinib-resistant NSCLC cells and inhibited tumor growth in *in vivo* murine models^[155,156]. This agent has also demonstrated cell-killing activity against ovarian and cervical cancer cells^[157]. Fedratinib inhibited mammosphere formation and in combination with carboplatin, inhibited breast cancer tumor growth in mice^[158]. In human papilloma virus (HPV)-positive cervical cancer cells, fedratinib treatment inhibited JAK2 and STAT3/5 activation, increased apoptosis, and reduced cyclin D1 expression, cell proliferation, and colony formation^[73]. In HNC cells, treatment with fedratinib increased susceptibility to natural killer cell killing^[159].

Filgotinib

Filgotinib is a selective JAK1 inhibitor currently being investigated in clinical trials for treatment of rheumatoid arthritis and inflammatory bowel disease; to date, this drug demonstrates a significant anti-inflammatory effect, as it reduces levels of cytokines such as IL-6^[160-162]. Findings from preclinical studies in solid tumors have been reported. The OSM-JAK-STAT pathway has been implicated in progression of several cancers, including NSCLC; treatment of NSCLC cells with filgotinib resulted in inhibition of STAT3

activation and reduced OSM receptor expression^[50]. Furthermore, treatment with filgotinib inhibited resistance to targeted therapy such as MEK, EGFR tyrosine kinase, and ALK inhibitors. In NCI-H889 lung cancer cells, derived from a metastatic site, filgotinib inhibited STAT3 activation^[163]. *In vivo*, filgotinib treatment reduced metastatic seeding of NCI-H889-derived tumors. In a breast cancer cell line, filgotinib inhibited STAT3 phosphorylation; in combination with a histone deacetylase inhibitor, there was increased apoptosis in breast cancer cells as well as tumor growth inhibition in mice harboring breast cancer tumors^[164].

Lestaurtinib

Lestaurtinib is a multitarget inhibitor that has activity against JAK2, in addition to fms-like tyrosine kinase tyrosine 3 (FLT3) and tropomyosin related kinase B (TrkB)^[165]. Its impact on the JAK/STAT pathway has been studied clinically in myeloproliferative disorders, but trials in solid tumors such as neuroblastoma focus on its activity against other targets such as TrkB. One preclinical study showed that lestaurtinib treatment of anaplastic thyroid cancer cell lines inhibited STAT5 phosphorylation/activation, cell proliferation, cell survival, and cell migration, in addition to tumor growth in *in vivo* models^[166].

Peficitinib

Peficitinib is a JAK1/2/3 and TYK2 inhibitor, approved in Japan in 2019 for rheumatoid arthritis after Phase III clinical trials demonstrated a reduction in symptoms and minimal toxicity compared to placebo in clinical trials^[167,168]. Only one study to date has reported its potential use in solid tumors^[169]. In ovarian cancer stem cells engineered to overexpress OCT4, peficitinib induced apoptosis and inhibited proliferation in conjunction with JAK1 inhibition.

CONCLUSION

Aberrant JAK/STAT signaling is associated with solid tumor development and progression. However, unlike hematopoietic malignancies which harbor activating JAK mutations that lead to increased JAK/STAT signaling, the majority of solid tumors that demonstrate increased JAK/STAT signaling lack somatic JAK mutations. Studies in preclinical cancer models of solid tumors collectively show that small molecule JAK inhibitors inhibit activation of STATs, particularly STAT3, in conjunction with inhibition of proliferation and tumor growth. The majority of JAK inhibitors tested in clinical trials, with the exception of AZD1480, were found to be safe and well-tolerated. Among these, ruxolitinib is the only inhibitor to date to demonstrate responses in early stage trials. While Phase II trial results in pancreatic cancer suggested an association between elevated CRP and response to ruxolitinib plus capecitabine, these findings were not seen in the Phase III trials^[89,90]. In patients with elevated CRP, ruxolitinib combined with capecitabine was associated with improved health-related quality of life in breast cancer^[91]. Additionally, treatment of patients with NSCLC with ruxolitinib plus afatinib resulted in partial responses and stable disease^[96]. However, most trials testing ruxolitinib exhibited disappointing results, and several were terminated early; this could possibly be explained by JAK inhibition impeding immune cell function, which may counteract some of the drug's other anti-cancer effects^[170]. It is clear that only a subset of solid tumors is likely to be sensitive to JAK inhibition. Candidate predictive biomarkers to date include elevated CRP in pancreatic and breast cancers, PTPRT/D mutations in HNC, and a JAK1 S703I mutation in HCC, and assessments of biologically plausible biomarkers that predict clinical responses are needed^[40,54,55,89,91]. The JAK inhibitors fedratinib, filgotinib, and peficitinib have been shown to abrogate JAK/STAT signaling and induce anti-tumor effects in solid tumor cell lines, but, to date, there are no clinical trials investigating these agents in solid tumors; lestaurtinib has been tested clinically in solid tumors for its activity against other targets not directly involved in the JAK/STAT pathway. Ruxolitinib, tofacitinib, fedratinib, and peficitinib are JAK inhibitors already approved for other indications, making them especially attractive options as they are known to be well-tolerated. Further investigation of JAK inhibitors in clinical trials is warranted to determine the therapeutic potential in solid tumors.

DECLARATIONS

Authors' contributions

Drafted and edited manuscript, figures, and tables: Qureshy Z, Johnson DE, Grandis JR

Availability of data and materials

Not applicable.

Financial support and sponsorship

This work was supported by NIH (R35CA231998) (to Grandis JR), (R01DE028289) (to Johnson DE, Grandis JR); and a Yearlong Research Fellowship awarded by the University of California San Francisco School of Medicine (to Qureshy Z).

Conflicts of interest

Johnson DE and Grandis JR are co-inventors of cyclic STAT3 decoy and have financial interests in STAT3 Therapeutics. STAT3 Therapeutics holds an interest in cyclic STAT3 decoy. Qureshy Z 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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Targeting epithelial-mesenchymal transition - an ongoing wild goose chase

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How to cite this article: Mokhamatam RB, Irlapati VK, Dravida S. Targeting epithelial-mesenchymal transition - an ongoing wild goose chase. *J Cancer Metastasis Treat* 2020;6:28. <http://dx.doi.org/10.20517/2394-4722.2020.38>

Received: 29 Apr 2020 **First Decision:** 9 Jun 2020 **Revised:** 8 Jul 2020 **Accepted:** 17 Aug 2020 **Published:** 28 Aug 2020

Academic Editor: Godefridus J. Peters **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Epithelial-mesenchymal transition (EMT) is a natural phenomenon that occurs during embryonic development. It is a phenomenon involving the transition of adherence-dependent stationary epithelial cells to adherence-independent migratory mesenchymal cells. Tumours reactivate this machinery and evade anti-tumour immunity and inhibition by cancer-specific drugs. EMT harnesses complex crosstalk among cancer cell signalling pathways that make it difficult to tackle therapeutically, and it plays a pivotal role in cancer metastasis. Most screening platforms and approved drugs are limited by their applicability to epithelial cancers. There is a significant need for developing new strategies targeting metastatic cancers. Here, we review the challenges with the current methods of screening and available drugs for EMT and shed some light on the key essentials needed for next-generation drug discovery attempts.

Keywords: Epithelial-mesenchymal transition, high-throughput screening, drug discovery, drug resistance, tumorspheres, organoids

INTRODUCTION

Epithelial-mesenchymal transition (EMT) is considered as a major phenomenon which contributes to cancer metastasis and drug resistance^[1], and many reviews are available for understanding the natural functions of EMT and its role in cancer progression^[2-4]. Our primary goal here is to discuss the drug screening methodologies developed by various groups and their limitations, while surrendering to the



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factual status of EMT's vital role in tumour biology, but is not declared to be the only mechanism for drug resistance. In this review, we start with a brief introduction of EMT, the signalling pathways involved in EMT, and the role of EMT in drug resistance, concluding with the need for developing new screening methodologies to overcome the limitations of the existing modalities.

OVERVIEW OF EMT

Epithelial cells are a differentiated type of cells that constitute the outer lining of human body organs, skin, urinary tract, and blood vessels. Mesenchymal cells originate from a type of connective tissue (called mesenchyme) found during embryonic development and therefore are understood to be stem cells. During embryogenesis, the process of EMT and mesenchymal to epithelial transition (MET) is a common observation^[5,6]. EMT is not expected once the epithelial cells reach terminal differentiation^[7,8], but the process is induced during tissue repair and unusual pathological stress^[7,9]. Most cancers arise from epithelial cells and grow indefinitely at that location. Epithelial cells interact laterally with other cells through junctional complexes and with the basal membrane via integrin receptors. These contribute to their apicobasal polarity and it is impossible to make them grow in isolation^[10]. Some of those epithelial cells undergo EMT, lose cell-cell junctions, and acquire mesenchymal phenotype, which makes them independent of these constraints^[11,12]. Having done this, these cells can migrate to other tissues, undergo MET, and establish successful metastases^[6]. Benign tumours of epithelial origin are not very harmful to the organism, but these metastases, especially when they are present in crucial tissues, are known to cause 90% of the mortality associated with these cancers^[13]. The role of EMT in metastasis is not clearly established. In fact, Fischer *et al.*^[14] showed that EMT is not required for successful breast to lung metastasis, as inhibition of EMT by inhibiting microRNA miR-200 did not inhibit the development of lung metastasis. EMT's role in developing chemoresistance, however, is indisputable^[14].

CELL SIGNALLING INVOLVED IN EMT

EMT has been an untargeted pathway of cancer progression resulting in significant mortality and morbidity in multiple cancers. This has led researchers internationally to focus investigations on EMT targeting pathways in the cell. EMT is a multistep process whereby epithelial marker genes are suppressed, and mesenchymal markers are upregulated. The most important epithelial marker is E-cadherin. The transcription factors which can suppress its transcription are Snail1, Snail2 (also called Slug), zinc finger E-box-binding homeobox 1 (ZEB1), and ZEB2^[15-17]. Twist, which is a master regulator of embryonic morphogenesis, is found to be essential for metastasis. High levels of Twist are observed in aggressive cancers and are associated with decreased E-cadherin^[18]. The lymphoid-enhancing factor is needed for EMT induced by transforming growth factor- β 3 (TGF- β 3)^[19]. Owing to their defined role in the process, all these transcription factors are termed EMT transcription factors (EMT-TFs). Recently, it was realised that EMT-TFs play key roles in almost all stages of cancer, i.e., initiation, primary tumour formation, invasion, dissemination, metastasis, and colonization at the secondary site^[4]. Above all, the expression of the combination of EMT-TFs is different in different cancers and even within a single tumour. Thus, the markers determining the stages of EMT change, leading to the possibility of false-positive or false-negative results in disease prognosis testing^[20,21]. Most of the transcription factors are regulated by receptor tyrosine kinases^[22], TGF- β family^[23], Wnt^[24], Notch^[25], and Hedgehog^[26] pathways. The activators of these pathways include ligands such as EGF, TGF- β , cytokines, tumour hypoxia, and components of the extracellular matrix^[27]. Of all these inducers, the TGF- β family has been most studied in the context of EMT. It consists of the TGF- β superfamily of ligands which include isoforms of TGF- β (TGF- β 1, β 2, and β 3) and bone morphogenetic proteins^[27]. The addition of exogenous TGF- β induces EMT in many cancer cell lines and is cell type-specific. Conversely, specific inhibition of TGF- β receptor-1 (TGF- β R1) abrogates this EMT induction^[28]. As EMT is activated by these growth factors and transcription factors, most of the drugs targeting EMT affect these signalling pathways^[29].

THE ROLE OF EMT IN TUMOUR IMMUNE EVASION AND DRUG RESISTANCE

EMT is a crucially important causative factor in tumour immune evasion and drug resistance. EMT has been shown to suppress cytotoxic T-lymphocyte mediated immunity in MCF7 cells by activating autophagy instead of apoptosis^[30]. EMT-activated MCF7 cells express high levels of programmed death-ligand 1 (PD-L1) which contributes to suppression of tumour immunity. A ZEB1 transcription factor is important in this process and miR200 family members negatively regulate it^[31]. EMT-related gene expression contributes to immunotherapy resistance against programmed cell death protein 1 inhibitors in urothelial cancers, non-small cell lung cancers (NSCLC) and triple negative breast cancers (TNBC)^[32,33]. EMT contributes in the development of chemoresistance in breast, pancreatic and bladder cancers^[14,29,34-36]. Both adriamycin- and vinblastine-resistant MCF-7 breast cancer cells lose epithelial markers like keratin-19 and uvomorulin expression, and overexpress mesenchymal markers such as vimentin. They also show reduced desmosome formation and tight junctions^[37,38]. This resistance is achieved by a variety of signalling events that contribute to EMT. Gottesman *et al.*^[39] reviewed the role of ATP-binding cassette (ABC) transporters in the multi-drug resistance of cancers. These are overexpressed in many cancers and the inhibition of them contributes to the reversal of resistance. MRP1 (ABCC1) is a multidrug ABC transporter that gives resistance to lung, breast, and prostate cancers, and neuroblastoma, and is expressed by the transcription factor N-myc proto-oncogene (MYCN)^[40]. Breast cancer resistance protein (BRCP/ABCG2) is one more example of an ABC transporter whose normal function removing toxins and xenobiotics in healthy tissues are repurposed in cancers towards multidrug resistance^[41]. Most of these ABC transporters are overexpressed by transcription factors such as Twist, Snail, and FOXC2, which contribute to the progression of EMT^[42]. Thus, targeting EMT via these transcription factors is a viable strategy to overcome drug resistance. Witta *et al.*^[43] proved that the reversal of EMT by overexpressing E-cadherin successfully sensitized NSCLC towards epidermal growth factor receptor (EGFR) inhibitors. They did this by pre-treating resistant cells with MS-275, a histone deacetylase (HDAC) inhibitor which activated E-cadherin, followed by treatment with gefitinib and erlotinib, which are EGFR inhibitors^[43]. Recent clinical work also suggests that EMT contributes to drug resistance by increasing cancer stem cell (CSC) markers. Treatment of a patient with metastatic prostate cancer with the PARP inhibitor talazoparib increased NANOG, CD133, CD44v6, and ALDH1, which are CSC markers^[44]. TGF- β 1-induced EMT increases ALDH expression and leads to the generation of CSCs. This contributes to decreased drug sensitivity, increased proliferation, invasion and metastasis, and poor prognosis^[45]. Owing to the fact that EMT is often regulated by many signalling pathways, it is nearly impossible to target it with single-agent therapies^[46]. Optimal combinatorial approaches are needed for specific types of cancer after understanding the molecular drivers of EMT.

EXISTING SCREENING TECHNOLOGIES FOR TARGETING EMT AND THEIR LIMITATIONS

Many drugs were tested and their variable efficiency against EMT has been demonstrated. These drugs encompass all the pathways which contribute to EMT development^[47]. The scope of this review is the methodologies for screening drugs against EMT. They can be broadly divided into two categories: 2-dimensional (2D) models, and 3-dimensional (3D) models. Along with increased dimensionality, the complexity of 3D models increases compared to 2D models, and there is decreased ease of handling.

2D models

As EMT is a 3-dimensional phenomenon, it is not possible to model it in 2-dimensions. Thus, these 2D models utilize only certain EMT features for designing the assays. These are easy to handle and well amenable to high throughput screening (HTS) systems. Most of these assays are based on reporter constructs which can measure the activation of CDH1 promoter (for E-cadherin protein) elements. Chua *et al.*^[48] developed a high throughput assay based on EMT spot migration, where they seeded epithelial cells as a spot and induced it with EMT inducers. The resulting mesenchymal cells will be loosely arranged and the area of the spot increases. They screened several drugs on inhibition of this spot area and found that ALK5,

MEK, and SRC inhibitors worked well as they are associated with signalling pathways that can activate EMT^[48]. Huang *et al.*^[49] made a cell line-based screening system for EMT inhibitors using 43 ovarian cancer cell lines. They categorized these cell lines into four subgroups, based on their levels of expression of EMT markers: epithelial, intermediate E, intermediate M, and mesenchymal types. Based on these types, the EMT inhibiting drugs can be promoters of epithelial markers in the epithelial and intermediate E groups, or inhibitors of mesenchymal markers in the other two groups. Using this approach, they identified an Src kinase inhibitor, Saracatinib (AZD0530) which reversed E-cadherin expression in the intermediate M subgroup^[49]. Zhang *et al.*^[50] developed a microfluid-based high throughput screening system, named mesenchymal migration chip. With this, many drugs can be screened for their inhibitory potential of mesenchymal migration, which might lead to the reversal of EMT. The migration velocity of individual cells and the total percentage of migrated cells can be quantified using this assay^[50].

The inhibition of E-cadherin expression is necessary for EMT progression. Using this feature, a bioluminescence-based epithelial marker promoter induction screening system was developed, whereby the promoter of E-cadherin or epithelial-specific epidermal growth factor receptor family member ERBB3 was cloned in a luciferase vector. Several HDAC inhibitors were identified using this system^[51]. A further high throughput study also utilized E-cadherin expression analysis with immunofluorescence in pancreatic cancer. It also identified a novel HDAC inhibitor 1-(benzylsulfonyl) indoline among 17 other compounds that worked in a dose-dependent manner. Positive hits were also validated for inhibiting tumorsphere formation^[52].

All the models discussed above can test drugs for EMT inhibition only when EMT is induced by ligands such as TGF- β , epidermal growth factor (EGF), and hepatocyte growth factor. However, none of them can measure the effect of physical and mechanical forces due to tumour growth which can also induce EMT. Nakanishi *et al.*^[53] recently developed a better assay for solving this problem. Here they used photoactivatable gold substrate which can change from non-cell-adhesive to cell-adhesive upon treatment with UV light. First, single irradiation with a specific pattern is performed, and cells will be seeded to confluency. Cells will grow tightly only in those irradiated regions. After the second irradiation for the remaining areas is given, cells can move into the surrounding regions, because of the mechanical force induced on the surrounding cells by the central cells. If EMT is successful, the spot size will increase and if the inhibitors were able to suppress EMT, the spot size will not increase. If the drugs can kill, then the spot size will decrease. By employing this assay, the group discovered nanaomycin H as a novel EMT inhibitor which can specifically kill EMT-induced cells^[53].

3D models

Notwithstanding the advantages with 2D models, they cannot completely mimic the 3-dimensional nature of the tumour. These 2D cultures are known to induce certain cellular features, which are different from the cells grown *in vivo*. 2D cultures in polystyrene plates enhance integrin signalling, as the cells are dependent on the surface attachment for growth. Because of this, growth factors like EGF and TGF- α cannot induce further growth, but induce proliferation in 3D and *in-vivo* models^[54]. Only 3D cultures can efficiently induce EMT-related transcription factors when compared with 2D cultures. This is achieved by the activation of nuclear factor- κ B in 3D cultures. The EMT-induced cells were able to form successful metastases^[55]. The 3D culture was first shown by Sutherland *et al.*^[56] in 1971 as multi-cell spheroids and it was suggested that the growth properties of these spheroids are more similar to *in vivo* tumours. Later, in 1990, the Bjerkgvig group showed the growth of multicellular organotypic spheroids to be similar to transplanted mouse tumours^[57]. It was subsequently discovered that a whole cancer can be regenerated using one cell type, which is termed CSC^[58]. This led to the development of tumorsphere cultures in almost all types of cancers and the development of drug screening systems for CSCs^[59]. EMT plays a crucial role in the development and maintenance of CSCs. Mesenchymal traits are common for normal stem cells as well as for CSCs^[60].

As the attachment of cells to the surface is too strong in 2D cultures using polystyrene plates, efforts were made to minimize the cell to surface attachment and to increase cell to cell contacts. One such effort was done by the Fujibayashi group, who designed nano-culture plates (NCP) using inorganic nanomaterial scaffolds and nanoimprinting technology. Increased cell migration and spheroid formation at different locations on the plates was demonstrated, unlike in 2D plates^[61]. Arai *et al.*^[62] used this NCP-based gel and soft-agar free 3D-HTS system for screening 1,330 compounds for spheroid EMT inhibitory (SEMTIN) activity. They found 9 compounds with significant activity. SB-525334, a TGF- β R1 inhibitor, and SU9516, a CDK2 inhibitor, were shown to have SEMTIN activity. This is also an example of the successful culture of A549 lung cancer cells on the NCP platform and its EMT features were established clearly. Aref *et al.*^[63] developed a 3D microfluidic assay that provides a 3D microenvironment for cells to mimic EMT using HUVEC cells, and the assay is quantifiable as well. They co-cultured A549 lung cancer cells with HUVEC cells in this system and compared several metastatic inhibiting drugs between 2D and 3D models. In their system, A83-01, which is a TGF- β R inhibitor, significantly lost potency in the 3D system (5 nmol/L *vs.* 2.5 μ mol/L)^[63]. 3D models of HNSCC expressed EMT markers better than 2D models. They also showed a decreased sensitivity to cisplatin and cetuximab, unlike in 2D models. This suggests that 3D models can provide better simulations of drug activity^[64]. These reports suggest that 3D models are far superior to 2D models in mimicking EMT phenomena and predicting the potency of the drugs.

NEXT-GENERATION DISCOVERY ATTEMPTS FOR CONTROLLING EMT

Though 3D assays are better than the 2D assays for efficient drug screening, they are limited by the ease of the experiment analysis for doing primary and secondary assays. Most of the 3D assays utilize artificial substrates or gel components, which in turn make those assays different from *in vivo* or clinical models. Although they can form 3D architects, they might be different from the original ones and might have been induced by the nature of the materials that were used. Tumorspheres are also no longer considered as exact replicas of the original tumour. They are only rich in the CSC population and lack the heterogeneity of the tumour *in vivo*^[65]. Maintenance of tumorspheres for long term with the same properties and composition is also challenging^[66].

Owing to all these problems with traditional 3D-HTS systems, organoid cultures are becoming more popular, as they are small pieces of original patient-derived tumours that were grown in laboratory conditions. They form a full tumour and express the markers and mimic the organ properties from which they were derived^[67]. Organoids use basement membrane components like Matrigel, which are closer to the natural system compared to ultralow attachment surfaces for tumorspheres which cannot be found in nature^[68]. Though the term organoid has been used in many different contexts, its actual popularity started when intestinal organoids were developed by the Hans Clevers group, using Lgr5⁺ stem cells^[69]. LGR5 was found to activate EMT in glioma stem cells and is a better therapeutic target for EMT control. It functions through the WNT/ β -catenin pathway^[70]. Because of the importance of organoids in many fields, it was considered as the “Method of 2017”^[71]. Fan *et al.*^[72] extensively reviewed the organoid models that were developed for different types of cancers.

Patient-derived tumour organoids are cheaper, faster, and easier to handle compared to patient-derived tumour xenografts, which were traditionally used. These can also be used for high throughput screening of drugs, that can be administered immediately to the corresponding patients^[72,73] (personalised cancer medicine). Despite the current lack of studies reporting on EMT using organoids, many more are expected soon. Hypoxic gradients that are much needed for EMT induction cannot be modelled by traditional 3D assays but can be done by using organoid models^[74]. Hahn *et al.*^[75] developed an intestinal organoid-based EMT model by inducing intestinal organoids with TGF- β 1 and TNF- α . This suggests that it is possible to get EMT models for different types of cancers, which can be further used for high throughput drug screening. Drugs that can come through the organoid screen are more likely to work better in patients and

it helps in improving personalised medicine^[73,76]. However, there are two main disadvantages of organoid models: (1) cost; and (2) better and easier assays need to be developed further for efficient screening. As these organoids harbour the heterogeneity of the tumour, classical assays based on a single type of cells are not enough. This research area is mainly dependent on omics studies, such as genomics, transcriptomics, and proteomics^[73,77]. Extensive research in this novel field might contribute to overcoming these limitations.

CONCLUSION

Decades of research and accumulating literature has revealed the complexity of EMT driven by its intricate network of physiology that is difficult to imitate under lab conditions as experimental model systems. Though many models are being used, they have their limitations and are currently unable to mimic the *in vivo* nature of EMT sufficiently. Researchers still struggle with false positive and false negative results due to these problems. Therefore, the problem should be addressed with a holistic approach including a combination of feasible 2D, 3D and organoid models along with omics studies to achieve meaningful outcomes for patient care. Given the modern progression of science and technology, with the merger of advanced interdisciplinary tools, we may soon see game-changing giant leaps of success in the precision targeting of EMT.

DECLARATIONS

Acknowledgments

We want to acknowledge Transcell Oncologics (www.transcellonco.science) investors for privately funding research and drug discovery in the company. We would also like to thank the Technology Business Incubator, University of Hyderabad, for providing lab space and facilities.

Authors' contributions

Wrote the manuscript with input received from Irlapati V and Dravida S: Mokhamatam RB

Contributed in supporting with references, summarizing the interpretation with critical feedback: Irlapati VK

Conceived the framework of the review, supported by providing overall direction with the flow, edited the manuscript: Dravida S

Availability and data and materials

Not applicable.

Financial support and sponsorship

None.

Conflict 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 systemic hallmarks of cancer

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How to cite this article: Paul D. The systemic hallmarks of cancer. *J Cancer Metastasis Treat* 2020;6:29.
<http://dx.doi.org/10.20517/2394-4722.2020.63>

Received: 19 Jun 2020 **First Decision:** 17 Jul 2020 **Revised:** 22 Jul 2020 **Accepted:** 6 Aug 2020 **Published:** 28 Aug 2020

Academic Editor: Ciro Isidoro **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Cancer is not just a lump of cells that divide, invade, and spread randomly, but rather a multi-layered precisely tuned process that requires the participation of the whole organism. There is an urgent need to zoom-out from the cellular and the local stromal view and broaden our perspective by including the whole organism level. Geographically separated cancer tissues communicate between themselves, forming a system that interacts with the rest of the organism through cancer induced systemic pathogenic networks. In the present paper, I introduce six systemic hallmarks of cancer that emerge as a result of these interactions. I also describe several potential therapeutic approaches that can be developed using the cancer system concept. Overall, I argue that the tumor-centric paradigm should be replaced with a broader approach that brings into focus the “cancerized” organism.

Keywords: Cancer system, metastasis, cancer hallmarks, organism, tissue, system of cancer, systemic networks, system biology

INTRODUCTION

The cancer system

From the systemic biology point of view, organisms are complex, embedded, multi-layered networks of interactions. At the cellular level, the networks are comprised of genes, metabolic intermediates, miRNA and signaling molecules (proteins, lipids, ions). At the tissular level, the networks are comprised of interactions between different cell types and between the cells and the supporting stroma. At the



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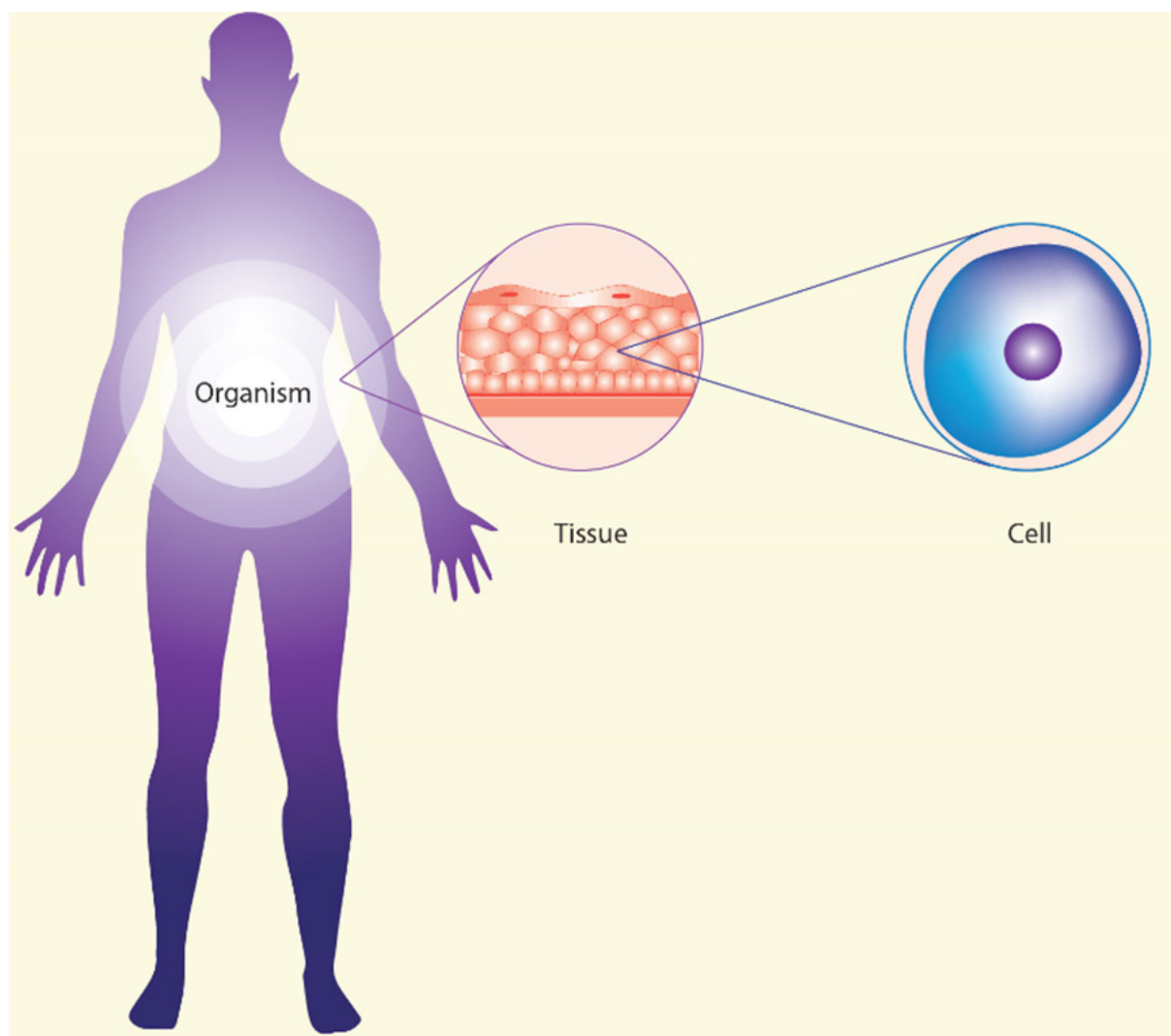


Figure 1. Three levels of organization

organismic level, the networks are comprised of interactions between different body systems (endocrine, nervous, immune, *etc.*). As described by the seminal work of Denis Noble^[1-3] these three levels of networks (cellular, tissular, organismic) [Figure 1] are co-dependent and there is no privileged level of causation. They interact and influence each other.

Cancer is also a multi-layered disease with multiple complex networks of interactions located at different levels, (i.e., cellular, tissular, organismic). The focus on the genome, the cancer cells, or even the cancer tissues, is too narrow and, in order to better understand the cancer process, there is an urgent need to zoom-out and broaden our perspective by including a broader, organismic level.

We define a system as a dynamic entity of several interacting components that are co-dependent and function in an integrated way. A single cell, an organ, the entire human body are all systems. In the present paper, I will focus on cancer at the macroscopic level. Macroscopically, experimental data accumulated over more than a decade, supports the concept of a cancer system formed by several geographically separated cancer tissues (the primary tumor, the local and the distant metastasis). The cancer system and the body systems are co-dependent and, through their interaction, new cancer induced pathologic systemic networks (CISPN) appear and the whole organism is “cancerized” to support cancer development [Figure 2].

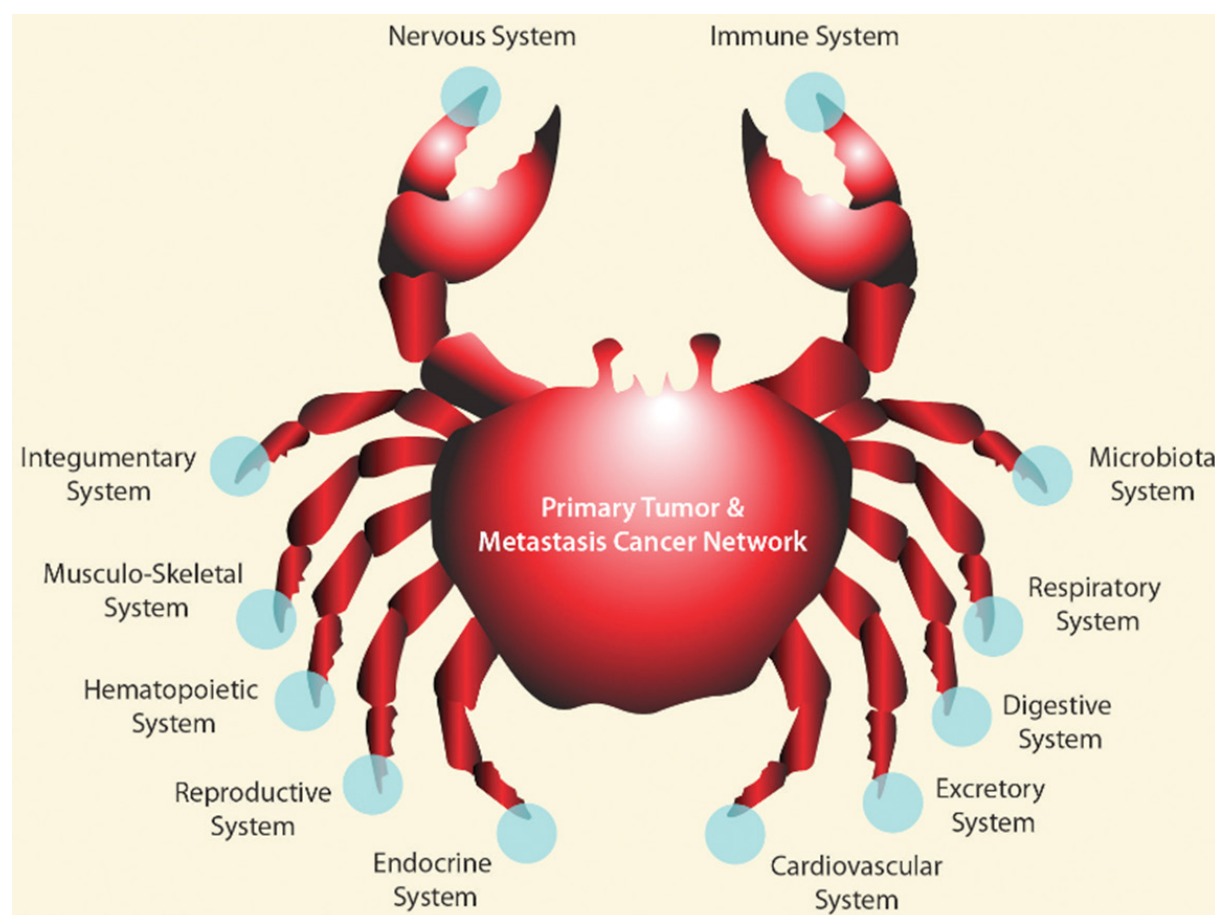


Figure 2. The cancer system and the body systems interact leading to novel cancer induced systemic pathologic networks

Cancer as a systemic disease

Over the last decade, several models of cancer as a systemic disease have been proposed^[4-8]. In 2010, Mikala Egeblad and her collaborators introduced the model of the tumor as an organ that may influence the immunity, the metabolism and the coagulation status of the host^[4]. In a paper published the same year, Sandra Mc Allister and Robert Weinberg suggested that tumor-host interactions extend well beyond the local tissue microenvironment and introduced the tumor “instigation” concept in which primary tumors perturb normal host organs and support the growth of metastatic tumors at distant anatomic sites^[5]. They listed several factors secreted by the tumors with systemic effects: vascular endothelial growth factor (VEGF), interleukin (IL)-6, IL-8, stromal cell-derived factor-1 (SDF1), fibroblast growth factor, growth-related oncogene- α (CXCL1), platelet-derived growth factor (PDGF), angiopoietins, transforming growth factor β (TGF- β), hepatocyte growth factor (HGF), angiogenin, leptin, sonic hedgehog homolog, regulated upon activation normal T-cell expressed (CCL5), and osteopontin^[5]. They also proposed a novel treatment of metastases by blocking their access to supporting stromal cells derived from the bone marrow. In a subsequent publication, the same authors^[7], refined their original model bringing additional support to the complementary idea that tumors can be also significantly influenced by systemic processes. In 2014, a research team from Austria, introduced the concept of tumor macroenvironment and described mainly the global metabolic changes that tumors exert on the whole organism^[6].

This systemic perspective should not be limited solely to clinically stage IV cancers. Often, systemic effects appear also when the tumors are localized and, in many instances, metastasis is present in a subclinical

form even when the tumor appears localized. This may be the reason why even when detected very early, a significant proportion of cancers is incurable. For example, the 5 year survival of stage I non-small cell lung cancer disease is approximately 60% (American Cancer Society Statistics, 2020). In 2019, a neuroscientist, Jeremy Borniger, published two papers^[8,9] focused on brain-tumor interactions, with special emphasis on the interaction of subcortical neural populations and cancer. Specifically, he and his team discovered that non-metastatic breast tumors may influence the endocrine and the immune systems of the hosts. Using a breast cancer mouse model, they demonstrated that by inhibiting the signaling of the lateral hypothalamic orexin/hypocretin neurons, the sleep quality and the metabolic dysregulations induced by the tumor were improved^[9].

We have also proposed previously the need for a broader, systemic perspective on cancer and introduced briefly the idea of the cancer system^[10]. The notion of the cancer system although closely related to the idea of the cancer-systemic disease is different in a fundamental way. The meaning of the word “systemic” refers to “affecting the body generally”. Metastatic and, sometimes, localized cancers, influence the whole organism and therefore they are classified as systemic diseases. On the other hand, macroscopically, cancer behaves like a sort of an organism within an organism and metastasis appears as a finely orchestrated process. The concept of a cancer system tries to capture precisely this deterministic behavior.

Cancer as a developmental disease

The idea that cancer represents an embryonal developmental program gone haywire has been around for more than four decades^[11,12]. As shown by a recent review^[13] there are some tissular and organismic genes that may play a role both in embryogenesis and cancer. It is important to point out that until approximately three weeks after fertilization, the embryo does not have a functioning circulatory system, and, therefore, the developmental programs involved in the embryo development are likely different from that of the metastatic process. A notable exception is the neural crest migration where epithelial-to-mesenchymal transition plays a critical role. Similar to the neural crest migration^[14], the metastatic process represents a transformed cellular program that once activated leads to the development of disseminated tumors at distance from the original site. The fact that the same genes (i.e., the nuclear hormone receptors, Hedgehog, Wnt, TGF- β , Notch) are involved in the generation and maintenance of multicellularity, and, they are also dysregulated in stem cells and metastasis^[15], suggest the striking idea that cancer, in general, and the metastatic process, in particular, may represent the activation of a developmental program that leads to the creation of a novel, pervasive, multicellular entity. As previously suggested by Mark Vincent, cancer appears to be much more than a simple dysregulated growth and may represent a form of multicellular life, with symbiotic properties^[16,17], that establishes a commensal relationship with the organism where it develops. In this process, the whole organism, “cancerized” through the development of CISP, as we will argue in this paper, far from being a passive bystander, becomes an active enabler of cancer progression and spread.

Metastasis as a finely orchestrated deterministic process

As opposed to the apparition of malignant tumors that is, in general, related to genetic mutations, metastasis appears to be mainly an epigenetic process. In the 2011 updated version of their original hallmarks article^[18], Hannahan and Weinberg, noted that the ability of cancer cells to invade and metastasize may not require additional genetic mutations in addition to those already present in the primary tumors^[19]. Also, the majority of the genes proposed by Massagué and collaborators more than a decade ago^[20,21] in their step by step model of metastasis are not mutated. In addition, Vogelstein *et al.*^[22] noted that despite considerable effort, specific genetic alterations that distinguish cancers that metastasize from cancers that do not metastasize have not been yet identified. The immediate conclusion, drawn by the Vogelstein team^[22], is that there are no specific metastasis genes.

This opinion of metastasis as a random, nondeterministic process has been challenged for 130 years since Paget asked the famous question: “What is what decides what organs suffer from disseminated cancer?” and launched the “seed and soil” hypothesis^[23]. By the late 70’s, metastasis started to be understood more and more as the result of non-random tumor-host interactions^[24], and the Paget’s “seed and soil” hypothesis has been strongly supported by the work of Fidler and Kripke^[25] and Price *et al.*^[26]. A recent study described widespread epigenetic reprogramming during the evolution of distant metastasis of pancreatic cancer in the absence of metastasis-specific driver mutations^[27]. This manifested as global reprogramming of histone H3K9 and DNA methylation within large heterochromatin domains (LOCKS) as well as regional changes in gene regulatory modifications. Interestingly, the authors found that the epigenetic changes were controlled by an anabolic glucose metabolism enzyme 6-phosphogluconate dehydrogenase (PGD). Glucose deprivation, RNA interference (RNAi) against PGD, and, treatment with 6-aminonicotinamide, reprogrammed the chromatin state of the distant metastasis. In addition, cells treated with RNAi against PGD did not form distal metastasis^[27]. Another recent study^[28], found also that in prostate cancer the master regulator genes of metastasis are genes involved in epigenetic regulation. Silencing a particular histone methyltransferase gene (Nuclear receptor binding SET Domain protein 2, NSD2) in vivo allografts, resulted in significant improvement in survival in the mice treated as well as a significant reduction in the metastatic burden without any effect on the primary tumor growth^[28].

These observations suggest a different view of metastasis. The plethora of genetic abnormalities present in established malignant tumors may not be the main driver of metastasis. No genetic mutation or mutations have been unequivocally shown to be associated with progression from localized to metastatic disease^[29]. As shown by several in vitro experiments, epigenetic factors present inside and outside tumor cells may control the metastatic process. The cytoplasm of human embryonic stem cells can epigenetically reprogram multipotent metastatic melanoma cells and made them to assume a melanocyte-like phenotype^[30]. Adam Telerman and Robert Amson, two researchers from École Normale Supérieure from Paris, France, who have been modeling tumor reversion for more than 20 years, stated^[31] that the “reversion process involves a reprogramming mechanism using epigenetic and probably genetic tools that will supersede the changes in cancer by assembling and triggering alternative ways leading to the suppression of tumorigenicity”. Some of the metastasis master regulators may not be even located inside the cancer cell. The work of Bissell and Radisky^[32] and Orimo and Weinberg^[33] demonstrated the crucial role of the tumor associated stroma in promoting tumor metastasis. Convincingly, a recent review^[34] illustrated how hyaluronan, an integrated component of the extracellular matrix (ECM), may modulate several key hallmarks of cancer: sustaining of the proliferative signaling, evasion of apoptosis, angiogenesis, activation of invasion and metastasis, reprogramming of energy metabolism and evasion of the immune response.

CANCER CELL PROGRAMS

If cancer cells switch back and forth between different programs, cancer may represent a controllable cellular state that can rerouted to a non-neoplastic phenotype. The model of cancer as a potentially reversible cellular program^[35] complements and refines the genetic model. It has been previously suggested that many of the properties associated with invasion and metastasis do not arise as purely cell autonomous processes^[36]. In most of the cases, the metastatic process seems to be due to adaptation and not to selection of the cancer cells^[37]. It is the secretion of factors such as TGF- β , HGF, tumor necrosis factor (TNF)- α , Wnt and PDGF by the surrounding tumor stroma, and, the activation in the tumor cells of several master regulators of embryogenesis, such as the transcription factors Twist, Snail, Slug, Zeb1 and Zeb2, regarded as the epithelial to mesenchymal transition (EMT) core regulators, that drive metastasis^[13,29]. These processes may be mediated by miRNAs^[38], that coordinate multiple genes at the same time, so it will be more appropriate to talk in terms of various cellular programs, i.e., a division program possibly controlling key mitotic genes^[39], an invasion program possibly controlling key invasion genes^[40,41], and, a metastatic program, possibly controlling key EMT genes^[13] [Figure 3]. The master regulators of these programs may

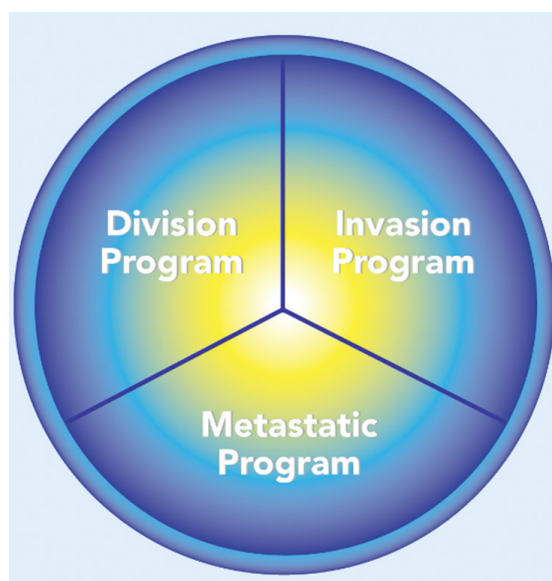


Figure 3. The cancer cell programs

or may not partially overlap. For example, miR-21 and miR-222 are involved in uncontrolled proliferation, miR-130 and miR-126 are involved in tumor angiogenesis and miR-373 and miR-155 are involved both in invasion and metastasis^[38]. The existence of specific cellular programs activated during the cancer process, suggests the possibility that the transformations induced by cancer both at the level of the tissue where it originally appears, and at distant sites where metastasis are formed, are not simple random by-products of malignancy but represent a well orchestrated process of local and global “cancerization”. In this paper, I argue that the metastatic phenotype is initiated and maintained by non-random CISP developed at the organismic level. The emergence of these CISP may result mainly from the finely regulated secretion by the tumors and their stroma of specific exosomes^[42,43]. Exosomes have been involved in the communication between the primary tumor and remote metastatic sites^[44] and, cancer cell-derived exosomes contain miRNAs that may regulate all the systemic hallmarks of cancer described below. In spite of the presence of RNase in blood, miRNAs survive due to their presence in exosomes^[45]. Both the nature of the proteins expressed on the surface of exosomes and the exosomes cargo are non-random as demonstrated by the work of David Lyden’s lab^[46,47]. Recently his team analyzed the protein exosomes from plasma of patients with five cancer types (breast, colorectal, lung, pancreatic, mesothelioma) and found that in cancer patients, the circulating plasma exosomes proteins, derive not only from the tumor itself, but also from the tumor environment, distant organs (i.e., liver) and the immune cells, this data supporting the “cancerized” organism model^[43].

CLINICAL IMPLICATIONS OF THE MODEL OF CANCER AS A MODIFIED CELLULAR PROGRAM

The division, invasion and metastatic programs may be activated differently and in different order in various cancer types, and this may explain the fact that cancers arising in different tissues have different propensity to grow locally, to invade the surrounding stroma and to metastasize. This might be related to distinct modified cellular programs present in Cancer Stem Cells (CSCs). CSCs by definition are a small subpopulation of cells within tumors with capabilities of self-renewal, differentiation, and tumorigenicity when transplanted into an animal host. When we think of CSC we mainly think of their ability to grow and form colonies but CSCs present in different tissues may not be the same qualitatively, and even, quantitatively^[48]. It is likely that due to these differences, programs for division, invasion and metastasis

are differently activated in different CSCs. Some CSCs may “instigate” or “educate” the stromal cells by secreting signals that induce changes in these cells that facilitate local invasion of the tumor^[49]. Other distinct population of stem cells, so called migrating cancer stem (MCS) cells, may be responsible for metastasis as proposed originally by Brabletz^[50]. Recently, this population of tumor cells with MCS properties was identified in a study conducted at Memorial Sloan Kettering Cancer Institute (MSKCI)^[51]. Using a colorectal cancer mouse model, the MSKCI investigators found two distinct population of stem cells: an adenoma forming stem cells population with oncogenic mutations and a L1CAM positive tumor-propagating metastasis-initiating stem cells without oncogenic mutations^[51].

A pervasive oncology dogma postulates that cancer develops in a linear way by initially growing locally, then subsequently invading the tissue where they appear and, finally, if given enough time, in the majority of cases, metastasize. Experimental data and clinical practice suggest that this assumption is incorrect. Some cancers, like breast or prostate, for example, sometimes behave as benign tumors that do not invade locally or metastasize and, maybe, this is why, the global, indiscriminately screening programs for breast and prostate cancers, may lead to over treatment of some patients. Another example is sarcoma, where roughly 50% of the sarcoma metastasize and 50% do not^[52]. As demonstrated by the work of Ganesh *et al.*^[51], the classical step by step genetic model of colorectal carcinogenesis of Fearon and Vogelstein^[53] may not apply to the metastatic process who does not involve a specific set of mutated genes. The different activation of different cancer cells programs in tumors of different types might explain the striking difference in clinical stage presentations of different cancer locations. For example, as many as 55% of squamous head and neck cancer presents as stage 4 most frequently with lymph nodes metastasis^[54], but only approximately 7% of thyroid cancers present as stage 4^[55]. The presence of distinct cellular programs in cancer may also solve the enigma of the existence of carcinomas of unknown origin where the primary tumor is never found. It is conceivable that in metastasis of cancers of unknown origin the metastatic program is activated before the division and invasive programs.

THE SYSTEMIC HALLMARKS OF CANCER

The hallmarks of cancer described by Hanahan and Weinberg in their two articles^[18,19], refer mainly to the cellular and tissular hallmarks of cancer. More recently^[56], Welch and Hurst proposed four cancer hallmarks specifically associated with the metastatic process: motility and invasion, colonization, plasticity and modulation but these four hallmarks are practically identical with the successive steps of the metastatic process described more than a decade ago by Joao Massagué^[20,21] and his collaborators. In this paper, we describe six novel systemic cancer hallmarks, that appear as a result of the interaction between cancer and the organism at the macroscopic level. The first systemic hallmark is the cancer system itself established through the connections between the primary tumor, the bone marrow and the distal metastasis. The five other systemic hallmarks are as following: the global inflammation, the immunity inhibition, the metabolic changes leading to cachexia, the propensity to thrombosis, and the neuro-endocrine changes [Figure 4].

Each of these six hallmarks is established through a different CISP. In the sections below, I will discuss one by one, the six CISP. The accompanying figures [Figures 5-10] are raw sketches illustrating the salient components of the different CISP. Apart from the nervous system, the connections between the CISP components are made through exosomes, cytokines and other soluble factors, represented by the dotted lines.

The primary tumor-metastasis network

Clinically, it has been noticed for a long time that the primary tumor and the distal metastases are interconnected and co-dependent. In some cases of renal cell cancer, for example, resecting the primary tumor induces a regression in the distal metastasis^[57]. On the contrary, in several experimental models, as shown by Folkman and his collaborators, resecting the primary tumor may accelerate the development of metastasis^[58,59]. Over the last two decades, it has been demonstrated also that besides this primary tumor-

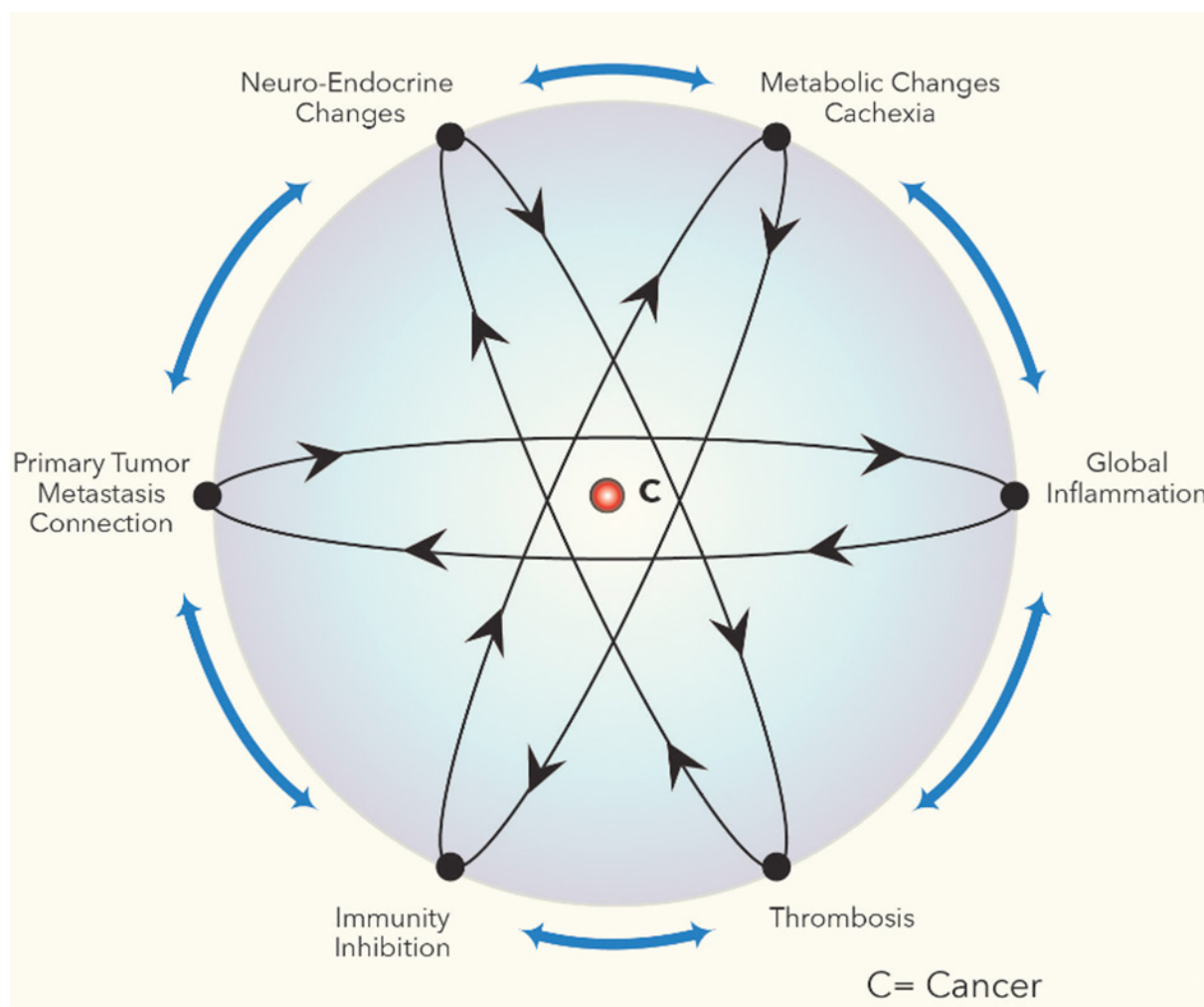


Figure 4. Systemic hallmarks of cancer

metastasis influence, there is a permanent “trialogue” between the primary tumor, the metastatic sites and the bone marrow [Figure 5]. Bone marrow can function as a source of hematogenic progenitor cells that prepare the niche for metastasis^[60,61] and, also possibly, as a source of malignant stem cells^[62].

Bone marrow-derived cells (BMDCs), which are frequently recruited to sites of tissue injury and inflammation, are crucial for the malignant process. In an elegant experiment, Houghton *et al.*^[63] demonstrated that in some cases, BMDCs may even represent the origin of malignant cells. These findings were subsequently confirmed by a different research team in a sarcoma mouse model^[64].

Kaplan *et al.*^[60] pioneered the work on the metastatic niche by demonstrating that BMDCs that express vascular endothelial growth factor receptor 1 (VEGFR-1) home to tumor-specific pre-metastatic sites and form cellular clusters before the arrival of tumor cells. Besides, BMDCs that facilitate the growth of tumor cells at distance from the site of origin and, cytokines and vesicles released into the circulation also contribute to the development of distal metastasis^[65-68]. In a recent review^[69], a comprehensive list of 34 primary-tumor, tumor stroma and myeloid derived factors that mobilize and recruit myeloid cells directly from the bone marrow to the pre-metastatic niche was compiled. The authors also proposed six characteristics of the pre-metastatic niche that empower the niche to favor tumor cell colonization and promote metastasis: angiogenesis and vascular permeability, lymphangiogenesis, inflammation,

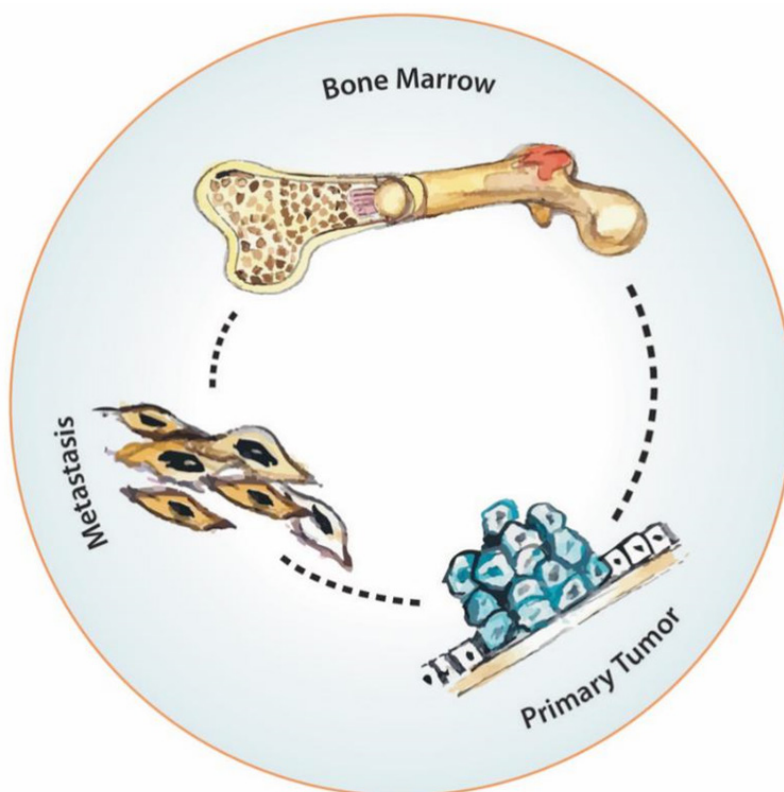


Figure 5. Primary tumor-metastasis-bone marrow network

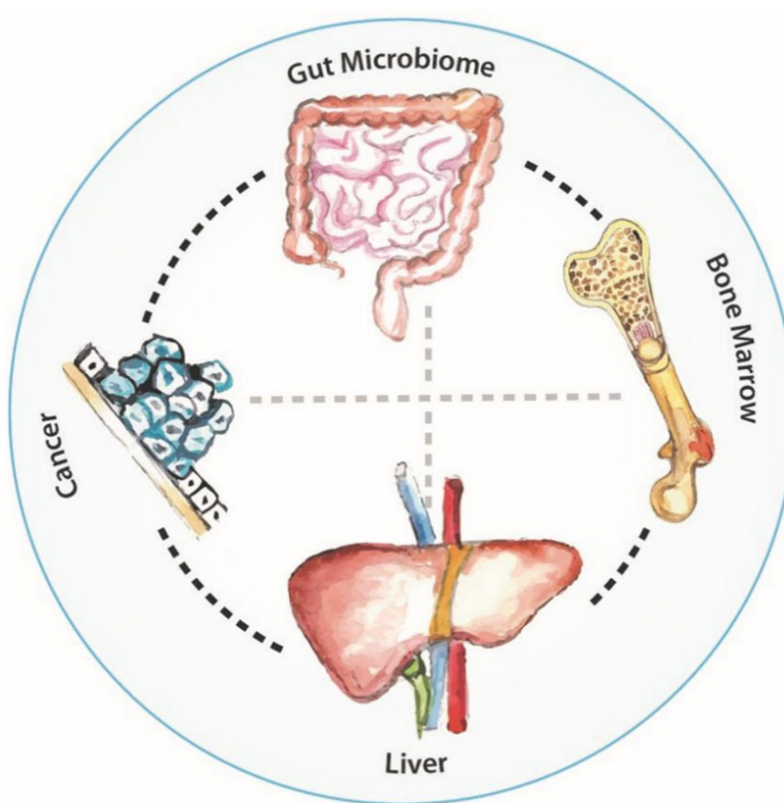


Figure 6. The systemic inflammation network

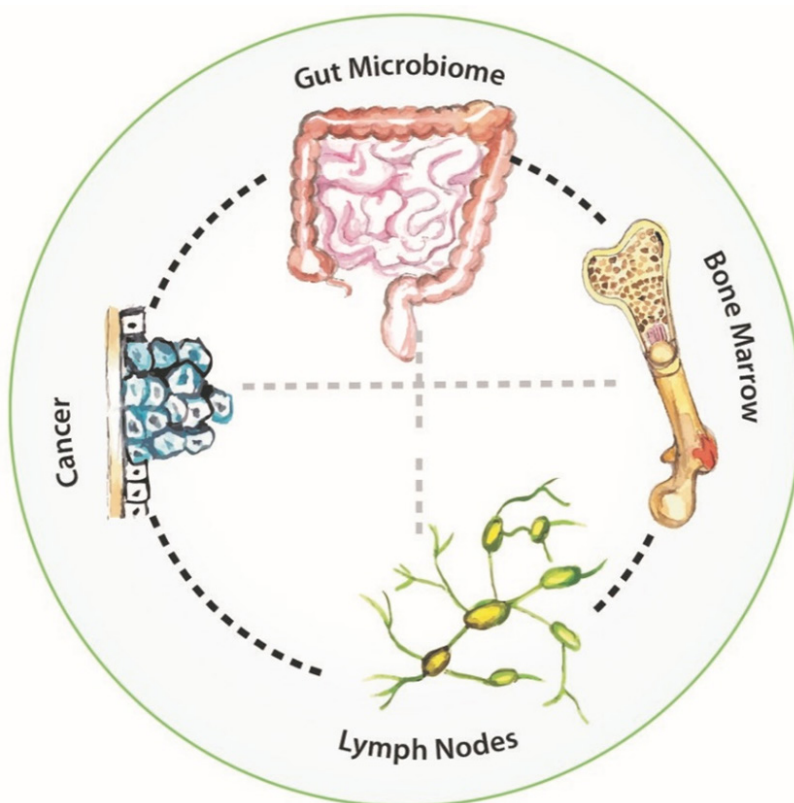


Figure 7. The immunity inhibition network

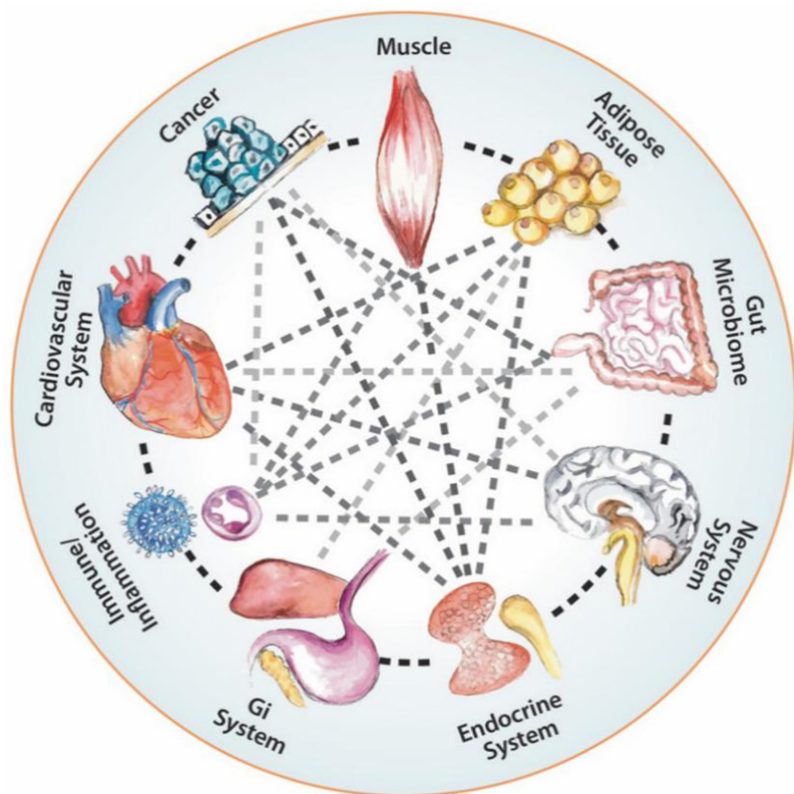


Figure 8. The global metabolism/cachexia network

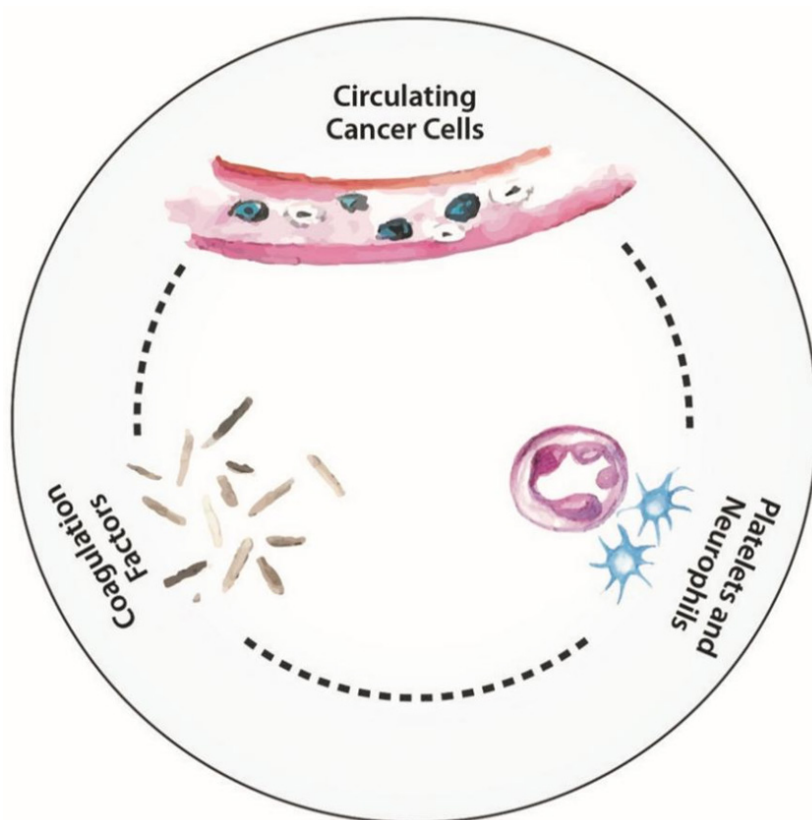


Figure 9. The thrombosis network

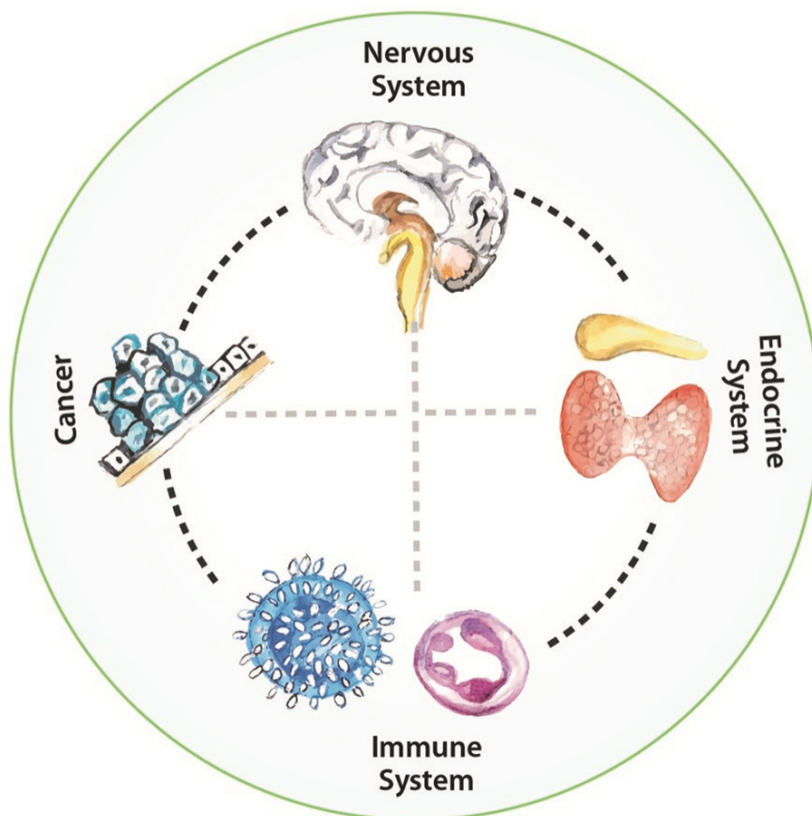


Figure 10. The neuro-endocrine network

immunosuppression, organotropism and reprogramming. These characteristics determine whether the metastatic cells present in the blood circulation can colonize and survive or become dormant after arrival^[69]. The best well described tumor secreted soluble molecules responsible for the niche formation and development are: VEGF-A, placental growth factor, and versican^[69]. Besides the cancer cells, there are also factors secreted by the tumor stroma that play a similar role: TGF- β , TNF- α , hypoxia-inducible factor-1 (HIF-1), granulocyte colony-stimulating factor (G-CSF). Some of these factors are secreted by the BDMCs themselves: VLA-4 (integrin $\alpha 4\beta 1$), matrix metalloproteinase (MMP)9 and ID3 protein (ID is a term that refers to its functional properties as both an inhibitor of DNA binding and an inhibitor of cell differentiation)^[70].

The exosomes are key factors responsible for the preparation of the pre-metastatic niche and the communication between the tumor, the bone marrow and the distal metastatic site^[41,71]. Different materials (proteins, mRNAs, DNA and miRNAs) carried inside the exosomes can be functionally delivered between different types of cells and transferred to distant locations, influencing the biological activities of tumor and non-tumor cells and promoting tumor growth, invasion, metastasis, angiogenesis, and drug resistance^[42,71]. A comprehensive review highlighted the key role played by the ECM components like syndecan in regulating exosome biogenesis, protein composition, function, and docking to recipient cells^[72]. Heparan sulphate chains of syndecans are essential for exosome formation within endosomal compartments, and trimming of heparan sulphate by heparanase activates the formation of an endosomal complex containing syndecan coupled to syntenin and ALIX^[73]. Reversely, tumor exosomes expressing high CD44 expression bind to hyaluronic acid and modulate the ECM as demonstrated for degradation of collagens, laminins, and fibronectin^[74]. Tumor-associated exosomes have been identified in biological (plasma, urine, saliva) and pathological (malignant effusions, pleural effusions, ascites) fluids from cancer patients. David Lyden's team from Weill Cornell Medical Center (WCMC), was able to demonstrate that in melanoma the transfer of the MET oncoprotein from tumor-derived exosomes to BM progenitor cells promoted the metastatic process and to describe quantitative and qualitative exosome signatures, along with specific BM progenitor cell populations mobilized as representative hallmarks of metastatic disease^[75]. Certain miRNAs are enriched in exosomes coming from the cancer cells, indicating that the exosomes composition seems to be controlled^[76]. Exosomes from mutant KRAS colorectal cancer cells for example show a distinct miRNA profile compared to wild type cells^[77]. The same WCMC team showed that in pancreatic cancer, the exosomes contained macrophage inhibitory factor (MIF) that is involved in the recruitment of bone marrow-derived macrophages and the blockade of MIF prevented liver pre-metastatic niche formation and metastasis. By releasing soluble factors, tumor cells were able to specifically direct bone marrow derived cells to the sites where they were supposed to go^[68]. An "integrin code" present on the surface of the exosomes seems to be responsible for the homing of future metastasis to precise prespecified distal organs^[46].

Redig and McAllister also included the participation of the bone marrow as a sine qua non component of the "instigation" model^[78]. These authors demonstrated a process of "systemic instigation" by injecting cells coming from an aggressive breast tumor in the flank of a mouse and analyzing the effect of these cells on the growth in the opposite flank of tumor cells coming from an indolent breast cancer tumor. The explanation for the influence at distance of one cancer tissue on the growth of another was established to be bone marrow-derived cells that were recruited to the distal sites and instigated the previously indolent tumor cells to grow^[78]. The importance of studying cancer as a systemic disease was underscored by another study done by a group of researchers from Harvard Medical School^[79]. The authors described a systemic cross-talk between lung tumors and bones. In an elegant mouse model, lung adenocarcinomas were able to remotely activate osteoblasts in bones even in the absence of local metastasis. In turn, these osteoblasts supplied tumors with neutrophils, which fostered cancer progression^[79].

Sometimes, the initiators of this "trialogue" are not the tumor cells themselves, but other stromal cells derived from the "cancerized" stroma. Cancer-associated fibroblasts have been shown, for example, to

release cytokines^[80] [i.e., SDF1] that once entered in the circulation may trigger the systemic release from the bone marrow of stem cells and haematopoietic progenitors that will support the formation of metastases^[81]. A research team from MD Anderson have shown that the origin of these cancer-associated fibroblasts is primarily the bone marrow^[82] but their origin and function within the tumor stroma varies^[83]. The existence of these networks of communication between geographically separated sites, brings experimental evidence to the cancer system model, and, supports the idea that metastasis is a non-random, finely regulated process.

The systemic inflammation network

The link between local stromal inflammation and cancer progression is well known and the molecular pathways responsible for this link have been well characterized^[84]. As any pathologist would certify, local inflammation is present in the stroma of many tumors and, inflammatory cells and molecules, may be involved in almost every aspect of cancer progression, including the tumour cells' ability to metastasize. Colotta *et al.*^[85] proposed that cancer related inflammation represents a seventh hallmark of cancer.

As described by Grivennikov *et al.*^[86], there are several types of inflammation that can promote cancer development and progression, differing by cause, mechanism, outcome, and intensity. Briefly, there is chronic inflammation associated with infections or autoimmune disease, there is inflammation due to prolonged exposure to environmental irritants or obesity, there is another distinct type of inflammation related to the tumor, and, finally, there is inflammation related to cancer therapies themselves. Approximately 20% of human cancers may be related to chronic inflammation caused by infections, exposure to irritants, or autoimmune disease^[87]. On the other hand, not only chronic inflammation may lead to cancer, but cancer may also cause local and systemic inflammation. Oncogene activation in cancer cells lead to expression of pro-inflammatory transcription factors within tumor cells [such as nuclear factor-kappaB (NF-κB), signal transducer and activator of transcription (STAT)3 or HIF-1α]. These activated transcription factors mediate the expression of key cytokines and chemokines as well as inflammatory enzymes within the tumor microenvironment. At the tissular level, different cytokines can either promote or inhibit tumor development and progression. Some of them may lead to tumor progression (IL-6, IL-17, IL-23), and also have direct effects on cancer cell growth and survival [TNF-related apoptosis-inducing ligand, Fas ligand, TNF-α, epidermal growth factor receptor (EGFR) ligands, TGF-β]. Others [IL-12, interferon (IFN)γ] may have an anti-tumor effect^[86].

Cancer inflammation is not only a local phenomenon [Figure 6]. High serum concentrations of inflammatory cytokines, (i.e., IL-1, IL-6) are found in many advanced malignancies^[88]. Circulating cytokines and small inflammatory molecules, such as chemokines and matrix-degrading proteins, are also involved in the systemic inflammation, and play a crucial role in the metastatic process^[88]. IL-1, for example, is involved in invasion and angiogenesis. IL-1 may enhance the invasiveness of already existing tumor cells by the induction of inflammatory molecules, such as MMPs, VEGF, heparanase, chemokines, and integrins on the malignant cells and endothelial cells, or by switching on the angiogenesis leading to tumor dissemination and metastasis^[89]. Systemic inhibition of IL-1 with anakinra (a recombinant derivative of IL-1RN) inhibits the growth and density of new vessels in IL-1-producing human tumor cell lines xenografted into immunodeficient mice, but not in their counterparts that do not produce IL-1^[90]. A recent review^[91], provided a comprehensive list of factors associated with colorectal systemic inflammation including cytokines, chemokines and growth factors (IL-6, C-C Motif Chemokine Ligand (CCL)2, CXCL (C-X-C Motif Chemokine Ligand)8, CSF1 [macrophage colony-stimulating factor (M-CSF)], and CSF2 [granulocyte-macrophage colony stimulating factor (GM-CSF)]). The authors noted that immune cells and fibroblasts are capable of producing many of these factors at much higher level than tumor cells and pointed out to the role of the stroma in systemic inflammation. Some of these immunomodulatory effects are modulated by exosomes that contain in their cargo a large variety of molecules^[92,93]. Exosomes secreted by tumors contain IL-8, CCL2, CCL3, CCL4, CCL5, CCL20 and TGF-β^[93].

The liver is a key player in the systemic inflammatory response. All the acute-phase proteins, including C-reactive protein (CRP), amyloid A, α_1 antitrypsin, and α_1 acid glycoprotein, are synthesised in the liver and secreted into the circulation^[94]. Classical studies have shown the deleterious role played by the cytokines secreted by the Kupfer cells in acute inflammation^[95]. For example, complete elimination of liver macrophages, decreased the mortality of mice challenged with zimosan, a potent inflammatory agent, from 27% to 0%^[96].

Another key component of the global inflammation network is the gut microbiota. The human body is in symbiosis with the gut microbiota, which outnumbers human cells by a 10-fold factor. As shown by two Science articles, gut microbiota modulates inflammation in both the tumor microenvironment and in the systemic circulation^[97,98]. Microbiota also regulates steady-state myelopoiesis and neutrophil homeostasis^[99]. Mouse models have shown that gut microbes promote the development of mammary carcinomas via a neutrophil-mediated mechanism^[100], and, microbiota-driven mobilization of myeloid-derived suppressor cells, favors malignant progression through systemic tumor promoting inflammation^[101].

Increase of the systemic markers of inflammation as neutrophils, lymphocytes and platelet counts and acute phase proteins, such as CRP and albumin or their combinations, computed in different scores, i.e., the neutrophil lymphocyte ratio, the platelet lymphocyte ratio and the Glasgow Prognostic Score, are associated with adverse prognosis in several malignancies^[102].

A systemic immune-inflammation index (SII), which is calculated as platelet (P) \times neutrophil (N)/lymphocyte (L) counts, has also been demonstrated to be closely associated with the prognosis of solid tumors especially lung cancer^[103].

In most advanced cancers, systemic inflammation is caused by cancer itself and indicate the aggressiveness of the tumor^[104]. Unfortunately, despite pre-clinical efficacy demonstrated in several animal studies, until present, agents used to manipulate systemic inflammation in the treatment of patients with advanced-stage cancer have only shown modest results^[105]. The clinical trials that used inhibitors of primary inflammatory cytokines (e.g., TNF- α , IL-6, IL-8), in the treatment of various types of human cancers (i.e., pancreas, renal) showed only limited benefit. This is not surprising as the function of cytokine varies with the clinical context and the same cytokine may promote or inhibit cancer progression. The same cytokine can be beneficial in some clinical context and detrimental in others, and the term yin-yang has been used for cytokine behavior^[106]. Currently there are multiple clinical studies in progress using agents that target cytokines (i.e., IL-1, CXCR4/CXCL12), transcription factors (i.e., JAK-STAT pathway inhibitors) or local immune/inflammatory cells (i.e., macrophages M2) and the field of cancer inflammation is currently a very active area of research^[105,107].

The immunity inhibition network

Tumor promoting inflammation and anti-tumor immunity are the two opposite factors that shape the evolution of tumors^[108]. As illustrated in the above section, tumors actively induce a global inflammatory state. They also inhibit the immune system, both locally and systemically [Figure 7]. The local inhibition of the immune system by the tumor checkpoint molecules has been well characterized and the use of checkpoint inhibitors is currently approved in many types of cancers. Tumors may also have a global inhibitory effect on the immune system as recently shown by a team from the University of Pennsylvania. The researchers described the release of exosomes carrying programmed death-ligand 1 (PD-L1) on their surface by metastatic melanoma cells. Stimulation with IFN- γ increased the amount of PD-L1 on these vesicles, which suppressed the function of CD8 T cells and facilitated tumor growth^[108]. Tumor cell-derived exosomes can also impair immunity through different mechanisms: exosomes containing miR-203 secreted by pancreatic cells may impair activation of the immune system through downregulation of toll-like

receptor 4 and IL-12^[109], exosomes can down regulate the functions of immune cells^[110,111], may promote Tregs expansion^[112], and inhibit the activity of natural killers (NK) cells^[113].

As is the case with inflammation, there is a co-dependent relationship between the immune system and the gut microbiome. The immune system plays an important role in defining the composition of the microbiota and preserving the ecology of the microbiota. Reversely, the microbiota influences all aspects of the immune system. Gut microbiome plays an important role in the training and the functional tuning of the immune system and can be seen as one of the key modulators of the immune system^[114]. In addition to influencing localized immune responses, microbiota also has broader effects contributing to innate and adaptive immunity at multiple levels^[115]. Myeloid cells respond to microbial signals, and initiate innate and adaptive immune responses^[99]. In 2013, it has been shown in two murine models that germfree or antibiotic-treated animals did not respond to chemotherapy, indicating that an intact microbiome was required for modulating the myeloid-derived immune cell responses in the tumor microenvironment^[97,98]. Alterations in the gut microbiome can affect response to immunotherapy in several cancer types. Matson *et al.*^[116] identified different bacterial species as being critical for response to therapy in their patients with advanced melanoma, with *Bifidobacterium longum*, *Collinsella aerofaciens*, and *Enterococcus faecium*, among others, found to be enriched in the feces of patients that responded to anti-PD-L1. Similar findings were reported by Routy *et al.*^[117] in patients with advanced urothelial carcinoma, non-small cell lung cancer, and renal cell carcinoma. Patients who have been treated with antibiotics within several months before, during, or after treatment with PD-1/PD-L1 blockade had shorter progression-free survival and lower overall survival rates compared with patients who had not received antibiotics. After sequencing fecal samples from these patients, the genera *Akkermansia* and *Alistipes* were enriched, and, the bacterial species *A. muciniphila*, specifically, was found to be highly represented in patients that responded to checkpoint blockade.

The immune cells play a dual role in cancer^[118,119]. Classically, some immune cells may promote cancer growth (M2 macrophages, T regs cells) and others fight cancer (M1 macrophages, CD8 cells). This is an over simplification as the same type of cells may play a pro, or anti-neoplastic role depending on the local and systemic context. For example, in the majority of cancers, an increased number of T regs in the tumor is associated with a poor prognostic, but in patients with colon or breast carcinomas, the presence and frequency of T reg in the tumor is correlated with an improved prognostic^[120]. A similar phenomenon has been shown for tumor associated macrophages^[121]. Like macrophages and T reg cells, tumor-associated neutrophils and NK cells may have both antitumoral and protumoral functions^[122]. As shown in by Labelle *et al.*^[123], platelets attract neutrophils into the tumor thrombi contributing to the metastatic niche development. Also, a high neutrophil to lymphocyte ratio, predicts poor outcome in several types of cancer including lung cancer, pancreatic cancer and colorectal cancer. There is new data showing direct involvement of neutrophils in different types of cancer and there is increasing evidence in preclinical models that granulocyte-CSF (G-CSF) can promote metastasis^[124,125]. Also, as shown by several research teams, metastatic cancer cells can induce neutrophils to form metastasis-supporting neutrophil extracellular traps (NETs) and drugs that degrade NETs have been shown to have a profound inhibitory effect on the development of metastatic disease in preclinical models^[126,127].

The global metabolism/cachexia network

In order to ensure sufficient biomass synthesis for their growth, cancer cells need to maintain high metabolic turnover rates. A large amount of energy is required to support this process. For example, an estimated ~17,700 kcal are required over 3 months to support metastatic colorectal cancer growth^[128]. Since the seminal work of Warburg^[129], it has been observed cancer cells have distinct metabolic programs than normal cells and metabolic reprogramming has been acknowledged as one of the classical hallmarks of cancer^[19]. The most distinctive metabolic differences of cancer tissues are increased aerobic glycolysis,

elevated glutaminolytic flux and enhanced amino acid and lipid metabolism. Some types of cancer cells utilize in excess glucose and, in some cases secrete lactate even in the presence of oxygen (the Warburg phenomenon). The propensity of cancer cells towards aerobic glycolysis does not seem to be related to an impairment of the respiration, as respiration is also needed for tumor growth^[130,131]. In some cancer patients, lactate is converted back to glucose in the liver, a process known as the oncogenic Cori cycle^[132-134] a process that is energetically very inefficient. Besides glucose and lactate, there are other nutriment needed for tumor growth for example, glutamine, glycine and aspartate for purine and pyrimidine synthesis, serine for membrane lipid component synthesis, branched amino acids, lipids, acetate and others^[135]. Not in all cancers the Warburg phenomenon is present, and, sometimes, high glycolytic rates in tumors and mitochondrial respiration often operate simultaneously in tumors^[136]. A sort of metabolic parasitism has been described at the tissular level by a group of French researchers^[137] who introduced the concept of the “reverse Warburg effect”^[138,139]. These authors proposed that aggressive cancer cells are “parasites” that use oxidative stress as a “weapon” to extract nutrients from surrounding stromal cells, forced to undergo aerobic glycolysis, and produce energy-rich nutrients (such as lactate and ketones) to “feed” cancer cells. They suggested that stromal catabolism, via autophagy and mitophagy, fuels the anabolic growth of tumor cells, promoting tumor progression and metastasis.

What is also becoming apparent, is that cancer cells or tissues have an altered metabolism, but, they also induce systemic changes of the whole body metabolism by secreting humoral factors (i.e., TNF- α , IL-1 and IL-6) and pro-cachectic factors (i.e., proteolysis-inducing factor and lipid mobilization factor) that lead to a generalized catabolic state followed by significant and progressive energy loss from host tissue in the final stages of cancer^[140,141]. A group of researchers from Taiwan metaphorically compared these influences of the tumor on the host's metabolism as a “metabolic dictatorship”, the tumors imposing their high demands on the normal host these metabolic changes, ultimately, in some types of cancers (i.e., pancreatic or gastric cancer) leading to cachexia^[142]. Basically, the metabolic parasitism described at the tissular level exists also at the level of the whole organism^[143].

Cachexia is a multi-organ syndrome involving changes in many tissues and organs besides the muscle, the adipose tissue and the tumor itself, other organs including the liver, the pancreas, the brain and the gut^[144] [Figure 8]. It involves up-regulated tissue catabolism and impaired anabolism, release of tumor-derived catabolic factors and inflammatory cytokines, and neuroendocrine dysfunction^[145]. As previously suggested by Al-Zoughbi and Porporato, the global metabolic changes that tumors exert on the whole organism are due to a precise reprogramming of the different key structures involved in the normal body energy expenditure balance and are not simply complications of tumor progression^[6,134]. This is why these authors introduced new terminology to describe this phenomenon, i.e., “macroenvironment”^[6] and “metabolic cancer syndrome”^[134]. In addition to the direct effects of tumor-derived cytokines on individual organs, there is also an interplay between muscle, fat, and liver involving several signaling pathways and metabolites leading to pathologic networks formation resulting in disruption of key metabolic pathways^[140] [Figure 8].

The incidence of cachexia among cancer patients is very high, especially in gastric and pancreatic cancer where the incidence is more than 80%. One of the main causes of cancer cachexia is inflammation. The cytokines secreted by the tumor may lead to the symptoms commonly associated with cachexia (loss of appetite, pain, fever, fatigue, cachexia) but, ultimately, cachexia is dependent on the patient response to tumor progression and the activation of the inflammatory response^[146]. One of the key cytokines involved in cachexia is IL-6, linked to both cachexia and metastasis events^[86], but, also other cytokines - such as TNF- α , IL-1 β , and TGF- β - are involved and they may induce inflammation and muscular and adipose tissue wasting^[147,148]. Pro-inflammatory cytokines promote also a shift in liver protein synthesis towards the production of CRP instead of albumin - which contributes to sustaining chronic inflammation^[149].

Inflammation is the key trigger of muscle wasting inducing alterations in protein and amino acid metabolism, together with activation of apoptosis and decreased regeneration^[144]. Besides muscles wasting, adipose tissue wasting is also present in the majority of cachexia patients. Indeed, cachectic patients manifest high levels of circulating free fatty acids, glycerol and triacylglycerol^[150] and the trigger of lipolysis may be also systemic inflammation^[151]. Another characteristic of cancer cachexia is the progressive switch from white adipose tissue to brown adipose tissue-brown adipose tissue derives its name from the darker color associated with the enrichment in mitochondria^[152]. Pro-inflammatory factors either derived from the host immune system or the tumor, contribute to this switch^[152]. Browning strongly contributes to the increased energy expenditure common in cachectic patients and, interestingly, cachectic lipid wasting occurs mostly in tumors actively secreting parathyroid hormone-related protein^[152,153]. Pro-inflammatory cytokines, such as IL-6, IL-1, TGF- β , and TNF- α , are common denominators both for metastasis and inflammation and for the metabolic reprogramming associated with cachexia^[86,122,133] and their underlying molecular pathways might overlap^[154]. One of these molecular pathways might be exosome secretion by tumour cells or adipose tissue. It has been shown recently that tumor related exosomes play a key role in activating the inflammatory process in cancer; thus, they might be involved in both host-wasting processes, as well as metastatic dissemination^[155-158]. As a direct proof of this concept, a study^[159] published in 2014, showed that cancer-derived microvesicles containing miR-21 induce apoptosis of skeletal muscle and lipolysis of the adipose tissue. Similarly, another study demonstrated that cancer associated microvesicles induce muscle wasting in mice through releasing extracellular Hsp70 and Hsp90^[155].

We will describe briefly below the contribution of different organs or body systems to cachexia. First, the liver plays a major role in cachexia. In the KRAS/P53 mouse model it has been shown that lung tumors act distally on the liver and reprograms hepatic metabolism through altered pro-inflammatory response via the STAT3-Socs3 pathway resulting in inhibition of hepatic insulin signaling, increased glucose production and a deregulated lipid synthesis^[160]. The authors suggested that tumor-secreted 'waste' such as lactate is converted to pyruvate and shunted through gluconeogenesis to produce glucose, which can further satisfy the heightened energetic demand of cancer cells. A research team coordinated by Douglas Fearon also demonstrated in two mouse models of cancer-induced cachexia that in pre-cachectic mice, even before the onset of the weight-losing phase of the syndrome, tumor-induced IL-6 has altered the capacity of the liver to respond to caloric deprivation^[141]. Tumors induce a reprogramming of the hepatic metabolism blocking the host's capacity to make available endogenous sources of energy that compensate for decreased caloric intake. Through suppressing ketogenesis, the tumor hampers the host's capacity to produce endogenous sources of energy that compensate for decreased caloric intake. This energy deficit magnifies the host stress response and leads to increased glucocorticoid levels that suppress the tumor immunity^[140]. Using an inducible lung cancer mouse model, a research group from WCMC, co-ordinated by Lewis Cantley, found that cachexia was associated with low ketones and increased glucocorticoid levels that suppresses tumor directed immunity^[161]. The low ketones level associated with reduced expression of hepatic peroxisome proliferator-activated receptor- α (PPAR α) targets that regulate fatty acid oxidation and ketogenesis. Treatment with fenofibrate, a PPAR α agonist restored hepatic ketogenesis, prevented the reliance on hepatic gluconeogenesis, and skeletal muscle wasting. This model was consistent with the hypothesis that global inflammation induced by the tumor signals the brain that increases corticotropin-releasing hormone (CRH) leading to glucocorticoid production that will induce type 2-skeletal muscle fibers breakdown^[161].

The pancreas also plays an important role in cachexia through secretion of insulin and glucagon. Insulin resistance is both a risk factor for cancer and is associated with cancer progression. The increase in insulin level driven by insulin resistance can drive cancer growth both directly through insulin receptors, and IGF-1 receptors present on the surface of cancer cells^[162,163] and indirectly by promoting liver gluconeogenesis and muscular wasting. Also, the increased production of glucagon in the alpha islet of pancreas during cancer progression, may also increase liver gluconeogenesis^[164]. Branched aminoacids released from the muscle

will be used in the liver for gluconeogenesis or protein synthesis in lung tumors^[165]. Interestingly, the increase of branched aminoacids blood levels may precede the clinical appearance of pancreatic cancer by several years^[166].

The impact of gut on cachexia is mostly through the gut microbiota. Alteration of the gut flora due to undernutrition and chemotherapy ultimately affects specific metabolite availability and absorption, which in turn affects tumor growth and cachexia^[167]. Host metabolism and energy balance are also influenced by an interplay between the intestinal microbiota, bile acids and nutrients that may have an impact on global inflammation, immune responses, gut hormone secretion and neuronal activity^[168].

Several hormones including insulin, catecholamines and atrial natriuretic peptide are involved in lipolysis^[169]. Besides the endocrine system, the brain is also actively involved in the cachectic syndrome by controlling food intake through appetite, satiation, taste and smell of food. Receptors of TNF- α and IL-1 are found in the hypothalamic areas of the brain, which regulate food intake. Anorexia induced by both TNF- α and IL-6 can be blocked by inhibitors of cyclooxygenase, suggesting that a prostaglandin, such as PGE2, may be the direct mediator of appetite suppression^[169,170]. Autonomic nervous system dysfunction has been also described in cancer patients with cachexia^[171]. IL-6 was found to stimulate hypothalamic release of CRH, and increase glucocorticoid production^[172].

Structural and functional heart changes similar to those found in cardiac failure are often associated with the cachexia syndrome. In addition to a loss of skeletal muscle mass and function, many patients with cancer cachexia also experience cardiac atrophy, remodeling, and dysfunction, which in the field of cancer cachexia is described as cardiac cachexia^[173,174]. It has been shown for more than two decades that cardiac cachexia is linked to raised plasma levels of TNF- α and other inflammatory cytokines and that the degree of body wasting is strongly correlated with neurohormonal and immune abnormalities^[175].

Israel and Schwartz^[176] postulated that cancer cells have hybrid metabolic features that take advantage of the catabolic state that they also initially induce. The two French authors proposed a comprehensive model of the systemic metabolic changes induced by cancer that I will describe briefly. Normally, in starvation, when blood glucose level decreases, glucagon and epinephrine activate gluconeogenesis and ketogenesis to form nutriment, mobilizing body stores. On the contrary, when glycemia is elevated, the pancreas releases insulin, activating anabolism and oxidative glycolysis, energy being required to form new molecules or refill stores. Usually, these two opposite physiological states exclude each other; when anabolism is triggered by insulin, catabolism is blocked and the normal organism metabolic configuration is finely regulated by the state of key enzymes. Depending on the needs, enzymes function like switches and direct the metabolism towards different pathways that are open or closed depending on their phosphorylation state. In cancer, some of their enzymes are phosphorylated as normally observed when catabolic hormones stimulate Gs-coupled receptors, whereas other enzymes adopt a configuration normally found in anabolic situations, mediated via tyrosine kinase receptors. Basically, despite the fact that the organism as a whole is in a starvation-like state induced by cancer, tumor cells have their anabolic pathways turned ON through tyrosine kinase receptors, sometimes constitutively activated through genetic mutations or amplifications. The pyruvate kinase (PK) and pyruvate dehydrogenase (PDH) of cancer cells are OFF in a phosphorylated form but the citrate synthase is ON pulling the glucose flux in the glycolytic direction. So, on one hand, cancer cells, have their PKs and PDHs inhibited by phosphorylation, like in gluconeogenesis, on the other hand they have an increased glycolysis that will be used for the synthesis of new molecular building blocks for new mitotic daughter cells. As a result, cancer cells burn glucose and increase the tumor mass, at the same time consuming the muscle proteins and the lipid stores of the organism. The outcome of this hybrid rewired metabolism gives them a selective advantage over normal cells^[176]. In subsequent publications, Israël proposed that the reason for this hybrid metabolism is an alteration of the GABA selection switch

between anabolism and catabolism in the pancreas, leading to a concomitant release of catabolic glucagon and anabolic insulin^[177]. According to him, the first cells that manifest a hybrid metabolism are the stem cells. Subsequently, this metabolic rewiring is stabilized through mutations or epigenetic changes selecting the most aggressive population and cancer cells arise. In his model, the pancreatic alteration is the *primum movens* of cancer followed by the metabolism switch of stem cells. The stem cells, initially committed to repair an organ, subsequently transform into cancer cells that use their metabolic advantage to compete for resources with the rest of the organism^[177-179]. The practical value of Israël's model is first the prediction that cancer could be detected several years before its clinical manifestations because of specific metabolome changes, and, second, the proposal of correcting the GABA switch pancreatic anomaly as a method for cancer prevention. In 2016, a group of German researchers analyzing data from the European Prospective Investigation into Cancer and Nutrition study found that abnormalities of two lipid metabolites (high levels of phosphatidylcholine PC ae C30:0 and low levels of lysophosphatidylcholines, C18:0, were consistently associated with increased risk of breast, prostate and colorectal cancer. These abnormalities were detected several years before the clinical apparition of cancer pointing to a global metabolic shift in phosphatidylcholine metabolism that may drive tumorigenesis^[180].

The thrombosis network

The role of different blood components^[181] and the lymphatic system^[182] in the metastatic process has been coming more and more into focus. Therapies targeted against other blood and lymphatic factors involved in cancer are in development^[181,182]. As many as 20% of cancer patients may have a thrombosis event during their lifetimes^[183]. As shown in a recent review^[184], a reciprocal connection exists between cancer and thrombosis, on one side cancer cells supporting clot formation, on the other side, clotting proteins support cancer growth and dissemination. Cancer is associated with a state of hypercoagulability, driven in part by the release of procoagulant factors, such as tissue factor (TF), released by the malignant tissue, as well as by inflammation-driven activation of endothelial cells, platelets, and leukocytes. Also, cancer cells are able to directly adhere to host cells (i.e., endothelial cells, monocytes, platelets, and neutrophils), thereby stimulating additional prothrombotic properties of the host thrombosis effector cells^[184] [Figure 9].

TF is considered to be the major molecular driver of cancer-associated coagulopathy and thromboembolic disorders. It is expressed either by cancer cells or its expression is induced by cancer cells in normal vascular tissues by both the release of soluble mediators and the direct cancer cell - host cell contact. Its expression is related to well defined oncogenic events: epidermal-to-mesenchymal transformation, TGF- β signaling, EGFR, phosphatase and tensin homolog (PTEN) and Src pathways, hypoxia induced signaling, *etc.* The majority of human epithelial cancers (lung, colorectal, prostate, breast, pancreatic, gastric, melanoma, *etc.*) are characterized by abundant levels of TF^[185]. Also, as shown by a Canadian group in a glioma model, TF may also control the state of tumor dormancy by recruiting to the tumor niche myeloid and blood vessels forming cells^[186]. Interestingly, the procoagulant and the signaling effect of TF in tumor biology can be targeted separately, and there are therapies under investigation that target solely the signaling effect of TF without affecting its homeostatic function^[187].

In addition, cancer cell interactions with platelets and neutrophils contribute to cancer cell adhesion, extravasation, and the establishment of metastatic lesions^[188]. Platelet-derived signals are required for the rapid intravascular recruitment of neutrophils to circulating tumor cells (CTCs) thrombi contributing to "early metastatic niches"^[123]. Also, TGF- β secreted by degranulating platelets may contribute to the activation the NF- κ B pathway in carcinoma cells, thereby inducing or sustaining the expression of EMT programs in the CTCs^[189]. Selectins are carbohydrate-binding molecules that bind to glycan structures, present on endothelial cells, platelets and leukocytes. There are three members of the selectin family: P-selectin expressed on activated platelets and endothelial cells, L-selectin present on leukocytes and E-selectin expressed on activated endothelial cells^[190]. P-selectin in particular seems to play a crucial role in

several types of cancer metastasis by mediating the aggregation of platelets with tumor cells forming clots. A recent study showed that intravenous injection of melanoma cells into WT mice resulted in multiple lung metastases, while in P-selectin-deficient mice pulmonary tumor metastasis and trapping of tumor cells in the lung was significantly reduced^[191]. Modulating the interaction between cancer cells and the circulating blood cells, and respectively, between cancer cells and the endothelial cells may represent novel therapeutic approaches. For example, there is experimental evidence that targeting specific types of the integrin receptors present on the surface of the platelets efficiently reduces tumor cell colonization into the lungs, suggesting that they could represent interesting targets for anti-metastatic drugs^[192].

A team from France characterized the microparticulosome, the repertoire of plasma membrane vesicles produced by different types of cells and was able to differentiate a microparticle signature associated with pancreatic and colorectal cancer^[193]. The same team showed in syngeneic ectopic and orthotopic mice models that treatment with the drug Clopidogrel prevented the binding of cancer cell-derived microparticles to fibrinogen-platelets aggregates at the site of thrombosis, and reduced the metastasis and the extent of thrombosis associated with cancer^[194]. Procoagulant factors associated with exosomes from tumors have been described for almost four decades^[195]. Tissue factor associated with exosomes has been found to be responsible for the Trousseau syndrome in one patient with lung cancer^[196] and there are several studies documenting the procoagulant effect of tumor exosomes^[197].

The neuro-endocrine network

Both the central nervous system and the neurovegetative nervous system are intimately involved in cancer. One of the most studied links between central nervous system and cancer is stress. The neuroendocrine mediators reach the cells of the immune system either through the peripheral circulation or through direct innervation of lymphoid organs [Figure 10]. As suggested by Claire Magnon^[198], a possible explanation for tumor formation associated with stress might rely on the activation of the sympathetic nervous system (SNS) through the sympathetic - adrenal - medullary axis, which controls the release of adrenergic neurotransmitters such as epinephrine or norepinephrine by the adrenals into the bloodstream in support of the fight-or-flight reflex. Catecholamine-mediated suppression of cellular immunity may play a role in increased growth of certain tumors^[199]. Also, primary and secondary lymphoid organs are innervated by sympathetic nerve fibers. Lymphocytes and monocytes express receptors for several stress hormones, including CRH, adrenocorticotrophic hormone (ACTH), cortisol, norepinephrine, and epinephrine. Therefore, it is possible that the neuroendocrine hormones released during a stressful event could alter immune function and subsequently alter the course of immune-based diseases^[200]. It has been reported that mice living in an enriched housing environment (EE) show reduced tumor growth and increased remission^[201]. This effect was described in melanoma and colon cancer models, and, it was proven that it was not caused by physical activity alone. Serum from animals held in an enriched environment (EE) inhibited cancer proliferation in vitro and was markedly lower in leptin. Hypothalamic brain derived neurotrophic factor (BDNF) was selectively upregulated by EE, its genetic overexpression reduced tumor burden, whereas BDNF knockdown blocked the effect of EE. The hypothalamic BDNF downregulated leptin production in adipocytes via sympathoneural β -adrenergic signaling^[201].

A key central nervous system structure involved in cancer is the hypothalamus. In the context of systemic inflammation, the hypothalamus integrates signals from peripheral systems, translating them into neuroendocrine perturbations, altered neuronal signaling, and global metabolic derangements^[202]. Cytokines, like IL-1 β and TNF- α , for example, generated in the periphery during cancer progression are amplified and modified within the hypothalamus, leading to hypothalamic inflammation and aberrant activity of weight- and activity-modulating neurons that may induce muscle atrophy via activation of the hypothalamic-pituitary-adrenal axis^[203,204]. Hypothalamic inflammation may be followed by dysregulation of homeostatic regulation of autonomic nerves (innervation of muscles, liver, fat tissue, endocrine glands

and other organs) that may further potentiate dysregulation of metabolism and enhance peripheral, pro-inflammatory reactions^[205]. Hypothalamus appears to be an important contributor in the development and maintenance of the cachectic state^[202]. Lower hypothalamic activity has been demonstrated by functional magnetic resonance imaging scans in patients with cachexia associated with advanced lung cancer^[206].

A unique crosstalk between the central nervous system and prostate tumours was recently revealed. In a striking experiment^[207], using a mouse model of prostate cancer, a French group demonstrated a process of tumour-associated neo-neurogenesis, in which neural progenitors leave the brain of the mouse and reach, through the systemic circulation, the primary tumour or the metastatic tissues. Once arrived there, they differentiated into new adrenergic neurons that are known to support the early stages of the development of cancer. The authors suggested the possibility that the tumour itself might deplete neurogenic niches in the brain by attracting neural progenitors to support its own development^[207].

Recent experiments suggest a direct relationship between the neurovegetative nervous system and certain tumors. As reviewed by Cole^[208], SNS activation modulates gene expression programs that promote metastasis of solid tumours by stimulating macrophage infiltration, inflammation, angiogenesis, epithelial-mesenchymal transition, and tumour invasion, and by inhibiting cellular immune responses and programmed cell death. SNS activation may also influence cancer progression via indirect pathways in which SNS innervation of distant tissues triggers secondary hormonal or cellular effects that subsequently affect the tumour microenvironment. For example, sympathetic innervation of bone marrow can stimulate the production of myeloid lineage immune cells which may infiltrate the tumoral microenvironment and promote metastasis^[209-211]. In prostate cancer, sympathetic nerve fibers may help tumors grow by interacting with beta-adrenergic receptors on stromal cells^[212]. Epidemiological studies showed that men with prostate adenocarcinoma who take non-selective beta-blockers have lower prostate cancer-specific mortality rates^[213]. A similar activity of beta-blockers has been described in melanoma or breast cancer patients indicating that adrenergic signaling might be involved in various types of cancer^[214,215].

If the role of the sympathetic system in cancer has been well documented, the contribution of the parasympathetic division of the autonomic nervous system is less clear. As shown by Kevin Tracey and his collaborators from the Feinstein Institute on Long Island, New York, the efferent vagus nerve-mediated cholinergic signaling controls immune function and pro-inflammatory responses via the inflammatory reflex^[216]. T and B cells express most cholinergic system components - e.g., acetylcholine, choline acetyltransferase, acetylcholinesterase, and, both muscarinic and nicotinic acetylcholine (ACh) receptors and the cholinergic signals generated by immune cells appear to be triggers of both the initiation and termination of cytokine synthesis (e.g., IL-2 in T cells and TNF- α in macrophages)^[217]. A recent study from the University of Sichuan, China, suggested that parasympathetic innervation may contribute to stomach cancer development via acetylcholine-mediated activation of muscarinic acetylcholine receptors^[218]. In a mouse model of stomach cancer, vagotomy suppressed gastric tumorigenesis^[219]. Also in a prostate cancer mouse model cholinergic-induced tumor invasion and metastasis were inhibited by pharmacological blockade of the stromal type 1 muscarinic receptor, leading to improved survival of the mice^[212]. However, as discussed by Cole *et al.*^[208], cholinergic blockade may stimulate indirectly the SNS promotion of cancer. An alternative strategy would be to target neurotrophic growth factors in cancer as many cancers are associated with nerve infiltration. An antineurotrophic antibody (tanezumab) has been developed by Pfizer and is currently used as an analgesic^[220].

As many as 8% of cancers might be associated with endocrine paraneoplastic syndromes^[221], but, a detailed discussion on these syndromes, is beyond the scope of this article. In the context of our discussion on the systemic hallmarks of cancer, it is clear, however, that production of specific hormones by tumors of particular types is not a random event^[222]. For example, squamous cell carcinomas typically produce

parathyroid hormone - related protein and small cell carcinomas (SCC) of the lung typically produce calcitonin, adrenocorticotropin (ACTH), or gastrin releasing peptide (GRP). In some case for example, bombesin (BBS)-like neuropeptides secreted by SCC can act as autocrine growth factors^[223]. Besides the involvement of catecholamines in cancer described before, several other hormones (i.e., estrogens, androgens) are well known to promote cancer development and metastasis^[224]. Also, the role of the thyroid hormones in promoting the metastatic process has been recently described^[225]. Other hormones, like melatonin, for example, may inhibit cancer metastasis^[226]. Patient with cancer have poor sleep and this may influence melatonin secretion. In a recent study done breast cancer women serum melatonin levels correlated significantly with self-reported sleep quality and psychometric profiles of depression^[227].

INTEROGATING THE SYSTEM: CANCER INDUCED SYSTEMIC PATHOLOGIC NETWORKS

In the recent years, liquid biopsies and “omics” became useful tools of the developing field of precision oncology. Through liquid biopsies and “omics” we can interrogate the global characteristics of the tumor itself, and obtain useful information that help us in the diagnostic, prognostic and treatment of cancer patients. In the near future, of great importance will be the characterization of the different CISPIN through specific biomarkers designed to analyze the systemic cancer hallmarks. This information might be used to refine the staging and prognostic of patients with metastatic cancer currently lumped indiscriminately under one large umbrella by the TNM staging and, also, design and monitor targeted interventions directed specifically against key CISPIN that behave as master regulators of the metastatic process. Circulating miRNA present inside the exosomes are plausible CISPIN master regulators^[42]. Exosomal proteins isolated from plasma of cancer patients have been recently characterized not only as useful biomarkers associated with several cancer types but also for dissecting different CISPIN involvement in the malignant process^[43]. Potential systemic biomarkers might be also found analysing metabolomics data. In order for a tumor to develop and spread needs energy and, global metabolic reprogramming, might well be one of the key systemic cancer hallmarks driving cancer from its emergence through its progression and metastasis. The Consortium of Metabolomics Studies (COMETS) was established in 2014 to facilitate large-scale collaborative research on the human metabolome and its relationship with disease etiology, diagnosis, and prognosis^[228]. Systemic metabolic changes in advanced cancers have been described in the past for several tumor types^[229,230]. The essential role of metabolism at the cellular level in controlling cancer hallmarks was recently proven. Using molecular data of 9,125 patient samples from The Cancer Genome Atlas, a group of researchers identified distinct metabolic expression subtypes in 27 cancer types based on mRNA expression patterns of seven major metabolic processes (amino acid metabolism, carbohydrate metabolism, integration of energy, lipid metabolism, nucleotide metabolism, tricarboxylic acid cycle and vitamin & cofactor metabolism)^[231]. The metabolic expression subtypes correlated with clinical outcomes: subtypes with upregulated carbohydrate, nucleotide, and vitamin/cofactor metabolism most consistently correlated with worse prognosis, whereas subtypes with upregulated lipid metabolism showed the opposite. The most interesting finding was that these metabolic subtypes were not related to specific genetic somatic drivers but were intrinsically coupled with cancer hallmark pathways (i.e., angiogenesis, cell division, *etc.*) and were modulated by highly recurrent master regulators across cancer types, ultimately leading to consistent survival patterns. As a proof-of-concept in vitro experiment, the authors also demonstrated that knockdown of two master regulators genes of carbohydrate metabolic subtypes (SNAI1 in a lung cancer cell line or RUNX1 in a sarcoma cell line) significantly decreased the concentrations of intracellular glucose. According to this model, the master metabolic regulators identified were key nodes with the greatest influence on systems-level metabolic activities and targeting these metabolic master regulators may inhibit tumor progression. Strikingly, all four master metabolism regulators genes identified in the 8 cancer types with significantly worse survival rates due to upregulated carbohydrate metabolism, SNAI1, RUNX1, RUNX2, and FOSL1^[231], play also a key role in embryonal development and EMT^[13,232,233] and might be also master regulators of the metastatic cellular program [Figure 3].

NOVEL THERAPEUTIC APPROACHES USING THE CANCER SYSTEM MODEL

Mark Vincent classified cancer treatments in two fundamentally different approaches: a “causality-inhibition” strategy, targeted towards the cancer cause, which at present is still a “moon shot”, remote from our current cancer treatment practices, and, an “acausal” approach that target a specific cancer marker or signature^[234]. At present, many aspects of cancer, in general, and, by large, the metastatic process are still incompletely charted territories, and, therefore, most of our current cancer treatments are not directed towards the specific cause that triggers the cancer process. In the near future, hopefully, once the mechanisms of the different cancer cellular programs are better described, we will be able to design effective causality-inhibition therapies.

For example, the immunomodulatory function of exosomes may be exploited for therapeutic effect. In 2008, a Chinese group from Guangxi University^[235] reported the results of a Phase I study in which 40 patients with advanced colorectal cancer received four weekly intravenous injections of ascites derived exosomes plus or minus GM-CSF. Stable disease and a minor response were observed in two of the patients treated. More recently, another Chinese group^[236], suggested that miRNA depleted pancreatic cancer exosomes might enhance the killing capacity of dendritic or cytokine-induced killer cells, and activate the immune system against pancreatic cancer. The exosomes packaging is closely regulated, and, different clones even from the same tumor may secrete exosomes carrying a different cargo with different properties^[237].

DISMANTELLING CANCER NETWORKS AT THE ORGANISM LEVEL

Networks, composed of various nodes and edges may be described at different levels in an organism. In a cell, nodes may be amino acids of cancer-related proteins, where edges are their distances in the 3D protein structure or nodes may represent protein/RNA molecules or DNA-segments, where edges are their physical or signaling contacts. In metabolic networks, nodes are metabolites and edges are the enzymes, which catalyze the reactions to convert them to each other. At the tissular levels, nodes can be the cancer cells and the stromal cells and the edges the different molecules through which they communicate. At the level of the whole organism, nodes may represent the different components of the cancer system and the different components of the normal body systems [Figure 2] and, the edges, cellular, exosomal or proteic signals exchanged between them. Cancer is a robust system that is able to maintain stable functioning despite various perturbations. The essential robustness of cancer is maintained through heterogeneous redundancy, i.e., the cancer tissue contains a heterogeneous distribution of genetically different cancer cells maintained by genetic instability^[238]. Communication is crucial for the development of the cancer system. In order to be able to dismantle such a complex multi-layered network as cancer, novel targeted multi-scale approaches are needed that target simultaneously key elements of the cellular, tissular and systemic cancer networks^[239]. Targeting the master genetic regulators or the hubs at the cellular level led to promising results^[28,240]. Also, a therapeutic approach based on game theory targeting the collaboration between cancer cells at the tissular level was recently proposed by Archetti and Pienta^[241]. It is conceivable that using similar mathematical tools, treatments targeting specific CISP elements at the organismic level can be designed. Evaluating the cancer system vulnerabilities through analysis of network topology and, especially, network dynamics can predict novel anti-cancer drug targets^[242]. In general, therapeutic approaches targeting levels above the cellular level may be less affected by cancer genetic instability and heterogeneity than treatments targeting the cancer cells themselves. A suggestive example is the improvement in the long term survival associated with check-point inhibitors that target cancer tissue as opposed to cancer cells^[243-245] as opposed to the almost universal development of acquired resistance associated to the use of tyrosine kinase inhibitors that target specific intra-cellular cancer networks^[246]. An attractive top-down regulator of cancer is the nervous system and novel therapies could be designed stimulating or inhibiting some of its components^[198]. As a proof of principle, amplying a single gene in the hypothalamus of obese mice through gene transfer of BDNF inhibited breast cancer progression and metastasis^[247].

“HORIZONTAL” VS. “VERTICAL” APPROACHES

Modalities to target cancer at the cellular level (i.e., tyrosine kinase inhibitors and antibodies directed to the antigens present on the surface of the cancer cells) have been already in place now for almost two decades. Immunotherapies with checkpoint inhibitors and CAR-T cell approaches have recently improved the quality and duration of life of many cancer patients. We envision that agents targeting the systemic hallmarks of cancer and interrupting the communication between the different components of the cancer system will represent the “new wave” of cancer treatments. In the multi-scale model of cancer a “horizontal” approach is considered targeting the cross-talk between the different components of the CISP and blocking the communication between its parts and a “vertical” approach would use drugs that act simultaneously at the cellular, tissular and systemic level in a particular sub-component of the CISP. An example of a “horizontal” approach is the finding that the blockade of the CXCL5/7 receptor CXCR2, or the transient depletion of either platelets or granulocytes, prevents the formation of early metastatic niches and significantly reduced metastatic seeding and progression^[123]. Granulocyte recruitment depends on the secretion of CXCL5 and CXCL7 chemokines by platelets upon contact with tumor cells^[123]. An example of a “vertical” approach using trans-level drugs acting simultaneously at the cellular, tissular and organismic levels simultaneously is the use of beta-blockers. Some beta-blockers, for example, like propranolol, at the tissular level have an immunomodulatory effect^[248] and at the organismic level alter the metastatic potential of cancer cells^[208]. A group of researchers at Penn State University found that melanoma patients who received immunotherapy while taking pan β -blockers lived longer than patients who received immunotherapy alone or patients that received immunotherapy and β 1-selective blockers^[249]. In a follow-up experiment with mice, the researchers saw the same results^[249]. Bisoprolol is another selective β 1-blocker commonly used to treat hypertension, cardiac ischemia, and congestive heart failure. Bisoprolol improved survival, increased total heart mass, and other heart parameters and, importantly, improved food intake and activity levels in an AH-130 tumor-bearing rats model^[250]. Clinical studies with Bisoprolol are planned in patients with cancer cachexia (Professor Anker, Charité Hospital, Berlin, personal communication).

CONCLUSION

Cancer is a multidimensional process with specific characteristics at the cellular, tissular and the organismic level. Basic research and clinical data obtained over the last decade suggests that, at the macroscopic level, cancer behaves like an evolving co-dependent system that interacts continuously through CISP with the modified body systems. Cancer cells and cancer stroma secreted exosomes, cytokines and other soluble factors together with the modified, cancer-supporting body systems, are responsible for establishing the CISP and the systemic hallmarks of cancer. Without taking into consideration this larger, organism-level picture, some of the current local treatments targeted towards the cancer cells or tissues may lead to cancer progression. For example, in some cases of head and neck cancer, (up to 29% in some series), checkpoint inhibitor treatments may induce cancer hyperprogression^[251]. Treatments targeted towards the cancer system and the systemic hallmarks of cancer are urgently needed. Moving to the organismic level and targeting the systemic hallmarks of cancer in concerted therapeutic approaches with currently existing therapies may further improve our cancer armamentarium in the immediate future.

DECLARATIONS

Authors' contributions

The author solely contributed to the article.

Availability of data and materials

Not applicable.

Financial support and sponsorship

Not applicable.

Conflicts of interest

The author declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

A written informed consent for publication was provided.

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Original Article

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Protection of small-cell lung cancer circulating tumor cells by cellular fragmentation

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How to cite this article: Rath B, Plangger A, Moser D, Hochmair M, Ulsperger E, Hamilton G. Protection of small-cell lung cancer circulating tumor cells by cellular fragmentation. *J Cancer Metastasis Treat* 2020;6:30. <http://dx.doi.org/10.20517/2394-4722.2020.51>

Received: 25 May 2020 **First Decision:** 14 Jul 2020 **Revised:** 17 Jul 2020 **Accepted:** 22 Jul 2020 **Published:** 17 Sep 2020

Academic Editor: Wei Zhang **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Aim: Small-cell lung cancer (SCLC) disseminates aggressively and may exhibit high chemoresistance and poor survival rates. In this study, we aimed to investigate a new mechanism of drug resistance for SCLC circulating tumor cells (CTCs).

Methods: SCLC CTC cell lines ($n = 4$) which shed cellular fragments (MAT), as demonstrated by light and scanning electron microscopy, are compared to permanent SCLC lines. Selected proteins are detected by proteome arrays and the functional impact of MAT is studied using cytotoxicity tests involving cisplatin and Topotecan.

Results: The SCLC CTC lines revealed layers of attached cellular fragments with a range of decreasing sizes from intact cells (approximately 12 μm) down to small debris (approximately 2 μm) which are not detectable in permanent SCLC lines. Intact SCLC CTC clusters represent cores of these fragment-coated spheroids. Proteome profiling of MAT revealed a protein pattern similar to intact cells. Chemosensitivity tests employing SCLC and SCLC CTC lines with chemotherapeutics used in therapy of SCLC demonstrated an inhibitory activity of MAT on the resulting cytotoxicity.

Conclusion: Generation of cell-associated debris by SCLC CTCs offers protective effects against cytotoxic drugs, representing a novel mechanism allowing survival of SCLC CTCs in patients.



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Keywords: Small cell lung cancer, circulating tumor cells, shedding, topotecan

INTRODUCTION

Small cell lung cancer (SCLC) constitutes approximately 15% of all lung cancers and is characterized by rapid metastasis, universal drug resistance upon recurrence which subsequently leads to a poor prognosis in patients^[1]. The initial choice of chemotherapy for patients with extended disease (ED)-SCLC includes using platinum-based regimens in combination with etoposide and immune checkpoint inhibitors. This yields high response rates but tumors may invariably relapse and exhibit increased chemoresistance resulting in a 2-year survival less than 10%^[2]. A host of chemically unique therapeutics failed in clinical trials of SCLC and the mechanisms of drug resistance have yet to be conclusively resolved thus far^[3]. The recent use of novel drug combinations yields a minor prolongation of the overall survival but at a cost of increasing side effects in light of frequent COPD exacerbations and sequelae of smoking. In contrast to NSCLC, SCLC does not have clear dependence on driver mutations and shows inactivation of the tumor suppressor genes p53 and RB1^[4]. Recurrent tumors grow rapidly and may show universal necrotic regions due to the lack of supply of blood vessels. Dissemination of SCLC appears to be correlated with a high count of circulating tumor cells (CTCs), exceeding the CTC numbers found in carcinomas of breast, colon and prostate by several magnitudes^[5]. Nevertheless, only a small fraction of the highly heterogeneous CTCs is competent to generate metastases. Several studies have reported a higher metastatic potential of CTC aggregates, defined as clusters of different cells^[6].

The extreme CTC count that is present in SCLC patients allowed us to expand eight permanent CTC lines from blood samples *in vitro*. Our data show these CTCs include typical SCLC markers such as CD56/NCAM, enolase-2 and chromogranin, which are EpCAM-positive^[7]. Furthermore, all of these CTC lines demonstrated spontaneous formation of larger spheroids under regular cell culture conditions, exhibiting high chemoresistance when compared with the corresponding single cell suspensions^[8]. The global drug resistance to structurally unrelated chemotherapeutics may be appropriately explained by a physical factor represented by the barrier of spheroids, limiting drug access to tumor cells in sufficient concentrations. A unique feature of SCLC CTCs is the release or shedding of cellular particles, which appear as free-floating debris or as coat containing cores of intact cell aggregates. Release of such cell fragments is not detectable in established cell lines from SCLC tumor tissues. The present work investigates the properties of these cell fragments and their possible role in protection of cells against chemotherapeutics.

METHODS

Cell lines and reagents

SCLC26A cell line was established in our laboratory from a pleural effusion fluid sample of an SCLC patient prior to treatment. NCI-H417, DMS153 and NCI-H69 cell lines were established from primary SCLCs before treatment and GLC16 from a recurrent tumor. These cell lines were obtained from the Finsen Center, Copenhagen, Denmark. The SCLC CTC cell lines BHGc7, 10, 16, 26 and 91 were established from blood samples of ED-SCLC patients at our institution^[7,9]. In brief, leucocytes and CTCs were isolated by gradient centrifugation (Ficoll-Hypaque; Sigma-Aldrich, St. Louis, MO, USA) and the cell preparation was cultivated in serum-free RPMI-1640 medium supplemented with insulin, IGF-1, transferrin, and selenite until appearance of clonal outgrowth. Following expansion of the CTC clones, cells were transferred to regular medium containing fetal bovine serum (FBS). Tissue culture medium consisted of RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FBS (Seromed, Berlin, Germany) and Penicillin-Streptomycin (Sigma-Aldrich). Single cell suspension of the CTC lines spontaneously form tumorspheres (TOS) in regular tissue culture. Blood collection and generation of cell lines was performed according to the Ethics Approval protocol #366/2003 by the Ethics Committee of the Medical University of Vienna, Vienna, Austria. All other chemicals were obtained from Sigma-Aldrich.

Cytotoxicity assays

1×10^4 cells in the form of single cells or TOS in 100 μ L medium were distributed to individual wells of 96-well microtiter plates (TPP, Trasadingen Switzerland) and ten 2-fold dilutions of the test compounds were added from stock dilutions as described previously. Assays were performed in triplicates. The plates were incubated for four days under tissue culture conditions and viable cells then detected using a modified MTT assay (EZ4U, Biomedica, Vienna, Austria). The respective dilutions of the compounds tested were present for the entire incubation period. IC_{50} values were determined from dose-response curves using Origin 9.1 software (OriginLab, Northampton, MA, USA).

Scanning electron microscopy

For scanning electron microscopy (SEM), samples were washed twice with phosphate-buffered saline, fixed in Karnovsky's fixative (Morphisto®, Frankfurt am Main, Germany) and dehydrated in a graded ethanol series. Ethanol dehydration was followed by hexamethyldisilazane drying (HMDS, Sigma-Aldrich) followed by gold sputtering (Sputter Coater, SC502, Polaron, Fisons Instruments®, England) and examination performed using a scanning electron microscope (JSM 6310, Jeol Ltd., Japan).

Western blot array

Biomarkers were analyzed using the ARY026 Proteome Profiler Array (R&D Systems, Minneapolis, MN, USA), which detects 84 cancer-related proteins according to manufacturer's instructions. Experiments were performed in duplicate and the different tests were calibrated using the six reference spots included for each individual membrane. Arrays were evaluated using ImageJ (NIH, Bethesda, MD, USA) and Origin 9.1 software (OriginLab, Northampton, MA, USA).

Statistical analysis

Statistical significance was tested by *t*-tests and $P < 0.05$ regarded as statistically significant difference using Origin software (Originlab, Northampton, MA, USA).

RESULTS

Physical structure of SCLC CTC spheroids

In our laboratory, SCLC CTC cell lines were obtained from blood samples of patients diagnosed with ED-SCLC prior to initiation of second-line chemotherapy. These cell lines show release of cellular fragments by intact cells or covering of the SCLC CTC spheroids by layers of such fragments. The cellular fragments were termed material (MAT). A CTC BHGc91 cluster encased in subcellular material is depicted in [Figure 1A](#) in light microscopy. Physical removal of the cover by vigorous pipetting releases a core assembly of intact tumor cells [[Figure 1B](#)].

Effects of MAT on the chemosensitivity of SCLC cell lines

MAT released into the cell supernatants were collected after centrifugation and added to chemosensitivity assays which employ cisplatin and Topotecan, respectively. Two typical experiments and their data, using BHGc10 CTCs are shown in [Figure 2A and B](#). The dose-response curves reveal that addition of MAT91 decreases the chemosensitivity of CTC cells to cisplatin and topotecan to a large extent. In the presence of MAT, a fraction of the SCLC CTC cells continue to survive at the highest concentrations of both chemotherapeutics tested.

In a subsequent chemosensitivity test, the viable cell content of a MAT91 fraction was eliminated by several freeze-thaw cycles. The absence of any contaminating living cells in this preparation resulted in a resistance-enhancing effect on the topotecan sensitivity of BHGc10 CTCs [[Figure 3](#)]. In this experiment, the protective effect was not exhibited for topotecan concentrations ranging from 2.5-10 μ g/mL.

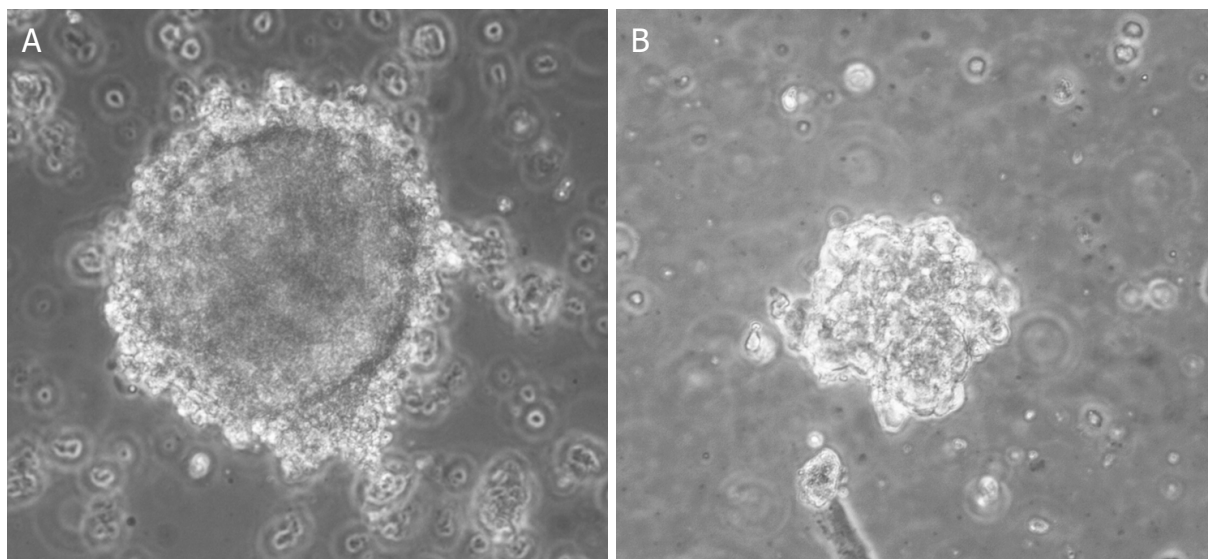


Figure 1. Light microscopy of a MAT-covered tumorsphere (A) and the same cell preparation after vigorous pipetting (B). MAT: material

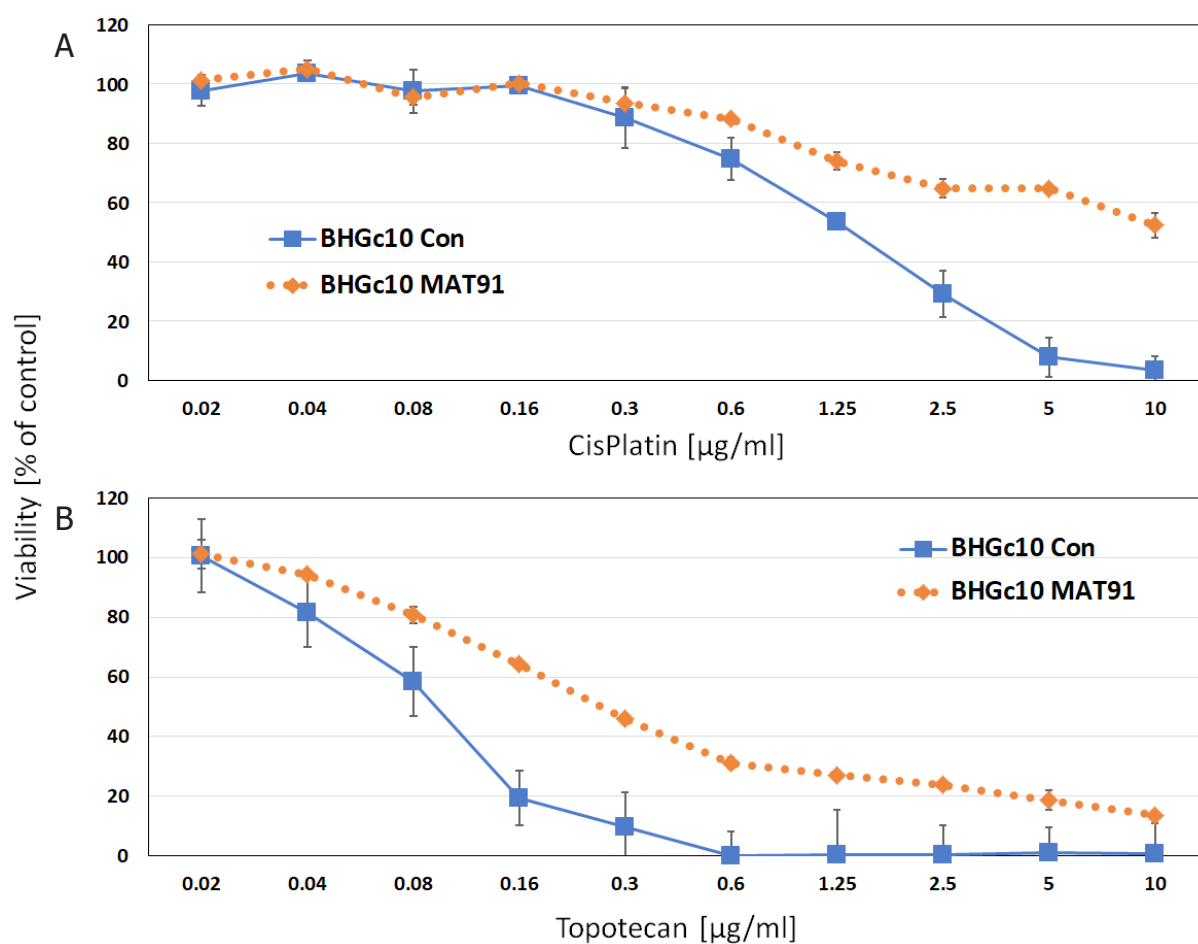


Figure 2. Cytotoxicity testing of BHGc10 SCLC CTCs employing cisplatin (A) or topotecan (B) showing controls and MAT supplementation, respectively (Error bars represent mean values \pm SD). CTCs: circulating tumor cells; SCLC: small-cell lung cancer; MAT: material

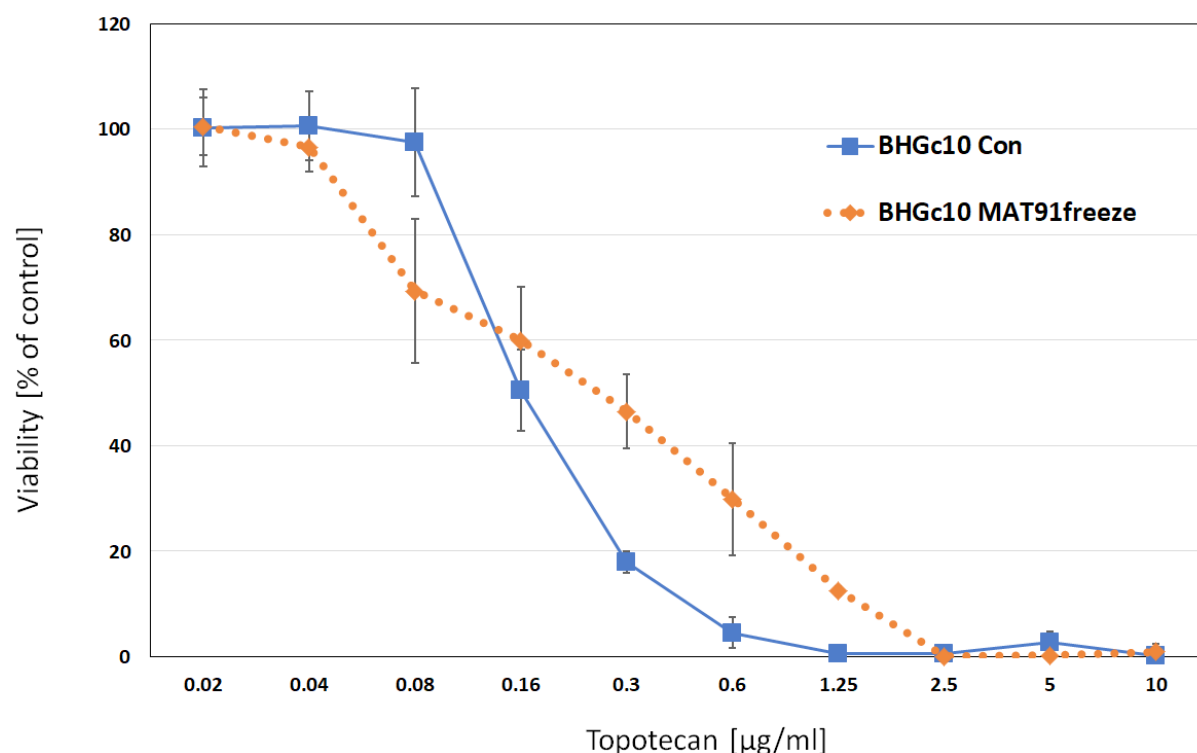


Figure 3. Cytotoxicity test of BHGc10 SCLC CTCs employing topotecan showing the control and MAT supplementation, respectively (Error bars represent mean values \pm SD). For this experiment MAT was pretreated with a freeze-thaw cycle. CTCs: circulating tumor cells; SCLC: small-cell lung cancer; MAT: material

Results of the chemosensitivity tests are summarized in Figure 4. Of the nine cell lines shown, only SCLC26A is derived from a pleural effusion and this demonstrated a decreased chemosensitivity to topotecan in the presence of MAT. All other cell lines exhibited increased resistance upon inclusion of MAT, including pleural cell line S457 and established cell lines NCI-69, NCI-H417 and DMS153.

Size distribution of MAT

MAT was analyzed for size distribution of its fragments and their appearances using SEM [Figure 5]. The results for BHGc7 CTCs show a continuous decrease in the size of their cellular fragments ranging from fully intact cells (10-12 μ m) down to small sizes of approximately 2 μ m. SEM also demonstrated that the cellular fragments exhibit a range of sizes and an appearance of degraded cells with remaining cytoskeletal structures.

For BHGc7, a wide range of cancer-related proteins were compared between the native cell line and MAT release. We used the ARY026 Proteome Profiler Array because in this particular cell line, its cell fragments demonstrate intact membranes in the beginning [Figure 6]. Although there was some variability in the expression of proteins, markers including p53, Enolase-2 and EpCAM were preserved upon release of the fragments. In general, MAT represents the normal cellular protein content of the cell of origin as far as testing with this specific array.

DISCUSSION

The prognosis of SCLC has not improved significantly in recent times despite the introduction of novel therapeutics and immunotherapy^[2,3]. The addition of anti-PD-L1 atezolizumab to chemotherapy for ED-SCLC has increased the median overall survival by 2 months and the median progression-free survival

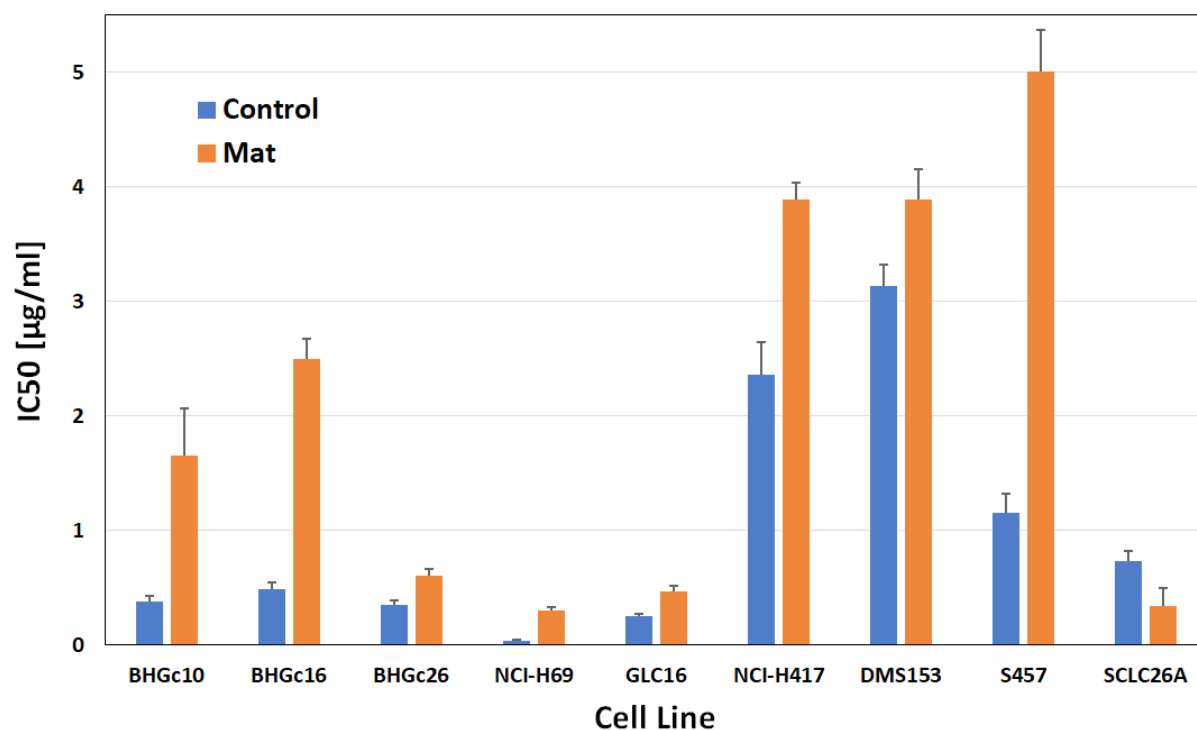
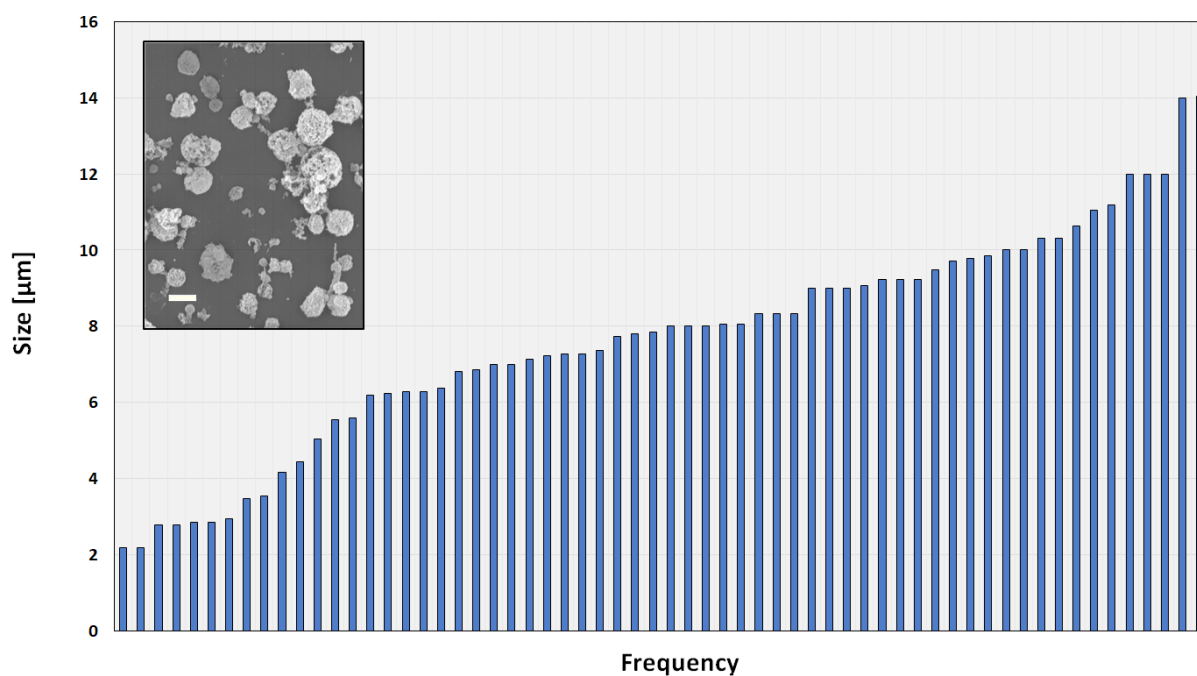


Figure 4. Summary of the IC₅₀ values for Topotecan and cell lines either as medium controls or supplemented with MAT (Error bars are represented by mean values \pm SD; all differences are statistically significant)



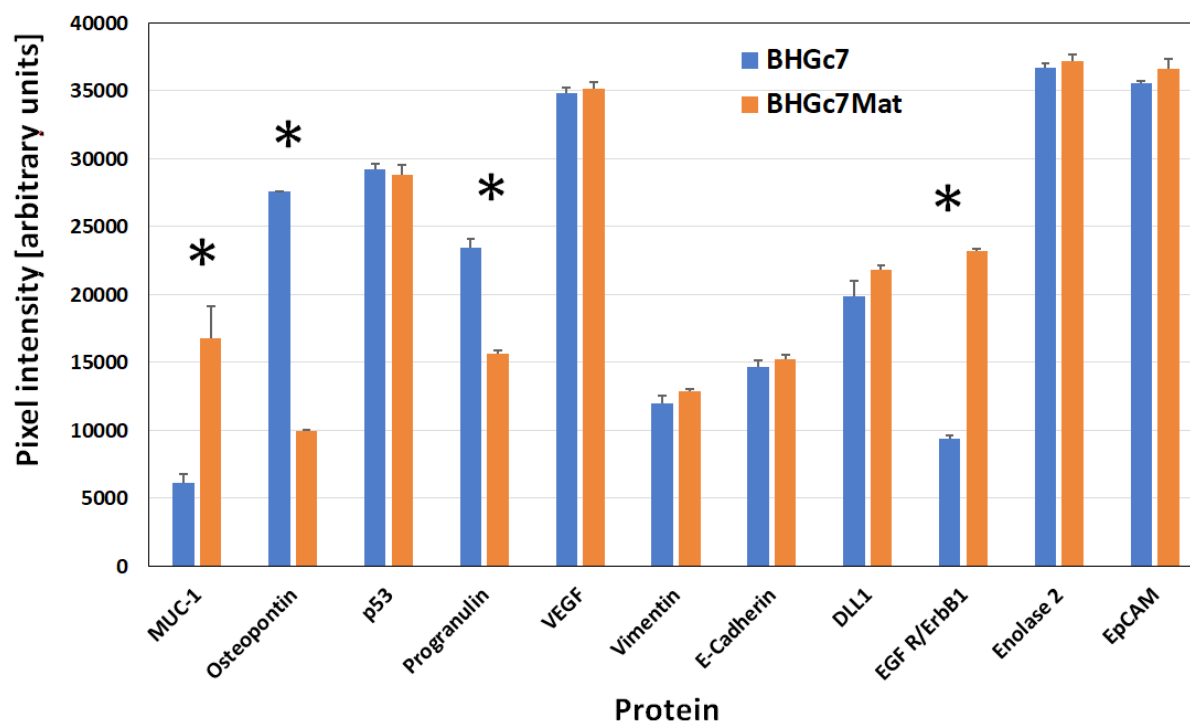


Figure 6. Comparison of selected proteins of the BHGc7 SCLC CTC cell line and its corresponding BHGc7 MAT. The ARY026 array used detects 84 cancer-related proteins *significantly different protein amounts are marked with an asterisk. Error bars represent mean values \pm SD. CTC: circulating tumor cell; SCLC: small-cell lung cancer; MAT: material

within approximately 1-2 years, often with a dismal prognosis. Second-line therapy consisting of topotecan or vincristine-epirubicin-cyclophosphamide regimens results in low response rates of short duration. Addition of further agents or administration of novel agents yields no major improvement with the exception of some progress with immunotherapy in responsive patients^[11].

The genome and gene expression of SCLC tumor tissue has been extensively characterized. Apart from inactivation of p53 and RB1, driver kinases have been detected in minor subpopulations of patients^[3]. The major tumor-promoting mechanism appears to be a general transcriptional de-regulation, which is difficult to target. Chemoresistance to a host of structurally unrelated compounds in SCLC is not feasibly explained by a matching range of individual counteracting mechanisms and global impairment of cell death has not been overcome, for example, by Bcl2 inhibitors^[12]. Therefore, the most likely mechanism of drug resistance remains to be presence of physical resistance at the tumor tissue level. The rapidly advancing SCLC recurrences may outgrow their vessel supply resulting in tumor cells near the necrotic area becoming increasingly resistant and aggressive. We have shown previously that SCLC CTCs form large aggregates, termed tumorspheres, spontaneously in tissue culture and that such spheroids exhibit significantly higher levels of resistance to chemotherapeutics as compared to single cell suspensions^[7]. Such tumorspheres may not exist in the circulation, but they form in capillaries prior to extravasation and generation of metastases^[13]. Furthermore, our collection of SCLC CTCs exhibit shedding of large quantities of cellular fragments in the presence of intact tumor cells, which can then be cultivated for unlimited passages. For certain SCLC CTC, such as BHGc7, BHGc10, BHGc16 and UHGc5lines, its fragments are released into the surrounding media but for other CTC lines as shown in Figure 1, tumor cell aggregates may be covered by fragments like a complete envelope. This superficially attached cover may be removed with the use of vigorous pipetting, demonstrating a loose association with cells.

Our present results indicate that the MAT has profound effects on chemosensitivity of SCLC CTC and SCLC cell lines through the increase of resistance to topotecan to a large degree^[14]. This effect is

largely independent of MAT containing viable material, since this effect is preserved during freeze-thaw cycles, except for very high topotecan concentrations which are not found in patients. Changes in the chemosensitivity of SCLC cells to cisplatin in the presence of MAT was tested but yielded less discernable effects (data not shown). In all likelihood, MAT is still able to bind topotecan via DNA and topoisomerases left, whereas the binding of cisplatin to DNA is impaired by the high sodium concentrations of the medium by preventing the exit of chlorides^[15,16]. SEM pictures of MAT depict cell fragments without membranes and a sponge-like cytoskeleton. Further analysis of the MAT demonstrates a gradual decrease of the size of the fragments down to approximately 2 μm . Nevertheless, this dimension remains too large for classical extracellular vesicles which are typically less than 1 μm ^[17].

The role of cellular debris in tumors, either by spontaneous formation or induction by chemotherapy is under investigation. Evidence from animal models indicates that chemotherapy may stimulate tumor initiation, growth, and metastasis partially by tumor-derived cellular debris (e.g., apoptotic/necrotic cells, and cell fragments)^[18-21]. Similarly, radiation-induced apoptotic tumor cells may promote tumor growth via the Révészphenomenon^[22-24]. In colon cancer, chemotherapy triggers tumor cell death and the resulting dead cells, or debris, may stimulate angiogenesis, inflammation, and growth^[24]. Debris induces the release of osteopontin (OPN), which is a marker of poor prognosis. OPN plays important roles in angiogenesis, cell proliferation and metastasis^[25-28]. Apoptotic tumor cells further stimulate chemotaxis of macrophage and the production of pro-inflammatory cytokines^[29]. In invasive breast cancers, patients with a high apoptotic index resulted in a shorter overall survival^[30]. Persistent apoptotic cells may progress to become necrotic cells that release macromolecules including that of degradative enzymes and inhibitory or protumor factors into the local microenvironment^[31]. The cancer-related protein array showed expression of OPN by BHGc7 and derived MAT, which may possibly play a role in modulating the tumor microenvironment.

Taken together, the SCLC CTCs shed cellular fragments spontaneously without exposure to cytotoxic drugs or environmental stress. In addition to the fragments described for the CTC BHGc91 cell line, other CTC lines which have been established in our laboratory show a comparable and reproducible release of such structures suggesting a general role in SCLC CTCs. The possible release of mediators by this MAT was not tested in these experiments, but the role of these fragments to decrease the chemosensitivity of SCLC cell lines has been established in other cytotoxicity assays. This novel characteristic of SCLC CTC cells may participate in clinical refractoriness to chemotherapy. Furthermore, SCLC can be monitored by the detection of routine neuroendocrine serum markers. In an extension of these assays, blood may be used in the search for particle-associated SCLC markers, which could be more indicative of tumor cell dissemination.

DECLARATIONS

Acknowledgments

We wish to thank Dr. T. Hohenheim for continuous endorsement.

Authors' contributions

Performed experimental work: Rath B, Plangger A

Made the SEM preparations: Moser D

Planned and discussed the investigation: Hochmair M, Ulsperger E

Supervised the study and the writing of the manuscript: Hamilton G

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

Not applicable.

Consent for publication

Not applicable.

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Review

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Exploiting the relevance of CA 19-9 in pancreatic cancer

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How to cite this article: Salleh S, Thyagarajan A, Sahu RP. Exploiting the relevance of CA 19-9 in pancreatic cancer. *J Cancer Metastasis Treat* 2020;6:31. <http://dx.doi.org/10.20517/2394-4722.2020.70>

Received: 1 Jul 2020 **First Decision:** 29 Jul 2020 **Revised:** 14 Aug 2020 **Accepted:** 27 Aug 2020 **Published:** 17 Sep 2020

Academic Editor: Lucio Miele **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is currently the fourth most common cause of cancer-related deaths in the United States. It has a poor prognosis and remains a difficulty to treat malignancy. Over the past several decades, significant efforts have been directed towards developing new approaches to enhance the efficacy of therapeutic regimens for PDAC treatment. In recent years, the measurement of serum carbohydrate antigen 19-9 (CA 19-9) has become one of the most validated and extensively used tumour biomarkers for PDAC. In particular, serum CA 19-9 levels have been explored as a validated tool to predict either the signs of disease progression or the response to treatment. However, despite its clinical relevance, the implications on diagnosis or accurately predicting tumour resectability, and monitoring disease symptoms in PDAC patients remains limited. This current review highlights the recent updates on the applicability of CA 19-9, its exploitation, and challenges in predicting the treatment efficacy and responses in PDAC patients.

Keywords: Pancreatic ductal adenocarcinoma, tumour biomarker, carbohydrate antigen 19-9, treatment efficacy

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) currently represents the fourth leading cause of cancer-related mortality, and is associated with a poor prognosis and a median survival of 6-9 months^[1-3]. Research into an effective treatment option for pancreatic cancer has long eluded the global research community. The majority of patients are usually diagnosed at advanced or metastatic stages where tumour cells have



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migrated from their local environment to distant sites^[4,5]. Importantly, this cancer type remains a hard-to-treat malignancy mostly due to the development of tumour resistance mechanisms to the conventional therapeutic approaches, which have been correlated with overall decreased survival rates^[4,5]. Despite these ongoing challenges, in recent years, incremental improvements were observed in the overall survival rate due to the emergence of combination chemotherapy regimens^[6,7]. For example, gemcitabine plus nab-paclitaxel, and FOLFIRINOX [a combination of 5-fluorouracil (5-FU), leucovorin, irinotecan, and oxaliplatin] show clinically meaningful improvements compared with gemcitabine monotherapy for PDAC patients^[6,7].

In the last decade, different biomarkers have been explored to identify a simpler tool for pancreatic cancer diagnosis and prognosis^[8,9]. The development of tumour biomarkers are essential to precisely detect patients at their early stage of tumour growth, as well as to evaluate the treatment responses so that the current therapeutic strategies can be further improved^[8-11]. Among various validated tools, alcohol dehydrogenase (ADH) isoenzymes, aldehyde dehydrogenase (ALDH) enzyme as well as carbohydrate antigen 19-9 (CA 19-9) have been explored as the most common tumour markers for pancreatic cancer^[8-14]. ADH and ALDH enzymes catalyse alcohol metabolism via the oxidative pathway, which eventually results in the generation of carcinogenic acetaldehyde. Given the importance of these biomarkers, studies by Jelski *et al.*^[12] compared the activities of ADH isozymes and ALDH in pancreatic cancer *vs.* normal tissues, as well as their differences between drinkers and non-drinkers. The authors observed that the class III ADH isoenzyme activity was significantly increased in pancreatic cancer tissue (14.03 mU/L) compared to the healthy tissue (11.45 mU/L). However, no significant differences were noticed in the activities of other ADH isoenzymes and ALDH between pancreatic cancer and normal cells^[12]. Overall, these findings indicated that ADH and ALDH mediated oxidative pathways of ethanol metabolism are not critical players in pancreatic carcinogenesis^[12]. However, considering the fact of increased ADH class III enzyme activity in pancreatic cancer, the same group analysed ADH isozymes and ALDH activities in the serum samples of pancreatic cancer patients^[13]. The authors observed a significant increase in ADH class III isoenzyme activity (13.52 mU/L) in pancreatic cancer patients compared to the control group (11.08 mU/L)^[13]. While total ADH activity was also significantly higher in cancer patients, no changes were noticed in other ADH isozymes as well as ALDH activities^[13]. Notably, a similar analysis in a large cohort of pancreatic cancer patients also demonstrated significantly increased activity of serum ADH class III isozyme (14.03 mU/L) compared to healthy controls (11.45 mU/L), respectively^[14]. Moreover, total serum ADH activity was also found to be increased compared to healthy controls^[14]. Overall, these studies suggested a potential role for ADH, particularly the class III isoenzyme as a biomarker of pancreatic cancer.

In recent years, CA 19-9, also known as Sialylated Lewis antigen (Sla) has emerged as one of the most-studied biomarkers for PDAC, which was characterized by a monoclonal antibody produced by hybridoma technology^[8-11]. As some mucins could contribute to cancer progression via mechanisms including the induction of T cell apoptosis, which later affects the anti-cancer immune response^[10,11], the human colorectal carcinoma cell line SW1116 was immunized with spleen cells of a mouse as a mucin-like product^[11]. The authors of this study characterized that CA 19-9 works by detecting an antigen of a carbohydrate on numerous protein carriers. Proteins that carry CA 19-9 were then identified by immunoprecipitating CA 19-9 from the pooled sera. In addition, mass spectrometry was also used to identify CA 19-9 associated proteins. Notably, various assays such as antibody arrays, western blot, kininogen, and apolipoprotein E by antibody arrays confirmed the presence of CA 19-9 antigen on apolipoprotein B-100^[10,11].

Mechanism of CA 19-9 biogenesis

CA 19-9 is generated by deregulated glycosylation, a process that enzymatically links glycan sugars to cellular lipids and proteins^[15-17]. In the normal pancreas, glycosylated proteins play various crucial functions including protecting and lubricating the pancreatic ducts^[15-17]. However, during cancer progression

aberrant glycosylation, one of the hallmarks of cancer, occurs due to numerous modifications that result in the formation of various glycosylated residues such as CA 19-9/(Sia) which has emerged as a potential biomarker^[15-17].

To emphasize the clinical relevance of this molecule, the survival rates of PDAC patients have been shown to be inversely related to the levels of CA 19-9^[18,19]. In these reports, increased levels of CA 19-9 were found to be associated with unresectable lesions or more numbers of advanced tumours, which indicates a poor prognosis for PDAC patients^[18,19]. Given the importance of this biomarker, studies conducted by Isacoff *et al.*^[20] have demonstrated that patients with normal levels of CA 19-9 (i.e., 36 U/mL or less) during the initial course of therapy had higher median survival rate compared to those who had elevated CA 19-9 levels (more than 36 U/mL). Furthermore, the median survival of PDAC patients on therapy who had a reduction in CA 19-9 levels by 90% was significantly longer than those patients with a less than 90% reduction in the level of this biomarker^[20]. However, since CA 19-9 is a tumour-associated but not a tumour-specific antigen, its implications are not pathognomonic for pancreatic cancer. It is known to be synthesized by the gall bladder, epithelial cells of the pancreas, stomach, and biliary ducts, which explains the increased level of CA 19-9 in the benign as well as advanced conditions of many malignancies^[21-24]. In this current review, we highlight the recent updates, and therapeutic potential, of CA 19-9, with an emphasis on experimental and clinical studies, as well as its importance as a prognostic biomarker in pancreatic cancer.

IMPLICATION OF CA 19-9 IN PDAC

In vivo studies

Several studies have highlighted the role of CA 19-9 in PDAC models^[25,26]. Wagner *et al.*^[27] conducted an *in vivo* study using three orthotopic xenograft mouse models to examine the efficacy of targeted immunocytokine L19-IL-2 in pancreatic cancer with the goal of assessing CA 19-9 as a biomarker for tumour progression when treated with L19-IL-2. Treatment with L19-IL-2 resulted in a significant reduction in the serum levels of CA 19-9, but no changes were observed with either IL-2 or L19 alone treatments. In contrast, there was a significant increase in the serum levels of CA 19-9 in the untreated control group of mice. This indicates a significant correlation between the serum levels of CA 19-9 and tumour volume of PDAC, and that a positive response of L19-IL-2 on CA 19-9 was observed against PDAC^[27]. Overall, these results corroborate the crucial role of CA 19-9 as a biomarker to assess the treatment responses for PDAC [Table 1].

Human studies

Applicability of CA 19-9 levels to determine therapeutic and prognostic responses in PDAC

There have been numerous studies investigating the clinical relevance of CA 19-9 in pancreatic cancer, and to define its applicability in predicting either the responses or resistance to various treatments^[28-30]. A summary of these investigations is given in Table 2. Kieler *et al.*^[28] evaluated CA 19-9 levels in response to chemotherapy with nanoliposome irinotecan (nal-IRI) plus 5-fluorouracil/leucovorin (5-FU/LV) as a second-line treatment for PDAC and compared it with oxaliplatin plus fluoropyrimidines. The findings revealed that at the start of therapy, the median progression-free survival (PFS) was significantly longer in patients who received nal-IRI plus 5 FU/LV compared to the median PFS in patients treated with oxaliplatin plus fluoropyrimidines. In addition, the median overall survival (OS) of patients with CA 19-9 levels over 772.8 kU/L receiving nal-IRI plus 5 FU/LV was significantly higher compared to those who were treated with oxaliplatin plus fluoropyrimidines. This study indicated the effectiveness of nal-IRI plus 5-FU/LV treatment in PDAC when compared with oxaliplatin plus fluoropyrimidines, and that increased CA 19-9 response was associated with better therapeutic outcomes.

In another report, Li *et al.*^[29] performed a prospective study to determine the therapeutic response to modified-FOLFIRINOX and correlated it with CA19-9 levels in patients with metastatic PDAC. The study

Table 1. Summary of *in vivo* studies defining the relevance of CA 19-9 in pancreatic cancer and treatment efficacy

Cell lines	Study design	Treatment(s)	Biomarker	Finding(s)	Ref.
Orthotopic xenograft mouse models, DanG and MiaPaca	To determine the efficacy of targeted immunocytokine L19-IL-2 in PDAC model by evaluating the CA 19-9 levels for assessing tumour progression	Targeted immunocytokine L19-IL-2	CA 19-9	There was a significant correlation between serum CA 19-9 concentrations and tumour volume. CA 19-9 levels were significantly reduced following L19-IL-2 treatment	[27]

PDAC: pancreatic ductal adenocarcinoma; CA 19-9: carbohydrate antigen 19-9

Table 2. Summary of human studies defining the relevance of CA 19-9 in pancreatic cancer and treatment efficacy

No.	Study design	Treatment(s)	Biomarker	Finding(s)	Ref.
1.	To assess serum CA 19-9 levels with the response rates of chemotherapeutic agents, nanoliposome irinotecan (nal-IRI) plus 5-fluorouracil / leucovorin (5-FU/LV) as a second-line treatment option, and compare the responses with oxaliplatin plus fluoropyrimidines	(1) Nanoliposome irinotecan (nal-IRI) plus 5-fluorouracil / leucovorin (5-FU/LV) (2) Oxaliplatin plus fluoropyrimidines	CA 19-9	CA 19-9 levels were significantly lower in patients receiving nal-IRI plus 5 FU/LV than those receiving oxaliplatin plus. Also, the median PFS and OS in patients who received nal-IRI plus 5 FU/LV were significantly longer compared to those receiving oxaliplatin plus fluoropyrimidines	[28]
2.	To evaluate CA 19-9 levels to determine modified-FOLFIRINOX responses in metastatic PDAC patients	Modified-FOLFIRINOX (FOL)	CA19-9	CA 19-9 levels were found to be decreased following modified-FOLFIRINOX treatment	[29]
3.	To determine the prognostic significance of CA 19-9 levels following neoadjuvant therapy (NT) in PDAC patients	Neoadjuvant therapy	CA 19-9	CA 19-9 stats indicated no increase following NT	[30]
4.	To study the changes in CA 19-9 levels as a predictor of OS in a randomized phase III trial	Nab-paclitaxel plus gemcitabine vs. Gemcitabine-alone	CA 19-9	Decreased CA 19-9 levels in each of the two treatments (nab-paclitaxel plus gemcitabine vs gemcitabine alone). Improved efficacy of combination treatment was observed with a decrease in CA 19-9 levels and patients had better outcomes compared with gemcitabine alone	[31]
5.	To investigate CA 19-9 levels and their dynamics during neoadjuvant treatment (NT) in predicting resectability and survival	FOLFIRINOX (FOL) vs. Gemcitabine-based NT	CA 19-9	Decreased CA19-9 levels were observed following treatment with FOL, and patients had significantly higher resection rates	[40]
6.	To determine CA 19-9 levels and correlate the results between borderline resectable pancreatic cancer (BRPC) vs. initially resectable pancreatic cancer (IRPC) receiving neoadjuvant therapy	Neoadjuvant therapy, FOLFIRINOX	CA 19-9	Patients with < 50% increase in CA19-9 levels had longer survival than patients with > 50% increase in CA19-9 levels. FOL treatment followed by neoadjuvant therapy significantly improved the survival response in BRPC patients	[41]
7.	To study the diagnostic accuracy of serum CA 19-9 in combination with RECIST-response on CT-imaging in predicting the resectability of locally advanced pancreatic cancer (LAPC) following induction chemotherapy	Induction chemotherapy	CA 19-9	There was a significant decrease in CA 19-9 levels following chemotherapy. An increase in the CA 19-9 level was significantly correlated with decreased survival	[42]
8.	To evaluate the efficacy of adjuvant chemoradiotherapy by assessing postoperative serum levels of CA 19-9	Adjuvant chemoradiotherapy	CA 19-9	Adjuvant chemoradiotherapy increased the surgical outcome in patients with increased levels of the CA 19-9 but not in patients with normal levels of CA 19-9	[43]
9.	To assess CA 19-9 serum levels as a biomarker in predicting the resectability of PDAC mainly in jaundiced patients	Any treatment	CA 19-9	The resectability in the majority of patients was accurately predicted by the serum CA 19-9 level. Also, the area under the ROC curve in patients with jaundice corresponded well with the findings obtained for non-jaundiced patients	[34]
10.	To develop an integrated predictive model to determine longer survival in locally advanced pancreatic cancer (LAPC) patients treated with chemoradiotherapy (CRT)	Chemoradiotherapy	CA 19-9	CA 19-9 was one of the significant factors which contributed to favourable PFS. In addition, decreased CA 19-9 levels and surgical resection following CRT treatment were significantly associated with higher OS	[44]

PDAC: pancreatic ductal adenocarcinoma; CA 19-9: carbohydrate antigen 19-9; TFFs: trefoil factors; LAPC: locally advanced pancreatic cancer; PFS: progression-free survival; RECIST: response evaluation criteria in solid tumours; CRT: chemo radiation therapy

demonstrated that patients receiving modified-FOLFIRINOX therapy had decreased levels of CA 19-9 and that this decrease in CA 19-9 was found to be associated with a significantly longer median OS of patients^[29]. In another study, Aoki *et al.*^[30] determined the prognostic significance of decreased CA 19-9 after receiving neoadjuvant therapy in patients with PDAC. The authors found no increase in CA 19-9 levels in patients receiving neoadjuvant therapy and that the responder group demonstrated a lower risk of hepatic recurrence compared to the non-responder group^[30]. Overall, these studies indicate the importance of evaluating CA 19-9 levels as a prognostic biomarker in monitoring the response to treatments for PDAC.

Chiorean *et al.*^[31] conducted a randomized phase III trial (MPACT) to evaluate the effect of weekly nab-paclitaxel plus gemcitabine over gemcitabine alone on CA 19-9 levels at eight-week intervals as a predictor of OS in metastatic pancreatic cancer patients. The authors observed that patients treated with nab-paclitaxel plus gemcitabine had better outcomes compared with gemcitabine alone and that the improved outcomes were associated with decreased levels of CA 19-9 compared to increased or static levels. In the same context, there was a statistically significant survival advantage for patients receiving nab-paclitaxel plus gemcitabine *vs.* gemcitabine alone^[31]. In addition, an abrupt decrease in CA 19-9 levels was observed in each of the two treatment arms during the initial eight weeks period of treatment^[31]. Moreover, prolonged OS and PFS has also been found to be associated with a decline in CA 19-9 levels^[31]. Overall, these findings confirmed the applicability of CA 19-9 as an early biomarker to assess the anti-tumour efficacy of therapeutic agents for metastatic pancreatic cancer patients.

Advantages of serum CA 19-9 levels as a biomarker for defining the surgical resectability in PDAC patients

Given that the CA 19-9 levels were positively correlated with the OS, several studies evaluated an association between CA 19-9 and tumour resectability. While surgical resection remains one of the potential curative options for PDAC, many cases are not amenable to surgical resection at the time of evaluation^[32-35]. For that reason, identifying unresectable patients preoperatively is vital to avoid undesired surgery^[32-35]. For this reason, the use of serum CA 19-9 levels pre-operatively has been extensively evaluated to determine resectability in pancreatic cancer patients^[32,33,36-40].

Heger *et al.*^[40] investigated the dynamics of CA 19-9 levels during neoadjuvant treatment (NT) in order to predict resectability and survival. The authors noticed a significant correlation between the reduced levels of CA 19-9 with resectability and OS. Importantly, a significant difference in CA 19-9 levels was observed following the treatment with FOLFIRINOX (FOL) as patients treated with this regimen had a significantly higher resection rate compared to those treated by gemcitabine-based NT^[40]. Furthermore, the combination of NT and FOL was found to be associated with a significantly improved survival rate among resected pancreatic cancer patients^[40]. This study corroborates findings by Bolton *et al.*^[41] who delineated their experience and correlated the findings between borderline resectable pancreatic cancer (BRPC) *vs.* initially resectable pancreatic cancer (IRPC) patients receiving neoadjuvant therapy. While no significant differences were observed in the OS responses between IRPC and BRPC prior to multiagent neoadjuvant therapy, BRPC patients treated with neoadjuvant therapy experienced increased OS^[41]. Also, patients with a high College of American Pathologists (CAP) score or a less than 50% increase in CA19-9 levels had longer survival than patients with a low CAP score and > 50% CA19-9 levels^[41]. Importantly, compared to all other treatments, FOLFIRINOX treatment followed by neoadjuvant therapy significantly improved the survival response in BRPC patients^[41]. Overall, these findings indicate that multiagent neoadjuvant therapy is associated with an increased survival rate, which is correlated with a decrease in CA 19-9 levels when compared to other neoadjuvant therapies.

Notably, these findings appear to be consistent with the studies by van Veldhuisen *et al.*^[42], who evaluated the diagnostic accuracy of serum CA 19-9 in combination with RECIST-response on CT imaging to predict the resectability of locally advanced pancreatic cancer (LAPC) following induction chemotherapy. The

authors reported that all patients with RECIST-regressive disease exhibited a significant decrease in the serum levels of CA 19-9 following induction chemotherapy, which indicated a positive correlation between CA 19-9 levels and RECIST-regressive disease. Importantly, an elevation in the CA 19-9 level was also found to be significantly associated with decreased survival^[42].

Another study conducted by Xu *et al.*^[43] evaluated the efficacy of adjuvant chemoradiotherapy by assessing the postoperative serum levels of CA 19-9. In this study, adjuvant chemoradiotherapy was found to be associated with improved surgical outcomes in patients with increased levels of this biomarker, but not in patients with the normal levels of CA 19-9^[43]. In addition, a significant improvement in surgical outcomes was noticed in patients with increased levels of serum CA 19-9 with negative lymph nodes^[43]. Overall, these findings suggest the importance of evaluating preoperative CA 19-9 levels for monitoring the therapeutic responses of various regimens, as well as determining the resectability of the disease.

Benefits of serum CA 19-9 levels as a biomarker for determining the surgical resectability in PDAC patients with jaundice

Santucci *et al.*^[34] performed a study to determine if serum CA 19-9 levels can be exploited as a biomarker for predicting the resectability of PDAC, mainly in jaundiced patients. The authors reported that the mean CA 19-9 level in patients with the resectable disease was significantly lower compared to those with locally-advanced or metastatic disease^[34]. It has also been observed that the ability of CA 19-9 in precisely predicting resectability was 0.886 when evaluated with the area under the Receiver Operating Characteristic (ROC) curve. This implies that the resectability in 88.6% of patients was accurately predicted by the serum CA 19-9 level. Concurrently, the area under the ROC curve in patients with jaundice corresponded well with data obtained for non-jaundiced patients^[34]. These findings suggested that CA 19-9 can be explored as an accurate predictive biomarker to assess the resectability of PDAC patients with jaundice.

In a separate study, Choi *et al.*^[44] developed an integrated predictive model to determine the long-term survival in LAPC patients treated with chemoradiotherapy (CRT). The authors reported that pre-CRT CA 19-9, post-CRT CA 19-9, and a decline in CA 19-9 levels were among the significant factors that contributed to the favourable PFS^[44]. It has been demonstrated that high-dose radiation, a decline in CA 19-9 levels, and surgical resection after receiving CRT, were all significantly correlated with longer OS^[44]. Overall, these findings imply the crucial role of CA 19-9 as a biomarker for developing a nomogram to help determine the patients for CRT, and aiding clinical decision making.

ONGOING CHALLENGES AND LIMITATIONS

There are a number of limitations that can confound the interpretation of CA 19-9 as a biomarker. Despite its remarkable contribution in clinical practice, the efficacy of CA 19-9 to be exploited as a biomarker remains controversial as it is not exclusively specific to this disease as numerous benign aetiologies can also deceptively increase the levels of CA 19-9^[43,45-51]. In addition, this tumour biomarker has been shown to have a modest and low sensitivity (79%-81%) in symptomatic patients. This also limits its diagnostic utility, keeping CA 19-9 in the category of those clinical biomarkers whose implications need to be precisely considered as a screening tool keeping other limitations in place^[43,45-51].

FUTURE PERSPECTIVES

Considering all the discussed limitations, several studies have suggested and evaluated the cut-off levels for serum CA 19-9 from 37 to 90 U/mL, which resulted in a potentially increased specificity of this biomarker to 95%^[43,44-51]. In other respects, Jahan *et al.*^[52] conducted a study to explore the individual and combined Trefoil factors (TFFs) alone and in combination with CA 19-9 as a promising panel for detecting pancreatic cancer. TFFs are defined as secretory products of cells that produce mucin. The authors reported that a

combination of TFFs with CA19-9 appeared to be a potential diagnostic tool for distinguishing between early-stage pancreatic cancer, benign controls, and chronic pancreatitis. Notably, the sensitivity of CA 19-9 was improved by its combination with TFFs^[52]. In addition, the combination of TFFs and CA 19-9 was associated with an increased overall efficacy of CA 19-9 to discriminate early pancreatic cancer from chronic pancreatitis, which indicated the exceptional capabilities of TFFs to differentiate this highly aggressive disease at the early stages of progression.

CONCLUSION

Based on the findings of various studies, CA 19-9 has proven to be an essential tool in the diagnosis of pancreatic cancer, and most importantly in monitoring patient responses to various treatment modalities. The validation of CA 19-9 as exhibiting excellent parameters, such as higher sensitivity and specificity compared to other biomarkers, has confirmed its potential as a promising clinical tool in this field. However, despite its undeniable benefits in the predictive analysis of treatment efficacy and responses in PDAC patients, there are still a number of limitations that warrant further investigation in the future.

DECLARATIONS

Authors' contributions

Involved in the writing, editing, revising, and approving the final version of the manuscript: Salleh S, Thyagarajan A, Sahu RP

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

Not applicable.

Consent for publication

Not applicable.

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Review

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Extracellular RNAs as potential biomarkers for cancer

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How to cite this article: Happel C, Ganguly A, Tagle DA. Extracellular RNAs as potential biomarkers for cancer. *J Cancer Metastasis Treat* 2020;6:32. <http://dx.doi.org/10.20517/2394-4722.2020.71>

Received: 7 Jul 2020 **Accepted:** 5 Aug 2020 **Published:** 17 Sep 2020

Academic Editor: Wei Zhang **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

The discovery that all cells secrete extracellular vesicles (EVs) to shuttle proteins and nucleic acids to recipient cells suggested they play an important role in intercellular communication. EVs are widely distributed in many body fluids, including blood, cerebrospinal fluid, urine and saliva. Exosomes are nano-sized EVs of endosomal origin that regulate many pathophysiological processes including immune responses, inflammation, tumour growth, and infection. Healthy individuals release exosomes with a cargo of different RNA, DNA, and protein contents into the circulation, which can be measured non-invasively as biomarkers of healthy and diseased states. Cancer-derived exosomes carry a unique set of DNA, RNA, protein and lipid reflecting the stage of tumour progression, and may serve as diagnostic and prognostic biomarkers for various cancers. However, many gaps in knowledge and technical challenges in EVs and extracellular RNA (exRNA) biology, such as mechanisms of EV biogenesis and uptake, exRNA cargo selection, and exRNA detection remain. The NIH Common Fund-supported exRNA Communication Consortium was launched in 2013 to address major scientific challenges in this field. This review focuses on scientific highlights in biomarker discovery of exosome-based exRNA in cancer and its possible clinical application as cancer biomarkers.

Keywords: Extracellular vesicles, exosomes, extracellular RNA, cancer, biomarker, liquid biopsy



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INTRODUCTION

Once thought to exist only within cells, RNA is now known to play a role in a variety of complex cellular functions. Recent research has shown that RNA can be exported from cells and plays a role in the molecular mechanisms of cell-to-cell communication^[1,2]. This paradigm-shifting observation launched the field of extracellular RNA (exRNA) biology and represents a fundamental change in our understanding of RNA in cell biology.

Extracellular RNA acts as a signalling molecule, traveling through body fluids carrying information from cell to cell. Types of exRNA include both longer messenger RNA (mRNA) and long non-coding RNA (lncRNA), as well as various types of small non-coding RNAs (ncRNAs). Non-coding RNAs can generally be broken down into two groups, regulatory ncRNAs and housekeeping ncRNAs, as outlined in Table 1. Regulatory ncRNAs include lncRNA, microRNA (miRNA), piwi-interacting RNA (piRNA), small interfering RNA (siRNA), tRNA-derived fragments and Y RNA. Regulatory small ncRNAs have emerged as vital players in various biological processes. They are known primarily for their role as regulators of gene expression at the post-transcriptional level; however, they have a wide range of functions. Further information on individual ncRNAs can be found in the review articles cited in Table 1. Housekeeping ncRNAs include ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), and small nucleolar RNA (snoRNA). Housekeeping ncRNAs are highly abundant and are essential for cellular activities such as the translation of RNA into proteins, and transcriptional splicing. The term exRNA includes many types of RNA. Small non-coding exRNAs are often the focus of studies due to their abundance, ease of detection, and regulatory function. miRNA are of particular interest due to their role in post-transcriptional regulation of gene expression. Changes in miRNA expression are associated with various pathological conditions and dysregulation of miRNA expression is a hallmark of human cancer^[3].

Extracellular RNA is secreted by all cell types and can be found in a variety of biofluids including plasma, serum, breast milk, saliva, cerebrospinal fluid (CSF), bile, semen, and urine^[4-7]. While many ncRNAs are found in human biofluids, miRNA, piRNA, snoRNA, tRNA-derived RNA fragments (tRF), and Y RNA represent the most prominent types of exRNA found within various human biofluids (Figure 1 and Table 1, asterisks)^[4,8]. Carriers of exRNA include extracellular vesicles (EVs), ribonucleoprotein complexes (RNPs), and lipoprotein complexes (LPPs). ExRNAs are either encased within extracellular vesicles, or, are tightly associated with proteins to avoid degradation by RNases. ExRNAs, in extracellular vesicles and/or associated with protein complexes, can then be transferred from donor cells to recipient cells, where they can elicit functional responses and regulate a number of biological processes^[9,10].

EVs, released by virtually all cell types, are small membrane-enclosed carriers of bioactive proteins, lipids, and nucleic acids (including exRNAs)^[11]. Cells release a variety of EVs to transfer biological cargo to local and distant recipient cells within the body to facilitate intercellular communication. The term extracellular vesicles is broadly used for particles released from the cell that are delineated by a lipid bilayer, however, there are multiple EVs subtypes which can be differentiated based on their size, biogenesis, release pathways, cargo, and function^[12]. The main EV subpopulations include microvesicles (MVs), and exosomes. MVs are approximately 100-1000 nm in size and are derived from outward blebbing of the plasma membrane. Exosomes are approximately 30-100 nm vesicles of endosomal origin^[13]. The biogenesis of exosomes begins with the formation of early endosomes by inward budding of the cell membrane, followed by second inward budding of the endosomal membrane creating intraluminal vesicles (ILVs) and the larger multivesicular bodies (MVBs). Fusion of the MVBs with the plasma membrane release ILVs as exosomes into the extracellular milieu. Cytosolic constituents such as proteins and nucleic acids can be sorted into both types of EVs as part of their respective biogenesis pathways [Figure 2].

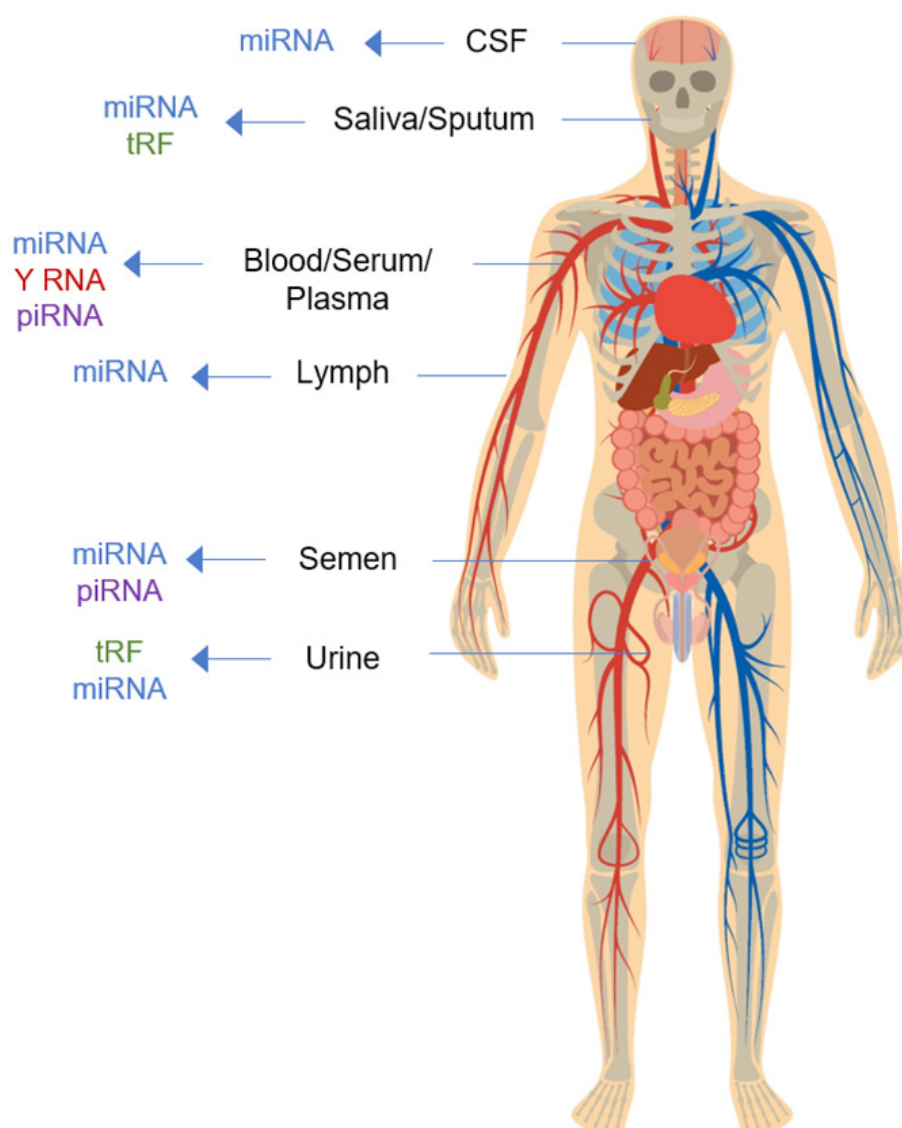


Figure 1. A schematic diagram showing exRNA types predominantly found in a representative set of human biofluids. miRNA: microRNA; piRNA: piwi-interacting RNA; tRF: tRNA-derived RNA fragments

Table 1. General classification of non-coding RNAs

Group	Abbreviation	Full Name	Size	ncRNA Review Article(s)
Housekeeping ncRNAs	rRNA	ribosomal RNA	120-4,500 nt	[72,73]
	snRNA	small nuclear RNA	100-300 nt	[74]
	snoRNA*	small nucleolar RNA*	60-300 nt*	[75,76]*
	tRNA	transfer RNA	76-90 nt	[77]
Regulatory ncRNAs	lncRNA	long non-coding RNA	> 200 nt	[78]
	miRNA*	microRNA*	21-22 nt*	[79,80]*
	piRNA*	piwi-interacting RNA*	23-31 nt*	[81]*
	siRNA	small interfering RNA	20-25 nt	[82]
	tRF*	tRNA-derived fragments*	17-26 nt*	[83]*
	Y RNA*	Y RNA*	?*	[84]*

*Asterisks represent the most prominent types of exRNAs found in human biofluids

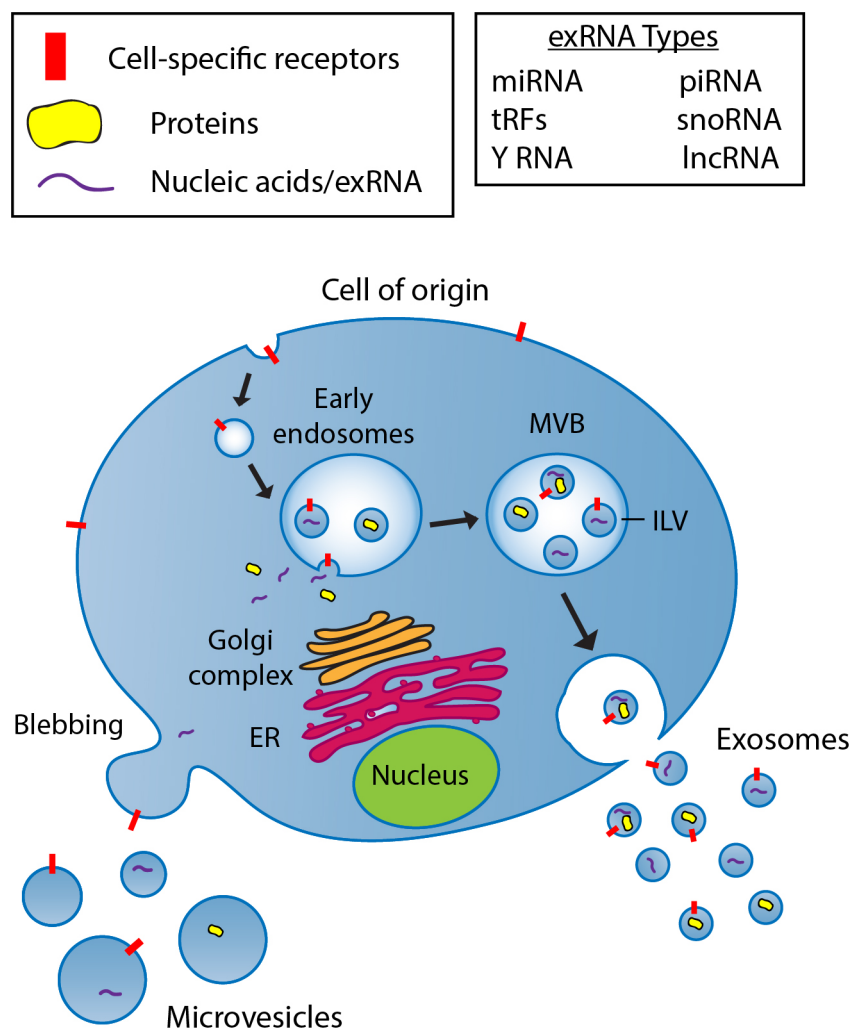


Figure 2. A schematic diagram showing the biogenesis pathway of microvesicles and exosomes. Microvesicles are formed by direct budding from the plasma membrane and are capable of encapsulating multiple forms of molecular cargo including proteins and nucleic acids. The biogenesis of exosomes begins with internalization of the cell membrane leading to the formation of early endosomes. Intraluminal vesicles (ILVs) are formed by the inward invagination of endosomal membranes, resulting in the formation of multivesicular bodies (MVBs). During this process, cytosolic constituents, including nucleic acids and proteins, can be sorted into ILVs. Upon fusion of MVBs with the plasma membrane, ILVs are released as exosomes into the extracellular milieu. Exosomes can include many different types of exRNA as listed in Table 1. ER: endoplasmic reticulum; MLV: multivesicular body; ILV: intraluminal vesicle; miRNA: microRNA; piRNA: piwi-interacting RNA; tRF: tRNA-derived RNA fragments; snoRNA: small nucleolar RNA; lncRNA: long non-coding RNA

Non-vesicle associated carriers include ribonucleoprotein (RNP) and lipoprotein (LPP) complexes. These non-membrane bound exRNA carriers have been shown to be present in human plasma and serum^[14,15]. The LPP family of complexes are classically regarded as carriers of lipids and can be further broken down into high-density lipoproteins (HDLs), low-density lipoproteins (LDLs), very LDLs (VLDLs), and chylomicrons based on their mass density. Recent studies have revealed that lipoproteins, such as HDLs and LDLs, can transport miRNAs and deliver them to recipient cells where they carry out their functional roles^[16,17]. One of the main interests in exRNA research is focused on their ability to mediate intercellular communication and act as signalling molecules in normal cell homeostasis, or as a consequence of pathological development. The exRNAs demonstrated potential as cancer biomarkers due to their function. There are published reports to support the use of exRNA for both cancer diagnosis and prognosis^[18].

This article focuses on exosome-derived exRNAs obtained non-invasively from liquid biopsy as potential biomarkers for the early detection and monitoring of cancers. Developing biomarkers based on exRNA is

relevant and important in the context of precision cancer therapy, since this approach will result in non-invasive procedures using body fluids as test samples, and essentially eliminate unnecessary repeat biopsies for diagnosis and monitoring effectiveness of a therapy^[19]. ExRNAs are now being evaluated as biomarkers in a variety of cancers and this review provides an understanding of the present status of exosome/exRNA-based cancer biomarker research, acknowledges challenges, and addresses the need to identify, develop, and validate clinically relevant exosomal exRNAs as cancer biomarkers.

LIQUID BIOPSY

Liquid biopsy is a term generally used to describe the collection of a body fluid to test for diagnostic information that will guide patient management. Various biological fluids can be used for liquid biopsies, but blood is one of the most accessible fluids along with urine and saliva^[20]. The ultimate goal of liquid biopsies in cancer patients is to be informative about the underlying tumour biology and establish biomarker clinical utility with clear prognostic value. Non-invasive measurement of cancer biomarkers using liquid biopsy allows for patient stratification, screening, monitoring treatment response, and detecting minimal residual disease following therapy/surgery and recurrence. The emergence of sensitive nucleic acid and protein biomarkers detection technologies have enabled the development of liquid biopsies with clinical applications in oncology. Currently, tumour biopsy is the preferred diagnostic tool available to clinicians to detect and monitor treatment for cancer. Since many tissues are difficult or impossible to biopsy or resect, and biopsies cannot provide information on treatment efficacy in real-time, RNA-based biomarkers are being developed to address these issues. A liquid biopsy platform that enables non-invasive real-time detection of cancer biomarkers may significantly reduce the need for tissue biopsy. Advancements in liquid biopsies are a key objective of precision oncology, with the goal of improving the diagnosis and treatment of cancer^[21].

Tumour derived liquid biopsy analytes in the blood include circulating tumour cells (CTC), circulating tumour DNA (ctDNA), exRNA, exosomes, and EVs^[21]. CTCs and ctDNA are the two analytes that have more reported utility than others as biomarkers in precision oncology. CTCs are tumour cells that have presumably been shed from the primary tumour and/or metastatic lesions into the bloodstream. CtDNA can be detected in the blood as part of the total cell-free DNA (cfDNA) pool, but is specifically derived from cancerous cells^[22]. Clinical applications for CTCs and cfDNA include prediction of cancer prognosis, selection and monitoring of therapeutic regimens, and drug target applications^[21].

Current challenges in cancer diagnostics using liquid biopsy

While liquid biopsies are increasingly being used for molecular diagnostics in oncology, challenges remain. One limitation in using CTCs for clinical applications is the scarcity of CTCs in the blood. The abundance of CTCs in the blood is low (approximately 1 cell per 1×10^9 blood cells in patients with metastatic cancer), and only a limited number of CTCs can be isolated from a single blood sample^[23-25]. Similarly, ctDNA concentration can vary from 0.01% to 90% of total cfDNA and, in general, the amount of ctDNA increases with tumour burden^[26,27]. These extreme low concentrations can make detection and analysis challenging. While CTCs can be analysed at the DNA, RNA, and protein levels, and provide information on functional cellular characteristics, analyses of CTCs provide limited information on tumour heterogeneity^[28,29].

CtDNA provides a more comprehensive view of the tumour genome as it reflects DNA released from multiple tumour regions or different tumour foci to capture tumour heterogeneity^[30-32]. However, due to the high fragmentation rate and low abundance of ctDNA, and high background levels of wild-type DNA in blood, the analysis is particularly challenging. Whole genome sequencing of cfDNA suggests both cfDNA and ctDNA are likely derived from apoptotic cells^[33]. While CTCs are shed from a tumour once it reaches a certain stage in development and ctDNA is released from dying cells, exRNA secretion (biogenesis) is a normal cellular process. This makes exRNA and EVs better candidates to provide insight into early stage cancers where cell death is not yet occurring.

LIQUID BIOPSY AND EXRNA

ExRNAs and EVs are among the liquid biopsy analytes that have demonstrated potential as cancer biomarkers due to their function, availability in most body fluids, and ability to be collected in a non-invasive manner, allowing frequent and longitudinal sampling. In cancer research, there is substantial evidence to support the use of exRNA for both diagnostic and prognostic purposes^[18]. Differential expression of cellular and extracellular miRNAs has been associated with a wide range of human diseases^[34]. While exRNA can include many diverse types of RNA species (as discussed above), most studies investigating the use of exRNAs as biomarkers have focused on miRNAs since its expression patterns are unique to individual tissues and differ between cancer and apparently uninvolved tissues^[35]. Y RNA is abundantly expressed in multiple body fluids and increased levels of Y RNA have been observed in the circulation of cancer patients^[5,36]. These observations have triggered interest in the potential use of Y RNA as a biomarker for cancer and other diseases. However, many other types for exRNAs are being explored as potential biomarkers, including mRNA mutations and other non-coding RNAs^[18].

There are opportunities for exRNA to be developed into reliable biomarker tests for cancer detection using liquid biopsy samples, since exRNA is remarkably stable and resists degradation mediated by ribonucleases^[15,37]. The discovery of stable RNA or exRNA outside of cells is continuously changing the fundamental understanding of intercellular signalling and of the role RNA may play in cell-to-cell communication and other complex biological processes. Because of their relative stability within vesicles or in association with RNPs and LPPs, and, marked differences between exRNAs secreted by apparently normal and tumour cells, exRNA molecules have high potential for development as biomarkers of various cancers including lung^[38], breast^[39] and prostate cancers^[40,41].

ADVANTAGES OF EXRNA IN LIQUID BIOPSY

The remarkable stability and relatively non-invasive access of different exRNA molecules makes them an interesting class of biomarkers. The stability of exRNAs have been tested *ex vivo* under various conditions including freeze-thaw cycles, extreme pH values, and storage at room temperature (RT)^[42]. Examination of exRNA from CSF or blood in the diagnosis of glioma revealed that the EV number and morphology remained largely unchanged if CSF was stored at RT^[43]. The total RNA and representative miRNA levels were well-preserved under this condition for up to a week, and a single cycle of freezing and thawing did not significantly alter EV number, morphology, RNA content, or miRNA levels, confirming its stability at RT. These findings demonstrated stability and the incredible ease and speed of obtaining specimens for testing compared to conventional biopsy. Measuring circulating RNA as liquid biopsy is a reliable alternative to conventional biopsies, offering a potentially cheaper, reliable, and non-invasive way of monitoring cancer development, progression, and remission.

Most of the tests for disease diagnosis and monitoring used in clinics are based on specific protein concentration changes in body fluids. In comparison to protein-based biomarkers, exRNA has several advantages including easier developed assays with specificity, and an amplifiable detection signal. Measuring low abundance RNA in biofluids also circumvents the inherent obstacle of high protein concentration and complexity in human body fluids in liquid biopsy.

In various diseases, normal EV cargo contents change as diseases initiate, and progress, altering the types of proteins and RNAs that are packaged. These changes are rapid and quantitative^[44]. Therefore, the exRNA profile of an individual may provide a snapshot of their health. Real-time changes in expression of exRNA offer prognostic values in predicting disease outcomes, monitoring treatment response and assessing treatment risk^[45].

EXRNA AS CLINICAL BIOMARKERS

In order to develop exRNAs as clinical biomarkers, the development process has to go through rigorous steps to define the intended target, examine clinical utility (must inform and guide patient treatment, management, and outcomes) and, validate the test both analytically (ensures specificity, accuracy, precision, and other characteristics of a biomarker test or device) and clinically (ensures that the test or device performs as intended) before clinical application. Since the U.S. Food and Drug Administration (FDA) is the regulatory body to qualify biomarkers for intended clinical studies, it is therefore relevant to understand the FDA definition of a biomarker and review various resources available for investigators.

FDA's definition of a biomarker

The FDA defines a biomarker as a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions^[46,47]. Qualified biomarkers have the potential to provide valuable information that may reduce uncertainty in regulatory decisions during drug development. When a biomarker is qualified, it means that it has undergone a formal regulatory process to ensure that it is reliable and reproducible for a specific interpretation and application in medical product development and regulatory review, within the stated context of use.

FDA BEST biomarker categories resource

It is essential to have effective, unambiguous communication for efficient translation of promising scientific discoveries into approved medical products. Unclear definitions and inconsistent use of key terms can hinder the evaluation and interpretation of scientific evidence and may pose significant obstacles to medical product development programs.

The FDA-NIH Joint Leadership Council identified harmonization of terms used in translational science and medical product development as a priority need, with a focus on terms related to study endpoints and biomarkers. Working together with the goals of improving communication, aligning expectations, and improving scientific understanding, the FDA and NIH developed the BEST (Biomarkers, EndpointS, and other Tools) resource for biomarker researchers^[46]. BEST defines seven biomarker categories: susceptibility/risk, diagnostic, monitoring, prognostic, predictive, pharmacodynamic/response, and safety. The BEST glossary aims to capture distinctions between biomarkers and clinical assessments and describes their distinct roles in biomedical research, clinical practice, and medical product development.

FDA center for drug evaluation and research biomarker qualification program

The mission of this program is to work with external stakeholders to develop biomarkers as drug development tools. Qualified biomarkers have the potential to advance public health by encouraging efficiencies and innovation in drug development. The goals of the biomarker qualification program (BQP) are to (1) support outreach to stakeholders for the identification and development of new biomarkers; (2) provide a framework for the review of biomarkers for use in regulatory decision-making; and (3) qualify biomarkers for specific contexts of use that address specified drug development needs.

Biomarker qualification is a process involving three stages that provide increasing levels of detail for the development of a biomarker for its proposed context of use. The processes to complete submissions to the center for drug evaluation and research (CDER) BQP are (1) a letter of intent (LOI); (2) qualification plan; and (3) full qualification package. More information about the FDA CDER BQP can be found on their website^[47]. A Pre-LOI meeting can be helpful for requesters to receive guidance from the FDA regarding their biomarker programs before submission to the program^[48]. Once a biomarker is qualified it can then be used in multiple drug development programs for the context of use without FDA re-review.

ExRNA as cancer biomarker

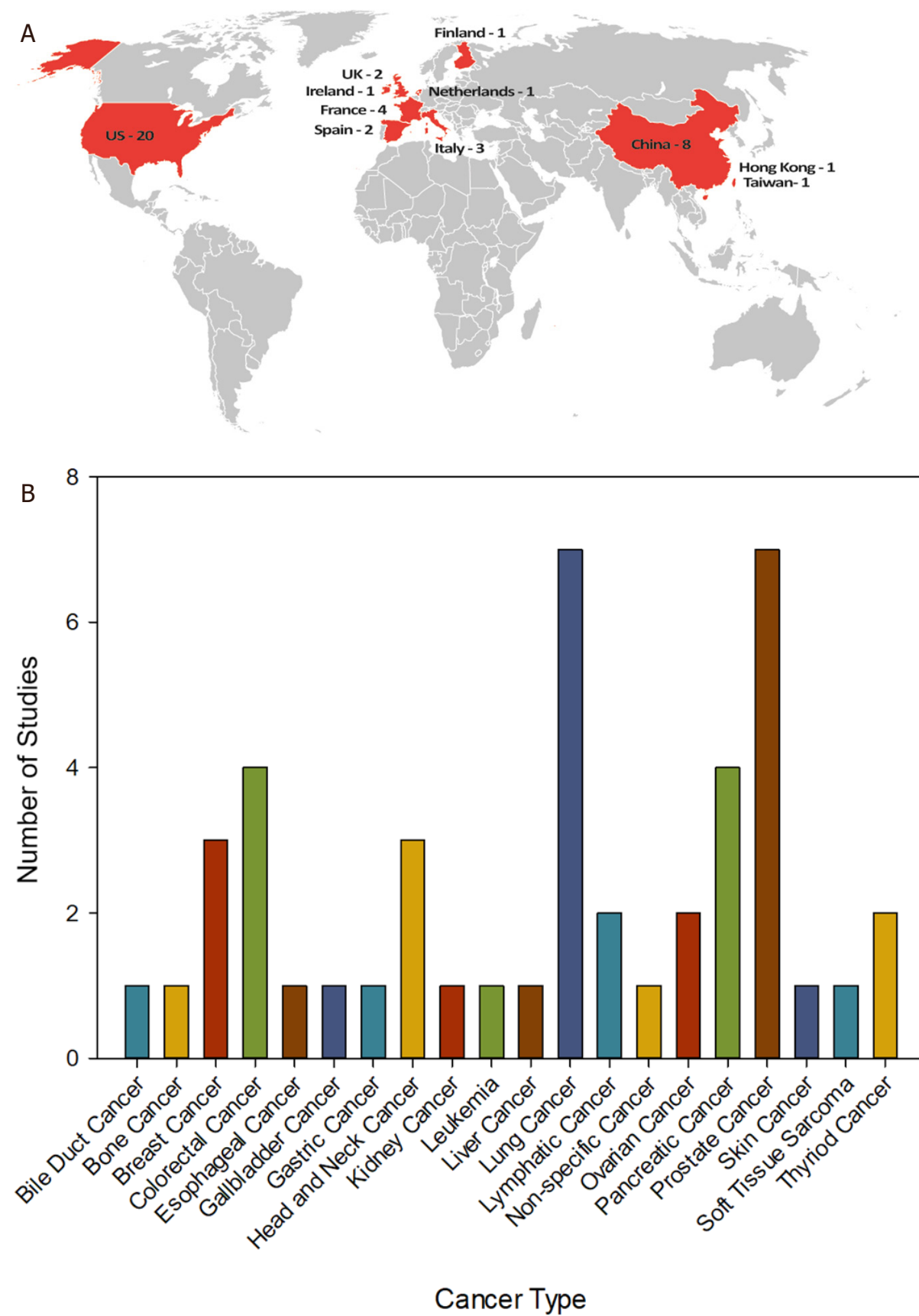
Medical oncologists have been using cancer biomarker tests to guide molecularly targeted therapies to achieve better therapeutic outcomes. In this regard, developing biomarkers and biomarker tests based on exRNA is relevant and important in the context of precision cancer therapy. Investigators have been assessing the current state-of-the-art methods for body fluid sample collection, exRNA isolation, and analysis, with exRNA biomarker discovery as the goal. This data has been unified in a report on the current state of knowledge of exRNA isolation and analysis techniques^[49]. To avoid loss of potential biomarkers, investigators have been using comprehensive methods, such as qRT-PCR and cutting-edge platforms for RNA sequencing, rather than selection methods for specific RNA species.

ExRNAs have already begun to demonstrate their utility as clinical biomarkers. A study by McKiernan *et al.*^[50] reported the development of a urine exosome-based non-invasive gene expression assay that discriminates high-grade from low-grade prostate cancer and benign disease. In another study Li *et al.*^[51] identified and validated a panel of salivary exRNA biomarkers for potential use in screening and risk assessment for gastric cancer. Using salivary gland secretions, investigators have identified 30 mRNA and 15 miRNA candidates whose expression patterns were associated with the presence of gastric cancer^[51]. These exRNA biomarkers were identified and validated with credible clinical performance for non-invasive detection of gastric cancer. Another recent study reported analysis of ctDNA and exRNA for monitoring tumour burden and therapeutic response in patients with multiple myeloma^[52]. This exploratory analysis has provided evidence of ctDNA for predicting disease outcome and the utility of exRNA as a biomarker of therapeutic response in multiple myeloma. It has been reported that an exosome-based detection of EGFR T790M in plasma from non-small cell lung cancer patients (NSCLC) may benefit from ALK (anaplastic lymphoma kinase) inhibitor therapy whose tissue samples are not available or who are unable or unwilling to undergo repeat biopsy^[53]. To address this need, Exosome Diagnostics developed an assay (ExoDx *Lung-ALK*) in a CLIA certified laboratory to isolate and analyse exosomal RNA from blood samples enabling sensitive, accurate and real-time detection of EML4-ALK mutations in patients with NSCLC.

Exosome Diagnostics has also developed a qPCR-based test (ExoDx *EGFR*) that interrogates mutations within the *EGFR* gene in NSCLC. The assay uses plasma derived exosomal RNA/DNA and cfDNA to detect *EGFR* mutations to inform clinical management^[53,54]. Castellanos-Rizaldos and colleagues compared this assay to the FDA approved companion diagnostic, cobas® *EGFR* Mutation Test v2 (Roche), that detects defined mutations within the *EGFR* gene from plasma cfDNA liquid biopsy samples of NSCLC patients and found increased sensitivity and specificity using the ExoDx *EGFR* assay which they attributed to the exRNA-based assay design^[53].

EXRNAS AS BIOMARKERS OF CLINICAL SIGNIFICANCE IN CANCER

To demonstrate the potential of exRNA and exosomes as clinical biomarkers, we mined data from current clinical trials exploring the utility of these liquid biopsy analytes in cancer. The ClinicalTrials.gov is a database for publicly and privately supported research studies conducted around the world. As of May 11th, 2020, there are 45 clinical trials on ClinicalTrials.gov that focus on the use of exRNA and exosomes as clinical biomarkers in cancer^[55]. The search results are summarized in Figure 3A-C. While a vast majority of the clinical trials are taking place in the U.S., there are many trials in other countries as well, including China, Italy, and Spain [Figure 3A]. These clinical trials span a large variety of cancer types [Figure 3B]. Lung and Prostate cancers are the most common disease models exploring the use of exRNA and exosomes as clinical biomarkers, as both cancer types are the focus of seven clinical studies. Overall, there is a large number of different cancer types represented in this data set. Fourteen out of the twenty different cancer types are the focus of one or two clinical studies, demonstrating the utility of exRNA and exosomes as clinical biomarkers. Blood is the primary biofluid utilized in these studies while urine is also a common source of biofluid used for liquid biopsies [Figure 3C]. The combination of blood/serum/



C

Biofluid	Number of studies
Blood	23
Bone Marrow	2
Lymph	1
Plasma	4
Serum	7
Saliva	1
Urine	7
Semen	1

Figure 3. Current clinical studies evaluating the use of exRNA and exosomes as cancer biomarkers. An advanced search for query terms ("extracellular RNA" OR exosome OR exRNA OR oncosome) AND biomarker focused on cancer as a disease model, was performed on ClinicalTrials.gov on 11 May 2020. The search was restricted to recruitment statuses on recruiting, not yet recruiting, active, not recruiting, completed, enrolling by invitation, and studies of unknown status. The search returned 45 studies which are summarized in [Figures 3A-C](#). A: A world map shows the locations (in red) of all clinical studies evaluating the use of exRNA and exosomes as cancer biomarkers. The numbers indicate the clinical studies in each location; B: clinical trials were grouped by general cancer type and the number of studies focused on each cancer type are shown. Projects that did not specify cancer type were grouped together as non-specific cancer; C: the clinical trial data was parsed for the types of biofluids used in each study. Some studies examined multiple types of biofluids while other did not include biofluid sampling. The table represents biofluids examined in all 45 clinical studies

plasma is utilized for 34 out of the 45 clinical trials investigating the potential of exRNA and exosomes as clinical biomarkers, representing 17 different cancer types. Not surprisingly, urine is the biofluid of choice when investigating biomarkers for prostate cancer. However, clinical studies are also exploring the use of urine as a biomarker for thyroid and kidney cancer. Saliva is an emerging biofluid that is inherently easy to collect, and, has been shown to reflect the spectrum of health and disease states found using serum^[56,57]. While there is only one clinical trial in this dataset using saliva for biomarker discovery, it is conceivable that emerging technological advancements will move saliva into the forefront as an accurate and reliable biofluid for molecular diagnostics.

Most studies investigating the use of exRNAs as biomarkers have focused on miRNAs, and indeed 12 of the 45 clinical trials in this dataset specifically examine the use of miRNAs as cancer biomarkers. However, two studies investigate mRNA as exosomal cargo and a molecular biomarker in cancer. Further, there is a clinical study looking at circular RNA (cRNA) in pancreatic cancer, and a study exploring exosome derived lncRNA in ovarian cancer. Notably, most of the clinical studies did not indicate a specific exRNA target. Overall, this data demonstrates widespread utility of exRNA and exosomes as clinical biomarkers across a spectrum of biofluids and cancer types.

NIH-supported research focused on exRNA and exosomes as biomarkers in cancer

The NIH supports many pre-clinical research projects focused on the use of exRNA and exosomes as biomarkers in cancer. To understand the breadth and type of research funded by NIH, we explored the Research Portfolio Online Reporting Tools Expenditures and Results Tool (RePORTER) using the website: <https://projectreporter.nih.gov/reporter.cfm>. An NIH RePORTER search for exRNA and exosomes as biomarkers in cancer found 138 projects that have been funded by NIH between 2010-2020, which is summarized in [Figure 4A and B](#). NIH funded projects were grouped by funding type and the number of awards for each funding type can be found in [Figure 4A](#). A large majority of these studies (87 out of 138) were research projects. However, the number of research training and career development awards indicate a growing number of trainees entering the field. The NIH Common Fund's Extracellular RNA Communication Program (ERCP) funded eight projects focused on exRNA and exosomes as biomarkers in cancer. NIH Small Business Innovation Research (SBIR) awards make up 10 of the 138 projects. These SBIR awards included funding to Tymora Analytical Operations, Cognext Diagnostics, Abtelum Biomedical, Nanomaterial Innovation, Biofluidica, Nanoview Diagnostics, Accure Health, Ascent Bio-Nano Technologies and Microsensor Labs.

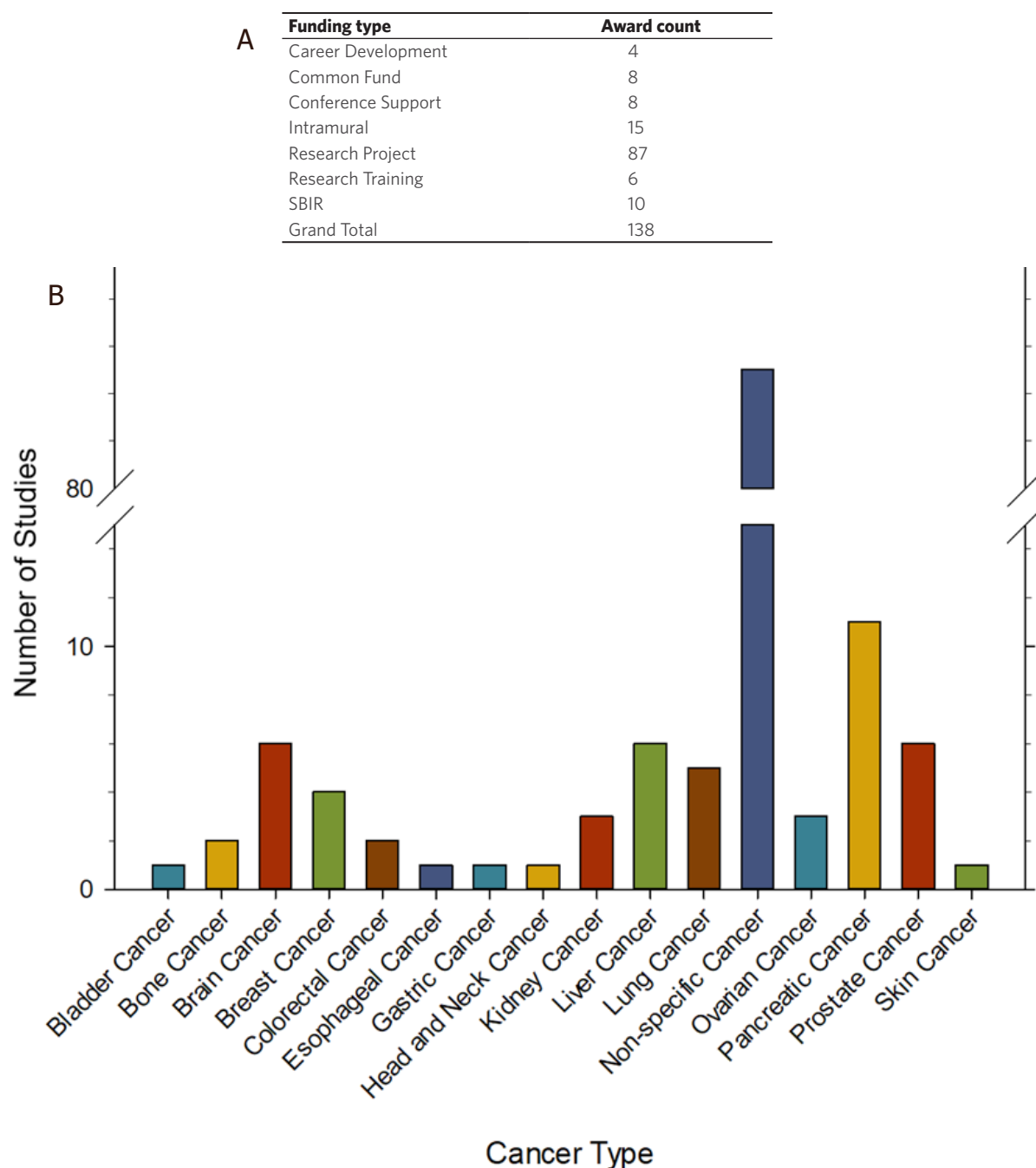


Figure 4. NIH supported research focused on exRNA and exosomes as cancer biomarkers. An advanced text search for (“extracellular RNA” OR exosome OR exRNA OR oncosome) AND cancer AND biomarker was performed on NIH RePORTER (<https://projectreporter.nih.gov/reporter.cfm>) on 9 May 2020. The text search was limited to project abstracts, project title, and project terms, and was focused on new awards only (excluding subprojects), funded by any NIH Institute or Center from 2010-2020. The search returned 138 projects, which are summarized in Figures 4A and B. A: NIH funded projects were grouped by general funding types; B: NIH funded projects were grouped by general cancer type and the number of projects focused on each cancer type are shown. Projects that did not specify cancer type were grouped together as non-specific cancer

A large majority (85 out of 138) of the NIH funded projects that focused on exRNA and exosomes as biomarkers in cancer were not directed toward any specific cancer type [Figure 4B]. Furthermore, the 53 remaining projects were spread across 15 different types of cancer.

Pancreatic cancer was the focus of 11 studies over the past 10 years and accounted for 8% of the total number of awards. Brain, liver, and prostate cancer were each investigated in 6 studies, and the remaining cancer types were each addressed 5 or less times. This data, along with the clinical trial data, suggest that exRNA and exosomes have great potential as biomarkers in a variety of cancer types and across many types of biofluids. The broad applicability, universal presence in human biofluids, general stability, and accessibility of exRNAs demonstrate their potential in disease detection, monitoring, and prognosis.

FDA-approved exosome-based clinical diagnostics

Exosome Diagnostics (a Bio-Techne brand) recognized an opportunity to utilize exRNA as a predictive marker for prostate cancer and developed a urine exosome gene expression assay that can identify higher-grade prostate cancer among patients with elevated prostate-specific antigen (PSA) levels. This simple, non-invasive, urine-based test provides an EXO106 score derived from exosome ERG and PCA RNA levels normalized to SPEDEF mRNA copy number^[50,58]. The U.S. FDA granted Bio-Techne Breakthrough Device Designation to this test [ExoDx Prostate IntelliScore (EPI)], making it the first exosome-based liquid biopsy test to receive this designation, and Medicare coverage in 2019. Further, a recent publication demonstrated that the EPI test influenced the overall decision to defer or proceed with a biopsy and improved patient stratification in a prospective, randomized, blinded, two-armed clinical utility study^[59].

CHALLENGES IN EXRNA RESEARCH

Even though the field of exRNA is very promising, there are challenges to this emerging area. A key barrier toward a comprehensive understanding of exRNA biology and function has been the heterogeneity of exRNA carriers, improved EV separation technologies, and EV targeting and cargo release.

EV biogenesis and cargo loading

ExRNA carriers include different particle subtypes such as EVs, RNPs, and LPPs, however, EVs have gained the most interest amongst these carriers. EVs are highly heterogeneous and can be further divided into different subpopulations that differ in size, density, morphology, and composition^[60]. EV subpopulations broadly include MVs and exosomes^[60]. An ongoing challenge in the field is to clearly discriminate between EVs, exosomes, and MVs.

Different EV biogenesis pathways also result in exRNA content that is extremely diverse and heterogeneous; and the intracellular sorting mechanisms that direct exRNAs to specific export pathways are not well understood^[61,62]. Furthermore, the nature and abundance of EV cargoes are cell-type-specific and often influenced by the physiological or pathological state of the donor cell and the stimuli that modulate their production^[63]. EV heterogeneity and the complexity of its exRNA cargo are likely sources of variability in exRNA profiling. Understanding the molecular mechanisms modulating EV biogenesis, the heterogeneity in EV subtypes, and the physiological relevance of their exRNA cargo will be crucial in harnessing their utility as cancer biomarkers.

Single vesicle EV isolation

A major challenge to the field of exRNA includes improved EV separation technologies. The heterogeneity of EVs, their nanoscale size, and the ambiguity of EV subpopulations that often have overlapping characteristics, are significant barriers to understanding the contribution of each specific EV subtype in different pathological systems^[60]. Due to a substantial overlap in the physio-chemical properties of exRNA carriers, many commonly used isolation protocols do not unambiguously separate EVs subtypes, or even EVs from non-EV exRNA carriers (such as RNPs or LPPs)^[64]. The lack of biophysical and biochemical markers for many different exRNA carriers makes the analysis and interpretation of exRNA data uniquely challenging. To address the variability in exRNA profiling studies, Murillo and colleagues applied computational deconvolution to exRNA-seq and exRNA qPCR profiles found in the Extracellular RNA

Atlas (<https://exrna-atlas.org>). Their analysis led to the identification of six exRNA cargo types found in multiple biofluids^[65]. While their findings suggest associations of cargo types with distinct carriers, it also demonstrated that the heterogeneity of exRNA carriers and cargo types exceeds the capabilities of current experimental methods to isolate and investigate specific carrier subpopulations and their cargo in a reproducible way^[65]. The generation and optimization of methods to isolate high purity exRNA subpopulations from biological samples, and, analyse the subsequent carrier exRNA contents, is a current goal in the field.

EV targeting and cargo release

To be functional in the context of cell-cell signalling, an EV must also be able to find its physiological target and release its cargo. But the question of how EVs target recipient cells can elicit a functional cellular response is still unknown. The specificity of targeting EVs to recipient cells is thought to occur through specific ligand-receptor interactions resulting in EV uptake. Mediators of these interactions include tetraspanins, integrins, lipids, lectins, heparan sulphate proteoglycans, and extracellular matrix components^[60,66]. Once EVs are bound to the recipient cells, many different types of endocytotic processes are known to mediate cellular uptake^[13,60]. Membrane fusion is an alternative entry method in cancer cells^[67]. However, different mechanisms of internalization have been described for different cell types, and the mode of EV entry into target cells is thought to play a role in the functional effects^[66]. It is possible that a population of EVs can simultaneously trigger a number of different methods of entry into a cell, with the primary entry points depending on the cell type and EV cargo^[66]. Understanding the mechanism of EV targeting and cargo release, and how this affects the functional fate of exRNA in recipient cells are outstanding questions in exRNA biology.

ERCP

The NIH Common Fund-supported Extracellular RNA Communication Program (ERCP) was launched in 2013 to accelerate progress in this new area of biomedical research. The overarching goal of the ERCP has been to accelerate progress in the field exRNA biology and establish exRNA, and their carriers, as mediators of intercellular communication. The first phase (stage 1) of the NIH Common Fund-supported Extracellular RNA Communication Consortium (ERCC1) addressed five major challenges in the exRNA field^[68]. The goals included: (1) to better understand the mechanisms of exRNA biogenesis, export and secretion from the cell of origin; (2) to develop reference profiles for exRNA species from healthy human biofluids; (3) to establish the utility of exRNA for biomarker development; (4) to establish the utility of exRNA for therapeutic development; and (5) to develop community-wide resources for exRNA standards, protocols, and data. The exRNA Portal (<https://exrna.org/>) is the central access point for ERCC resources including descriptions of all ERCC projects, exRNA data and data standards, protocols, and computational tools.

While significant advances were made during ERCC Stage 1, the exRNA field still faces many challenges, in part due to both the inherent diversity of exRNA and the heterogeneity of exRNA carriers^[61]. In September 2019, the ExRNA Communication Program stage 2 (ERCC2) commenced to tackle the complexity of exRNA molecules and the diverse array of exRNA carriers. ERCC2 researchers will develop tools to efficiently and reproducibly isolate, identify, and analyse different carrier types and their exRNA cargos and allow analysis of one carrier and its cargo at a time. The three major initiatives addressed in Stage 2 of the ERCC include: (1) Improved Isolation and Analysis of exRNA-Carrier Subclasses; (2) Towards Single Extracellular Vesicle (EV) Sorting, Isolation, and Analysis of Cargo; and (3) to serve as a community-wide resource for exRNA standards, protocols, and data. The purpose of these initiatives is to further characterize the cell or tissue from which their respective exRNAs originate and shed light on the diversity of exRNAs carried by EVs. This will allow for a better understanding of the precise role of exRNAs as signalling molecules for both physiological and pathophysiological processes, ultimately accelerating the development of exRNAs for diagnostics.

CHALLENGES OF EXRNA IN LIQUID BIOPSY

Although exRNAs are more sensitive and specific biomarkers than proteins, and better reflect the cell dynamic than DNA does, there are limitations in the use of exRNA as biomarkers. EV heterogeneity and the complexity of its exRNA cargo, are sources of variability in exRNA profiling within and across studies, which has been a significant hinderance. To address the lack of consistency and reproducibility, Srinivasan *et al.*^[69] demonstrated that exRNA sequencing reproducibility varies across isolation methods and that the performance of exRNA isolation methods can vary across biofluids and RNA species. To stimulate exRNA biomarker development, they developed miRDar (<https://exrna.shinyapps.io/mirdar/>), an interactive web-based application to help investigators select the optimal exRNA isolation method for their studies based on the biofluid of interest. The development of standardized sample isolation and analysis procedures would allow a more meaningful comparison and integration of data from different studies, which may facilitate the development of exRNA based clinical applications.

EVs are heterogeneous in nature and technical challenges remain in EV isolation. Current methods for isolating EVs from complex biofluids cannot clearly identify EV cellular origins within a pool of highly abundant vesicles. As such, there is no way to clearly differentiate cancer-derived EVs from healthy host cell-derived EVs in biofluids. However, ERCC2 efforts should be able to address this pressing challenge. A recent report described a process for EV enrichment by identifying cancer cell membrane proteins compared with healthy cell membrane proteins using TCGA Human Protein Atlas and GTEx, and presented isolation of tumour derived EVs from animal serum^[70]. This finding is encouraging to pursue exRNA biomarker research for detecting cancer at a very early stage. Better characterization of the differences between exRNA profiles of diseased and healthy individuals will allow the diagnostic and prognostic utility of exRNA-based profiling to increasingly becoming a reality^[18,50,71].

CURRENT STATUS OF EXRNA AS BIOMARKER

It is conceivable that EVs, exosomes, and exRNA are important resources for developing cancer biomarkers. In this regard, a growing number of scientific reports suggest exRNA as a reliable non-invasive alternative to the invasive approaches for diagnosis, treatment and prognosis of cancer. Recently, a U.S.A.-based diagnostics company utilized exRNA as a predictive marker for prostate cancer, and developed a urine exosome gene expression assay to identify higher-grade prostate cancer among patients with elevated PSA levels^[50,58]. U.S. FDA granted Bio-Techne Breakthrough Device Designation to this test (ExoDx Prostate IntelliScore, EPI), which is the first exosome-based liquid biopsy test to receive this Designation. The National Comprehensive Cancer Center Network included EPI as a recommended test in their Clinical Practice Guidelines for Oncology for Prostate Cancer Early Detection (Version 1.2019). While this is a significant step forward in exosome/exRNA-based test development, advancement in this technology is required to address all types of cancers.

The explosion of technological advancements including sophisticated bioinformatics and availability of better tools offer a wide spectrum of opportunities to explore exosomes/exRNA for developing reliable biomarker tests using liquid biopsy samples to accelerate real-time cancer diagnosis and molecularly guided therapy.

However, there are challenges to isolate tumour-specific exRNA and use as biomarkers for clinical oncology due to inadequate separation technology and heterogeneity of exRNA carriers. Current methods for isolating EVs from complex biofluids does not clearly define the cell-of-origin or target cell of exRNA cargo and, therefore, are unable to determine with certainty the tissue of origin. This warrants improvement in EV separation technology, and better understanding of EV targeting and cargo release.

The expectation is to develop liquid biopsy-based analytical assays using circulating exRNAs specific for the tumour type and to identify clinically relevant biomarkers useful as a diagnostic, prognostic or treatment response markers for cancer patients to fully appreciate its clinical potential as cancer biomarkers.

FUTURE PERSPECTIVES

The potential for the use of EVs, exosomes, and exRNAs in cancer biomarker development are starting to yield clinical utilities for diagnosing cancer, and as indicators of progression and/or treatment response. EVs derived from cancer cells appear to modulate the function and may induce epigenetic changes in distant recipient cells. Results from several studies as indicated in this review have already shown a prominent role of exRNAs associated with exosomes in instituting these changes. EVs can retain the molecular signature of the cell of origin, and its exRNA cargo has tremendous diagnostic potential. Since the identification of exRNAs in various human bio-fluids, an increasing number of studies have positioned exRNA as a new type of non-invasive biomarker with wide-ranging clinical potential.

While significant advances have been made, the use of exosomes and exRNAs as cancer biomarkers faces remaining challenges that slows down its full potential from being realized. The NIH-led ERCC has supported research into the important roles of exRNAs in biological processes and its potential in molecular diagnosis, and to advance the technologies of exRNA identification and isolation from different types of bio-fluid. The ERCC has played critical roles in unmasking the mechanism of exRNA biogenesis, delivery and function; in defining a reference catalogue of exRNA in normal individual body fluids; in developing the clinical utility of exRNA as biomarkers of disease or as therapeutic molecules. The ERCC have also led the field in addressing major challenges in the field and providing valuable tools and technologies in this emerging field.

Although a few exRNA biomarkers have been discovered individually for cancer diagnosis, a systematic identification of novel exRNA biomarkers will need to be further pursued through better isolation of homogeneous populations of exosomes and comprehensive analyses of their cargo. Currently, there are only limited mature exRNA biomarkers that could guide clinical decision making. Large cohorts with matched clinical information, including survival time, disease recurrence, response for drug usage or other information can be catalytic in the identification of novel exRNA biomarkers. Sufficient clinical cohorts are also required to validate the performance of biomarkers for early-diagnosis, prognosis and drug usage for precision oncology.

In the future, it is also possible to target exRNAs as cancer therapeutic methods. The secretion and circulation of EVs that contain regulatory exRNAs can be blocked to prevent cancer from progressing and metastasis developing. In addition, exosomes could be used as a transmitter of specific regulatory elements into target cells, inhibiting the development of tumour. Some regulatory exRNAs that play roles in pivotal processes in tumour development could be repressed or sequestered to lower their abundance and inhibit their functions. In summary, exRNA is useful not only for liquid biopsies to diagnose various cancer types, but it also provides potential avenues for therapy.

DECLARATIONS

Authors' contributions

Conception and preparation of the manuscript: Happel C, Ganguly A, Tagle DA

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

Not applicable.

Consent for publication

Not applicable.

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Review

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Role of autophagic response induced by major phytochemicals in cancer prevention and treatment

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How to cite this article: Wenner C, Stewart R, McCabe A, Pepe J, Oberlin J, VanWert A, Bommareddy A. Role of autophagic response induced by major phytochemicals in cancer prevention and treatment. *J Cancer Metastasis Treat* 2020;6:33. <http://dx.doi.org/10.20517/2394-4722.2020.73>

Received: 14 Jul 2020 **First Decision:** 10 Aug 2020 **Revised:** 21 Aug 2020 **Accepted:** 27 Aug 2020 **Published:** 17 Sep 2020

Academic Editor: Sanjay Gupta **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Phytochemicals derived from dietary sources and natural products have gained significant attention in the scientific community due to their ability to modulate various pharmacological and biological activities. Understanding the molecular mechanisms by which natural products protect against various diseases including cancer will provide the basis for both clinical use and further chemical modification to develop targeted therapy. Autophagy, an evolutionarily conserved self-digestion process that employs lysosomal-mediated enzymatic degradation has a functional role in a wide range of pathological disorders, and has attracted oncology scientists over the past two decades. Studies employing natural products have shown that induction of autophagy may be either cytoprotective or cytotoxic governed by different molecular pathways. In this review, we summarize four major phytochemicals namely phenethyl isothiocyanate, capsaicin, withaferin A, genistein and their association with autophagy in cancer chemoprevention. We also discuss ideas for further investigation essential to understanding their mechanisms, which will guide their clinical applications for cancer prevention and treatment.

Keywords: Cancer, autophagy, capsaicin, phenethyl isothiocyanate, genistein, withaferin A



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INTRODUCTION

Autophagy/macroautophagy is an evolutionarily conserved catabolic process that provides energy and macromolecular precursors through lysosomal degradation of cellular material^[1,2]. Autophagy occurs at an elementary level in all eukaryotic cells during their normal growth and development. Although the role of autophagy in energy homeostasis is quintessential, its role in cellular processes such as degradation of cytoplasmic organelles and proteins is also well established. The process of autophagy typically begins with the formation of a phagophore, followed by recruitment and processing of light chain 3 I (LC3) to LC3-II occurring in the presence of an autophagic stimulus with eventual sequestration into an autophagosome. Subsequently, the newly developed autophagosome engulfs cellular proteins and organelles, fuses with a lysosome, forming an autolysosome, where autophagy occurs^[2]. Intracellular components including mitochondria and endoplasmic reticulum are engulfed by the newly formed phagophore before undergoing degradation upon fusion with lysosomes. This process facilitates the recycling of bioactive ingredients for cellular sustenance^[3-5]. The degradative products, which include simple carbohydrates and amino acids are recycled back into the cytoplasm. The mammalian target of rapamycin (mTOR), a highly conserved serine/threonine kinase senses signals from growth factors, energy status and stressful environment and serves as a key regulator of cell growth and division. mTOR's ability in mediating cellular responses is known to depend on the availability of amino acids and growth factors. It is usually active in an amino acid-rich environment and usually regulates protein translation but inhibits autophagy. When the levels of extracellular amino acids are very low, the process of autophagy recycles intracellular components to restore amino acid levels^[6]. Upregulation of mTOR results in excessive cell proliferation and eventually leads to the development of cancerous growth. Inhibition of mTOR results in induction of autophagy. The deregulation of autophagy has been clearly established in various chronic conditions including cancer. On the basis of the stimuli and tumor type, autophagy has been reported to be tumor promoting, or tumor-suppressive, indicative of the context-dependent role of autophagy in cancer^[4,5]. Studies also suggest that pharmacological or genetic inhibition of the autophagic response increases the chemotherapeutic efficacy of conventional agents, suggesting that autophagy inhibition in situations where it promotes cell survival may be an appropriate strategy in the treatment of cancer.

A large body of evidence including several epidemiological studies shows an inverse association of a diet rich in fruits and vegetables and the development of various cancers^[7-9]. In the last couple of decades, scientists and especially cancer biologists have started exploring the role of major dietary phytochemicals consumed by people across the world. Studies have identified the ability of these phytochemicals to modulate important signaling pathways and oncogenes associated with cancer development and progression. To that end, various studies have also looked into the role of plant-derived phytochemicals in the autophagic response and have reported important findings in their ability to modulate this cellular process as a way to affect the development of chronic conditions including cancer^[10-12]. However, further research is essential to understand the mechanistic details associated with induction and/or inhibition of autophagy by these bioactive compounds. The current review summarizes established findings related to the autophagic response to four major phytochemicals widely consumed by different cultures across the world.

PHENETHYL ISOTHIOCYANATE

Phenethyl isothiocyanate (PEITC) is a naturally occurring isothiocyanate containing a phenethyl group attached to its nitrogen. PEITC is found in various cruciferous vegetables in the form of gluconasturtiin. Myrosinase, an important enzyme located in the cellular matrix, catalyzes the conversion of PEITC from gluconasturtiin. When cruciferous vegetables, such as watercress, broccoli, radishes, or turnips are chewed or crushed, myrosinase is activated^[13]. Myrosinase can also be activated in the digestive system, which allows PEITC to be released after the vegetable has been ingested. Both myrosinase and PEITC

are thermolabile, and therefore cooked forms of cruciferous vegetables provide considerably less PEITC to the body than raw forms^[13]. Recent studies of PEITC have focused on the phytochemical's possible role in cancer chemoprevention. Specifically, prostate cancer prevention has received attention, owing to its long latency and because dietary exposure to PEITC is noninvasive and rather promising. Androgen receptor signaling plays a significant role in many aspects of prostate cancer cell growth^[14], suggesting the utility of androgen responsive LNCaP cells, which are commonly used in many different prostate cancer studies^[15]. The mitochondria's role in metabolism makes it a major source of cellular energy. Therefore, inhibiting mitochondrial function also depletes the available cellular energy, forcing the cells to destroy themselves and use their contents for energy^[16]. Studies explored the mitochondrial structural alterations and formation of autophagosomes upon treatment with PEITC in prostate cancer cells^[17,18]. PEITC-induced autophagy along with apoptosis, contributing to growth suppression of prostate cancer cells both in cell culture and in a transgenic mouse model of prostate cancer^[19,20].

Glycolysis refers to the aerobic or anaerobic catabolism of glucose, which results in pyruvate, NADH, and ATP. Certain metabolic intermediates of glycolysis can also benefit cancer cells, as they promote macromolecular biosynthesis, which enhances the available nutrient supply^[21]. Without the products of glycolysis, cancer cells utilize the autophagic response, where they degrade cytosolic organelles to generate energy for survival^[22]. The effect of PEITC on glycolysis, miRNA, and the matrix metalloproteinase (MMP), MMP2/MMP9 signaling pathway was shown to slow the spread of prostate cancer. This study further explored how PEITC could affect c-Myc-regulated glycolysis. PEITC exposure increased the level of c-Myc proteins, known to inhibit the expression of the enzymes hexokinase 2 (HKII), pyruvate kinase isozyme 2 (PKM2), and lactate dehydrogenase A (LDHA), which promote glycolysis^[23]. PEITC exposure was shown to inhibit glycolysis, as revealed by suppressed expression of important markers including HKII, PKM2, and LDHA in LNCaP and 22Rv1 cells^[23]. This is a revolutionary finding, as PEITC-mediated inhibition of glycolysis in cancer cells would eliminate a major energy store for prostate cancer cells, allowing for a new type of chemotherapy, utilizing the cell's natural autophagic response system. In addition, by inhibiting glycolysis in cancer cells, PEITC has the potential to eliminate the common problem of multidrug-resistant (MDR) hypoxia^[24,25], which is a major complication for other chemopreventive treatments. Hypoxia, a known factor that transforms cancer cells into MDR cancer cells, also promotes cellular glycolysis through what is known as the Pasteur effect. Hypoxia does this by manipulating cancer cells' metabolism, resulting in the induction of cellular quiescence^[25]. Therefore, hypoxia is a major cause of chemotherapeutic drug resistance. Thus, PEITC's role in inhibiting glycolysis in cancer cells may limit the hypoxic environment and minimize the transformation of MDR cancer cells.

PEITC was also shown to induce autophagic response through the regulation of MMP2/MMP9 signaling pathways. Both MMP2 and MMP9 proteins are responsible for extracellular matrix collagen/protein degradation. Activation of MMP proteins can promote angiogenesis and limit cellular apoptosis, allowing cancers to proliferate^[26,27]. MMP2, MMP9, and c-myc work together in cancer progression. PEITC was shown to downregulate the expression of MMP2, MMP9 and c-myc proteins and simultaneously induce autophagy^[26]. A study using hepatoma-derived growth factor resulted in tumor growth inhibition, and proved a linkage between autophagy and MMP2, MMP9, and c-myc proteins. It also proved that by modulating the signal pathway of the MMP2 and MMP9 proteins, prostate cancer cell migration and invasion could be inhibited^[28]. MMP2 is also a downstream effector of the JAK/STAT pathway, involved in tumorigenesis and metastasis. PEITC was shown to suppress the JAK/STAT-MMP2 pathway and induce autophagy *in vitro* and in a lung cancer mouse xenograft model. However, autophagy induced by PEITC preserved metastasis potential, which was abrogated in the presence of chloroquine^[29]. A different study that explored PEITC's epigenetic regulatory effects on miRNA in prostate cancer cells found that PEITC influenced miR-194 levels and downregulated the expression of oncogenic MMP2 and MMP9 proteins. The downregulation of MMP2 and MMP9 proteins ultimately reduced prostate cancer metastasis^[30]. It has

Table 1. Summary of autophagic response induced by PEITC in various cancer models

Author	Year	Specimen	Cancer	Treatment	Results
Wang <i>et al.</i> ^[29]	2018	Cell culture: A549, H661 and SK-MES-1; lung cancer xenograft	Lung	PEITC, 3-MA, chloroquine	Combination therapy enhanced the inhibitory effect of PEITC on metastasis potential of lung cancer cells
Singh <i>et al.</i> ^[23]	2018	Cell culture, LNCaP and 22Rv1 cells	Prostate	PEITC	Inhibited PCa growth by c-Myc overexpression
Zhang <i>et al.</i> ^[30]	2016	Cell culture, LNCaP cells	Prostate	PEITC	Suppressed cell proliferation through the MMP2/MMP9 pathway
Xue <i>et al.</i> ^[19]	2014	Cell culture, LNCaP cells	Prostate	PEITC	PEITC increased the number of smooth ER vacuoles. Within 18 h of exposure, mitochondrial membrane potential was disrupted
Yu <i>et al.</i> ^[14]	2013	Cell culture, LNCaP cells	Prostate	PEITC	Inhibited AR-regulated transcriptional activity and growth of PCa cells
Powolny <i>et al.</i> ^[18]	2011	Transgenic mouse model (TRAM)	Prostate	PEITC	Suppressed prostate cancer progression by induction of autophagic cell death
Xiao <i>et al.</i> ^[20]	2010	Cell culture, LNCaP and PC-3 cells	Prostate	PEITC	Induced autophagy through ROS
Bommareddy <i>et al.</i> ^[17]	2009	Cell culture, PC-3 and LNCaP cells	Prostate	PEITC, 3-MA	Induced apoptotic and autophagic cell death

PEITC: phenethyl isothiocyanate; ROS: reactive oxygen species; ER: estrogen receptor; AR: androgen receptor

also been shown that MMP2 and MMP9 proteins are both highly expressed in breast cancer tissues and are related to lymph node metastasis and tumor staging^[27]. These results further prove that the regulation of miR-194 and MMP2 and MMP9 proteins is critical in cancer research and that PEITC's ability to modulate the expression of these factors is vital in the induction of autophagy and prevention of metastasis. Table 1 summarizes the PEITC-induced autophagic response in different cancer models.

CAPSAICIN

Plants belonging to the genus *Capsicum* synthesize the alkaloid capsaicin (CAP), known to give chili peppers their hot, spicy flavor^[31]. Despite being an important cultural ingredient in many cuisines, CAP possesses anticancer properties, which have become an area of scientific interest in regard to chemoprevention. Studies have aimed at elucidating the mechanisms in which CAP works to promote autophagy in cancer cells to foster clinical applications for cancer treatment.

Researchers have discovered that coupling CAP with other chemotherapeutic drugs can minimize cellular chemoresistance to conventional therapies. A study investigating the combination of CAP and cisplatin against human osteosarcoma (HOS) cells showed that CAP at lower concentrations and cisplatin displayed a synergistic reduction of cellular viability in MG63, 143B and HOS cell lines^[32]. This study further showed cell cycle arrest in the G0/G1 phase and also examined the association of MMPs with inhibition of cellular invasion. The combination treatment resulted in decreased expression of MMP-2 and MMP-9 and reduced gelatin degradation by these enzymes. The study also investigated whether CAP/cisplatin treatment induced autophagy. Expression of the autophagy-associated proteins Beclin 1, Atg3, Atg5, Atg16, and LC3-II was increased along with the accumulation of autophagic vacuoles in the cytoplasm, indicating autophagy induction. CAP/cisplatin treatment was shown to target the reactive oxygen species (ROS)/AKT/mTOR pathway, a crucial mediator of cellular autophagy, by reducing p-AKT and p-mTOR levels. Upon treatment with the autophagy inhibitor bafilomycin (BAF), cells exhibited upregulation of apoptotic genes, suggesting that autophagy may be a pro-survival mechanism in osteosarcoma (OS) cells. CAP/cisplatin treatment significantly increased the generation of ROS, found to mediate the activation of the JNK pathway. In addition to reducing the cytotoxic effects of the combination treatment, pretreatment of HOS cells with N-acetyl cysteine (NAC), an ROS scavenger, reduced JNK phosphorylation and partially suppressed autophagy by reversing p-AKT and p-mTOR reduction^[32]. Another study examined the effects of CAP on the sensitivity of cholangiocarcinoma (CCA) cells to common chemotherapeutic drugs. The

CCA cellsexamined, QBC939, SK-ChA-1, and MZ-ChA-1, exhibited MDR to the chemotherapeutic drugs cisplatin, vincristine, and 5-fluorouracil (5-FU) as well as CAP. *In vitro*, combined treatment of 5-FU with low concentrations of CAP displayed synergistic effects in 5-FU-induced anti-proliferation, increasing sensitivity to apoptosis. The autophagy inhibitor 3-methyladenine (3-MA) enhanced the effectiveness of 5-FU inQBC939 cells, while the autophagy activator retinoic acid receptor alpha (RARA) promoted resistance to 5-FU. Studies have shown that the growth and survival effects of autophagy typically promote chemotherapeutic drug resistance. 5-FU treatment increased the expression of Beclin 1, LC3-II, and Atg5 genes as evidenced by acridine orange staining, PCR, and Western blot analysis. CAP/5-FU co-treatment inhibited 5-FU-mediated autophagy through phosphorylation of the Akt/mTOR pathway. Hence, CAP hindered chemotherapeutic drug resistance, proving its potential as an adjunct to conventional therapies^[33].

In response to environmental stresses such as DNA damage, CAP increases cellular viability through autophagy in certain cell lines. One study explored the impact of CAP on glioblastoma and breast cancer cell lines with mutated p53 oncosuppressor genes. Mutated p53 cells are known to express resistance to common therapies and promote tumor growth. The study noted that CAP increased levels of LC3-II, reduced p62 protein, and reduced mutant p53 by autophagy-mediated protein degradation. In addition, treatment caused p53 to transactivate damage-regulate autophagy modulator, a gene that induces autophagy through lysosomal protein expression, among other apoptotic genes. Western blotting confirmed that poly ADP-ribose polymerase (PARP) cleavage occurred after CAP treatment in both U373 and DKBR3 cells. The loss of mutant p53 inevitably altered the ratio of folded to misfolded p53 proteins, consequently restoring wild-type p53 protein activity in target cells^[34]. CAP-induced autophagy has also been shown to repair DNA in MCF-7 breast cancer cells. Autophagy was initiated by the AMPK α -mTOR pathway and the concentration of p53 protein increased in both the nucleus and cytosol. Moreover, cells experienced an alteration of mitochondrial membrane potential and blockingS-phase. In response to cancerous gene damage, CAP initiated DNA repair by activating δ -H2AX, ATM, DNA-PKcs, and PARP-1. Inactivation of the Atg5 gene in M059K cells resulted in phosphorylation of ATM and DNA-PKcs and increased expression of p53 and LC3-II. ATM and p53 inhibition resulted in PARP cleavage and prevented DNA-PKcs phosphorylation without changing LC3-II levels. Therefore, autophagy inducedby genetic damage improved cellular viability by repairing DNA through ATM regulation of DNA-PKcs and PARP-1^[35].

Inhibition of CAP-induced autophagy can enhance cellular sensitivity to apoptosis in a variety of human cancers. CAP decreased cellular viability in U251 glioma cells by increasing the expression of Beclin1, p62, and PUMA. Treatment with 3-MA, a known inhibitor of PI3K that blocks the formation of autophagosomes, reduced procaspase-3 and Beclin1 while increasing the expression of p53 and PUMA. Inhibition of p53 increased the expression of autophagic proteins. Therefore, inhibition of CAP-induced autophagy in glioma cells contributed to apoptosis^[36]. As discussed above, CAP synergistically restricted proliferation, increasing CCA cell sensitivity to apoptosis and upregulated apoptotic gene expression upon autophagy inhibition in OS cells^[32,33]. Multiple studies have demonstrated that CAP promotes apoptosis in a time-dependent manner upon inhibition of autophagy in hepatocellular carcinoma, melanoma and bladder cancercells^[37-39]. CAP was shown to target the ROS/signal transducer and activator of transcription 3 (Stat3) pathway in hepatocellular carcinoma cells. Western blotting indicated increased expression of autophagy markers LC3-II and Beclin1, GFP-LC3 autophagosome formation, ROS generation, and upregulated phosphorylation of Stat3, a known regulator of autophagy. In addition, NAC reversed the effects of CAP on ROS/Stat3-dependent autophagy, consequently inducing apoptosis^[36]. CAP stimulated LC3-II levels, formation of acidic vesicular organelles, and expression of ubiquitin-binding protein p62, which transports other proteins to the phagophore complex for selective autophagy, in 5,637 and T24 bladder cancer cells. To test if CAP could induce autophagy at varying levels of p62, cells were co-treated with CAP and BAF, a lysosomal inhibitor. Cells were found to generate p62, proving that CAP can activate autophagic flux. It was noted that CAP treatment generated ROS production resulting in time-dependent depolarization of

the mitochondrial membrane potential and an increase in the ADP/ATP ratio, both indicative of decreased cellular metabolic energy and autophagy. In addition, CAP increased concentrations of Atg4C, a protein responsible for autophagosome development and upregulated autophagic genes: GABA Type A Receptor Associated Protein Like 1 (GABARAPL1), LC3B, Sequestosome-1 (SQSTM1), immunity related GTPase M (IRGM), Unc-51 like autophagy activating kinase 1 (ULK1), tumor necrosis factor (TNF) and phosphatase and tensin homolog (PTEN). CAP-resistant cells underwent epithelial mesenchymal transition (EMT) and autophagy through activation of the Hedgehog pathway when exposed to CAP. Interestingly, activation of this pathway caused CAP-induced EMT cells to develop chemotherapeutic drug resistance to mitomycin C, gemcitabine, and doxorubicin^[39].

Studies have indicated that multiple signaling pathways mediate CAP-induced autophagy. Dihydrocapsaicin (DHC), an analog of capsaicin, mediated autophagy in a catalase-dependent manner. Treatment upregulated the expression of Atg5, a gene necessary for autophagosome formation, in addition to Atg4 and Atg7 consequently increasing LC3-I conversion to LC3-II. Catalase induction by DHC decreased baseline levels of ROS, consequently increasing production of LC3-II protein and caspase-3 activation. Upon exposure to 3-amino-1, 2, 4-triazole (3AT), a catalase inhibitor, LC3-II levels from DHC treatment were decreased. Conversely, overexpression of the catalase gene resulted in increased expression of LC3-II^[40]. Lysosomes co-localized with LC3-II to form autolysosomes in treated LNCaP and PC-3 cells; however, blockade of autophagy prevented their formation. High intracellular levels of ROS were observed in LNCaP cells compared to PC-3 cells. Upon treatment with NAC, ROS production by CAP was decreased in both cell lines. Confocal microscopy confirmed that cells treated with NAC did not accumulate autolysosomes and reduced inhibition of the PI3K/Akt pathway, indicating the critical role of ROS in autophagy^[41]. Another study confirmed that treated NPC-TW01 cells displayed reduced interaction between Beclin1 and Bcl-2, suggesting that autophagy activation may be regulated by the Beclin1/Bcl-2 complex and the class III PI3K/Beclin1/Bcl-2 pathway. Inhibition of the PI3K/Akt/mTOR pathway suggested a correlation between CAP and cellular proliferation^[42]. Table 2 summarizes the capsaicin-induced autophagic response in different cancer models.

WITHA FERIN A

Withaferin A (WA), a steroidal lactone, is derived from a number of plants belonging to the family Solanaceae, including *Acnistus arborescens* and *Withania somnifera*. It has been used in Indian medicine for centuries and is currently being investigated in the Western world for its anticancer, antitumor, and anti-inflammatory properties; however, its mechanism of action is unclear and is currently being investigated.

ROS generation was shown to be one of the main mechanisms by which WA prevents the growth of cancer cells. For example, WA used alone or in combination with cisplatin has proven to induce cell death by generating ROS and subsequent DNA damage in ovarian cancer and non-small cell lung cancer (NSCLC) cells^[43,44]. The combination of doxorubicin and WA in the treatment of ovarian cancer cell lines A2780, A2780/CP70, and CaOV3 resulted in a time- and dose-dependent synergistic effect on the inhibition of cell replication and induction of apoptosis. The combination treatment also showed enhancement of ROS production, causing DNA damage^[45]. Colorectal cancer cells resistant to 5-FU that were treated with the combination of WA and 5-FU showed induction of ER stress, which eventually led to cell death. In these cells, in addition to inducing G2M phase arrest, WA worked by upregulating stress sensors including BiP, PERK, CHOP, ATF-4, and eIF2a^[46].

Hyperpolarization of the mitochondrial membrane is another mechanism of action of WA. Targeting the mitochondria would influence mitochondrial metabolic activity, which could eventually lead to paraptosis. Cells treated with WA exhibited features (fusion of mitochondria and ER, expansion of vacuoles) that are indicative of paraptosis. Another notable observation in the treated cells was a decrease in the expression of Alix, an endogenous inhibitor of paraptosis^[47]. WA was shown to induce mitochondrial apoptosis

Table 2. Summary of autophagic response induced by Capsaicin in various cancer models

Author	Year	Specimen	Cancer	Treatment	Results
Chu <i>et al.</i> ^[38]	2019	Cell culture, A375, C8161	Skin: Melanoma	CAP	Induction of apoptosis and autophagy
Wang <i>et al.</i> ^[32]	2018	Cell culture, MG63, 143B, HOS, xenograft	Osteosarcoma	CAP and DDP	G0/G1 cell cycle arrest. Decreased tumor invasion and growth. Apoptosis and autophagy initiated through the ROS/AKT/mTOR and ROS/JNK pathways
Lin <i>et al.</i> ^[42]	2017	Cell culture, NPC-TW01	Nasopharyngeal	CAP	Inhibited proliferation through the PI3K/Akt/mTOR pathway. CAP activated autophagy at the elongation phase through the PI3K/Beclin-1/Bcl-2 pathway
Ramos-Torres <i>et al.</i> ^[41]	2016	Cell culture, LNCaP, PC-3	Prostate	CAP, NAC and 3-MA	Induced autophagy through inhibition of the Akt/mTOR pathway. Enhanced ROS generation induced autophagy
Garufi <i>et al.</i> ^[34]	2016	Cell culture, H1299, U373, SKBR3	Lung, Breast, and Glioblastoma	CAP	Autophagy restored wild type p53 through mutant p53 degradation
Chen <i>et al.</i> ^[37]	2016	Cell culture, HepG2	Liver: Hepatocellular Carcinoma	CAP	Inhibition of autophagy increased cellular sensitivity to apoptosis. CAP increased ROS generation to activate STAT3 autophagy
Amantini <i>et al.</i> ^[39]	2016	Cell culture, 5637, T24	Bladder	CAP	ROS generation decreased cellular metabolic energy. CAP stimulated EMT through the Hedgehog signaling pathway, causing chemotherapeutic drug resistance
Liu <i>et al.</i> ^[36]	2016	Cell culture, U251	Glioma	CAP	Inhibition of autophagy induced apoptosis
Hong <i>et al.</i> ^[33]	2015	Cell culture, QBC939, SK-ChA-1, MZ-ChA-1, xenograft	Cholangiocarcinoma	CAP and 5-FU	Inhibited 5-FU mediated drug resistance. CAP/5-FU treatment restricted proliferation, increasing apoptosis sensitivity. Notably decreased tumor volume and growth
Yoon <i>et al.</i> ^[35]	2012	Cell culture, MCF-7, M059K, M059J	Breast, Glioblastoma	CAP	Initiated autophagy through the AMPK α -mTOR pathway. Autophagy promoted cellular viability by repairing DNA through ATM regulation of DNA-PKcs and PARP-1
Oh <i>et al.</i> ^[40]	2008	Cell culture, WI38, HCT116, MCF-7	Lung, Colorectal, and Breast	DHC and CAP	Induced G0/G1 cell cycle arrest and apoptosis. Autophagy mediated in a catalase dependent manner

CAP: capsaicin; mTOR: mammalian target of rapamycin; PARP: poly ADP-ribose polymerase; DNA-PKcs: DNA-dependent protein kinase catalytic subunit

and decrease the spread of cancer cells into surrounding tissues in drug-resistant breast cancer cells. It also led to the suppression of the Nf-kappaB/m-TOR signaling pathway^[48]. WA was shown to impair the proteolytic activity of lysosomes causing blockade of autophagic flux, a decrease in the substrates required for the TCA cycle and impaired oxidative phosphorylation, resulting in apoptosis in breast cancer cells^[49]. Similarly, another study concluded that the inhibition of proteasome activity and induction of impaired autophagy constituted the main mechanism associated with the antitumor effects of WA in breast cancer cells^[50]. WA was shown to induce G2/M cell cycle arrest in myelodysplasia and leukemia cells^[51]. Another study analyzed the effect of WA on pancreatic cancer cells, one of the most difficult forms of cancer to treat due to cell resistance to treatment. WA was shown to increase the number of autophagosomes, while simultaneously inhibiting the SNARE pathway, specifically the STX17 and SNAP29 receptors, which prevents autophagosome and lysosome fusion. WA was also shown to increase ER stress and inhibit proteasome activity, leading to an increase in ubiquitinated proteins in the cell. Pretreating the cells with TUDCA partially reduced WA-induced LC3-II accumulation, suggesting that ER stress precedes WA-induced autophagy. This study also investigated autophagosome formation by analyzing the encoding of the GFP-LC3-II protein with the lentivirus vector. Pancreatic cancer cell treated with WA showed increased GFP-LC3-II in a dose-dependent manner, concluding that there was an increase in autophagosome formation^[52]. Another study sought to determine why WA was successful in inducing apoptosis in PC-3 and DU-145 but not in TIG-1 or LNCaP cells. In PC-3 and DU-145 cells, WA increased mRNA and protein levels of c-Fos. This did not occur in TIG-1 or LNCaP cells, which resulted in the ER stress response, eventually leading to cell death. A decrease in the expression of the anti-apoptotic protein

Table 3. Summary of autophagic response induced by Withaferin A in various cancer models

Author	Year	Specimen	Cancer	Treatment	Results
Alnuqaydan <i>et al.</i> ^[46]	2020	Cell culture, WS480, HT-29, HCT-116, NCM-460	Colorectal	WA with 5-FU	Inhibited β -catenin pathway and promoted G2M cell cycle arrest
Liu <i>et al.</i> ^[48]	2019	Cell culture, MDA-MB-231	Breast	WA	Induced mitochondrial apoptosis by increasing Bax levels and decreased Bcl-2
Hsu <i>et al.</i> ^[44]	2019	Xenograft model; Cell culture, A549, CL141, H441, CL97, H1975, CL152, H1299	Lung	WA and pemetrexed, cisplatin, or gemcitabine	Synergistic effect of WA and pemetrexed, cisplatin, or gemcitabine. WA downregulated mTOR/STAT3 signaling
Siddharth <i>et al.</i> ^[57]	2019	Cell culture, Huh7, HepG2, MHCC97H, MHCC97L	Liver	WA and WA + Chloroquine or Bafilomycin	WA induced autophagy. Combination of WA with Chloroquine or Bafilomycin had a higher efficacy than monotherapy
Muniraj <i>et al.</i> ^[50]	2019	Cell Culture, MDA-MB-231	Breast	WA	WA inhibited autophagy flux leading to decreased substrates for the TCA cycle
Ghosh <i>et al.</i> ^[49]	2017	Cell culture, MCF-7, MDA-MB-231	Breast	WA	WA inhibited tubulin polymerization
Ghosh <i>et al.</i> ^[47]	2016	Cell culture, MCF-7, MDA-MB-231	Breast	WA	WA promoted paraptosis
Okamoto <i>et al.</i> ^[51]	2016	Cell culture, MDS92, MDS-L	Myelodysplasia and Leukemia	WA	G2/M phase cell cycle arrest
Li <i>et al.</i> ^[52]	2016	Cell culture, Panc-1, SW1990, MIA PaCa-2, AsPC-1, BxPc-3	Pancreas	WA with cisplatin, paclitaxel, epirubicin or TNFSF10	WA induced ER stress-mediated apoptosis. When combined with cisplatin, paclitaxel, epirubicin or TNFSF10, this effect was increased
Nishikawa <i>et al.</i> ^[53]	2015	Cell culture, PC-3, DU-145, TIG-1, KD, LNCaP	Prostate	WA	WA showed an increase in mRNA and protein levels of c-Fos
Rah <i>et al.</i> ^[54]	2015	Cell culture, PC-3, DU-145	Prostate	AWA- Derived from WA	WA treatment resulted in autophagy and apoptosis
Vyas <i>et al.</i> ^[55]	2014	Cell culture, PC-3, MDA-MB-231, DRO81-1, HT-1080, 4T1, CaSki, AB12, Panc-1	Prostate, breast, thyroid, soft tissue sarcoma, cervical, pancreatic, mesothelioma	WA	Inhibited oncogenic signaling pathways, specifically F- κ B, Akt, signal transducer and activator of transcription 3 (Stat3) and estrogen receptor- α (ER- α)
Hahm <i>et al.</i> ^[56]	2013	Cell culture, MDA-MB-231, MCF-7, MCF-10A	Breast	WA	WA treatment resulted in autophagy
Kakar <i>et al.</i> ^[43]	2012	Cell culture, A2780, A2780/ CP70	Ovarian	WA and cisplatin	The combination of treatment synergistically enhanced antitumor effects of cisplatin
Fong <i>et al.</i> ^[45]	2012	Cell culture, A2780, A2780/ CP70, CAOV3	Ovarian	WA and Doxorubicin	The combination of treatment induced apoptosis and enhancement of ROS production causing DNA damage

WA: Withaferin A; mTOR: mammalian target of rapamycin; ROS: reactive oxygen species

c-FLIP (L) was also observed in the study^[53]. When prostate cancer cells were treated with 3-AWA, an azido derivative from WA, LC3B-I was converted to LC3B-II, stimulating autophagy and eventually, apoptosis. Prostateapoptosis response-4 (PAWR or Par-4) is a protein closely associated with tumor-suppressing activity, and right before apoptosis, PAWR levels are elevated. When PAWR is overexpressed in a cell, it induces apoptosis by inhibiting the antiapoptotic protein BCL2 by binding to Wilms tumor 1 protein (WT1). This study found that because PAWR binds to WT1, it indirectly downregulates BCL2 expression and suppresses Beclin 1, an essential component in autophagy^[54]. WA has been shown to target oncogenic signaling pathways, specifically NF- κ B, Akt, Stat3, and estrogen receptor- α (ER- α). In human cancer cells, these pathways are often hyperactive; however, WA has been shown to inhibit their activity. WA was shown to inhibit the NF- κ B pathway by nuclear translocation of the p65 subunit of NF- κ B and or downregulation of p6 in prostate cancer cells and soft tissue sarcoma cells. Suppression of ER- α by WA in breast cancer cells can lead to apoptosis. Cells treated with WA showed downregulated ER- α protein, leading to apoptosis^[55]. Upon exposure to WA, MDA-MB-231 and MCF-7 breast cancer cells underwent autophagy, which was confirmed by analysis of acidic vesicular organelles and cleavage and recruitment to autophagosomes of LC3-II^[56]. WA-induced autophagy was shown to be cytoprotective in hepatic cells, and by inhibiting its cytoprotective effects with chloroquine, tumor cells became more responsive to therapy^[57]. Table 3 summarizes the WA-induced autophagic response in different cancer models.

GENISTEIN

Genistein is a polyphenolic isoflavone compound derived from soy-based foods. The chemical structure of genistein is similar to that of estradiol, indicating its ability to bind to estrogen receptors. Genistein is soluble in polar organic solvents and has a lower solubility in water. Epidemiological studies have shown an inverse relation of soy-food intake and cancer development, especially in Asian countries where the consumption of soy-based foods is high compared to Western countries. Several meta-analyses have found that intake of soy foods and soy-based isoflavones is associated with prostate cancer reduction in men and breast cancer reduction in both pre- and postmenopausal women. Epidemiological evidence-guided research studies aimed at determining the molecular circuitry altered by genistein in cancer prevention by employing relevant cellular and animal models. Genistein is typically present in its glycosylated form during its biosynthesis in soybeans. After ingestion, deglycosylation occurs in the small intestine, and the free form of genistein is absorbed in the body resulting in its various pharmacological effects^[58].

Genistein's antitumor effects have been extensively investigated in various cancer models including, ovarian, breast, prostate, and lung cancers and leukemia and melanoma^[10,59]. One of the earlier studies that investigated the antitumor properties of genistein in ovarian cancer cells reported the induction of apoptosis and autophagocytosis as potential mechanisms of cell death^[60]. In that study, A2780 ovarian cancer cells were transfected with a GFP-LC3 plasmid and subjected to glucose deprivation or different concentrations of genistein. Analysis of cells using fluorescence microscopy revealed recruitment and localization of LC3-II to autophagosomes, indicative of an autophagic response. Further experiments in the same study demonstrated that nutrient deprivation was an important part of autophagic cell death induced by genistein in ovarian cancer cells^[60]. A study focusing on elucidating the antitumor effects of genistein employing A549 lung adenocarcinoma cells concluded that genistein, by inhibition of autophagic flux, enhanced tumor necrosis factor-related apoptosis, inducing ligand (TRAIL)-induced cell death compared to genistein or TRAIL alone treated cells^[61]. Another study using pancreatic cancer cells showed that genistein potentiated the antitumor effect of 5-FU by inducing apoptotic and autophagic cell death^[62]. That study used both cell culture and pancreatic tumor xenograft models and demonstrated that the combination of 5-FU and genistein had superior antitumor effects against the growth of pancreatic cancer cells when compared to the groups treated with 5-FU and genistein alone. Analysis of molecular mechanisms revealed increased expression of LC3-II, Beclin 1 and decreased levels of Bcl2, a key negative regulator of autophagy, which binds to Beclin1^[61]. In addition, the study also confirmed the induction of autophagic cell death in the group treated with both genistein and 5-FU by using chloroquine, a known inhibitor of autophagy^[62]. Prietsch *et al.*^[63] investigated the antiproliferative effects of genistein using MCF-7 human breast cancer cells. The study demonstrated that the antitumor effect of genistein involved the generation of free radicals, increase in BAX/Bcl-2 ratio, and downregulation of survivin, which ultimately resulted in induction of apoptosis and autophagy. Even though the study did not demonstrate the exact role of autophagy, LC3-II immunostaining of the cells treated with genistein clearly revealed the induction of autophagy in breast cancer cells^[63]. Consumption of genistein (soy products or dietary supplements) was shown to have a differential effect in breast cancer cells with a varied ER α /ER β ratio^[64]. Cells with a low ratio were shown to be more susceptible to genistein and increased effectiveness was seen in combination with tamoxifen-treated cells by increasing autophagic cell death^[64]. In contrast, a high ratio was shown to have counterproductive effect in anticancer treatment due to a decrease in ROS production, one of the main mechanisms of action of cisplatin (apoptosis) and tamoxifen-treated cells (autophagic cell death)^[64]. A study employing a preclinical model for estrogen receptor positive (ER+) breast cancer showed that lifelong genistein intake reduces the risk of *de novo* and acquired tamoxifen resistance and recurrence of tumors. The findings from the study also showed that prepubertal and lifetime genistein consumption improved responsiveness to tamoxifen^[65]. The antitumor benefits associated with genistein intake was linked to its potential in boosting immunity against tumors, reducing unfolded protein response and pro-survival effects of autophagy^[65]. Phytochemicals' ability to work synergistically and/or additively in the presence of

a different compound in lowering tumor burden has been reported for various cancers. Nakamura *et al.*^[66] showed synergistic antitumor effect by co-treatment with indole-3 carbinol and genistein against human colon cancer HT-29 cells. The antitumor effects were attributed to induction of apoptosis via inhibition of Akt phosphorylation and progression of autophagy. In a different study that used a combination of genistein and LC3 shRNA plasmid transfection, it was shown that the combination inhibited rapamycin-induced autophagy and promoted apoptosis in human malignant neuroblastoma SK-N-BE2 and IMR-32 cells both *in vitro* and *in vivo*^[67]. Malignant neuroblastoma is an extracranial solid tumor that usually occurs in children. Autophagy serves as a pro-survival mechanism in malignant neuroblastoma and usually deters the efficacy of conventional chemotherapeutic agents. The study revealed a decrease in expression of autophagy-related markers including LC3 expression and Beclin1 levels, and also modulated the expression of the apoptosis regulatory proteins Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic) essentially to increase the Bax: Bcl-2 ratio to trigger the mitochondrial pathway of apoptosis^[67]. Most recently, a study that developed genistein-PEGylated silica hybrid nanoparticles against colorectal cancer showed that the genistein-based nanoparticles exerted improved antiproliferative effects by modulating antioxidant enzymatic activity and oxidative stress levels in human colon cancer HT29 cells. Furthermore, cells treated with the nanoparticles exhibited characteristics of autophagy and resulted in autophagic as well as apoptotic cell death without any side effects^[68].

Radiotherapy continues to be an important treatment approach for several cancers. Genistein was shown to enhance the efficacy of radiation therapy in various cancer types. In a study employing non-small cell lung cancer (NSCLC) cells, genistein was shown to enhance cell radiosensitivity by inducing apoptotic and autophagic cell death. The findings revealed the role of genistein in enhancing radiosensitivity by increasing DNA damage, inhibiting cytoplasmic distribution of Bcl-xL and dissociation of Beclin1 from Bcl-xL to induce apoptosis and autophagy, respectively^[69].

In addition to the beneficial effects discussed above, either alone, with estrogen receptor modulator (tamoxifen) or radiotherapy, genistein has also been found to increase the potency of several traditional cytotoxic chemotherapeutic agents. For example, a recent study showed that genistein potentiated arsenic trioxide (As_2O_3) treatment of acute promyelocytic leukemia induced apoptosis and autophagy with the combination treatment. The study revealed that genistein in combination with As_2O_3 was able to increase the total release of ROS and also the expression ratio of LC3-II/LC3-I in NB4 cells^[70]. In a similar study, both cisplatin-sensitive A2780 and cisplatin-resistant ovarian cancer cells were treated with genistein alone. The findings revealed that genistein inhibited AKT kinase, which is an oncogenic kinase that plays an important part in glucose uptake. The resulting low glucose intracellular environment induced autophagy, which was evident by an increase in LC3-II levels^[71]. In a different study, genistein was used to treat uterine leiomyoma (UtLM) cells, in which MAP1LC3A was used as a marker to determine an autophagic response. The findings revealed induction of autophagy and subsequent apoptotic cell death by genistein in UtLM cells 72 hours after treatment^[72]. Table 4 summarizes the genistein-induced autophagic response in different cancer models.

Other major phytochemicals known to induce autophagic response

In addition to the four phytochemicals discussed in the current review, several other phytochemicals have been found to induce autophagic response in various cancer models. For example, curcumin^[11], a polyphenolic compound present in turmeric, was shown to induce autophagic cell death in malignant glioma cells by inhibiting the Akt/mTOR/p70S6k pathway and activating the ERK1/ERK2 pathway^[73,74]. A most recent study showed that combination of mTORC1/2 inhibitor with curcumin induced autophagy-mediated cell death by reducing the expression of Rictor and Akt in renal carcinoma, *in vitro* and in a xenograft model^[75]. Similarly, resveratrol^[12], another major polyphenolic compound present in various natural products including grapes, is well known for its antitumor effects in various cancer models, both *in vitro*

Table 4. Summary of autophagic response induced by Genistein in various cancer models

Author	Year	Specimen	Cancer	Treatment	Results
Pool <i>et al.</i> ^[68]	2018	Cell culture, HT-29 cells	Colon cancer	Genistein-PEGylated silica hybrid nanoparticles	Induction of autophagic and apoptotic cell death
Zhang <i>et al.</i> ^[69]	2018	Cell culture, NSCLC	Lung cancer	Genistein and radiotherapy	Induction of autophagic and apoptotic cell death
Zhang <i>et al.</i> ^[65]	2017	Animal model, S-D rats (DMBA chemical carcinogenesis)	Mammary tumors	Genistein	Protective benefits of lifelong intake of genistein included immune response, blockade of pro-survival effects of autophagy
Castro <i>et al.</i> ^[72]	2016	GM10964	Uterine leiomyoma	Genistein	Increased autophagy leading to apoptosis
Pons <i>et al.</i> ^[64]	2016	Cell culture, MCF-7 and T47D	Breast cancer	Genistein with cisplatin and tamoxifen	Genistein's therapeutic effects were altered by the ratio of ER α /ER β
Gali-Muhtasib <i>et al.</i> ^[71]	2015	Cell culture, CaOV3 and ES2 cells	Ovarian	Genistein	Autophagy induced by Akt kinase inhibition
Nazim <i>et al.</i> ^[61]	2015	Cell culture, A549 cells	Lung adenocarcinoma	Genistein and TRAIL	Increased apoptotic cell death
Prietsch <i>et al.</i> ^[63]	2014	Cell culture, MCF-7	Breast cancer	Genistein	Induction of apoptosis and autophagy through ROS generation and increase in BAX/Bcl2 ratio
Suzuki <i>et al.</i> ^[62]	2014	Cell culture and xenograft;	Pancreatic cancer	Genistein and 5-FU	Induction of autophagic cell death
Fan <i>et al.</i> ^[70]	2014	Cell culture, NB4 and NLRVMS cells	leukemia	Genistein + As ₂ O ₃	Increased autophagy and apoptosis in with combination therapy
Mohan <i>et al.</i> ^[67]	2013	Cell culture and xenograft; SK-N-BE2 and IMR-32 cells	Neuroblastoma	Genistein and LC3 shRNA plasmid	Treatment resulted inhibition of rapamycin-induced autophagy and promoted apoptosis
Nakamura <i>et al.</i> ^[66]	2009	Cell culture, HT-29 cells	Colon cancer	Genistein and Indole-3 carbinol	Inhibition of Akt pathway to induce apoptosis and progression to autophagy
Gossner <i>et al.</i> ^[60]	2007	Cell culture, A2780 cell	Ovarian cancer	Genistein	Genistein induced autophagic cell death due to nutrient deprivation

ROS: reactive oxygen species; NSCLC: non-small cell lung cancer

and *in vivo*. For example, resveratrol was shown to induce autophagy-mediated cell death in ovarian cancer, breast cancer and leukemia cells^[76-78]. It was shown that genetic or pharmacological modulation of autophagic proteins could play a major role in resveratrol-mediated cell death^[79]. A recent study with ovarian cancer cells showed that treatment of the cells with resveratrol resulted in growth suppression through epigenetic modulation of genes and microRNAs that are associated with the invasive phenotype^[80]. Honokiol, a phenolic compound from the magnolia tree was studied for its protective effects against chronic ailments including cancer. Numerous studies identified the potential of Honokiol to induce autophagic cell death in various cancer models^[81-84]. For example, a recent study showed that generation of intracellular ROS, activation of ERKs and upregulation of Atg7 were essential to Honokiol-mediated autophagic cell death in OS^[85].

A schematic diagram focusing on the mechanism by which different phytochemicals induce autophagy is presented in [Figure 1](#).

Limitations of phytochemicals

A large body of evidence suggests the inverse association of consumption dietary compounds rich in phytochemicals discussed in this review and the development of various malignancies including cancer. Despite the promising preclinical evidence, clinical development of these phytochemicals has faced severe roadblocks owing to their pharmacokinetic profile. The genetic profile of individuals is known to play a major role in the tissue distribution and elimination of various phytochemicals. For example, in a randomized study, glutathione S-transferase (GSTM-1)-positive individuals who consumed broccoli, a dietary source of sulforaphane (isothiocyanate) were shown to excrete sulforaphane metabolites at a

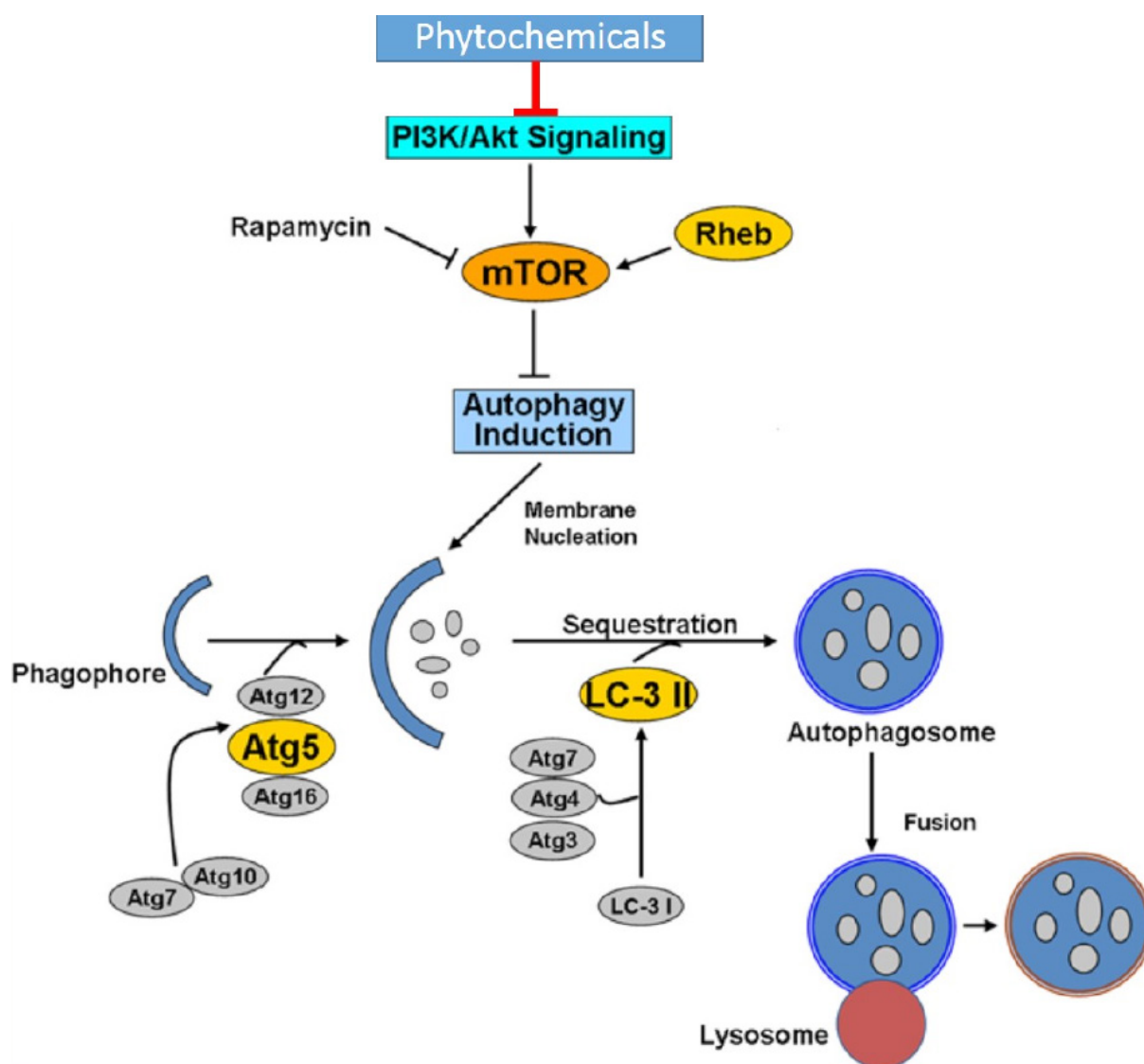


Figure 1. Schematic diagram representing the mechanism by which phytochemicals inhibit PI3k/mTOR pathway to induce autophagy. Induction of autophagy ensues upon negative regulation of mTOR that leads to formation of a phagophore and subsequent sequestration of cellular constituents in the presence of important autophagy proteins (Atg proteins) and cleaved LC3-II. Fusion of the autophagosome with lysosome results in lysis and generation of energy that is typically expended for cell survival or cell death (Type II). mTOR: mammalian target of rapamycin

highest rate. Higher and rapid elimination of phytochemicals/metabolites indicates decreased biological benefits at the tissue level in the individuals who are positive for the enzyme involved in its metabolism^[86]. Similarly, studies identified that isothiocyanates (especially sulforaphane) are not effective as therapeutic agents against chronic conditions that are known to be associated with oxidative stress^[86]. Pharmacokinetic studies in preclinical models and human trials have shown that phytochemicals are avidly metabolized in the body, which could limit the availability of these compounds or their associated active metabolites at the target organs distinct from the site of absorption. The therapeutic benefits of phytochemicals could only be realized through repeated administration, which would eventually produce systemic concentrations at the target sites. In addition, several studies including those discussed in the current review are exploring the possibility of increasing the bioavailability of these compounds through pharmaceutical modifications. Another important limitation of the administration of phytochemicals is their ability to cause the generation of ROS, which plays a major role in their pharmacological effects. It is important to limit the

oxidative damage caused by ROS in the body. If uncontrolled, ROS may lead to the development of various chronic diseases and contribute to toxic side effects. It is because of these limitations that phytochemicals are not having much success in clinical trials and hence are being investigated extensively to overcome these obstacles.

FUTURE PERSPECTIVES AND CONCLUDING REMARKS

The relevance of autophagy in chronic diseases has been well established and shown to have a major role in the development of these ailments, including cancer. Autophagic response in cancer development is both tumor-suppressive and tumor promoting depending on such factors as cancer type, energy status and metabolic processes within a cell. Moreover, the regulation of autophagy by phytochemicals in cancer cells also depends on epigenetic mechanisms that modulate the expression of key proteins involved in its induction^[87]. The overall chemopreventive effects of various phytochemicals^[88], including those discussed in the current review, were also attributed to their ability in regulating microRNA levels. Understanding the molecular mechanisms by which autophagy plays a role in cancer stem cells is critical in developing novel therapies^[89]. Cancer cells can stimulate the process of autophagy for accessing nutrients in their microenvironment for their growth and proliferation, and hence, understanding the role of autophagy in its microenvironment could result in radical approaches for a better therapeutic outcome. In this review, we discussed four major phytochemicals that are abundantly present in various dietary sources and consumed by different cultures across the world. These phytochemicals are shown to induce autophagic response in different cancer models. The autophagic response evoked by phytochemicals not only can mitigate resistance to conventional therapies but also serve as a type (type II) of cell death. The induction of autophagy by different phytochemicals depends on various factors, including the type of cancer. For example, genistein was shown to potentiate the antitumor effect of 5-FU by inducing apoptotic and autophagic cell death. Similarly, CAP and cisplatin combination induced autophagy that served as a pro-survival mechanism in OS cells, which when inhibited by bafilomycin resulted in upregulation of apoptotic genes. WA-induced autophagy was shown to be cytoprotective in hepatocellular carcinoma, which was abrogated in the presence of autophagy inhibitors, increasing the efficacy of combination therapy. The autophagic response induced by PEITC increased overall cell death in prostate cancer cells, while the response preserved the metastatic potential of lung cancer cells, which was abrogated in the presence of chloroquine. In addition, PEITC's ability to modulate the expression of miR-194, MMP2, and MMP9 proteins was vital in the induction of autophagy and prevention of metastatic potential in prostate cancer cells.

A continued progress in understanding the pharmacology of these phytochemicals and advances made to overcome the challenges associated with their pharmacokinetic profile have enabled their commercialization. The market for various phytochemicals continues to grow as more and more people are looking for alternate approaches in promoting their health and preventing disease development. Several phytochemicals are marketed as dietary supplements and are widely available in various forms. In conclusion, the utility of phytochemicals as therapeutic agents when used either alone or in combination with other conventional therapies can only be achieved after addressing the pitfalls and understanding the disease progression.

DECLARATIONS

Acknowledgments

The authors would like to acknowledge the faculty development funds awarded to Bommareddy A by Wilkes University and to thank Dr. Paul S. Adams and the mentoring task force at Wilkes University for providing summer research stipends to undergraduate students.

Authors' contributions

Responsible for compiling information along with the tables related to PEITC, capsaicin, withaferin A and genistein A: Wenner C, Stewart R, McCabe A, Pepe J, Oberlin J

Helped with the figures and future perspectives section: VanWert A

Conceived the idea and compiled information related to introduction, limitations to phytochemicals and future perspectives & conclusions and was responsible for the preparation of the final article: Bommareddy A

Availability of data and materials

Not applicable.

Financial support and sponsorship

Not applicable.

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 endometrial cancer

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How to cite this article: Malentacchi F, Sgromo C, Antonuzzo L, Pillozzi S. Liquid Biopsy in endometrial cancer. *J Cancer Metastasis Treat* 2020;6:34. <http://dx.doi.org/10.20517/2394-4722.2020.34>

Received: 16 Apr 2020 **First Decision:** 9 Jul 2020 **Revised:** 19 Jul 2020 **Accepted:** 11 Aug 2020 **Published:** 27 Sep 2020

Academic Editor: Wei Zhang **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Liquid biopsy (LB) is an emerging tool for the evaluation of relapse in several cancers and nowadays is used in lung cancer for primary detection and molecular characterization when tumoral tissue is not available. It can represent an innovative biospecimen for the screening, diagnosis, and monitoring of all types of cancer and for monitoring of therapeutic efficacy. LB includes several biofluids such as blood, urine, peritoneal fluid/lavage, and analytes (circulating tumor cells, circulating tumor DNA, long noncoding RNA, microRNA, vesicles, mRNA, and protein) that can play different roles in diagnosis, prognosis, and patient management as well as in the improvement of the knowledge of cancer evolution. Endometrial cancer (EC) is a tumor usually detected at low stage with a good prognosis, but few low risk cases, unexpectedly, can evolve to bad prognosis. Up to now, no molecular target exists to treat advanced stage or to define the evolution of low stage EC. This review focuses on how the LB may help in the management and characterization of patients affected by EC.

Keywords: Endometrial cancer, liquid biopsy, long noncoding RNA, circular RNA, circulating tumor DNA, circulating tumor cell, extracellular vesicle, peritoneal lavage

INTRODUCTION

The current use of traditional biopsy in the management of cancer has several limitations in the developing era of precision medicine, with cancer treatment mainly due to the progression of cancer and the onset of resistance to therapies. Sometimes tissue biopsy cannot reflect the heterogeneity of the primary tumor and the evolution of tumor due to the natural course of the disease and under the pressure of treatment.



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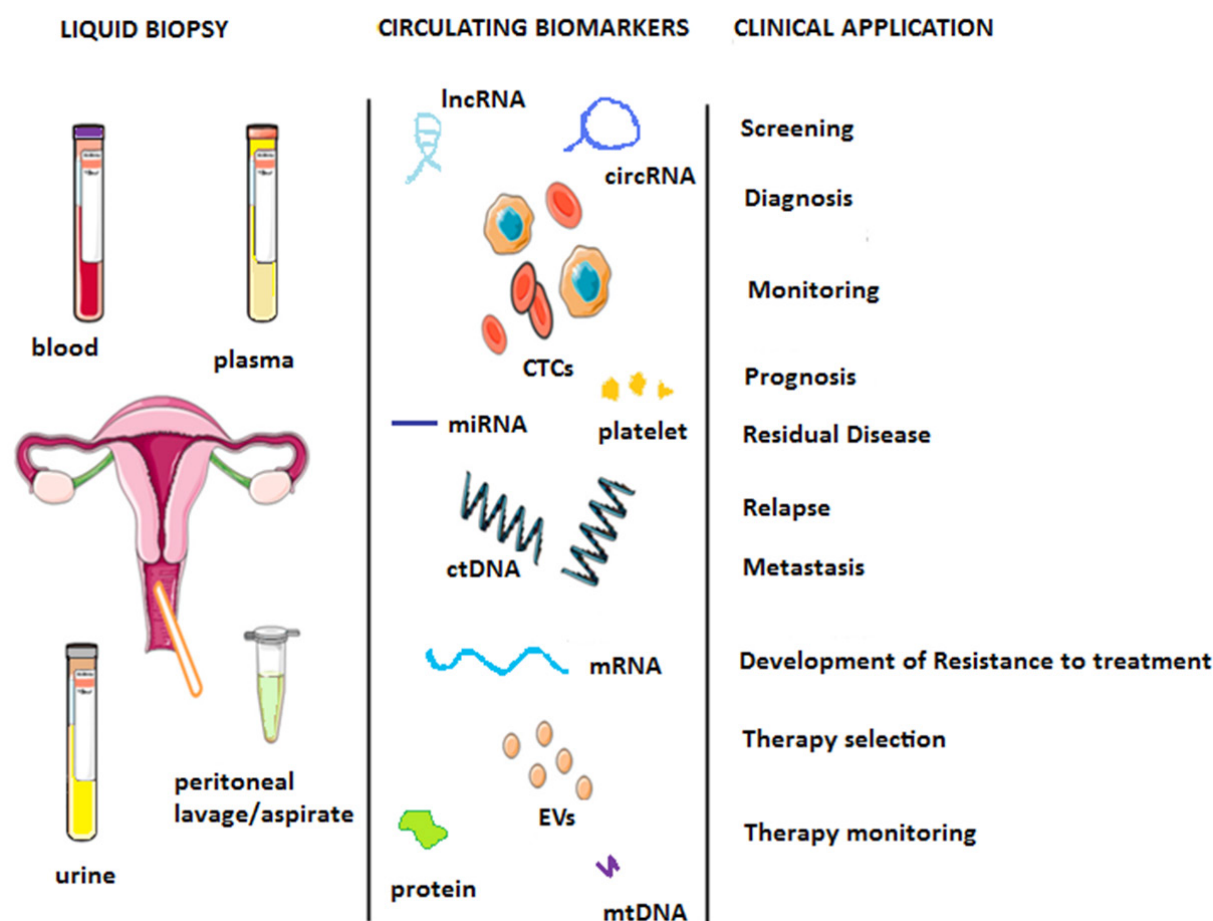


Figure 1. Schematic overview of LB biospecimen, biomarkers, and their clinical applications in EC. Modified from Muinelo-Romay et al.^[24]. LB: liquid biopsy; EC: endometrial cancer

Moreover, traditional biopsy is invasive, it is neither feasible nor practical to perform serial biopsies to guide treatment in real time, and, depending on the site of tumor, biopsy cannot be performed.

Liquid biopsy (LB) has the potential to overcome many of the limitations of traditional biopsy since it is highly tailored and minimally invasive, can be repeated several times, is a cost-effective method, and can be used to screen and monitor response to treatment and to identify the clones involved in the relapse, metastasis, or resistance to treatment. However, many challenges still need to be overcome before LB becomes a reliable and widely available option^[1].

LB is represented by fluids that can be collected from the body such as blood, urine, cerebrospinal fluid, lavages, peritoneal fluids, and saliva^[2], and the related biomarkers are, consequently, circulating tumor markers such as circulating tumor cells (CTCs), nucleic acids, proteins, and metabolites deriving from tumors.

LB usually refers to blood or blood compounds, and the principal biospecimens analyzed are circulating tumor DNA (ctDNA), CTCs, and proteins. Proteins were the first molecules analyzed within blood and other body fluids before the invention of LB [e.g., CA125 in peripheral blood (PB) for ovarian cancer]. Nowadays, the target biomolecules are increasing and include microRNA (miRNA), lncRNA, miRNAs, vesicles, and platelets [Figure 1].

Blood circulating biomarkers are currently used in research and have been suggested for tumor characterization to set a personalized target therapy [e.g., epidermal growth factor receptor (EGFR) mutation profile in lung carcinoma] in clinical practice. Despite the great development of droplet-based digital PCR (ddPCR) and the various optimizations of Next Generation Sequencing technologies (NGS), for both CTCs and ctDNA assays, there is no standardization starting from the pre-analytical phase through the analytical procedures to platform/technological methods^[3-5].

Endometrial cancer (EC) is the most frequent gynecological malignant disease affecting the inner lining of the uterus^[6]. The incidence has increased between 1980 and 2010, due to an increment in average age and obesity^[7]. Eighty percent of this type of cancer is diagnosed in an early stage [by International Federation of Gynecology and Obstetrics (FIGO) 2009 classification: stage IA], with five-year survival rates above 80%. EC is classified into two main histological types: Type I, which is more frequent, and Type II, comprising several subtypes such as serous and undifferentiated carcinomas^[8,9]. In 2013, the Cancer Genome Atlas Research Network (TCGA) suggested implementing classification based on molecular characterization^[10], which can be useful to define targeted therapy and monitor cancer development and treatment. Moreover, there are currently no targeted therapies: in fact, there is no molecular target for treatment, detection, or monitoring. Finally, 75% of patients have early-stage, low-grade endometrial endometrioid carcinoma (EEC), which can be treated by surgery; however, about 15% of patients develop recurrence, which cannot be correctly predicted at diagnosis^[11].

In this paper, we investigate the classical biomarkers in blood LB and the new ones in other biofluids for EC [Table 1] by reviewing the data in journal databases.

MATERIALS AND METHODS

The evaluation of papers was performed in PubMed using the following key words: “miRNA”, “circulating miRNA”, “cell-free DNA/ctDNA”, “lncRNA”, “vesicles/exosomes”, “messenger RNA (mRNA)”, “circular/circRNA”, and “CTC/circulating Tumor cells” “LB” (blood, urine, saliva, and peritoneal lavage/fluid); AND “Endometrial cancer/gynecological malignancies”. The search retrieved 130 articles from 2015 to 2020, of which about 100 were included in this review.

BLOOD-LIQUID BIOPSY

When Liquid biopsy is cited in relation to tumor, the main evaluated biomarkers are CTCs and ctDNA in blood. There is no consensus when comparing ctDNA, CTCs, and primary tumor^[12]; the results change depending on the tumor analyzed and the procedure adopted for biomarkers isolation and characterization. For example, these results include 92% for KRAS in colon cancer comparing ctDNA and primary tissue^[13], “good correlation” between CTs and ctDNA in colon^[14], and “higher accuracy of ctDNA as prognostic biomarker” in comparison to CTC in overall evaluation in cancer^[15].

Concerning EC, no studies have been reported comparing CTCs, but an interesting study compares the somatic mutation profile evaluated on ctDNA extracted from plasma to that derived from peritoneal lavage on 50 patients by NGS, which shows a concordance on KRAS of 42% between primary tissue and peritoneal lavage ctDNA and 18% for PIK3CA; for plasma ctDNA, this concordance decreases to 7% and 4%, respectively^[16].

Circulating tumor cells

CTCs were discovered as early as 1869 by Thomas Asworth, who found tumor cells in the blood of a person who died from metastatic cancer. CTCs were mainly investigated in prostate, breast, lung, and thyroid carcinomas. Some studies are based on the identification of cells, while other on the characterization of

Table 1. Liquid biopsy biospecimen and molecules in endometrial cancer

Biospecimen	Biomolecule	Biomarker	Suggested for	Not assayed in EC	Sample analyzed	Ref.
Bloodn	CTCs	Protein mRNA	Diagnosis		10	[81]
					62	[24]
					24	[28]
					92	[29]
					24	[30]
	DTCs	DNA	Diagnosis		311	[31]
					34	[22]
					401	[24]
					13	[27]
					24	[28]
	Single CTC Ct/cfDNA	Mutation	Diagnosis	x		[30]
					44	[37]
					109	[50]
					48	[51]
					5	[52]
	mtDNA	Integrity/ amount CNV Presence	Biomarkers		60	[53]
					53	[55]
					53	[54]
					59	[56]
					3580	[24]
	miRNA	Expression	Diagnosis/ prognosis		44	[63]
					32	[64]
					77	[66]
					42	[67]
					267	[72]
	mRNA	Methylation	Biomarkers		93	[73]
					3568	[75]
					45	[76]
					56	[79]
					10	[77]
	Protein	Expression (hTER) Expression (leptin) HE4	Biomarkers		5	[78]
						[101]
	circRNA			x		
	lncRNA			x		
	snRNA			x		
	snoRNA			x		
	piRNA			x		
	Extracellular vesicle	DNA miRNA mRNA circRNA Protein		x		
			Biomarkers		3	[84]
					10	[81]
						[85]
Platelet	miRNA			x		
	mRNA			x		
	circRNA			x		
	Protein			x		
Urine	lncRNA			x		
	Ct/cfDNA			x		
	miRNA		Biomarkers		20	[24,88]
	mRNA			x		
	Protein			x		
	circRNA			x		
	Extracellular vesicle	DNA miRNA mRNA circRNA Protein		x		

Uterine/ peritoneal lavage	Cells (CTC)		Biomarkers/ prognosis	2	[89]
	Ct/cfDNA (uterine)		Biomarkers/ prognosis	382	[24]
				5	[96]
				1	[97]
				107	[98]
	miRNA		x		
	mRNA		x		
	circRNA		x		
	Protein	IL11 (uterine)	Biomarkers/ prognosis	16	[95]
	Extracellular Vesicle	DNA	x		
		miRNA (Exosome)		25	[99]
		mRNA	Biomarkers	25	[94]
		circRNA	x		
		Protein	x		
	Cytology (peritoneal)	Diagnosis/ prognosis/ screening		46	[89]
				5	[90]
				22	[91]
				198	[92]
				30	[93]
				25	[94]

CTCs: circulating tumor cells; cfDNA: cell-free DNA; DTCs: disseminated tumor cells; ctDNA: circulating tumor DNA; lncRNA: long noncoding RNA; circRNAs: circular RNAs; mRNA: messenger RNA; miRNA: microRNA; snRNA: small nuclear RNA; snoRNA: small nucleolar RNA; piRNA: piwiRNA

CTCs by the identification of specific biomarkers related to primary cancer. There are several methods for identification of CTCs:

(1) Immunoaffinity: This method uses specific antigens present on the surfaces of CTCs but not expressed on other cells. In the negative enrichment, the CD45 antigen is generally targeted to capture normal cells. In the positive enrichment, these antigens are used for identification and separation of CTCs from other blood cells. Usually, these antigens are represented by EpCAM, but they may result in false-positives; thus, it is also important to consider epithelial-to-mesenchymal transition (EMT) and stem cell markers. To overcome this challenge and to include more CTC subpopulations during separation, multiple surface markers such as EGFR, human epidermal growth factor receptor 2 (HER2), mucin 1 (MUC1), and CXCR4-SDF^[17] have recently been developed as specific biomarkers related to CTC-plasticity phenotype, stemness, and epithelial-to-mesenchymal transition (EMT) features that may provide an advantage in the promotion of metastasis for CTC dissemination and homing (by the CellSearch system and CTC-chip and ISET)^[18] or to set immunotherapy^[19]. Even if they can be related to specific surface proteins characterizing the primary tumors, there is a chance that they may be lost during tumor development in some tumor clones. Two of the main disadvantages and challenges of immunoaffinity-based CTC isolation methods can be addressed to the heterogeneity of CTCs, which can cause a loss of CTC subpopulations.

(2) Immunomagnetic positive enrichment: Immunomagnetic strategies are based on the previous strategy and the antigens for CTC identification are bound to magnetic beads (not to a surface as for immunoaffinity), even if this can cause a reduction in the CTC capture efficiency and result in CTC loss. Consequently, this technology has recently been improved with microfluidic and nanoparticles (e.g., graphene oxide, silica nanoparticle, and gold nanoparticle-thiol exchange reaction).

(3) Size-based technique: It is a physical method based on cell-sized: usually, CTCs have a higher size (9-19 μm) in comparison to other blood cells (about 8 μm). Sized-based techniques use membrane microfilters (typically polycarbonate films containing controlled nano- to micron-sized pores) and microfluidics under controlled pressure to prevent mechanical damage to cells during filtration. This method avoids the inaccuracy of heterogeneous antigen expression observed in CTCs. The main advantage of this technique is that it is label-free (e.g., ISET, ScreenCellCyto, and Parasortix).

(4) Membrane capacitance: Dielectrophoretic field-flow fractionation (DEP-FFF) employs separation by size and polarizability using membrane capacitance, which can process 30 million cells within 30 min

with high recovery rates. However, it requires very specific parameters such as cell type and electric field frequency (e.g., DEPArray)^[20].

(5) Density based: This procedure is based on centrifugation, which uses the specific density of RBCs, leukocytes, and cancer cells on specific buffer. This method is one of the first reported.

(6) Reactive ion etching: This procedure is based on photolithography to make specific patterns on the glass surface that favor CTC attachment to normal cell attachment on the basis of adhesion preferences^[20].

(7) Acoustic-wave fields inside microchannels are used to capture CTCs based on the principle that all cells experience different acoustic radiation forces that result in varying movement trajectories, ultimately separating the cells. In most cases, this procedure is associated with others, including size, density, and compressibility.

(8) Combined method: It is the combination of more than one of the above-listed procedures (e.g., CTC-iChip1 and CTC-iChip2)^[21,22].

All the aforementioned technologies have been designed for cell capture *ex vivo*. Nowadays, a new technology, known as GILUPICellCollector®, applies an anti-EpCAM wire directly into the peripheral arm vein and captures CTCs with remarkable efficiency, processing approximately 1.5 L of blood in 30 min^[20].

A new definition was recently introduced related to classical CTCs. It is not known if all CTCs are able to induce relapse or metastasis. Consequently, the term CTCs refers to tumor cells that can be found in circulation. Those CTCs able to induce metastasis and disseminate are defined disseminated tumor cells (DTCs) based on the presence of specific antigens on their surface, identified by immunocytochemistry (ICC) or via their corresponding mRNA by reverse transcription quantitative polymerase chain reaction (RT-qPCR)^[21,23]. Generally, CTCs, after enrichment, are evaluated to identify specific cancer-related profile biomarkers by fluorescence *in situ* hybridization for genome amplification detection, ICC for protein markers identification, and RT-PCR/RT-qPCR/NGS for quantifying specific RNA and DNA sequence analysis^[20]. It is necessary to underline that most of the procedures for CTC isolation, characterization, and count were designed for cancers other than EC, therefore they may not be appropriate for recognizing specific antigens related to EC-CTC.

Only some of the above-mentioned technologies for CTC isolation are currently approved for *in vitro* diagnostics and are mainly not specific for EC. These technologies may not be suitable to properly identify EC-CTCs and could give different results. Moreover, these techniques may be inappropriate to identify different biomarker profiles based on DNA mutations^[24], DNA methylation^[25], mRNA expression^[26-28], or protein^[28].

Count of CTCs

There are few articles related to CTC count in EC. One is based on CTC enumeration by MetaCell® technology, which has been detected in 75% of patients with EC^[29]. Another one is based on the identification of circulating progenitor cell number (characterized by CD34, VEGFR2, and KDR expression) in the PB of women with early EC. Those numbers result significantly augmented compared with the ones coming from healthy control women^[24]. Another study evidenced that the detection of more than two CTCs is useful for a preoperative diagnosis of grade 3 EC: patients with poorly differentiated endometrioid EC have higher CTC number. As suggested by Bogani *et al.*^[30], the discrepant results are mainly due to different biomarkers used for CTC characterization. Regarding DTCs, it has been reported that they are present in 16% of bone marrow aspirates of women affected by EC^[31].

Biomarkers of CTC

Some studies focused on the identification of the expression of single specific biomarkers such as Mig7, CK19^[27], and thyroid transcription factor-1, which were found to be correlated with TNM staging, vascular

infiltration, lymphatic metastasis, progression-free survival, and the decrease of median survival time; consequently, they are proposed as good markers for endometrial carcinoma and metastasis^[26]. Other studies have proposed mRNA multi-markers based on the isolation of CTCs or by CellSearch followed by RT-qPCR analysis. Bao *et al.*^[28] proposed the following pan: CK20, CEA, AGR2, MGB2, DLL4, EphA2, Her3, and PDGFR α . Obermayr *et al.*^[32] proposed another pan focused on: CCNE2, DKFZp762E1312, EMP2, MAL2, PPIC, and SLC6A8.

Another study explored the presence of specific mutations EC-related genes such as *CTNNB1*, *STS*, *GDF15*, *RELA*, *RUNX1*, *BRAF*, and *PIK3CA*, which may be suggested as potential therapeutic targets^[24].

A consensus panel profile based on DNA or mRNA CTC analysis has not been defined, due to the low number of studies performed, the different technologies used for CTC isolation and biomarker analysis, and the different biomarkers analyzed and procedures used for identification. Moreover, another challenge is represented by the need of specific databases and algorithms able to integrate several sources of information derived from analyzing multiparametric data as well as multiple biospecimens (e.g., CTC and ctDNA)^[33].

Count and biomarkers of CTCs

Several studies have analyzed both the count of CTCs and their molecular characterization, in which the antigen for isolation was used as a marker as well as for enumeration, e.g., EpCAM and stathmin [as confirmed by immunohistochemistry (IHC)], showing an increase in CTC number and stathmin IHC in non-endometrioid versus endometrioid histology, tumor size ≥ 5 cm vs. < 5 cm, higher-stage disease, and worse survival^[28].

An investigation performed on patients affected by EC with and without recurrence, ranging from Grade 3 Stage IB to Stage IV carcinomas and recurrences, by EpCAM-based immunoisolation using the CELLectionTM Epithelial Enrich kit (Invitrogen, Dynal) followed by RT-qPCR analysis, associated the presence of CTC with high-risk EC, evidencing the CTC-plasticity phenotype with stemness and EMT gene-expression profile^[22].

CTC in vitro for drug response model

The ability to isolate live CTCs by specific device (e.g., MetaCell®), as reported by Kolostova, might be useful to test *in vitro* specific drug treatments^[34].

Single CTC analysis

Although limited in sample size and number of studies, due to the highly technical and expensive procedures, for characterizing the profile of CTCs, it may be useful to perform genetic and expression profiles of single CTC, which have demonstrated superior diagnostic accuracy in defining lineage identity in other tumors such as multiple myeloma and prostate cancer^[35]. The main procedure adopted is the evaluation of genetic profile by NGS after whole genome amplification^[20].

ctDNA

Biology of ctDNA

The presence of ctDNA was first described more than 30 years ago, even if intensive research began only in the 2010s. Active and passive mechanisms were proposed for the origin of cell-free DNA (cfDNA). Through passive mechanism, cfDNA is released into the blood *via* apoptosis and necrosis performed by macrophages and phagosomes from hematopoietic cells. Through active mechanism, tumor cells secrete cytoplasmic fragmented DNA to communicate with distant tissue through exosomes.

The fragment size of cfDNA is 170-200 bp, reflecting the structure of nucleosomes, sustained by nuclease digestion of genomic DNA (gDNA) during apoptosis. The fragment size profile differs between cfDNA and ctDNA from tumor cells. Shorter (< 100 bp) or longer (> 10,000 bp) fragments are frequently observed in ctDNA from cancer patients as the majority of the cfDNA from normal cells is released after apoptosis, while ctDNA can also be released by necrosis. Necrosis-based fragmentation appears to create longer or shorter DNA fragments in comparison to physiological apoptotic origin in healthy subjects. Short ctDNA fragments are more frequently observed in patients with metastatic disease than in those with early-stage cancer.

After surgical resection, the half-life of ctDNA was estimated to be 114 min. The cfDNA amount was estimated to be three-fold higher in cancer patients compared with that in healthy individuals^[36]. However, pregnancy and some disorders, such as infectious and autoimmune diseases, stroke, infarction, and trauma, induce an increase in cfDNA^[37]. The ctDNA amount shows wide variation among cancers, differs among stages, and may correlated with tumor burden^[36]. The evaluation cfDNA in term of amount and integrity as well as studying specific mutations is cheaper and easier than CTC evaluation.

ctDNA has already been implemented in routine clinical practice after European Medicines Agency approval of the EGFR mutation test (Therascreen EGFR Plasma, Qiagen) in plasma of patients with non-small cell lung cancer^[24].

Nevertheless, no consensus or standardized analytical procedures have been defined to harmonize cfDNA procedures (although there is agreement concerning the isolation of cfDNA and ctDNA from plasma, being serum preferred) or the analytical phase (methodologies, reference genes for integrity, and total amount evaluation).

Levels of cfDNA in serum are higher than in plasma due to contamination of gDNA from leucocytes during the clotting process; therefore, plasma is preferable. For the same reason, cfDNA needs to be isolated from standard EDTA-blood tubes within 2-4 h. Alternatively, tubes containing stabilizers that prevent cell lysis should be used for blood collection^[38]. However, two centrifugations should be performed, the first within 2-4 h for separating plasma from whole blood at 1000-2000 g and 4 °C for 10-15 min and the second performed on plasma to separate plasma from platelets or cellular debris for the same time and temperature but at about 15,000 g^[38]. ctDNA assays can identify tumor-specific genetic alterations (e.g., somatic point mutations, loss of heterozygosity, gene fusions, gene copy number variations, and DNA methylation changes)^[3,36,39,40], allow the monitoring of cancer evolution and the setting of specific personalized targeted therapy^[41], and provide suggestions for immunotherapy^[42]; they can allow detect minimal residual disease, monitor mutations that are related to tumor burden, and define the appearance of resistance to targeted therapy^[43].

Methodologies for detection of ctDNA

The methodologies depend on the purpose of ctDNA^[44-48]:

- (1) Quantitative PCR (qPCR): This procedure is used for the evaluation of total cfDNA amount and/or cfDNA integrity. To be sure to identify properly ctDNA coming from EC (and not from others tumors or pathologies), it is necessary to refer to specific mutations present in the tumor. Instead, for the evaluation of total cfDNA amount and integrity, it is necessary to use a specific gene that is not amplified or differentially fragmented in other tumors or pathologies.
- (2) ddPCR: This procedure is mainly related to the evaluation of the presence of specific tumor-related mutations. The concept of this procedure is based on Poisson distribution and allows the absolute quantification of each allele or mutation considering the number of wells containing the target genes in comparison to wild type (wt) taking into consideration that a single molecule is present in each well.

Standard PCR or qPCR instead evaluate the median distribution of a mixed mutated and wt solution, which may decrease the accuracy and sensitivity for the detection of rare mutations. Several instruments allow ddPCR, which can be distinguished depending on the size of generated droplets.

(3) NGS: This procedure is mainly related to the evaluation of the presence of specific tumor-related mutations, although it can also be used to evaluate copy number variation (CNV) and quantification of total amount. Deep massive paralleling allows the evaluation of multiple mutations (several mutations in the same gene and several genes) and more than one sample in the same run^[49].

Mutations of ctDNA

Several studies have evidenced the presence of ctDNA in gynecological malignancies and the detection of specific mutations related to EC.

One of the first studies was performed by Dobrzycka *et al.*^[50], using PCR-restriction fragment length polymorphism in a cohort of 109 patients with EC to analyze *TP53* and *KRAS* mutations, confirming the data reported for primary tumors that there is a higher percentage of *TP53* mutation in serous carcinomas as well as a high frequency of *KRAS* mutations in grade 2 endometrioid tumors. Pereira *et al.*^[37] observed the presence of mutations in *TP53*, *PTEN*, *PIK3CA*, *MET*, *KRAS*, *FBXW7* and *BRAF* in ctDNA, and these detections were useful to predict the tumor recurrence, with an average of seven months, before the radiologic evidence.

Moreover, Bolivar *et al.*^[51] evidenced the presence of mutations on *CTNNB1*, *KRAS*, *PTEN*, or *PIK3CA* genes, independently of total cfDNA amount, in association with advanced stage, deep myometrial invasion, lymphatic/vascular invasion, and primary tumor size.

As discussed below, even for ctDNA of EC, there is evidence of discrepancy between the mutations found in plasma compared to those evaluated in primary tissue sample with an agreement of only 33%^[52].

Integrity index of ctDNA

Only one study has evaluated the integrity index performed on ctDNA extracted from serum and based on evaluation of Alu repeats. The authors registered an increase in ctDNA integrity and amount in high grade compared to G1 ECs, suggesting a role of cfDNA as potential prognostic biomarker in EC^[53].

Copy number variation in ctDNA

The same findings were obtained for the evaluation of CNV in ECs by NGS by Nakabayashi *et al.*^[54] on three patients on the following loci: 1p36-p31 and 1q12-q44 or 8q24.

Total amount of cell free DNA

Regarding the total cfDNA evaluation, the two reported articles are based on the evaluation of Alu repeats: the previous citation^[53] suggests a relationship between integrity and EC grade, while another^[55] reports no significant difference in cell-free DNA among stage or histological grade of EC, as well as no significant change in cell-free DNA before and after operation.

Mitochondrial ctDNA

Recent studies have demonstrated a potential link between circulating mitochondrial cell-free DNA (cfmtDNA) content and cancer. In particular, the evaluation of aberrant changes and altered content of cfmtDNA represents an important approach for early cancer diagnosis with some unique advantages over circulating nuclear cell free DNA (cfnDNA), such as the much shorter and more simply organized mitochondrial genome and the higher number of mtDNA copy. These characteristics make screening much easier and more cost-effective, with respect to the nuclear genome one, in body fluid samples such as

plasma and serum. mtDNA does not have protective histones and sophisticated DNA repair mechanisms, which underscore its susceptibility to oxidative stress and other genotoxic insults. The abnormal alteration of mtDNA copy number is well documented for numerous malignancies. Recently, Cicchillitti *et al.*^[56] evidenced an association among cfmtDNA (by qPCR), EC grading, and hypertension and inflammation markers, suggesting its role as a predictive biomarker^[57-62].

miRNA

miRNA expression

miRNAs are involved in the pathogenesis of various human cancers, such as lung, prostate, colorectal, and leukemia, as either oncogenes or onco-suppressors. The main emerging evidence of miRNAs is related to their use as biomarkers due to their higher stability in comparison to longer RNAs and their relatively high concentration in body fluids, such as serum, plasma, saliva, and urine, initiating a new era of disease research.

Circulatory miRNAs are reported mainly from monocytes, plasma, and exosomes, and they are resistant to degradation by RNase enzyme, thus stable in the blood and urine. Consequently, the potential roles of miRNAs in clinical practice are related to early diagnosis and classification of tumors, in the identification of poorly differentiated malignancies, and in the use as biomarkers potential use in the prediction of survival and response to treatment.

Recent studies have expanded our knowledge of the roles of miRNA in the pathology of gynecologic malignancies: in ovarian cancer, miRNAs participate in the development of drug resistance, while, in EC, they play essential roles in oncogenic processes, including cell proliferation, migration, and metastasis. The most critical aspect, more than in CTCs and ctDNA, is the lack of standard procedures for the analytical and pre-analytical phases (e.g., the absence of housekeeping genes for relative quantification) that leads to the identification of a broad range of miRNAs but none of them with strong confirmation for clinical use.

Tan *et al.*^[63] showed a higher expression of the serum level of miR-155 in differentiated EEC in comparison to healthy controls and associated it with cancer stage, lymph node involvement, and metastasis. Zhai obtained the same results for miR-194^[64]. Torres found that the expression of miR-99a, miR-100, and miR-199b was upregulated in plasma of EEC patients, and a combination of miR-99a and miR-199b was more accurate in distinguishing EEC disease when compared with single miRNAs^[65]. Another genome-wide serum miRNA expression profile identified a combination of four serum miRNAs (miR-222, miR-223, miR-186, and miR-204) as a fingerprint for EEC detection^[66].

Other investigations of miRNA profiling in EEC have tried to find associations between circulating miRNAs and clinic-pathological characteristics such as FIGO stage, grade, relapse, and nodal metastases. The miRNAs expression is mostly linked to that in corresponding tumor tissue^[24,67-72]. In this regard, Wang *et al.*^[73] found that miR-15b, -27a, -223, miR-3145, and miR-4638, obtained by genome-wide miRNA expression profiles, are differentially expressed in the EEC plasma between the two cohorts^[73,74].

Nevertheless, only one meta-analysis was performed on the discovered miRNAs related to EC. However, inconsistencies or discrepancies about diagnostic accuracy of circulating miR-21 remain. EC patients showed higher miR-21 expression compared with benign lesion patients^[75].

miRNA methylation

Mainly focusing on their role as biomarkers, several miRNAs have been explored for their methylation profile in tissue and their correspondent expression in serum, but it has only been confirmed for miRNA-203^[76].

Protein circulating than CA125

Only two studies investigated proteins related to EC. Tessitore *et al.*^[77] reported an increase of circulating leptin in gynecological malignancies and breast cancer related to the increase of corresponding mRNA in primary tumor tissue. This increase was related to cachexia and hormonal markers such as estrogen receptor (ER) and progesterone receptor and, only in post-menopause, to an increase in circulating estradiol.

Qu *et al.*^[78] evidenced the increase of serum epididymis protein 4 (HE4) level and suggested its use as a biomarker for the management of ovarian and endometrial cancer patients.

mRNA

Recently, specific mRNAs have been investigated in LB of EC patients. Few studies have been conducted, and a single article reports that hTERT mRNA was detected only in cancer patients in comparison to healthy subjects and that levels increased with tumor stage^[79].

Extracellular vesicles

Extracellular micro- and nano-membrane vesicles produced by different cells progressively attract the attention of the scientific community. They function as mediators of intercellular communication transporters of genetic material and signaling molecules between cells. In the context of keeping homeostasis, extracellular vesicles contribute to the regulation of various systemic and local processes. Exosomes, microvesicles, also referred to as microparticles or ectosomes, and large oncosomes were defined as actively released vesicles.

Because extracellular vesicles (EVs) contents reflect the contents of the cell of origin, multiple studies on EVs from body fluids, in the context of cancer diagnosis, prediction, and prognosis, have been performed. EV-based LB provided an overview of the main EV constituents as potential biomarkers: surface proteins, intravesicular soluble proteins, lipids, DNA, and RNAs, including mRNA (intact and fragmented), miRNA, piwiRNA (piRNA), transfer RNA (tRNA), fragments of ribosomal RNA (rRNA), long noncoding RNA (lncRNA), and circular RNAs (circRNAs)^[80].

EVs have been identified as the main mediators of cell-to-cell communication between tumor and stromal cells in local and distant microenvironments participating in the formation of the premetastatic niche prior to CTC colonization^[81]. EVs can be involved in the response to immunotherapy^[82], and, moreover, they may be used as drug delivery systems^[83]. They can be isolated from several biospecimens such as blood, urine, CSF, lymphatics, tears, saliva, nasal secretions, ascites, and semen.

Few studies have dealt with the investigation of EV levels in EC, and these studies were performed on circulating EVs. In particular, Xu *et al.*^[84] explored the role of circRNAs in EVs isolated from the serum of affected patients. They found 275 circRNAs to be differentially expressed, among which 209 were upregulated and 66 downregulated. All circRNAs have been identified thanks to wide-expression analysis, and two of them (hsa_circ_0109046 and hsa_circ_0002577) were confirmed by RT-qPCR.

An interesting article evidenced in advanced EC, via CellSearch® technology, the presence of CTCs in high-risk EC patients. Those CTCs were characterized by an EMT-expression profile correlated with the increase of EVs that have been found containing extracellular-matrix-like collagens (COL18A1), proteoglycans (VCAN, AGRN, and HSPG2), glycoproteins (TNC), constituents of the cytoskeleton (ACTG1, TUBA1B, and TUBB), integrins (ITGA3), and laminins (LAMA5). The above-cited proteins are all associated with tumor cell adhesion to the endothelium and to the promotion of their adhesion and colonization at distant sites. In particular, the authors found, by means of targeted proteomics, that the adhesion protein

LGALS3BP was significantly enriched in circulating EVs from an EC patient cohort with a high risk of recurrence^[81].

Platelet

Platelets contain many different RNA species including miRNAs, circRNAs, and mRNAs that are altered in cancer. No studies regarding platelets have been performed on EC^[85].

URINE

miRNA

The improvement of high throughput technologies such as NGS and qPCR allowed the evaluation of specific biomarkers, such as cell-free miRNAs, in urine^[86] and methylation profile of cell free tumor^[87].

A study reported a specific downregulation of miR-106b, as well as in serum and plasma samples, in comparison with healthy donors^[24].

Recently, Ye reported the identification of several miRNA involved in Grade 3 EC (miR-9, miR-92a, miR-99a, miR-100, miR-199b, miR-1228, miR-9, miR-1228, miR-9, miR-92a, miR-21, miR-222, miR-223, miR-186 and miR-204, miR-203, miR-21, miR-887-5p, miR-106b, and miR-200c-3p)^[88].

UTERINE/PERITONEAL LAVAGE CYTOLOGY

Surgical staging of gynecologic neoplasms, mainly in ovarian cancer, include the collection of peritoneal washings coming from the abdomen and pelvis. The aim of taking peritoneal washings is to identify occult disease. Peritoneal cytology is supposed to add information on the spread of microscopic peritoneal disease. However, the peritoneal washing cytology examination may give false positivity in benign diseases and false negativity in the early stages.

Less is known about the prognostic impact of peritoneal cytology in EC and published data show inconsistent results, mainly in relation to the prognostic importance of positive cytology to predict relapse or metastasis^[89]. Recently, the use of cytology PAP-test, usually performed for cervical cancer evaluation, has been proposed to analyzed endometrial cancer, in particular the liquid cytology approach (LC)^[90]. This procedure is worth mentioning in this overview, even if this procedure is not a true LB but a procedure in between biopsy and LB, mainly because the collection procedure is minimally invasive and allows collecting tumor cells derived from gynecological districts. After uterine lavage with saline solution, the collection of cancer cells can allow identifying genetic variations related to endometrial cancer by the washing of the uterine cavity. The data suggest that LC is a feasible and reproducible adjuvant method for screening endometrial lesions and in combination with classical biopsy can improve the diagnostic accuracy of endometrial lesions^[90-94].

To identify other biomarkers for implementing cytology, recent studies demonstrated the role of IL-11 protein in uterine fluids related with the amount in endometrial tumor epithelial cells in women with grade 1 EC^[95]. In a case report, in cells deriving from uterine lavage, mutations on *PTEN*, *TP53*, *PIK3CA*, *PIK3R1*, *KRAS*, *CTNNB1*, *FGFR2*, *RNF43*, *PPP2R1A*, *POLE*, *APC*, and *FBXW7* were observed; these are genes that are some of the most frequently mutated in EC^[24,96-98]. In addition, exosome contents, mainly referring to miRNAs, showed that 114 miRNAs (by mRNA array) were significantly dysregulated in EC patients. Eight miRNAs (miRNA-383-5p, miRNA-10b-5p, miRNA-34c-3p, miRNA-449b-5p, miRNA-34c-5p, miRNA-200b-3p, miRNA-2110, and miRNA-34b-3p) demonstrated classification performance according to the area under the receiver operating characteristic curve^[99].

lncRNA AND circRNA AND OTHER small nuclear/nucleolar RNA IN LB

There are currently no studies available relating other circulating RNAs, namely lncRNA, piRNA, small nuclear (snRNA), and small nucleolar (snoRNA), to EC. The last three classes are also known as snc/snRNAs due to the fact that they are located in the cell nucleus and are fundamental in RNA-RNA remodeling, spliceosome assembly, and translation processes (i.e., post-transcriptional modification of rRNA). The class of snoRNAs named “U(n)” (i.e., U1 and U2-U12), due to their high Uridyl content, is involved in the spliceosome complex. These particular RNAs are 60-300 nt long and are transcribed from intronic sequences of coding and noncoding genes. Some of them are used to normalize the relative quantification of miRNA in tissues and fluids. Although they do not properly represent “housekeeping” due to their change and different pre-analytical phase, no specific studies have been conducted on these biomolecules to evaluate their possible function as biomarkers^[88].

Concerning lncRNAs, they are involved in chromatin remodeling, gene expression and transcription, and protein-protein interaction, and their role in cancer is known with respect to snRNAs. Since this is a new field, for EC, pilot studies concerning circulating lncRNA are only relative to colon cancer and their prospective use^[100].

Noteworthy, a peculiar class of lncRNA is represented by circRNAs characterized by a covalent linkage, which gives them a specific circular form and makes them biologically stable and resistant to RNases. They were recently investigated in several cancer tissues including EC. A recent paper highlights their differential display in a pilot study aiming to analyze three samples of EC in comparison to corresponding adjacent non-cancerous tissue and focused on has-circ_0039569, which has been significantly correlated with tumor differentiation^[88].

Recently, circRNAs - hsa_circ_0109046 and hsa_circ_0002577 - were suggested as potentially investigable biomarkers in LB of EC^[101].

ROLE OF LB IN THE MONITORING AND TREATMENT OF ENDOMETRIAL CANCER

Patients with EC progression after first-line chemotherapy have a poor prognosis. Until now, no targeted therapies are in use for ECC treatment, although several phosphoinositide 3-kinase inhibitors are under investigation and in clinical trials. As for lung cancer, the evaluation of specific circulating mutations may give information concerning the success of the therapy and related to the development of specific molecular resistance or new clones^[102]. Several studies have found that the genotyping results derived from tissue biopsy analysis differ from those derived from LB. The concordance rates for metastatic cancer patients and for patients with primary tumors were 83.3% and 78.3%, respectively. These discrepancies may be related to intra-tumor heterogeneity, indicating that the assessment on tissue can cause misinterpretations, while LB may reflect mutations and changes occurring in tumor that cannot be revealed in the primary biopsy. Thereby, LB may offer new prospective for monitoring tumor development, the efficacy of therapy, and the arise of treatment resistance^[36].

CONCLUSION

In 2013, the Cancer Genome Atlas Research Network (TCGA) suggested implementing a classification with molecular characterization^[10], which can be useful to define targeted therapy and monitor cancer development and treatment; nevertheless, today, there are no targeted therapies: in fact, there is no molecular target for treatment, detection, or monitoring^[11]. LB represents a novel tool, due to the minimally invasive- or non-invasive procedure for biomarker collection, overcoming the limit of classical tissue biopsy and allowing the monitoring of tumor burden, the efficacy of therapy, the arise of resistance, and the development of cancer change, relapse, and metastasis.

As discussed in this review, the investigation of biomarkers in LB of EC evidenced that only experimental approaches have been proposed, and none of them have reached clinical application. In particular, most of the results concerning LB in EC are referred to circulating cell free DNA/ctDNA and CTCs in blood. This fact may be related to the statement that most ECs have a good prognosis and are diagnosed at early stage, and thus little effort has been made in this direction despite the improvement of new technologies (e.g., NGS and ddPCR) and new isolation systems for emerging biospecimen for LB in cancer detection (e.g., CTC and EV). The overview of LB biomarkers in EC shows that blood is the most investigated biospecimen and the main biomolecules are miRNA and ctDNA. Nevertheless, for those biomarkers evaluated in EC, some discrepancies were observed among studies, mainly related to the lack of standard procedures for pre-analytical and analytical phases. This aspect plays a critical role in EC, principally due to the limited number of studies or patients recruited within each study performed on this topic. Nevertheless, to underline the efforts performed in this direction, in this review, we present, albeit isolated, studies that for the first time performed analyses of specific biomarkers in LB of EC (e.g., circulating mRNA in blood) and we provocatively suggest to extend them to biomarkers (lncRNA, methylation of circulating miRNA, and single CTC analysis) not yet analyzed in EC but investigated in other cancers. The purpose is to suggest that LB of EC represents a fascinating tool and field of study to improve the knowledge of this type of tumor and to improve the life-quality of patients.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the study: Malentacchi F, Pillozzi S

Data research: Malentacchi F, Sgromo C

Revision of the manuscript: Antonuzzo L

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

Not applicable.

Consent for publication

Not applicable.

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Review

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The good and bad sides of exosomes: pre-metastatic niche formation, cancer biomarker and therapy carriers

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How to cite this article: Panfoli I, Bruschi M. The good and bad sides of exosomes: pre-metastatic niche formation, cancer biomarker and therapy carriers. *J Cancer Metastasis Treat* 2020;6:35. <http://dx.doi.org/10.20517/2394-4722.2020.50>

Received: 25 May 2020 **First Decision:** 18 Jun 2020 **Revised:** 2 Jul 2020 **Accepted:** 21 Jul 2020 **Published:** 27 Sep 2020

Academic Editor: Robert J. Griffin **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Exosomes, nanovesicles of endocytic origin, are secreted by most cell types; cancer cells representing no exception. Exosomes facilitate intercellular communication as they deliver diverse proteins, mRNA, miRNA and lipids. In this review, we discuss how exosomes represent one of the main risks associated with cancer but also one of the most promising new tools to fight it. Exosomes appear to function as signalling molecules between the tumour microenvironment, i.e., the complex of both cancer and stromal cells, and the rest of the body. Cancer-derived exosomes have been shown to drive the initiation and progression of metastasis, by transporting their cargoes to target tissues. In this respect, exosomes are implicated in cancer progression, dissemination and therapy resistance. However, exosomes are also emerging as a key tool in precision medicine, pivotal for cancer liquid biopsy in early diagnosis and for assessing when there is a recurrence. Profiling exosomal cancer-derived nucleic acids by ultrasensitive next-generation sequencing along with mapping the protein profile utilizing high-throughput proteomics will allow earlier cancer detection, therapeutic stratification and monitoring of response to therapy. Exosomes are also a promising new tool for cancer immunotherapy. Clinically utilizing exosomes for these applications in cancer diagnosis and therapeutics will be the next challenge.

Keywords: Biomarkers, cancer, exosomes, immunotherapy, liquid biopsy, precision medicine, proteomics



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INTRODUCTION

Exosomes (Exo) are nanovesicles secreted by virtually any cell and found in all human body fluids^[1-4]. They play important roles in intercellular communication both in physiology and disease^[4-6]. After being released into the extracellular space, Exo can enter the circulation and ultimately be taken up by recipient cells to which they can convey their protein, nucleic acid and lipid cargo in turn modulating their activity^[7,8]. By proteomic and biochemical analyses, we have reported a novel characteristic of human Exo, i.e., they can consume oxygen to aerobically synthesize ATP^[9-11]. Proteomic and statistical analyses of both human mesenchymal stem cell (MSC) and urinary Exo showed enrichment of proteins related to aerobic metabolism, including the redox complexes I, IV, and V in their membranes^[9-11]. Such aerobic metabolic capacity appears consistent with the Exo need to maintain their cup-shaped structure and functionality in the face of a prolonged permanence in the circulation^[10-12] and with their ability to rescue bioenergetics of damaged cells^[13,14].

Research in the field of cancer is benefiting from the growing understanding of the biogenesis and functions of Exo released from cancer cells^[15-18]. However, the role of Exo in the pathophysiology of cancer is multifaceted^[19]. Cancer cell-derived Exo have long been known to be involved in processes such as cancer progression, metastasis, immune escape, angiogenesis and therapy resistance^[20,21]. Exo function as signalling molecules between the tumour microenvironment, i.e., the complex of both cancer and stromal cells, and the rest of the body^[16]. Tumour-derived Exo can drive the metastatic process by targeting specific organs setting a pre-metastatic environment^[17]. The tumour environment reacts by amplifying oncogenic pathways in which Exo signalling is proposed to play a role^[22].

Nonetheless, Exo also contain biomarkers, and can be considered biomarkers themselves^[23,24]. For example, urinary Exo deriving from every cell of the urinary tract and kidney, may represent a promising reservoir of cancer biomarkers to assess disease progression in urologic cancers^[25,26]. Exo can be noninvasively isolated from human urine to detect biomarkers, with promising applications in patient stratification, monitoring of therapy response or use as vehicles for therapeutic delivery^[27].

Thanks to high throughput mass spectrometry techniques, it can be envisioned that Exo will help in earlier diagnosis, directing therapeutic targets and assessing therapy resistance upon detection of signature cargo biomarker biomolecules^[1,16]. In the landscape of precision medicine, cancer liquid biopsies, i.e., the analysis of circulating cell-free tumour DNA and tumour cells (CTC)^[28], can exploit examining Exo signatures, when they are derived from biological fluids^[29]. Exo can also potentially be used as tumour-targeting vehicles for cancer^[17,18,21]. There is also the exciting possibility of engineering the content of Exo and targeting them to tissues of interest^[30,31].

This review focuses on the dual role of Exo in cancer. The good side of Exo is their clinical potential. The ability of Exo to mediate cell-to-cell communication as stable carriers of molecular messages can be exploited for cancer diagnosis and therapy, with novel exciting perspectives of translation to the clinical setting^[16,32]. The bad side of Exo is the long-recognized ability of their cargo to modify the cancer microenvironment promoting cancer progression and metastasis by recruiting target cells, as is the case for melanoma^[33].

EXOSOMES

Exo, a subset of extracellular vesicles (EVs) of endocytic origin, actively shed from multivesicular bodies (MVBs) which carry proteins, RNAs (mRNA, miRNA, transfer RNA, ribosomal RNA, nucleolar RNA, and noncoding RNA) and DNA fragments^[1,3,7,34]. Exo biogenesis involves the formation of early endosomal vesicles, that develop into late endosomes undergoing inward budding forming intraluminal vesicles

(ILVs) in MVBs^[6,35]. Exo acquire their luminal and membrane contents through both direct and stochastic sorting^[6]. If nascent ILVs are not degraded by fusion with lysosomes, they fuse with the plasma membrane and are released into the extracellular space^[36]. Once in the circulation, Exo are directed to other cells via an unclear mechanism, probably involving members of the tetraspannin family (CD9, CD63 and CD81), where they are taken up by the target tissues through membrane fusion, endocytosis, or receptor-ligand interaction^[2,34,37,38].

Exo isolation involves a variety of techniques^[39,40]. Several commercial exosome isolation kits are in use, although their ability to yield pure and functional Exo is uncertain^[41]. To date, differential ultracentrifugation is considered the gold standard, and it is much preferred when pure Exo are required. Low-speed centrifugation subsequent to polyethylene glycol precipitation has been utilized to enrich Exo from large sample volumes, but there is the possibility of polymer contamination^[42]. The other Exo isolation methods are size-based, antibody capture-based, microfluidics-based techniques or precipitation by altering Exo solubility with water-excluding polymers such as polyethylene glycol, all of which do not require high-speed centrifugation^[40]. Fluidic techniques such as exosome total isolation chip, can sort Exo from a heterogeneous population of EVs based on their size, with the advantage of allowing the handling of small sample volumes^[43]. Label-free efficient separation of Exo from human blood has been reported, using an integrated continuous-flow acoustofluidic device^[44]. This last automated isolation technique is useful in case of biohazard, and for integration with downstream Exo analysis systems^[44]. Notably, most novel isolation methods are limited by the requirement of sophisticated instrumentation or costly reagents. Therefore, notwithstanding their promising potential, a concern about the clinical applications of Exo appears to be the standardization of isolation techniques.

THE GOOD SIDE OF EXOSOMES

Exo are emerging as promising and sensitive cancer biomarkers for disease diagnosis^[21,23,24,26]. Once released from cancer cells, Exo enter the circulation and are transported in biological fluids carrying their functional cargoes^[1]. Profiling Exo cancer-derived nucleic acids by ultrasensitive next-generation sequencing and proteins by large-scale high-throughput proteomics could allow early cancer detection, therapeutic stratification and response to therapy monitoring^[17,18,38,45,46].

Liquid biopsy has drawn attention as a minimally invasive and cost-effective method for sampling of genetic, proteomic and metabolic material from different types of cancer^[47]. While traditional cancer liquid biopsies utilize cell free DNA or CTC^[48], Exo are emerging as a novel tool in the field of precision medicine in the early diagnostic and recurrence assessment applications^[24,49]. This has proven true especially for urologic tumours^[29,50]. The advantage of Exo over existing approaches is their stability in body fluids allowing the specific cancer-derived proteins and nucleic acids to be preserved^[1,4]. For example, a cell membrane-anchored proteoglycan, glypican-1 (GPC1), overexpressed in a variety of cancers such as breast and pancreatic cancer, was also identified in the circulating Exo from the serum of pancreatic cancer-bearing patients. Detection of GPC1⁺ Exo in the serum can be utilized to distinguish healthy subjects from pancreatic cancer patients with very high specificity and sensitivity, findings that can then be correlated with survival rates^[51]. Considering its cited role in the progression of human pancreatic cancer, miR-301a-3p was proposed as a biomarker in the diagnosis of this type of cancer^[52]. An Exo-specific “melanoma signature” with prognostic and therapeutic potential has been proposed^[33].

Exo can also become stable and reliable engineered nanocarriers^[23]. In fact, the benefits of the use of Exo as therapeutic agents include their biocompatibility and ability to deliver their content to specific target cells, an unfavourable feature *per se*, that can however be of advantage. The characteristic exosomal membrane stability, which is enriched in cholesterol, sphingomyelin and ceramide, along with its tumour-targeting capacity, has suggested the use of Exo in anticancer therapy delivering therapeutic miRNAs

and proteins or as drug delivery system for cancer therapy^[53]. Drug delivery with Exo has been utilized for the treatment of breast, pancreatic, lung, and prostate cancers and glioblastoma^[54]. Drug loading into Exo can be accomplished by both active and passive encapsulation approaches^[55,56]. Passive loading can be accomplished by simple incubation of the Exo with the drug or miRNAs to let them diffuse along the concentration gradient or treating cells with a drug, and using Exo secreted by these donor cells. Active encapsulation can be performed by freeze-thawing cycles, electroporation of the Exo membranes, or sonication in the presence of the drug^[56]. Sonication can be also used to encapsulate proteins. Freeze-thawing method has also been utilized to fuse Exo with liposomes, to develop exosome-liposome hybrids carrying specific proteins^[57]. By manipulating the shedding process of Exo, these can also be engineered using viral vectors, such as retroviruses and adenoviruses to engineer the parent cells to secrete modified Exo, or alternatively, to directly manipulate the content Exo following secretion^[30,56]. The use of Exo also shows significant safety and low toxicity benefits as compared to cell-based therapies, which bear the risk of activation of a host immune response. By contrast, allogenic Exo elicit lower immune response^[30]. The use of MSC-derived Exo for delivery of anticancer therapy was shown to hold more promise^[58] than the use of MSC themselves^[59]. The fact that MSC Exo carry an aerobic metabolic ability^[11] may also be taken into closer consideration.

Exo are also a promising new tool of cancer immunotherapy^[15,60]. In lieu of dendritic cell (DC)-based cancer immunotherapy, still in its infancy due to problems associated with culture and storage, the use of DC-derived Exo to generate immune responses against tumours, has been proposed^[31]. DC-derived Exo possess immunostimulatory properties thanks to the expression of integrins, ICAM-1 and MHC class II and class I molecules as well. Exo stability would maintain the DC-derived Exo cancer antigen composition, including surface MHC-peptide complexes and the CD80 and CD86 costimulatory molecules, thus triggering the desired cancer antigen-specific immune response^[31]. DC-derived Exo-based phase I and II clinical trials have demonstrated their ability to target cancer sites^[61].

By genetic modification of the parental cell, Exo can be loaded with a variety of biological molecules, such as miRNAs and proteins. Exo surface modification by addition of ligands or proteins to the Exo surface can direct them to target cancer cells^[32]. In this respect, the features that allow Exo to reach their targets (i.e., ligands expressed onto their surface) allow their positive applications. Targeting ligands on the surface of Exo can also be engineered. For example, HEK293T cells were engineered to express the protein Lamp2b, fused to a fragment of interleukin 3 in their Exo membrane, and it was found that these Exo loaded with imatinib or with a specific siRNA were able to target cancer cells and inhibit their growth *in vitro* and *in vivo*^[62]. Exo released by macrophages, loaded with paclitaxel (PTX) increased the cytotoxicity of PTX by about 50-fold. *In vivo*, those Exo co-localized with lung metastases causing a significant inhibition of their growth^[63].

The hallmark of the double nature of Exo is their ability to deliver miRNA, small noncoding RNAs that target mRNAs and change their expression. Exo can be manipulated to have them deliver anti-miRNAs, or tumour-suppressing miRNAs for cancer treatment. It was shown that endothelial cells can transfer exogenous miRNA to cancer cells via Exo^[64].

THE BAD SIDE OF EXOSOMES

Cancer cells secrete more Exo than healthy cells^[65]. Moreover, their contents appear to be tailored to mediate cancer dissemination^[8,16,17,23]. Being potent signalling mediators, Exo shed from cancer cells act in facilitating cancer initiation and progression^[20,21]. Exo are also secreted by cells in the tumour microenvironment (TME) such as stromal and immune cells^[22,66]. TME is the complex setting of the interplay between cells and signalling events, of which Exo along with cytokines and chemokines are key players^[8]. In particular, Exo can precondition the TME setting the pre-metastatic niche, which increases

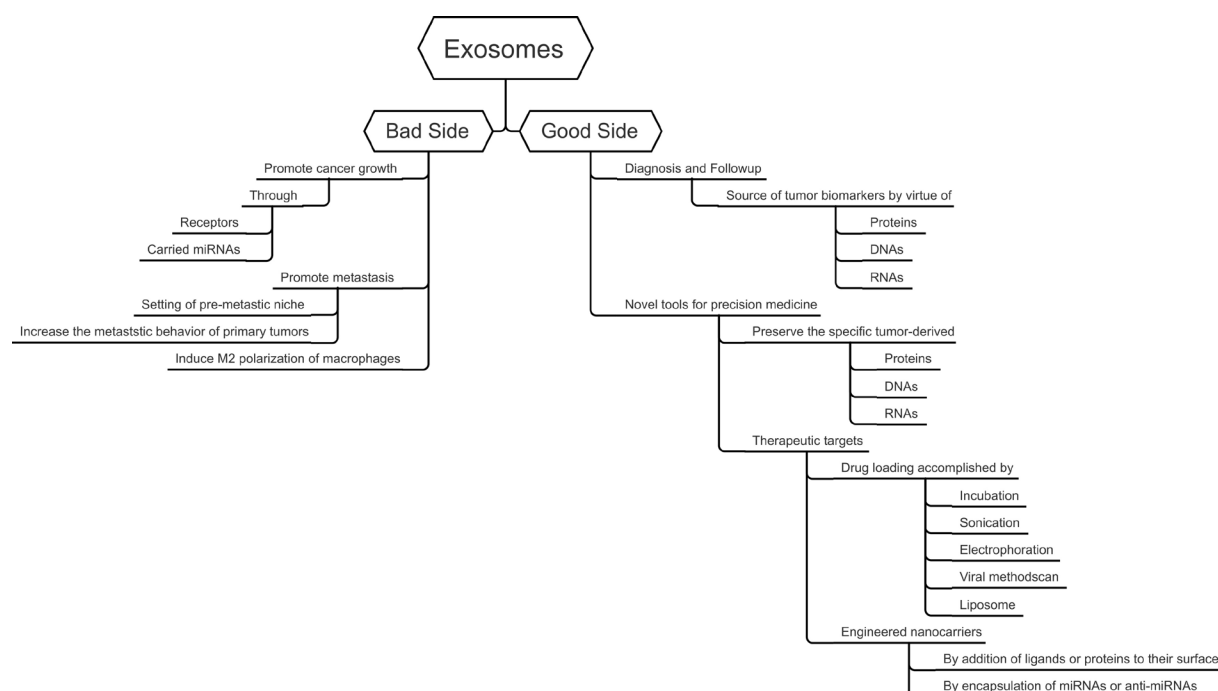


Figure 1. Schematic graphical representation of the “good” and “bad” sides of exosomes

metastatic success^[16]. Evidence suggests that the contents of Exo modulate immune cell activity, enabling immune surveillance and treatment evasion. For example, Exo were shown to express programmed death-1 (PD-1), which interacts with programmed death-ligand 1 (PD-L1)^[61]. Upregulation of the expression of PD-L1 receptor on T cells mediates PD-L1-dependent immune evasion, by actively suppressing the function of CD8. It was shown that exosomes expressing PD-L1 shed from metastatic melanomas helping the tumour to evade immune surveillance. Assessment of exosomal PD-L1 content has been proposed to stratify patients for therapy with anti-PD-1 antibodies, a promising treatment for metastatic melanoma^[61]. While remaining a poorly understood process, metastasis is the cause of most cancer-related deaths with miRNA transfer to endothelial cells, mediated by Exo, facilitating this metastatic process^[16].

Exosomal mRNA and proteins from prostate cancer cells were shown to modulate the prostatic TME^[21,67]. The formation of melanoma lung metastases is preceded by the recruitment of bone marrow progenitors primed through receptor tyrosine kinase MET activation by Exo^[33]. Pancreatic cancer cell-derived Exo can induce stellate cells to recruit macrophage subpopulations, establishing a pre-metastatic environment inside the liver^[17,33]. Pancreatic cancer cell Exo promote metastasis by inducing the M2 polarization of macrophages through activation of the PTEN/PI3Kg pathway^[52]. It was shown that Exo miR301a-3p overexpression is associated with poor survival^[52].

Given that Exo have “good” and “bad” roles (see Schematic in Figure 1), a prerequisite for the successful implementation of their use for cancer therapy requires rigorous isolation, and characterisation. In an attempt to support the standardisation of Extracellular vesicles (EV) in research and clinical applications, an international consortium was established, named “EV-TRACK” (<http://evtrack.org>). EV-TRACK is a knowledgebase intended to gather and centralize reports on EV biology and methodology^[68].

CONCLUSION

Exo are nano-sized vesicles secreted from living cells into all body fluids. They bear a dual role in cancer biology, being supportive of cancer progression, by setting the tumour metastatic niche, but are also

carriers of cancer cell-specific protein and nucleic acid contents. Exo are viewed as promising tools for cancer diagnosis and therapy. We highlight how current research is exploiting both these utilities and the strategies used to enhance the potential of Exo in the treatment of cancer. If understood in more detail, the identification of cancer-specific Exo would become vital in early diagnosis and assessing response to therapeutic intervention(s) when monitoring cancer progression and its treatment. The unique stability, biocompatibility, homing ability and low immunogenicity of Exo, can also be exploited as a delivery system for therapeutic agents since Exo can also be loaded with a variety of molecules. As our understanding of Exo biology and the profiling of their protein and RNA cargoes as potential biomarkers is expanded, the ability to engineer content of Exo opens new possibilities for their use as selective carriers of therapeutic miRNAs and/or drug carriers to target tumour cells. It is foreseeable that the next years will witness the challenging clinical applications of Exo in cancer diagnosis and therapeutics.

DECLARATIONS

Authors' contributions

Made substantial contributions to the conception and design of this study and wrote the manuscript: Panfoli I

Revised this work substantially: Bruschi M

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

Not applicable.

Consent for publication

Not applicable.

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Review

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Epithelial-mesenchymal transition: a hallmark in pancreatic cancer stem cell migration, metastasis formation, and drug resistance

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How to cite this article: Safa AR. Epithelial-mesenchymal transition: a hallmark in pancreatic cancer stem cell migration, metastasis formation, and drug resistance. *J Cancer Metastasis Treat* 2020;6:36. <http://dx.doi.org/10.20517/2394-4722.2020.55>

Received: 1 Jun 2020 **First Decision:** 24 Jul 2020 **Revised:** 18 Aug 2020 **Accepted:** 9 Sep 2020 **Published:** 27 Sep 2020

Academic Editor: Godefridus J. Peters **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Metastasis, tumor progression, and chemoresistance are the major causes of death in patients with pancreatic ductal adenocarcinoma (PDAC). Tumor dissemination is associated with the activation of an epithelial-to-mesenchymal transition (EMT) process, a program by which epithelial cells lose their cell polarity and cell-to-cell adhesion, and acquire migratory and invasive abilities to become mesenchymal stem cells (MSC). These MSCs are multipotent stromal cells capable of differentiating into various cell types and trigger the phenotypic transition from an epithelial to a mesenchymal state. Therefore, EMT promotes migration and survival during cancer metastasis and confers stemness features to particular subsets of cells. Furthermore, a major problem limiting our ability to treat PDAC is the existence of rare populations of pancreatic cancer stem cells (PCSCs) or cancer-initiating cells in pancreatic tumors. PCSCs may represent sub-populations of tumor cells resistant to therapy which are most crucial for driving invasive tumor growth. These cells are capable of regenerating the cellular heterogeneity associated with the primary tumor when xenografted into mice. Therefore, the presence of PCSCs has prognostic relevance and influences the therapeutic response of tumors. PCSCs express markers of cancer stem cells (CSCs) including CD24, CD133, CD44, and epithelial specific antigen as well as the drug transporter ABCG2 grow as spheroids in a defined growth medium. A major difficulty in studying tumor cell dissemination and metastasis has been the identification of markers that distinguish metastatic cancer cells from cells that are normally circulating in the bloodstream or at sites where these cells metastasize. Evidence highlights a linkage between CSC and EMT. In this review, The current understanding of the PCSCs, signaling pathways regulating these cells, PDAC heterogeneity, EMT mechanism, and links between EMT and metastasis in PCSCs



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are summarised. This information may provide potential therapeutic strategies to prevent EMT and trigger CSC growth inhibition and cell death.

Keywords: Pancreatic cancer, cancer stem cells, epithelial-mesenchymal transition, metastasis, drug resistance

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related death in the world with a low probability of early diagnosis^[1]. KRAS, CDKN2A, TP53, and SMAD4 are frequently mutated genes that define the genetic landscape of PDAC. PDAC is one of the most lethal cancers due to its high metastatic potential and delayed detection^[2,3]. The median survival time following diagnosis remains at less than 6 months, with an overall survival rate of less than 4%^[1-3]. Gemcitabine (GEM) treatment has only increased the median survival of PDAC patients from 3-4 months to 6-7 months^[4-6]. Recent evidence shows that using FOLFIRINOX (leucovorin, fluorouracil, irinotecan, oxaliplatin) in PDAC patients was more effective than GEM as it demonstrated longer survival in pancreatic cancer patients (11.1 months vs. 6.8 months)^[6,7]. Resistance to apoptosis is a common feature of PDAC and a major reason why this devastating disease is resistant to various treatment strategies including GEM and FOLFIRINOX^[8]. Another major problem limiting our ability to treat PDAC is the existence of rare populations of pancreatic cancer stem cells (PCSCs) or cancer-initiating cells in pancreatic tumors; PCSCs may represent sub-populations of tumor cells resistant to therapy which are most crucial for driving invasive tumor growth^[9-13]. The PCSCs express a wide array of markers such as CD44, CD24, epithelial specific antigen (ESA), CD133, c-mesenchymal to epithelial transition (c-MET), CXCR4, PD2/Paf1, and ALDH1^[14-17]. These cells are capable of regenerating the cellular heterogeneity associated with the primary tumor when xenografted into mice^[13-17]. Therefore, the presence of PCSCs has prognostic relevance and influences the therapeutic response of tumors.

Metastasis is the major cause of high PDAC mortality. As Mu *et al.*^[15] recently described, tumor progression is driven by the cross-interaction between tumor cells, primarily cancer stem cells (CSCs) (or cancer-initiating cells) and surrounding stromal cells as well as distant organs, in which tumor-derived extracellular vesicles (TEX) play a major and important role. Mu *et al.*^[15] report that the PCSC markers Tspan8, alpha6beta4, CD44v6, CXCR4, LRP5/6, LRG5, claudin7, EpCAM, and CD133, participate in a metastatic cascade at various steps, often via PDAC CSC-TEX.

In this review, the models of CSCs in PDAC and their cell-intrinsic and - extrinsic regulatory pathways are described. Insights into the heterogeneity of cell sub-populations of PDAC, plasticity, cancer stemness, and the involvement of epithelial-mesenchymal transition (EMT), which participates in metastasis are highlighted. These properties may account for the unsuccessful clinical trials that test therapeutics designed to directly target CSCs.

PCSCS

CSCs from epithelial tissues were first identified in breast cancer in 2003 by Al-Hajj *et al.*^[18], who found that a distinct sub-population of cancer cells expressing CD44+CD24-/low ESA+ develop into tumors in immunodeficient mice. In PDAC, the presence of CSCs was reported in 2007 by Shah *et al.*^[19] who demonstrated that CD44+CD24+ESA+ cells exhibit high tumorigenic potential.

Two models are proposed to describe the origin of CSCs [Figure 1] and their role in tumorigenesis^[20-22]. The first one is the hierarchical model in which the CSCs represent a small distinct sub-population within the tumor with capacity for self-renewals and also the ability to differentiate into progeny cells; the progeny

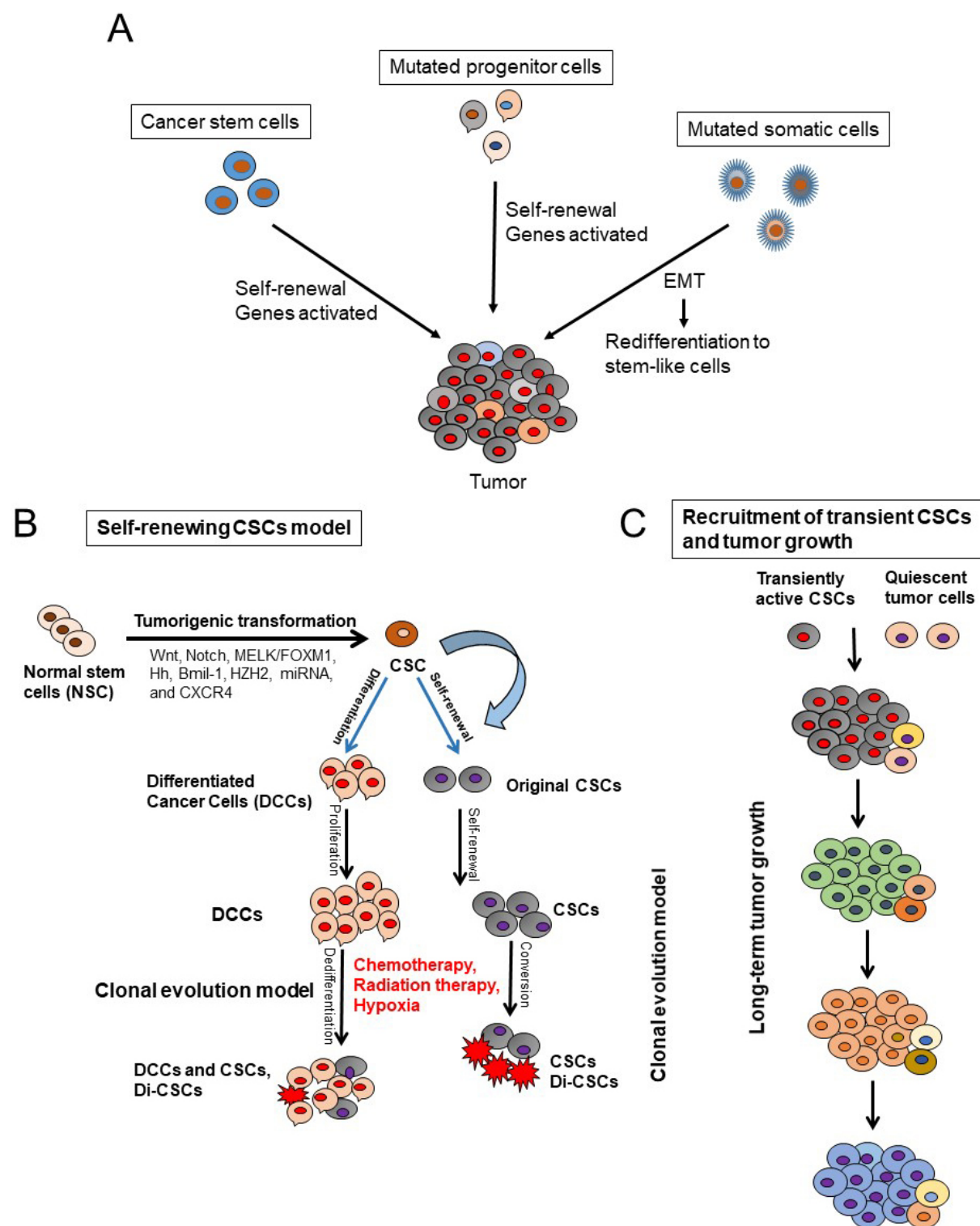


Figure 1. Models that explain pancreatic cancer tumor development and roles of cancer stem cells (CSCs) in tumor initiation and progression. A: stochastic model shows that every cell has the potential to be the tumor initiator; B: CSC model shows that CSCs originate from normal stem cells (NSCs) through mutations and tumorigenic transformation of several potential pathways including Hh: hedgehog, epithelial-to-mesenchymal transition (EMT), and the reverse process mesenchymal-to-epithelial transition (MET). CSCs are also generated by de-differentiation of differentiated malignant cells by chemotherapeutic agents, ionizing radiation, and hypoxia. CSCs and drug-induced CSCs (Di-CSCs) are enriched following conventional chemotherapy treatment; C: the model of long-term tumor growth in PDAC states that a succession of CSC or tumor initiating cells (TIC) clones and drives tumor progression in serial xenotransplantation. Individual TICs contribute to tumor formation transiently and generate mainly non-tumorigenic progeny expressing little or no self-renewal

cells then proliferate to initiate and sustain tumor development and progression. Therefore, this model indicates that in the clinical setting, eradication of CSCs may prevent tumor recurrence.

The second model is stochastic model in which every cell within a tumor is capable of promoting tumor initiation and progression^[21]. Furthermore, the heterogeneity within the tumor cell population is due to accumulated genetic mutations^[21]. These CSCs models may express plasticity in the presence of tumor microenvironmental stimuli including oxidative and nutritional stress, low oxygen tension, and cytotoxic drugs to which the tumor can be subjected to^[16,21-25]. These factors influence the inter-conversion of a non-CSC population to a CSCs^[21,22] [Figure 1]. Previous studies have demonstrated that extracellular matrix components like collagen can influence clonogenic tumor growth, tumor initiation, and invasion of PDAC due to activation of the FAK signaling pathway^[26,27]. Additionally, a hypoxic tumor microenvironment is known to promote the conversion of non-CSC to CSC populations in PDAC^[8]. These data corroborate with observations that the CSCs within the tumor are the cells' survival advantage, capable of living under stress conditions and to express resistance to cancer therapies.

Accumulated evidence obtained through large-scale genomic studies and single cell RNA sequencing analysis has indicated the existence of CSCs in hematological malignancies and solid tumors including PDAC, breast cancer, malignant glioma, prostate cancer, non-small cell lung cancer, colorectal cancer, and hepatocellular carcinoma^[28-37]. Substantial *in vitro* and *in vivo* studies have provided convincing evidence supporting CSCs as the critical cause of cancer initiation, growth, metastasis, and treatment resistance in cancers^[20-24]. CSCs were also found to exhibit characteristics of an EMT, a process known to enable cancer cell dissemination and metastasis to other organs^[35-41]. Additionally, significant and convincing evidence shows that inducing EMT in cancer cells confers cancer stem cell-like characteristics, which promote their metastatic and tumor-initiating abilities^[37,38]. While PCSCs self-renewal is essential in the progression, migration, and metastasis of PDAC, clonal evolution and plasticity of PCSCs within PDAC tumors are not well understood^[38-46]. In this regard, Ball *et al.*^[47] showed that long-term progression of PDAC in serial xenotransplantation happens by a succession of transiently active PCSCs producing tumor cells in a temporally restricted manner with little overlap between subsequent xenograft generations. Therefore, the clonal PCSC activity in PDAC differs from the continuous activity of limited numbers of self-renewing PCSCs with a defined and rigid cellular hierarchy. Indeed, PCSCs heterogeneity and plasticity make therapeutic targeting of PCSCs very difficult and challenging^[24]. Furthermore, several investigations have suggested that CSCs exist in dynamic equilibrium with more differentiated cancer cells via bi-directional regeneration or interconversion of differentiated cancer cells to CSCs, caused by various factors^[23,24,48,49,50].

A distinct characteristic of PDAC is its desmoplasia, consisting of a significant amount of cancer-associated fibroblasts (CAFs) and a very dense fibrotic stroma^[51] [Figure 2]. The CAFs are pro-inflammatory due to activation of several signaling factors including nuclear factor kappa B (NF- κ B), signal transducer and activator of transcription (STAT)-1 and STAT-3, and transforming growth factor (TGF)- β /SMAD^[51-54]. These signaling factors cooperate in active cross-talk with cancer cells through paracrine signaling factors including chemokines, insulin-like growth factor, and proteases^[52-56]. Furthermore, several pro-stemness paracrine factors are secreted by distinct CAFs^[56-62] and support the self-renewal and the stemness properties of initial PCSCs in tumors or promote the conversion of cancer cells into PCSCs^[63]. Additionally, chemotherapy (e.g., GEM), can affect CAFs in PDAC, which then acquire a senescence-like secretory phenotype and increase the production of pro-stemness chemokines which enhance tumorstemness and aggressiveness of PDAC after therapy^[13]. It is known that CAFs consist of a heterogeneous population, with specific functions within tumors and in the process of metastasis^[64].

Interestingly, besides CAFs, the PDAC stroma also contains bone marrow-derived mesenchymal stem cells (MSCs)^[65]. The MSCs significantly contribute to tumor progression and promote cancer stemness

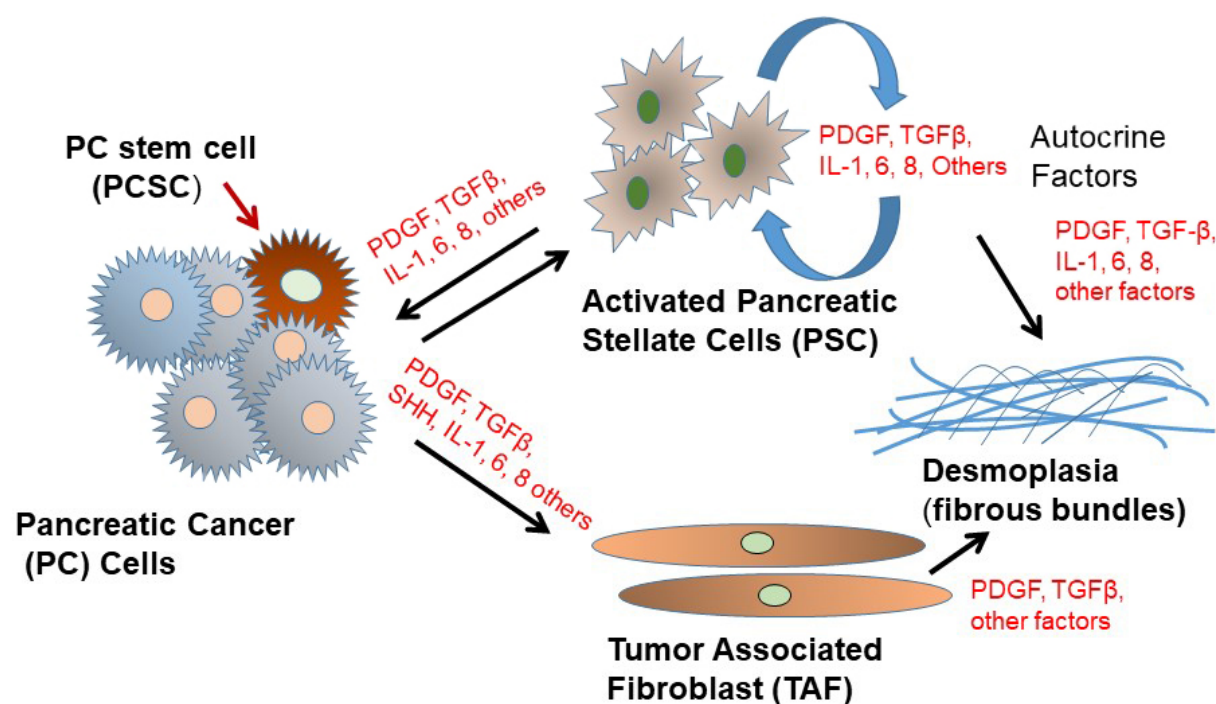


Figure 2. Schematic depiction of pancreatic cancer cell interactions with tumor-associated fibroblasts (TAF) and activated pancreatic stellate cells (PSC) in the tumor microenvironment. PDGF: platelet-derived growth factor; TGFβ: transforming growth factor β

by secreting pro-stemness cytokines, chemokines, and growth factors or by differentiating into pro-stemness CAFs^[62,63,66]. MSCs also produce pro-stemness niches in the stroma of PDAC, and infiltrating immune cells produce pro-stemness factors that form pro-PCSC niches^[67-70]. Waghray *et al.*^[71] identified and characterized mesenchymal stem cells (MSC) within the human PDAC tumor microenvironment (TME). These cancer-associated MSCs (CA-MSCs) increase the growth, invasion, and metastatic potential of PDAC cancer cells^[71], and CA-MSCs secrete the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) that is required for tumor cell proliferation, invasion, and trans-endothelial migration. The depletion of GM-CSF in CA-MSCs inhibited the ability of these cells to promote tumor cell growth and metastasis^[71]. Therefore, CA-MSCs may provide a potential strategy for a PDAC therapeutic approach. Since the desmoplastic stroma in PDAC is believed to be a major barrier for the efficient penetration of anti-cancer agents into the tumor, efficient anti-PCSC therapeutics plus anti-stromal or stromal remodeling therapeutics may be able to penetrate through the thick layer of stroma to reach PCSCs and the bulk of tumor cells, to trigger their cell death or growth inhibitory effects and effectively inhibit growth and metastasis of PDAC.

In addition to PCSCs and the previously discussed distinct stromal cells, the PDAC immune system also plays a critical and complex role in the development and progression of PDAC. It is well-established that myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs) alter the immune environment of the TME and help tumor proliferation as well as metastatic and immunotherapy resistance^[72]. Inhibitors of the CSF-1R were shown to reprogram the TME and TAMs and lead to enhanced T-cell-mediated tumor elimination^[72]. Furthermore, FAK inhibitors reduced the infiltration of MDSCs, TAMs, and regulatory T-cells^[72]. Moreover, C-C motif chemokine receptor (CCR)-2 has been shown to mediate the recruitment of TAMs to the tumor^[73]. Bone marrow mesenchymal stem cells (BM-MPCs) display self-renewal, differentiation, dormancy, and hematopoiesis properties; BM-HPCs also secrete cytokines and extracellular matrix for the growth of metastases^[74-77]. BM-MSCs are major players in the tumor microenvironment^[78] and affect inflammation, the tumor environment, immunity, and cancer metastasis^[79].

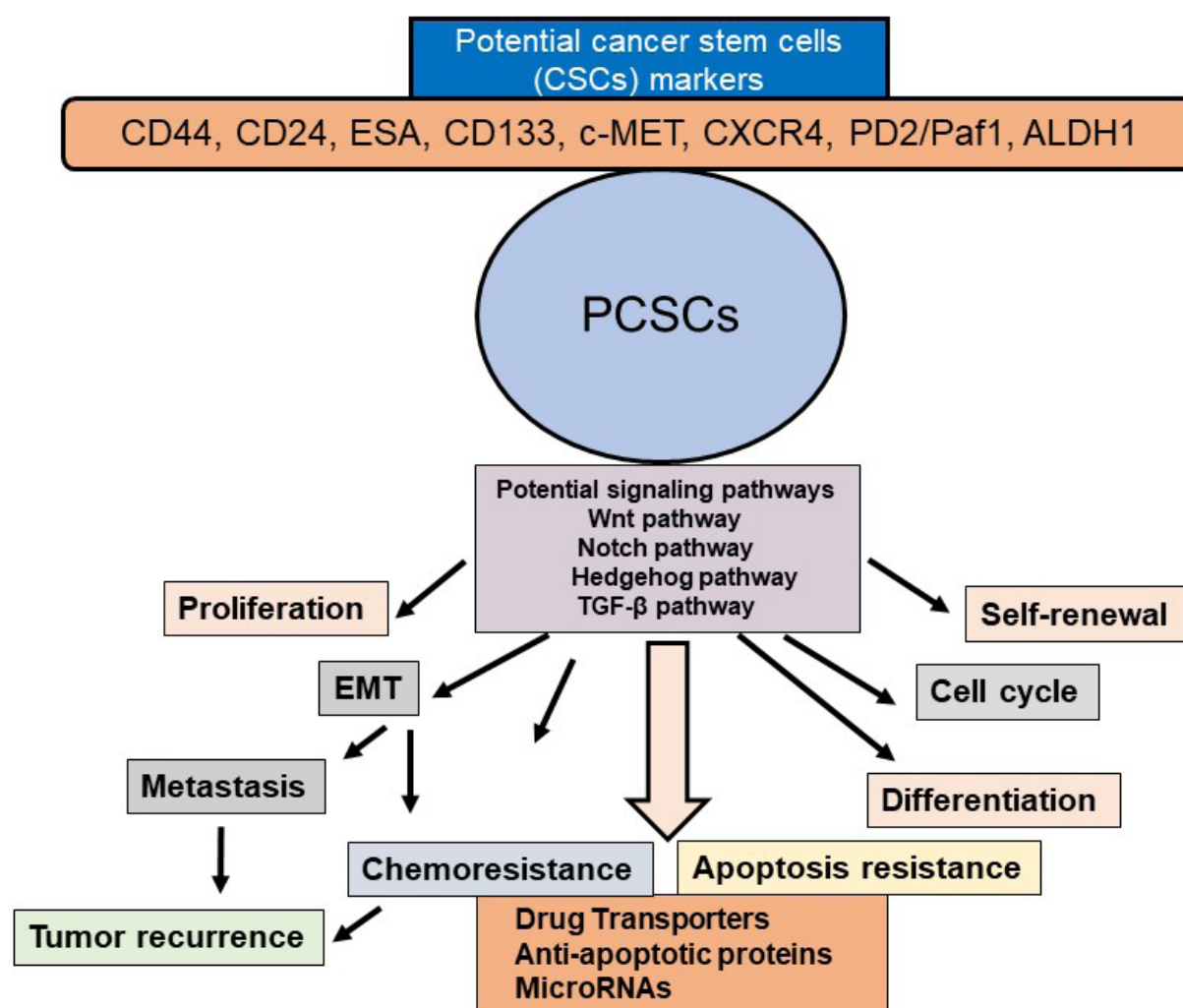


Figure 3. Schematic presentation of PCSC properties and their contribution to EMT, tumor metastasis and recurrence, chemoresistance, and apoptosis resistance. Potential CSC markers and signaling pathways in PCSCs and activation of several signaling pathways in the self-renewal, maintenance, and tumor recurrence in PDAC are also shown. PCSC: pancreatic cancer stem cell; EMT: epithelial-mesenchymal transition

Signaling pathways in PCSCs

Various signaling pathways are altered in PCSCs and EMT cells including Hedgehog, Notch, Wnt, NF-κB, and AKT [Figure 3]. Among these, Hedgehog, Notch, and Wnt play particularly important roles in PCSCs^[51]. These signaling pathways are critical regulators of PCSC self-renewal, tumor growth, invasion, metastasis, and therapy resistance^[16,40,80,81]. Furthermore, miRNAs play a significant role in the regulation of PCSCs^[82,83].

Notch signaling regulates cell proliferation, survival, apoptosis, and the differentiation of various cancers including pancreatic cancer cells and PCSCs as well as promoting EMT by controlling some transcription factors and growth factors including Snail, Slug, and TGF-β. Notch targets many genes which play critical roles in the development and progression of human malignancies^[84-86]. Several studies have demonstrated that resistance to chemotherapy in PCSCs is linked to the active Notch signaling pathway^[63,87,88].

Another self-renewal pathway in PCSCs is Hedgehog signaling, which is involved in tumor initiation, progression, and metastasis^[63,89,90]. The three hedgehog genes include Sonic hedgehog (Shh), Indian

hedgehog, and the Desert hedgehog homolog^[90,91]. It has been shown that one of these three ligands binds to the receptor Patched1 and releases the protein smoothened (Smo)^[63,89,90]. Smo triggers the activation of downstream target genes such as the GLI family of transcription factors and PTCH. Yamasaki *et al.*^[92] have reported that a nine-fold increase in Shh mRNA levels has been found in CD44+CD24+ESA+ PCSCs when compared to the bulk of unsorted pancreatic cancer cells. Inhibition of Hedgehog signaling by Smo suppression has been shown to reverse EMT and suppress the invasion of pancreatic cancer cells^[93-95].

Substantial evidence has shown that Wnt/ β -catenin signaling is involved in cell proliferation, migration, apoptosis, differentiation, and self-renewal of CSCs in several types of cancers^[96-98]. Dysregulation of the Wnt/ β -catenin signaling pathway is associated with chemotherapy resistance in PDAC, and significant evidence suggests that nuclear β -catenin plays an essential role in EMT^[97,99]. The Wnt signaling pathway also plays a significant role in regulating PCSCs^[99,100]. Additionally, Wnt signals significantly regulate CSCs in solid tumors including PDAC in the niche environments^[98,101,102]. Because of dysregulation in the Wnt signaling pathway, PCSCs are significantly susceptible to Wnt signal inhibitors^[98,99,103,104]. Hence, Wnt in the PDAC niche of PCSCs offers a critical therapeutic target in PDAC.

A significantly activated signaling pathway in CSCs is the NF- κ B pathway; its inhibition triggers the loss of CSC properties^[105,106]. Interestingly, the CCL21/CCR7 axis known to facilitate metastasis to distant organs promoted the metastasis and survival of CD133+ PCSCs and regulated their metastasis by modulating EMT and the Erk/NF- κ B pathway^[107]. Moreover, NF- κ B-mediated invasiveness in CD133+ PCSCs is regulated by autocrine and paracrine activation of IL1 signaling^[108]. Furthermore, the crucial role of PCSCs in developing resistance to gemcitabine treatment through the Nox/ROS/NF- κ B/STAT3 signaling pathway was demonstrated by Zhang *et al.*^[109]. Significantly, therapeutic targeting of the FGFR1/Src/NF- κ B signaling axis has been shown to inhibit PCSCs and oncogenicity^[110]. These findings will provide new directions for identifying potential targets that regulate NF- κ B-mediated invasiveness of PCSCs and can be used to sensitize pancreatic cells to chemotherapy. In addition to the above major signaling pathways, the mTOR pathway has been shown to be essential for the self-renewal of PCSCs^[111,112].

Like other cancers, miRNA expression is dysregulated in PDAC^[113,114]. Two classes of miRNAs play crucial roles in cancer cells, oncogenic miRNAs and tumor suppressor miRNAs^[23,115,116]. Jung *et al.*^[117] demonstrated that PCSCs exhibit differential expression of miR-99a, miR-100, miR-125b, miR-192, and miR-429 compared with controls. Another study reported the loss of miR-34 in CD44+CD133+ PCSCs, while miR-34 restoration led to the inhibition of spheroidal growth of CSCs and tumor formation^[118]. Wellner *et al.*^[119] showed that miR-200c, miR-203, and miR-183 down regulate stem cell factors and described a regulatory feedback loop between miRNAs and CSC in pancreatic cancer. Moreover, they demonstrated that ZEB1 represses expression of stemness-inhibiting miR-203 and that ZEB1 links EMT activation and stemness maintenance by suppressing stemness-inhibiting miRNA expression, and therefore promotes mobile migrating CSCs. These authors concluded that targeting the ZEB1-miR-200 feedback loop may potentially be a valid and promising therapeutic approach for PDAC.

While this review discusses abnormalities in the intracellular signaling pathways known to be involved in carcinogenesis, growth, metastasis, EMT, and chemoresistance in PDAC, the significance of these regulatory pathways to the major risk factors for pancreatic cancer including diabetes, smoking, alcoholism, and psychological stress remains to be discovered.

EPITHELIAL-MESENCHYMAL (EMT) TRANSITION

A small number of cancer cells in the primary tumor are capable of undergoing EMT, which critically promotes tumor invasion and metastatic dissemination^[120,121] in human cancer patients including PDAC^[40,41,122], as well as in PCSCs and circulating tumor cells^[122-124]. Metastatic seeding is frequently

initiated before diagnosis of the primary tumor in cancer patients, and disseminated tumor cells can remain dormant in secondary sites before forming metastatic tumors^[125-128]. The majority of pancreatic cancer-related mortality is due to metastatic disease.

EMT is critical for this rapid metastatic tumor progression, and it is a multi-stage trans-differentiation cellular process that allows epithelial cells to undergo multiple biochemical changes to gain a mesenchymal phenotype^[128,129]. During this process, epithelial cells lose their epithelial markers (such as E-cadherin, occludin, claudin, and laminin 1) and gain mesenchymal markers such as N-cadherin, vimentin, and fibronectin^[130]. These changes are caused by the activation of specific EMT transcriptional programs. Some transcriptional regulators such as TWIST, SNAI1, SNAI2, ZEB1, and ZEB2 repress E-cadherin expression, while others play roles in promoting the expression markers of mesenchymal differentiation markers including N- and/or R-cadherin and vimentin, as well as cellular matrix and focal adhesion proteins involved in promoting motility^[124,131,132]. Furthermore, EMT is associated with enhanced activity of matrix metalloproteinases^[124,131,132].

After the invading cancer cells reach metastatic sites, they undergo the reverse EMT process, MET, and adapt to proliferating in the invaded tissue microenvironment^[133-136]. Since metastasis is the major cause of cancer mortality, a detailed understanding of EMT could potentially lead to more effective therapeutic strategies for PDAC.

METASTASIS AND CSC POPULATION

Emerging evidence indicates that as the tumor microenvironment opts for a CSC population, these cells can also metastasize and initiate cells that participate in metastasis^[137]. Several cellular signaling pathways regulate the self-renewal capacity of CSCs and control their inter-conversion between the dormant state and subsequent re-activation upon metastasis^[137-140]. These re-activated cells are nurtured by extracellular niches, which crosstalk and support positive cytoprotective signals such as Wnt and Notch^[96,141-145]. As Mu *et al.*^[15] described, tumor progression is driven by the cross-interaction between tumor cells, primarily CSCs and surrounding stromal cells as well as distant organs, in which tumor-derived extracellular vesicles (TEX) play a major and important role. Mu *et al.*^[15] report that the PCSC markers Tspan8, alpha6beta4, CD44v6, CXCR4, LRP5/6, LRG5, claudin, EpCAM, and CD133 participate in the metastatic cascade at different stages, often via PDAC TEX. In PDAC tumors, the PCSC population shows over-expression of genes involved in EMT and the distinct ability to metastasize and colonize distant tissues.

CD44 is overexpressed in CSCs, frequently shows alternative spliced variants, and plays a role in cancer development and progression. The CD44 major ligand, hyaluronan, binds to and activates CD44 resulting in stimulation of several cell signaling pathways that trigger cell proliferation, survival, and increased cellular motility^[146]. Cancer cells that undergo an EMT acquire properties of CSC and show enhanced CD44 expression^[43]. CD44 consists of two isoforms, standard (CD44s) and CD44 variants (CD44v), but the different functional roles of these variants are not well known. CD44v may play a role in regulating EMT and the plasticity of cancer cells^[146-148]. Moreover, evidence shows that CD44 activates the MT1-MMP-SNAI1 axis to promote metastasis in PDAC, and CD133 activates the IL1 β -NF- κ B pathway leading to invasiveness and metastasis^[108,149]. Interestingly, Zhang *et al.*^[107] demonstrated that the CCR7-CCL21 axis in PCSCs mediates EMT phenotype via activation of ERK/NF- κ B signaling pathways. Additionally, it has been shown that the Hh pathway is implicated in playing a role in EMT in PCSCs. Inhibition of Hh signaling in CSCs reduced self-renewal, EMT tumorigenesis, invasiveness, drug resistance, and metastasis^[150]. Activation of the PI3K/AKT/mTOR pathway^[151] alone or in combination with the Hh pathway^[152] in PCSCs also increased metastasis in these cells.

RELEVANCE OF PCSCS IN PDAC DEVELOPMENT

The foregoing discussion provided ample evidence related to the role of PCSCs in carcinogenesis, growth, metastasis, EMT, and chemoresistance in PDAC. The above aberrant signaling pathways govern cancer cell plasticity, which give rise to tumor cellular heterogeneity, EMT, therapeutic resistance, and recurrence through clonal replacement and activation of dormant CSCs in PDAC as well as other cancers^[80,88,153,154]. PDAC is characterized by molecular alterations regulating PCSCs, including mutations of K-RAS, TP53, transforming growth factor- β , Hedgehog, WNT and NOTCH signaling pathways. Many genetic alterations were defined in PDAC such as earlier events including K-ras point mutation^[155], INK4a/Arf deficiency^[156], the epidermal growth factor receptor (EGFR) over-expression, gene amplification and HER2/neu over-expression and TP53, transforming growth factor- β , Hedgehog, WNT and NOTCH signaling pathways maintain PCSCs. Furthermore, the roles of EGFR, Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTORC1/GSK-3 pathways in PCSCs and their relationship to PDAC tumor initiation, EMT, malignant development has been investigated^[157]. As discussed above, recent advances in the role of EMT and PCSCs in tumor progression, metastasis, chemo-resistance, and the mechanisms integrated with aberrant biochemical signals and the underlying pathways have been surveyed in this review. Furthermore, WNT signaling cascades cross-interaction with the FGF, Notch, Hedgehog and TGF β /BMP signaling cascades and regulate expression of functional CSC markers, such as CD44, CD133 (PROM1), EPCAM and LGR5 (GPR49)^[40,153-158]. Aberrant canonical and non-canonical WNT signaling in human malignancies, including PDAC are involved in CSC survival, bulk-tumor expansion and invasion/metastasis^[158]. Despite these advances, the significance of these regulatory pathways to the major risk factors for pancreatic cancer including diabetes, smoking, alcoholism, and psychological stress remain to be studied in detail in the future.

CONCLUSION

Substantial evidence has demonstrated that CSCs including PCSCs trigger the characteristic hallmarks of various tumors including self-renewal, invasiveness, tumor recurrence, resistance, metastasis, and resistance to chemotherapeutic agents and radiotherapy. Moreover, the bulk of cancer cell population displays plasticity in most tumors including PDAC, which enables them to dynamically inter-convert between non-CSC and CSC states. Another critically important and intriguing characteristic of CSCs including PCSCs is their capacity to disseminate, migrate, and form metastatic lesions expressing resistance to therapies. In some tumors including PDAC, this plasticity has been associated with the EMT process. Furthermore, cytokines and growth factors, provided by the CSC niche containing CAFs, MSCs, endothelial cells and specific immune cells, and hypoxia, trigger transcriptional and epigenetic regulations leading to the induction of plasticity, stemness, EMT, and metastasis. Taken together, the foregoing discussion in this review provides a better understanding of the molecular mechanisms underlying these behaviours in CSCs, including PCSCs, and may lead to the identification of specific therapeutics and novel strategies to prevent EMT and metastasis, trigger CSC growth inhibition and cell death, and increase the sensitivity of tumors including PDAC to cancer therapeutics.

DECLARATIONS

Acknowledgments

I would like to thank Dr. Mary D. Kraeszig for her excellent editorial assistance.

Authors' contributions

The author contributed solely to the article.

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

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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Overview of genetic signaling pathway interactions within cutaneous malignancies

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How to cite this article: Maner BS, Dupuis L, Su A, Jueng JJ, Harding TP, Meisenheimer VII J, Siddiqui FS, Hardack MR, Aneja S, Solomon JA. Overview of genetic signaling pathway interactions within cutaneous malignancies. *J Cancer Metastasis Treat* 2020;6:37. <http://dx.doi.org/10.20517/2394-4722.2020.60>

Received: 16 Jun 2020 **First Decision:** 16 Jul 2020 **Revised:** 17 Aug 2020 **Accepted:** 26 Aug 2020 **Published:** 27 Sep 2020

Academic Editor: Lucio Miele **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Melanoma and non-melanoma cutaneous malignancies are some of the leading causes of cancer-related death in the United States. Though melanoma is more known to have a high mortality rate, the total mortality per year is nearly equal for between melanoma and non-melanoma skin cancer. Moreover, the non-melanoma types of cutaneous malignancies have potential to become locally invasive and even metastasize with very little to no treatment options when advanced. The development of these malignancies involves various genetic pathways through the four hallmarks of cancer development: malignant cell growth, apoptosis evasion, the use of supporting stroma and vascularization, and modulating and promoting an inadequate immune response. The genetic signaling pathways of basal cell carcinoma, squamous cell carcinoma, verrucous carcinoma, basosquamous cell carcinoma, melanoma, and cutaneous T-cell lymphoma interact with each other through genetic predisposition as well as with environmental exposures. Furthermore, solar ultraviolet radiation and chronic inflammatory states are found to initiate the progression of many of these cutaneous malignancies. This paper includes validated models of genetic pathways, emerging pathways, and crosstalk between genetic pathways through the four hallmarks of cancer development. Moreover, unlike most reviews addressing oncogenetics of the well-recognized, as well as newly



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discovered, genetic pathway mutations, this review stresses that these pathways are not fixed but rather exist in dynamic, interrelated, interactive, complex, and adaptive flux states.

Keywords: Basal cell carcinoma, squamous cell carcinoma, verrucous carcinoma, basosquamous cell carcinoma, melanoma, cutaneous T-cell lymphoma, genetic pathways, cancer hallmarks

INTRODUCTION

The molecular and genetic basis of the development of cutaneous malignancies involve multiple pathways which continue to evolve over time. Tumor initiation, promotion, and progression leading to the multiplication of abnormal cells are all effected by genetics, environmental factors, acute and chronic exposures, diet, trauma, and many other factors. Angiogenesis and metastasis are likely influenced by host dependent factors such as age and immunological status. Many skin malignancies environmental factors, such as UV radiation that causes photocarcinogenesis, are coupled with genetic and epigenetic alterations or extrinsic factors that contribute to local inflammation and dysregulation of normal pathways. Emerging evidence supports the role of chronic inflammation in skin carcinogenesis mediated by factors including nuclear factor-kappa B (NF- κ B), signal transducer and activator of transcription 3 (STAT3), and hypoxia-inducible factor-1 alpha (HIF-1 α). In this article, we review validated models including the hedgehog pathway for basal cell carcinomas, p53 (TP53) pathway for squamous cell carcinomas, and BRAF pathway for cutaneous melanomas, among others. Additionally, we address emerging pathways that have not been completely elucidated including those implicated in cutaneous T-cell lymphoma. We also review the presumptive biology of less common skin cancers including basosquamous cell carcinoma and verrucous carcinoma.

In this review we approach the role of genetic events in relationship to four interactive processes referred to as “cancer hallmarks”. These signals must interact for cells which have undergone a carcinogenic event to survive, proliferate, maintain a footing, and spread. The four hallmarks we review are: (1) malignant cell growth; (2) prevention of apoptosis; (3) promoting use of supporting stroma and vascularization; and (4) modulating and promoting an inadequate immune response. The genetic pathways of cutaneous malignancies will be grouped as related to basal cell carcinoma, squamous cell carcinoma, verrucous carcinoma, basosquamous cell carcinoma, melanoma, and cutaneous T-cell lymphoma. Nonetheless, this review differs from most discussions concerning oncogenetics because we stress that these well-recognized, as well as newly discovered, genetic pathway mutations are not fixed but rather exist in dynamic, interrelated, interactive, complex, and adaptive flux states.

GENETICS OF BASAL CELL CARCINOMA

Nonmelanoma skin cancers (NMSC) include cancers affecting keratinocytes such as basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). This term also includes Merkel cell carcinoma (MCC), a rare neuroendocrine tumor, and cutaneous T-cell lymphoma (CTCL). Four interactive processes referred to as the four hallmarks must interact for cells which have undergone a carcinogenic event to survive, proliferate, maintain a footing, and spread.

Malignant cell growth

Ultraviolet radiation

The most prevalent carcinogenic event promoting NMSC is ultraviolet radiation (UV) from sun exposure and/or from other UV sources such as tanning beds^[1]. Skin damage from sun exposure modulates tumorigenesis by damaging DNA, creating an inflammatory state, and activating oxidative stress responses, receptor tyrosine kinases (RTK), and pro-apoptotic pathways^[2]. UV, a recognized carcinogen, penetrates

through the skin and damages keratinocytes of the basal epidermal layer. UV is emitted from the sun as well as from some man-made light. UVA (315-400 nm) compared to UVB (280-315 nm) accounts for 95% of sunlight. UVA is the primary source of light in tanning beds and it is less mutagenic^[3,4]. UVA penetrates deeper into the epithelium affecting dermal stroma, while UVB is absorbed into the stratum corneum layer^[5]. Primarily, UV causes DNA damage by generating cyclobutene pyrimidine dimers and 6-4 photoproducts^[6]. Under stress, Sestrin2, an antioxidant, is activated by tumor suppressor p53 and inhibits positive cell growth regulator mammalian target of rapamycin (mTOR)^[7,8]. The activity and skin penetration of UV depends on its wavelength with UVA penetrating between 700 nm - 1 mm and UVB penetrating between 280-320 nm^[9].

Damage to chromosome 9 hedgehog pathway

BCC is the most common NMSC and accounts for 80% of NMSC diagnoses^[10]. BCC-specific risk factors include intermittent/recreational sun exposure, other sources of UV light, ionizing radiation, and skin phototype^[11]. The most critical pathway in BCC tumorigenesis appears to be the Hedgehog [*sonic hedgehog* (SHH)] pathway composed of three genes: *SHH*, *DHH*, and *IHH* as well as two *patched* genes: *PTCH1* and *PTCH2*. The SHH pathway is important for the patterning, growth, and development of vertebrates. The *patched* genes encode SHH pathway receptors responsible for suppressing Smoothed (SMO), a transmembrane protein/proto-oncogene capable of activating Glioma-associated oncogene (Gli) transcription factors^[12]. In the SHH pathway, a SHH ligand binds to its receptor, PTCH (a transmembrane protein) to disinhibit SMO^[13]. The activation of SMO, which occurs in the primary cilium, causes the accumulation of intracellular calcium ions resulting in a disruption in calcium homeostasis^[14,15]. The transcription of Gli proteins has been shown to be sufficient to induce BCC development^[16]. After the SHH pathway, *TP53* gene point mutations are the second most common genetic mutation in BCCs^[11,17]. Traditional advanced BCC therapy consists of surgical resection, but the advent of small-molecule inhibitors of the SHH pathway allowed for new therapeutic options for patients with locally advanced or metastatic BCC^[13,18].

Activation of the SHH pathway at the level of PTCH involves SHH interaction with PTCH through two distinct interfaces, the interface between PTCH and the calcium and zinc binding surfaces of SHH and the interface between PTCH and the *N*-terminal palmitoyl and *C*-terminal cholesteryl moiety of SHH. Mutations at these interfaces that increase SHH binding to PTCH may subsequently increase the signaling strength of the SHH pathway and may drive tumorigenesis in BCC. Activation of the SHH pathway at the level of SMO involves side-chain oxysterols (endogenous cholesterol metabolites) that induce SMO accumulation in primary cilia even in the absence of SHH ligands. Excess side-chain oxysterols may lead to overactivation of the SHH pathway while pharmaceutical and genetic approaches aimed at reducing cellular cholesterol levels have been shown to attenuate SHH signaling in target cells, highlighting a potential role of excess side-chain oxysterols in the pathogenesis of BCC^[19] [Figure 1].

DNA damage repair

Damaged keratinocytes must depend on DNA repair mechanisms such as ataxia-telangiectasia-mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) proteins, members of the PIKK family of proteins. After the cell experiences a UV-induced double-stranded DNA break, ATM undergoes autophosphorylation which empowers ATM to phosphorylate checkpoint kinase (CHK) 2^[20,21]. CHK2 inhibits CD25 phosphatases which then prohibit the cell from undergoing mitosis^[22]. ATM also can phosphorylate p53, arresting the cell in G₁^[23]. If the UV induces a single-stranded DNA break, ATR will be activated. ATR will autophosphorylate and then CHK1 which goes on to phosphorylate CDC25^[24,25]. This, once again, prevents the cell from completing mitosis. In addition, ATR is involved in the p21 pathway which can modulate various cyclins/CDKs to inhibit the cell cycle^[26]. ATR phosphorylates murine double minute 2 (MDM2) to then inactivate p53. The inactivation of p53 results in the uncontrolled proliferation

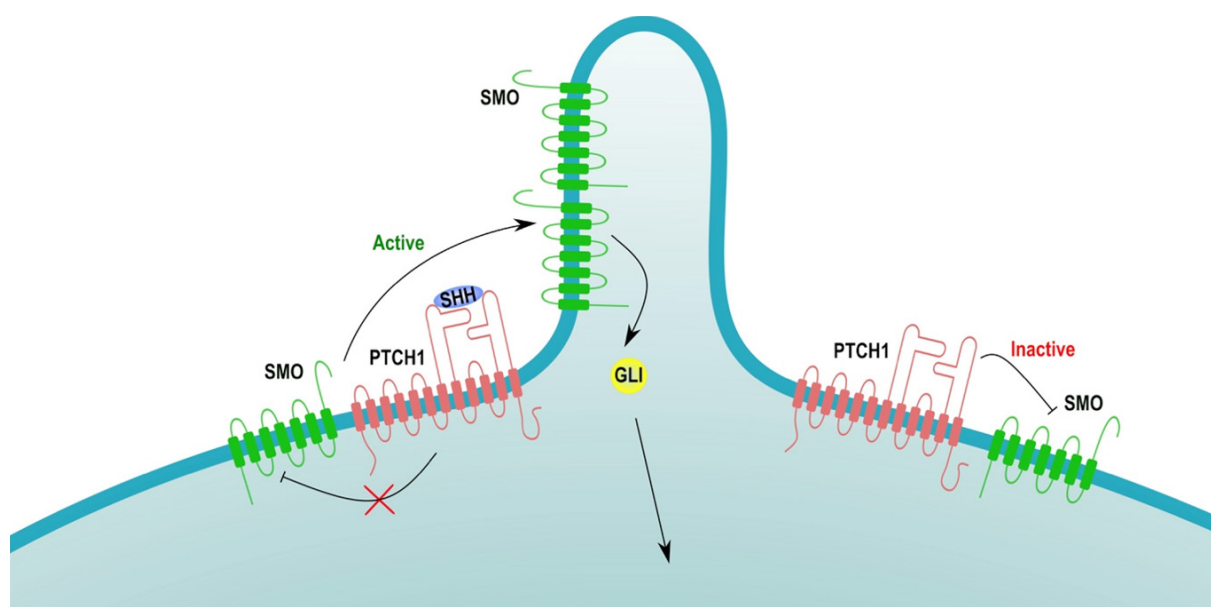


Figure 1. Sonic hedgehog signaling pathway. This figure describes the genetic signaling pathway of SHH. SHH: sonic hedgehog; SMO: smoothened

of cells with damaged DNA. On the other hand, ATR and ATM can directly phosphorylate E3 ubiquitin ligase SIAH1 which activates p53. The activation of p53 sends cells that normally should be permitted to proliferate to go into apoptosis. Thus, the dysregulation of ATR and ATM results in unpredictable cell cycle activity, especially at the p53 checkpoint which is known as “the guardian of the genome”.

Apoptosis evasion

Role of UV

UV exposure activates stress responses in the epidermis such as reactive oxygen species production which damages lipids, proteins, and DNA while also invoking antioxidant defense by suppressing tumorigenesis and initiating apoptotic pathways^[27]. The conflicting evidence forces the consideration that antioxidative therapies may be counterintuitive in the treatment of certain cancers. When observing the tumors of melanoma and non-melanoma patients, NMSC had lower levels of superoxide dismutase and catalase when compared to the melanoma samples. This observation suggests that NMSC are associated with weaker antioxidative defenses during tumorigenesis^[28]. To examine the signaling involved in oxidative stress, we will explore the p38 mitogen-activated protein kinases (p38) and c-Jun N-terminal kinases (JNK) pathways, both of which have been shown to be involved in pro- and anti-apoptotic mechanisms^[29].

P38 signaling

P38 is a Raf-mitogen activated protein kinase (MAPK) protein that can respond to oxidative stress by triggering apoptosis. When oxidative stress activates the p38 system in keratinocytes, apoptosis signal-regulating kinase 1 may become activated^[30]. Oxidative stress also inhibits MAPK phosphatases resulting in increased p38 activation^[31]. UV alone suffices in activating the p38 signaling pathway^[30,32].

JNK signaling

JNK is another member of the MAPK family that can be activated in as little as 5 min post-UV exposure. JNK targets activator protein-1 (AP-1) which is an oncogenic transcription factor involved in cell cycle regulation^[33]. Although associated with pro-apoptotic activity, AP-1 can promote survival through crosstalk with the NF-κB pathway. Pharmacological studies have shown that the inhibition of JNK in human keratinocytes *in vitro* results in greater UV-induced apoptosis^[34]. The same effect was shown *in vivo* when inhibiting AP-1 in dominant negative c-jun hairless mice^[35].

Stroma vascularization effects

RTK activation

RTKs are a group of receptors activated in response to UV exposure^[36]. Some of the more well-known RTKs include the IGF1-R, EGFR, FGFR, and VEGFR^[37]. RTK activation and RTK downstream pathways are targets for drug development^[38]. In NMSC, cetuximab (an EGFR inhibitor) is available as a pharmaceutical therapy^[39]. There are two main RTK activation pathways in NMSC, PI3K/mTOR signaling and RAF/MEK/ERK signaling^[37] [Figure 2].

PI3K/mTOR signaling

There are two forms of mTOR, mTORC1 and mTORC2, and both forms are involved in the development of NMSC secondary to sun exposure^[40]. PI3K and tensin homolog (PTEN) mutations are common in NMSC^[41,42]. To activate mTORC1, PI3K is recruited to the receptor which then leads to the phosphorylation of the p85 subunit. This allows PI3K to phosphorylate PIP2, converting it to PIP3. PIP3 then recruits phosphoinositide-dependent kinase 1 and AKT to the receptor to activate AKT. AKT then phosphorylates the mTORC1 negative regulator which leads to the activation of mTORC1, signaling for cell growth and proliferation^[43,44] [Figure 3].

Activation of mTORC2 occurs once PI3K phosphorylates PIP2 to PIP3^[45]. Part of the SIN1 protein is recruited to the cell membrane where it binds to PIP3, resulting in a conformational change in mTORC2 that reveals an active kinase site^[24]. PIP3 then recruits AKT to mTORC2 which results in the phosphorylation of AKT at the active kinase site^[46].

FOXO3 signaling

The FOXO family of proteins are transcription factors known to regulate tumor longevity and suppression^[47,48]. FOXO3a transcriptionally targets apoptotic genes such as *FasL*, *TNF-related apoptosis-inducing ligand gene (TRAIL)*, *BIM*, and *PUMA*, all of which are involved in UV-induced apoptosis^[49-51]. AKT is a negative regulator of FOXO3a that acts by translocating into the cell nucleus with a chaperone protein, 14-3-3^[52]. Together, AKT and its chaperone reduce FOXO3a activity, sensitizing keratinocytes to UVB-induced apoptosis^[49-51,53].

Raf/MEK/ERK signaling

The Raf/MEK/ERK pathway can be activated secondary to UV exposure. Raf binds MEK1 and MEK2, phosphorylating them. MEK1 and MEK2 then phosphorylate ERK^[54]. ERK1 is involved in the regulation of cell growth, malignant transformation, and drug resistance^[55]. A study found that cells treated with SMO inhibitors (a hedgehog pathway BCC treatment) have a tendency to increase RAS/MEK/ERK signaling, leading to the development of SCC^[56]. Thus, therapies should consider targeting the mTORC2/AKT and RAF/MEK/ERK pathways to simultaneously suppress BCC and SCC malignancies^[57].

Isoelectric signaling

There appears to be a relationship between the isoelectric signaling and gene modulation. One study showed that IFN- γ can lead to the loss of EGF's inhibition of basolateral K⁺ channel activity^[58]. IFN- γ levels are often elevated during periods of inflammation which leads to the shift in signaling to favor the EGF-stimulated pathways (such as Raf/MEK/ERK and PI3K/mTOR described above)^[59].

Modifying immune response

NF- κ B pathway

Aside from the direct effects of sunlight, skin injury resulting in inflammatory response may also lead to tumorigenesis in NMSC. When the skin is damaged, such as in UV-induced sunburns, the microenvironment of the damaged area changes to allow for the extravasation of leukocytes to the tissue

injury site. NF κ B is an established pathway in the mediation of inflammation. NF κ B is a heterodimer of p65 and p50 subunits who are both bound to I κ B, an inhibitory protein. Toll-like receptors, tumor necrosis factor (TNF) receptors, and RTK can all cause NF κ B activation by activating I κ B via phosphorylation^[60]. This process removes the inhibition of NF κ B, allowing the protein to move into the nucleus and promote the transcription of pro-inflammatory genes such as TNF- α , IL-1 IL-6, IL-8, and various other cytokines and interferons^[61]. Furthermore, p65-dependent NF κ B signaling nurtures a pro-inflammatory environment inducing SCC tumor initiation and promotion^[60,61].

STAT3 pathway

Pro-inflammatory growth factors and cytokines induce the expression of the STAT family of transcription factor. STAT3 enhances the transcription of factors related to inflammation, tumor promotion, cell survival, and metastasis^[62]. In mice exposed to UVB, the overexpression of STAT3 results in accelerated skin tumorigenesis whereas the removal of STAT3 genes confers a resistance to skin tumor formation^[63].

Once thought of as independent determinants of tumorigenesis, the four hallmarks of cancer form an interdependent network of signals that promote successful tumor growth. The study of skin cancer occurs under various conditions and laboratory settings. The high degree of variability in models, UV dosage, dimensions (2D vs. 3D), and methodologies creates potential confounds in the comparison of cancer signaling pathway.

GENETICS OF SQUAMOUS CELL CARCINOMA

Cutaneous squamous cell carcinoma (cSCC) is one of the most common forms of skin cancer worldwide. cSCCs most commonly present on the head and neck, particularly on the forehead, face, ears, and cheeks. Although melanoma is categorically perceived as a higher threat in society, more than 15,000 patients die from metastatic cSCC each year in the United States; this figure exceeds the total mortalities attributed annually to melanoma^[64]. In addition, the mortality rate of metastatic cSCC is over 70%^[65]. While multiple treatment modalities have been developed for advanced melanoma, advanced cSCC continues to have poor prognosis and limit options for treatment besides a newer immune checkpoint inhibitor, Cemiplimab^[66].

The factors evoking the carcinogenesis of cSCC are multiple and varied; the primary contributor is exposure to solar UV radiation. cSCC may also be initiated by industrial and inorganic exposures to tar, crude paraffin oil, fuel oil, creosote, lubricating oil, nitrosoureas, and arsenic in medications, foods, and drinking water^[67]. Organ transplant recipients and other immunosuppressed patients have increased risk for cSCC, especially locally advanced cSCC (lacSCC) and metastatic cSCC. Additionally, cSCC can develop in the setting of chronic inflammation. For example, cSCCs may develop from chronic ulcers, psoriasis, cutaneous lupus erythematosus, radiation dermatitis, porokeratosis, and lichen sclerosus. Understanding the various genetic pathways in which cSCC emerges will provide insight into improved and individualized treatments of cSCCs. The underlying genetic processes involved in the initiation, promotion, maintenance, and establishment of aggressive growth patterns resemble the pathophysiology hallmarks common to all cancers: cell growth, prevention of apoptosis, development of supportive stroma and vascularization, and modulation of the immune response.

Thus, each of the carcinogenic pathways for cSCC will follow complex genetic crosstalk between pathways for each of these hallmark systems as well as between these components. These signaling pathways may be unique to a hallmark component of the genetic pathway or be present in multiple parts of the genetic pathway. These genetic pathways include primary, well recognized, genetic mutations of signaling pathways, initiation of alternative genetic pathways, or modulation to and between alternative genetic pathways. Each of these signaling pathways may vary in how it affects the overall status of cSCC through one or more hallmarks systems.

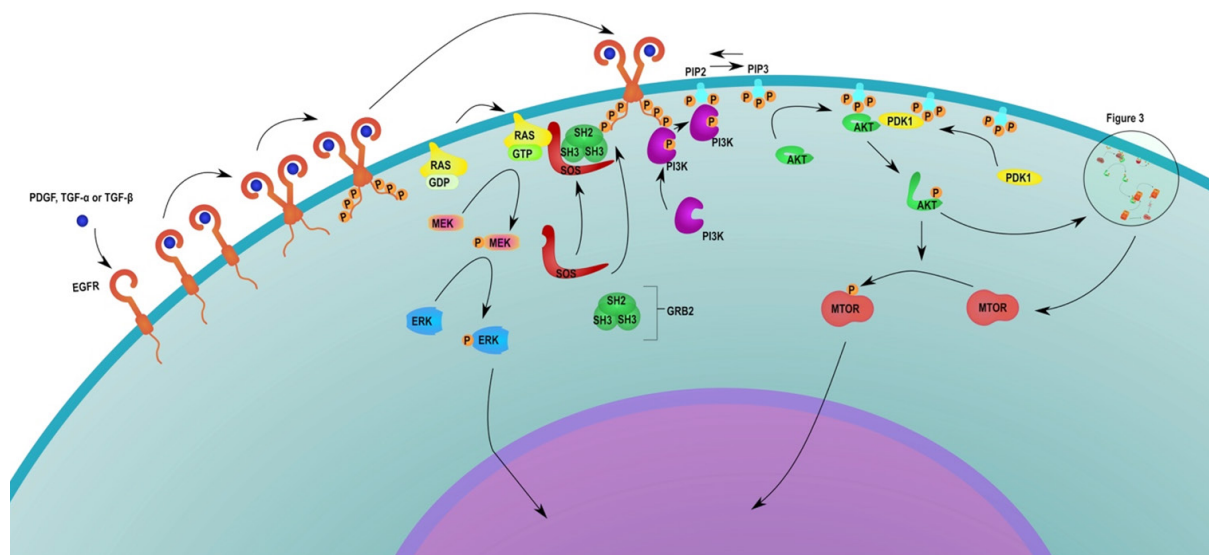


Figure 2. Receptor tyrosine kinase genetic signaling pathway. This figure shows the epidermal growth factor receptor/receptor tyrosine kinase pathways. PDGF: platelet derived growth factor; TGF: transforming growth factor

UV radiation

Cell growth

The EGFR pathway is important for keratinocyte proliferation, turnover, and wound repair. In normal cells, EGF, transforming growth factor- α (TGF- α), TGF- β , and platelet derived growth factor (PDGF) are four of many ligands that must bind to EGFR for its activation. After binding, the receptor undergoes dimerization and autophosphorylation, followed by the signaling proteins with the SH2 domain attaching to the Grb2 adapter protein and SOS complex^[68]. This cascade of events continues with phosphorylation of the Ras/Raf/Map kinase pathway which then activates mTOR pathways^[69]. This cascade of events is similar to that described in the BCC section. mTOR causes a cascade of signaling events that culminate in combinations of DNA replication, protein synthesis, and lipogenesis. Hyperproliferative lesions, including psoriatic plaques, seborrheic keratoses, and cutaneous malignancies, have been found to have mutations in EGFR^[70]. Often this is due to the uncontrolled activation of the receptor without the necessary ligands present (i.e., EGF, TGF- α and β , PDGF). In addition, UV mutates the EGFR, promoting uncontrolled keratinocyte replication and eventually cSCC showing that these chronic inflammatory conditions also have malignant potential [Figure 2]. The similarities in pathway mutations between BCCs and cSCC demonstrate that BCCs and cSCCs have the potential to have modulating and communicating pathways. Rarely discussed, is this concept that the homeostatic flux of signals is imbalanced, allowing malignant keratinocytes to behave like benign keratinocytes through the option to differentiate into a BCC or cSCC.

Genetic crosstalk

In addition, crosstalk between these genetic events plays a marked role in cSCC. The EGF ligand promotes the activation of the EGFR pathways stimulating wound repair and eventually promotes hyperproliferative disease if uncontrolled. In addition, the ligands that activate EGFR can also activate the SHH pathway. It has been found that some BCCs have the potential of mutating to SCCs. The exact pathophysiology behind this event will be discussed in the basosquamous cell carcinoma section. Given that the mutation of the EGFR signaling pathway is known for producing SCCs, this pathway also has crosstalk with the SHH pathway, recognized as a culprit for the growth of BCCs. The pathway has been shown to increase intracellular calcium through activation of protein kinase C and MMPs, which incites further EGFR pathway activation^[71] [Figure 4]. However, the extracellular environment found within the mouse embryonic stem cells could be a hypercalcemic environment. A hypocalcemic environment may cause

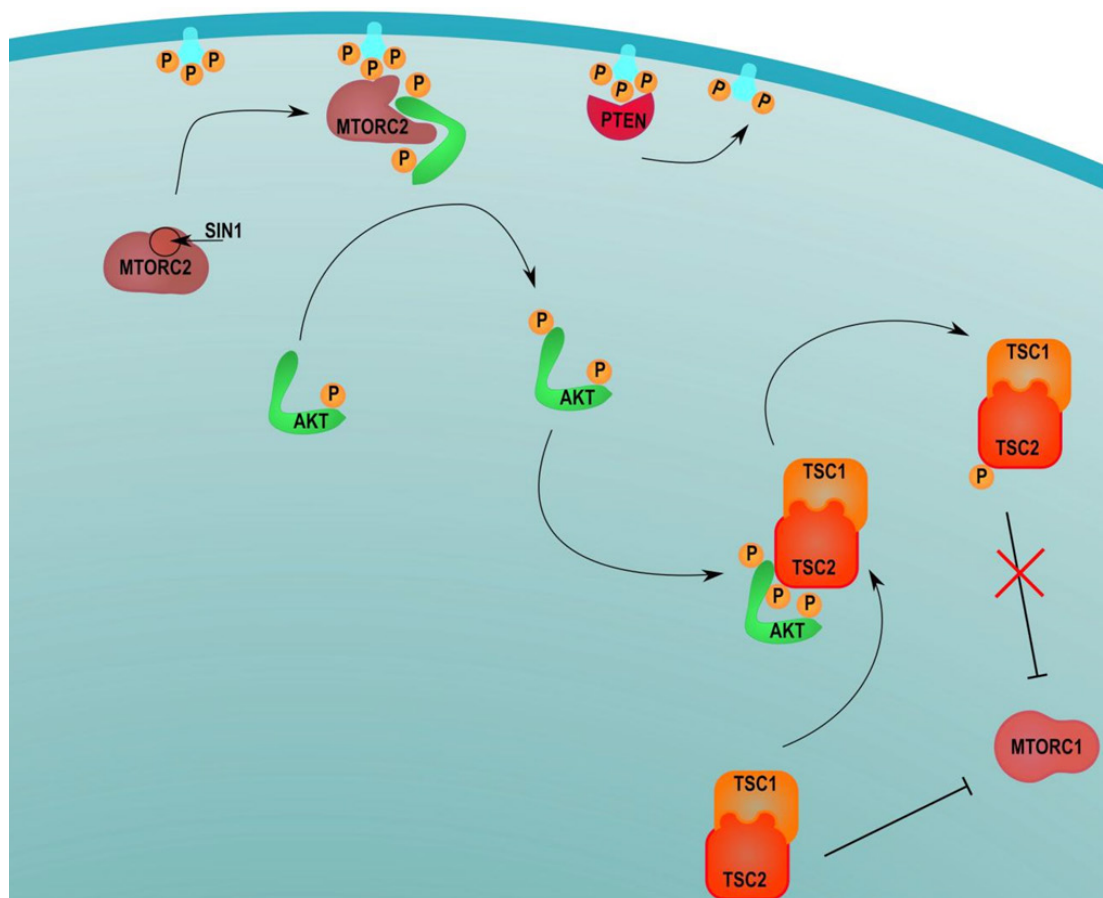


Figure 3. mTORC1 and two signaling pathways. This figure describes the transcriptional mTORC1 and mTORC2 pathways

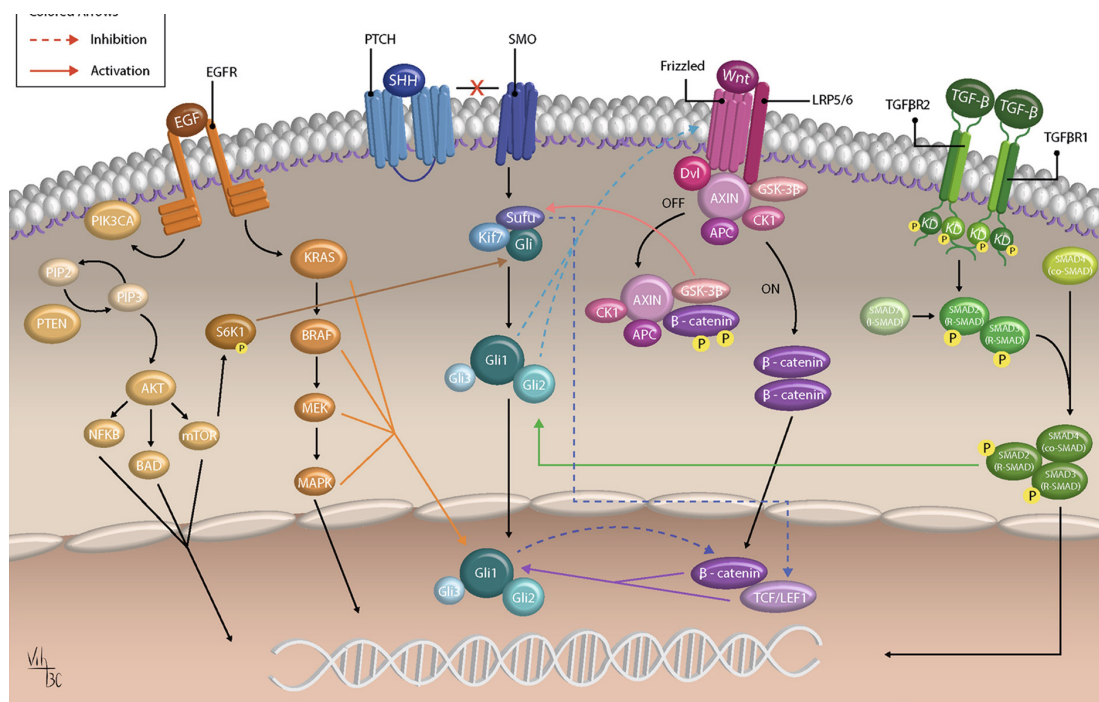


Figure 4. Crosstalk with SHH pathway: this image describes the crosstalk between multiple pathways. SHH: sonic hedgehog; SMO: smoothed; TGF: transforming growth factor

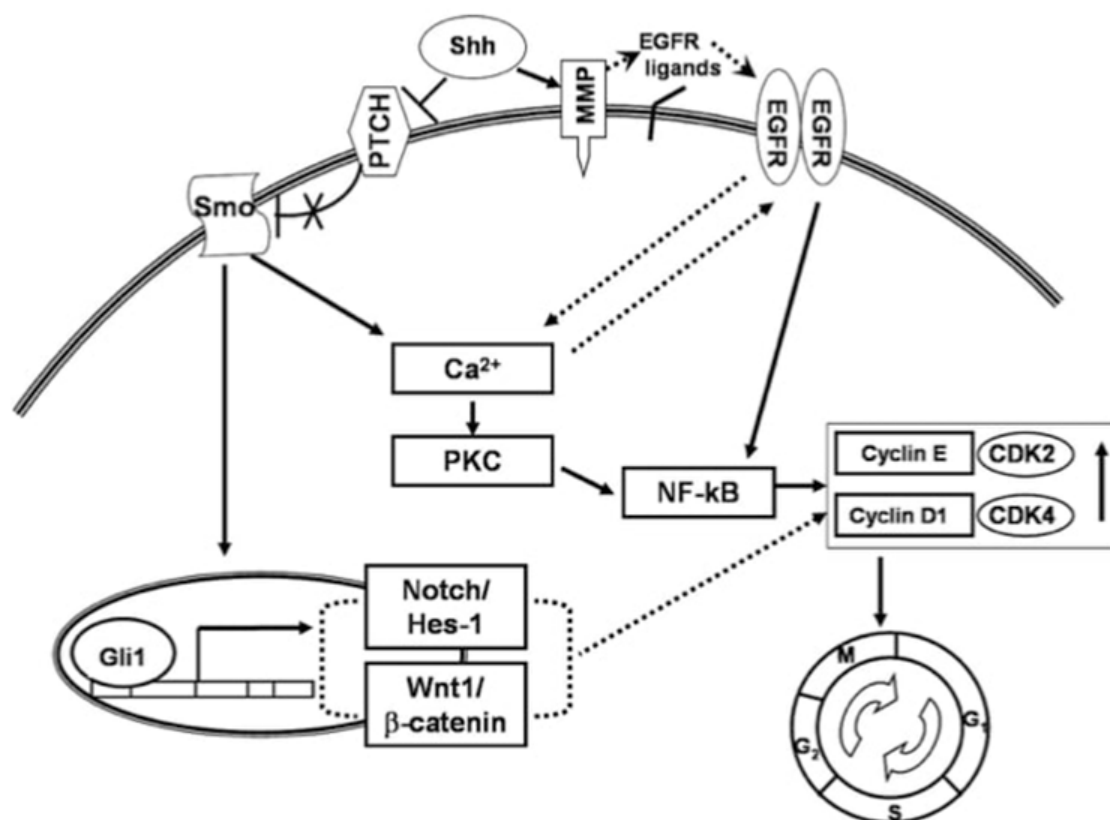


Figure 5. SHH pathway increases intracellular calcium, which can also increase the activation of EGFR. SHH also activates MMPs, which will activate the EGFR and increase cellular growth^[71]. SHH: sonic hedgehog; EGFR: epidermal growth factor receptor; MMP: metalloproteinases; NF-κB: nuclear factor-kappa B

an imbalance within the SHH pathway leading to atypical signaling and activation of other modulating receptors^[71] [Figure 5]. Because the crosstalk between the EGFR pathway and SHH pathway occurs early in the activation of the SHH pathway, the blockade of SHH can lead to shunting of the SHH pathway to the EGFR pathway causing upregulation in the EGFR pathway and increased proliferation of keratinocytes. Eventually, these tumors have the potential to mutate to a different type of skin cancer [Figure 4]^[72].

Moreover, *NOTCH* gene mutation contributes to cSCC development through its role in chronic inflammation, trauma, or both. *NOTCH* genes are responsible for the production of NOTCH proteins involved in cell proliferation and regulation of apoptosis^[73]. Cytochrome P450 family 1 subfamily B member 1 (CYP1B1), a heme-thiolate monooxygenase, is involved in estrogen metabolism and biosynthesis as well as a catalyst to hydroxylation of E2 to 4-hydroxyestradiol. Normal keratinocytes have been found to express this enzyme. In fact, recent studies have found that CYP1B1 antagonizes the signaling of NOTCH1, which, in turn, blocks keratinocyte proliferation and differentiation. Western blot and immunofluorescence display that there is increased involucrin, keratin 10, and ki67 (a proliferation marker) after downregulation and knock out of CYP1B1, demonstrating augmentation of keratinocyte proliferation and differentiation^[74]. More research is being performed on the effect of CYP1B1 and NOTCH1 within SCC cells. This innovative research can lead to a new creation of a pharmacologic agent that can treat lacSCC in the future^[74].

Other *NOTCH* pathway genes include the recombination signal binding protein for immunoglobulin J gene (*RBPJ*). This gene codes for RBP-Jκ also known as “CBF1, Suppressor of Hairless, Lag-1” (CSL) transcription factor within keratinocytes. Upper epithelial cells downregulate the production of CSL.

However, pre-malignant and cSCCs demonstrate increased levels of CSL, leading to the excessive proliferation of normal epithelial cells underlying the development of actinic keratoses and cSCCs^[75].

Role of microRNAs

MicroRNAs (miRNAs) play an important role in cell growth of cSCCs. However, the function of the many miRNAs varies. Within various signaling pathways, some miRNAs are upregulated, while others are downregulated, causing various responses. However, all these miRNAs increase cell growth of cSCC malignant lesions. For example, miR-21 works as an oncogene that targets transcription factor GRHL3, an important element in the PTEN pathway. When miR-21 is upregulated, the PTEN and PI3K/Akt/mTOR signaling pathways are overexpressed leading to increased cell growth^[76]. This is one of the many miRNAs that influence cell growth of cSCCs. In fact, some of the upregulated miRNAs determine the aggressiveness of the cSCC lesion. Because miRNAs influence both the PTEN and mTOR pathways, this information always shows potential of crosstalk between the BCC and cSCC pathways.

Apoptosis evasion

An important cause of DNA instability is solar UV. Both UVA and UVB radiation induce the most common types of cSCC. It is well-established that UVB radiation causes direct keratinocyte DNA damage leading to cSCC, but exposure to UVA radiation has also been shown to foster tumorigenesis through DNA damage resulting from photosensitizers within the body causing indirect production of ROS. In natural sunlight, the ratio of UVA:UVB varies depending on the day, season, and latitude^[77]. It is not well understood how exposure to variable ratios of UVA:UVB radiation affects progression through these oncogenic pathways. However, use of sunscreen that blocks UVB without blocking UVA, as well as chronic or acute UV exposure through window glass, can possibly increase the ratio. Foods containing furocoumarins (psoralens) also may increase the absorption of UVA and thus alter the UVA:UVB ratio^[78]. Moreover, patients should be cautioned that substances applied to the skin such as retinoids, tanning oils and products with methyl and benzyl nicotinate may have the opposite effect of sunscreen and enhance the cutaneous absorption of UV radiation^[79].

Solar UV radiation can induce DNA mutations within the cyclobutene pyrimidine dimers and 6-4 photoproducts^[80]. These mutations can activate the ataxia telangiectasia and Rad3 (ATR) DNA repair system that, in turn, activate TP53, a tumor suppressor important for apoptosis. The BCC sections elaborate on the mechanisms of ATR. Mutation in either or both genes can lead to apoptotic escape and resultant overactive cell growth.

Mutation of cutaneous TP53 thus evokes an uncontrolled activation of the cell cycle allowing mutated cells to progress to the synthesis phase (S phase) of the cell cycle prematurely. Wildtype CDKN2A/p16 is responsible for generating cyclin dependent kinase (cdk) inhibitors INK4A and p14, both of which are TP53 degradation inhibitors. INK4A binds to cdk4 and cdk6, inhibiting cell cycle progression into the S phase. When the oncogene CDKN2A/p16 is mutated, INK4A is mutated, resulting in a lack of inhibition of cdk4 and cdk6 within the cell cycle. This mechanism resembles that of TP53 in that the cell cycle prematurely progresses into the S phase allowing unregulated DNA replication and advancement into the mitotic phase of the cell cycle. Recent studies have discovered with flow cytometry targeting ATR-activated cells can eradicate the number of cells with 6-4 photoproducts. Eliminating these cells can block solar UV radiation-induced damage from occurring and potentially cSCC tumorigenesis [Figure 6].

Downregulation of certain miRNAs can affect apoptosis; specifically, the downregulation of miR-34a decreases the function of TP53 leading to avoidance of apoptosis. Select solar UV radiated keratinocytes appear to have the downregulation of miR-34a, leading to the proapoptotic effect of these tumor cells^[76].

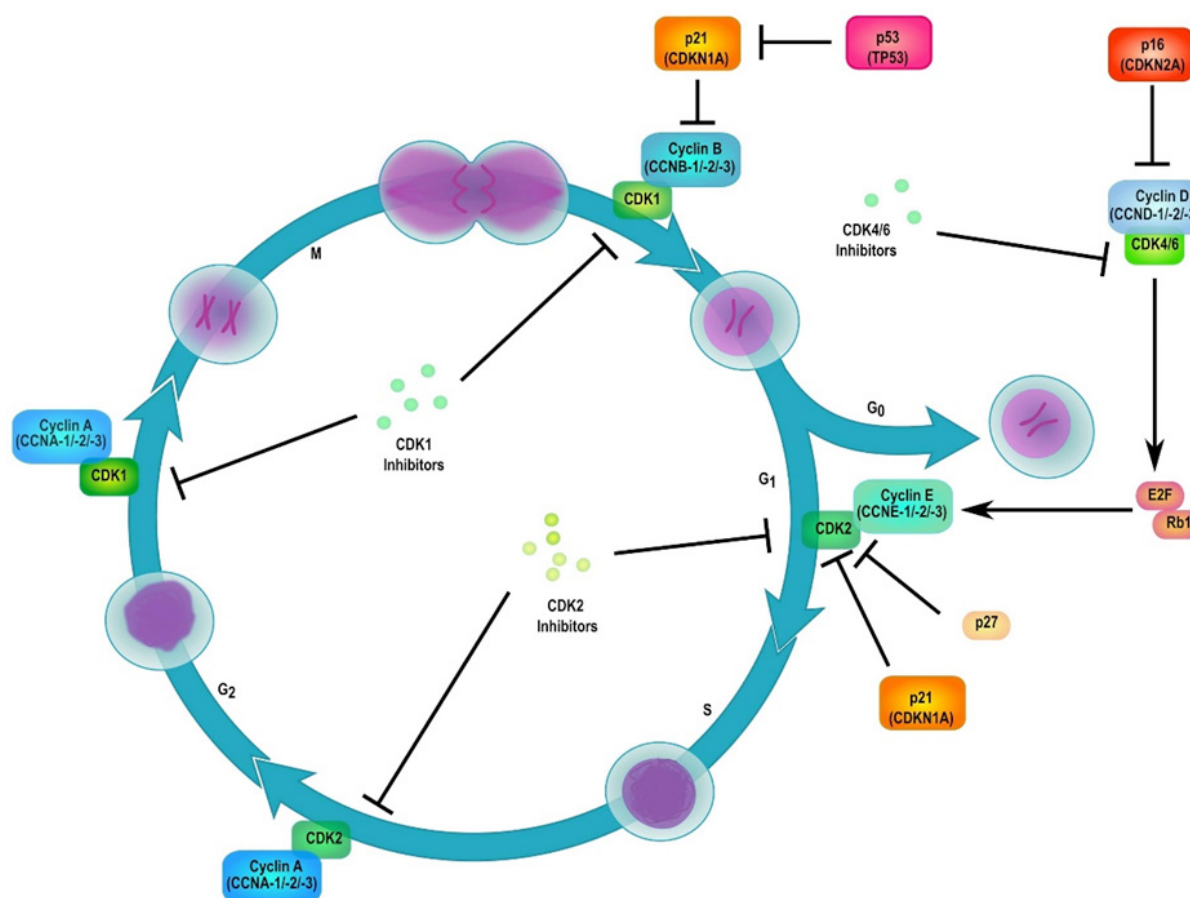


Figure 6. Cell cycle displaying TP53 inhibition of cell proliferation. G1 to S phase by CDKN2A blockade of cdk4 and cdk6^[81]

Keratoacanthomas (KA) are controversial lesions. These lesions are classified as cSCCs or lesions with cSCC potential that can spontaneously resolve. Nevertheless, taking the risk of waiting for a KA to spontaneously resolve can be detrimental, since not all lesions regress. Some KAs continue to grow rapidly; a subset may become aggressive and metastasize. There are many gene mutations that cause the transformation of signaling pathways that eventually lead to the appearance of these lesions. The most common type of genetic pathway that is mutated is the apoptotic pathway. The most noteworthy upregulated genes found through microarray analysis in KAs were *MALAT-1*, *S100A8*, and *EHF*. These genes modulate caspases, Bax, and Bcl-2, leading to avoidance of apoptosis^[82]. Microarrays show thousands of 1449 genes that vary from those of cSCC, yet there are many similar gross and histological features between cSCC and KA. Regardless of this debate, KAs have a highly malignant potential similar to those of cSCC and should be treated as soon as they are discovered.

Immune response evasion

UVB radiation (and to a lesser extent, UVA radiation) modulates the immune response over time. Actinic keratosis (AK) is a pre-cancerous lesion that may evolve into cSCC. Whether AK is to be viewed as a dysplastic, precancerous lesion of keratinocytes or if they should rather be understood to be highly scattered, disorganized cSCC *in situ* has been contested in the realms of dermatopathology and histopathology^[83]. Further research will be required to elucidate the proper classification of AKs. For this discussion, AKs will be considered precancerous lesions. The progression from AK to cSCC is associated with UVA and UVB modulation of immune signaling pathways. Chronic UV radiation causes an increase in p53 associated inflammation affecting apoptosis of keratinocytes; however, only roughly

10% of AKs progress to cSCC. In one school of thought, the fate of AK is dependent a stepwise progression through several stages, driven by complex immunologic mechanisms. In many cases, AKs may present asymptotically with little or no inflammation. These lesions may be subclassified as asymptomatic actinic keratoses (AAK). Although initially asymptomatic, some AKs become inflamed and present symptomatically and thus may be classified as inflamed actinic keratoses (IAKs).

It is thought that some AAKs progress to IAKs as cells undergo molecular and immunologic changes that promote increased growth and inflammation. For example, Fas ligand (FasL) site desensitization may lead to the inhibition of CD8⁺ T lymphocytes resulting in impaired CD8⁺-mediated apoptosis and thus promote lesion progression^[76]. Other AAKs may not undergo the changes necessary for progression and remain asymptomatic. It has also been shown that IAKs recruit immune cells, including CD3⁺, CD4⁺, CD8⁺ T lymphocytes, and Langerhans cells, producing further inflammation^[84]. Should an IAK experience sufficient growth and keratinocyte damage, it may progress to cSCC *in situ* or Bowen's Disease. Bowen's Disease is slow-growing and shows displays only partial-thickness atypia. With further inflammation, damage, and mutations of keratinocytes, Bowen's disease can evolve into cSCC with full thickness atypia and potential to invade and metastasize. The role of inflammation in the progression from AAK to IAK to cSCC is also supported by a stepwise increase in the expression of Bcl-2. Overall, the UV damage of keratinocyte DNA leads to the progression of a pre-cancerous asymptomatic lesion into a malignancy.

Secondary to UV induced cSCC are those induced through keratinocyte signaling mutation induced by chronic inflammation. Injury to the skin causes a wound healing cascade to occur, activating keratinocyte proliferation and many of the cell signaling pathways that are involved in tumorigenesis. The EGFR pathway, the intrinsic pathway, and the extrinsic inflammatory pathways are all included in the cell signaling pathways causing tumorigenesis. Chronic inflammatory conditions, including lichen sclerosus, may activate the intrinsic pathways causing chronic inflammation, increased cell turn over, and possible tumorigenesis by activating wound healing and repair simultaneously^[85]. The extrinsic pathway includes the influence of environmental factors and viral causes of inflammation, including human papillomavirus (HPV)^[85] [Figure 7]. The viral mechanisms of HPV will be discussed later in the verrucous carcinoma section. This pathway involves the release of a cascade of inflammatory cytokines and chemokines which promote the migration of lymphocytes to the injury site and subsequent repair. Chemokines can enhance cell turnover causing increased DNA replication, predisposing cells to genetic mutations that foster the formation of tumors such as cSCC. In addition, cutaneous unilateral linear porokeratosis, another chronic inflammatory disease, has high potential for malignant transformation due to its mutations in psoriasin, p16^{INK4a}, and involucrin, similar to that of cSCC but at a lower level^[87]. However, when the AKs and cSCC lesions do appear, they produce chemokines that increase metastatic potential.

Chemically induced mutagenesis

Cell growth and apoptosis

Chemicals, including arsenic and pharmacologic agents, may be implicated in the development of cSCCs. Chronic exposure to high concentrations of arsenic in food and drinking water is a common cause of cSCC, especially cSCC on the palms. Arsenic levels in drinking water as low as 300 µg/L have been found to cause cSCC^[88]. Levels between 200-1000 µg/L have been identified in 2013 in the ground water in multiple communities in Massachusetts^[89]. There are many medications and vaccinations that include the use of arsenic as adjuvants, active and inactive ingredients depending on the medication or vaccine; however, the amount within these pharmacologic agents is not enough to evoke malignant transformation of keratinocytes. Nonetheless, there is a need for further research on the chronic exposure of small amounts of arsenic and possible malignancies. Arsenic-exposed hyperkeratotic epithelial cells express elevated levels of keratin-1, keratin-10, involucrin, and loricrin, biochemical mediators important to the proliferation of keratinocytes^[88]. These lesions can further progress to become Bowen's disease and later cSCC with full

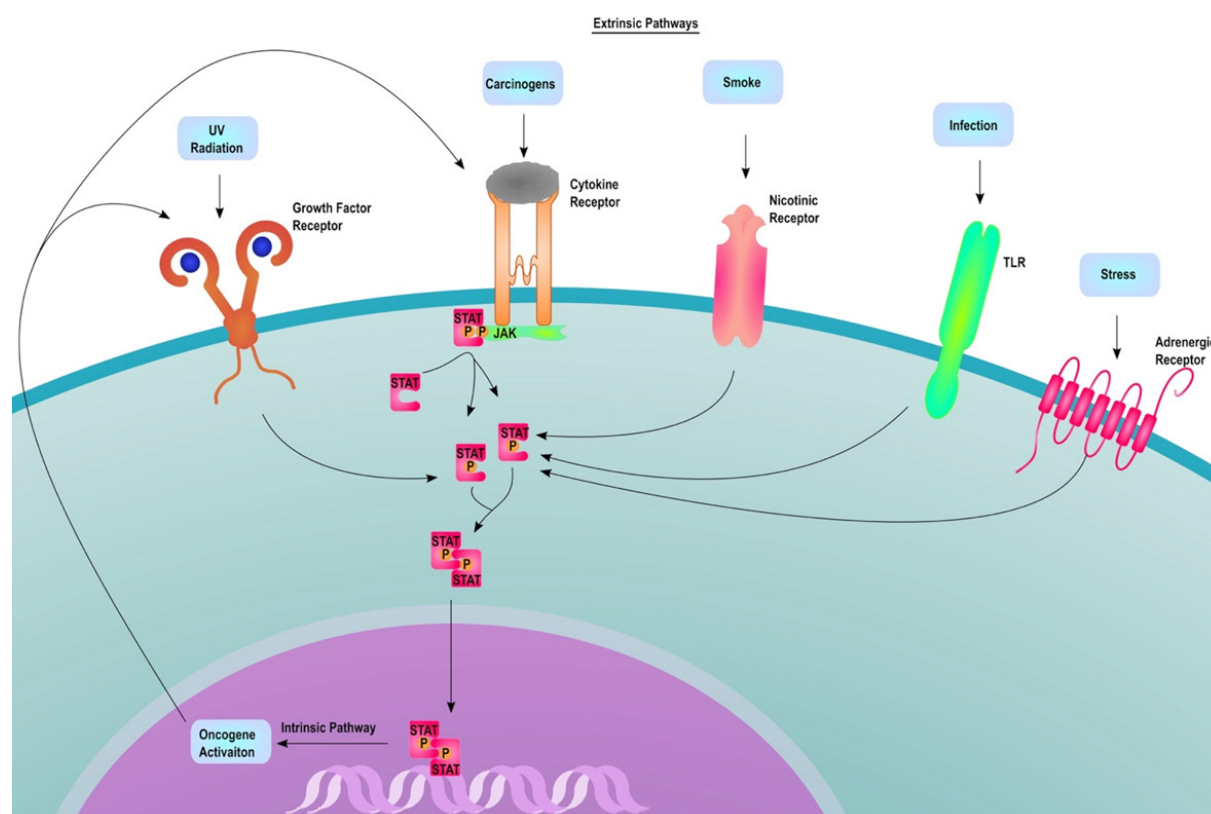


Figure 7. This image shows the extrinsic pathway insults including infection, stress, UV radiation, and carcinogens while the intrinsic pathway is due to oncogene activation^[86]. UV: ultraviolet radiation; JAK: Janus kinase

atypia. Arsenic has also been found to change the functionality of transcription factors and transcriptional co-activators that affect cell growth and the stromal environment in which keratinocytes reside. Genetic pathways of UV-induced cSCCs commonly express *TP53*, *HRAS*, or other tumor suppressor gene mutations leading to evasion of apoptosis. However, when analyzing the signaling pathway modulation caused by arsenic, many of the genes mutated are directly involved with transcription. Nrf-2, a transcription factor important to the homeostasis of redox reactions within keratinocytes, keeps inflammation and oxidative stress at bay. Gene suppression of Nrf-2 in chronically exposed arsenic human epithelial cell lines produces arsenic-induced malignant conversion of keratinocytes^[88]. Additionally, the transcription co-activator, Yap, effects keratinocytes leading to malignant transformation. Yap is only activated via phosphorylation by Phospho-LATS kinase in wild-type keratinocytes. With a high level of chronic arsenic exposure, Yap is translocated to the nucleus of keratinocytes causing increased proliferation and the eventual appearance of dysplasia and malignancy^[90].

BRAF inhibitors can also affect the appearance of cSCCs. During treatment with BRAF inhibitors, cSCCs can spontaneously appear. There are many case reports showing that BRAF inhibitor, Sorafenib, inhibits PI3K, MAP kinase, and NFκB, which can reduce the release of cytokines from Langerhans cells, decreasing T lymphocyte response to new cSCCs^[91]. As a result, the presence of IAKs or small cSCCs on patients taking BRAF inhibitors has the potential to cause rapid growth of cSCCs to become lacSCC due to the lack of immune response against these lesions.

Supporting stroma and vascularization

Invasion of the stroma that supports keratinocytes is a process that maintains and even evokes aggressive malignant behavior in cSCC. Arsenic and UV radiation affect keratinocyte stroma similarly. Chronic

arsenic exposure leads to hypersecretion of MMPs, which degrade type IV collagen in the basement membrane. This promotes the diapedesis of immune cells and the release of inflammatory markers and chemokines, causing further cell growth and increased malignant potential. In addition to arsenic, UV also affects the stroma of underlying keratinocytes. UV radiation leads to genetic changes within the cell affecting dermal fibroblasts and the underlying basement membranes. The structure of the microenvironment, including the epidermal basement membrane and dermal extracellular matrix, can be altered by precancerous lesions. These precancerous lesions cause a release of chemokines, leading to an invasion of inflammatory cells and encourage the growth of cSCCs. These AKs and cSCCs have been found to have increases in MMPs, disintegrin-like metalloproteinases domain (ADAMs), tissue inhibitors of metalloproteinases, and other extracellular matrix degrading enzymes. Furthermore, tumor cells change the molecular function of cell-cell adhesion and prevent the integrins of damaged basement membrane from binding to extracellular matrix and binding to tumor cells. Tumor cells upregulate complement factor H and factor H-like protein 1, leading to further insult and invasion. The upregulation of these inflammatory signals by tumor cells directly affects the microenvironments of keratinocytes, causing further insult to the stroma^[92].

Trauma and chronic inflammatory conditions

Chronic inflammation and trauma can change the epigenetics of the composition of the underlying stroma on which the epithelial cells lie, allowing these cells to have greater metastatic potential. The upregulation of *STAT3*, *p63*, *FGFR2*, and other genes coding for and upregulating chemokines can be the change necessary for conferring invasive potential^[92]. The chemokine SDF-1 and its endothelial cell receptor C-X-C chemokine receptor type 4 (CXCR4), both involved in angiogenesis, are upregulated in chronic inflammatory states. Once premalignant lesions are formed, SDF-1 and CXCR4 may affect chemotaxis, upregulation of metalloproteins, and activation of stromal fibroblasts, leading to loss of collagen types in the basement membrane^[93]. Marjolin ulcers (seen in burn patients), chronic decubitus ulcers, and diabetic ulcers can all undergo processes that lead to stromal changes and subsequent keratinocyte differentiation. The signaling pathways associated with chronic inflammatory ulcer transformation can appear from a spontaneously new pathway that is also associated with the epithelial cell growth. One signaling pathway is linked with inhibiting the cdk, PDGF, and SHH pathway modulations. However, an additional pathway deals with the suppression of the proapoptotic WNT/ β catenin pathway. Both pathways downregulate the extracellular matrix genes and upregulate MMP gene activation, affecting many of the same pathways affected by UV radiation. Nevertheless, Marjolin ulcers trigger a fibrotic change in the basement membrane causing a transformation in the function of adhesion molecules between keratinocytes^[94]. Marjolin ulcers display decreased expression of IL-18, suggesting that overexpression and utilization of the immune system in chronic inflammation can eventually cause suppression of the immune response towards infections and cancerous cells^[83]. This information can guide further research into chronic inflammatory conditions and trauma causing cutaneous malignancies.

Not all cSCCs are preceded by precancerous lesions. Some cSCCs can appear *de novo*. This is especially true in the cSCCs developed by transplant patients. Transplant patients often have weakened immune systems due to both immunosuppressive therapy and the underlying conditions that necessitated the transplant. Post-transplant patients with cSCC have been found to have *de novo* T lymphocyte mutations in *ZNF577* coding for zinc finger proteins and *FLOT* gene coding for T-cell migration, dampening the immune response against tumor cells and subsequent overgrowth^[95]. These types of genetic mutation pathways that lead to cSCC can be investigated as innovative treatment approaches for patients with more advanced forms of cSCC.

The tumorigenesis of cSCC is complex. UV exposure, exposure to carcinogenic substances, alteration of the stromal environment of keratinocytes, and chronic inflammatory states may all be implicated in

the development of a tumor, either independently or in combination. There are multiple possible genetic drivers capable of fostering the development of cSCC. Each of these drivers may influence the development of a malignancy by impacting one of the four hallmark pathophysiological processes common to all cancers. Depending on the provoking carcinogenesis, signaling mutations of the provoking carcinogenesis, and crosstalk between the signaling of the hallmark processes, cSCC may have various aggressive potentials and require various biogenic signaling-related therapies. This is a complex multi-factorial individualized process overlapping signaling mutations that can be individually addressed therapeutically for cSCC. It is important to note that all tumor cells are not created equally. In fact, the processes, mutations, and pathways discussed above can occur at once within one tumor between multiple cells. GEP testing by Castle Biosciences, Phoenix AZ, is being developed for SCC to assess gene upregulation and downregulation within a given tumor for high and low risk patterns. This test will help determine metastasis risk among high risk cSCC patients. Thus, the GEP test, when available, should help improve prognosis projections for thus tumors labelled as poor prognosis cSCC^[96].

GENETICS OF VERRUCOUS CARCINOMA

Verrucous carcinoma (VC), first described by Dr. Lauren Ackerman in 1948, is a rare variant of SCC, usually in the oral mucosa. Risk factors for VC include HPV infection, smoking, chronic inflammation, and repeated trauma. While the tumor generally has a good survival prognosis, it can be locally aggressive and may recur after treatment. The mortality with VC is more often due to local invasion rather than metastases^[97]. VC usually presents as a warty, exophytic, non-ulcerating lesion with a red/white surface, making it difficult to clinically distinguish from other similar appearing dermatologic conditions. This ambiguity leads to a lack of objective criteria for diagnosis, as well as challenges in establishing a definitive method of treatment.

Malignant cell growth

While a definitive association has not yet been described, HPV infection may be a significant contributor to VC. Plantar VC was shown to be associated with HPV type 16, while VC of the scalp was associated with HPV type 33^[98,99]. E6 and E7 oncoprotein production by high-risk strains of HPV cause p53 and retinoblastoma (RB) tumor suppressor inactivation, leading to cell cycle disruption and contributing to the progression of VC^[100]. The E6 protein forms a trimeric complex with p53, a tumor suppressor, and E6-AP, a cellular ubiquitination enzyme [Figure 8]. This complex interferes with the functions of p53, leading to uncontrolled cell growth. The RB protein normally binds to transcription factors of the E2F family, which allows it to suppress replication enzyme genes like origin recognition complex 1, mini-chromosome maintenance proteins, and cell division cycle 6^[101]. The E7 protein inhibits this interaction between E2F and RB, allowing for uninhibited cell division and replication^[102].

Chronic inflammation is another hypothesized cause of cutaneous VC. VC has arisen in cases of decubitus ulcers, hidradenitis suppurativa, epidermolysis bullosa, as well as chronic bladder inflammation by schistosomiasis^[103,104]. Chronic inflammation causes oxidative stress due to the release of cytokines, prostaglandins, and TNF. This leads to genetic and epigenetic modifications including the inhibition of DNA repair, modification of transcription, prevention of apoptosis, and stimulation of angiogenesis. Inflammatory cells such as macrophages and T lymphocytes may express migration inhibitory factor (MIF), which inhibits p53 transcriptional activity, leading to a loss of cell cycle regulation. The Cys⁸¹ residue of MIF is thought to play a critical role in this association between MIF and p53, leading to the inhibition of p53 mediated gene activation and apoptosis^[105]. The loss of p53 contributes to a lack of response to DNA damage, which increases the likelihood of carcinogenesis.

Repeated mechanical irritation also leads to DNA damage and resultant carcinogenesis^[103]. Specifically, in the case of epidermolysis bullosa, VC was thought to arise due to a mutation in the keratin 5 gene (KRT5).

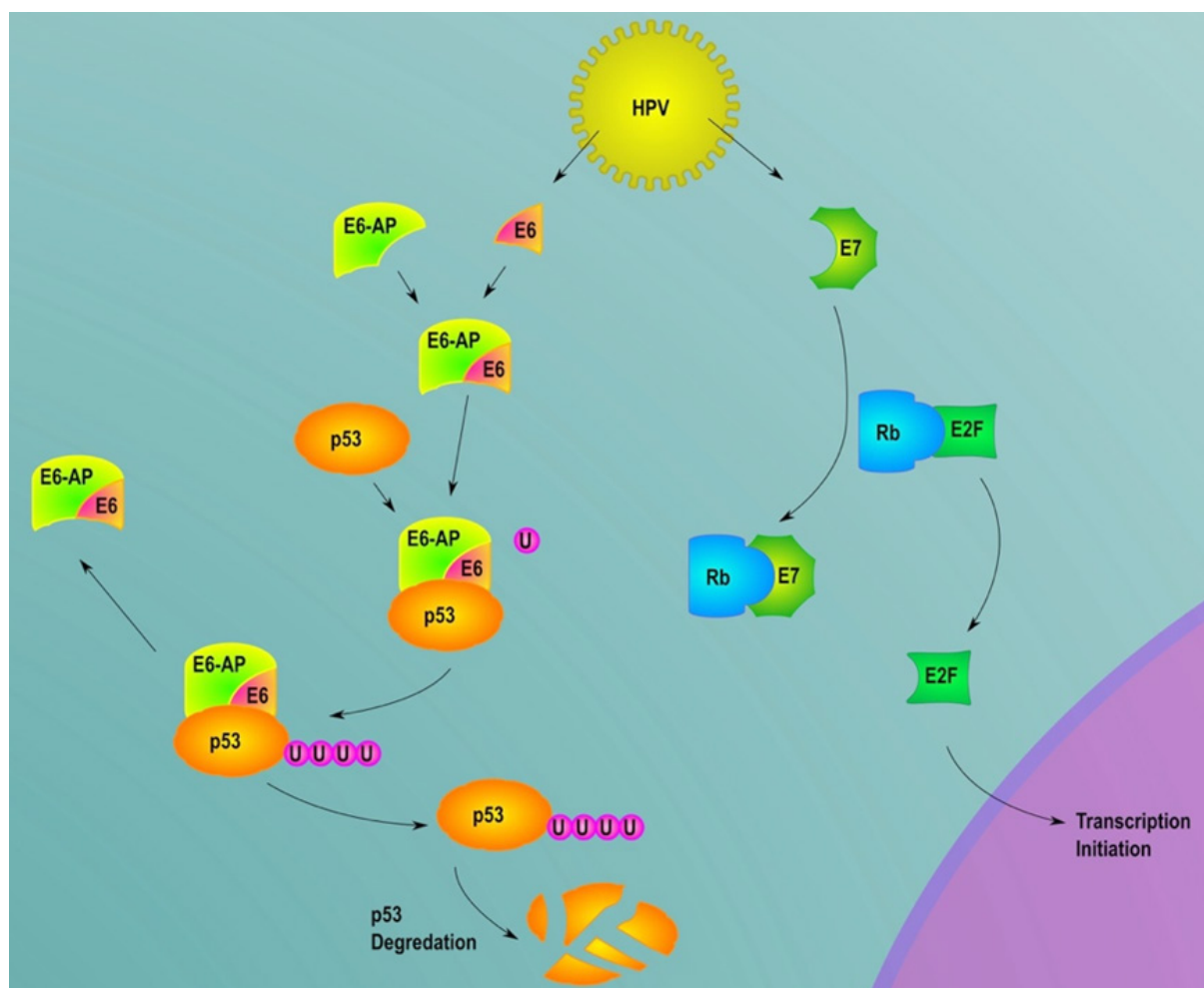


Figure 8. The pathway of human papilloma virus (HPV). This figure describes the effects of HPV on cells involving the E7 and E7 proteins

In keratinocytes, KRT5 and KRT14 normally copolymerize to form intermediate filaments which help anchor the epidermis^[106]. Mutations of helix boundary motifs of KRT5 are thought to lead to the most severe phenotype of epidermolysis bullosa^[107]. These mutations lead to fragile keratinocytes with unstable cell-to-cell junctions. This instability causes keratinocyte-mediated inflammation, which may contribute to the pathogenesis of VC^[104].

Oral verrucous carcinoma (OVC) is more commonly seen than the cutaneous subtype. The PI3K/AKT/mTOR pathway was shown to be upregulated in OVC, suggesting a role for this pathway in the progression of OVC^[59]. In this pathway, PI3K phosphorylates AKT, which confines AKT to the plasma membrane [Figure 2]. This is followed by many downstream effects of AKT, one of which is the activation of mTOR. mTOR is a serine/threonine kinase expressed in human cells, and its action is executed by two complexes, TORC1 and TORC2. TORC1 leads to the downstream activation of 4EBP1 and P70S6K, which are involved in the translation of mRNA into proteins for cell growth^[108]. This pathway leads to increased proliferation of tumor cells, as well as resistance to treatment. The PI3K/Akt/mTOR pathway is also upregulated in oral SCC.

Furthermore, the downregulation of miRNAs contributes to the pathogenesis of OVC. Specifically, miR-195 is found to be significantly downregulated in OVC^[109]. miR-195 serves as either an oncogene or a tumor

suppressor, and is involved in tumor proliferation, metastasis, apoptosis, and angiogenesis. miR-195 exerts its effects through various target genes, one of which is cdk6. cdk6 is activated in the G₁ phase of the cell-cycle and plays a role in the development of various cancers; cdk6 is found to be significantly upregulated in OVC^[110]. This negative correlation between the expression of cdk6 and miR-195 in OVC serves as confirmation of the interaction between the two. However, the specific role of miR-195 and cdk6 in OVC pathogenesis is an area requiring further investigation.

Apoptosis evasion

The diagnostic value of expression of the apoptosis-related regulatory proteins p16, p53, p21, and RBGP (retinoblastoma gene product) in cases of oral, penile, and cutaneous VC has been studied. The variation in expression of these proteins may be helpful in differentiating VC from classic SCC^[111].

P16 is a cyclin D-dependent kinase inhibitor that is inactivated in a variety of cancers. This protein blocks the progression of the cell from the G₁ to S phase^[112]. Interestingly, p16 additionally plays a role in the regulation of UV-induced apoptosis of cells^[113]. In cases of VC, some level of p16 expression is found in 79% of cases^[111]. p21, a mediator of p53 function, is upregulated by p53 in response to DNA damage. Similar to the actions of p16, p21 upregulation leads to the inhibition of cdk2 and CD4 complexes, causing cell cycle arrest in the G₁ phase^[111]. In 58% of VC cases, p21 expression is seen in the lower third of the epithelium, while the remaining 42% of cases show full-thickness expression^[111].

While p53 mutations are one of the most frequently reported tumor suppressor mutations in human cancers, p53 expression is significantly different in VC vs. SCC. All cases of SCC showed p53 expression throughout the entire thickness of the epithelium, yet cases of VC showed p53 expression exclusively in the lower third of the epithelium^[111]. Interestingly, the Ki67 protein showed a very similar pattern of expression in VC vs. SCC, with the former showing expression only in the lower third of the epithelium. The exact function of Ki67 is yet to be understood; it is thought to play a role in regulation of the G₁, S, G₂, and M phases of the cell cycle^[114]. Both p53 and Ki67 showed a statistically significant difference in expression between VC and SCC and may play a role in differentiating these tumors.

Supporting stroma and vascularization

The differences in angiogenesis between SCC and VC have been studied using measures such as microvascular density (MVD) and endothelial proliferative index (EPI). MVD is measured by scanning CD34 immunostained sections to find areas with a high density of microvascularization. EPI is estimated using MIB-1 stained slides to locate MIB-1 positive endothelial cells. Both MVD and EPI were found to be significantly lower in VC vs. SCC; it is hypothesized that the less aggressive nature of VC may be attributable to these lower values^[115].

The release of chemokines due to chronic inflammation also plays a role in angiogenesis. Members of the CXC chemokine family that are glutamate-leucine-arginine motif positive (ELR+) exert their pro-angiogenic effects by activating the CXCR2 receptor. This activation leads to increased levels of VEGF and decreased levels of thrombospondin-1, an antiangiogenic glycoprotein^[116]. Stromal cell derived factor known as SCDF1 or CXCL12 plays a role in increasing endothelial VEGF expression, as well as the chemotaxis of cancer cells^[117].

Modulating immunity

Chronic inflammation correlates with a lack of tumor immune surveillance. The cytokine IL-23 is upregulated in many human tumors. While IL-23 increases angiogenesis and upregulates matrix metalloproteases, one of its functions is to inhibit CD8⁺ T-cells, which are an essential component of tumor surveillance. Deletion of IL-23 was shown to restrict tumor growth, leading to protection against carcinogenesis^[118].

The use of a TNF inhibitor, specifically etanercept, is believed to cause immune modification that may ultimately lead to the development of cutaneous VC in a patient^[119]. Patients undergoing TNF- α inhibitor therapy may be susceptible to complex effects on the regulation of inflammation, autoimmunity, and apoptosis. These drugs have the potential to inhibit the extrinsic apoptotic pathway by modulating the activation of death receptors 4 and 5 (DR4 and DR5) [Figure 7]. DR4 and DR5 are normally activated by TRAIL, resulting in the apoptosis of cancer cells^[119]. Drugs like etanercept inhibit this process, potentiating carcinogenesis. Nonetheless, a systematic review and metanalysis of patients treated with anti-TNF- α agents failed to demonstrate any increased risk of malignancy^[120].

Furthermore, there is a possible effect of anti-TNF therapy on hypermethylation of the ASC/TMS1 protein. ASC/TMS1 is responsible for the activation of phagocytes through the secretion of cytokines and activation of the inflammasome^[119]. ASC/TMS1 also indirectly potentiates apoptosis through its interaction with p53 and the Bcl-2-associated X protein (BAX). BAX induces apoptosis, due to the activation of caspase 8. ASC/TMS1 may be responsible for translocation of BAX to mitochondria by serving as its carrier protein^[119]. Hypermethylation of ASC/TMS1 inhibits these processes, enhancing cancer development. The effect of anti-TNF drugs on ASC/TMS1 function still requires further investigation. With these known genetic signaling pathways, pharmacogenomic treatment plans can be focused on the patient and/or lesions specific genetic pathways in conjunction with the anti-TNF drugs to target all mutated signaling that can appear within patients' varying flux states.

GENETICS OF BASOSQUAMOUS CELL CARCINOMA

Basosquamous cell carcinoma (BSC) is a rare, aggressive skin neoplasm that has histopathological characteristics of both BCC and SCC^[121]. The majority of BSC cases are found in the head and neck region, with older Caucasian males most commonly affected^[122]. Clinically, BSC is indistinguishable from BCC and is commonly referred to as "metatypical basal cell carcinoma"^[122,123]. Diagnosis of BSC is typically made with a biopsy of a lesion suspected of being a BCC or an SCC^[124]. It should be noted that BSC is distinct from a collision tumor in that BSC is more of a "mixed tumor" consisting of BCC with areas of SCC differentiation^[125], whereas a collision tumor has distinct BCC and SCC entities that are present in close proximity^[126]. This can be seen histologically by the presence of a transition zone of atypia in BSC, an uncommon feature for a collision tumor^[125]. Compared to BCC, BSC has a higher tendency for local recurrence and a higher propensity for lymph node and distant metastases^[121,127]. BSC comprises approximately 2% of all skin cancers with a metastasis rate of about 7%^[128] compared with a metastasis rate of BCC of 0.0028%-0.55%^[127,129] and of SCC of 2%-5%^[130]. The BSC recurrence rate is 12%-51% after standard surgical excision^[128]. This contrasts with post-standard surgical excision recurrence rates of BCC and SCC at 5%-14%^[131] and ~8%,^[132,133] respectively. BSC has an approximately 4% recurrence rate following Mohs micrographic surgery compared with recurrence rates post-Mohs micrographic surgery (MMS) of BCC (1%-2%)^[131] and SCC (3%), respectively, although there was some variability in the literature^[128,131,134]. Interestingly, the recurrence rates of BSC post-MMS were reported to be about 4% regardless of site (head and neck, trunk, lower limb, or upper limb). In BSC larger than 2 cm, the rate of recurrence is believed to be slightly increased^[128]; however, these results were not statistically significant, likely due to small sample size^[134]. Thus, MMS is currently the preferred treatment for BSC independent of body location.

Malignant cell growth

BCC genetic drivers' role in BSC

BSCs are frequently associated with SHH pathway mutations, implicating SHH deregulation as the primary driver in BSC and providing evidence that BSCs shares similar cancer drivers to BCC^[135]. Loss of function in *PTCH1* and gain of function in G-protein-coupled receptor *SMO* in the BCC pathway are the most common mutations that cause SHH deregulation in the BCC pathway. Similarly, 45% of BSCs were shown to have deleterious mutations in *PTCH1* compared to 44% of BCCs and 10% of SCCs. About 5% of BSCs

contain SMO oncogenic mutations compared with 25% of BCCs and 0% of SCCs^[135]. BSCs also contained all the other known BCC cancer drivers, MYCN, PP6C, GRIN2A, CSMD3, DCC, PREX2, APC, and ARID1A at a frequency statistically similar to that of BCCs^[135].

SCC genetic drivers' role in BSC

BSCs lack the classical SCC driver mutations, NOTCH1/2, HRAS/KRAS, and CDK2NA, at a frequency expressed by SCCs. Instead, BSC contain SCC driver mutations at a significantly lower rate that is closer to that seen in BCCs. The presence of classical BCC cancer drivers in BSCs and the lack of classical SCC driver mutations suggests that BSCs have a mutational landscape similar to that of BCC, and that BSC cancer drivers likely arise through the deregulation of SHH signaling^[135].

Genetics of cells modulating between BCC to SCC cells

It is believed that BSC originally derive from BCC due to their similar mutational landscape; they subsequently acquire mutations that lead to squamatization^[121]. Of the 20 cancer genes identified in BSC, ARID1A is mutated in 45% of BSCs compared to 19% of BCC and 19% of SCC^[125]. ARID1A normally plays a role in the differentiation of several cancer types because it encodes a component of the chromatin remodeling complex, SWI/SNF. SWI/SNF plays an important role in repairing damaged DNA, so ARID1A impairment can cause defects in DNA repair. Disruption of ARID1A reduces the restrictive nature of chromatin remodeling in terminally differentiated cells, imparting a plasticity that increases cell survival, regeneration, and proliferation. Under selective pressure, such as a SMO inhibitor treatment, ARID1A mutations allow keratinocytes to undergo squamatization, promoting *de novo* SCC development from BCC^[125]. Further research could be done to see if mutations within other components of chromatin remodeling could confer plasticity in BSC.

Additionally, switching from the SHH pathway to the RAS/MAPK pathway is a form of crosstalk between the pathways and another way that BCC can avoid selective pressures such as SMO inhibitors, leading to squamatization. As discussed in the SCC section, an upregulated RAS/MAPK pathway can lead to SCC. Pathway switching is believed to be driven by a loss of primary cilia, a microtubule-based signaling organelle that is essential for high SHH pathway signaling, promoting RAS/MAPK signaling and subsequent squamatization as seen in Figure 9. Furthermore, Gli1 is active in the nucleus and is typically elevated in BCC. Stained BSC shows that basaloid keratinocytes demonstrate high Gli1 and low MAPK staining which is similar to that of BCC. Squamatized keratinocytes in BSC demonstrate low Gli1 and high MAPK staining which is similar to that of SCC. Interestingly, the transition zone shows a moderate MAPK expression and high Gli1 expression, indicating that RAS-MAPK pathway activation drive squamatization in BSC with subsequent loss of Gli1 expression as a secondary event. This discovery indicates the activation of the RAS-MAPK pathway and subsequent reduction in SHH signaling as a potential modulator of tumor plasticity in BSC^[136]. Overall, tumor fate in BSC is believed to be dictated by a balance between SHH and RAS/MAPK signaling^[125].

Calcium flux is a potential modulator of the balance between SHH and RAS/MAPK signaling in BSC. The degree of extracellular calcium is a regulator of the SHH pathway. In rat gastric mucosal cells, SHH is unable to activate extracellular signal-related kinases in calcium-free conditions. Conversely, cells in a calcium-rich media show increased intracellular calcium levels, a marker for SHH pathway activation^[137]. Altogether, it is possible that calcium flux shifts the balance between SHH and RAS/MAPK signaling in BSC, contributing to tumor plasticity^[138]. More research is needed to establish a causal relationship between calcium flux and the balance of SHH and RAS/MAPK. Future studies could be done to identify additional modulators and to understand the interplay between existing modulators in conferring tumor plasticity.

Though it is known that BSC appear from BCC, it is unknown if these cutaneous lesions can also appear from a SCC. However, due to the consistent state of flux that the body undergoes and crosstalk between

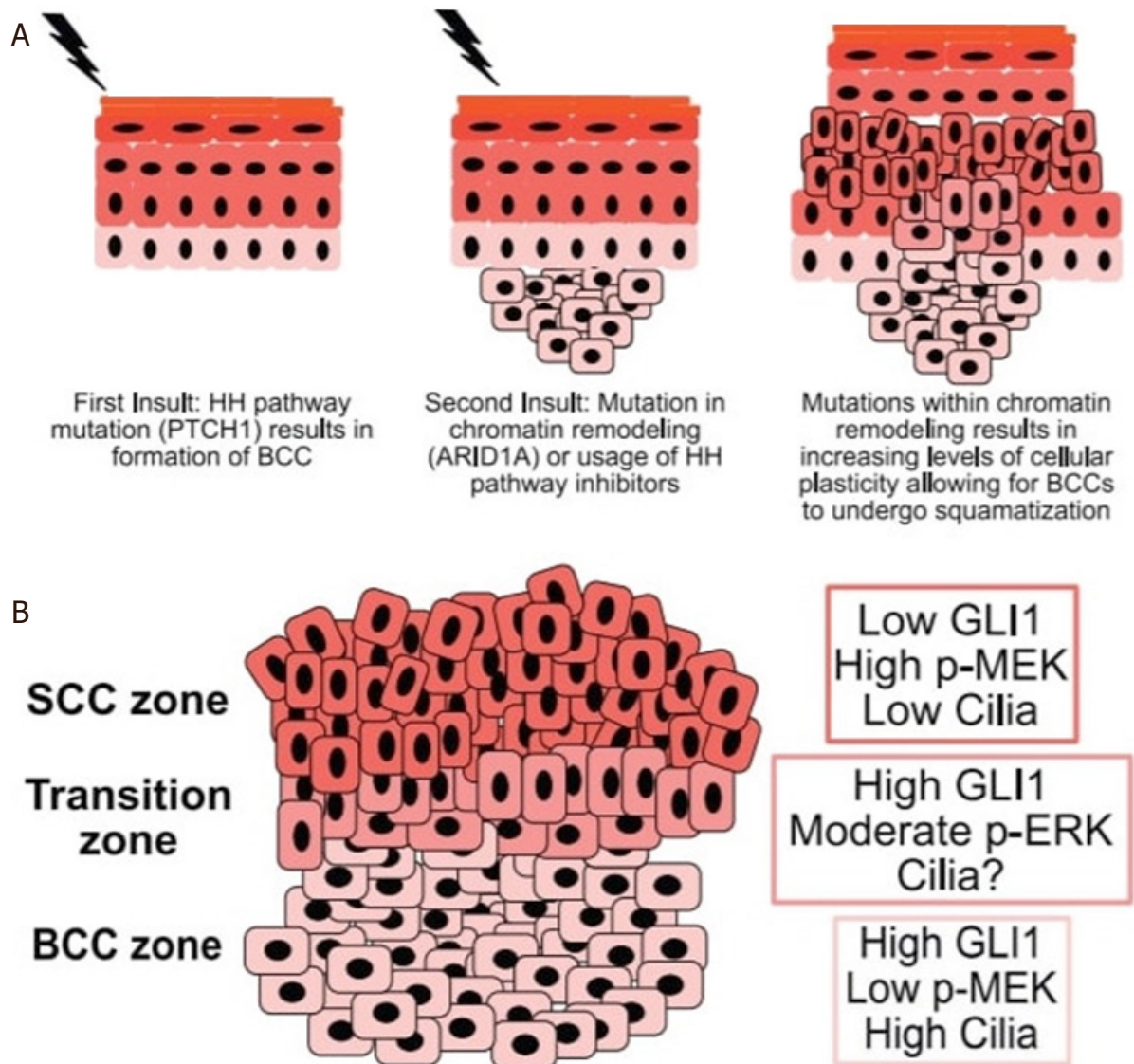


Figure 9. Genetic lineage of BSC. A: activating SHH pathway mutations initially drive formation of BCCs. Acquisition of *de novo* ARID1A mutations or other chromatin remodeling mutations under pharmacological SMO inhibition drive cellular plasticity, pushing basal cells to undergo squamatization and leading to BSC formation; B: within the BCC zone, high levels of SHH signaling, low RAS/MAPK pathway activity, and high levels of ciliation drive tumor growth. Within the SCC zone, RAS/MAPK signaling increases with a concomitant reduction in SHH pathway activity and ciliation. Within the transition zone, cells begin to show higher levels of RAS/MAPK pathway activity while maintaining SHH signaling. The levels of ciliation are unknown. SHH: sonic hedgehog; BCC: basal cell carcinoma; SMO: Smoothed; BSC: basosquamous cell carcinoma

these genetic pathways, there is a possibility that SCCs could become BCCs and BCCs can become SCCs. There is a need for further research finding data to address this issue.

Prevention of apoptosis

Strong expression of FasL in both BCC and SCC is believed to allow the tumor to avoid apoptosis. FasL mediates apoptosis in cells that express Fas receptor and is a member of the tumor necrosis family. FasL on tumor cells can bind to the Fas receptor on T-cells, macrophages, and natural killer cells which make these effector cells unable to infiltrate the tumor nodules and trigger the extrinsic apoptotic pathway^[139]. Although there have not been studies confirming that strong expression of FasL is an important mode for how BSCs prevent apoptosis, BSCs' genetic similarity to BCC and histopathologic characteristics of both

BCC and SCC suggest that strong FasL expression plays a role in allowing BSC to avoid apoptosis. BSC that do not have strong expression of FasL may not be able to avoid apoptosis or may avoid apoptosis through other means.

Additionally, bcl-2 promotes cell survival without stimulating cell proliferation. Bcl-2 is associated with the intrinsic apoptotic pathway, triggered by intracellular stress^[140]. Studies indicate that bcl-2 positive tumors have a better prognosis and slower course of progression. In BSC, immunohistochemical studies for bcl-2 yield a positive result in the BCC regions but a negative result in both the transition zone and SCC region^[141]. Studies show only low-grade bcl-2 positivity in the BCC regions of BSC, similar to that of aggressive BCC. In contrast, non-aggressive BCC such as nodular and superficial BCC have high grade bcl-2 positivity. This suggests that BSC may downregulate bcl-2 in the BCC regions, stimulating uncontrolled cell proliferation and contributing to its more aggressive behavior compared to non-aggressive BCC^[141].

Crosstalk

Crosstalk between various signaling pathways can be a determining factor as to why some patients respond to cancer treatments and others fail treatments. In fact, the BSC pathways and hair follicle pathways appear to have significant crosstalk; both of these pathways are dependent on SHH, PTCH1, SMO, and Gli^[142]. In normal hair follicle development, PTCH1 acts as a receptor for SHH, and their interaction triggers SMO which modulates a signaling cascade. Activation of the SMO cascade results in subsequent translocation of Gli to the nucleus. Gli is a key modulator of the SHH pathway and causes upregulation of several downstream target genes that modulate hair follicle development^[143]. Injury to skin enlists hair follicle stem cells in the healing process, namely through the SHH pathway. However, if cells that carry a mutation in SMO are recruited to the injury site, this mutation will trigger unregulated downstream SHH pathway signaling which is implicated in BCC and BSC^[144]. This shared SHH pathway involvement suggests that the mechanisms of tumor formation and hair follicle proliferation are interlinked and essential for the normal development of hair follicles with dysregulation or mutation in this pathway leading to cancer^[143]. It is possible that BSC originates as BCC from pluripotent basal cells and initiates in a similar manner as BCC, typically from hair follicle stem cells in the hair follicle bulge^[145], as seen in the previous BCC section. Through modulating pathways conferring plasticity as described above, squamatization occurs to form BSC.

Vismodegib is a SHH pathway inhibitor that is used for the treatment of BCC. There have been many cases of SCC developing from BCC following treatment with Vismodegib. The appearance of SCC may indicate a *de novo* SCC adjacent to the BCC which is proliferating independently, an SCC that develops as a result of stem cell differentiation during SHH inhibition, or an SCC that was present as part of a metatypical BCC such as BSC that has decreased SHH signaling due to SHH inhibition and subsequently increased RAS/MAPK signaling^[146]. There fails to be many reported cases of SCC transforming to BCC following use of Vismodegib. Thus, it may be difficult to determine if SHH inhibitors could play a role in the desquamatization of cSCC to BCC. Nevertheless, because BSC has a high tendency for recurrence and has a high rate of metastasis, Moh's micrographic surgery is currently the preferred treatment for BSC^[128].

Supporting stroma and vascularization

When comparing supporting stroma of BCC and BSC, there is a significant difference between the stroma of BSC, and high risk (HR) BCCs (micronodular and/or infiltrative) compared to that of low risk (LR) BCCs (nodular and/or superficial). LR BCC group lesions have fibromyxoid stroma without desmoplastic stroma, whereas 80% of the HR BCC and 61.8% of the BSC group lesions have desmoplastic stroma^[147].

The stromal differences in LR and HR BCCs and BSC further suggests variety between the tumor types. Fibromyxoid stroma is most often observed in lesions with mild inflammation. However, desmoplastic

stroma is associated with dense inflammation which attributes to stroma-derived factors that recruit additional inflammatory cells or inflammatory cell secreting factors that change stromal characteristics^[147]. Inflammatory cell/secreting factor recruitment of collagenolytic enzymes that cause destruction of the surrounding collagen and matrix metalloproteinases that cause proteolysis of extracellular matrix components are implicated in SCC and likely BSC to cause desmoplastic stroma, facilitating tumor growth and metastasis^[148]. Further research needs to be done to identify the types and functions of immune cells in peritumoral inflammation in order to understand the interactions between peritumoral inflammation and stroma specifically in BSC. Additionally, activated fibroblasts and inflammatory cells of peritumoral stroma secrete extracellular matrix proteins and growth factors in a paracrine fashion which can change the expression of genes affecting angiogenesis, tumor growth, and metastasis. Though the precise mechanisms underlying the complex interactions between the stroma and tumor in BSC is unknown, the crosstalk communication between various genetic signaling pathways indicates the flux state amongst signaling pathways and keratinocyte homeostasis.

Modulating immune response

Dense peritumoral/perivascular inflammation in BSC is a marker of host immune response and may be associated with recruitment of protumor immune cells. For LR BCC, 83.3% of the lesions exhibit only mild peritumoral inflammation whereas of the HR BCC, 76% of the lesions exhibit dense peritumoral inflammation. Of the BSCs, 51.4% demonstrate dense peritumoral inflammation and 37.1% demonstrate moderate peritumoral inflammation, frequencies that most closely approximated those of the HR BCC group^[147]. Similarly, perivascular inflammation in adjacent dermis was observed in 91.4% of BSC group lesions, 97.5% of HR BCC group lesions, and 55% of LR BSC group lesions. Only the BSC and HR BCC group lesions were found to have dense perivascular inflammation^[147].

The host immune response between BSC and HR BCC shows some similarities based on peritumoral and perivascular inflammation density, both in contrast to LR BCC. Firstly, differences in inflammation density between BSC and LR BCC may be attributed to an increased number of regulatory T-cells in BSC that suppress anti-tumor T-cell response and are associated with a worse prognosis compared to LR BCC that do not have increased regulatory T-cells^[147]. Secondly, immature dendritic cells (IDCs) are involved in attenuating the immune response in BCC and perhaps also in BSC. Attenuation of the immune response occurs through the induction of peripheral T-cell tolerance by IDCs and through regulatory DC secretion of IL-10 that suppresses T-cell proliferation. IDCs are also believed to contribute directly to tumor proliferation through unknown mechanisms. Lastly, an increase in expression of Th2 cytokines, specifically IL-4 and IL-10 contribute to an immunosuppressive tumor environment that favors proliferation in BCC and perhaps also BSC^[149]. It should be noted that the tumor permissive mechanisms described above likely occur concurrently with immune responses directed towards tumor eradication. Anti-tumor immune responses include: CD8⁺ T-cells for a specific adaptive antitumor response, IL-23 for enhanced proliferation of memory T-cells, IL-12 for activation of mature DCs and Th1 antitumor immunity^[149]. Overall, BSC modulation of immune response appears to occur dynamically with a protumor, attenuated immune state only partially compensated for by the host antitumor response. This dynamic state shows myriad fluctuations and crosstalk signaling between genetic signaling pathways, which can further personalized BSC treatment for advanced or metastatic BSCs.

GENETICS OF MELANOMA

Although NMSC are the most common type of skin cancer, cutaneous melanoma is the most aggressive, accounting for greater than 80% of skin cancer deaths largely due to its susceptibility to metastasize to other organs^[150]. Close to 200,000 cases of melanoma will be diagnosed in the United States in 2020 with a rate of one American dying from melanoma every hour^[151]. There are 4 main types of melanoma: superficial spreading melanoma (SSM), nodular melanoma (NM), lentigo maligna melanoma (LMM), and acral

lentiginous melanoma (ALM)^[152]. Cutaneous SSMs account for 70% of melanomas, while NMs account for 15%-30% of melanomas. Both SSM and NM subtypes are often associated with the *BRAF* V600E mutation^[152]. ALM (2%-10%) and LMM (5%) are rarer types that are both associated with mutations in the *C-kit* gene^[152].

The transformation of melanocytes into melanoma cells and further progression to metastasis involves the complex interaction of signaling pathways with multiple environmental, genetic, and host factors. The primary contributor is DNA damage from UV light exposure, although genetic disorders like xeroderma pigmentosum and familial history of melanoma can strongly increase a person's risk^[153]. Occupational exposure to ionizing radiation among radiologic technologists has been shown to increase risk to developing skin cancer and melanoma^[154]. A recent literature review suggests airline pilots and cabin crew may have twice the risk of melanoma compared to the general population and increased melanoma mortality among pilots from possible cumulative cosmic radiation^[155]. However, much of the relevant evidence is considered out-of-date as it reflects environment and behaviors in the late twentieth century and is incongruent with modern day standards. Immunosuppressed populations like organ-transplant recipients have an increased risk of many malignant cancers, with skin cancer being the most common^[156]. Understanding the mechanisms in various signaling pathways can provide insight into providing personalized and effective treatment. This amalgamation of genetic crosstalk modulates the transformation process and involves the hallmarks of cancers: (1) cell growth; (2) prevention of apoptosis; (3) supporting stroma and vascularization; and (4) modulating immune response.

Cell growth

UV-induced damage

Within hours, UVA causes immediate and lasting hyperpigmentation (tanning) due to induction of oxidative stress in melanocytes. UVA damages the extracellular matrix and induces an immune response facilitating invasion and metastasis of skin cancer cells^[113]. In contrast, UVB directly causes skin cancer genesis and induces a slower delayed tanning through a nascent melanin synthesis pathway and melanocortin receptor-1 (MC1R) signaling^[3]. MC1R activates the DNA damage response causing the formation of cyclobutene pyrimidine dimers and 6-4 photoproducts that distort the DNA helix and when unrepaired lead to mutations^[157]. In addition, UVB triggers an inflammatory response by recruiting neutrophils and macrophages and promotion of angiogenesis contributing to melanoma cell survival and metastasis^[3].

CDKN2A/p16 deletion

The CDKN2A/p16 protein is a crucial cell cycle gatekeeper at the G₁-S checkpoint and its location encodes for tumor suppressors p16^{INK4a} and p14^{ARF}^[158]. As mentioned in the cSCC section, P16^{INK4a} inhibits cdk4 and cdk6 [Figure 10], activating RB protein and preventing cell cycle progression into S from G₁ phase^[159,160]. On the other hand, p14^{ARF} positively regulates p53 [Figure 10] by inhibiting negative regulator MDM2^[161,162]. Deletions in the CDKN2A locus was found in 50% of all melanomas and had high penetrance in familial melanoma^[153,163]. The inactivation of CDKN2A and p53 inactivation leads to uncontrolled cell proliferation of melanocytes.

Apoptosis evasion

BRAF in RAS/RAF/MAPK/ERK pathway

The most prevalent and highly studied oncogenic melanoma mutation is *BRAF* with the most common mutation substituting glutamic acid for valine (V600E)^[164]. An estimated 40%-50% of mutated melanomas are of *BRAF* V600E mutation^[165]. In the MAPK pathway [Figure 10], the presence of the oncogenic *BRAF* V600E mutation drives constitutive phosphorylation of MEK1 and 2, activating ERK 1 and 2 and reprogramming cellular metabolism to sustain cell survival and growth^[166]. In major melanoma subtypes

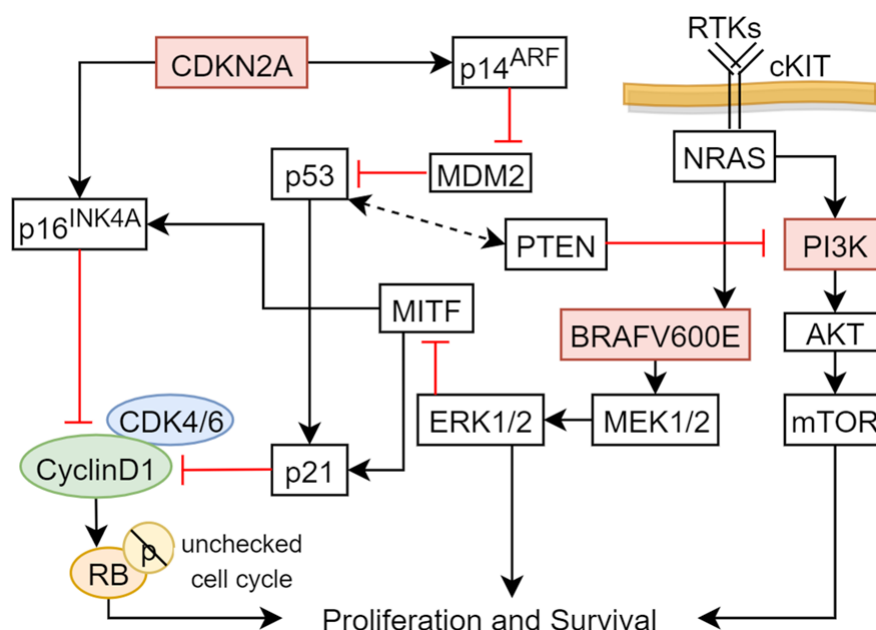


Figure 10. Significant signaling pathways in melanoma. Schematic of major pathways associated with cell survival, differentiation, and proliferation. Arrows represent active signals and red lines represent inhibitory signals. mTOR: mammalian target of rapamycin; MITF: microphthalmia-associated transcription factor

of NRAS, BRAF, and c-kit pathways, proteins expressed in the MAPK signaling pathway has been found suggesting crosstalk between pathways^[167]. In addition, several mutations of key upstream proteins of the aforementioned MAPK cascade includes NRAS, KIT, GNAQ, and GNA11^[168]. In over 80% of uveal melanomas, GNAQ or GNA11 is activated using the MAPK pathway^[169].

PI3K-AKT pathway

The neuroblastoma RAS viral oncogene (*NRAS*) is mutated in approximately 30% of melanomas^[168]. Ras binds to PI3K activating secondary messenger PIP3 and binds to serine threonine kinase AKT^[170]. An important downstream effector of PI3K-AKT is mTOR [Figure 10], which functions to initiate a cascade that inhibits autophagy^[160,171]. PTEN is an antagonist of PI3K-mediated signaling. Loss of functional tumor suppressor gene *PTEN* increases AKT phosphorylation leading to decreased apoptosis and increased melanoma survivability^[165]. *PTEN* mutation was also found in nearly 44% of *BRAF* mutated melanomas in contrast to 4% of *NRAS* mutated melanomas^[168]. These mutational patterns suggest that *BRAF* and *NRAS* mutations appear to be mutually exclusive and distinct oncogenic drivers^[170].

c-KIT inhibition

c-KIT encodes for transmembrane tyrosine kinase KIT which binds to stem cell factor and activates cellular proliferation pathways including RAS-ERK and PI3K/AKT [Figure 10]^[162,172]. KIT mutations were more frequently in mucosal, acral, and chronically sun-damaged skin melanoma than in non-chronic sun-damaged skin melanoma in the United States^[173]. Of note, KIT mutations are believed to be more frequent in non-white populations and reported in higher mutation rates in East Asian patients^[174,175].

Supporting stroma and vascularization

HIF-1 α /VEGF

Tumors require vascularization to grow, metastasize, and invade. Angiogenesis correlates with the progression of melanoma tumor growth by supplying oxygen and nutrients and providing an opportunistic route to spread into blood circulation. VEGF is an important angiogenic factor and leads to secretion of

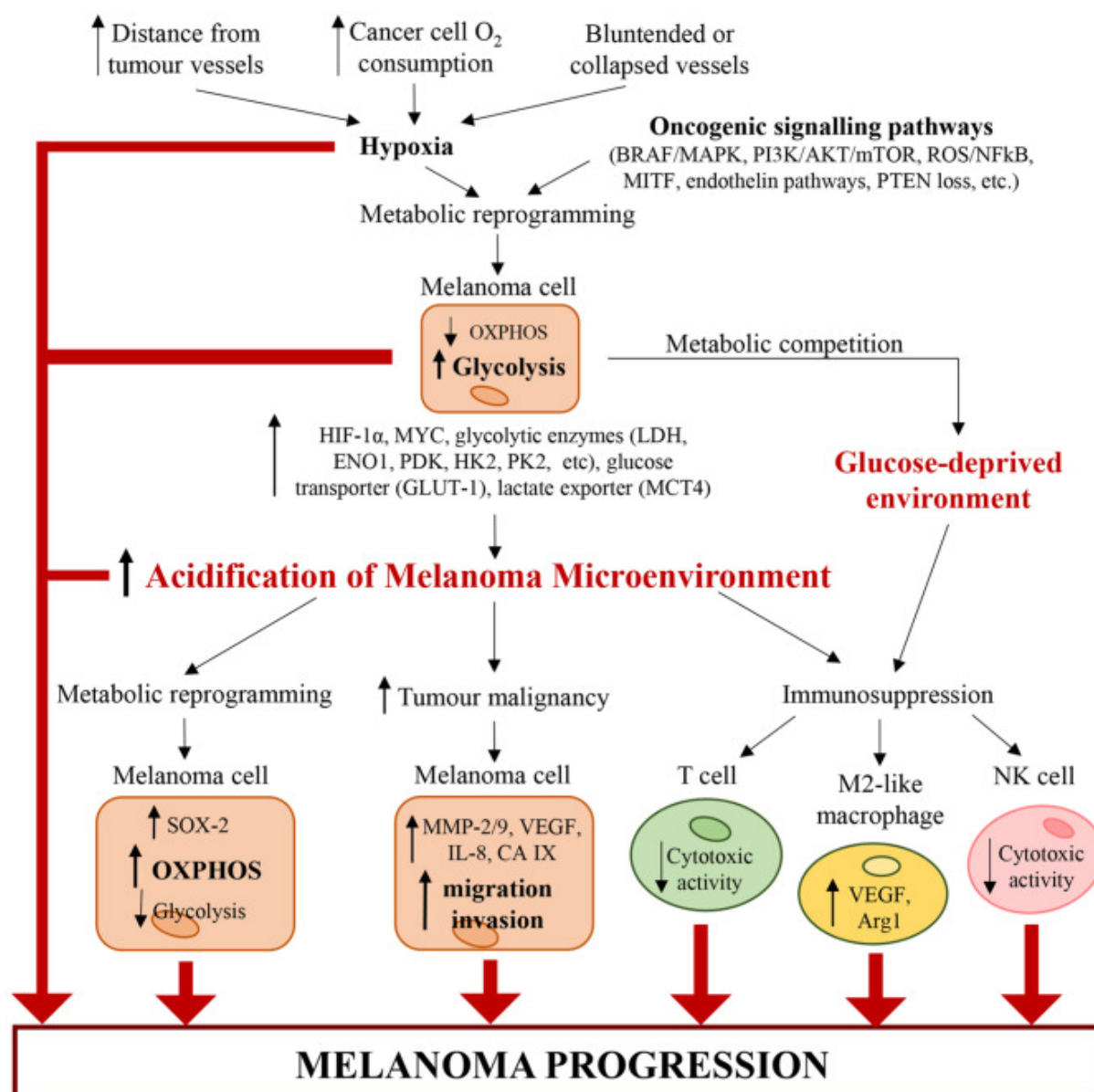


Figure 11. Multifactorial reprogramming to acidification of melanoma tumor microenvironment. The pH and oxygen microenvironment work together with genetic and metabolic pathways to promote melanoma proliferation and metastasis progression^[176]. HIF-1α: hypoxia-inducible factor-1 alpha; mTOR: mammalian target of rapamycin

MMPs degrading extracellular matrix and allowing for openings for cells to invade neighboring tissues^[174]. HIF-1α initiates VEGF-A expression and key to downregulating mitochondrial energy metabolism by switching to glycolysis^[163]. This Warburg effect of lactic acidosis contributes to the acidification of the tumor microenvironment triggering metabolic reprogramming in melanoma cells [Figure 11] with increased pro-angiogenic VEGF and altered immunosuppression^[167].

Melanocyte-lineage

Microphthalmia-associated transcription factor

The microphthalmia-associated transcription factor (*MITF*) gene is a key regulator for the development and differentiation of melanocytes. Melanoma cell phenotype switching is characterized in the ERK/MAP-kinase pathway [Figure 10] targeting MITF phosphorylation in either transient or sustained activation^[177].

Sustained ERK phosphorylation inhibits MITF keeping its levels low leading to proliferation. High MITF levels along with transient ERK phosphorylation triggers melanocyte differentiation^[178]. cAMP signaling activates HIF-1 α and increases MITF levels^[179]. HIF-1 α and VEGF promotion triggers microenvironment changes that promote melanoma progression [Figure 11]. Cellular crosstalk in signaling pathways also plays a driving role. MITF activates anti-apoptotic gene *BCL2*, promoting melanocyte survival^[180]. Cell cycle inhibitors p21 has been implicated along with p16INK4A and p14ARF [Figure 10] in the senescence of melanocytes^[181]. As the fate regulator of the melanocyte lineage, MITF activity fluctuates through modification of signaling pathways and microenvironmental dynamics.

Modulating immune response

Melanoma utilizes co-inhibitory pathways to avoid surveillance by the immune system and its ability to respond and possibly eradicate the tumor cells. Two well-characterized co-inhibitory pathways involve the cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) and programmed death-1 (PD-1) T-cell co-receptor^[182]. CTLA-4 outcompetes CD28 for B7 binding on antigen presenting cells, limiting IL-2 production and lowered survivability of the T-cell^[183]. PD-1 is induced later during T-cell activation and ligation with PDL1 and PDL2 promote tumor growth^[184]. Immunotherapy for melanoma blocks PD-1 and CTLA-4 checkpoints and elicits a therapeutic response^[183]. The role of mechanical trauma in the development of melanoma remains unclear. One retrospective analysis on acral melanoma accounting for the majority of melanoma in the Chinese population provided epidemiological evidence for potential association between trauma and acral melanoma^[185]. For dark-skinned and East-Asian populations, patients have reported direct trauma related to subungual melanoma^[186]. However, there is not enough literature to support an established correlation.

Future directions

Many patients with good prognosis indicated by early stage through traditional clinicopathologic histology staging methods still decline from metastases^[187]. Recent advances in personalized medicine could be instrumental in capturing a more accurate and detailed micro-staging of melanoma through next-generation sequencing and genetic profiling. Validation studies of the 31-GEP, which categorizes risk as low or high by class, has indicated the 31-GEP test as an accurate predictor of metastasis of cutaneous melanoma^[188-190]. Systematic meta-analysis demonstrated the 31-GEP test consistently identifies melanoma patients at increased risk of metastasis independent of clinicopathologic factors and improves on current staging^[191]. The identification of early stage low-risk patients vs. late stage high-risk stage patients would significantly help in determining life-saving adjuvant therapies or avoiding unnecessary treatments and costs. Analysis of profiles of gene expression modification induced by different signaling pathways in melanoma cells has considerable potential in understanding clinically relevant molecular mechanisms and biomarkers for non-melanoma skin cancers to create personalized treatment plans for each patient with melanoma. Unfortunately, the use of 31-GEP testing on thin melanomas is controversial. A recent article believes that the testing should not be done on people with thin melanomas if there is no evidence on change of outcome and treatment plan. Once further studies on 31-GEP testing are done, the evidence of effectiveness in thin melanomas will be apparent^[192].

Summary

Recent endeavors into understanding the pathways involved in the development and metastasis of skin cancer into melanoma has led to some degree of hope in therapy against this historically difficult to treat cancer. It is clear melanoma is not homogenous, but a complex multifactorial disease characterized by crosstalk of several different pathways and elucidation of mechanisms that contribute to tumor growth and chemoresistance. Hence, understanding these complex mechanisms is crucial and will lead to more optimal treatments for each patient.

CUTANEOUS T-CELL LYMPHOMA

Cutaneous T-cell lymphoma (CTCL) is a type of T-cell mediated dermatologic neoplasm which falls under non-Hodgkin's lymphoma^[193]. Mycosis fungoides (MF) and Sézary syndrome (SS), the most frequently diagnosed forms of CTCL, have become some of the most researched forms of non-melanoma skin cancers. CTCLs are often misdiagnosed as benign skin conditions, such as atopic dermatitis or psoriasis^[194]. Understanding the various signaling pathways of CTCL is conducive to forming proper treatment plans and prognoses. The goal of this section is to discuss the pathways in which CTCL arises and the treatment options utilized for this form of non-melanoma skin cancer.

There are various theorized and studied pathways in which CTCL arises. The majority of these cases do not progress to systemic malignancy, rather, it remains a mostly cutaneous cancer. Clinical features of CTCL can be fairly variable. From erythrodermic exanthems to flesh colored papules, CTCL can present in a number of cutaneous manifestations. There are a few exogenous factors to note that have been studied to have an antigen induced CTCL reaction. A common pathway in which CTCL evades the body is via the antigen-antibody mechanism. In the antigen-antibody mechanism, T-cells are consistently exposed to specific antigens resulting in proliferation and failure to induce cellular apoptosis^[195]. Not only are medications thought to be provoking factors in antigens causing T-cell proliferation, but viral, bacterial, and fungal antigens are also factors causing uncontrolled T-cell proliferation^[195]. Some of these viral antigens include human T-cell lymphoma virus, Epstein-Barr virus, and herpes simplex virus. In addition, methicillin resistant *Staphylococcus aureus* (MRSA), *Mycobacterium leprae*, *Chlamydia pneumoniae*, and even fungal dermatophytes can be inducing factors causing CTCL^[196,197]. Furthermore, there is an association between vitamin D deficiency, and even vitamin D receptor single nucleotide polymorphisms, causing deficiencies within the system. Vitamin D is important for the function of many immune cells including those of T cells. Paracrine communication between B and T lymphocytes, *Cathleidin* gene regulation and innate immunity all function with the use of Vitamin D. Without properly regulated innate and adaptive immunity, *Staphylococcus aureus* and other bacteria and viruses have a higher probability of colonizing the skin while continuously priming and activating T lymphocytes. As mentioned earlier, *Staphylococcus aureus* has a known association with CTCL meaning that Vitamin D and polymorphisms in the VDR can indirectly have an association with CTCL^[198].

Some studies have found association between atopy and CTCL with an IgE increase found in biopsies. However, there is a need for further research in the subject of atopy and allergen-induced CTCL.

Extrinsic exposures

Various studies have determined that if the antigen producing agent is removed, then the CTCL response will eventually eliminate itself. When comparing patients with CTCL and hypertension taking hydrochlorothiazide to those with CTCL that are not taking hydrochlorothiazide, complete clearance or improvement of their CTCL cutaneous lesions has been observed when hydrochlorothiazide is withdrawn^[195]. What is most important is that it is understood the different ways in which CTCL can be treated. Disruption of specific pathways via monoclonal antibody addition, as well as removing an antagonizing antigen, are just a couple examples in how this research has allowed for the elimination of this cutaneous neoplasm.

MRSA has also been found to be an insult leading to CTCL. Moreover, the treatment of MRSA can lead to improvement of CTCL. Patients with erythrodermic CTCL were discovered to have a decreased body surface area affected when being treated for a MRSA infection^[196]. This finding leads to the conclusion that patients with erythrodermic CTCL have obtained a coinfection MRSA. Once treated, the cutaneous manifestations dramatically decrease, thus, it is recommended that patients with CTCL be empirically treated for a MRSA infection^[196].

While many of the signaling pathways discussed are being studied to better understand CTCL progression and initiation, there are various other pathways continuing to be discovered and evaluated in association with this cutaneous neoplastic disease. Cutaneous T-cell lymphoma is a result of malignant T-lymphocytes proliferation and invasion^[194].

Apoptosis evasion

Epigenetics

Many of the identified common mutations in CTCL are in genes coding for proteins that alter epigenetic factors, including AT-Rich Interaction Domain 1A (*ARID1A*) and *DNMT3A*^[199]. *ARID1A* alters histone structure, exerting its effects through epigenetic mechanisms. Mutations in *ARID1A* have led to the downregulation of *PTEN* in other types of cancer^[200]. The effect of *ARID1A* downregulation leads to decreased apoptosis and uncontrolled cell growth between the G1 and S phase of the cell cycle. *DNMT3A* is a gene that encodes for DNA methyl transferase 3 α , and mutations in *DNMT3A* are linked to aberrant cytosine methylation and potentially the downregulation or silencing of important oncogenes^[201].

One commonly researched target for CTCL is the NOTCH family of proteins. NOTCH1 activity is increased in both SS and tumor stage MF. The activity of NOTCH1 is typically upregulated through increased levels of binding ligand, Jagged1. Jagged1 ligand binds through a juxtacellular mechanism to NOTCH1. NOTCH1 is then cleaved into ICN1 by proteases. ICN1 exerts downstream effects, one of these being the expression of hairy and enhancer of split-1 (HES1) [Figure 12]^[202]. In tumor stage MF, the suspected upregulated ligand is Jagged1. This is thought to occur through an epigenetic pathway involving DNA hypermethylation of the miR-200c promoter region^[202]. miR-200c can function as an oncogene that inhibits activation of NOTCH1 through decreasing expression of the NOTCH1 ligand Jagged1. However, hypermethylation in the promoter sequence of miR-200c decreases expression of miR-200c is seen in MF. This results in increased NOTCH1 activity through an increase in levels of Jagged1^[202]. Overactivity of NOTCH1 has been linked to decreased expression of *PTEN* and degradation of p53 in T-cell acute lymphoblastic leukemia^[203]. In addition, HES1 expression is also linked to the upregulation of the NF- κ B pathway in T-cell acute lymphoblastic leukemia^[204]. There is a need for more research on the cause of hypermethylation of these miRNA regions that cause downregulation.

NOTCH1 is also linked to the AKT pathway through *PTEN* and p53 regulation by miR-122. miR-122 activates AKT^[205]. TP53 mutations are found in CTCL^[199]. miR-122 is primarily known for causing apoptosis within hepatocytes, yet recent studies have found that miR-122 is also found throughout human skin, especially in patients with MF. Lesioned skin has been found to express higher levels of miR-122 in patients with MF and SS compared to healthy skin. Though the normal function of miR-122 is found to induce apoptosis in hepatocytes, when upregulated, miR-122 blocks apoptosis. Cells with upregulation of miR-122 have been discovered to have phosphorylated AKT and downstream transcription factors in the pathway. As a result, AKT is then overactivated, leading to decreased apoptosis from increased TP53 inhibition. This lack of apoptosis leads to the uncontrolled cell growth of cutaneous T-cells^[205].

Malignant cell growth

Mutations upregulating the Janus kinase (JAK) and signal transducer and activator of transcription proteins (STAT) are implicated in CTCL. JAK1, JAK3, STAT3, and STAT5B are mutated in certain CTCL cell lines^[206]. Mutations in JAK1, JAK3, and STAT5B have also been implicated in T-cell prolymphocytic leukemia^[207]. The effects of JAK/STAT signaling in CTCL are thought to be driven partially by the production of cytokines and angiogenic factors^[194]. Generally, the mechanism of JAK/STAT signaling is as follows. Specific ligands or cytokines bind to the RTK, causing activation and dimerization of the receptor. This dimerization allows JAK proteins to come into close proximity to each other. JAK-JAK phosphorylation occurs, and JAK then phosphorylates STAT proteins. STAT proteins then dimerize and translocate to the nucleus, where they act as transcription factors [Figure 12]^[208].

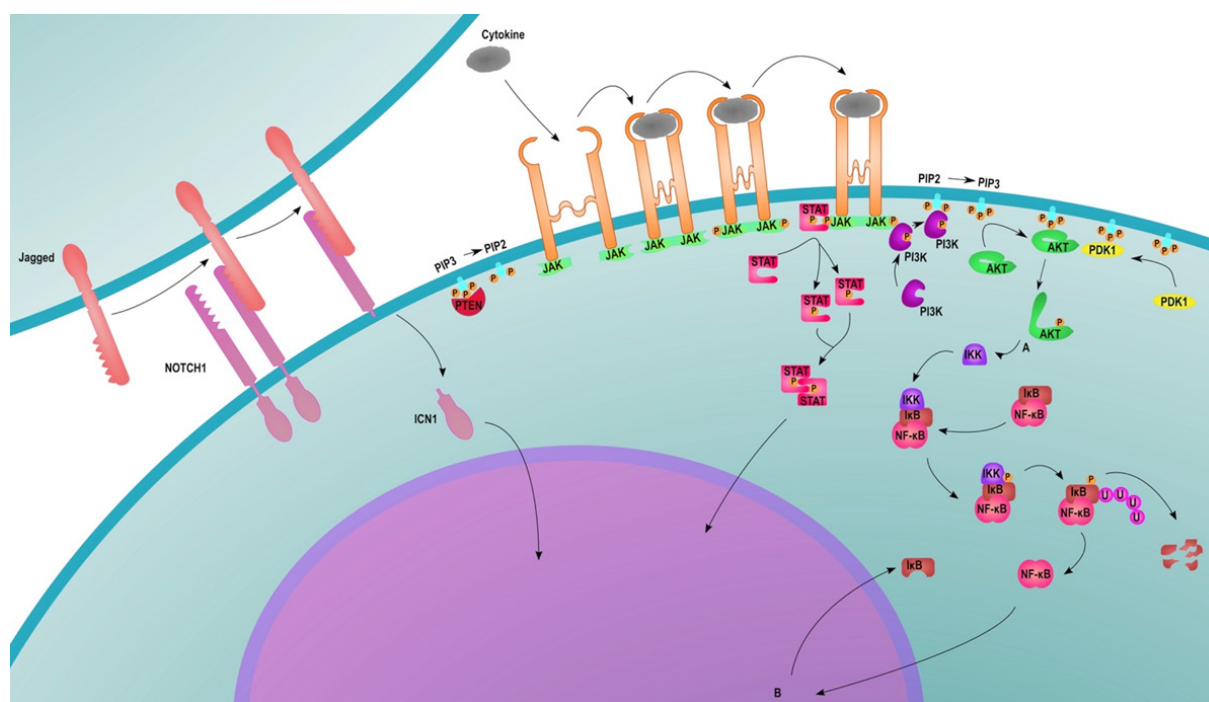


Figure 12. Common pathways in cutaneous T-cell lymphoma. This figure describes the genetic signaling pathway of NOTCH1. NF-κB: nuclear factor-kappa B; JAK: Janus kinase

Activated JAK is also known to activate PI3K, resulting in eventual activation of AKT [Figure 12]^[208]. Activation of AKT has also been implicated to occur in CTCL through mutations in the PI3K pathway relating to TCR-CD28^[209]. Additionally, loss of heterozygosity in PTEN, the negative regulator of PI3K, have been observed in MF, and PTEN activity is shown to be altered in CTCL^[194]. The PI3K pathway is explained in greater detail in the SCC section of this paper. Activated JAK is also known to activate PI3K, resulting in eventual activation of AKT [Figure 12]^[208]. Activation of AKT has also been implicated to occur in CTCL through mutations in the PI3K pathway relating to TCR-CD28^[209].

NF-κB pathway activity has also been shown to be increased in CTCL^[206]. The canonical NF-κB pathway occurs as follows. IκB kinase (IKK) acts to phosphorylate the IκB element of NF-κB-IKB. Phosphorylated IκB is ubiquitinated, which allows for the degradation of IκB by proteasomes. NF-κB is then able to enter the nucleus to act as a transcription factor^[204]. One of the effects of NF-κB signaling is an upregulation of its negative regulator IκB. This serves as a negative feedback mechanism of NF-κB signaling [Figure 12]^[210]. The NF-κB pathway is also linked to the activation of AKT. One of the actions of AKT is the phosphorylation of IKKα, which is part of the IKK complex of the canonical NF-κB pathway [Figure 12].

The non-canonical NF-κB pathway is implicated also in CTCL. Mutations are seen in the C terminal of the gene *NFκB2*, which is an area encoding for autoinhibitory functions in NF-κB2. Typically, the C terminal of inactive NF-κB2 assists in suppressing the canonical NF-κB pathway. Mutations in the *NFκB2* gene are, therefore, thought to contribute to activation of the canonical NF-κB pathway in CTCL^[199].

The genes *phospholipase C, gamma 1* (*PLGC1*), and *caspase recruitment domain family member 11* (*CARD11*) are amplified in CTCL and are thought to contribute to abnormal TCR signaling^[199]. *PLGC1* activates the NF-κB pathway and nuclear factor of activated T-cells through diacylglycerol and inositol 1,4,5-trisphosphate (IP3), respectively^[211]. *CARD11* is linked to the induction of the NF-κB pathway, although the mechanism of action is not yet completely understood^[212].

Other mutations in tumor suppressor genes, *CDKN2A*, *TNF- α Induced Protein 3 (TNFAIP3)*, *zinc finger E-box binding homeobox 1 (ZEB1)* and *FAS*, have been identified in CTCL^[199]. TNFAIP3 protein works to inactivate IKK when the NF- κ B pathway is overactivated by TCR^[213]. ZEB1 is a zinc finger transcription repressor protein, and the loss of ZEB1 in CTCL is linked to the overexpression of cytokines like IL-2. Mutations in *ZEB1* also increases the expression of *GATA3*, which binds to the promoter site of the gene encoding for thymocyte selection-associated HMG box protein (TOX)^[199,214]. TOX is a mediator of T-cell maturation from CD4⁺ and CD8⁺ to CD4⁺^[214]. The loss of *FAS* in CTCL cell lines most likely also contributes to apoptosis resistance^[199].

As mentioned previously, the pathogenesis and progression of CTCL are believed to be influenced by alterations to the local cytokine milieu. These changes are thought to occur in part due to dysfunction in CXCR4^[215]. CXCR4 mediates downstream effects of various receptor tyrosine kinases, one of which is TCR. When TCR is activated, CXCR4 is able to ligate with TCR, triggering a process that enhances the stability of mRNA transcripts for IL-2, IL-4, and IL-10. This process occurs in the presence and in the absence of CXCL12, the sole endogenous ligand for CXCR4. Furthermore, blocking the association between CXCR4 and TCR decreases mRNA transcript stability for both TCR-dependent and TCR-independent cytokine production in SS cell lines, without altering other downstream effects of TCR^[215].

Supporting stroma and vascularization

More recently, CTCL progression via angiogenesis is being studied. Angiogenesis via the VEGF pathway results in the congregation of tumor cells^[216]. The stromal involvement of CTCL involves a CXCL12^[216]. The stromal factor and its adjacent receptor work to create a signaling network for bone marrow stem cells, which is found in earlier disease states^[216].

Summary

Though there is a need for further research on CTCL, there are many treatment options available. Historically, first line treatment for CTCL has been psoralen and UVA (PUVA) therapy^[217]. When used in conjunction with interferon- α , efficacy has been shown to increase due to elevated levels of apoptosis^[217]. PUVA upregulates p53 allowing increased apoptosis within the tumor cells^[217]. With the addition of interferon- α , apoptosis is increasingly regulated via the JAK1 signaling pathway^[217].

Brentuximab vedotin is also a potential treatment for CTCL^[218]. This monoclonal antibody has been shown to have efficacy against refractory cases of CTCL, as well as peripheral T-cell lymphoma^[218]. During the studies performed using this medication, it was found that success is greatest in those CTCL cases where CD30⁺ positive cells are present. CD30⁺ falls within the TNF receptor class, which is involved in cell proliferation^[218]. Thus, explaining the mechanism of this monoclonal antibody to inhibit specifically CD30⁺ expression^[218]. Another monoclonal antibody used in the treatment of MF and SS is Alemtuzumab^[219]. Thus, research of immunomodulatory treatments is on the rise for CTCL leading to personalized treatments targeted to the genetic signaling pathways, which are dependent on the person's specific flux state.

CONCLUSION

Melanoma and non-melanoma skin cancer are some of the most prevalent cancers that can lead to death if not properly managed. However, knowing the genetic signaling pathways of these conditions can further help researchers and providers give more options to patients suffering from these conditions. With this information, the provoking genetic factors can be blocked with the use of pharmaco- and immunotherapy allowing for patients with treatment resistant, locally advanced and even metastatic lesions to be treated due to the different options of therapy blocking multiple signaling pathways. Though some information is known about the genetic pathways of cutaneous malignancies, there is still a demand for research that can be done to improve treatment options to decrease mortality rate and recurrence.

Initiators and promoters

There are different provoking aspects that cause cutaneous malignancies. Promoters are products or a low-level exposure a carcinogen that can begin the process of tumorigenesis but not quite reach the threshold to create full DNA mutations. Exposome is the combined exposure to all factors that cause internal chemical environment change^[220]. Promoters, however, can change gene expression that leads uncontrolled growth. Initiators are factors that begin the process of DNA mutation and alteration in structure. It is not the exposure to one carcinogenic chemical or product that immediately causes DNA mutation and tumorigenesis, but it is the initial exposure that primes the tumor cells to change the function of certain genes. Eventually, the exposome can promote tumors to form^[220]. It is also not just the exposure to one carcinogen in low doses but the repeated exposure to multiple carcinogens. In the case of cutaneous malignancies, solar UV is seen as a carcinogen that can either work as a promoter, an initiator, or both a promoter and initiator depending on the amount of exposure and protective measures. Moreover, studies have found that children exposed to the high-level UV from Western Australian sun have been found to have varying risk of cutaneous malignancies. Patients that arrive to Western Australia prior to age 10 have a 50% increased risk of dysplastic nevi or melanoma compared to those who arrive after the age of 10 regardless of their time spent sunbathing. This factor is due to the solar UV in Western Australia being higher of many other countries from where patients immigrate. Solar UV acting as an initiator and/or promoter for cutaneous malignancies explain the importance of protecting the skin from solar UV at all costs regardless of Fitzpatrick skin type. There is a need for further research to find other carcinogens that can act as initiators and/or promoters that to help patients prevent from these cancer-causing factors^[221].

Effects of mi-RNAs

Genetic testing and gene therapy might be the future for patients with cutaneous malignancies. As previously stated, Castle Biosciences has already created testing to further determine staging and implication for sentinel node testing for melanoma and metastatic risk for SCC. However, the genetic testing does not stop there. miRNA plays an important role in signaling pathways determining the activation and suppression of specific genes for transcription factors. As mentioned in the previous sections, miRNAs can cause hypomethylation of genes leading to increased transcription, translation, and uncontrolled proliferation. Many of these genes are upregulated and downregulated having different effects on the genes; these effects can also lead to the appearance and progression of these cutaneous cancers. Moreover, SCC, VC, and CTCL have all been found to be incited by miRNAs. There are hundreds to thousands of miRNAs that can transform the function of genetic pathways and transcription factors from a normal functioning pathway to a malignant pathway. With this information, miRNAs can be a subject of research to help further test patients and possibly place them in complete remission as well as prophylaxis to prevent the next cutaneous malignancy from appearing.

Further genetic testing

In addition to genetic testing and therapy, there are many more topics within genetics that have room for further research. Many of the genetic tests performed are through somatic genetic testing and germline genetic testing. The germline testing is done through either saliva or lymphocytes. However, studies have found that within the saliva of patients with systemic lupus erythematosus, their salivary DNA has adapted the DNA of their food products^[222]. “You are what you eat”. This information can be helpful; however, with determining the somatic genetic susceptibility of cutaneous malignancies, there must be a way to determine the difference between the genetic makeup of the food consumed and that of the patient’s unmutated, unincorporated somatic cell DNA and RNA. This can be a problem with lymphocytes as well. T- and B-lymphocytes have specific genetic makeup that turns them into the subtype of mature T- and B-lymphocytes. Yet, when T- and B-lymphocytes are presented with a specific antigen or allergen, their genetic makeup can also transform to program the lymphocyte for either attack of the antigen or produce antibodies towards the antigen, respectively. Additionally, lymphocytes can change their genetic code

depending on their antigen priming, thus cancer cells may promote a change in lymphocyte DNA to avoid recognizing the cells as foreign^[223]. There also will need to be consideration for the use of inactive mature B- and T-lymphocytes that have not yet been presented with antigen compared to that of the lymphocytes that have been presented with an antigen. It is possible that the human body is a state of active dynamic mosaicism with genes turning on and off that have adapted genetic material to food products and other antigens within the body. This information can ensure further accuracy when determining the somatic genetics of patients with cutaneous malignancies and help with their treatment and prognosis.

Cellular electrical charges

Another important factor to consider with skin cancer genetics is that tumors tend to also have an electrical charge as do many cells within the body. This is also a way to evade immune system attack. Tumor cells are negatively charged. Lymphocytes that have been primed with tumor antigen are also negatively charged. In the world of chemistry, similar charges are opposed to each other while opposite charges attract each other. However, antibodies are positively charged. The positive charge of antibodies assists with T-helper lymphocyte eradication of tumor cells. However, it is known that natural killer cells and CTLs are important in immune killing of tumor cells, so there is more research that needs to be done in terms of the effect of charge between immune cells and tumor cells as well as on genetic signaling.

Communication between immune system and nervous system

The central and peripheral nervous systems are important for regulating the systems in the body. In fact, the nervous system directly affects the function of the immune system against fighting malignancies. Chronic exposure to stressful events stimulates the adrenal gland, a neuroendocrine organ, to release sympathetic nervous system stimulating hormones which disturbs the normal function and balance of the immune system. In addition, this stress can also increase lymphatic vascularization in current tumors that increases the number of chemokines released and promotes diapedesis of tumor cells. It is important to note and follow patients who have chronic stressors without stress relief exercises to maintain homeostasis. This neuroendocrine modulation also can influence the appearance of cutaneous tumors. As a result, decreasing stressful events in the lives of patients can help prevent cutaneous malignancies from occurring^[224].

The body as a flux state

It is important to remember that tumors are not always a collection of cells that function the same way undergoing the same processes at the same time. Many of these tumors function as organs, like that of the heart or lungs with various cell types and cell functions. Likewise, there can be tumor cells that are evading apoptosis by downregulating tumor suppressors, other cancer cells that are promoting cell growth through the mutation of oncogenes while others are invading the stroma by damaging the underlying adhesive molecules and releasing proteinases. In addition, cancer cells also can communicate amongst each other with autocrine and paracrine signaling. This signaling can activate specific genetic pathways to even mutate a normal, non-cancerous cell to that of a cancerous cell and change the genetic pathway of those that are already malignant. All these processes can occur simultaneously especially in the faster growing, more aggressive types of skin cancer. This hypothesis can explain why there are patients that can go into complete remission from a treatment type and others are treatment resistant. The multiple genetic signaling pathways activated, crosstalk and switching from one genetic pathway to another can be the reason as to why there is treatment resistance. Tumor cells, like immune cells, may have an ability to release chemokines leading to autocrine and paracrine communication between the cells. This process can also form a communication system between the cells telling each of them their function and even invade benign cells to become malignant. Adjuvant therapy might be considered in the earlier stages of cutaneous malignancies depending on the number of genetic pathways that are discovered within these tumor cells. These discoveries give providers further information to continuously perform full body skin examinations and surveillance patient chronic inflammatory conditions for transformation into malignant conditions.

Furthermore, the incorporation of artificial intelligence data analysis of trends between the various pathways can help improve management of cutaneous malignancies by clinicians. Once these conditions are elucidated, oncologists can develop prospective personalized dynamic genetic testing for identifying the critical set of active oncogenetic pathways that need to be disrupted at particular moments. This process would then transform the lives of individual patients suffering from multiple cutaneous malignancies around the world.

DECLARATIONS

Authors' contributions

Wrote, final edited and revised the manuscript: Maner BS, Dupuis L, Su A, Jueng JJ, Harding TP, Meisenheimer VII J, Siddiqui FS, Hardack MR, Aneja S, Solomon JA

Contributed to [Figure 10](#): Su A

Contributed to [Figures 1, 2, 3, 6, 7, 8, 12](#) and reference organization: Meisenheimer VII J

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

None that may be perceived as influencing the representation or interpretation of review other than Dr. James A. Solomon was a clinical investigator of Vismodegib clinical trials and expanded access program-funds paid to his employer and has been a paid consultant by Genentech, Sun Pharmaceuticals, Mayne Pharmaceuticals.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Editorial

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Special issue on “Immunotherapy of cancer: future possible therapy for metastatic cancer”

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How to cite this article: Potdar PD. Special issue on “Immunotherapy of cancer: future possible therapy for metastatic cancer”. *J Cancer Metastasis Treat* 2020;6:38. <http://dx.doi.org/10.20517/2394-4722.2020.75>

Received: 22 Jul 2020 **Accepted:** 22 Jul 2020 **Published:** 27 Sep 2020

Academic Editor: JCMT Editorial Office **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

The global burden of cancer incidence is estimated to be up to 18.1 million people with 9.6 million deaths in 2018. The major cause of death is due to the high toxicity of chemotherapeutic drugs and drug resistance developed by cancer cells. Thus, there is an unmet need for appropriate therapy which can kill cancer cell alone and reduce drug resistance. Immunotherapy is an alternative therapy for cancer which mainly boosts the immune response of these patients and kills cancer cells alone with fewer side effects. Since Professor James Allison from M.D. Anderson Cancer Centre, Texas, USA, received the Noble prize for his outstanding contribution to the field of immunotherapy, there has been great excitement in this field. Immunotherapy has become a powerful branch of cancer therapy for the treatment of various types of cancers due to milder side effects than the presently available chemotherapy or radiotherapy. It mainly works by strengthening the host immune responses against tumor cells by supplementation of modified immune system components. There are four different types of immunotherapies: monoclonal antibodies, cancer vaccines, immune checkpoint inhibitors, and Chimeric Antigen Receptor-T cell therapies. Immunotherapy can be given in combination with mild doses of chemotherapy or radiotherapy obtain effective and non-toxic treatment for most cancer patients.

With the development of several innovative technologies in the field of cancer medicine, molecular biology, stem cell biology, and bioinformatics, there has been a rapid increase in our understanding of the human immune system. Similarly, there is an extensive program planned to discover small molecules, peptides, recombinant antibodies, vaccines, and cellular therapeutics, which are being applied to manipulate the immune response of cancer patients to cure cancer. These immune therapies have provided significant



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benefits against cancer, especially the application of immune checkpoint inhibitors, cell therapies, and vaccines. In this Special Issue on immunotherapy of cancer, we have invited pioneer scientists and clinicians involved in the latest work in this area to contribute their original manuscripts, case reports, clinical studies, and reviews that focus on immunotherapy for curing cancer.

There is an interesting paper written by Dr. Thibault Gauduchon from Lyon France, who explained that there is a great improvement in the cure of squamous cell carcinoma of head and neck cancer by changing the therapeutic management with immunotherapy using various checkpoint inhibitors or small molecules. They further highlighted that the checkpoint inhibitors are the first to be validated in second-line treatment with programmed death-1 and programmed death-ligand 1 (PD-L1) inhibitors to increase their effectiveness. The major goal of this paper by Dr. Gauduchon et al. is to provide an update on the possibilities of current immunotherapy in the management of Head and Neck Squamous Cell Carcinoma.

Snaar-Jagalska et al. from Leiden University, Netherlands, demonstrated the importance of inhibition of CXCR4 signaling in cancer metastasis by using the zebrafish xenograft model as a translational tool for anti-cancer discovery. This is a very important paper for scientists who are working on experimental cancer biology for drug discovery programs in cancer immunotherapy. Finally, they addressed the translational impact of targeting CXCR4 signaling in the tumor microenvironment for the treatment of metastatic cancer.

Matthew Spector et al. from the University of Michigan, Michigan, USA, reviewed novel immunotherapeutic approaches in head and neck cancer. They further stated that pembrolizumab is now approved as first-line immunotherapy for head and neck cancer. However, there are still low response rates in the cure of this cancer and therefore additional strategies need to be implemented. This review summarizes the most common immune disruptions, checkpoint inhibitors identified in head and neck cancer, and discusses ongoing approaches aimed at targeting the tumor immune microenvironment.

Professor Giovanni LO Re, a well-known oncologist from CRO Oncology Pordenone Aviano, Italy, documented his experience with an exceptional young inoperable patient for LA head metastatic pancreatic ductal adenocarcinoma (PDAC), treated with seven cycles of Nab-Paclitaxel-Gemcitabine (Nab-PCT-GEM) and RT 50 Gy/15 fractions combined to biweekly GEM and salvage FOLFIRINOX having five-year survival. He further stated that this is because this patient has travelled 15,000 km on foot, suggestive that his “walking therapy” may have helped this PDAC patient to survive more time. Dr. Giovanni well explained how a physical activity such as walking has helped in boosting the immunity of PDAC patients and increased survival rate. It is an interesting observation to note for many oncologists who are treating PDAC and other types of cancer: by implementing this simple strategy in their patients, they may have better survival.

In this Special Issue, we are lucky to have received a paper from Professor Porunelloor A. Mathew from UNT Health Centre, Texas, USA, who reviewed his experience working on Lectin-Like transcript 1 (LLT1) as an immunotherapeutic target for triple-negative breast and prostate cancer. Dr. Mathew suggested that blocking inhibitory signals to NK cells using monoclonal antibodies to LLT1 could enhance the lysis of prostate cancer and triple-negative breast cancer (TNBC) cells by NK cells. His lab further demonstrated that higher expression of LLT1 in TNBC and prostate cancer cells increased lysis of cancer cells after blocking LLT1 with monoclonal antibodies and may offer a potential target for breast and prostate cancer treatment. I hope this will be useful to many of our special issue readers when implementing this work in the cure of prostate and triple-negative breast cancers.

In this special issue, we included an article from Professor Kimberly Webster, a Medical Social Science expert from Northwestern University, Feinberg School of Medicine, USA who developed the Functional

Assessment of Chronic Illness Therapy (FACIT) item library with patient-reported adverse events associated with immune checkpoint modulators (ICM). This FACIT item library will allow clinicians to monitor the value of ICM treatment for patients with cancer by looking at the clinically relevant measurement of symptoms. Thus, the use of item selection from FACIT item library will be encouraged in clinical research and clinical practice evaluations.

We are fortunate to obtain a very interesting review article on immune checkpoint inhibitor therapy in EBV-associated Gastric Cancer (EBVaGC) from Dr. Frank J. Slack from Beth Israel Deaconess Medical Centre, Harvard Medical School, Boston, USA. Several studies have shown that EBVaGC is commonly characterized by high lymphocytic infiltration in the tumor microenvironment, coupled with overexpression of immune checkpoint-related genes, including PD-L1. However, the regulation of PD-L1 expression in EBVaGC is poorly understood and further studies are needed to explain how EBV and host factors contribute to it. Dr. Slack highlighted that EBVaGC is a strong candidate for immune checkpoint blockade therapy after proper research in this area.

Thus, overall, we have to state here that this special issue on Immunotherapy of Cancer has included a number of original research articles, clinical studies, and systematic reviews of cancer immunotherapy from different angles contributed by various distinguished scientists and clinicians from prime institutes and medical centers all over the world. We hope that this special issue can provide valuable information to cancer researchers, clinicians, and academic professors for the enhancement of knowledge in the field of immunotherapy as well as better therapy of metastatic cancers.

DECLARATIONS

Acknowledgments

We would like to thank all the authors of this special issue for their excellent research stories and stimulating ideas. We would also like to express our great appreciation to all the special issue reviewers and editors, whose efforts substantially contributed to the improvement of the overall quality of this issue.

Authors' contributions

Wrote and reviewed the manuscript: Potdar PD

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

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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Exosomes as a promising diagnostic tool in head and neck squamous cell carcinoma?

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How to cite this article: Pastor K, Benecke L, Muller L. Exosomes as a promising diagnostic tool in head and neck squamous cell carcinoma? *J Cancer Metastasis Treat* 2020;6:39. <http://dx.doi.org/10.20517/2394-4722.2020.57>

Received: 7 Jun 2020 **First Decision:** 30 Jun 2020 **Revised:** 27 Aug 2020 **Accepted:** 14 Sep 2020 **Published:** 24 Oct 2020

Academic Editor: Robert J. Griffin **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Head and neck squamous cell carcinoma (HNSCC) is the 6th most frequently diagnosed malignancy and accounts for about 5% of all malignancies worldwide. There is a lack of biomarkers to monitor the status and progress of the disease. Therefore, it is of great importance to develop non-invasive diagnostic tools such as exosomes that monitor tumor changes and provide molecular information about the malignancy to identify the metastatic disease earlier and allow better therapeutic management. Thus, we aimed to review whether tumor-derived exosomes can predict disease progression in HNSCC and if and especially how they can be used as a diagnostic tool.

Keywords: Exosomes, head and neck squamous cell carcinoma, tumor-derived exosomes, liquid biopsy

CONSIDERATIONS ABOUT EXOSOMES AS BIOMARKERS IN HNSCC

Exosomes are virus-sized extracellular vesicles ranging from 30 to 150 nm in diameter that originate from the endosomal compartment of most eukaryotic cells^[1-4]. By fusion of cytosolic multivesicular bodies with the cell surface, they are released into the extracellular environment. Detectable in biological fluids such as serum/plasma, urine, saliva, cerebrospinal fluid, and cell culture medium, exosomes accumulate in the tumor microenvironment since tumor cells are highly active in producing exosomes^[5]. HNSCC patients with early or advanced disease severity show a significantly higher exosome number in their plasma in comparison to healthy patients^[6,7]. We can compare exosomes to hemerodromes in ancient Greece^[8].



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Pheidippides, one of the first marathon runners in 490 BC, is said to have run from Marathon to Athens acting as a messenger to provide news of the victory of the battle of Marathon. He ran approximately 240 km in two days from Athens to Sparta. Similar to the hemerodromes, exosomes serve as an intercellular communication system between different cells^[9]. Pheidippides died after completing his mission. Most probably, the exosomes are depleted as well once the message has been sent^[8]. They are produced by all cell types, both healthy and malignant cells^[9]. They contain a variety of functional proteins and genetic cargo such as DNA, mRNAs, miRNAs, and long non-coding RNAs.

Tumor-derived exosomes (TEX) share the characteristics of their parental cell but are known to enrich certain factors. It is thought that there is a new chapter in biology behind this: exosomes seem to be part of a messaging system and can deliver specific signals to distant cells^[10]. Because of these features, exosomes are ideal candidates as non-invasive biomarkers, also called “liquid biopsy”^[10]. It is essential to mention that exosome levels rise during pathological changes and decrease during convalescence^[11], and therefore, their protein content is highly informative^[12]. Thus far, therapy monitoring tools lag in oncology. As such, PET/CT-scan is said to be one of the most sensitive imaging modalities in clinical routine: cancer detection is only visible on the scan above 4 mm, and it cannot be performed monthly. Besides, other current diagnostic tools such as endoscopy are invasive and may put the patient at risk of potential complications. These facts point towards the use of exosomes as a diagnostic tool as non-invasive biomarkers to set the patient at minimal risk^[11].

TEX are highly enriched in HNSCC patients' plasma. They carry several immunosuppressive proteins and molecules, facilitating the epithelial-to-mesenchymal transition (EMT). Recent analysis alludes that cancer therapies can affect the morphology and behaviour of tumor cells, somewhat encouraging rather than inhibiting EMT. Moreover, TEX participate in the regulation of EMT, as shown by Franzen *et al.*^[13], Min *et al.*^[14] and Theodoraki *et al.*^[15]. Most of the experiments on showing exosome involvement in EMT have been conducted with cell lines or in mouse models. Besides, it has been shown that TEX isolated from HNSCC patients' plasma are highly immunosuppressive. TEX provoke immune cell dysfunction by acting on immune cells through several mechanisms: they hinder the functions needed for antitumor responses, programmed cell death of activated T effector cells, proliferation of suppressive activity in regulatory T and B cells, manipulation of cellular differentiation and transfer of cells to the tumor^[16]. They bear immune checkpoint proteins such as PD-1, PD-L1, and CTLA-4. PD-L1 exosomes produced by either normal or tumor cells can alter immune responses and therefore affect disease activity^[17]. Since PD-1 and especially PD-L1 inform about disease activity, it proves that exosomes can function as disease biomarkers.

TEX portray the communication network of the tumor to trigger autocrine, juxtacrine, and paracrine signaling between several cells^[12]. They convey autocrine signals that lead to tumor progression. Furthermore, they induce the production of cytokines, chemokines, growth factors and tumor necrosis factor alpha (TNF- α). These factors represent juxtacrine and paracrine signaling which is the communication with immune cells infiltrating tumors and stimulate the production of soluble factors enhancing tumor growth^[12].

There is an immune response modulation from exosomes released by cancer cells. These exosomes carry damage-associated molecular patterns (DAMPs) such as DNA and RNA to myeloid cells as shown by Kurywachak *et al.*^[18]: “Cancer cells release exosomes that carry DAMPs such as DNA and RNA to myeloid cells which activate the intracellular virus-sensing pathways cyclic GMP-AMP synthase - stimulator of interferon genes, retinoic acid-inducible gene I, and absent in melanoma 2 (AIM-2), and stimulate the production of inflammatory cytokines such as interleukin (IL)-6, TNF- α , IL-8, and IL-1 β .”^[18]

Additionally, cancer exosomes have the characteristic to generate new vessel formation, which allows an influx of oxygen and nutrients and waste removal and impact the metabolic reprogramming of cancer

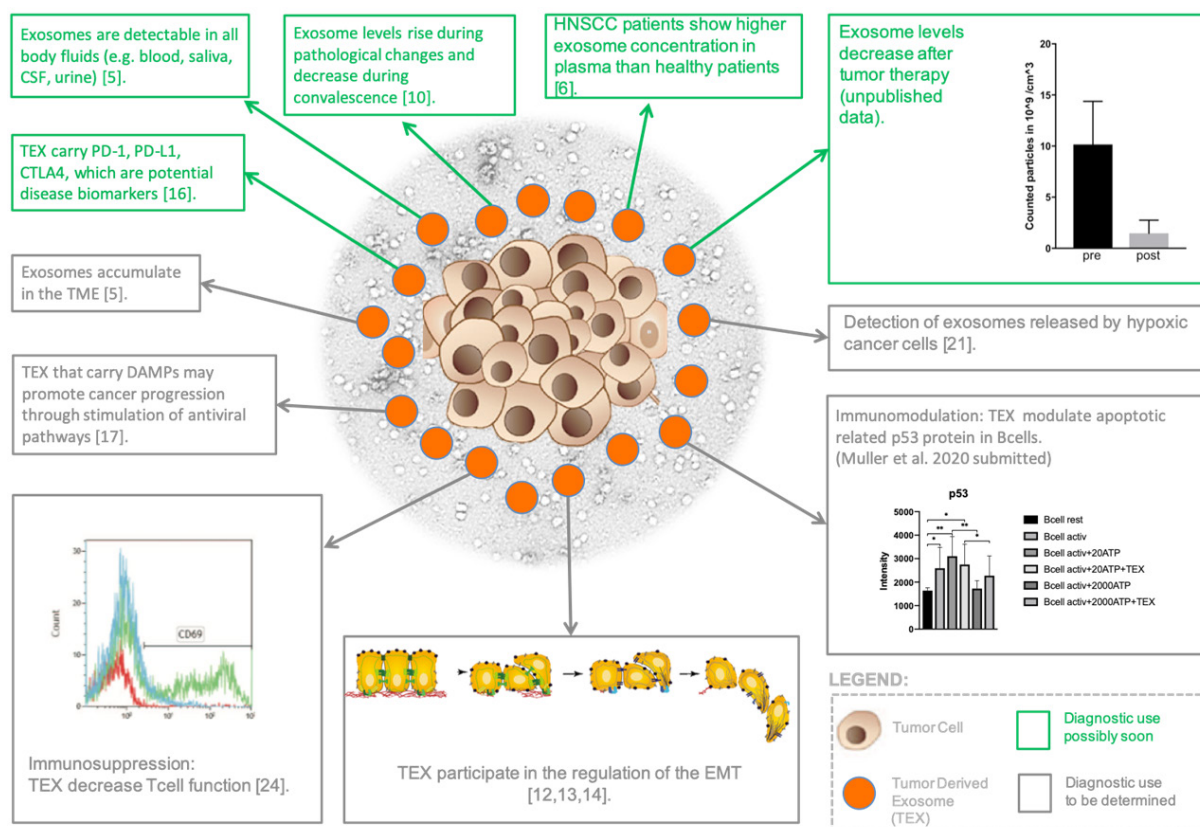


Figure 1. Exosomes and some of their properties with their potential use as diagnostic tools: this figure illustrates tumor cells centrally surrounded by exosomes, including tumor-derived exosomes (TEX) and some of their properties as prescribed above applicable in the clinical routine soon. It also shows the diagnostic value and applicability in the clinical routine not yet determined. For example, TEX participate in the regulation of the epithelial-to-mesenchymal transition (EMT), TEX carrying damage-associated molecular patterns (DAMPs) may promote cancer progression through stimulation of antiviral pathways, exosomes accumulate in the tumor microenvironment (TME), and TEX promote immunomodulation. Some typical exosome analyses have been included: TEX cause a decrease in CD69 in T-cells by flow cytometry (lower left), nanoparticle analyses by Zetaviewer before and after primary radiochemotherapy in head and neck squamous cell carcinoma (HNSCC) (upper right) and the influence of TEX on the protein expression profile of p53 in B-cells by dot blots (lower right)

cells^[19]. Exosome features such as the capability to modulate the host immune response, communication network of exosomes with the tumour microenvironment and involvement of exosomes in tumor progression and metastasis show that they play an important role in the tumor microenvironment^[19].

Tumor hypoxia is considered a negative predictive factor in HNSCC^[20,21]. Hypoxic cancer cells are considered to be radioresistant. Interestingly, hypoxia-derived exosomes have been described to express a different phenotype, and consequently, profile change of exosomes toward hypoxia could be used as a biomarker to guide further therapy^[22].

The aspects of exosomes mentioned above given their use as diagnostic tools are summarized in the following Figure 1.

PROS AND CONS OF EXOSOME USE IN LIQUID BIOPSY

For the assessment of tumor profiling, a tissue biopsy is one of the methods standardly used in which a sample of tissue is taken from the body by using specific needles or surgery. This procedure is not only an invasive method, but it also shows the disadvantage that the anatomical location of some oncogenic mutations is not always accessible by tissue biopsy. Another disadvantage is that the tissue extraction may

cause an expansion of the metastatic lesion^[23]. In addition, it is time-consuming since several surgeries are needed to follow up on tumor progression, making it more expensive^[9]. Exosomes are steady in the circulation and are found in almost every body fluid which makes them easy to use as a diagnostic tool. In comparison to tissue biopsy, liquid biopsy has the advantage of being a non-invasive method, which allows real-time monitoring of disease progression as well as detection of any tumor in any anatomical location with no risk of augmenting the metastatic lesion. Moreover, it improves the chance of an early diagnosis of cancer. Exosomes are presently emerging as promising non-invasive biomarkers of tumor progression and promotion of malignancy.

In the circulatory system of a patient with HNSCC, circulating tumor DNA (ctDNA, from tumor cells) is detected^[4,24]. ctDNA may be detected in various body fluids^[25]. During the formation of a tumor, circulating tumor cells (CTC) can be detected in the bloodstream through different techniques, which allows CTC lines to be used for drug sensitivity analysis and extraction of information at the cellular level^[4,26]. Therefore, CTC and ctDNA can be used as a diagnostic tool for real-time monitoring of tumor progression. However, it requires fresh samples, demands larger amounts of blood and requires the processing of samples within hours.

The advantage of exosomes is that one can use not only fresh samples but frozen samples as well. Thus, we were able to conduct exosome analyses on long-term frozen samples from a vaccination trial. It was possible to show the antitumor response and correlate the results with the patients' survival^[27]. Exosomes among other cancer-related particles are present in higher concentrations and are detectable earlier in metastatic disease than CTCs^[28]. As a result, another considerable advantage of exosomes is the small amount of blood needed to detect exosomes. Around one milliliter of blood may be enough depending on the extent of the analyses planned. Exosomes are more profuse and more specific since they contain highly informative protein content and genetic cargo such as DNA and mRNA^[12]. Besides, exosomes reveal specific markers such as HSP70 and Alix. They can reveal the original cell markers by imagining particular surface proteins and their target cells, making the isolation easy for tissue and target cell-specific exosomes^[29]. The content of nucleic acids in exosomes is a qualified source for cancer analysis. The DNA content of exosomes can reveal the mutational status of the original cell^[29].

HNSCC patients during an active disease state show a significant rise in circulating exosomes with exceptionally high levels of TEX and immunosuppressive factors^[7,30]. Not only TEX but also T-cell derived exosomes bear comparatively high levels of immunosuppressive factors in HNSCC patients^[31]. In case of advanced disease (stage III/IV), T-cell derived exosomes conveyed much higher levels of CD15s, a functional Treg marker, than in patients in the first stage^[31]. This is another indicator that plasma-derived exosomes of HNSCC patients could function as an easily accessible, non-invasive "liquid biopsy" of disease progression.

There is a way to transport drugs to cells since exosomes are taken up by various cells. Packaging paclitaxel in exosomes facilitates the allocation of the pharmacological agent^[32]. Many more studies are needed regarding this issue, and time will reveal which of these approaches will lead to a better outcome in patient care.

The disadvantage might be that the enhancement of exosomes by ultracentrifugation, ultrafiltration, or density gradient centrifugation is not only time-consuming but also requires considerable equipment, thus being cost-intensive^[29]. In the future, faster isolation methods should be developed for clinical application and standardization of results and diagnostic reference levels as well.

TECHNIQUES FOR EXOSOME CHARACTERIZATION

There are various isolation procedures of exosomes from body fluids and cell culture supernatants, including immunoaffinity capture, polyethylene glycol-mediated precipitation, ultracentrifugation, and microfluidics. To be able to use exosomes as a tumor biomarker, it is crucial to preserve their origin, structural integrity, molecular and genetic substance, and their functional properties as well to be suitable for DNA, RNA, and protein analysis.

Nevertheless, the simultaneous purification of exosomes is essential since plasma-derived exosomes have a high abundance of proteins^[33]. From current data, the mini-SEC method so far seems to be a favorable method compared to other isolation methods due to its better recovery and consistency of vesicles, because there is a risk of protein complex aggregation and loss of vesicles and biological functions as a result of ultracentrifugation.

In the following section, we give an overview of commonly used exosome characterization procedures.

Electron microscopy

Electron microscopy (EM) is the gold standard for exosome identification and verification of exosome preparation quality since it was one of the first methods used. It is also the only technique for direct exosome visualization. However, this method is difficult to use on an everyday basis. One of the reasons is that EM results in exosome loss during dehydration and embedding^[34]. Other variations of this technique may improve the results such as scanning EM and cryoEM, but further quantification of exosomes is a bigger issue.

Nanoparticle tracking analysis

This method provides an assembled analysis of Brownian motion via light dispersion to rank and size nanoparticles in a liquid suspension^[35-38]. Based on Brownian motion, analyzing over time light scatter produced by a laser beam that hits the particles yields a size distribution and concentration^[39]. Although good and regular calibration is essential, it is one of the more favorable methods used because it does not depend on detecting a definite marker and also due to the suspension of exosomes in a broad range of solutions^[39].

Tunable resistive pulse sensing

Tunable resistive pulse sensing discovers the passage of distinct particles through a membrane's pores. This happens when temporary electrical resistance is measured. It correlates with the amount and size of particles present. Pore size can be modified to improve the measurement. The analysis gives a concentration and particle distribution similar to nanoparticle tracking analysis^[39].

Developing a concordance between techniques for quantification of exosomes plays an important role in establishing the future of exosome biomarkers in clinical use^[39].

Immunodetection methods

These are analyses that based on the detection of mono- or polyclonal antibody bound to the antigen in the sample.

Western blots are used for the study of exosome cargo, and specifically, the semi-quantitative densitometry analysis of protein bands is insightful for disease progression^[12]. It also reveals that PD-L1 or TGF- β /LAP are significantly linked with disease progression^[12].

Flow cytometry, another valuable quantitative analysis of exosome cargo, is used for exosome capture on streptavidin-coated magnetic beads. It was also demonstrated for the collection of CD63+ exosomes utilizing biotin-labelled anti-CD63 Ab, starting with fraction number four of exosomes separated by mini-SEC^[40]. This method demonstrates that the levels of PD-1 and PD-L1 in exosomes obtained from HNSCC patients' plasma is significantly associated with pathological changes such as disease progression^[40].

PD-L1 is a ligand with the potential of inducing immune suppression in activated T cells that express PD-1. When PD-L1-bearing exosomes were obtained with CD3 and CD69 T lymphocytes, the down-regulation of CD69 expression was blocked. Anti-PD1 antibodies inactivate this inhibition. Likewise, when plasma-derived exosomes are obtained with activated CD8 T-cells, fast programmed cell death of T cells takes place. This reveals that TEX modulate immune cell activity and are able to function as immune biomarkers^[30,40,41].

Clinical analyses need to be simple, reliable and quick. To meet these criteria, we are currently working on a new isolation technique using immunoaffinity that allows us to have results in flow cytometry within a few hours. This will enable a high-throughput analysis of patient samples.

One interesting method described could be the photoacoustic and fluorescence flow cytometry platform, as described by Nolan *et al.*^[28]. This is able to perform *in vivo* flow cytometry on tumor-associated exosomes in mouse models. Also, more research is needed to bring this technique into human diagnostics, where it seems to be a challenging but promising path.

Mass spectrometry

MS is one of the most favorable techniques for proteome characterization, where analytes are converted to gaseous ions and are classified recording to their mass-to-charge ratio. It offers various advantages such as its specificity, throughput, sensitivity and cost-effective testing. It uses three different proteomic MS techniques: digestion by trypsin (bottom-up), digestion by a protein with less-frequent cleavage sites (middle-down), and analysis of native protein (top-down). MS has also been linked with ChIP to quantify histone modifications, transcription factors and other chromatin-associated proteins.

As already mentioned above, exosomes contain a variety of functional proteins and genetic cargo such as DNA, mRNAs, miRNAs and long non-coding RNAs. Tang *et al.*^[42] state: "Sucrose density gradients, ultrafiltration, high performance liquid chromatography-based protocols and immunoaffinity-capture methods, singly or combined with the application of ultracentrifugation, can provide high enrichment and purity of exosomes". Besides, a number of alternative methods for analysis of exosome RNA extraction methods have been used: phenol-based techniques (TRIzol) and combined phenol and pure column-based techniques. The Total Exosome RNA and Protein Isolation kit (TER) has been particularly selected for RNA isolation^[42].

Global proteomics is an identification method using as many proteins as possible within one sample. There are two different strategies: via data dependent acquisition (DDA) or data independent acquisition (DIA), with DDA being used more commonly than DIA^[43].

CONCLUSION

Cancer is one of the biggest challenges of medicine, and we still cannot defeat it as a whole. However, liquid biopsy, the analysis of exosomes, ctDNA and CTCs may give us the possibility for non-invasive diagnosis, real-time monitoring of disease progression and developing a treatment according to gene profile as well. The special features of exosomes make them ideal for minimally invasive liquid biopsy. Exosomes are unique; they express not only "dead" particles such as ctDNA but they also show actual biological activity

that helps us study the tumor more accurately. Liquid biopsy is one of the advanced methods that will soon help to diagnose tumors earlier and also to follow their morphological development, making treatment more precise than in the past. It might not replace the X-ray, MRI and CT scan because it is still essential to know where precisely the tumor grows, but once the tumor is visible, it is more likely to be associated with an adverse prognosis. With the help of liquid biopsy, we can diagnose the tumor much earlier and thus decrease the need for chemotherapy, which may be useful for cancer therapy in the advanced stage but may have many side effects for the patient. Thus, we could achieve a better result with the help of a liquid biopsy.

Every tumor is individually different. The liquid biopsy helps us track down its morphological development earlier and, in this way, helps us to control it better and enables us to develop a treatment according to its genetic profile. This is what we call precision medicine. Exosomes can be released by both immune cells and non-immune cells. They have immunomodulatory properties. EX lead to signalling in T-cells and in other immune cells. The primary mechanism is the uptake of exosomes and the release of non-coding RNAs, especially microRNAs which play a part in the regulation of RNA translation^[8]. TEX regulate immune cells by the direct interaction between Fas and Fas-ligand, which causes cell death of activated CD8 cytotoxic T-cells^[8]. Exosomes seem to have multiple effects on the immune system. As such they transport pro- and anti-tumorigenic as well as pro- and anti-inflammatory signals to target cells. Depending on the target cell, for example, dendritic cells, T lymphocytes or B cells, exosomes may send clear signals^[8].

Due to their various properties, exosomes are an exciting target for therapeutic and diagnostic aims. Tumor cells are known to produce numerous exosomes. In the plasma of HNSCC patients, exosomes are enormously increased in comparison to healthy patients. Their profile difference makes them attractive to be used in liquid biopsy analysis^[8]. Cancer treatment such as immunotherapy in HNSCC patients is being conducted in clinical practice currently. First data on using exosomes as tumor biomarkers in HNSCC have been reported^[17,30]. However, more data are needed on the features of circulating exosomes in HNSCC patients. Many investigations on exosomes in cancer treatment as well as the first keen results have been published^[44]. Many more studies will be carried out on this topic and time will tell which of these approaches will lead to a better outcome in patient care.

DECLARATIONS

Authors' contributions

Literature research and writing the paper: Pastor K

Figure design: Benecke L

Study idea in design: Muller L

Availability of data and materials

Not applicable.

Financial support and sponsorship

This work was supported by Propatient (pp18-08).

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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Case Report

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Nivolumab-induced severe bullous pemphigoid in a patient with renal cancer: a case report and literature review

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How to cite this article: Wu X, Palvai S, Jalil A. Nivolumab-induced severe bullous pemphigoid in a patient with renal cancer: a case report and literature review. *J Cancer Metastasis Treat* 2020;6:40. <http://dx.doi.org/10.20517/2394-4722.2020.77>.

Received: 30 Jul 2020 **Accepted:** 23 Oct 2020 **Published:** 29 Oct 2020

Academic Editor: Godefridus J. Peters **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

With the widespread use of immunotherapy in numerous solid tumours, immunotherapy-related adverse events (irAEs) have started to emerge and bring new challenges for clinicians to manage. Among established irAEs, dermatologic toxicity is one of the most common toxicities; it is often mild but can be severe and potentially life-threatening, such as bullous pemphigoid. Here, we report a case of nivolumab-mediated severe, extensive, refractory bullous pemphigoid involving both skin and oral mucosa in a patient with metastatic renal cancer. We also summarise a list of selected case reports of immunotherapy-induced bullous pemphigoid by literature review. We highlight various presentations, investigations and managements of this type of skin irAEs. Meantime, we would like to discuss the correlation of skin irAEs incidence rate with immunotherapy drug benefit and resistance.

Keywords: Bullous pemphigoid, mucous membrane pemphigoid, immunotherapy, dermatologic toxicity, nivolumab

INTRODUCTION

Immunotherapy, such as anti-programmed death-1 (PD-1)/programmed death ligand 1 (PD-L1) antibody or anti-CTLA 4 antibody or combination, has significantly improved survival of patients with various malignancies. However, immunotherapy-related adverse events (irAEs) have started to emerge and bring new challenges for oncologists to manage. Among the known irAEs, dermatologic toxicity is one of the



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most common toxicities^[1]. Most immunotherapy-induced cutaneous toxicities are mild, such as non-specific rashes or pruritus. However, some manifestations of skin irAEs can progress to high-grade and potentially life-threatening situations, such as bullous pemphigoid (BP)^[2]. Since 2015, more than 40 cases of immunotherapy-induced BP have been reported with the majority involving either the skin or mucous membrane only. Presentations of both cutaneous and oral mucous BP are rare (less than seven cases).

Here, we report a severe, extensive (involving both skin and oral mucosa), refractory case of BP that occurred 9 months after initiation of nivolumab in a patient with metastatic renal clear cell cancer. We also summarise a list of selected case reports of checkpoint inhibitor-induced BP by literature review. We highlight various presentations, investigations and management approaches of immunotherapy-induced BP. Meantime, we would like to discuss the correlation of skin irAE incidence rate with immunotherapy drug benefit and resistance.

CASE REPORT

A 66-year-old gentleman with no known history of autoimmune disease was diagnosed with right clear renal cancer in 2014. He was initially treated with radical nephrectomy with excision of tumour thrombus in the renal vein and retro-hepatic inferior vena cava. In March 2017, his cancer relapsed with a single spinal metastasis, which was treated with excision of the intradural tumour and laminectomy and radiotherapy as well, which resulted in complete neurological recovery. Post-radiotherapy, he opted for active tyrosine kinase inhibitor, sunitinib, in light of relapsed renal cancer in his spine. In October 2017, sunitinib was discontinued due to spontaneous haematoma in his right calf and thigh, which was likely due to combination of sunitinib and low-molecular-weight heparin (LMWH) for his atrial fibrillation. He then had a long period of surveillance, during which he gradually developed asymptomatic relapsed metastatic renal cancer in his left adrenal gland, followed by relapsed cancer in his left kidney. In March 2019, he commenced immunotherapy infusion with 2 weekly nivolumab, considering further disease progression with multiple lung metastatic disease. His other medical history was only atrial fibrillation, for which he was on LMWH and bisoprolol.

From August 2019, five months post-initiation of nivolumab, he developed a mild pruritic erythematous skin rash scattered mainly over his chest and upper limbs. At that time, there were no blisters reported. He continued nivolumab with the rash controlled mainly by topical steroids or short courses of oral steroids. In November 2019, he developed large, tense, haemorrhagic blisters, which were exacerbated following a 3-week holiday in Australia, leading to an emergency hospital admission. On clinical examination, he had a severe widespread bullous eruption affecting his chest, abdomen, inner thighs and upper arms. Active blisters and erosions were seen on the roof of his mouth and scrotal skin as well. Some blisters had burst leaving severely eroded skin with signs of superimposed infection. Bullae at various stages were seen, including intact ones, burst ones with an eroded base, and a few areas of early re-epithelialisation as [Figure 1](#). He had no involvement of the scalp region and external eye examination was normal.

An incisional skin biopsy from an active blister with haematoxylin and eosin examination revealed epidermal hyperplasia and subepidermal splitting. There were few eosinophils, scattered plasma cells and neutrophils with a subjacent chronic inflammation. Direct immunofluorescence (DIF) showed clearly positive staining for IgG in the basement membrane zone, and a faint positive IgA staining. Subsequent serum antibody analysis confirmed positive pemphigoid antibodies consistent with BP see [Figures 2 and 3](#).

The patient was started immediately on intravenous methylprednisolone 1 mg/kg as well as flucloxacillin for 4 days and then switched to oral prednisolone and flucloxacillin when no new blisters developed. Most of the blisters gradually re-epithelialised within 4-6 weeks of oral steroids. Two months later, the corticosteroid treatment was tapered. However, the blisters reappeared when prednisolone was stepped



Figure 1. Clinical presentations. Multiple, grouped, large, tense haemorrhagic blisters were seen on the first clinical presentation, which affected the chest, abdomen, inner thighs and upper arms (A, B and C); active blisters and erosions were seen on the roof of the mouth (D)

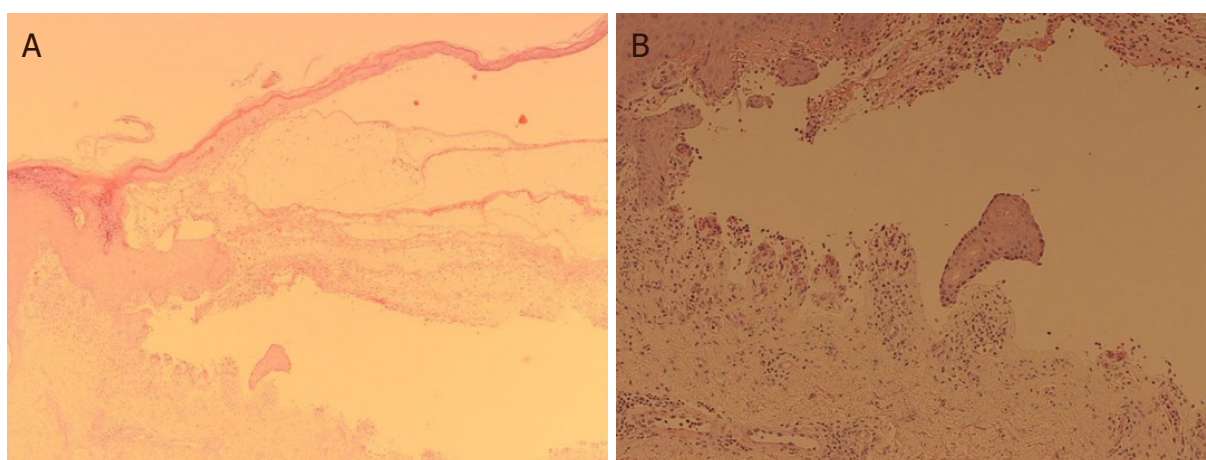


Figure 2. Haematoxylin and eosin staining of edge (A) and base (B) of blisters. Epidermal hyperplasia and subepidermal splitting were seen with few eosinophils, scattered plasma cells, neutrophils and a subjacent chronic inflammation

down to 10 mg per day, requiring re-introduction of high-dose prednisolone. Consequently, nivolumab had been permanently discontinued in view of refractory BP and further disease progression in his adrenal metastatic disease.

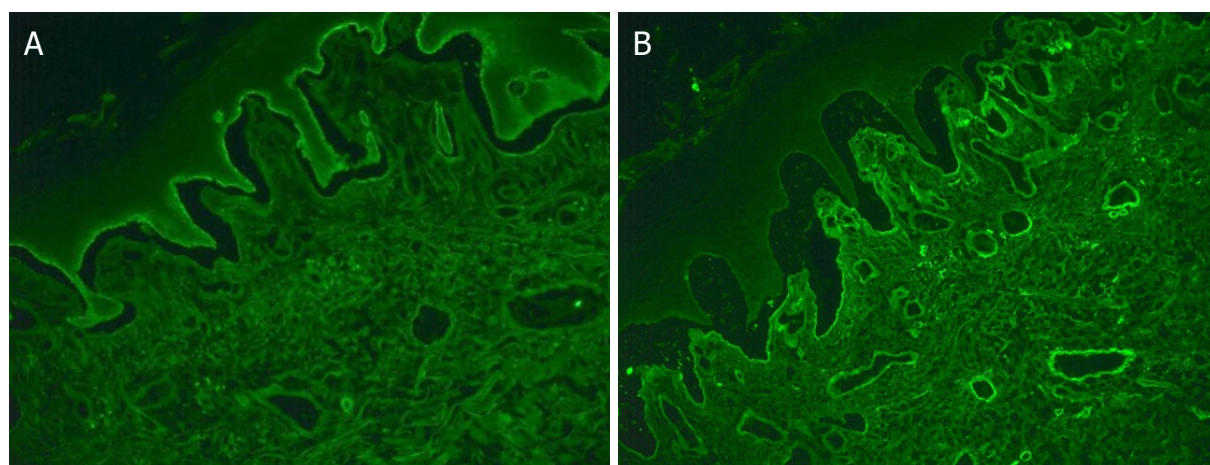


Figure 3. Direct immunofluorescence showed clearly positive staining for IgG in the basement membrane zone (A), and a faint positive IgA staining (B)

DISCUSSION

BP is an autoimmune blistering disorder that is caused by autoantibodies against hemidesmosomal protein BP180 and BP230 at the skin basement membrane^[1]. The causes of BP in oncologic patients could be idiopathic, paraneoplastic^[2,3], secondary to cancer therapy or related to another medication. With the widespread use of immunotherapy in cancer management, BP has become a well-established cutaneous toxicity associated with immunotherapy. The cause of BP in our case is most likely nivolumab, as the patient denied a history of BP, and as his other medications were generally tolerated for years. The timing of his renal cancer diagnosis (5 years prior) and nivolumab initiation (9 months prior) makes the paraneoplastic phenomenon highly unlikely.

Here, we summarise a list of selected case reports of immunotherapy induced BP by literature review in Table 1. Most reported immunotherapy-induced BP involving either skin or mucous membrane only Table 1; combination of cutaneous BP and mucous membrane pemphigoid (MMP) is rare^[4]. Cutaneous BP typically presents with pruritic blisters that arise on erythematous or urticarial plaques. Gradually, the bullous lesion can erode and leave haemorrhagic crust^[5]. MMP usually presents with oral erosions, blistering or desquamative gingivitis, which are associated with significant morbidity and severe complications, such as tissue destruction, fibrosis and loss of function (e.g., laryngeal stenosis). In addition, MMP could be initially indistinguishable from a gingivitis due to poor oral hygiene practices^[6]. Thus, oncologists should be aware of cutaneous BP and MMP in all patients who have been treated with immunotherapy.

Onset of BP post-initiation of immunotherapy varies from weeks to several months [Table 1]. Previous evidence showed that the median number of weeks of immunotherapy prior to onset of BP was 17 (range of 3 to 91)^[7]. However, delayed cases have been reported, even several months after discontinuation of immunotherapy^[8,9].

Diagnostic workup usually includes skin biopsy for histology and immunofluorescence staining. Histopathology normally shows subepidermal clefting with an eosinophilic inflammatory infiltration^[10]. DIF characteristically shows linear deposits of IgG and/or C3 along the epidermal basement membrane zone. Indirect immunofluorescence (IIF) or enzyme-linked immunosorbent assays will confirm the deposit of autoantibodies specific for BP230 (also known as BPAG1) and/or BP180 (also known as BPAG2; collagen XVII)^[6,11]. Several cases have been reported with raised serum anti-BP180 associated with immunotherapy-induced BP as well^[12]. Interestingly, in one case, it was demonstrated that immunotherapy-induced BP

Table 1. Selected reported cases of immunotherapy-induced bullous pemphigoid

Case number	Immunotherapy	Primary tumour	Clinical presentation	Time to develop BP (weeks)	Treatment	Ref.
1	Pembrolizumab	Melanoma	Cutaneous BP	22	Oral steroids	[27]
2	Nivolumab	Tongue SCC	Oral and cutaneous BP	8	Topical/oral steroids	[9]
3	Pembrolizumab	Melanoma	Cutaneous BP	16	Topical/oral steroids	[9]
4	Nivolumab	Urothelial Ca.	Cutaneous BP	18	IV/oral steroids	[9]
5	Nivolumab	NSCLC	Cutaneous BP	6	Oral and topical steroids and nicotinamide	[9]
6	Nivolumab	Melanoma	Cutaneous BP	3	IV/oral steroids	[9]
7	Pembrolizumab	Melanoma	Severe BP exacerbation		Oral steroids	[28]
8	Nivolumab	Lung cancer	Cutaneous BP	6	Oral steroids and omalizumab	[29]
9	Pembrolizumab	Melanoma	Cutaneous BP	84	Topical and oral steroids	[30]
10	Ipilimumab then nivolumab	Melanoma	Cutaneous BP	24	Topical and oral steroids	[23]
11	Durvalumab then nivolumab	Melanoma	Cutaneous BP	52	Topical steroids	[23]
12	Nivolumab	SCC of lung	Cutaneous BP	18	Oral and topical steroids	[23]
13	Pembrolizumab after ipilimumab	Melanoma	Cutaneous BP	32	Oral and topical steroids	[31]
14	Pembrolizumab	Melanoma	Oral and cutaneous BP	78	Topical steroid	[32]
15	Pembrolizumab	Melanoma	Oral and cutaneous BP	18	Oral steroid and methotrexate	[32]
16	Pembrolizumab	Melanoma	Recurrent BP		Oral steroid	[32]
17	Pembrolizumab then nivolumab	Melanoma	Localised BP		Monitoring	[33]
18	Nivolumab	Lung	Oral and cutaneous BP	80	IV Methylpred+ rituximab	[17]
19	Pembrolizumab	Melanoma	Prolonged BP	44	Oral and topical steroids	[34]
20	Nivolumab	Renal cell Ca.	Cutaneous BP	12	Oral and topical steroids	[35]
21	Pembrolizumab	Melanoma	Cutaneous BP	24	Niacinamide	[36]
22	Nivolumab	Melanoma	Cutaneous BP	12	Oral and topical steroids	[36]
23	Pembrolizumab and ipilimumab	Melanoma	Cutaneous BP	24	IV and oral steroids and methotrexate	[37]
24	Pembrolizumab	Melanoma	Cutaneous BP	51	Oral steroids	[38]
25	Nivolumab	Skin SCC	Cutaneous BP		Oral steroids and dapsone	[7]
26	Nivolumab	Melanoma	Cutaneous BP		Topical steroids	[7]
27	Atezolizumab	Urothelial Ca.	Cutaneous BP	22	Topical/oral steroids and omalizumab, methotrexate	[12]
28	Nivolumab	NSCLC	Cutaneous BP		Topical/oral steroids	[12]
29	Nivolumab	NSCLC	Cutaneous BP		Topical/oral steroids and omalizumab	[12]
30	Nivolumab then Pembrolizumab	NSCLC	Cutaneous BP		Topical and oral steroids and nicotinamide	[12]
31	Pembrolizumab then ipilimumab and nivolumab	Melanoma	Cutaneous BP		Topical and oral steroids and nicotinamide	[12]
32	Nivolumab	Renal cell Ca.	Cutaneous BP		Oral steroids and nicotinamide	[12]
33	Pembrolizumab	NSCLC	Cutaneous and oral BP		Topical and oral steroids	[12]
34	Pembrolizumab	Melanoma	MMP	66	Doxycycline only	[39]
35	Pembrolizumab	Melanoma	Cutaneous BP	12	Topical and oral steroids	[14]
36	Ipilimumab then pembrolizumab	Melanoma	Cutaneous BP	29	Topical and oral steroids	[14]
37	Nivolumab	NSCLC	Cutaneous BP	7	Topical and oral steroids and niacinamide	[26]
38	Ipilimumab after pembrolizumab	Melanoma	BP		Oral and topical steroid	[40]
39	Nivolumab	NSCLC	BP		Oral steroids	[41]
40	Ipilimumab after nivolumab	Melanoma	BP	50	Topical steroids	[42]
41	Pembrolizumab	Merkel cell Ca.	MMP	13	Topical steroids	[43]
42	Nivolumab	Melanoma	Oral and cutaneous BP		Topical steroids	[44]
43	Nivolumab	Renal cell Ca.	BP on higher dose immunotherapy		Oral steroids	[18]

44	Nivolumab	Melanoma	Cutaneous BP		Topical/oral steroids and dapsone	[13]
45	Nivolumab	Melanoma	MMP		Topical steroids	[45]
46	Pembrolizumab	Ovarian Ca.	Severe MMP	3	Topical and oral steroids	[46]
47	Nivolumab	Lung	Oral and cutaneous BP		Oral steroid	[8]
48	Atezolizumab	Penile SCC	Photodistributed BP		Oral steroids	[47]
49	Atezolizumab	Urothelial cell Ca.	Cutaneous BP	77	Topical steroids, doxycycline and niacinamide	[15]
50	Nivolumab	Renal cell Ca.	Cutaneous BP	52	IV and oral steroids	[48]
51	Durvalumab and tremelimumab	NSCLC	BP	42	Oral steroids	[49]
52	Cemiplimab	Cutaneous SCC	BP		Oral steroids and rituximab	[50]

SCC: squamous cell cancer; Ca.: carcinoma; NSCLC: non-small cell lung cancer; MMP: mucous membrane pemphigoid; BP: bullous pemphigoid

may develop in the presence of anti-LAD-1 IgG antibodies alone but absence of anti-BP180 or anti-BP230 autoantibodies^[13].

Treatment regimens generally include steroids (oral or topical or combination), often in conjunction with discontinuation of immunotherapy. As steroids can potentially diminish immunomodulatory actions, topical steroids are first-line treatment for BP and are largely safe due to their limited systemic absorption. Meanwhile, oral nicotinamide and tetracycline (doxycycline or minocycline) have demonstrated a good effect in mild or moderate cases whilst completely avoiding systemic steroids^[14,15]. The role of nicotinamide in treating BP is modulating inflammatory cytokines and acting as poly adenosine diphosphate ribose polymerase inhibitor^[16]. However, severe cases have frequently required intravenous methylprednisolone (1-2 mg/kg). Refractory immunotherapy-induced BP shows response to rituximab or omalizumab^[12,17], which are started after immunotherapy discontinuation and lead to resolution of BP. Future studies are warranted to explore targeted immunosuppressant agents for severe steroid-refractory BP.

The question of resuming immunotherapy after resolution of BP remains under discussion. In the situations where immunotherapy is ineffective, discontinuation is clearly warranted. While in the context of a clinical response to immunotherapy, the discontinuation of immunotherapy is challenging, and the risks of continued treatment must be carefully weighed against its benefits. As such, the decision of resuming immunotherapy should be made as a part of multidisciplinary team and on an individual basis. In one case report BP developed after the patient was transitioned to a higher dose of nivolumab. Another case report showed improvement in BP after switching from nivolumab to pembrolizumab^[18]. Consequently, immunotherapy should not be permanently discontinued without the trial of the previously tolerated dose or frequency or different immunotherapy.

Understanding the correlation between the incidence rate of skin irAEs and oncology benefit or immunotherapy resistance is critical for clinical practice. There is some evidence showing potential positive association between development of cutaneous irAEs and improved survival in patients on immunotherapy, particularly vitiligo^[19,20]. In addition, a systematic review and meta-analysis suggested that the skin, gastrointestinal and endocrine irAEs might be positively associated with clinical benefit, while the pulmonary irAEs might be associated with immunotherapy resistance^[21]. However, the development of severe BP in our case is not a marker of good response to immunotherapy, as the patient failed to respond to nivolumab despite the extensive, refractory, severe BP. Immunotherapy resistance can be classified as primary, such as in never-responders, or acquired, which emerges after a period of response^[22]. Our patient had continuously slow disease progression in his metastatic left adrenal and left kidney mass since nivolumab, which could be primary immunotherapy resistance. However, we could not totally exclude the possibility of pseudo-progression, as his other metastatic diseases were largely stable. In a later phase, the

extra use of steroids and cessation of nivolumab certainly further contributed to his disease progression. Consequently, understanding the correlation between skin irAEs and clinical outcome could provide valuable information in distinguishing between pseudo-progression and immunotherapy resistance, which will assist oncologists in the decision of resuming immunotherapy after the recovery from manageable skin irAEs.

The mechanism of immunotherapy induced BP is currently unclear. While BP is associated with both a humoral and a cellular response, it is widely believed that immunotherapy induced BP could be provoked by an autoantibody targeted to a shared antigen and the dysfunction in T-regulatory cells, which is inhibited by immunotherapy^[23]. The shared target antigen is located both at the dermoepidermal junction and on the surface of tumour cells, such as BP 180, which can be expressed on the surface of melanoma cells, non-small cell lung cancer cells and basement membrane skin cancer^[24,25]. Meanwhile, dysregulation of PD-1/PD-L1 and CTLA-4 signal pathway induced by immunotherapy can impair the balance within the immune system, resulting in the development of off-target effects and autoimmunity^[26]. Further studies are required to determine the underlying mechanisms of immunotherapy induced BP.

Conclusion

Here, we report a rare case of severe, extensive and refractory nivolumab-induced BP in a patient with advanced renal cancer. With the widespread use of immunotherapy, it has become increasingly important to document cases of immunotherapy-induced BP to provide more information in diagnosing and treating these cutaneous adverse events. Moreover, the close collaboration between dermatologists and oncologists is essential to allow the patients with cutaneous irAEs to continue antitumor therapy, avoiding unnecessary discontinuation of immunotherapy.

DECLARATIONS

Acknowledgments

All authors would like to acknowledge Dr. Dalila Malek, Dr. Paul Gatt, Dr. Ravi Suchak and Dr. Marice Sudaerson (Southend Hospital) for support and advice.

Authors' contributions

Designed the study and wrote the manuscript: Wu X, Palvai S

Reviewed and approved the manuscript: Jalil A

Availability of data and materials

Not applicable.

Financial support and sponsorship

This study supported by National Health Service Funding.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Written informed consent was obtained from the patient.

Consent for publication

Written informed consent was obtained from the patient for publication of this case report and any accompanying images.

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Review

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Leptomeningeal metastases from non-small cell lung cancer: state of the art and recent advances

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How to cite this article: Pellerino A, Internò V, Muscolino E, Mo F, Bruno F, Pronello E, Franchino F, Soffietti R, Rudà R. Leptomeningeal metastases from non-small cell lung cancer: state of the art and recent advances. *J Cancer Metastasis Treat* 2020;6:41. <http://dx.doi.org/10.20517/2394-4722.2020.80>

Received: 11 Aug 2020 **First Decision:** 15 Sep 2020 **Revised:** 30 Sep 2020 **Accepted:** 14 Oct 2020 **Published:** 29 Oct 2020

Academic Editor: Lombardi Giuseppe **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Patients with leptomeningeal metastases (LM) from non-small cell lung cancer (NSCLC) have a poor outcome with survival of less than 1 year regardless of advancements in treatment strategy. In the past, some randomized clinical trials have been conducted with heterogeneous inclusion criteria, diagnostic parameters, response evaluation and primary endpoints. Efforts to develop a standardized magnetic resonance imaging (MRI) assessment and liquid biopsy techniques to monitor disease evolution in plasma or cerebrospinal fluid (CSF) are underway. This review aims to cover the main clinical and diagnostic challenges of LM from NSCLC, in particular the role of MRI, CSF cytology and liquid biopsy for the diagnosis and monitoring of the disease, as well as the most recent clinical trials on targeted therapies. Targeted therapy, such as epidermal growth factor receptor tyrosine kinase inhibitors and anaplastic lymphoma kinase rearranged inhibitors, represent a feasible treatment with encouraging results in terms of disease control and survival. For ineligible patients, immune checkpoint inhibitors could represent a therapeutic option with acceptable tolerance, although clinical trials focused on LM from NSCLC are lacking and represent a research focus for the future.

Keywords: Anaplastic lymphoma kinase inhibitors, epidermal growth factor receptor tyrosine kinase inhibitors, clinical trials, immunotherapy, leptomeningeal metastases, liquid biopsy



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INTRODUCTION

Leptomeningeal metastases (LM) represent an end-stage complication of advanced systemic cancer in approximately 5% of patients. Autopsy series have revealed a high prevalence of undiagnosed or asymptomatic LM in 19% of patients with solid tumors^[1], representing the third most common metastatic complication of the nervous system after brain metastases (BM) and epidural metastases. The incidence of LM in patients with cancer is increasing due to better tools for diagnosis and monitoring and more effective targeted therapies that lead to prolonged survival^[2]. However, overall survival remains in the order of weeks to months regardless of treatment type.

A recent cohort study of 163 patients with LM has shown a median age of 57 years, and LM was the initial presentation of cancer in 19 patients (11.7%), while in 28 patients (19%) LM was diagnosed during the initial treatment course of primary tumor, and in the remaining 116 patients (81%) LM was diagnosed at recurrence. The most common primary solid tumors in this cohort are non-small cell lung adenocarcinoma (NSCLC - 52%), followed by small cell lung carcinoma (SCLC - 18%), and breast cancer (16%)^[3].

NSCLC has a significant risk LM (20% of patients)^[4] with a median development time of 12 months (range 2-18 months) from diagnosis of the primary tumor^[3]. Epidermal growth factor receptor (EGFR) mutated and anaplastic lymphoma kinase (ALK) gene rearranged NSCLC are more prone to recur with LM^[5,6]. Thus, there is need to improve diagnostic tools, validate biomarkers to monitor disease progression, and search for new treatment regimens for LM patients.

Here, we review the clinical and diagnostic challenges of LM from NSCLC, the role of magnetic resonance imaging (MRI), cerebrospinal fluid (CSF) cytology and liquid biopsy, as well as the most recent clinical trials on targeted therapies.

THE ROLE OF CNS BARRIERS IN DRUG DELIVERY

The CNS is considered as a sanctuary site which is protected by different barriers from neurotoxic agents. The blood-brain barrier (BBB) consists of tight junctions (TJs) linked to the endothelial cells of the brain, creating a physical barrier that limits the passage of molecules^[7]. The specialized endothelial cells maintain a continuous, non-fenestrated basal lamina and interact with other perivascular cells, such as astrocytes, pericytes, and perivascular macrophages, which contribute to the integrity of the BBB^[8]. Molecules may cross the BBB by two mechanisms. The paracellular transport consists of a diffuse and passive flow between the endothelial cells, and is regulated by physicochemical properties, including molecular weight, electrical charge, and lipophilicity. In general, the TJs reduce the paracellular transport of molecules when the BBB is intact; thus, the paracellular transport is limited to small, lipophilic molecules that are less than 500 Daltons^[9]. The transcellular transport consists of a flow of molecules across the luminal side of the endothelial cell, through the cytoplasm, and then to the abluminal side into the brain interstitium. Some active transport mechanisms are typical of transcellular transport for larger and less lipophilic molecules, such as glucose, insulin, albumin, blood cells, infectious agents, and potential neurotoxins^[10].

Similarly, the BBB impacts the ability of therapeutic agents to penetrate the CNS. In fact, more than 90% of all small-molecules, and nearly 100% of large compounds, have poor penetration through the BBB^[11], leading to a decreased efficacy on CNS disease control from chemotherapy and targeted agents. Notably, the BBB is normal in pre-metastatic niche and micrometastases (< 1 mm), and protects them from most of anticancer agents that are employed in the adjuvant treatment of NSCLC^[12,13]. The most recent generations of anaplastic lymphoma kinase (ALK) inhibitors and EGFR tyrosine kinase inhibitors (TKIs) display an increased ability to cross the BBB, reaching significant CSF concentrations [Table 1], but these drugs may be actively transported back into the cerebral blood flow by efflux pumps. The most important is the

Table 1. CSF concentration and penetration of targeted agents in LM from NSCLC

Drug	Target	Dosage/day	CSF level (nmol/L)	Penetration (CSF/plasma) %
First generation TKIs				
Erlotinib ^[6,14]	EGFR	150 mg	66.9	2.8-3.3
Gefitinib ^[6]	EGFR	250 mg	8.2	1.13
Second-generation TKIs				
Afatinib ^[15]	EGFR	40 mg/m ²	1.0	1.65
Third-generation TKIs				
Osimertinib ^[16,17]	EGFR mutated Thr790Met	160 mg	7.51	2.5-16.0
AZD3759 ^[18]	EGFR	300 mg bid	25.2	100.0
First-generation inhibitors				
Crizotinib ^[19]	ALK, MET, ROS1	250 mg	0.14	0.26
Second-generation inhibitors				
Ceritinib ^[20]	ALK, ROS1	450-600 mg	NA	15.0
Alectinib ^[21,22]	ALK, RET	60 mg/kg	2.69	63.0-94.0
Brigatinib ^[20]	ALK, ROS1, EGFR	90-180 mg	NA	NA
Third-generation inhibitors				
Lorlatinib ^[23]	ALK, ROS1	100 mg	6.5-308	31.0-96.0

EGFR: epidermal growth factor receptor; ALK: anaplastic lymphoma kinase; ROS1: proto-oncogene tyrosine-protein kinase type 1; MET: proto-oncogene c-MET; RET: proto-oncogene REarranged during Transfection; TKIs: tyrosine kinase inhibitors; bid: twice/daily; NA: not available

P-glycoprotein 1 (P-gp1), a member of the ATP binding cassette family, which recognizes a wide range of compounds employed in the adjuvant setting of NSCLC (with the exception of AZD3759 and alectinib), and contribute to drug resistance^[24].

The blood-tumor barrier (BTB) lacks TJs and astrocyte-endothelial contacts, but is enriched with P-gp1 along the luminal and plasma membranes of tumor cells compared with BBB, and contributes to limiting the drug penetration on BM and LM^[13]. More importantly, the permeability of BTB and BBB in BM and LM varies widely between lesions and regions of concern, resulting in non-homogeneous drug distribution^[13,24,25].

The space between the CSF and the CSF-producing choroid plexus is known as the blood-CSF barrier, which determines the adequate concentration of molecules by primarily active transports^[26]. Since the BBB and blood-CSF barrier use non comparable active transport mechanisms, and CSF drug concentrations strictly depend on blood-CSF barrier, the distribution of drug into CSF cannot be considered as a reliable measure of BBB permeability or surrogate of drug concentration in BM or LM^[27]. With this regard, future phase 0 trials are strongly encouraged to analyze drug-target effects and pharmacokinetic-pharmacodynamic relationships in the early clinical development of new drugs^[28]. Lastly, drug concentrations in the CSF are correlated with the free drug concentration in plasma: clinicians may modulate the concentration of free drug in plasma by changing the dose and schedules of treatment, including the use of high-dose or pulse administration^[29-32].

Pathogenesis of leptomeningeal metastases from NSCLC

Tumor cells may reach the leptomeninges in different ways, such as hematogenous spread through the vessels of the arachnoid and choroid plexi, along peripheral nervous system by nerve and vascular sheaths, through lymphatic dissemination or invasion by contiguity^[33]. Furthermore, access of the ventricular system or using a piecemeal compared with en-block tumor resection, have been suggested as risk factors for leptomeningeal dissemination^[34,35]. Post-operative SRS is an effective adjunct to reduce the risk of local recurrence^[36]. However, some studies have suggested that SRS may also be associated with increased rates of LM recurrence, with reported incidence of up to 31% in 1 year^[37,38].

Complement component 3 (C3), which is produced by cancer cells in the CSF, is upregulated in LM models from lung and breast cancer. In particular, C3 promotes the disruption of blood-CSF barrier, leading to the passage of some mitogens, such as amphiregulin, that promotes tumor cell growth within leptomeninges^[39]. Similarly, Matrix Metalloproteases (MMPs) type 9, A Disintegrin and Metalloproteases (ADAMs) type 8 and 17 interfere with the integrity of the blood-CSF barrier, facilitating the passage of tumor cells into the subarachnoid space^[40]. Some driver mutations select clonal tumor cells making them more prone to metastasize to the CNS. Brastianos *et al.*^[41] showed that distinct genetic alterations were not detected in the matched primary-tumor sample in 53% of 86 patients with BM, while spatially and temporally separated BM were genetically homogenous and shared similar druggable pathways, including PI3K/AKT/mTOR, CDK, and HER2/EGFR. Further investigations have revealed three regions with significantly higher amplification in BM from NSCLC, including MYC, YAP1, and MMP13, and deletions in CDKN2A/B^[42]. Thus, the response to targeted therapies in BM or LM not necessarily recapitulate the response of the primary tumor: profiling the BM and LM might be advantageous in planning therapeutic interventions, predicting response, and discovering new targets that could be absent in the primary disease.

The acquired resistance to first-generation targeted therapy in NSCLC has been suggested to promote LM. Nanjo *et al.*^[43] described that acquired resistance to gefitinib is characterized by an upregulation of MET and absence of T790M mutation. Moreover, the T790M mutation has not been identified neither in BM or LM^[44,45], nor in CSF of patients who have developed LM following EGFR TKIs^[46]. Jiang *et al.*^[47] reported a lower frequency of T790M mutation (21%) and a higher frequency of MET amplification (39%) in the CSF, suggesting that MET amplification could confer a major risk of leptomeningeal invasion^[48].

Clinical and radiological diagnosis of leptomeningeal metastases

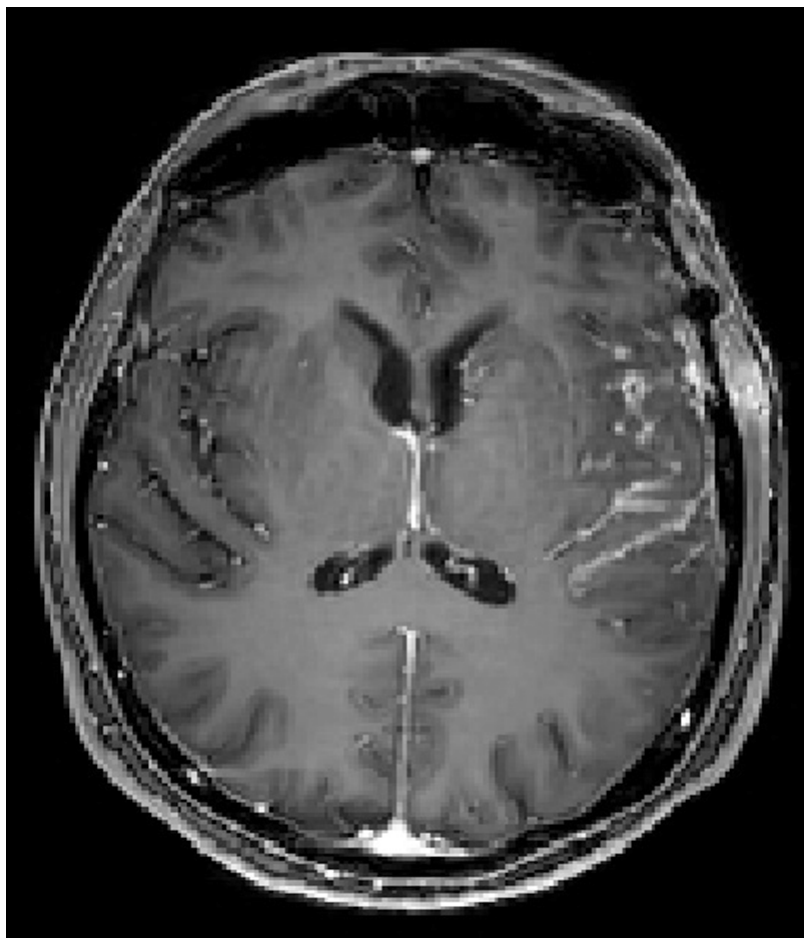
Symptoms from LM are typically multifocal due to the involvement of different segments of the neuroaxis. Spinal cord and nerve roots are the most frequent sites of LM (60%), followed by cranial nerves (35%) and cerebrum (15%)^[49]. Table 2 summarizes the most common symptoms from LM. As any site in the CNS may be involved, the evaluation of a patient with suspected LM is difficult, and signs and symptoms may be shared by BM, or mimic treatment-related toxicities or neurological paraneoplastic syndromes. Therefore, the neurological examination is crucial and should be performed by an expert neurologist. However, LM is a complication of solid tumors that is primarily being treated by medical oncologists, thus a standardized clinical evaluation is needed especially during follow up. The Neurologic Assessment in Neuro-Oncology (NANO) scale was drafted by a group of physicians, including neurologists, medical oncologists, radiation oncologists, and neurosurgeons, with expertise in neuro-oncology, and was tested during routine examination, reporting an objective clinician-reported outcome of neurologic function with high inter-observer agreement^[50,51]. Unfortunately, the NANO scale is not useful enough, due to the low sensitivity to detect the multilevel involvement of the CNS typically seen in LM. With this regard, the Leptomeningeal Assessment for Neuro-Oncology (LANO) group has developed a standardized assessment with several domains, such as gait, strength, sensation, vision, eye movement, facial strength, hearing, swallowing, level of consciousness and behavior, which may be graded as 0 (normal), 1 (slight abnormal), 2 (moderate abnormal) and 3 (severe abnormal)^[52]. However, the LANO scale needs to be validated, and other tools are being used to improve the diagnosis of LM.

Brain and spinal cord MRI with contrast enhancement is mandatory for the assessment of suspected LM^[53]. Contrast-enhanced T1-weighted and fluid-attenuated inversion recovery (FLAIR) sequences are the most sensitive to show LM^[54]. Linear or nodular enhancing lesions of the cranial nerves and spinal nerve roots (e.g., cauda equina), brain sulci and cerebellar folia are the most common findings^[55] [Figures 1 and 2]. Nodular lesions typically are small (< 5 mm) with a complex geometry, and to measure the tumor burden is difficult. As a result, the RANO Leptomeningeal Metastasis Group proposed a LANO scorecard for diagnosis of MRI in LM patients, but most of the raters experienced problems with the instructions on the

Table 2. Most common clinical manifestations of LM

Headache	Related to an increased intracranial pressure, blockage of the CSF flow and obstructive hydrocephalus
Spinal symptoms	Lower motor neuron weakness, sensory loss, radicular and back/neck pain, bladder, sexual and bowel dysfunctions
Cranial nerve palsies	Diplopia and visual impairment (II), ophthalmoparesis (III-IV-VI), hearing loss (VIII), facial weakness (VII), trigeminal sensory impairment (V), dysphagia (XI-X)
Impaired consciousness	Mood and mental changes (especially in case of encephalopathy), seizures
Gait disturbances	Due to cerebellar (coupled with nystagmus, dysmetria, dysarthria) or sensitive ataxia
Nausea/vomiting	involvement of the vestibular nerve and floor of the fourth ventricle floor

CSF: cerebrospinal fluid; LM: leptomeningeal metastases

**Figure 1.** Linear enhancement of left temporal sulci from epidermal growth factor receptor mutated non-small-cell lung cancer

scorecard, and discordance for the rating of single items at baseline and follow-up was observed. A new simplified RANO-LM score is now under development^[56]. Similarly, the European Association for Neuro-Oncology and the European Society of Medical Oncology (EANO-ESMO) have proposed a classification of the radiological findings in LM: linear lesions (type A), nodular lesions (type B), both linear and nodular lesions (type C), absence of enhancing lesions in presence of hydrocephalus (type D)^[57]. Overall, both LANO and EANO/ESMO groups have proposed a tentative diagnostic workup that include clinical symptoms, imaging, and CSF cytology for diagnosis and assessment of treatment response for LM; however, a major issue is to define measurable versus non-measurable lesions, and changes in the measurement that qualify for response. Due to these caveats, their application in daily clinical practice remains limited.



Figure 2. Diffuse linear spinal leptomeningeal enhancement from epidermal growth factor receptor and anaplastic lymphoma kinase wild-type non-small-cell lung cancer

In general, the sensitivity of contrast-enhanced MRI in detecting LM is about 70%-85% with a specificity of approximately 75%-90%^[58]. Freilich *et al.*^[59] have shown that contrast-enhanced MRI is altered in approximately 90% of patients with LM from solid tumors and positive CSF, while about 20%-30% of patients with LM may present a false-negative MRI^[4]. Therefore, a negative MRI does not exclude a diagnosis of LM in a patient with typical neurological symptoms. No other alternative imaging techniques have been used to validate negative MRI results in cases of high index of suspicion of LM. For instance, there are no studies on the sensitivity and specificity of 18F-fluorodeoxyglucose positron emission tomography-computed tomography (FDG-PET-CT) for LM diagnosis, due to resolution issues. In one patient only a LM from NSCLC was detected using FDG-PET-CT^[60].

CSF analysis

Some biochemical alterations may be found in the CSF of LM patients, such as an increased pressure ($> 200 \text{ mm H}_2\text{O}$) in 21%-42% of patients, high level of proteins ($> 50 \text{ mg/dL}$) in 56%-91%, decreased level of glucose ($< 60 \text{ mg/dL}$), and elevated leucocyte count ($> 4/\text{mm}^3$) in 48%-77.5%^[61]. All these findings are not pathognomonic of LM, and a CSF cytology positive for neoplastic cells remains the gold standard for diagnosis. CSF cytology does not allow a quantitative analysis and has a low sensitivity with 30%-50% of LM patients with negative CSF^[57]. In case of negative results after the first CSF tap, the EANO/ESMO Guidelines recommend performing a second lumbar puncture to improve the sensitivity up to 80%. Moreover, there is no evidence that a conversion to a negative CSF is correlated with disease control in leptomeninges and with a prolonged progression-free survival (PFS); thus, the cytological clearance of CSF is not a reliable method to monitor leptomeningeal response.

Liquid biopsy in leptomeningeal metastases from NSCLC

Liquid biopsy consists of detecting tumor biomarkers in body fluids, such as blood, plasma, CSF, urine, saliva, ascites, with the aim to diagnose and monitor disease. Different biomarkers may be detected,

including circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), or exosomes. CSF liquid biopsy has been suggested as a more sensitive tool to achieve a diagnosis of LM than conventional CSF cytology. CTCs can be found using rare cell capture technology and immune flow cytometry assay with antibodies against epithelial cell adhesion molecule (EpCAM), reporting a sensitivity of 76%-100% and a specificity of 84%-100% for diagnosis of LM^[62]. van Bussel *et al.*^[63] reported a sensitivity of 94% (95%CI: 80-99) and a specificity of 100% (95%CI: 91-100), with a cutoff of 0.9 CTC/mL, when using EpCAM immunoflow cytometry. Several studies have shown that the CellSearch technique with immunomagnetic identification and quantification of CSF CTCs improves the ability to diagnose LM from NSCLC, particularly in those patients with conventional acquired negative CSF cytology^[64-66]. Moreover, the addition of CSF ctDNA analysis may improve the diagnosis of LM in cases with a low amount of CSF CTCs^[67]. Plasma liquid biopsy has been investigated as a surrogate tool for diagnosis of LM from NSCLC. Unfortunately, poor concordance has been reported between plasma and CSF in LM from NSCLC regardless of the type of driver mutation and liquid biopsy technique. Zheng *et al.*^[68] have reported that next-generation sequencing of paired plasma and CSF samples of 11 patients with LM from ALK rearranged NSCLC identified driver mutations in 81.8% of CSF and 45.5% only of plasma. Similarly, Ying *et al.*^[69] have compared CSF and plasma samples of 92 patients with LM from EGFR mutated NSCLC reporting a high mutation rate in CSF (81.5%) with an overall amount of 197 mutations, whereas plasma displayed a lower mutation rate (62.5%) and amount of mutations (68%). Furthermore, a significant discordance of mutation profiles between CSF and plasma has been reported: a further analysis of EGFR showed an activating mutation in 51.4% of CSF and 38.9% of plasma samples with a concordance of 47.7%. Notably, the EGFR T790M resistance mutation was detected in CSF of 2 patients only (2.8%), denoting that mutation occurs more frequently in extracranial sites^[67,70,71]. Huang *et al.*^[72] reported similar results when evaluating EGFR status in CSF and plasma (75% *vs.* 36.4%, respectively) in a cohort of 11 LM cases from EGFR mutated NSCLC, and EGFR T790M mutation was found more frequently in plasma (39%) compared with CSF (13%). Li *et al.*^[73] have found targetable EGFR mutations in CSF of 26/26 patients (100%) and in plasma of 19/26 patients (73.1%). In particular, TP53 loss of heterozygosity (LOH) was identified in CSF of 19/26 patients (73.1%) and in plasma of 2/26 patients only (7.7%), and T790M mutation in 8/26 CSF samples (30.4%) and in 6/26 plasma samples (21.7%). Lastly, Ma *et al.*^[74] reported that 7/13 patients (53.8%), who received TKIs, developed uncommon EGFR mutations in ctDNA of CSF. Interestingly, these uncommon EGFR mutations, including G719A, L861Q, L703P, and G575R mutations, were more frequent in LM (54.5%) than in patients with BM (10%). Overall, CSF liquid biopsy appears to be more sensitive than plasma in detecting druggable mutations in LM. Moreover, CSF has a significant number of specific mutations, such as TP53 LOH, MET amplification, CDKN2A, NTRK1 and CDK4 mutations, that contribute to the tumorigenesis and development of LM from NSCLC^[69,75].

Integrated assessment for diagnosis and monitoring of LM from NSCLC

The combination of CSF analysis, including cytology and liquid biopsy, with MRI assessment may improve the ability to diagnose LM. Hyun *et al.*^[58] studied the diagnosis of LM in a cohort of 519 patients with advanced NSCLC; by MRI alone in 35% of patients, by CSF cytology alone in 22%, and by both techniques in 42%^[4]. However, the absence of a standardized quantification of LM disease burden represents a challenge. Recently, Nevel *et al.*^[76] have investigated whether MRI disease burden assessment and CSF analysis can be employed to predict survival in LM from NSCLC. For the MRI assessment, the Authors have scored the MRI using 8 predefined anatomic locations, such as cerebrum, ventricles, brainstem, cerebellum, cranial nerves, cervical, thoracic, and lumbosacral spinal cord. One point has been awarded for each affected location regardless of the number of lesions. The presence of ≥ 3 sites of disease was statistically correlated with a risk of death compared with < 3 sites (HR = 1.95; 95%CI: 1.16-3.30; $P = 0.01$), while anatomic locations of LM were not associated with OS. Regarding CSF analysis, protein level above the upper limit was not correlated with OS, while CSF white blood cell count and low glucose at diagnosis of LM were significantly correlated with OS ($P = 0.04$ and $P < 0.0001$, respectively). Importantly, Nevel *et al.*^[76] reported that patients with ≥ 50 CTCs/3 mL had an increased risk of death in comparison with those with $<$

50 CTCs/3 mL (HR = 3.39; 95%CI: 1.01-11.37; $P = 0.048$). A further analysis revealed that increased values of ctDNA concentrations (median concentrations of 0.022 ng/ μ L) were associated with an increased risk of death (HR = 16.33; 95%CI: 0.69-384; $P = 0.08$). Overall, they suggested that a significant advantage from CTCs count and ctDNA analysis in CSF coupled with a simplified MRI assessment, may help to predict survival. However, only a small number of samples of CSF has been analyzed for CTCs and ctDNA. Thus, it will be important to validate the prognostic value of MRI assessment and advanced CSF techniques in a larger and multicenter cohort of LM from NSCLC.

Treatment options for leptomeningeal metastases from NSCLC

Radiotherapy

Different radiation techniques are investigated in BM, such as stereotactic radiosurgery (SRS), whole-brain radiotherapy (WBRT), intensity modulated radiation therapy (IMRT), or proton beam therapy. Radiotherapy (RT) does not represent the first line treatment in LM for different reasons. First, a retrospective analysis has demonstrated a major impact of systemic chemotherapy and targeted agents in LM control and OS^[77]. Moreover, randomized clinical trials evaluating the efficacy and safety of RT in LM have not been conducted thus far. Focal RT, such as involved field or SRS, are considered in patients with local, circumscribed and symptomatic lesions, or in those with CSF flow obstructions due to spinal or intracranial blocks in order to improve the distribution of intra-CSF therapy. Wolf *et al.*^[78] retrospectively analyzed 16 patients with LM from solid tumor (8 NSCLC), treated with SRS, reporting a disease control of 57.1% (partial response in 8 patients) with a median OS of 10 months (6-month and 1-year OS of 60% and 26%, respectively). The Authors suggested that SRS could be added to treat bulky LM in patients also eligible for systemic therapy, including immuno-therapies and targeted therapies, with the aim to prolong OS. WBRT may be considered as palliative treatment in patients with symptomatic extensive nodular or linear LM. Gani *et al.*^[79] reported a median OS of 2 months following WBRT in 27 patients with LM from solid tumors (7 NSCLC). Ozdemir *et al.*^[80] reported a median OS 3.9 months after WBRT in a cohort of 51 LM from NSCLC, and a longer OS (11.3 months) in patients with ECOG 0-1 and without BM. Brower *et al.*^[81] retrospectively analyzed 124 patients with LM from solid tumors (32 NSCLC) and showed a median OS of 9.2 months when WBRT was utilized in conjunction with systemic chemotherapy, with a major benefit in patients with good KPS (KPS ≤ 50 : 1.1 months; KPS 60-80: 2.0 months; KPS 90-100: 5.9 months). Notably, Ozdemir and Brower identified some prognostic factors (KPS ≥ 90 and absence of BM) in patients with prolonged OS as compared with historical controls. Craniospinal RT (CSI) is not recommended because of the poor benefit and the significant risk of developing severe adverse effects (myelotoxicity, enteritis and mucositis). Hermann *et al.*^[82] have conducted a retrospective study on 16 patients with LM (5 from NSCLC) treated with CSI alone (6 patients) or in association with intrathecal methotrexate (10 patients), reporting a median OS of 2 months after CSI alone, and 4 months after combined treatment. Interestingly, most of the patients (11/16 - 68%) experienced significant neurologic improvement (improvement in walking in 7 patients, pain relief in 6 patients, reduction of bladder and bowel incontinence in 3 patients). Devecká *et al.*^[83] reported OS rates in a cohort of 19 patients with LM (5 from NSCLC); a median OS of 7.3 months, 3.3 months and 1.5 months for patients with 0, 1 and 2 risk factors according to the proposed prognostic score (KPS < 70 and the presence of extra-CNS disease), respectively. Recently, Yang *et al.*^[84] have investigated the tolerability of proton CSI in 19 patients with LM (11 from NSCLC) in a phase I trial, reporting a median OS of 8 months (95%CI: 6 to not reached), of whom 4 patients (19%) were disease free ≥ 12 months. Two patients only reported grade 4 lymphopenia, grade 4 thrombocytopenia, and grade 3 fatigue.

The US National Comprehensive Cancer Network (NCCN) 2020 guidelines for management of LM recommend focal RT in association with intrathecal chemotherapy in patients with favorable prognostic factors (KPS ≥ 60 , mild neurologic deficits, stable systemic disease, available therapeutic options for systemic disease). For patients who do not meet these criteria, focal RT to symptomatic lesions or best supportive care, are the suggested options^[85] [Figure 3]. Lastly, the use of IMRT or proton therapy for treatment of LM should not be considered as usual therapy.

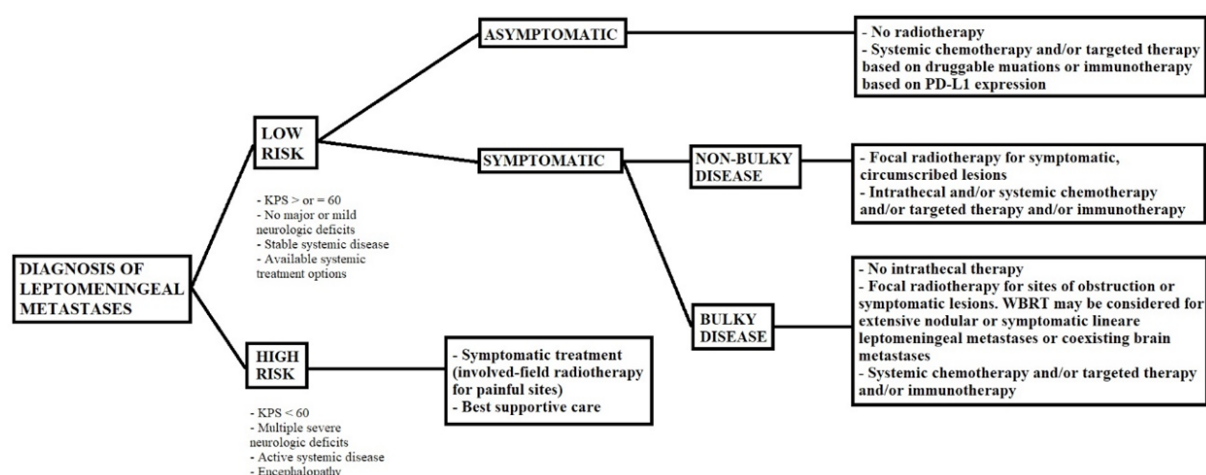


Figure 3. Suggested flowchart on management of leptomeningeal metastases

Table 3. Randomised clinical trials on intrathecal chemotherapy in LM from NSCLC

Study	No. of patients	Intrathecal therapy	Results
Grossman <i>et al.</i> ^[88] , 1993	59 (15 NSCLC)	Arm 1: MTX 10 mg twice weekly Arm 2: thioTEPA 10 mg twice weekly	Median OS: MTX: 3.9 months thioTEPA: 3.5 months No patients have neurological improvement, and 75% had neurologic improvement
Hitchins <i>et al.</i> ^[89] , 1987	44 (13 NSCLC)	Arm 1: MTX 15 mg twice weekly Arm 2: MTX 15 mg twice weekly plus Ara-C 50 mg/m ² every 2 weeks	Median OS: MTX: 4.5 months MTX/Ara-C: 1.7 months Radiological response to MTX was superior to combined MTX/Ara-C (61% vs. 45%), but not statistically significant
Glantz <i>et al.</i> ^[90] , 1999	61 (6 NSCLC)	Arm 1: Ara-C 50 mg/m ² every 2 weeks Arm 2: MTX 10 mg twice weekly	Median OS: MTX: 3.3 months Ara-C: 11 months Radiological response to Ara-C was superior to combined MTX (26% vs. 20%), but not statistically significant

NSCLC: non-small-cell lung cancer; MTX: methotrexate; Ara-C: liposomal cytarabine; LM: leptomeningeal metastases

Intrathecal therapy

Intrathecal therapy can be administered by lumbar puncture or an intraventricular route. Prolonged survival has been demonstrated using intraventricular route (e.g., Ommaya reservoir)^[86], but the management of the device may be difficult, and careful handling is required to avoid obstruction^[87]. Furthermore, some complications may occur following intrathecal therapy, including aseptic or chemical meningitis, arachnoiditis, and delayed leukoencephalopathy with seizures^[87]. In general, intrathecal compounds can only penetrate the tissue for 2-3 millimeters, thus it is preferred in patients with linear leptomeningeal lesions and non-bulky disease. Three drugs are commonly used: methotrexate (MTX), liposomal cytarabine (Ara-C) and thioTEPA. Three clinical trials have investigated the efficacy of intrathecal therapy in LM from solid tumors, including NSCLC, reporting OS between 1.7-11 months [Table 3]. Younger age (< 55 years), absence of systemic metastases or cranial nerve involvement, normal value of CSF glucose and proteins are considered favorable prognostic factors^[91]. Lin *et al.*^[92] reported a case of LM from NSCLC, who received intrathecal chemotherapy with pemetrexed via Ommaya reservoir. The local treatment led to an improvement of the quality of life, as well as the clearing of CSF cytology and stable LM disease for 17 months. Wu *et al.*^[93] have conducted a pooled analysis, that evaluated intrathecal chemotherapy in NSCLC patients. Overall, 4 prospective studies and 5 retrospective studies were included.

Thirty-seven patients were treated with intrathecal chemotherapy alone, and 552 patients received multiple interventions (intrathecal chemotherapy, WBRT, EGFR TKIs, traditional chemotherapy, and supportive care). The clinical response of the patients receiving intrathecal chemotherapy alone ranged between 71% to 79% with a median OS longer (7.5 months) than that of patients who received combined treatments (3.0-5.0 months). Overall, the efficacy of intrathecal therapy is modest, and careful evaluation of clinical factors helps clinicians to identify the subgroups of patients who may benefit.

Systemic chemotherapy for LM from NSCLC

A standard treatment is not validated thus far, but platinum based-chemotherapy with or without RT is recommended in patients with LM from NSCLC, who have no druggable mutations or programmed death-ligand 1 (PD-L1) tumor proportion score < 50^[3]. The median OS in patients with good prognostic factors is approximately 11.5 months following traditional chemotherapy^[94]. Bevacizumab has been investigated in LM after failure of first-generation EGFR TKIs with clinical and radiological response in 2 patients with LM from EGFR mutated NSCLC, who progressed after first-line treatment with erlotinib^[95].

Targeted agents

Approximately 20%-25% of patients with NSCLC have oncogene driver mutations: the most frequent is the EGFR (10%-15%) followed by the ALK rearrangement (3%-5%), while PD-L1 expression ranges from 21.9% to 32.9%. Less frequent mutations are KRAS, MET, ROS1, BRAF, and HER2. Targeting some of these mutations has shown a significant advantage in BM from NSCLC. Now, the activity of targeted therapy in LM is under investigation in clinical trials.

Role of EGFR tyrosine kinase inhibitors in LM from NSCLC

Two retrospective studies have shown that LM is more common in patients with EGFR mutations (9.4%) than in EGFR wild-type (1.7%)^[96,97]. Different studies have investigated the efficacy of first- and second-generation EGFR TKIs in LM [Table 4]. A low CSF level (1%-3%) of the first-generation EGFR TKIs has been found, suggesting an inability to adequately penetrate the BBB. Therefore, higher or “pulsatile” doses of either gefitinib or erlotinib have been administered in order to achieve adequate therapeutic concentrations, reporting a median OS ranging from 3 to 12 months^[31,98-101]. The second-generation EGFR TKI, afatinib, has shown some activity in 11 LM patients with uncommon EGFR mutation (Gly719X) that were pre-treated with erlotinib or gefitinib. Twenty-seven percent of patients achieved significant radiological response, with a median PFS and OS of 2 months and 3.8 months, respectively^[15]. The third-generation EGFR TKI osimertinib has demonstrated remarkable activity to control systemic and CNS disease^[102]. In light of that, osimertinib represents the first-line treatment, regardless of T790M mutation, and it is considered the preferred initial therapy when feasible^[103]. The increased ability to cross the BBB makes osimertinib an attractive compound to be investigated in LM. Nanjo *et al.*^[17] have investigated the standard dose of osimertinib (80 mg) in LM after failure of first- and second-generation TKIs. The Authors reported a CSF clearance in 2 patients out of 5 with a definitive diagnosis of LM, and a median PFS of 7.2 months. Notably, osimertinib was active on CSF malignant cells either with T790M or Leu858Arg mutations^[17]. The phase I BLOOM study demonstrated good activity of high-dose osimertinib (160 mg/day) in 41 patients with LM who were heavily pretreated with TKIs. The intracranial objective response rate (ORR) was 62% (95%CI: 45-78), the median OS was 11.0 months (95%CI: 8.0-18.0 months), and a CSF tumor cell clearance was confirmed in 11 patients (28%)^[104]. Saboundji *et al.*^[105] retrospectively analyzed a cohort of 20 patients treated with osimertinib: all patients (100%) displayed neurological improvement, and 5 patients (20%) showed prompt radiological response within 15 days from the start of treatment. For this cohort, median PFS and OS were of 17.2 and 18 months, respectively. Similar results were reported by Ahn *et al.*^[106], retrospectively analyzing 22 patients with LM from EGFR T790M mutated NSCLC treated with osimertinib 80 mg/day: ORR was 55% (95%CI: 32-76), and median OS was 18.8 months (95%CI: 6.3-NC). Park *et al.*^[107] also reported an intracranial ORR of 55.0%, median PFS of 7.6 months (95%CI: 5.0-16.6),

Table 4. Studies on EGFR TKIs in patients with LM from non-small-cell lung cancer

Study	No. of patients	Treatment	Outcomes
Grommes <i>et al.</i> ^[31] , 2011 Retrospective	9	Pulsatile high-dose erlotinib (1500 mg weekly)	Radiological response in 6/9 patients (66.7%) Median OS: 12 months
Lee <i>et al.</i> ^[98] , 2013 Retrospective	25	Arm 1: gefitinib 250 mg/day Arm 2: erlotinib 150 mg/day	CSF cytology in 10/25 patients (40%) Erlotinib led to CSF cytology conversion in 64.3% of patients, while 9.1% only following gefitinib
Yang <i>et al.</i> ^[99] , 2015 Retrospective	6	Pemetrexed 500 mg/m ² day 1; cisplatin 30 mg day 1-2; erlotinib 150 mg day 3-21	Response rate: CR 1/6 (16.6%); PR 2/6 (33.3%); SD 2/6 (33.3%) Median OS: 9 months
Kawamura <i>et al.</i> ^[100] , 2015 Retrospective	35	Arm 1: high-dose erlotinib (200-600 mg/day every 2-4 days) Arm 2: standard dose erlotinib (150 mg/day)	High-dose erlotinib: radiological response in 3/10 patients (30%), neurological improvement in 6/12 patients (50%) Median OS: - high-dose group: 6.2 months Standard dose group: 5.9 months
Jackman <i>et al.</i> ^[101] , 2015 Phase I	7	2 weeks of high-dose of gefitinib (750-1000 mg/day) and 2 weeks of 500 mg/day	Median OS: 3.5 months Median PFS: 2.3 months CSF cytology clearance in 1/7 patients (14.3%) Neurological improvement in 4/7 patients (57.1%)
Liao <i>et al.</i> ^[102] , 2015 Retrospective	75	Arm A: Gefitinib + CT Arm B: Erlotinib + CT Arm C: Afatinib + CT Regimen details not available	The association of TKI plus chemotherapy is correlated with prolonged survival in both univariate and multivariate analysis
Tamiya <i>et al.</i> ^[15] , 2017 Prospective	11	Afatinib 40 mg/m ² daily	Median CSF penetration: 1.65% Median CSF concentration: 1.4 ng/mL (2.9 nmol/L) Radiological response: 27.3% Median PFS: 2 months Median OS: 3.8 months
Yang <i>et al.</i> ^[16] , 2017 Phase I	32	Osimertinib 160 mg daily	20/23 patients (86.9%) had neurological improvement 23/32 (72%) had radiological response
Nanjo <i>et al.</i> ^[17] , 2017 Prospective	13 (3 definitive LM and 8 possible LM)	Osimertinib 80 mg daily	CSF penetration: 2.5% Median PFS: 7.2 months
Yang <i>et al.</i> ^[104] , 2020 Prospective	41	Osimertinib 160 mg daily	ORR 62% Median OS 15.2 months
Saboundji <i>et al.</i> ^[105] , 2018 Retrospective	20	Osimertinib 80 mg daily	100% of patients experienced neurological improvement Median PFS: 17.2 months Median OS: 18 months
Ahn <i>et al.</i> ^[106] , 2020 Retrospective	22	Osimertinib 80 mg daily	ORR 55% Median OS 18.8 months
Park <i>et al.</i> ^[107] , 2020 Phase 2	40	Osimertinib 160 mg daily	ORR 55% Median PFS 7.6 months Median OS 16.9 months
Ahn <i>et al.</i> ^[109] , 2016 Prospective	29 (4 with LM)	AZD3759	3/4 patients (75%) had a significant reduction of EGFR expression 1/4 patients (25%) had a CSF conversion in two consecutive samples
Cho <i>et al.</i> ^[110] , 2017 Prospective	18	Arm 1: AZD3759 200 mg daily Arm 2: AZD3759 300 mg daily	5/18 patients (27.8%) had a radiological response, while 9/18 patients (50%) a stable disease
Xu <i>et al.</i> ^[112] , 2020 Prospective	3	Erlotinib (150 mg/day) plus nimotuzumab (200 mg/m ²) weekly	Rapid clinical response within 6-8 weeks from the start of treatment 2/3 patients reported a radiological response

ALK: anaplastic lymphoma kinase; LM: leptomeningeal metastases; EGFR: epidermal growth factor receptor; TKIs: tyrosine kinase inhibitors; PFS: progression-free survival; ORR: objective response rate; CR: complete response; PR: partial response; SD: stable disease; OS: overall survival; TKI: tyrosine kinase inhibitor; CSF: cerebrospinal fluid

and median OS of 16.9 months (95%CI: 7.9-not reached) in a phase II trial cohort of 40 patients with LM treated with osimertinib 160 mg daily. Interestingly, osimertinib is not only active against T790M mutation, but may also target uncommon mutations, such as S768I mutation^[108].

AZD3759 is a novel compound with excellent BBB penetration, which is active against EGFR mutations, with the exception of T790M mutation. The efficacy and tolerability of AZD3759 have been investigated in 29 patients in a phase I trial. Of the four patients with LM who were enrolled, 3 displayed a significant

Table 5. Studies on ALK inhibitors in patients with LM from non-small-cell lung cancer

Study	No. of patients	Treatment	Outcomes
Costa <i>et al.</i> ^[128] , 2011	1	WBRT plus crizotinib 250 mg twice daily	PFS: 9 months
Ahn <i>et al.</i> ^[114] , 2012	2	Intrathecal MTX plus crizotinib 250 mg twice daily	PFS 5 and 10 months, respectively
Arrondeau <i>et al.</i> ^[117] , 2014	1	Ceritinib 750 mg daily	PFS: 5.5 months
Dudnik <i>et al.</i> ^[118] , 2015	3	WBRT plus ceritinib 500 mg/daily	PFS - Patients 1: 18 months Patient 2 and 3: 7 months
Gainor <i>et al.</i> ^[122] , 2015	4	Alectinib 600 mg twice daily	Radiological and neurological improvement in 4/4 patients (75%)
Ou <i>et al.</i> ^[123] , 2015	1	Alectinib 600-750 mg twice daily	Long-lasting complete response (15 months)
Gainor <i>et al.</i> ^[124] , 2016	2	Alectinib 900 mg twice daily	Radiological and neurological improvement for 3.5 and 6 months, respectively
Gaye <i>et al.</i> ^[126] , 2019	1	Brigatinib 180 mg once daily with a 7-day lead-in period at 90 mg	PFS 14 months
Pellerino <i>et al.</i> ^[128] , 2019	1	Lorlatinib 100 mg once daily	PFS 12 months Complete radiological response

ALK: anaplastic lymphoma kinase; LM: leptomeningeal metastases; CR: complete response; PR: partial response; SD: stable disease; PFS: progression-free survival; OS: overall survival; MTX: methotrexate; WBRT: whole-brain radiotherapy

reduction of EGFR expression on the cell surface, and one patient had a CSF conversion in two consecutive samples^[109]. Cho *et al.*^[110] have investigated the efficacy of AZD3759 at two different doses (200 mg or 300 mg) reporting in 5/18 patients (27.8%) a radiological response, and in 9/18 patients (50%) a stable disease. A new anti-EGFR monoclonal antibody, nimotuzumab, has demonstrated some activity in BM from NSCLC^[111]. Xu *et al.*^[112] have used nimotuzumab in association with erlotinib in 3 patients with advanced LM reporting clinical improvement within 6-8 months from the start of treatment and a radiological response in 2/3 patients.

Role of ALK inhibitors in LM from NSCLC

LM in ALK rearranged NSCLC tends to occur in approximately 5% of patients as a late complication after a median time of 9 months from the diagnosis of the systemic tumor^[6]. Although the benefit from ALK inhibitors has been established in BM, data regarding the activity in LM are limited to case-reports. The first-generation ALK, ROS1, and MET inhibitor crizotinib has demonstrated remarkable CNS disease control rate (55% and 65% at 12 and 24 weeks, respectively) in patients with BM^[23,113]. Three case reports described the activity from the association of crizotinib and WBRT or intrathecal methotrexate in LM, reporting a PFS of 6-10 months [Table 5]^[19,114]. The second-generation ALK/ROS1 inhibitor ceritinib displayed significant systemic and intracranial activity in patients with ALK rearrangement who were pretreated with crizotinib^[115,116]. Ceritinib has been also reported to be active in LM in association with either traditional chemotherapy or WBRT in patients who progressed after failure of crizotinib, with a median PFS of 5-18 months^[117,118]. The phase II ASCEND 7 trial has investigated ceritinib in BM and LM from ALK rearranged NSCLC. Forty-two patients previously treated with radiotherapy and an ALK inhibitor were assigned to arm 1; 40 patients with prior ALK inhibitor alone were assigned to arm 2; 12 patients with prior radiotherapy alone were assigned to arm 3; and 44 patients not previously treated with radiotherapy or an ALK inhibitor were assigned to arm 4. Evaluation of the intracranial response was done in 28, 29, 7, and 33 patients in the respective arms having measurable BM at baseline, and showed an intracranial objective response rate (ORR) of 39.3%, 27.6%, 28.6%, and 51.5% in arms 1, 2, 3, and 4, respectively. Unfortunately, the trial reported the intracranial ORR for BM only, without any details regarding LM response^[119]. Alectinib, which is a second-generation ALK/RET inhibitor, has been approved either after crizotinib and as a first-line treatment for ALK rearranged NSCLC^[120,121]. Different case series on LM have reported significant and durable radiological responses with both standard (600 mg twice daily) and increased dose (900 mg twice daily)^[122-124]. Moreover, the J-ALEX trial has compared the efficacy of alectinib or crizotinib as first line-treatment in BM and asymptomatic LM; however, results for the last

subgroup have not been reported^[120]. Brigatinib, a potent ALK/ROS/EGFR inhibitor, had an impressive intracranial ORR of 53%-67% with a median PFS > 12 months when used in BM^[125]. Gaye et al.^[126] reported a case of LM pretreated with first- and second-generation ALK inhibitors, and achieved a median PFS of 14 months following brigatinib. Overall, the efficacy of brigatinib in LM needs to be further investigated, and the phase 3 ALTA-1L trial includes patients with any CNS recurrence. Preliminarily, the intracranial ORR was 67% with a median PFS of 11 months, but response of LM was not analyzed separately from that of BM^[127]. Lorlatinib, which is an ALK/ROS1 inhibitor with an excellent BBB penetration, led to an intracranial ORR of 44% in ALK rearranged NSCLC patients heavily pretreated with ALK inhibitors^[23]. Moreover, a recent case report described a significant and long-lasting response of a spinal LM, achieving a PFS of 12 months from the start of treatment^[128].

Immunotherapy for LM from NSCLC

Inhibitors of the programmed death-1 (PD-1)/PD-ligand 1 pathways, such nivolumab and pembrolizumab, have shown some efficacy in patients with advanced NSCLC patients with pretreated BM^[129-132]. The PD-L1 expression and tumor-infiltrating lymphocytes have been suggested to be predictive factors for response to immune checkpoint inhibitors (ICIs)^[133], but their expression in LM remain unknown. Gion et al.^[134] described neurological improvement lasting 7 months in patients with LM treated with nivolumab, and Dudnik et al.^[135] reported 1 partial response and 1 stable disease lasting > 21 and 10 weeks, respectively, in 2 patients with LM after a treatment with nivolumab. A prospective evaluation of 19 patients with LM from NSCLC, who were treated with ICIs (13 with nivolumab and 6 with pembrolizumab), showed a median PFS of 3.7 months, and a 6- and 12-months OS of 36.8 and 21.1%, respectively^[136]. Brastianos et al.^[137] have investigated the efficacy of pembrolizumab in a phase 2 trial of 20 patients with LM (2 from NSCLC). The study met the primary endpoint, as 12/20 (60%, 90%CI: 0.39-0.78) patients were alive at 3 months after enrollment. The activity of pembrolizumab is now being investigated in a phase 2 study focused on LM from NSCLC (NCT03091478). The Table 6 summarizes the ongoing clinical trials on LM from NSCLC.

Steroids in the management of LM

Steroids are frequently used in daily clinical practice for the treatment of neurological symptoms from LM. Considering the long half-life that allows the administration in a single daily dose, dexamethasone is the most used steroid. The main advantage is represented by the significant glucocorticoid potency, associated with the virtual absence of mineralocorticoid effects, resulting in a decreased risk of electrolyte imbalances compared with other steroids. The main effects are to decrease the permeability of the BBB and limit the extravasation of fluid^[138,139], and antiemetic properties by reducing the cellular 5-HT₃ receptor expression on the medulla oblongata^[140,141]. In general, steroids are employed to reduce meningeal irritation and radicular pain from LM or chemical meningitis following intrathecal chemotherapy^[57]. Studies on dosing and tapering of dexamethasone in LM have not been performed thus far, therefore the dose should be tailored to each patient's individual needs. In general, the lowest dose of steroids should be used for the shortest time possible to limit adverse events, such as arterial hypertension, increased risk of fungal infections, osteoporosis, diabetes, myopathy, and psychiatric effects (e.g., insomnia, emotional lability, hypomanic and manic episodes)^[142].

Prognostic factors

In general, there are two models used for predicting outcomes in patients with LM. The first is based on the general condition of patients detected by Karnofsky performance score (KPS), neurologic symptoms, and presence of extracranial metastases. In this regard, the US National Comprehensive Cancer Network (NCCN) guidelines (version 2, 2020) for patients with LM stratifies patients as "good risk" or "poor risk"^[85]. Good risk patients have KPS ≥ 60, mild neurologic deficits, no bulky disease, stable systemic disease, and available therapeutic options for systemic disease, resulting in a prolonged survival compared with "poor risk patients. The MRI presentation of LM has been suggested to impact the survival: in particular,

Table 6. Ongoing clinical trials on LM from NSCLC

Study	No. of patients	Treatment	Outcomes
NCT04356118 Phase 4	30	Recombinant human endostatin 7.5 mg/m ² /day once a day for 2 weeks and 1 week off plus intrathecal MTX plus targeted therapy (EGFR TKIs or ALK inhibitors)	Primary: OS Neurological PFS Adverse events Secondary: ORR
NCT04343573 Phase 2	100	Arm 1: proton CSI (30 Gy/30 fr) plus standard of care for LM per physician choice Arm 2: proton CSI (30 Gy/10 fr) alone	Primary: CNS PFS Secondary: OS
NCT04356222 Phase 4	30	Durvalumab 10 mg/kg every 2 weeks plus intrathecal MTX	Primary: OS Neurological PFS Adverse events Secondary: ORR
NCT04192981 Phase 1	36	WBRT (30 Gy/10 fr) plus GDC-0084 in 3+3 dose-escalation cohort: 45, 60, 75 mg daily, with a potential dose de-escalation cohort to 30 mg	Primary: MTD Secondary: Local recurrence rate
NCT04425681 Phase 2	20	Osimertinib 80 mg daily plus bevacizumab 7.5 mg/kg every 3 weeks	Primary: PFS ORR Secondary: Adverse events
NCT04148898 Phase 2	80	Arm 1: Osimertinib 80 mg daily alone Arm 2: Osimertinib 80 mg daily plus bevacizumab 7.5 mg/kg every 3 weeks	Primary: PFS ORR Secondary: OS Adverse events
NCT03719768 Phase 1b	23	Avelumab 800 mg iv every 2 weeks plus WBRT (30 Gy/10 fr)	Primary: Safety and dose limiting toxicity Secondary: ORR Number of T cells in CSF Activation status of T cells in CSF
NCT03091478 Phase 2	13	Pembrolizumab 200 mg every 3 weeks	Primary: ORR

MTX: methotrexate; EGFR TKIs: epidermal growth factor receptor tyrosine kinase inhibitors; ALK: anaplastic lymphoma kinase; OS: overall survival; PFS: progression-free survival; ORR: objective response rate; CSI: craniospinal irradiation; WBRT: whole-brain radiotherapy; MTD: maximum tolerated dose; LM: leptomeningeal metastases; NSCLC: non-small-cell lung cancer

a diffuse linear enhancement LM has been correlated with a prolonged OS compared with nodular LM from NSCLC^[4,57]. Those 2 models may not be reliable to predict the prognosis of LM from NSCLC without an integration of molecular markers, especially in patients with druggable mutations. Some authors have suggested a prognostic assessment integrated with molecular alterations (molGPA) to predict the outcome of LM patients from NSCLC. In particular, 301 patients with LM from NSCLC were scored using the molGPA and classified them into 3 prognostic groups of high, intermediate and low risk (molGPA score of 0, 0.5-1.0 and 1.5-2.0, respectively). The median OS of high, intermediate and low risk LM patients were 0.3, 3.5 and 15.9 months, respectively ($P < 0.001$). Moreover, EGFR/ALK positivity, KPS ≥ 60 , and absence of extracranial metastases are independent predictive factors for better OS^[143].

CONCLUSION

The leptomeningeal space remains a sanctuary site, with limited penetration of drugs. Recent advances in diagnosis and treatment have been made, but several issues are still unaddressed. A standardized MRI assessment for evaluating LM at diagnosis and during follow needs to be validated in prospective cohorts. Similarly, CSF liquid biopsy could be a useful tool for diagnosis and monitoring of LM, especially in those

patients with equivocal MRI findings, but sensitivity and specificity of different liquid biopsy techniques have to be compared, and cut-off values should be identified. The third-generation EGFR inhibitor osimertinib has demonstrated significant CNS penetration and survival advantage in EGFR-mutated LM compared with standard therapies, such as RT, traditional chemotherapy, first- and second-generation TKIs. The combination of third-generation TKIs with RT or traditional chemotherapy could provide an additional advantage in terms of quality of life and disease control. Several case-reports have reported some efficacy of ALK inhibitors in LM, but clinical trials should be designed to confirm this benefit. For those patients who do not have druggable mutations, ICIs could represent a therapeutic option with acceptable tolerability, but clinical trials focused on LM from NSCLC are lacking and represent a research focus for the future.

DECLARATIONS

Authors' contributions

Conceptualization, writing original draft and preparation: Pellerino A, Soffietti R, Rudà R

Data collection and curation: Pellerino A, Internò V, Muscolino E, Mo F, Bruno F, Pronello E, Franchino F, Soffietti R, Rudà R

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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

Not applicable

Consent for publication

Not applicable

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Review

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A review on exosome-based cancer therapy

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How to cite this article: Schulze AV, Deng F. A review on exosome-based cancer therapy. *J Cancer Metastasis Treat* 2020;6:42. <http://dx.doi.org/10.20517/2394-4722.2020.79>

Received: 6 Aug 2020 **First Decision:** 21 Sep 2020 **Revised:** 18 Oct 2020 **Accepted:** 27 Oct 2020 **Published:** 6 Nov 2020

Academic Editor: Robert J Griffin **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Cancer is one of the leading causes of mortality and morbidity globally. Many types of cancer treatments have been developed, such as chemotherapy, surgery, radiotherapy, and immunotherapy. However, these therapies can also kill healthy cells and lead to severe side effects. Therefore, scientists are looking for new strategies to eliminate cancerous cells specifically. Exosomes, nanometer-sized lipid bilayer-enclosed vesicles secreted from various cell types, exist in nearly all body fluids, including blood, breast milk, saliva, urine, bile, pancreatic juice, cerebrospinal, and peritoneal fluids. They carry myriad donor cell-derived bioactive molecules such as proteins, lipids, and RNAs (including microRNA and lncRNA) and can deliver them to both nearby and distant recipient cells. Due to these characteristics, exosomes have attracted great interest in cancer treatment (especially serving as a biological carrier for some drugs, microRNA, lncRNA, inhibitors, and antibodies). In this paper, we will review the current knowledge of exosome therapeutic applications in cancer.

Keywords: Exosomes, cancer, cancer therapy, gene carrier, drug carrier

INTRODUCTION

Cancer remains the leading cause of death globally. There are over 200 types of cancer which claim more than 10 million lives annually^[1]. Despite a lot of research focusing on this grave disease, cancer therapeutics still have the lowest clinical trial success rate of all major diseases. This is likely due to the fact that it is hard for our immune system to distinguish cancerous cells from healthy cells. For instance, current therapeutics like radiotherapy and chemotherapy not only kill the cancerous cells, but also healthy cells^[1]. Therefore, developing new therapeutic strategies in order to precisely eliminate cancerous cells is an urgent need.



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Exosomes are nanosized extracellular vesicles which are secreted by various cells throughout the body^[2-5]. They carry important bioactive molecules including proteins, lipids, DNA (e.g., mitochondrial DNA and genomic DNA), and RNAs [e.g., microRNAs (miRNAs), long non coding RNAs (lncRNAs), and messenger RNA (mRNA)], and can transfer them to the recipient cells - thus playing a crucial role in cell-cell communication^[2-6]. Exosomes are present in most bodily fluids including blood, breast milk, saliva, urine, bile, pancreatic juice, cerebrospinal, and peritoneal fluids^[2-6]. Through the circulating flow, they can transfer from their original cells to distant tissues where they localize to target cells by binding their surface molecules to receptors on the surface of the target cells^[2-6]. Due to the advantage of their unique biocompatibility and high stability, exosomes have attracted great interest in cancer treatment as an effective anti-cancer drug delivery carrier or gene carrier. In this review, we provide an overview for studies of exosomes application in cancer therapies, as well as their advantages and challenges.

DUAL ROLES OF EXOSOMES IN TUMORIGENESIS

According to the exosome database ExoCarta (www.exocarta.org), 9,769 proteins, 3,408 mRNA, 2,838 miRNA, and 1,116 lipids have been identified in exosomes from different organisms and bodily fluids^[7]. By transferring bioactive molecules from the donor cells to the recipient cells, exosomes play crucial roles in cell-cell communication^[2-5]. Many studies have shown that cancer cells release a larger number of exosomes to exchange information with other cells both nearby and at distance^[8-10]. Through delivering their bioactive cargoes (including proteins, miRNAs, and lncRNAs), cancer cell-derived exosomes contribute to the formation of the pre-metastatic microenvironment, tumor growth and progression, immune escape, angiogenesis, anti-apoptotic signaling, drug-resistance, and so on^[8-10] [Figure 1]. Meanwhile, exosomes from healthy cells, such as dendritic cells (DCs), B cells, and T cells, play an important role in inhibiting tumor growth^[11-14] [Figure 1]. To date, numerous miRNAs, lncRNAs, and proteins have been found to play important roles in cancer progression [Table 1]. Therefore, depending on their cell of origin and their bioactive cargo, exosomes can play dual roles in cancer regulation, either inhibiting or promoting growth.

EXOSOME-BASED CANCER THERAPIES

Since exosomes can unload bioactive cargo to cancer cells, they have attracted great interest in cancer treatment^[66-72]. Currently, several different methods have been developed for cancer therapies: (1) using naturally derived exosomes from immune cells to suppress cancer cells^[66]; (2) inhibiting the release of cancer-derived exosomes; (3) using exosomes as gene carriers^[69]; and (4) using exosomes as anti-cancer drug carriers^[68,71] [Figure 2].

Naturally derived exosomes for cancer therapy

In cancer-immunity, DCs are involved in the first step of tumor cell growth inhibition by capturing neoantigens and triggering the tumor-specific cytotoxic lymphocyte response^[73]. DC-derived exosomes (Dex) contain various bioactive cargoes responsible for antigen presentation, making them ideal for the treatment of cancer^[74,75]. In 1998, Zitvogel *et al.*^[76] found that tumor peptide-pulsed Dex were able to activate the antigen-specific cytotoxic T lymphocytes response *in vivo* and eradicate or suppress growth of established murine tumors in a T cell-dependent manner. Moreover, Munich *et al.*^[77] found that Dex can directly kill tumor cells and activate naturel killer (NK) cells through TNF superfamily ligands. It has also been found that cancer cell-derived exosomes have an immunostimulatory effect on anti-tumor DCs^[66]. Thus, Dex represent an important strategy for cancer therapy.

Interfering with cancer cell-derived exosomes for cancer therapy

Cancer cell-derived exosomes are considered to accelerate cancer pathogenesis by contributing to the formation of the pre-metastatic microenvironment, tumor growth and progression, immune escape, angiogenesis, anti-apoptotic signaling, and drug-resistance^[8-10]. Therefore, inhibition of the cancer cell-

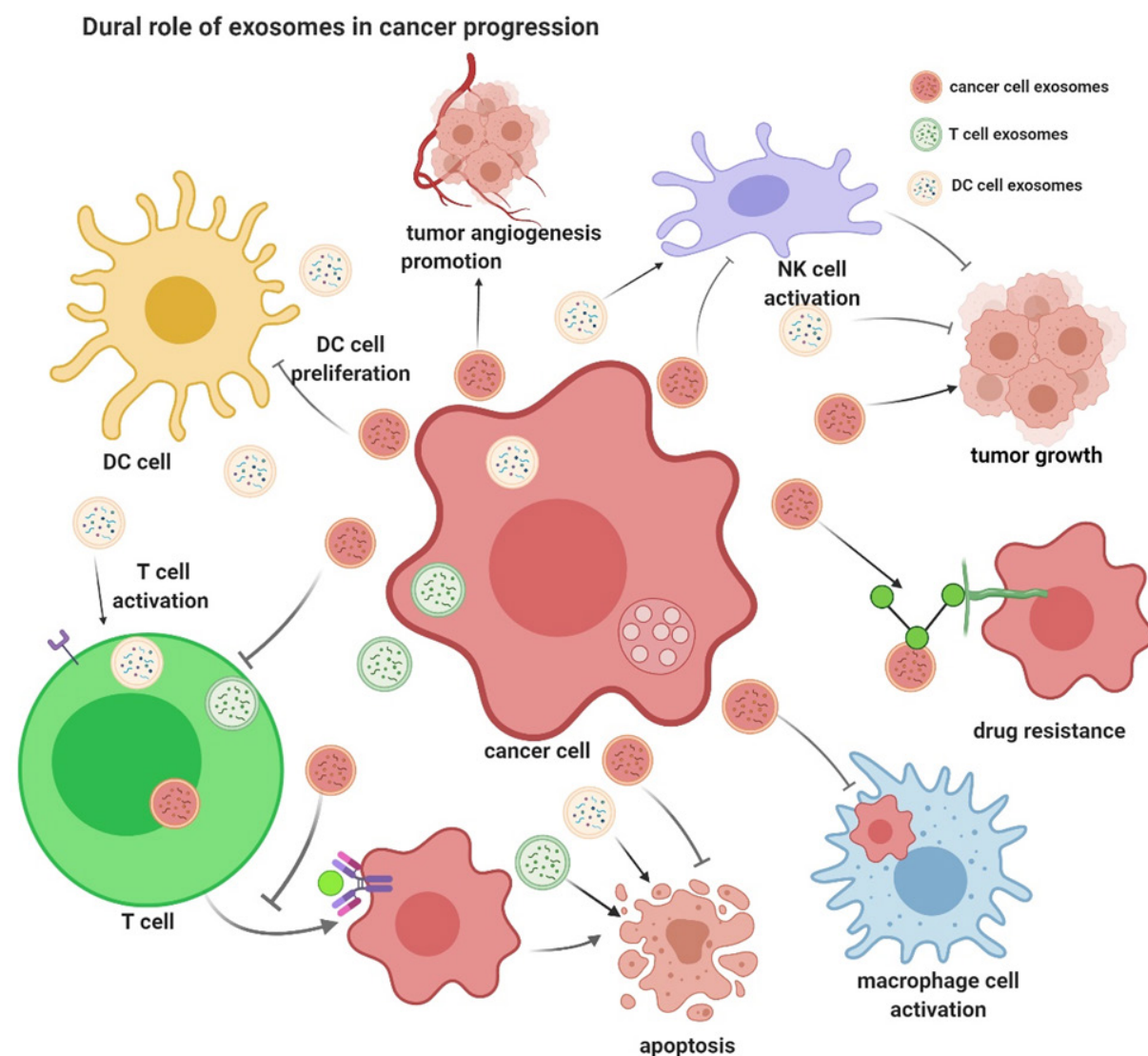


Figure 1. Dual role of exosomes in cancer progression. Cancer cell-derived exosomes contribute to the formation of the pre-metastatic microenvironment, tumor growth and progression, immune escape, angiogenesis, anti-apoptotic signaling, drug-resistance, and so on. While exosomes from healthy cells including dendritic cells, B cells, and T cells, play crucial role in inhibiting tumor growth (This figure was created with BioRender.com)

derived exosomes synthesis, release, and uptake may serve as an effective cancer therapy^[78]. With the use of a mouse model, Bobrie *et al.*^[78] found that blockade of Rab27a, a key mediator of exosome secretion, resulted in decreased primary tumor growth and lung dissemination of metastatic carcinoma (4T1) cells. It was found that miR-494 is enriched in melanoma-derived exosomes, and that exosomal transport of miR-494 promotes metastasis^[79]. More specifically, inhibiting the function of Rab27a blocked exosomal transfer of miR-494 and resulted in inhibiting melanoma growth and metastasis^[79]. Another possible way to mitigate tumor growth is to simply remove circulating exosomes via a hemofiltration system (such as the Aethlon ADAPTTM system). This alternative may also serve as another possible therapeutic approach for reversing immune dysfunction and improving the immune responses to tumor growth^[80]. Finally, multiple studies have discovered that blocking exosomal uptake is another potentially effective method for cancer therapy^[81,82]. For example, the heparan sulfate (HS) proteoglycans has been discovered as internalizing receptors of cancer cell-derived exosomes^[82]. HSPG deficiency, or the HS mimetic (heparin) treatment, significantly reduced exosome uptake and exosome-mediated stimulation of cancer cell migration^[82]. In all three cases (blocking exosomal secretion, exosomal removal, and blocking exosomal uptake), disrupting

Table 1. List of exosomal bioactive molecules involved in various cancer progression

Exosome cargos	Function in cancer progression
MicroRNAs	
miR-122	Promotes metastasis ^[15]
miR-9	Promotes tumor angiogenesis ^[16]
miR-105	Induces vascular leakiness and promoting metastasis ^[17]
miR-135b	Enhances angiogenesis ^[18]
miR-93-5P	Promotes the proliferation of esophageal cancer cells ^[19]
miR-132	Promotes angiogenesis ^[20]
miR-23	Enhances angiogenesis and vascular permeability ^[21]
miR-200	Promotes cancer cell metastasis ^[22]
miR-126	Promotes the anti-tumor response ^[23]
miR-92a	Regulates hepatic pre-metastatic niche in lung cancer ^[24]
miR-210	Increases angiogenesis and tumor progression ^[25]
miR-221	Promotes oncogenic activity in gastric cancer ^[26]
miR-141	Associates with the presence of cancer ^[27]
miR-375	Associates with the presence of cancer ^[27]
miR-494	Enhances angiogenesis through the suppression of PTEN and the Akt/eNOS pathway ^[28,29]
miR-21	Promotes hepatocellular carcinoma growth ^[30]
miR-103	Promotes tumor progression and angiogenesis ^[31]
miR-542-3p	Regulates tumor growth/Wnt signaling through targeting cdh17 and TRAF4 ^[29]
miR-19a	Metastatic colonization through specifically silencing PTEN ^[32]
miR-92	Metastatic colonization through specifically silencing PTEN ^[32]
miR-17	Metastatic colonization through specifically silencing PTEN ^[32]
miR-373	Raises the invasion and metastasis of tumor ^[33]
miR-501	confers doxorubicin resistance and tumorigenesis ^[34]
miR-1247-3p	Promotes cancer progression ^[35]
hsa-miR-940	Induces extensive osteoblastic lesions in the resulting tumors ^[36]
Long noncoding RNAs	
lncRNA-HOTTIP	Promotes cisplatin resistance ^[37]
lncRNA-ZFAS1	Enhances gastric cancer cell proliferation and migration ^[38]
lncRNAs-MALAT-1	Promotes cell proliferation and migration in non-small cell lung cancer ^[39]
lncRNA-TUC339	Promotes cell proliferation, clonogenic growth and tumor growth ^[40]
lncRNA-PART1	Induces gefitinib resistance ^[41]
lncRNA-UCA1	Enhances UCA1 expression and resistance to cetuximab ^[42] ; Induce tamoxifen resistance ^[43]
lncRNA-Sox2ot	Promotes EMT and stemness ^[44]
lincRNA-ROR	Suppresses cell apoptosis ^[45]
lncRNA-AFAP1-AS1	Induces resistance to trastuzumab ^[46]
lncRNA-ARSR	Induces resistance to the sunitinib ^[47]
lncRNA-SNHG14	Induces resistance to trastuzumab ^[48]
lncRNA PCSEAT	Enhances cellular proliferation and motility ^[49]
lncRNA-SBF2-AS1	Enhances temozolomide resistance ^[50]
lncRNA-H19	Enhances resistance of doxorubicin ^[51]
lncRNA-RP11838N2.4	Promotes erlotinib resistance ^[52]
Proteins	
MIF	Initiates pro-metastatic effect ^[53]
EGFR vIII	Activates the VEGF signaling ^[54]
TGF- β	Suppresses T cell proliferation ^[55]
ANXA6	Facilitates the establishment of lung metastasis ^[56]
Integrins	Promotes cancer progression ^[57]
Tspan8	Essential for the crosstalk between cancer initiating cells and their surrounding ^[58]
CD151	Essential for the crosstalk between cancer initiating cells and their surrounding ^[58]
MICA	Prevents NK cell-mediated tumor elimination ^[59]
TNF- α	Induces immune cells death ^[60]
CEMIP	Promotes cancer cell colonization in brain metastasis ^[61]
PD-L1	Inhibits immune responses ^[62,63] , promotes tumor growth ^[64,65]

PTEN: phosphatase and tensin homolog; EMT: epithelial-mesenchymal transition; VEGF: vascular endothelial growth factor; TNF- α : tumor necrosis factor- α ; TGF- β : transforming growth factor- β ; PD-L1: programmed death-ligand 1; EGFR: epidermal growth factor receptor

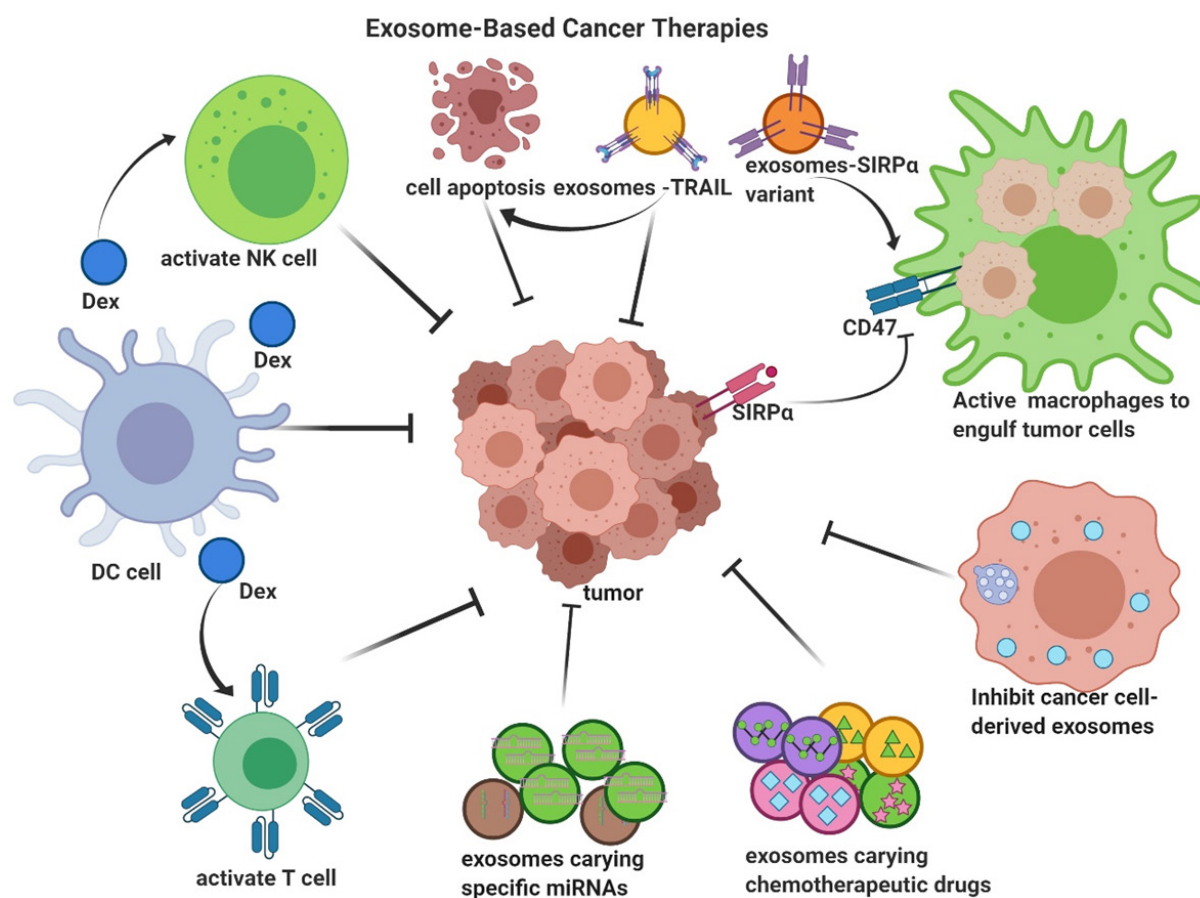


Figure 2. Exosome-based cancer therapies. Currently, four different methods have been developed for cancer therapies: (1) natural exosomes from some immune cells to suppress cancer cells; (2) inhibition of cancer cells-derived exosomes; (3) exosomes as gene carriers; and (4) exosomes as anti-cancer drug carriers (This figure was created with BioRender.com)

cell-cell communication from cancer-cell derived exosomes appears to offer an exciting new way to treat cancer.

Exosomes as gene carriers for cancer therapy

Although, there is great potential for exosomes in cancer therapy, the use of natural exosomes is hard and rarely achieves the expected therapeutic result. Fortunately, engineered exosomes carrying specific proteins, RNAs, or drugs have been found to possess great potential for effective cancer treatment.

Exosomes as miRNA carrier for cancer therapy

miRNAs are endogenous, small, non-coding RNAs that can regulate gene expression by binding to target mRNAs. Therefore, miRNAs could be a powerful tool for cancer therapy. However, miRNAs are easily degraded *in vivo* and delivery of miRNAs to their specific target cells/tissue/organ is a major challenge. As exosomes are stable small vesicles that can carry functional bioactive molecules long distances with a high degree of target specificity^[2-6], they have been suggested as a potential carrier for miRNAs in cancer therapy. Over the last few years, many scientists have focused on exosomal-based delivery of miRNAs and miRNA inhibitors for cancer therapy^[83-88]. For instance, in 2013, Katakowski *et al.*^[83] reported that exosomes enriched with the anti-glioma miRNA (miRNA-146b) can suppress glioma growth *in vitro* and can also significantly reduce glioma xenograft growth in rats. Similarly, miR-101-enriched exosomes can suppress osteosarcoma cell invasion/migration *in vitro* and suppress metastasis *in vivo*^[84]. Wang *et al.*^[85] also reported that exosomes loaded with miR-335-5p can decrease cancer growth and invasion both *in vitro* and *in vivo*.

Interestingly, O'Brien *et al.*^[86] used engineered mesenchymal stem cells to secrete exosomes enriched with miR-379 for *in vivo* therapy of breast cancer. Importantly, they found that systemic administration of miR-379 enriched exosomes can significantly reduce tumor activity. Another miRNA, miR-145-5p, was also found to inhibit pancreatic ductal adenocarcinoma cell proliferation and invasion, as well as increased apoptosis. Moreover, exosomes transfected with miR-145-5p were able to inhibit pancreatic ductal adenocarcinoma cell proliferation and invasion through TGF- β /Smad3 pathways^[87]. On the other hand, inhibitors of exosomal miRNAs, which play key roles in cancer progression through exosome, have become another effective method for cancer therapy. For example, exosomal miR-25-3p is a metastasis-promoting miRNA of colorectal cancer. Exosomes enriched with miR-25-3p dramatically promoted vascular permeability and colorectal cancer metastasis in mice liver and lung. Nevertheless, these effects can be rescued by blockage of exosomal miR-25-3p by a miR-25-3p inhibitor^[88].

Exosomes as protein carrier for cancer therapy

Recently, many scientists have begun to develop an exosomal-based cancer vaccine^[89-91]. For example, TNF-alpha-related-apoptosis-inducing-ligand (TRAIL), a cytokine, functions as a ligand that induces cell apoptosis^[92,93]. Rivoltini *et al.*^[94] reported that TRAIL-armed exosomes could promote apoptosis in cancer cells and control tumor progression *in vivo*. Furthermore, IL-18 enriched exosomes enhance Th1 cytokine release and proliferation of peripheral blood mononuclear cells, suggesting that IL-18 enriched exosomes harbor more capability to induce specific anti-tumor immunity as they trigger a bigger immune response^[95]. Yang *et al.*^[96] also found that IL-2 enriched exosomes induce the antigen-specific Th1-polarized immune response and cytotoxic T lymphocyte response more efficiently, leading a significant inhibition of tumor growth in mice. Exosomes can also be used as carriers of protein antagonists. For example, signal regulatory protein α (SIRP α) can interact with CD47, a “don't eat me” signal that limits the ability of macrophages to engulf tumor cells. Exosomes carrying SIRP α antagonists could significantly increase tumor phagocytosis, as has been observed in tumor-bearing mice^[97].

Exosomes as chemotherapeutic drug carriers for cancer therapy

Anti-tumor chemotherapeutic drugs can effectively kill fast-growing tumor cells. However, these drugs can also kill the normal, healthy cells that are fast-growing, causing serious side effects. Besides, for some hydrophobic drugs, it is a challenge for them to target tumor cells with any kind of specificity. Therefore, an effective carrier for these drugs is badly needed. Due to their naturally derived origin and their stable lipid bilayer structure, exosomes have the great potential to serve as an effective carrier for chemotherapeutic agents. As early as 2012, Tang *et al.*^[98] reported that tumor cell-derived microparticles can be used as chemotherapeutic drug carriers. They found that chemotherapeutic drugs loaded onto microparticles had a potent anti-tumor effect both *in vitro* and *in vivo*^[98]. In 2015, Kim *et al.*^[99] developed a exosome-based formulation of paclitaxel (PTX), a commonly used chemotherapeutic agent, to overcome multiple drug resistance (MDR) in cancer cells. Three methods including incubation at room temperature, electroporation, and mild sonication were used to incorporate PTX into exosomes; among which, the mild sonication method resulted in the highest loading efficiency and sustained drug release^[99]. PTX-loaded exosomes (exoPTX) increased cytotoxicity more than 50 times in drug resistant MDCK_{MDR1} (Pgp +) cells^[99]. Furthermore, through a similar *in vivo* mice model, Kim *et al.*^[99] found that exoPTX can efficiently target cancer cells and produce strong antineoplastic effects in mice with lung metastases. Similarly, Saari *et al.*^[100] also found that delivery of PTX via cancer cell-derived exosomes enhances the cytotoxicity of PTX in autologous prostate cancer cells. Furthermore, by modifying the exosome surface proteins, exosomes can deliver chemotherapeutic drugs to cancer cells with a high degree of specificity. For example, Tian *et al.*^[101] engineered Lamp2b-iRGD peptide (α v integrin-specific) expressing mouse immature DCs (imDCs), isolated their exosomes and used them to deliver doxorubicin (Dox). Using this approach, they found that iRGD-exosomes can efficiently target and deliver Dox to α v integrin-positive breast cancer cells *in vitro*, and specifically to tumor tissues, resulting in inhibition of tumor growth *in vivo*^[101].

CONCLUSION AND FUTURE DIRECTIONS

Due to their role in cancer progression and biological features, exosomes possess promising potential for cancer therapy. To date, numerous exosomes-based cancer therapies have been studied and developed including applying naturally derived immune cell exosomes to suppress cancer cells, inhibiting cancer cells-derived exosomal activity, and using exosomes as gene/drug carriers. However, there are considerable challenges to be solved. First, the difference among exosomes from different sources is still not clear. Second, the exosome number to get a therapeutic effect may be significantly different among different cancers. Third, scalability and heterogeneity of tumor may influence therapeutic outcome. Moreover, different functions of exosomes derived from different sources are not fully studied. Furthermore, knowledge on how exosomes can be modified so that they possess a high degree of specificity to particular cancer cells remains unclear. Finally, the storage and the stability of exosomes remains ill-defined. With these challenges, there is a vital need to systematically characterize exosomes derived from different cells/tissue to choose the most efficient cells for specific cancer therapy. There is a further need to identify the specific cancer cell surface markers for designing exosomes as drug carriers with high specificity to cancer cells. Moreover, because of the complexity and the heterogeneity of the tumor, exosome-based cancer may need to combine with other approaches. Finally, clinical trials of exosome-based cancer therapy are urgently needed to determine the efficacy of the application. While there are still challenges ahead, it is clear that exosomes offer novel and important applications for the treatment of cancer.

DECLARATIONS

Acknowledgments

All figures were created with BioRender.com.

Authors' contributions

Conceived the general idea of the review, made up the structure and searched the literature: Deng F

Drafted the manuscript: Schulze AV, Deng F

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

Not applicable.

Consent for publication

Not applicable.

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Review

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Recent advances in immunotherapy for pancreatic cancer

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How to cite this article: Chi J, Patel R, Rehman H, Goyal S, Saif MW. Recent advances in immunotherapy for pancreatic cancer. *J Cancer Metastasis Treat* 2020;6:43. <http://dx.doi.org/10.20517/2394-4722.2020.90>

Received: 31 Aug 2020 **Accepted:** 20 Oct 2020 **Published:** 6 Nov 2020

Academic Editor: Godefridus J. Peters **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Pancreatic ductal adenocarcinoma (PDAC) remains a disease with a dismal prognosis. Since 1996 there have only been two upfront regimens found to be superior to gemcitabine: FOLFIRINOX (5-fluorouracil, leucovorin, irinotecan and oxaliplatin), and gemcitabine plus nab-paclitaxel. Despite the improvement noted in these trials, PDAC is highly chemo-resistant and patients who respond will inevitably develop resistance. The unique immunosuppressive tumor microenvironment with extensive desmoplasia has posed challenges to developing new and effective treatments. Therapeutic vaccines, combination treatments, adoptive T cell transfer, as well as immunomodulators are being explored. With the emerging use of immunotherapy and immunomodulators, the scope of this review is to present the current data on these agents as well as focus on the advancements in the treatment of PDAC. Overall, results in this realm have been disappointing to date reflecting the non-immunogenic and complex tumor microenvironment of PDAC.

Keywords: Pancreatic ductal adenocarcinoma, chemoresistance, desmoplasia, tumor microenvironment, extracellular matrix, immunogenicity, immunotherapy, vaccines, immune checkpoint inhibitors, immunomodulation

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) has a grim prognosis and is now the third leading cause of cancer-related deaths in the United States^[1]. Furthermore, it is estimated that pancreatic cancer will become



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the second leading cause of cancer-related deaths in the United States by 2030^[2]. It is estimated that 57,600 Americans will be diagnosed with pancreatic cancer and more than 47,050 will die of the disease in 2020 making it the most lethal malignancy of all major cancers^[1]. Most patients present with unresectable or metastatic disease leading to an abysmal 5-year-overall survival (OS) rate of only 7%. Even when surgery is feasible in 15%-20% of the patients, the 5-year survival remains only about 10%^[3]. Current front-line treatment options include traditional chemotherapies that only provide modest survival benefits.

Gemcitabine was considered the backbone of management for metastatic PDAC as it provided a survival benefit as well as alleviation of symptoms compared to fluorouracil (5-FU)^[4]. Many trials after this failed to show improvement from this integral study in 1997. Various combination treatments have been explored since then to see if any headway could be made in the treatment of this lethal disease. Notably, combination treatments with epidermal growth factor receptors (EGFR) as well as anti-vascular endothelial growth factors (VEGF) did not produce clinically meaningful results^[4-6]. Additionally, combination treatments exploring gemcitabine with various cytotoxic agents including the addition of oxaliplatin, 5-FU, capecitabine, and irinotecan failed to demonstrate a statistically significant OS advantage^[7-10]. Two chemotherapy combination regimens have shown superiority in patients with metastatic disease since then. In the PRODIGE/ACCORD and MPACT trials, FOLFIRINOX and gemcitabine plus nab-paclitaxel, respectively, showed an OS benefit at the cost of increased toxicity^[11,12]. OS has improved with the addition of chemotherapy in the adjuvant setting as well, however, there is much room for improvement^[13,14]. Despite the improvement noted in these trials, pancreatic cancer is highly chemo-resistant and patients who respond will inevitably develop resistance to these therapeutic modalities^[15]. Based on this historical data, providers can attempt treatment with varying success, but there is still a dire need to develop newer agents and incorporate newer strategies to improve outcomes in pancreatic cancer.

Recently, immunotherapy has revolutionized cancer care and has garnered approval in many different solid tumors, including melanoma, lung cancer, and urothelial cancers, among others^[16]. Therefore, it has been of great interest to explore the role of various immunotherapies in PDAC. Overall, results in this realm have been disappointing to date, reflecting the non-immunogenic and complex tumor microenvironment of PDAC. To overcome these challenges, therapeutic vaccines, combination treatments, adoptive T cell transfer, as well as immunomodulators are being explored. With the emerging use of immunotherapy and immunomodulators, the scope of this review is to present the current data on these agents as well as focus on the advancements in the treatment of PDAC.

MECHANISMS OF CHEMORESISTANCE AND TUMORIGENESIS

Pancreatic cancer is highly resistant to chemotherapy and radiotherapy, making treatment less effective compared to other solid tumors. The dense desmoplasia surrounding the pancreatic tumor microenvironment (TME) plays a major role in immune modulation and chemotherapy resistance. Pancreatic TME is comprised of the tumor and its surrounding stroma. The development and progression of PDAC is associated with the interplay of inflammatory cells, mediators, pancreatic stellate cells (PSCs), and the extracellular matrix (ECM) that gives rise to the tumor micro environment favoring tumorigenesis^[17,18] [Figure 1].

PSCs are part of normal pancreatic stroma and involved in vitamin A storage, exocrine/endocrine secretion, phagocytosis and maintenance of pancreatic stroma^[19]. In PDAC, the malignant cells secrete transforming growth factor beta-1 (TGFβ-1), fibroblast growth factor-2 (FGF2), sonic hedgehog and platelet derive growth factor (PDGF) that drive PSC proliferation and increase ECM deposition leading to desmoplastic reaction^[19,20]. The PSCs also contribute to the chemo resistance by actively entrapping gemcitabine in their cytoplasm lessening the effect of gemcitabine on pancreatic cancer cells^[21]. Dense ECM deposition is thought to promote proliferation, chemoresistance, and limit T-cells accumulation near cancer

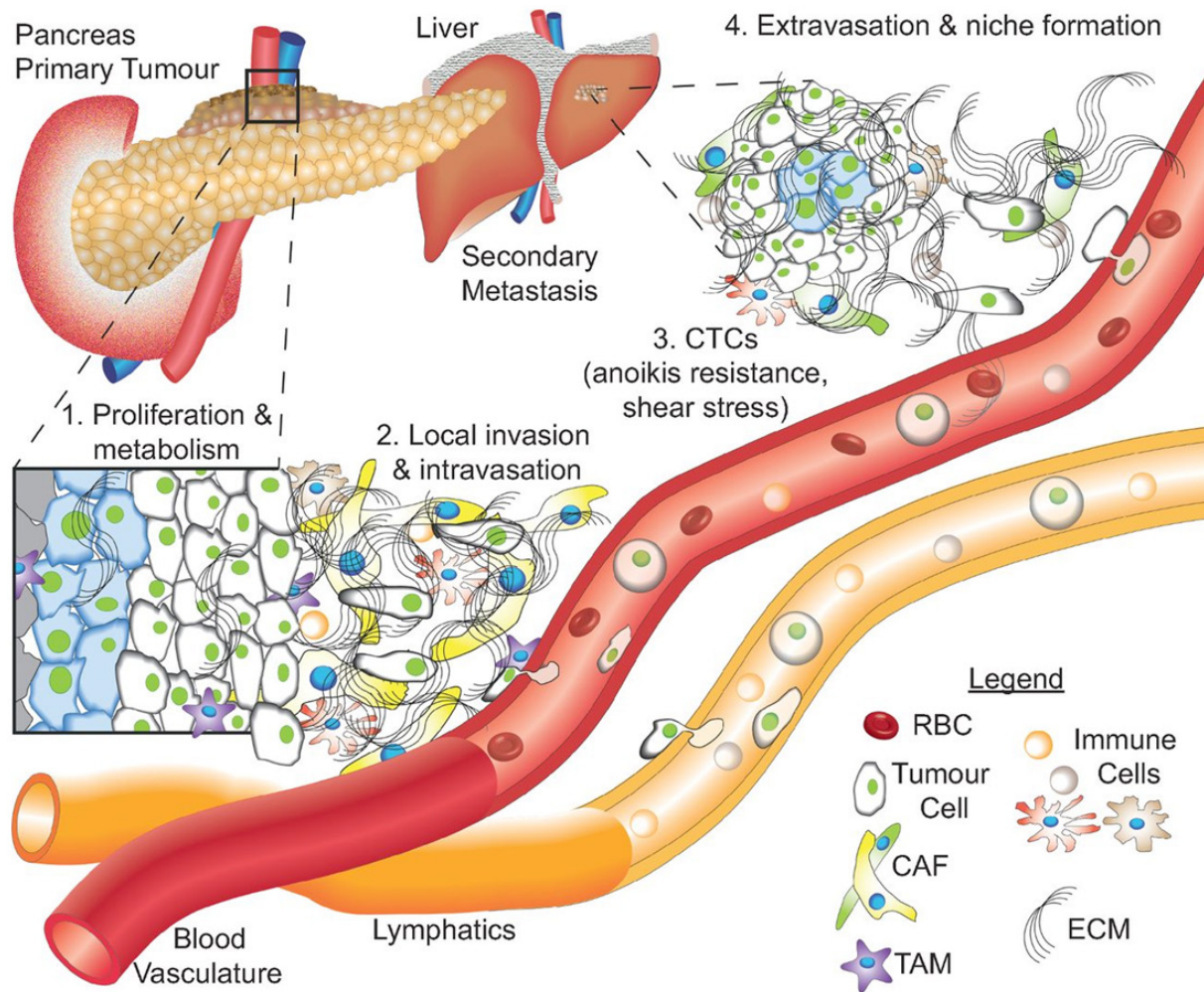


Figure 1. PDAC progression model and interaction with extracellular matrix (ECM). (1) Pancreatic cancer cells proliferate in the primary tumour, metabolising nutrients delivered by the blood vasculature and surrounding stroma; (2) cancer cells invade through the ECM, including cancer-associated fibroblasts (CAFs) and tumour-associated macrophages (TAMs), among other cancer-associated cell types, eventually intravasating or invading into the lymph and travelling to distant sites; (3) circulating tumour cells (CTCs) must develop resistance to anoikis, as well as shear stress, in order to survive in the circulation with red blood cells (RBCs) and leucocytes; (4) after travelling through the circulation, CTCs extravasate at secondary sites, commonly the liver, establishing a new niche. Re-use permitted under Creative Commons CC BY Attribution 4.0 Unported (CC BY 4.0) license, which permits others to copy, redistribute, remix, transform and build upon this work for any purpose, provided the original work is properly cited, a link to the license is given, and indication of whether changes were made. Link to license: <https://creativecommons.org/licenses/by/4.0/>. You are not required to obtain permission to reuse this article^[18]

cells^[22,23]. ECM also increases intra-tumor interstitial fluid pressure, which leads to vascular dysfunction, limits accumulation of chemotherapeutic agents within the tumor and increases tumor hypoxia^[24,25]. Hypoxic TME contributes to selection of more aggressive tumor cells that survive and proliferate^[26]. These studies demonstrated the important role of desmoplastic reaction in the progression of PDAC making it an attractive target for therapeutic agents. However, studies with PDAC animal models lacking ability to form desmoplastic reaction demonstrated accelerated tumor progression, suggesting a more complex role of desmoplasia in PDAC. The recent HALO 301 trial using pegvorhialuronidase alfa (PEGPH20), an enzyme that degrades hyaluronic acid in the ECM, in combination with gemcitabine and nab-paclitaxel, failed to improve survival further substantiating a more complex role of desmoplasia^[27].

The PSCs stimulated by PDAC cells in the TME can further differentiate into different populations of cancer associated fibroblasts (CAFs). Öhlund *et al.*^[28] demonstrated that alpha-smooth muscle actin

expressing CAFs is more prevalent in close proximity to PDAC cells whereas CAFs further away from the tumor cells have increased expression of fibroblast activating protein (FAP). The two populations of fibroblasts are mutually exclusive. FAP-positive CAFs secrete increased levels of cytokines [interleukin (IL)-1, IL-6, IL-8, IL-10] and growth factors such as VEGF, insulin-like-growth factor (IGF1), PDGF, connective tissue growth factor (CTGF) and FGF which stimulate angiogenesis, proliferation and metastasis^[19,29]. The PSCs also promote immunosuppression by recruiting myeloid-derived suppressor cells (MDSCs) through the signal transducer and activator of transcription 3 (STAT3) pathway^[30,31]. The MDSCs express programmed death-ligand 1 (PD-L1) and the cytotoxic T-lymphocyte antigen 4 (CTLA-4) receptors resulting in T cell tolerance. MDSCs also promote the development of regulatory T cells (Treg) through the CD40 engagement in presence of interleukin-10 (IL-10) and TGF β . Besides immune regulation, MDSCs also favor tumor progression via a non-immune mechanism by promoting tumor angiogenesis, cancer cell stemness, aggressiveness, and invasiveness^[32].

The complex interactions among the components of the PDAC TME make the tumor highly inductive to angiogenesis, metastasis and treatment resistance.

THE CHALLENGING IMMUNE ENVIRONMENT IN PDAC

Immunogenicity is related to the degree of epitope structural difference between the tumor and normal cells. Neoantigens, peptides generated from non-silent coding mutations in the cancer cell genome, are highly immunogenic^[33]. Studies have shown that tumor mutation load is proportional to neoantigen burden, which positively correlates with response to immunotherapy^[34,35]. However, PDAC is characterized by low tumor mutational burden (TMB) ranging from 10-60 encoded neoantigens in contrast to 100-1500 mutations per megabase expressed by other solid tumors that respond to immunotherapy^[36]. The limited expression of neoantigens by PDAC leads to poor immune surveillance.

Additionally, the TME of PDAC is known to be immunosuppressive with reduced cytotoxic T cells (CD8+) and T helper cells (CD4+) with increased Tregs, tumor-associated macrophages (TAMs), and MDSCs^[37]. The location of T cells in PDAC also has important implications in resistance mechanisms. For example, CD3+ T cells, which are either CD4+ or CD8+ T cells, have been identified more commonly at the invasive front of PDAC with fewer cells detected in the center thereby excluded by malignant cells. Moreover, the tumor infiltrating CD3+ T cells also cluster next to nests of malignant cells but are unable to interact with tumor cells since they are trapped within the stromal tissue^[38]. Therefore, the overall regulatory immune population of cells with exclusion of cytotoxic T cells along with the physical barrier created by a dense stromal environment creates a non-immunogenic tumor that is often resistant to immune recognition and killing. Nevertheless, the impact of T-cell infiltration on prognosis has shown inconsistent results with some studies showing improved OS with increased intratumoral CD3+ T-cells, whereas other studies have not shown an association between T-cell density and patient survival^[38,39].

The combination of an immunosuppressive environment and low TMB makes PDAC an “immune desert” that is resistant to immunotherapies. However, it also suggests a potential treatment strategy by overcoming low immunogenicity.

Immune check point inhibitors

The immune recruitment and response from T cells (CD4+ and CD8+) via antigen recognition in the presence of malignant cells are controlled by inhibitory and stimulatory signals called immune checkpoints. By expressing inhibitory ligands, tumor cells can evade immune surveillance^[40,41]. The first immune checkpoints discovered were CTLA4 and its ligands B7-1 and B7-2, and programmed cell death receptor 1 (PD1) and its ligands PD-L1 and PD-L2^[42,43]. Although immune checkpoint inhibitors (ICIs) such as ipilimumab (CTLA4 inhibitor), pembrolizumab (PD1 inhibitor) and durvalumab (PD-L1 inhibitors) have

benefitted patients with a wide variety of solid tumors, they have shown limited success in patients with PDAC^[44,45].

One of the first studies to test single agent immunotherapy for the treatment of PDAC was a phase II trial with ipilimumab, a CTLA4 inhibitor. It was given to 27 patients with PDAC including 20 patients with metastatic disease and 7 patients with locally advanced disease. There were no responders by response evaluation criteria in solid tumors criteria (RECIST ver.1.1) and three subjects experienced grade 3 or higher immune-mediated adverse events (colitis, encephalitis, and hypophysitis) leading to early discontinuation of the trial^[45]. In a larger phase I study in 2012 with 207 patients, 14 of whom had PDAC, patients were treated with anti-PD-L1 antibody, and no responses were seen in patients with metastatic PDAC^[46]. In 2015, another smaller phase I study of pembrolizumab (PD-1 inhibitor) in patients with advanced solid tumors showed similar results in 1 out of 30 patients with PDAC who did not respond^[47].

ICIs have shown benefit in a small group of PDAC patients whose tumor harbors mismatch repair (MMR) deficiency, which comprise 1%-3% of PDAC diagnoses^[48,49]. In a phase II trial, which included 8 patients with PDAC, who were MMR deficient and treated with pembrolizumab, there was a 62% objective response among PDAC patients^[50]. There has been one case report for the benefit of pembrolizumab in MMR-proficient PDAC, but with high TMB potentially highlighting a new population that would benefit from immunotherapy^[51]. Pembrolizumab is the first ICI to receive approval from FDA for tumor agnostic indication for use in patients with MMR-deficient malignancies^[50]. Based on the success of pembrolizumab in patients with refractory, metastatic cancers with MMR deficiency, the National Comprehensive Cancer Network (NCCN) now recommends consideration of MSI or MMR testing in patients with locally advanced or metastatic PDAC.

Combination of chemotherapy, immunotherapy, and radiation

Given the lack of response with single agent ICIs in treatment of PDAC, combination with chemotherapy or with other immunotherapies has also been studied.

Phase I studies

A phase Ib dose finding study of ipilimumab and gemcitabine showed partial response in 15% (2 patients) and stable disease in 38% (5 patients)^[52]. In a follow up phase Ib dose expansion study 2 out of 16 patients had partial response and 5 out of 16 had stable disease. Median PFS was 2.5 months (95%CI: 0.8-4.8 months) and median OS of 8.5 months (95%CI: 2.2-10.3 months)^[53].

Phase II studies

One of the largest phase II trials was a study comparing durvalumab (PD-L1 inhibitor) in combination with tremilimumab (CTLA-4 inhibitor) vs. durvalumab alone in 65 patients with metastatic PDAC. The objective response rate (ORR) was 3.1% and median OS of 3.1 months in the combination arm and ORR of 0% with median OS of 3.6 months in the monotherapy arm. Median PFS was 1.5 months in both arms^[54]. Due to the low patient numbers, the trial was not powered to observe the association between treatment response and PD-L1 expression or MSI status. Therefore, combination ICIs thus far have shown minimal benefit in treatment of PDAC.

Combination with chemotherapy and radiation

The combination of radiation with chemotherapy (chemoXRT) and immunotherapy is also being explored in the treatment of PDAC. The rationale of combining radiation is based on a previously recognized phenomenon called the abscopal effect whereby a local treatment (i.e., ionizing radiation) results in systemic or off-target shrinkage of tumor. The abscopal effect is postulated to be induced by anti-tumor T cell response mediated by immunogenic cell death after radiation^[55,56]. Therefore, it has been proposed that

Table 1. Ongoing clinical trials investigating immune check point inhibitors

Study drug	Combination	Study phase	Treatment setting	Sponsor	Clinicaltrials.gov identifier
Anti-PD-1 antibodies					
Nivolumab	Nab-paclitaxel ± gemcitabine	I	Any	Celgene	NCT02309177
	± Ipilimumab	I/II	Any	Bristol-Myers Squibb	NCT01928394
	Radiotherapy ± ipilimumab	II	Second line	Herlev Hospital	NCT02866383
Pembrolizumab	Radiotherapy	I	Second line	University of Pennsylvania	NCT02303990
	None	I	Any	Merck Sharp & Dohme	NCT02054806
	Paricalcitol	II	Any	Translational Genomics Research Institute	NCT03331562
	BL-8040 (CXCR4 antagonist)	II	Second line	MD Anderson	NCT02907099
	Azacitidine	II	Second line	Columbia University	NCT03264404
Anti-PD-L1 antibodies					
Atezolizumab	None	I	Any	Genentech	NCT01375842
	None	II	Any	Hoffmann La Roche	NCT02458638
Avelumab	Binimetinib (MEK inhibitor) and talazoparib	II	Second line	Pfizer	NCT03637491
Durvalumab	Pexidartinib (CSF1R inhibitor)	I	Second line	Centre Leon Berard	NCT02777710
	Galunisertib (TGFβ antagonist)	I	Second line	Eli Lilly	NCT02734160
	None	I/II	Any	MedImmune	NCT01693562
	Radiotherapy ± tremelimumab	I/II	Second line	National Cancer Institute	NCT02311361

CXCR4: C-X-C chemokine receptor type 4; MEK: mitogen-activated protein kinase kinase enzymes; CSF1R: colony stimulating factor 1 receptor; TGFβ: transforming growth factor β

combining chemoXRT with immune therapy may increase tumor immunogenicity and increase pancreatic tumor response to immunotherapy^[57]. This hypothesis is supported by various studies demonstrating that PDAC tumors treated with neoadjuvant chemoXRT had increased numbers of CD4+ and CD8+ T-lymphocytes compared to patients who did not receive neoadjuvant chemoXRT^[58]. In another study, neoadjuvant chemoXRT showed significantly lower numbers of immunosuppressive regulatory T cells although there was no difference in the number of CD4+ and CD8+ T cells^[57].

In pre-clinical studies, radiation has shown mixed results. In a mouse model of PDAC, a combination of radiation with dual blockade of PD-L1 and CTLA-4 resulted in improved survival and tumor responses compared to dual blockade without radiation or radiation alone^[59]. Another mouse model suggested an immunosuppressive T cell effect where radiation exposure induced macrophage immunosuppressive phenotype with reduction in CD8+ T-cells and increased Tregs^[60]. The role of radiation in clinical studies is still being explored. A phase Ib/II study with neoadjuvant pembrolizumab with chemoXRT is ongoing. The combination appears to be safe but efficacy data have not been reported^[61]. Other ongoing clinical trials [Table 1] include a pilot study evaluating SBRT in combination with tremilimumab (anti-CTLA-4) and PD-L1 monoclonal antibody MEDI4736 (NCT02311361) and an open label phase II study combining radiation with nivolumab with or without ipilimumab (NCT02866383).

Vaccines

Another strategy to overcome the immune desert of pancreatic cancer that is under investigation is the use of therapeutic vaccines [Table 2]. Vaccines may potentially turn PDAC into more immunogenic tumors by activating specific T cells with the ability to migrate into PDAC tumors^[62]. Tumors harbor driver and passenger mutations that may lead to changes in amino acid sequences, which in turn produces mutant proteins that are expressed by the tumors. These mutant proteins are processed into short polypeptides and

Table 2. Ongoing clinical trials investigating vaccines

Study drug	Combination	Study phase	Treatment setting	Sponsor	Clinicaltrials.gov identifier
Whole cell vaccines					
GVAX (+cyclophosphamide)	Nivolumab, Urelumab	I/II	Neoadjuvant and Adjuvant Treatment	Johns Hopkins	NCT02451982
	Nivolumab + SBRT	II	Neoadjuvant	Johns Hopkins	NCT03161379
	Ipilimumab, nivolumab, CRS-207	II	Second line	Johns Hopkins	NCT03190265
	Pembrolizumab + SBRT	II	First line	Johns Hopkins	NCT02648282
	Pembrolizumab, IMC-CS4	early phase I	First line	Johns Hopkins	NCT03153410
Algenpantucel-L	None	n/a	Any	NewLink Genetics Corporation	NCT03165188
Bacterial-based vaccines					
Listeria based vaccine CRS-207	Ipilimumab, nivolumab with or without GVAX + cyclophosphamide	II	Second line	Johns Hopkins	NCT03190265
	Epacadostat, pembrolizumab ± GVAX and cyclophosphamide	II	Second line	Johns Hopkins	NCT03006302
Yeast-based vaccines					
GI-4000	Aldoxorubicin, cyclophosphamide, oxaliplatin, capecitabine, fluorouracil, leucovorin, lovaza, nab-paclitaxel, bevacizumab, avelumab, ALT-803, aNK, ETBx-011, SBRT	I/II	Second line	NantKwest, Inc.	NCT03387098
	Cyclophosphamide, oxaliplatin, capecitabine, fluorouracil, leucovorin, nab-paclitaxel, bevacizumab, avelumab, ALT-803, aNK, ETBx-011, SBRT	I/II	Second line	NantKwest, Inc.	NCT03329248
	Cyclophosphamide, oxaliplatin, capecitabine, fluorouracil, leucovorin, nab-paclitaxel, bevacizumab, avelumab, ALT-803, aNK and ETBx-011	I/II	Second line	NantKwest, Inc.	NCT03136406
YE-NEO-001	None	I	First line	NantBioScience, Inc.	NCT03552718
Viral vector-based vaccines					
Ankara Vaccine Expressing p53	Pembrolizumab	I	Second line	City of Hope Medical Center	NCT02432963
Peptide vaccines					
Personalized mesothelin epitopes	Adjuvant chemotherapy + Polyinosinic-polycytidylic acid, and poly-L-lysine (poly-ICLC)	I	First line	Washington University School of Medicine	NCT03956056
KRAS peptide vaccine iNeo-Vac-PO1	ipilimumab, nivolumab	I	First line	Johns Hopkins	NCT04117087
	GMCSF	I	Second line	Zhejiang Provincial People's Hospital	NCT03645148
Personalized neoantigen vaccine	Adjuvant chemotherapy	I	First line	Changhai Hospital	NCT03558945
DNA based vaccines					
Personalized neoantigen DNA vaccine		I		Washington University School of Medicine	NCT03122106
Dendritic cell-based vaccines					
Autologous DC vaccine	None	I	First line	Baylor College of Medicine	NCT04157127
mDC3/8-KRAS Vaccine	None	I	Any	University of Pennsylvania	NCT03592888

displayed on the cell surface by major histocompatibility complex (MHC) and are recognized by T cells as foreign antigens^[63]. This distinguishing feature of a malignant cell from a normal cell can provide a method of targeting the tumor cells while sparing the normal cells by the immune system.

Antigens expressed by PDAC cells can be grouped into two categories: tumor-specific antigens (TSA), which are only expressed by the tumor cells, and tumor-associated antigens (TAA), which are mostly restricted to malignant cells with limited expression on normal cells^[64]. TSAs are neoantigens unique to

each patient. A vaccine that targets TSAs needs to be personalized to the individual patient. However, TSAs such as MUC1 or KRAS which is expressed by more than 90% of PDAC cells are an exception to this rule. Currently there are a number of vaccines targeting shared antigens and patient specific antigens that are under investigation. Examples of TAAs expressed by PDAC cells include EGFR family, carcinoembryonic antigen (CEA), mesothelin, VEGF family, and Wilms' tumor 1 (WT-1); however, since they may also be expressed by normal cells, off-target toxicity can occur^[65,66].

Whole cell vaccines

GVAX is the most extensively evaluated whole cell vaccine consisting of two human allogeneic pancreatic tumor cells lines irradiated to release antigens. It is also genetically engineered to release granulocyte-macrophage colony-stimulating factor (GM-CSF) at the vaccination site^[67]. Dendritic cells are attracted to GM-CSF site where they phagocytose antigens released from apoptotic PDAC cells. These dendritic cells then travel to draining lymph nodes, present tumor antigens found in vaccine PDAC cell lines to effector T-cell and activate them.

In an early phase clinical trial, GVAX demonstrated improved disease-free survival (DFS) in PDAC patients who displayed delayed-type hypersensitivity responses to autologous tumor cells^[68]. In a phase II trial of 60 patients with resected PDAC, GVAX was studied in combination with chemoXRT. Results demonstrated a DFS of 17.3 months with an OS of 24.8 months, and induction of mesothelin-specific CD8+ T cells was seen in those treated with GVAX, which correlated with DFS^[69]. Another trial studied GVAX in the neoadjuvant setting where patients received cyclophosphamide and GVAX prior to pancreaticoduodenectomy. Surgical resection samples from these patients were noted to have novel vaccine-induced upregulation of PD-1/PD-L1 pathways in the intratumoral tertiary lymphoid aggregates^[62]. In the metastatic setting, GVAX has also been studied with or without ipilimumab in patients that were refractory to gemcitabine-based therapy. This study enrolled 15 patients in each arm and results showed a median OS of 3.6 months vs. 5.7 months ($P = 0.072$) for single agent ipilimumab versus combination ipilimumab with GVAX^[70]. Currently, there are ongoing phase I and II clinical trials of GVAX in various combinations with nivolumab and urelumab (NCT02451982), nivolumab and radiation therapy (NCT03161379), ipilimumab, nivolumab and CRS-207 (Listeria based vaccine) (NCT03190265), pembrolizumab and radiation therapy (NCT02648282), pembrolizumab and IMC-SC4 (NCT03153410).

Another whole cell vaccine is Algenpantucel-L which contains two irradiated allogeneic PDAC tumor cell lines transfected to express alpha-1, 3-galactosyltransferase epitopes, a cell surface carbohydrate. A phase III IMPRESS trial comparing chemoXRT + algenpantucel-L vs. chemoXRT alone in the adjuvant setting showed no improvement in OS. Unfortunately, no further trials are planned at this time due to limited clinical benefit^[71].

Bacterial-based vaccines

Vectors based on bacteria, including *Listeria monocytogens*, salmonella, *Lactobacillus Plantarum*, and Bacillus Calmette-Guerin, have been studied as they can elicit both innate and adaptive immune responses to TAAs. The bacterial vectors are modified to express specific antigens and elicit potent CD8+ and CD4+ responses via MHC I and MHC II antigen processing pathways. CRS-207, a live attenuated listeria vaccine genetically modified to secrete mesothelin was studied in combination with GVAX^[72,73]. In a phase II trial with metastatic PDAC patients, GVAX with cyclophosphamide (Cy/GVAX) was compared to Cy/GVAX followed by CRS-207. The Cy/GVAX plus CRS-207 arm showed an OS benefit of 6.1 months vs. 3.9 months in the Cy/GVAX alone arm^[74]. It was also noted that patients who elicited mesothelin specific CD8+ T-cell responses also had longer OS. However, a more recent phase IIb trial in which previously treated patients with metastatic PDAC who failed > 2 lines of chemotherapy were randomized 1:1:1 to receive Cy/GVAX + CRS 207 (arm A), CRS-207 (arm B), or a physician's choice of single-agent chemotherapy (arm C),

showed Cy/GVAX + CRS-207 did not improve OS compared to chemotherapy^[75]. CRS-207 is now being studied in combination with indoleamine 2, 3 dioxygenase-1 (IDO1) inhibitor and ICIs (NCT03190265, NCT03006302).

Yeast-based vaccines

Yeast cells have also been used as vaccine vectors given their ability to elicit robust T cell responses. Yeast cells can be genetically engineered to express TSAs. GI-4000 is a combination of four vaccines made up of heat-inactivated *S. cerevisiae* expressing the three most common Ras mutations of human cancers. A phase II randomized trial in which GI-4000 was given together with gemcitabine in PDAC showed a modest OS benefit of 524 days vs. 444 days^[76]. Another phase I trial (NCT03552718) is evaluating a personalized neoepitope yeast vaccine in patients with resected pancreas, liver and lung cancers.

Viral vector based vaccines

Another strategy to elicit T-cell response is using viral vectors modified to encode TAAs/TSAs. These vectors include adenovirus (AdV), adeno-associated virus (AAV), vaccinia virus (VV), and alphavirus. Phase I results were encouraging. However, a phase III trial with PANVAC-VF, a recombinant attenuated vaccinia and fowlpox vector expressing CEA, MUC-1 and immunostimulatory molecules ICAM-1, B7.1 LFA-3, did not show statistical significance in overall survival^[77]. A phase I trial (NCT02432963) is evaluating a combination of vaccinia virus expressing p53 (MVA-p54) and pembrolizumab in PDAC and other solid tumors.

Peptide vaccines

Antigenic peptide vaccines are phagocytosed by dendritic cells and presented to T-cells to trigger a response. These vaccines have the potential to be personalized to individual patients with improved selection of immunogenic epitopes and have been shown to have some benefit in high risk melanoma patients^[78]. In PDAC, despite demonstration of T-cell immune response by several peptide vaccines (elpamotide-VEGFR2 vaccine, KIF202A-66-member of kinesin super family protein 20A, and RAS peptide) in clinical trials, no OS benefit was observed^[79-81].

DNA-based vaccines

DNA vaccines contain a DNA plasmid that encodes for highly immunogenic TAA that are specific to a patient's PDAC including epitopes of mesothelin. After administration, the DNA plasmid is electroporated into cells. The expressed neoantigens are taken up by antigen presenting dendritic cells to elicit T-cells response. A phase I double blind, placebo controlled trial of VXM01, a DNA vaccine, in 71 patients with advanced PDAC showed that it was well tolerated and led to activation of VEGFR2-specific cytotoxic T-cells^[82]. A phase I DNA vaccine trial (NCT03122106) of resected PDAC patients is ongoing.

Dendritic cell-based vaccines

Dendritic cells are specialized antigen presenting cells that process antigens to prime T cells. They can be loaded with tumor cell lysate and antigens, expanded ex-vivo and administered into patients^[83,84]. Two phase I trials with autologous dendritic cells pulsed with mutant KRAS peptides (NCT03592888) and dendritic cells loaded with tumor cell lysate and RNA (NCT04157127) corresponding to the patient's specific tumor mutation and HLA type in resected PDAC patients are currently ongoing.

Adoptive T-cell therapy

Adoptive T-cell therapy is another area of interest in treatment of PDAC given its success in number of hematologic malignancies^[63]. T-cells are genetically engineered to express chimeric antigen receptor (CAR) which targets specific tumor antigens. The receptors are made up of an extracellular antigen recognition domain to an intracellular signaling domain of CD28, 4-1BB, and other co-stimulatory molecules which

Table 3. Ongoing clinical trials investigating CART cells immunotherapy

Study drug	Combination	Study phase	Treatment setting	Sponsor	Clinicaltrials.gov identifier
CART-meso	None	I	Second line	University of Pennsylvania	NCT03323944
	None	N/A	Second line	Shenzhen BinDeBio	NCT03638193
	None	I/II	Second line	National Cancer Institute	NCT01583686
CART-Nectin4	None	I	Second line	The Sixth Affiliated Hospital of Wenzhou Medical University	NCT03932565
CART-EpCAM	None	I/II	Any	First Affiliated Hospital of Chengdu Medical College	NCT03013712
CART-CD70	Cyclophosphamide, Fludarabine, Aldesleukin	I/II	Second line	National Cancer Institute (NCI)	NCT02830724
CART-CLD18	Cylophosphamide, Fludarabine	N/A	Any	Kang YU, First Affiliated Hospital of Wenzhou Medical University	NCT03302403
CART-TnMUC1	Cylophosphamide, Fludarabine	I	Second line	Tmunity Therapeutics	NCT04025216
CART-PSCA	Rimiducid	I/II	Second line	Bellicum Pharmaceuticals	NCT02744287

activate the T cell when an antigen is bound. These cells are expanded ex-vivo and re-infused into patients. In a phase I trial in which mesothelin-specific CAR-T cells were given to 6 patients with chemotherapy-refractory metastatic PDAC, 2 patients had stable disease with PFS of 3.8 and 5.4 months. Additionally, one patient had 69.2% decrease in metabolic activity on PET (positron emission tomography) scan with complete reduction in FDG (F-2-fluoro-2-deoxy-D-glucose) uptake in all liver lesions at 1 month but no effect was seen on primary PDAC^[85]. Only small numbers of patients have been treated with adoptive T-cell therapy and there have been variable results in metastatic sites versus primary tumor. There are currently various other preclinical and phase I/II trials with CAR T cells targeting various tumor antigens such as Nectin4/FAP, EpCAM, CD70, CLD18, TnMUC1, PSCA (NCT03932565, NCT03013712, NCT02830724, NCT03302403, NCT04025216, NCT02744287) [Table 3].

Although clinical data suggests that vaccines and CAR T-cell therapy can elicit anti-tumor T cell responses in PDAC with suggestion of clinical benefit, no clinically meaningful responses have been reported with CAR T-cell treatment of PDAC. These approaches have the potential to target the PDAC specifically; however, the tumor fighting cells may not be able to reach their target due to the desmoplasia and immunosuppressive tumor microenvironment. There may be also be other barriers to effective anti-tumor immune therapy. Further studies are needed to better understand and target these barriers.

OTHER TARGETED AGENTS

There are also strategies using agents that target DNA repair, pathway inhibitors, metabolism and extracellular matrix. Some of the notable ones are discussed below [Table 4].

PARP inhibitors

Poly (ADP-ribose) polymerase (PARP) enzymes repair single stranded DNA breaks and play crucial roles in DNA damage repair (DDR). PARP inhibitors are small molecules that trap PARP enzymes on DNA and prevent the process of DDR. Cancer cells with deficient in DNA repair via homologous recombination due to mutations in *BRCA 1/2* are very sensitive to PARP inhibitors^[86]. The presence of PARP inhibitors lead to death of *BRCA 1/2* mutated cancer cells due to a markedly reduced capacity for DDR. The first randomized, phase III trial, POLO found that maintenance therapy with a PARP inhibitor, Olaparib, significantly prolonged the progression of disease in advanced PDAC with germline *BRCA* gene mutations compared to placebo (PFS 7.3 months vs. 3.8 months)^[87]. Currently, there are several clinical trials at varying phases studying the efficacy of different PARP inhibitors in advanced PDAC.

Table 4. Ongoing clinical trials investigating novel targeting agents

Study drug	Combination	Study phase	Treatment setting	Sponsor	Clinicaltrials.gov identifier
PARP inhibitors					
Olaparib	None	II	Any	AstraZeneca	NCT01078662
	None	III	Maintenance	AstraZeneca	NCT02184195
	None	II	Second line	AstraZeneca	NCT02677038
Rucaparib	Cediranib (VEGF inhibitor)	II	Second line	National Cancer Institute	NCT02498613
	None	II	Maintenance	Abramson Cancer Center, UPenn	NCT03140670
	Liposomal irinotecan, 5-fluorouracil and leucovorin	I/II	Any	Academic and Community Cancer Research United	NCT03337087
Niraparib	Ipilimumab or nivolumab	I/II	Second line	Upenn	NCT03404960
	None	III	Any	Anup Kasi	NCT03553004
Veliparib	FOLFIRI or mFOLFIRI	II	Second line	National Cancer Institute	NCT02890355
	mFOLFOX6	I/II	Any	Georgetown University	NCT01489865
	± Cisplatin and gemcitabine	II	Any	National Cancer Institute	NCT01585805
Talazoparib	Avelumab and binimetinib	Ib/II	Any	Pfizer	NCT03637491
NTRK inhibitors					
Larotrectinib	None	II	Any	Bayer	NCT 02576431
Entrectinib	None	II	Any	Hoffmann-La Roche	NCT02568267
Mitochondrial inhibitor					
Devimistat	Gemcitabine plus nab-paclitaxel mFOLFIRINOX	I	First line	Atlantic Health System	NCT03435289
		III	Any	Rafael Pharmaceuticals	NCT03504423
FAK inhibitor					
PF-00562271	None	I	Second line	Verastem	NCT00666926
Connective tissue growth factor inhibitor					
Pamrevlumab	Gemcitabine plus nab-paclitaxel	I/II	Neoadjuvant	FibroGen	NCT02210559
	Gemcitabine plus nab-paclitaxel	III	First line	FibroGen	NCT03941093

NTRK: Neurotrophic tropomyosin-related kinase; FOLFIRINOX: leucovorin, fluorouracil, irinotecan, oxaliplatin; FAK: focal adhesion kinase

NTRK inhibitors

Topomyosin receptor kinase (TRK) transmembrane proteins plays an important role in neural development. When different partners fuse with TRK encoding genes (*NTRK1*, *NTRK2* or *NTRK3*) the resulting gene encode for oncogenic proteins that constitutively activate the RAS-MEK-ERK, PI3k-AKT, and diacylglycerol-inositol-1,4,5-triphosphate signaling pathways^[88]. NTRK fusions are found in < 1% of PDAC. In the STARTRK-2 trial (NCT02568267) with entrectinib, a TRK A/B/C inhibitor, 3 patients with PDAC had PR based on RECIST criteria. Another ongoing trial NAVIGATE (NCT 02576431) showed response rate was as high as 75% among 55 patients with NTRK fusion positive solid tumors treated with larotrectinib. However, only one patient with PDAC was included in the cohort. Currently, entrectinib and larotrectinib are approved by FDA for a tissue-agnostic indication in solid tumors that harbor *NTRK* fusion gene.

Devimistat

Devimistat selectively inhibits the tricarboxylic acid cycle in the mitochondria and is hypothesized to work synergistically with cytotoxic agents by decreasing mitochondria metabolism, impairing production of intermediates required for DNA damage repair. Devimistat was studied in a phase I trial in combination with mFOLFIRINOX in 20 patients with metastatic PDAC. The response rate was 61% with 3 patients achieving CR^[89]. Currently Devimistat is being studied in a phase III trial in combination with mFOLFIRINOX (NCT03504423) and in a phase I trial in combination with gemcitabine and nab-paclitaxel (NCT03435289).

Focal adhesion kinase inhibitors

Focal adhesion kinase (FAK) is a tyrosine kinase that promotes tumor growth, invasion and metastasis by interacting with tumor cells and stromal cells in various types of cancer^[90]. In preclinical research, FAK

is shown to be overexpressed in pancreatic tumor tissues. FAK promotes metastasis by regulating focal adhesions, matrix metalloproteinase surface expressions and enhancing tumor growth by promoting anti-apoptotic functions. The combination of FAK inhibitors together with gemcitabine and nab-paclitaxel has been shown to delay tumor growth in patient derived xenograph models, relative to chemotherapy alone^[90]. Currently, a phase I clinical trial with FAK inhibitor PF00562271 is under investigation in patients with advanced pancreatic cancer (NCT00666926).

Connective tissue growth factor inhibitors

CTGF is a protein is highly expressed in PDAC that contributes to the dense desmoplasia. It is also thought to protect the cells from hypoxia-mediated apoptosis and drive more aggressive tumor cells selection^[91]. In preclinical study with mouse model of PDAC, pamrevlumab, a monoclonal antibody against CTGF in combination with gemcitabine has been shown to prolonged survival^[92]. A phase I/II trial involving patients with unresectable PDAC with pamrevlumab and gemcitabine/nab-paclitaxel (NCT02210559) showed higher resection rate in the arm with patients who received pamrevlumab. Currently, FDA granted fast track designation to pamrevlumab for the treatment of unresectable locally advanced PDAC^[93]. A phase III trial with this agent is recruiting (NCT03941093).

EXPERT OPINION

Surgery, chemotherapy and radiation therapy are three of the most commonly used modalities to treat pancreatic cancer. However, even with intense chemotherapy regimens, such as FOLFIRINOX, outcomes for these patients remains dismal with conventional therapies. Cancer cells prevent immune cells from recognizing them as a threat, thereby, allowing cancer cells to evade the immune system resulting in continued growth and metastasis. Immunotherapy restores the ability of the immune system to detect and destroy cancer cells by overcoming mechanisms by which tumors evade and suppress the immune response.

Recent negative findings from studies evaluating immune checkpoint inhibitors, whether alone or in combination, have suggested that they may not solely be ideal agents for treatment of pancreatic cancer. Instead, additional agents that prime the immune microenvironment may be needed to see efficacy. This may be attributed to the fact that immune checkpoint inhibitor combination therapy has shown efficacy for immunogenic, “hot” tumors, such as melanoma and lung cancer, while pancreatic cancer on the other hand, is not an immunogenic tumor, commonly called a “cold” tumor.

It is now known that pancreatic cancer does not contain a lot of immune cells and actually also has immunosuppressive signals. Therefore, we may have to adopt a “multi-pronged approach”. Such an approach may include different types of agents, including oncolytic viruses, or vaccines that can prime the immune microenvironment so that the checkpoint inhibitors can then impart their efficacy. Investigators have reported feasibility and clinical activity of T-cell therapy in patients with pancreatic cancer. The question is how do we facilitate bringing in these immune cells?

Pancreatic cancer tumors are well known for the presence of dense desmoplastic stroma that is made up of pancreatic stellate cells, fibroblasts, immune cells, and extracellular matrix proteins. The tumor microenvironment is immunosuppressive and dominated by myeloid-derived suppressor cells and regulatory T cells. Though recent data has shown disappointing results with agents that promised to deplete the stroma, such as agents that inhibit the sonic Hedgehog signaling pathway and pegylated hyaluronic acid, it is important to note that the tumor microenvironment does play a significant role in modulating the immune recognition of PDAC. It is now postulated that extracellular matrix depletion may actually lead to tumor progression further substantiates a more complex role of desmoplasia in tumor biology.

In summary, PDAC responds poorly to immune checkpoint blockade with both anti-CTLA-4 and anti-PD1/anti-PD-L1 immunotherapies. This could be due to the presence of dense desmoplasia and tumor microenvironment made up of extracellular matrix proteins, fibroblasts, endothelial cells, and immune cells. It has been clearly indicated by preclinical studies that the tumor microenvironment has abundant immunosuppressive cell types and few effector T cells.

In addition, cancer associated fibroblasts (CAF) especially FAP positive CAF have been shown to be important in mediating immunosuppression in the PDAC tumor microenvironment. Although treating PDAC with immunotherapy alone has not been very successful, various combination strategies of immunotherapies (including CAR-cells and immune check point inhibitors) with therapeutic vaccine and/or radiation therapies to improve immunogenicity of PDAC are ongoing. The multipronged approach to treating PDAC has led to various trials targeting tumor DNA repair (PARP inhibitors), and tumor metabolism (mitochondrial inhibitor). Novel agents such as FAK inhibitors, CTGFs are also being studied in early phase trials. The results of these studies are highly anticipated and some trials hold promise to advance into later-phase clinical trials. It is hoped that the results from these studies will eventually improve the outcome for pancreatic cancer patients.

TAKE HOME POINTS

Patients with pancreatic cancer show poor response to chemotherapies and ICI due to its unique immunosuppressive tumor microenvironment with extensive desmoplasia.

The complex interactions among the components of PDAC TME (fibroblasts, endothelial cells, and extracellular matrix proteins, tumor-associated macrophages, MDSC, and Treg cells) lead to tumorigenesis, chemo-resistance, and immune suppression although some recent studies also show evidence of tumor-restraining role of the desmoplasia.

PDAC is characterized by low TMB due to limited expression of neoantigens and leads to poor immune surveillance and poor response to ICIs. However, a small subset (1%-3%) of PDAC patients with dMMR or MSI-H tumors showed high response rate to single agent pembrolizumab.

- (1) ChemoXRT may increase immunogenicity in PDAC, which can sensitize tumors to immunotherapy. Multiple phase I/II trials with combination chemoXRT and immunotherapy are ongoing.
- (2) GVAX, vaccine developed from PDAC tumor associated antigens, showed T-cell mediated antitumor activity in preclinical and early phase clinical trials. It is now under investigation in combinations with ICIs. There are various ongoing early phase trials using viral vector, peptide based vaccines, DNA based vaccines, yeast-based vaccines and dendritic cell-based vaccines.
- (3) Strategies with various targeted therapies are being explored in clinical trials targeting tumor DNA repair (PARP inhibitors), and tumor metabolism (mitochondrial inhibitors).
- (4) Novel agents such as FAK inhibitors and CTGF are also being studied in early phase trials.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: Chi J, Patel R, Rehman H, Goyal S, Saif MW

Performed data acquisition, as well as provided administrative, technical, and material support: Chi J, Patel R, Rehman H, Goyal S, Saif MW

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

Not applicable.

Consent for publication

Not applicable.

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Review

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Is there a role for resection of oligometastatic disease in pancreatic ductal adenocarcinoma?

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How to cite this article: Puckett Y, Eskander MF, Aquina CT, Pawlik TM. Is there a role for resection of oligometastatic disease in setting of pancreatic ductal adenocarcinoma? *J Cancer Metastasis Treat* 2020;6:44.
<http://dx.doi.org/10.20517/2394-4722.2020.98>

Received: 11 Aug 2020 **First Decision:** 15 Sep 2020 **Revised:** 30 Sep 2020 **Accepted:** 14 Oct 2020 **Published:** 11 Nov 2020

Academic Editor: Lucio Miele **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

It is estimated that approximately 80%-90% of pancreatic ductal adenocarcinoma (PDAC) will present with unresectable disease with about half of patients presenting with distant metastases. The prognosis of these patients is generally poor with an average life expectancy of approximately 6 months and a median 5-year survival of 1%. The current standard of care for metastatic PDAC patients is palliative chemotherapy, as surgery in this setting does not lead to better survival. More recently, some centers have utilized very specific patient selection to perform resection of oligometastatic disease with reported improvement in survival - with many centers using response to systemic therapy as a sign of favorable biology. We performed a literature review investigating the role of surgical resection of oligometastatic disease of the lung and liver in PDAC.

Keywords: Pancreatic cancer, pancreatic ductal adenocarcinoma, liver metastasis, oligometastatic disease, M1, surgery, metastasectomy

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) accounts for approximately 3% of all cancers and about 8% of all cancer deaths in the United States^[1]. Despite improvements in surgical technique and chemotherapy regimens, the 5-year relative survival for pancreatic cancer has only increased from 3% to 9% over the last



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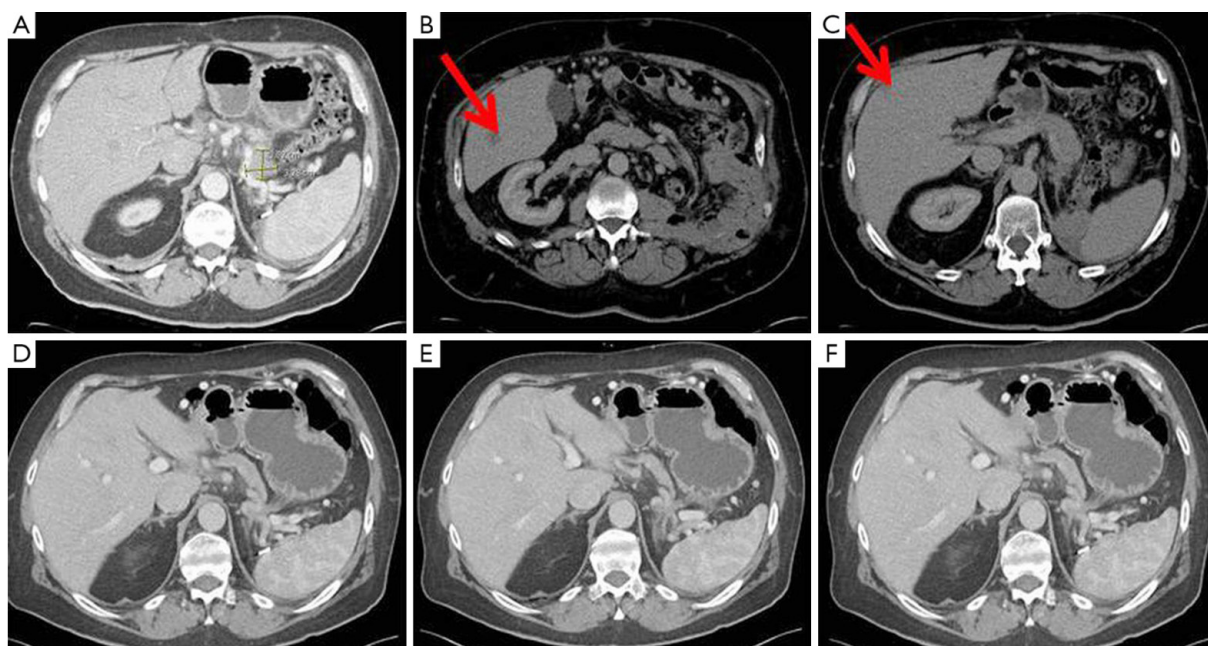


Figure 1. Sixty-six year-old man diagnosed with: biopsy-proven PDAC seen on CT (A); the patient also had synchronous biopsy-proven liver metastases (B and C, red arrows); he underwent neoadjuvant chemotherapy with 5-FU, leucovorin, oxaliplatin, and irinotecan with radiologic response 24 months after diagnosis (D-F). Surgical resection of the pancreas and liver lesions was performed as a one-stage procedure. The patient remained disease-free 30 months post-resection^[7]. PDAC: pancreatic ductal adenocarcinoma; CT: computed tomography; 5-FU: 5-fluorouracil

35 years^[1,2]. The majority of PDAC is diagnosed when the cancer has spread to distant sites or has become unresectable^[3,4]. The term *oligometastases*, coined in 1995 by Hellman and Weichselbaum, refers to the existence of metastases that are few in number^[5]. In 2019, Damanakis *et al.*^[6] proposed a definition for oligometastatic disease in pancreatic cancer that included anatomic as well as biological criteria to identify a subset of patients with favorable biology and improved prognosis who may be candidates for surgery. Damanakis restricted the cohort to patients with “limited disease,” defined as 4 or fewer hepatic or pulmonary metastatic lesions, as well as only individuals with a Carbohydrate antigen 19-9 (CA 19-9) of less than 1,000 U/mol. In addition, only patients who responded to systemic chemotherapy were included. With these restricted criteria, the investigators identified a subset of about 8% of patients from the original cohort with metastatic disease who had improved overall survival with resection.

Traditionally, oligometastatic disease in the context of PDAC has been associated with a poor prognosis with an average life expectancy of approximately 6 months and a median 5-year overall survival of 1%^[7]. Surgical resection of oligometastatic disease in pancreatic cancer is generally not associated with prolonged survival. The National Comprehensive Cancer Network treatment guidelines do not, therefore, consider these patients to be surgical candidates^[8]. However, the topic has been much debated, and some centers have performed resection of PDAC metastases and have published data advocating for this approach in very select patients [Figure 1]^[5,8-10].

A review on resection of PDAC metastasis published in 2006 reported a median survival ranging from 4 to 6 months with a 5-year survival of zero^[4]. Nevertheless, potential utility of resecting oligometastatic disease has been suggested based on outcomes after palliative R2 resection. For example, a study by Bockhorn *et al.*^[11] demonstrated a survival benefit for patients who underwent an R2 resection versus *versus* bypassed patients (11.5 vs. 7.5 months). However, patients who underwent an resection experienced increased morbidity such as longer hospital stay, and 2 patients out of 40 died perioperatively. These data suggested that debulking of

disease in select patients may be associated with a possible prolonged and have served to encourage other investigators to consider surgical removal of oligometastatic disease.

Metastatic PDAC most commonly involves the liver, followed by inter-aortocaval lymph nodes (ILN), lung, and peritoneum^[7,12]. However, distant metastasis in patients with PDAC has been documented in almost every organ based on autopsy studies^[13-19]. Surgical resection of liver and lung oligometastases with negative margins can be technically feasible depending on the anatomic location; however, decisions regarding oligometastasectomy should be based on predictions about tumor biology and careful patient selection. Surgical resection of liver metastases may be safely accomplished at the time of pancreatic resection in select patients, while pulmonary metastases can often be performed in a minimally invasive fashion after primary pancreatic resection. In general, resection of oligometastatic PDAC lesions should be done in a staged fashion after systemic chemotherapy that has demonstrated stable or responsive disease.

We herein performed a literature review of studies investigating whether there is a role for surgical resection with curative intent of limited PDAC metastases to the lung or liver. The focus of this literature review was on pulmonary and hepatic metastases given that the majority of available literature on oligometastasectomy in PDAC is focused primarily on those two organs.

HEPATIC METASTASECTOMY

The liver is the most common location of PDAC metastases due to the proximity of the liver and blood supply to the pancreas^[20,21]. Approximately 70% of patients with metastatic PDAC have disease in the liver. Among these patients, 30% have limited disease^[22]. While resection of colorectal liver metastasis is safe and provides a survival benefit, the resection of liver metastases in non-colorectal patients, including pancreatic cancer patients, has not been widely accepted^[22]. Because national guidelines do not recommend resection of a primary tumor along with synchronous liver metastases, research on the topic is lacking^[23]. The general treatment approach to liver metastases in PDAC is systemic chemotherapy and/or radiation therapy^[24]. In addition, locoregional ablative techniques such as percutaneous radiofrequency ablation and microwave ablation have been recognized as safe and less invasive alternatives to surgery with comparable recurrence and survival outcomes^[25-27].

In a study by Hackert *et al.*^[9], a total of 128 patients underwent surgery for oligometastatic PDAC with spread to either the liver (85 patients) or ILN (43 patients) [Table 1]. Among these patients, 20 had received neoadjuvant treatment. The patients underwent a variety of procedures including pancreaticoduodenectomy, distal pancreatectomy, total pancreatectomy with splenectomy, extended lymphadenectomy, bisegmentectomy, right hepatectomy, segmental liver resection ranging from 1-4 segments, and extended right hepatectomy. Overall, 72.9% of the liver resections were performed at the time of primary pancreatic resection with the rest performed following pancreatic resection at an average of 18.4 months later. The majority of the liver metastases resected were less than 2 cm in diameter, and 96.4% of patients had more than three metastatic lesions in the liver. Twenty patients had undergone neoadjuvant treatment prior to resection; data on adjuvant therapy was only available for 95 patients, 73 of whom completed adjuvant therapy. Surgical morbidity and 30-day mortality rates were 45.0% and 2.9% for the whole cohort, respectively. Median overall survival was 12.3 months with a 5-year overall survival of 8.1% after liver resection and 10.1% after ILN resection. There was no survival difference detected between patients that received neoadjuvant treatment and those that did not. The number of liver metastases, size of liver metastases, and pre-operative CA 19-9 levels were not significantly associated with survival. There was also no significant difference in overall survival between synchronously and metachronously resected patients when survival was analyzed from time of liver resection.

A multi-institutional European study performed by Tachezy *et al.*^[28] examined a total of 69 patients with PDAC and synchronous liver metastases who underwent combined pancreas and liver resections compared

Table 1. Summary of most recent studies of PDAC patients undergoing metastasectomy for liver metastases

Authors	Type of Study	Year	Chemotherapy	n	Morbidity (%)	30-day mortality (%)	Median OS (months)	2-year survival (months)	5-year survival (months)
Hackert <i>et al.</i> ^[9]	Single Center Retrospective	2017	20 patients received neoadjuvant chemotherapy; 73 patients completed adjuvant chemotherapy	85 (liver) 43 (ILN)	45.0	2.9	12.3 12.3	N/A	8.1% 10.1%
Michalski <i>et al.</i> ^[10]	Systematic review of 3 case reports and 18 studies	2008	N/A	103	24.1-26.0	0.0-4.3	5.8-11.4	N/A	N/A
Tachezy <i>et al.</i> ^[28]	Retrospective review of 6 centers	2015	100% received neoadjuvant chemotherapy	69	68.0	1.0	13.6	N/A	N/A
Frigerio <i>et al.</i> ^[29]	Retrospective review of 2 centers	2017	100% received neoadjuvant chemotherapy	24	62.0	0.0	56.0	N/A	N/A
Klein <i>et al.</i> ^[30]	Single Center Retrospective	2012	100% received adjuvant chemotherapy	22	18.0	0	7.6	5.0	0.0

PDAC: pancreatic ductal adenocarcinoma; ILN: intraaortocaval lymph nodes; N/A: not applicable; OS: overall survival

to patients who were explored but not resected. The overall survival was longer in the resected group (13.6 vs. 7 months, $P < 0.001$) for pancreatic head tumors. However, there was no survival benefit for distal pancreatic tumors (14 vs. 15 months, $P = 0.312$).

A 2017 retrospective study from Italy used the strategy of downstaging via neoadjuvant chemotherapy^[29]. About 5% of the initial cohort who had synchronous liver oligometastatic disease met the criteria of disappearance of liver metastasis and decreased CA 19-9. R0 resection was achieved in 88% of patients with a 17% complete pathologic response. Overall survival was 56 months and disease-free survival 27 months. The authors concluded that carefully selected patients fully responsive to neoadjuvant chemotherapy may be cautiously selected for surgery.

Alternatively, some studies have not shown a survival benefit. A single-center study by Klein *et al.*^[30] Germany studied the outcomes of 22 patients with PDAC and synchronous hepatic metastases who underwent combined pancreatic resection and liver-directed therapy compared to matched non-metastatic PDAC patients who underwent surgical resection. A third of the patients with liver metastasis underwent segmentectomy and the remainder enucleation. The matched groups had similar complication rates; however, the two-year survival rate for the metastatic group was a dismal 5% (one patient), and no patients survived 5 years.

The only systematic review to date on resection of isolated hepatic metastases in PDAC was published in 2008 and therefore included older studies than this current review. The study was comprised of 3 case reports and 18 retrospective single center studies for a pooled number of 103 cases. The great majority of the studies included less than 10 patients. The overall morbidity and mortality ranged between 24.1%-26.0% and 0-4.3%, respectively. Median survival ranged between 5.8 and 11.4 months^[10].

PULMONARY METASTASECTOMY

The lung is another frequent area of oligometastases in PDAC with potential for surgical resection to achieve negative margins [Figure 2]. While there is a clear survival benefit to pulmonary metastasectomy in colorectal cancer patients^[31-35], the data is not certain for PDAC patients [Table 2].

A study by Arnaoutakis *et al.*^[34] reported improved median survival for patients with isolated pulmonary metastases from PDAC undergoing pulmonary resections with low morbidity and mortality. This study

Table 2. Summary of most recent studies of PDAC patients undergoing metastasectomy for lung metastases

Authors	Type of Study	Year	n	Morbidity (%)	30-day mortality (%)	Median OS (months)	2-year survival (months)	5-year survival (months)
Arnaotakis <i>et al.</i> ^[34]	Single Center Retrospective	2011	9	0%	0%	51.0	40%	N/A
Robinson <i>et al.</i> ^[36]	Single Center Retrospective	2016	29	0%	0%	29.0	N/A	N/A
Downs-Canner <i>et al.</i> ^[37]	Single Center Retrospective	2015	78	N/A	N/A	35.6	N/A	18.3
Yamashita <i>et al.</i> ^[38]	Single Center Retrospective	2015	2	N/A	N/A	70.0	N/A	N/A

PDAC: pancreatic ductal adenocarcinoma; N/A: not applicable; OS: overall survival

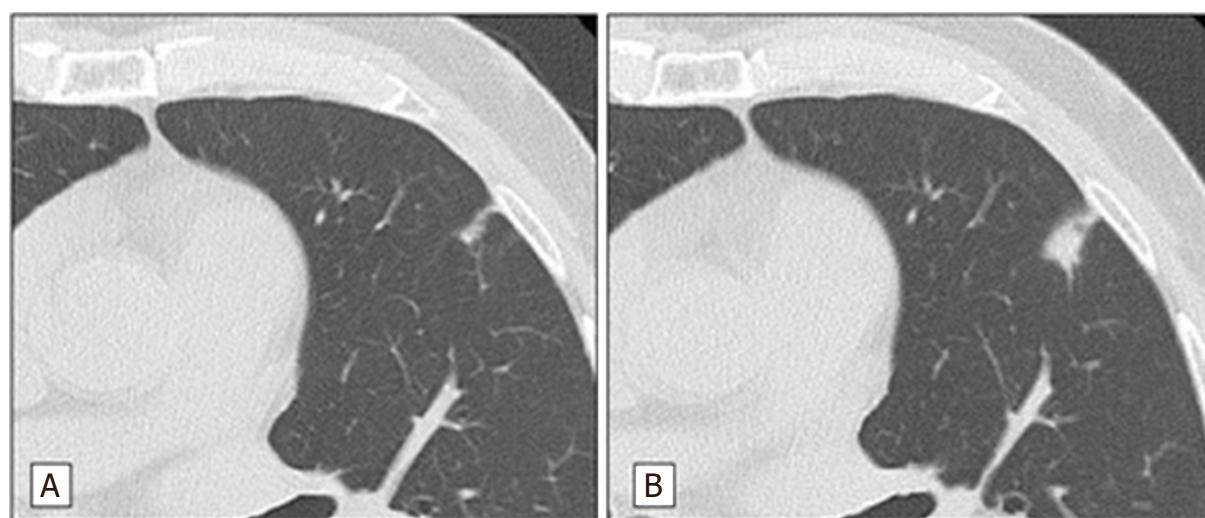


Figure 2. CT chest showing isolated pulmonary metastases initially diagnosed (A) 98 months after curative resection of PDAC and then (B) 108 months after curative resection of PDAC, showing that the nodule increased in size. The patient underwent wedge resection and received gemcitabine-based adjuvant chemotherapy for 6 months. The patient remained asymptomatic and tumor-free for a total of 4 years since last pulmonary metastasectomy^[40]. CT: computed tomography; PDAC: pancreatic ductal adenocarcinoma

included 9 patients who underwent pulmonary metastasectomy after initial pancreatectomy for stage I or II PDAC (median time from pancreatectomy to pulmonary metastasectomy of 34 months) compared to a matched reference group who did not undergo lung resection. The majority of the patients received adjuvant chemotherapy and radiation. Median overall survival for the pulmonary metastasectomy group was 51 months compared to 23 months for the reference group, $P = 0.04$. There were no complications or in-hospital mortality after pulmonary metastasectomy.

A more recent study on isolated pulmonary metastasectomy for PDAC showed similar results. The cohort included 29 patients who had previously undergone resection of pancreatic cancer who subsequently underwent lung resection, 55% for isolated pancreatic cancer lung metastases and 45% for primary lung cancer. Median disease-free interval was 24 months for the pulmonary metastasectomy group vs. 8 months for the non-metastasectomy patients. Median overall survival after lung resection was 28 months for the pulmonary metastasectomy group, corresponding to an estimated 5-year overall survival of 37%. There was no post-operative morbidity or mortality^[36].

Downs-Canner *et al.*^[37] evaluated all patients with pulmonary metastases from PDAC at a high-volume institution in the United States to determine the natural history of this disease process. Median survival for patients with lung-first metastasis was significantly longer than for patients with synchronous abdominal-lung metastases or abdominal-first disease. Among patients who underwent pancreatic resection and then

developed lung-only disease, survival was significantly longer (67.5 months) for patients who received surgical resection or stereotactic radiosurgery ($n = 8$) compared to chemotherapy (33.8 months) or observation (29.9 months).

Similarly, another study by Yamashita *et al.*^[38] identified 142 resected PDAC patients, 14 of whom developed isolated pulmonary recurrence. Patients who had isolated pulmonary recurrence had significantly longer survival than those with metastatic disease to other locations (40.3 vs. 20.9 months, $P = 0.0156$). The two patients who underwent pulmonary metastasectomy survived for 70 months after resection.

Overall, a small amount of retrospective data shows improved survival for staged resection of isolated pulmonary metastases in PDAC, especially if combined with adjuvant chemotherapy and/or radiation. The data also suggests that lung-only metastatic disease may be more indolent than metastatic disease in other locations making it a reasonable target for metastasectomy in select patients.

CONCLUSION

As recurrence and liver metastasis associated with resected pancreatic cancer is quite high and most systemic regimens only improve survival by a few months, resection of oligometastatic disease may be reasonable in very carefully selected patients. Furthermore, as systemic chemotherapy becomes more effective, the ability to treat patients with oligometastatic disease, as well as select patients who may benefit from resection may improve. As molecular data emerge, targeted approaches informed by the underlying genetic profile of a specific pancreatic tumor may also help to tailor more personalized approaches to therapy.

Although the current standard of care for metastatic pancreatic adenocarcinoma is palliative chemotherapy with no role for surgical resection, single institution experiences suggest that there may be a survival benefit for resection of oligometastatic disease in select patients. Some centers have created algorithms to select patients with more favorable tumor biology who might benefit from resection, applying such parameters as response to chemotherapy and CA 19-9 values. It appears that morbidity and mortality is slightly higher for hepatic compared with pulmonary metastatic resection. If surgery is being considered for oligometastatic disease, surgical resection should be combined with systemic and patients need to very carefully selected.

There are no randomized controlled trials exist, and current data are based on small, retrospective observational studies with varying definitions of oligometastasis and different inclusion criteria, extent of metastasectomy, timing and regimens of systemic therapy, and reference groups for comparison. As such, the level of evidence to suggest a benefit for resection of oligometastatic disease remains low.

Of note, there is an ongoing multicenter, randomized, controlled phase III trial called CSPAC-1 (NCT03398291) in China evaluating the possible benefit of simultaneous resection of pancreatic cancer and liver oligometastases after induction chemotherapy^[39]. Inclusion criteria include age 18-75, Eastern Cooperative Oncology Group performance status 0-1, and histologically-confirmed stage IV pancreatic cancer with no more than 3 liver metastases. Patients who meet inclusion criteria for intervention, including resectable primary tumor and liver lesions, no new metastatic disease, and a drop in abnormal tumor markers by more than 50% after chemotherapy, are eligible for synchronous resection of the primary pancreatic cancer and the liver metastasis. Patients will randomized to chemotherapy and resection versus standard chemotherapy. The primary outcome measure is overall survival from start of induction chemotherapy, and secondary outcomes include overall survival after completing induction chemotherapy, quality of life, and post-operative morbidity and mortality. The expected completion date of the study is 2025. Until randomized data are available, resection of oligometastatic disease of the liver and lung in PDAC should only be done in a multi-disciplinary setting for highly selected patients and, preferably, on a protocol basis.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the literature review: Puckett Y, Pawlik TM
Performed literature review and manuscript preparation: Puckett Y, Eskander MF, Aquina CT
Responsible for conception and design of literature review: Pawlik TM

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

Not applicable.

Consent for publication

Not applicable.

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Review

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Signaling pathways downstream to receptor tyrosine kinases: targets for cancer treatment

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How to cite this article: Cordover E, Minden A. Signaling pathways downstream to receptor tyrosine kinases: targets for cancer treatment. *J Cancer Metastasis Treat* 2020;6:45. <http://dx.doi.org/10.20517/2394-4722.2020.101>

Received: 9 Sep 2020 **First Decision:** 26 Oct 2020 **Revised:** 9 Nov 2020 **Accepted:** 19 Nov 2020 **Published:** 30 Nov 2020

Academic Editor: Xuefen Le Bourhis **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Mammalian cells have the ability to respond to a myriad of diverse extracellular stimuli that modulate cell function. This often involves ligands binding to cell surface receptors and subsequent activation of intracellular signaling pathways. These pathways can lead to changes in gene expression patterns that in turn regulate cell growth, differentiation, migration, and function. One important type of cell surface receptor is the receptor tyrosine kinase (RTK). In response to ligand binding, RTKs dimerize, then trans-phosphorylate each other, leading to activation of downstream pathways. While the signaling proteins in these pathways are important for normal cell growth control, when improperly regulated they can lead to uncontrolled growth and sometimes cancer. For this reason, they are often considered to be good candidates for drug targets for chemotherapeutic drugs. RTKs can activate multiple different signaling pathways. Some of the signaling proteins in these pathways can have crosstalk with other RTK activated pathways, and some of them can be activated by multiple mechanisms in addition to activation by RTKs. While there is a wide array of different signaling proteins and pathways activated by RTKs, in this review we will discuss components of several key pathways including the MAPK pathway, the Her2/Neu pathway, mTOR, and Pak kinases. We provide an overview of the roles for these pathways in cell signaling and discuss how different components of these pathways are being considered as targets for cancer treatment.

Keywords: Receptor tyrosine kinases, signaling pathways, targeted cancer therapeutics, oncogenes, cell signaling



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INTRODUCTION

Cells within multicellular organisms depend upon complex ways of communicating with their surroundings and other cells and tissues. An important method by which cells communicate is by responding to signals from extracellular stimuli. Growth factors are small extracellular ligands that transmit signals between cells and their environment. They can control cell function by binding to transmembrane receptors, activating a myriad of different intracellular signaling pathways. Growth factors commonly bind to the receptor tyrosine kinase family of transmembrane receptors (RTKs). RTKs have an extracellular domain that binds to ligand, a transmembrane domain, and an intracellular domain that has tyrosine kinase activity. Once bound to ligand, the RTKs dimerize, and transphosphorylate on tyrosine residues. This usually leads to binding of adapter proteins to the phospho-tyrosine residues, leading in turn to a cascade of events within the cell^[1]. This includes activation of GTP binding proteins, a cascade of serine/threonine phosphorylation events, and translocation of certain proteins to the nucleus. Ultimately this can lead to the regulation of transcription factors which in turn result in changes in gene expression patterns. The result is the regulation of genes that control cell growth, differentiation, survival, and function^[2]. In order to understand how normal cell function and communication is regulated, it is critical to understand how those intracellular events occur in normal cells.

In addition to their important roles in controlling normal cell fate, it is also important to understand the roles that signaling pathways play in cancer. Intracellular signaling pathways are often improperly regulated in cancer^[3]. As a result, cancer cells often become independent from their control by extracellular stimuli. Instead, they can gain the ability to grow, proliferate, and survive, without external regulation. This is an important feature of many cancer cells, and one that is important to understand in order to develop individualized treatments.

Understanding which intracellular signaling proteins are most important for controlling cellular behavior is important for understanding the molecular basis of cancer. There are multiple types of extracellular stimuli and intracellular signaling pathways that are important for controlling cell growth. These include not only pathways regulated by RTKs, but also pathways that are regulated by other types of receptors such as trimeric G protein coupled receptors and cytokine receptors. The study of RTKs has taken a prominent role in studying cancer. This is in part due to being linked to the Ras family of proteins, which are mutated in a large percentage of cancers^[4]. Furthermore, multiple components of RTK activated signaling cascades have been linked to cancer when they are aberrantly regulated. In this review we will focus on the signaling pathways that are mediated by RTKs, which have been implicated in cancer when improperly regulated. While the number of different pathways that are associated with cancer is large, we will focus on several RTK regulated pathways in this review, and we will discuss their importance in cancer research. We then discuss some of the drugs and drug candidates that are designed to target signaling proteins within these pathways.

MITOGEN-ACTIVATED PROTEIN KINASE PATHWAY

The mitogen-activated protein kinase (MAPK) signaling pathway was discovered over 30 years ago. It is one of the most well studied intracellular signaling pathways and it often serves as a model for RTK activated signaling pathways. The MAPK pathway is frequently implicated in cancer and serves as an important target for cancer therapies. The MAPK signaling cascade plays several key physiological roles in healthy cells. Specifically, MAPKs mediate cellular growth, proliferation, and survival processes^[5]. MAPK operates by modulating transcription factors, which in turn leads to regulation of gene expression in response to extracellular signals^[6]. Abnormal MAPK signaling is shown to be associated with several types of cancer such as breast cancer, prostate cancer, colorectal cancers, melanoma, and leukemia. Dysregulation of MAPK signaling in cancer is associated with evasion of apoptosis, uncontrolled cell proliferation, and resistance to chemotherapy and targeted therapies^[7].

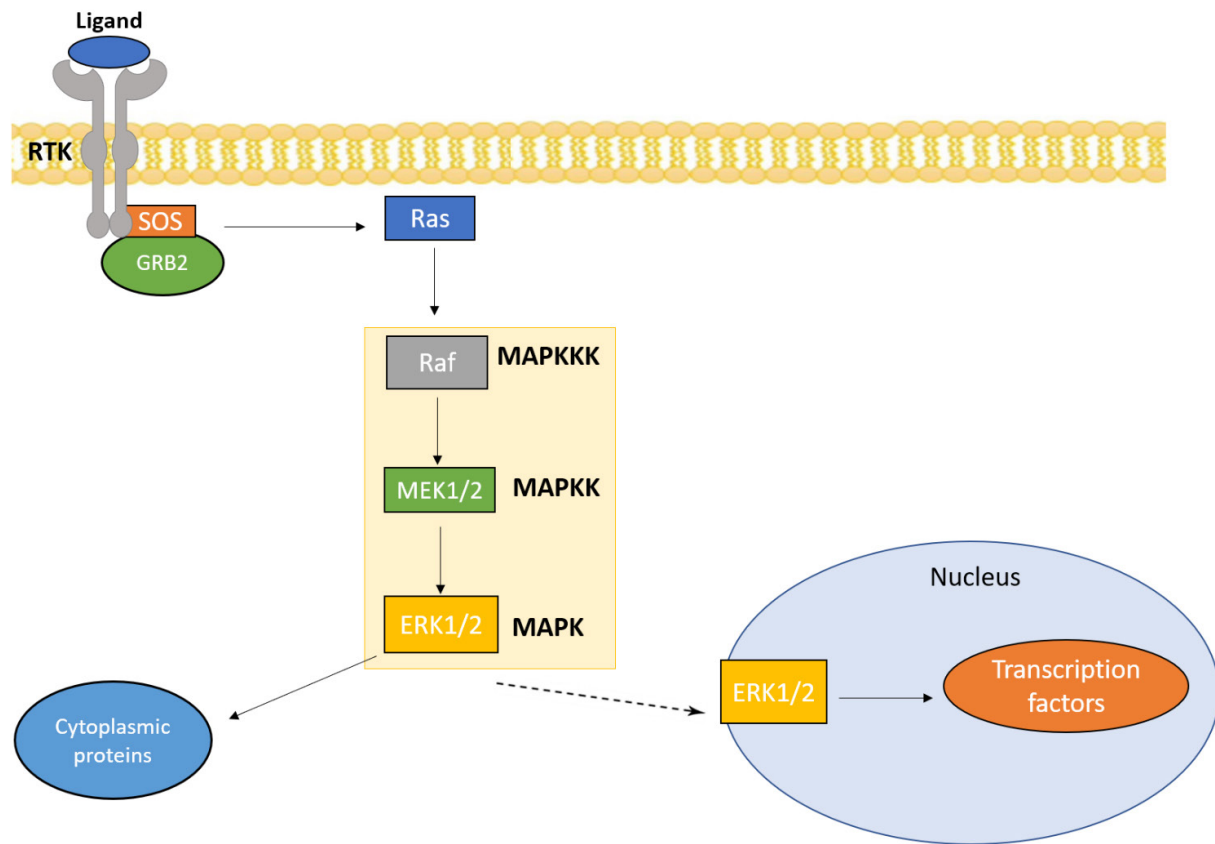


Figure 1. MAPK/ERK pathway: the MAPK/ERK pathway is activated by a variety of extracellular signals, such as growth factors and other mitogens. The main components of this pathway are Ras, Raf, and MEK which lead to the activation of ERK. ERK subsequently activates several cytoplasmic proteins and transcription factors that influence cell proliferation and survival. MAPK: mitogen-activated protein kinase; ERK: extracellular signal-regulated kinases; MAPKK: MAPK kinase; MAPKKK: MAPK kinase kinase

The MAPK pathway can be activated by multiple mechanisms, including activation of RTKs in response to extracellular stimuli. For example, binding of the epidermal growth factor receptor (EGFR), an RTK, to its ligand epidermal growth factor leads to EGFR dimerization and activation. This in turn can trigger activation of the MAPK pathway [Figure 1]. The canonical MAPK cascade is composed of three successive serine/threonine kinases: MAPK kinase kinase (MAPKKK), which phosphorylates MAPK kinase (MAPKK) which in turn phosphorylates and activates MAPK^[8]. MAPKKK itself is regulated in response to small GTPases, typically Ras, which are activated by exchange factors in response to the activated RTK. In humans, the MAPK family includes the extracellular signal-regulated kinases (ERK1/2), Jun N-terminal kinases (JNK1, JNK2, and JNK3), and p38^[9].

Dysregulation of the ERK pathway is found in approximately one-third of all human cancers^[10]. ERK, as a MAPK, operates downstream to the Ras small GTPase. The Ras/ERK pathway is activated by various stimuli, including growth factors and mitogens. Growth factors and mitogens depend on this cascade to transmit signals for regulating gene expression which in turn leads to regulation of cell growth and apoptosis^[11]. Other important components of this pathway include Raf and MEK [Figure 1]. One important way the pathway is activated in quiescent cells is through the binding of a growth factor (GF) to receptor tyrosine kinases (RTKs) in the cell membrane. The binding of GFs causes the dimerization and autophosphorylation of RTKs, which leads to the recruitment of signaling molecules including SOS, the Ras GTPase exchange factor. SOS exchanges GDP for GTP to activate Ras which then recruits Raf. Raf in turn activates MEK, culminating in activation of ERK. Activated ERK phosphorylates several substrates

such as transcription factors and cytoplasmic signaling proteins which have key roles in the regulation of cell growth and function^[8]. Due to the alteration of the Ras/ERK pathway in many cancers, components of the pathway are considered to be promising drug targets for cancer therapies.

Ras inhibitors

The RAS genes are among the most common oncogenes in human cancer and are of particular interest for targeting the ERK signaling cascade in cancer. The Ras family of proteins consists of three different isoforms - K-Ras, N-Ras, and H-Ras. Point mutations in all three of the RAS genes are associated with different types of cancer. For instance, K-RAS mutations are seen in over half of pancreatic tumors and N-RAS mutations are present in almost 20% of skin cancers^[12]. As Ras is inactive in quiescent cells, its constitutive activation drives tumor formation. Previous efforts to target Ras in cancer therapies have proven to be unsuccessful. However, the development of new direct Ras inhibitors is a promising area of research.

One way to inhibit Ras is by targeting Ras membrane localization. In order to exert its oncogenic effects, Ras must localize to the plasma membrane. The farnesyltransferase inhibitors (FTIs) were created to block Ras membrane localization. The C-terminal CaaX box is a motif that is conserved in all Ras proteins and is essential for the addition of a farnesyl lipid group. The addition of a farnesyl group to a targeted cysteine in the CaaX motif is catalyzed by farnesyltransferase (FTase) and allows Ras to interact with the membrane^[13]. FTIs have been shown to be ineffective in the treatment of solid tumors with K-RAS and N-RAS mutations, but are promising in treating certain malignancies with H-RAS mutations, such as thyroid and bladder cancers^[14]. The failure of FTIs in blocking K-Ras membrane localization is attributed to K-Ras having a higher affinity for FTase than H-Ras. Additionally, K-Ras can interact with geranylgeranyltransferase (GGTase) which allows for the covalent attachment of a geranylgeranyl lipid group to the CaaX domain, thus allowing K-Ras to localize to the membrane without farnesylation by FTase. Nonetheless, researchers have designed a neo-substrate that binds to the farnesylated cysteine in the CaaX domain to prevent geranylgeranylation by GTTase and subsequently, K-Ras membrane localization^[13].

Another advancement is to exploit the G12C mutation in K-Ras to suppress oncogenic activity. Targeting tumors with the K-Ras^{G12C} mutant is of clinical importance because this mutation is frequently expressed in lung and colon adenocarcinomas^[15]. In addition, there were few known mechanisms for inhibiting K-Ras activity in cancer cells until the recent discovery of a druggable pocket in K-Ras^{G12C}. This target, called the switch-II pocket, has initiated the innovation of several irreversible inhibitors to lock K-Ras^{G12C} in its inactive GDP state. There are currently several K-RAS^{G12C} inhibitors that are in Phase I/II of clinical trials. For instance, AMG510, developed by Amgen, interacts with the His95 small groove within the switch-II pocket. Preclinical studies have shown regression of K-RAS^{G12C} tumors and elevated T cell infiltration at tumor sites in mice treated with AMG510^[16]. Likewise, MRTX849 (Mirati Therapeutics) also binds K-Ras in the switch II pocket. MRTX849 has proved to be as effective as a K-Ras^{G12C} inhibitor by inhibiting GTP-loading^[16]. AMG10 and MRTX849 have both been shown to lead to inhibition of ERK phosphorylation and signaling, thus contributing to anti-tumor activity in humans. Further work is being done to determine the efficacy of K-Ras^{G12C} inhibitors in combination with other anticancer drugs^[17].

Another area of interest is to investigate Ras dimerization as a potential therapeutic target for cancer. Previous studies show that monomeric Ras can bind Raf, but numerous studies indicate that dimerization of Ras is necessary for the activation of Raf. Under normal cellular conditions, Ras-GTP is recruited to the plasma membrane where it activates the Raf kinase domain. Raf in turn activates the MAP kinase pathway. As such, all three isoforms of Ras activate Raf, and K-Ras is the most powerful activating agent of Raf^[18]. Multiple studies have found evidence for the role of Ras homodimerization in Raf activation. For instance, by using a K-Ras mutant that impairs dimerization, K-Ras dimerization was shown to be critical for the

abnormal Ras signaling that is seen in many cancers. While more research needs to be done to determine the mechanism behind Ras dimerization, current data suggests that disruption of Ras dimerization has the capacity to suppress Ras-Raf signaling in cancer^[19].

Raf and MEK inhibitors

The Raf family of protein kinases are central components of the ERK cascade as they interact with Ras and initiate the activation of the kinase cascade. When Raf is dysregulated, it can exert oncogenic effects, including metastasis, invasion, and uncontrolled cellular proliferation^[20]. Researchers have discovered mutations that affect the catalytic activity of Raf to be present in several human tumors. Of the three different isoforms of Raf, A-, B-, and C-Raf, B-Raf was found to be commonly mutated in numerous cancers, specifically melanoma. In fact, B-Raf mutations are found in 66% of all melanomas^[21]. In wild type cells, Ras prompts the dimerization of B-Raf and its subsequent activation. In cancer cells with mutated B-RAF, activating mutations cause B-RAF to be expressed constitutively, which promotes cell growth and inhibits apoptosis^[22].

In particular, the B-Raf^{V600E} mutation, present in melanoma and colon cancer, is shown to drive cancer development, and serves as an important diagnostic and prognostic biomarker^[23]. Inhibitors that target B-RAF^{V600E} have been clinically proven to prolong the survival of advanced stage melanoma patients. Nonetheless, these agents display adverse effects, and patients often relapse due to acquired resistance. A major problem with first generation B-RAF inhibitors is that drug treatment provokes increased wild-type Raf membrane localization and B-/C-Raf dimerization. This means that when the inhibitor is under non-saturating conditions, drug bound B-Raf can dimerize with drug-free Raf, resulting in the activation of MEK and ERK in the presence of active Ras. This contributes to the limited activity of these first-generation inhibitors in cancers with upregulated Ras despite having the V600E B-Raf mutation. As such, this poses a challenge for next generation B-RAF^{V600E} inhibitors. One way in which researchers are looking to combat the dilemma of Raf dimerization and drug resistance is by creating inhibitors that target both monomeric and dimeric Raf with equal affinity. These agents, known as pan-Raf inhibitors, have been shown to inhibit Raf signaling and paradoxical ERK activation in colorectal cancer and melanomas. Another approach that is being studied to combat paradoxical ERK activation is through an inhibitor that binds to the ATP binding pocket of B-Raf and impedes Raf dimerization^[20].

In addition to Ras and Raf, MEK is another kinase that has an important role in the ERK pathway and serves as a promising drug target. Once it is activated, MEK phosphorylates ERK. ERK in turn plays critical roles in the regulation of cellular proliferation and apoptosis. As such, the inhibition of both isoforms of MEK, MEK1 and MEK2, have the potential to cause growth inhibition in tumors where the ERK pathway is activated. Interestingly, trametinib, the FDA approved MEK1 and MEK2 inhibitor, is shown to inhibit tumor proliferation in advanced stage melanomas with the B-RAF^{V600E} mutation. Compared to standard chemotherapy, trametinib given as a single agent showed statistically significant improvement in terms of disease progression-free survival during treatment^[24]. The other FDA-approved MEK inhibitor, cobimetinib, proved successful in blocking ERK/MAPK signaling in B-RAF and K-RAS mutated cell lines. Clinically, cobimetinib is well tolerated in patients with advanced solid tumors with the B-RAF^{V600E} mutation^[25]. Further, several preclinical and clinical studies show that the use of a MEK inhibitor alongside a BRAF inhibitor is a promising form of treatment for advanced stage melanomas. As previously mentioned, melanoma patients treated with first generation BRAF inhibitors often relapsed due to acquired drug resistance. The dual inhibition of both BRAF and MEK leads to more significant tumor response than BRAF therapy alone^[26].

ERK inhibitors

ERKs are important effectors in the Ras/ERK pathway. There are two structurally similar isoforms of ERK, ERK1 and ERK2, and they belong to the MAPK family of protein kinases that are central to signal

transduction. ERK is involved in the regulation of many cellular processes, such as proliferation and differentiation, and its increased activity is often implicated in several human cancers^[27]. ERK is activated by its upstream effector MEK through phosphorylation [Figure 1]. ERK has roles in both the cytoplasm and the nucleus. In the cytoplasm, ERK modulates key cytoskeletal proteins that affect motility, metabolism, and cell adhesion^[28]. ERK exerts its nuclear functions by dimerizing and localizing to the nucleus once it is activated. In the nucleus, ERK phosphorylates and subsequently activates a number of transcription factors that are involved in cell proliferation and survival^[29]. Due to its aberrant expression in several cancers, it is being studied as a potential therapeutic option in cancers with B-Raf and N/K-Ras mutations^[27].

As discussed earlier in this review, BRAF mutations are common in an array of human cancers, most notably in melanoma. BRAF inhibitors are shown to be promising in the short-term, but ultimately, are ineffective at blocking cancer cell survival and proliferation. BRAF/MEK dual inhibition is associated with an increased anti-tumor response. Unfortunately, up to 70% of tumors treated with a BRAF/MEK dual inhibition strategy experience ERK/MAPK pathway reactivation and evade treatment^[30]. Hence, novel strategies that target the Ras/ERK pathway in cancers with BRAF mutations are sought. Once such novel inhibitor that targets this pathway is the ERK inhibitor ulixertinib. Recent studies report on the efficacy of ulixertinib in solid malignancies in a phase I clinical trial. Ulixertinib is a highly potent, reversible, and selective ATP-competitive inhibitor of ERK1/2. Previous evidence shows that ulixertinib reduces tumorigenesis in BRAF- and Ras-mutant xenograft models^[27]. The results of the phase I clinical trial provide evidence that ulixertinib is effective in cancers with a diverse assortment of BRAF mutations. In addition, in melanoma patients whose tumors were resistant to BRAF/MEK dual inhibition, ulixertinib is shown to exert anticancer effects^[31]. Due to the success of ulixertinib thus far, the inhibitor is being investigated in combination with other inhibitors, such as BRAF inhibitor dabrafenib^[31].

Despite the initial success of ulixertinib, challenges remain with ERK inhibitors. A 2018 study by Jaiswal *et al.*^[32] conveyed that ERK inhibitors, when used as a single agent, are likely to confer drug-acquired resistance. The researchers looked at five different ATP-competitive ERK inhibitors, including ulixertinib, to determine the mechanism behind acquired resistance to ERK inhibition. Upon treatment with ERK inhibitors, RAS-/RAF-mutant cell lines acquired several mutations that affect the binding of ERK inhibitors to their target, preventing the hindrance of ERK1/2 activity^[32]. The authors also displayed the novel finding that ERK2 amplification is a mechanism of resistance to ERK inhibitors^[32]. Hence, due to the diverse mechanisms of resistance to ERK inhibition, ERK inhibitors are being looked at in combination with other inhibitors of the ERK/MAPK pathway. Jaiswal *et al.*^[32] suggested that ERK inhibitors in combination with MEK inhibitors may prove advantageous in treating malignancies with acquired resistance to RAF/MEK inhibitors. In addition, RTKs and components of the PI3K/AKT/mTOR pathway were identified as mediators of acquired resistance. As such, combination therapy involving ERK inhibitors with either inhibitors of the PI3K/AKT/mTOR pathway or RTK inhibitors, such as those that target members of the HER family, may prevent the development of resistance^[32]. Overall, the use of ERK inhibitors as part of combination therapy is promising for the treatment of cancers with mutations in the ERK/MAPK pathway.

HER2/NEU/ERBB2

The RTK EGFR consists of four members, also known as the human epidermal growth factor receptor (HER) or ErbB family. These receptors include HER1 (also called ErbB1 or EGFR), HER2 (also called ErbB2), HER3 (ErbB3), and HER4 (ErbB4). Under normal conditions, the members of this family of RTKs are known to have important roles in development. Like other RTKs, the HER/ErbB family of receptors are activated by binding to ligands on their extracellular domains, which leads them in turn to form dimers with each other or with other HER/ErbB family members. Upon dimerization, intracellular signaling pathways are activated, leading to a response to the signal. HER2/ErbB2 functions differently from the other members because it does not require a ligand to form a dimer. Although it does not directly bind any

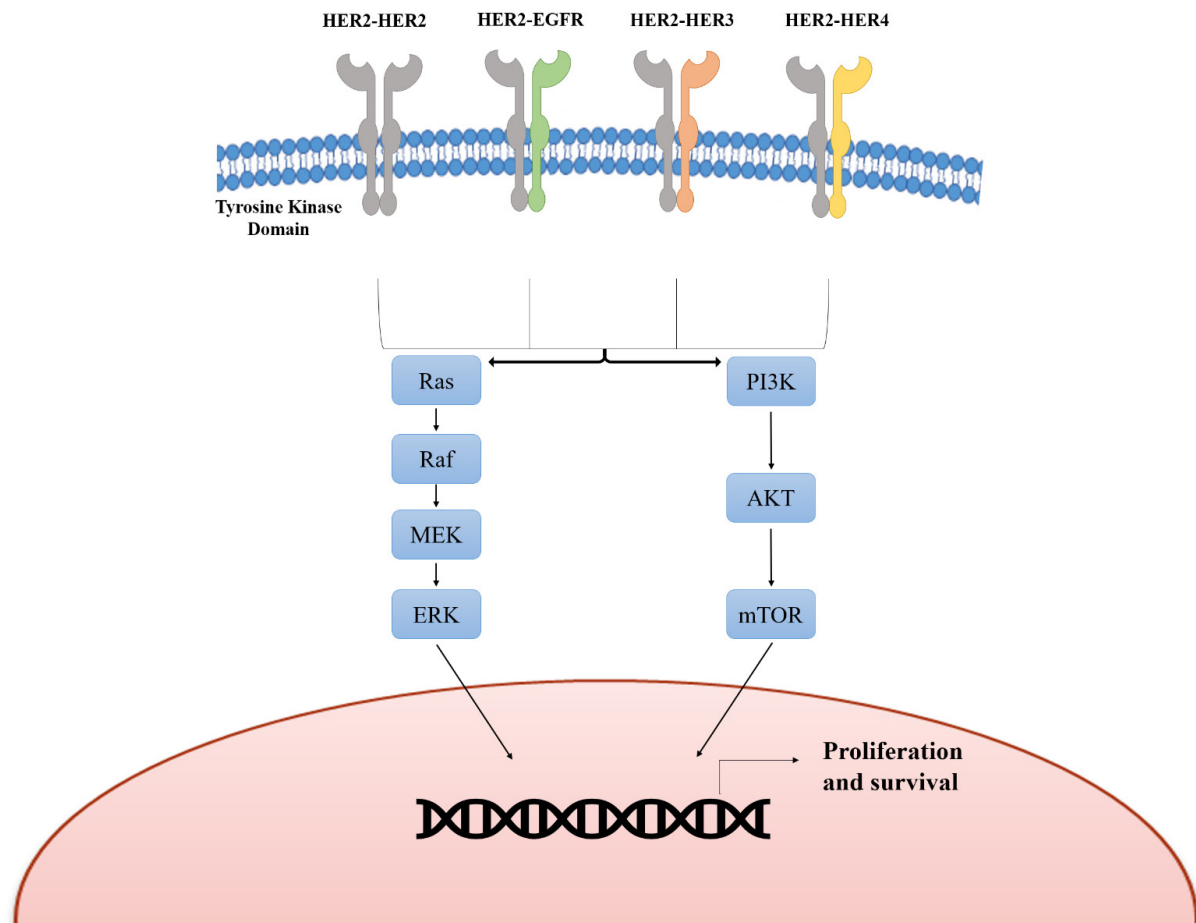


Figure 2. Her2 signaling: HER2 is a member of the HER/EGFR family of RTKs. HER2 signaling has important roles in regulating normal cell function. However, its dysregulation is often implicated in cancer. HER2 can homodimerize with itself or heterodimerize with the other members of the HER family. Overexpression of HER2 can lead to the overactivation of the MAPK/ERK and PI3K/AKT/mTOR pathways, leading to uncontrolled cell growth and evasion of apoptosis. HER: human epidermal growth factor receptor; EGFR: epidermal growth factor receptor; RTK: receptor tyrosine kinase; MAPK: mitogen-activated protein kinase; ERK: extracellular signal-regulated kinases; PI3K: phosphoinositide 3-kinase; AKT: protein kinase B; mTOR: mammalian target of rapamycin

ligands, HER2 is activated by homodimerization or heterodimerization with HER1, HER3, or HER4^[33,34] [Figure 2].

The signaling pathways regulated by the HER receptors have important roles in responding to extracellular signals and in turn regulating normal cell function. Improper regulation of the HER receptors, especially HER1 and HER2, can be associated with cancer. In healthy cells, HER2 activation triggers signaling pathways that control normal cell growth, differentiation, motility, and adhesion. Overexpression of HER2 leads to the over-activation of downstream pathways, including the PI3K/Akt/mTOR and MAPK/ERK signaling cascades, which are known to be involved in malignant transformation^[35]. The overexpression of HER2 signaling is shown to promote the loss of cell adhesion and polarity, defining features of epithelial cancers^[36,37]. As such, HER2 dysregulation is associated with ovarian cancer, gastric cancer, and perhaps most notably, breast cancer.

HER2 in breast cancer

Breast cancer is the most common type of cancer among women in the United States, and 20-30% of breast cancers are thought to be HER2-positive. Amplification of the *HER2* gene in breast cancer is associated with invasiveness, large tumor size, and late clinical stage. HER2 overexpression is also associated

with higher rates of recurrence and shorter rates of survival in patients with breast cancer^[38,39]. Once activated, HER2 is shown to recruit proto-oncogene Src. The constitutive activation of the Src kinase by the overexpression of HER2 has a role in tumor progression and metastasis and is implicated in breast cancer^[40]. Moreover, one way in which HER2 promotes metastasis is by its upregulation of the chemokine receptor CXCR4. HER2-mediated CXCR4 expression in breast cancer is shown to be instrumental in the movement of malignant cells to specific tissues, which contributes to poor survival rates^[41,42]. In addition, HER2 overexpression in epithelial breast cancer cells is shown to deregulate the G1/S cell cycle checkpoint. HER2 exerts its effects on G1/S control by upregulating cyclins D1 and E and cyclin-dependent kinase CDK6, and by enhancing the degradation of cyclin-dependent kinase inhibitor p27^{Kip1}. These changes in cell cycle progression contribute to the enhanced signaling of the PI3K/Akt and MAPK pathways^[43].

In addition to CXCR4, HER2 interacts with several other tumor markers. For instance, estrogen receptor (ER) and progesterone receptor (PR) are important for the prognosis and diagnosis of breast cancer. The overexpression of ER and PR in breast cancer is associated with a better clinical outcome when compared to tumors that overexpress HER2. Notably, ER and PR expression are inversely correlated to HER2 expression. There are several explanations for this occurrence. For example, the hyperactivation of the MAPK pathway in response to HER2 overexpression contributes to the downregulation of ER. Hence, the overexpression of HER2 leads to decreased ER and PR expression and promotes oncogenesis^[33,44]. Likewise, breast cancers with HER2 amplification have been shown to overexpress the angiogenic growth factor VEGF. The HER2-mediated upregulation of VEGF expression is associated with aggressiveness and high rates of recurrence in HER2-positive breast cancers^[45]. Lastly, recent evidence shows that HER2 disrupts the function of the tumor suppressor E-cadherin. E-cadherin is a transmembrane protein that mediates epithelial cell adhesion and tissue formation. Cell-cell adhesion by E-cadherin is maintained through its interaction with β -catenin and the cytoskeleton. HER2 disrupts the E-cadherin/ β -catenin complex by binding and phosphorylating β -catenin. This blocks the interaction between E-cadherin and β -catenin, resulting in the loss of E-cadherin expression. As such, the loss of E-cadherin is found in over half of invasive lobular carcinomas at both the primary tumor and metastatic sites^[46-48]. Accordingly, HER2 overexpression is shown to have a prominent role in the invasiveness of breast tumors and serves as a critical drug target.

Targeting HER2 in breast cancer

The advent of anti-HER2 therapies, along with the increase of early cancer screenings, have led to significant improvements in the survival rates of HER2-positive breast cancer over the past two decades. Anti-HER2 therapies, such as the monoclonal antibody trastuzumab (Herceptin) and the tyrosine kinase inhibitor lapatinib (Tykerb), have shown to be clinically successful in treating HER2-positive breast cancers. Nonetheless, a significant proportion of HER2-positive breast cancer patients who were treated with trastuzumab relapse and develop metastatic disease. This indicates that tumors possess or develop resistance to anti-HER2 therapies^[43]. In order to discern the many mechanisms of intrinsic and acquired resistance of HER2-positive tumors, it is important to understand how trastuzumab works to inhibit HER2. Trastuzumab is shown to downregulate HER2 by binding its extracellular domain, and to block HER2-HER3 dimerization^[49,50]. Consequently, trastuzumab exercises several anti-tumor effects in HER2-overexpressing cells by downregulating PI3K/AKT pathway signaling along with downregulating mediators of cell cycle progression, such as cyclin D1, and upregulating the p27 cell cycle inhibitor^[51,52].

It is critical to understand the various mechanisms behind trastuzumab resistance in order to develop novel anti-HER2 therapies. One way in which HER2-positive cancer cells become resistant to trastuzumab is through the constitutive activation of the PI3K/Akt/mTOR signaling cascade. Loss of function mutations in tumor suppressor PTEN and activating mutations in PI3K lead to aggressive cell growth. These alterations in the PI3K/Akt pathway are present in patients who have been treated with trastuzumab and are thought to be associated with drug resistance^[51]. Another leading way that HER2 overexpressing tumor

cells escape anti-HER2 therapies is through the expression of p95HER2. Approximately 30% of HER2-positive breast cancers express the p95HER2 fragment, which is a truncated form of HER2 that is especially oncogenic, as it spontaneously homodimerizes into a constitutively active form. Tumors with p95HER2 fragments are resistant to trastuzumab because they lack the extracellular domain and the binding site for trastuzumab^[53,54]. Unlike trastuzumab, HER2 RTK inhibitor lapatinib can inhibit p95HER2 along with HER2, as it binds the ATP active site present in both proteins. This inhibition blocks phosphorylation and inhibits downstream signaling^[55].

Although lapatinib has some advantages, cancer cells can still develop resistance. When HER2 signaling is suppressed by lapatinib and trastuzumab alike, alternative survival mechanisms can be activated. Even though HER2 and p95HER2 are shown to be inhibited by lapatinib, the suppression of HER2 signaling can lead to the upregulation of other HER family members and RTKs, such as the fibroblast growth factor receptor-2 and insulin-like growth factor-1. Alternately, lapatinib resistance can be attributed to the hyperactivation of the PI3K pathway by the activation of Src kinases through insulin receptor substrate 4. Recently, another anti-HER2 agent, neratinib, was evaluated in clinical trials. Unlike lapatinib, neratinib is an irreversible pan-HER inhibitor^[56]. A recent phase III clinical trial compared the effects of neratinib versus lapatinib in combination with capecitabine, an antimetabolite, in patients with HER2-positive metastatic breast cancer. The results indicate that neratinib + capecitabine significantly improved progression-free survival over lapatinib + capecitabine. These results offer a promising outlook on the use of neratinib combination therapy in the treatment of aggressive HER2-positive breast cancers^[57].

Trastuzumab and lapatinib-resistant cells continue to depend on HER2 to evade drug treatment. One way in which these late stage drug-resistant cancer cells continue to depend on HER2 is by expressing MUC4, a membrane-associated mucin that masks HER2. This is shown to prevent the binding of trastuzumab to HER2 and to maintain HER2 phosphorylation. Knockdown of MUC4 with RNA interference (RNAi) led to an increased binding of HER2 to trastuzumab^[58]. Studies have also been designed to investigate the use of RNA interference directly against HER2. The results of a recent study demonstrate that RNAi of HER2 by siRNA has antiproliferative effects and decreases HER2 mRNA and protein levels *in vitro*^[59,60]. In addition, another study looked at the effects of siRNA on BT474 cells (ductal carcinoma). The authors report that siRNA treated BT474 cells do not acquire resistance against the treatment. This work has not yet been translated to *in vivo* studies, but these preliminary results demonstrate that siRNA poses as a promising option for patients with drug-resistant HER2-positive breast cancer^[61].

PI3K/AKT/MTOR PATHWAY

The mammalian target of rapamycin (mTOR) pathway is another example of an important signaling protein that can operate activated downstream to RTKs [Figure 3]. mTOR is a serine/threonine kinase. Together with other proteins, it forms two different complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is activated by RTKs as well as by other types of stimuli. It is part of a signaling pathway that includes phosphoinositide 3-kinase (PI3K) and AKT, which are implicated in cell survival [Figure 3]. The mTOR pathway is activated in a wide variety of solid tumors and is one of the main causes of resistance to anti-tumor therapies. The mTORC1 signaling cascade is involved in growth, proliferation, motility, metabolism, and immune response regulation. Hyperactivation of the PI3K/AKT/mTOR pathway leads to genomic instability and uncontrolled proliferation of cancer cells. Thus, due to its role in tumorigenesis and drug resistance, the PI3K/AKT/mTOR cascade is a prominent therapeutic target for several types of cancers^[62].

PI3K is an important upstream activator of mTORC1. The PI3K family is a group of lipid kinases that regulates a broad array of cellular functions in signaling and metabolism. Of the 3 classes of PI3Ks, the class I PI3Ks are most often linked to oncogenesis^[63]. The activation of G-coupled protein receptors

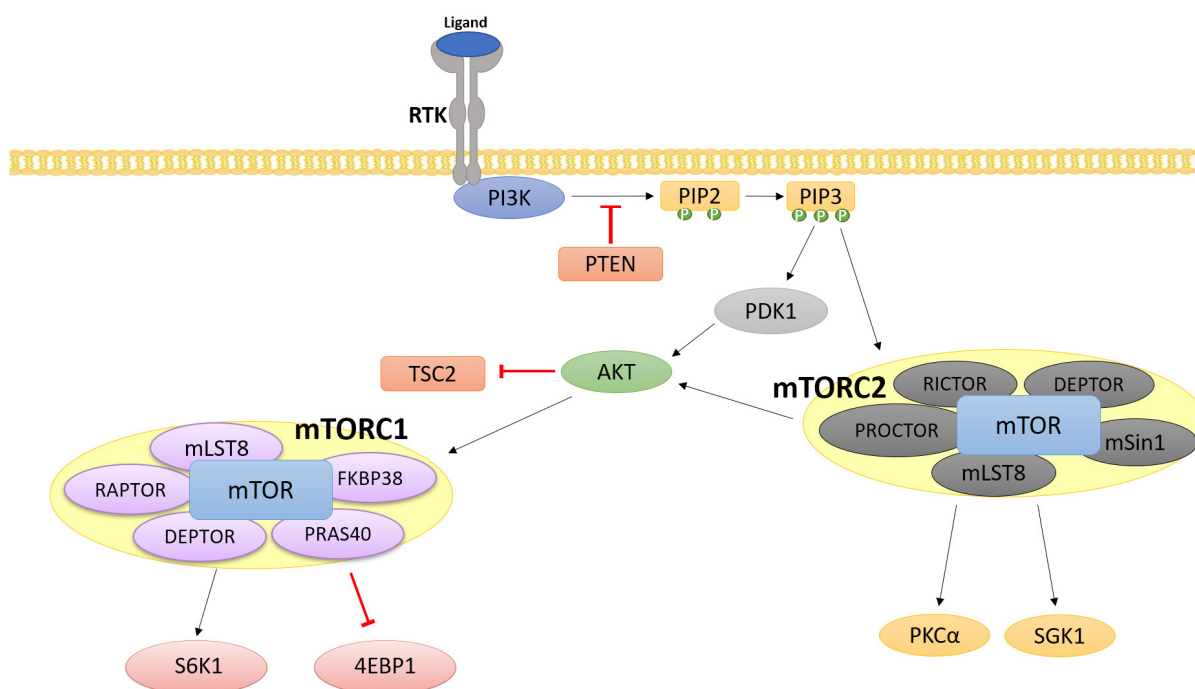


Figure 3. PI3K/AKT/mTOR signaling cascade: The PI3K/AKT/mTOR pathway is activated through RTK signaling, and hyperactivation of this pathway is implicated in several cancers. The main effectors of this pathway, mTORC1 and mTORC2, are major regulators of translation, cell metabolism, and cell proliferation. RTK: receptor tyrosine kinase; PI3K: phosphoinositide 3-kinase; AKT: protein kinase B; mTOR: mammalian target of rapamycin

and autophosphorylation of tyrosine residues of RTKs recruit PI3K to the membrane and activate its catalytic subunit. PI3K activation leads to the phosphorylation of PIP2 to produce secondary messenger phosphatidylinositol (3, 4, 5)- trisphosphate (PIP3). PIP3 amplifies the initial signal from PI3K and subsequently recruits 3'-phosphoinositide-dependent kinase-1 (PDK1) and protein kinase B/Akt to the membrane. PDK1 is responsible for the activation of Akt, and Akt in turn regulates several downstream effectors. Notably, Akt activates the Forkhead Box O proteins and the mTOR pathway^[64].

The mTOR pathway is a major regulator of translation and consists of two complexes, mTORC1 and mTORC2. AKT can both directly and indirectly activate the mTOR pathway. AKT is shown to activate the mTORC1 complex through the phosphorylation and subsequent inactivation of two negative regulators of the mTOR pathway - tuberous sclerosis complex 2 and PRAS40 (proline-rich Akt substrate of 40kDa). In mTORC1, mTOR associates with Raptor, which regulates mTOR activity and recruits mTORC1 substrates. mTORC1 regulates ribosomal proteins S6K and 4E-BP1, which have roles in regulating protein synthesis and metabolism^[65] [Figure 3]. The phosphorylation and activation of S6K by mTORC1 leads to the phosphorylation of S6, which advances mRNA translation and further promotes the synthesis of rRNAs, tRNAs, and transcription factors. On the other hand, mTORC1 inactivates 4E-BP1 which plays a role in the inhibition of translation. Hence, when 4E-BP1 is phosphorylated by mTORC1, translation initiation is promoted^[66]. Unlike mTORC1, less is known about the activation and regulation of mTORC2. However, evidence shows that mTORC2 is activated by PIP3 and growth signals that can promote cell proliferation and survival [Figure 3]. Similar to the role that Raptor has in mTORC1 regulation, Rictor associates with mTORC2 and is important in stabilizing and monitoring mTORC2 activity. Additionally, mTORC2 is shown to regulate small GTPases and members of the AGC kinase family, which includes AKT, PKC, and SGK1 (protein kinase C)^[64,67]. As such, the dysregulation of both mTORC1 and mTORC2 have been shown to exert oncogenic effects.

All known major effectors of the PI3K/AKT/mTOR pathway are frequently mutated in cancer. As previously mentioned, PI3K depends upon RTKs for its activation. Because the majority of PI3K is inactive in the cytoplasm, even miniscule changes in RTK activity can lead to overactivation of PI3K. For instance, mutations that lead to increased activation of EGFR (ERBB1) and HER2 are associated with cancers with aberrant PI3K pathway signaling^[68]. In addition, activating mutations in the platelet derived growth factor (PDGFR α) RTKs are found to be present in several cancers, including gastrointestinal stromal tumors and mammary carcinomas^[69,70]. Further, activating missense mutations in *PIK3CA*, the gene that encodes for the p110 α catalytic subunit of PI3K, are found in a wide variety of cancer types. Tumor suppressor phosphatase and tensin homolog (PTEN) regulates and represses PI3K activity by the dephosphorylation of secondary messenger PIP3. PTEN, however, is found to be mutated in several tumors which results in the constitutive activation of PI3K. Activating mutations in *PI3KCA* and inactivating mutations in PTEN are often found to coexist in many cancers, such as breast cancer and colon cancer. Amplification of AKT is also seen in cancer as a result of mutations in the *AKT1* gene^[71,72]. Lastly, there are various cancer-related mutations in the *MTOR* gene. These mutations affect the activity and assembly of both mTORC1 and mTORC2^[73].

PI3K inhibitors

There are three main classes of PI3K inhibitors - pan-PI3K inhibitors, isoform specific PI3K inhibitors, and dual PI3K/mTOR inhibitors. Several pan-class I PI3K inhibitors are currently in clinical development^[74]. Pan-class I PI3K inhibitors exert inhibitory effects against each isoform of PI3KCA, the gene that encodes for p110 α , the catalytic domain of PI3K. Buparlisib, for instance, binds to the ATP binding site of the lipid domain of PI3K and exhibits potent activity in cancers with *PI3KCA* activating mutations that are found in a wide range of human cancers. In addition, copanlisib, another pan-PI3K inhibitor, also shows significant anti-tumor activity in cancers with *PI3KCA* mutations and in cancers with HER2 overexpression. Pan-class I PI3K inhibitors have shown anticancer effects when administered as single agents. Nonetheless, there are multiple side effects that limit the efficacy of class-I inhibitors, such as hyperglycemia and fatigue^[75-77]. Further, isoform specific PI3K inhibitors have advantages over pan-PI3K inhibitors because they only bind to one isoform of PI3K. There are four class-I PI3K isoforms (PI3K α , PI3K β , PI3K γ , and PI3K δ), and each isoform plays non-redundant roles in particular tumor types. Hence, by targeting one isoform, undesirable side effects can be limited^[78].

As previously mentioned, mutations in the *PI3KCA* gene are recurrent in solid tumors. There are several selective and highly specific inhibitors that target the PI3K α isoform, which is composed of the p110 α catalytic subunit encoded by *PI3KCA*. Preclinical studies have shown that HER2- and KRAS-driven tumors rely on PI3K α for tumor survival. Hence, the inhibition of this isoform by selective PI3K α inhibitors acts as a promising therapeutic target. These inhibitors have been shown to have potency against wild-type PI3K α and E545K and H1047R gain-of-function mutants. Such inhibitors, such as NVP-BYL719, are shown to block the phosphorylation of AKT, which is highly dependent on p110 α activity. In addition, isoform-specific PI3K α inhibitors are shown to decrease glucose consumption and blood supply to cancer cells, contributing to their anti-tumor effects. As such, further studies are being carried out to determine whether using isoform-specific PI3K α inhibitors in combination with other agents enhances tumor regression^[79,80].

In addition to PI3K α , targeting the PI3K δ isoform may be beneficial for certain cancers. PI3K δ is largely seen in leukocytes and serves as an important target for cancer and immune-related diseases. As it is downstream to several B-cell receptors, PI3K δ plays an integral role in B-cell signaling and is seen in B-cell related malignancies. Mutations in the *PI3KD* gene have not been found in patients with chronic lymphocytic leukemia, but previous studies report that increased PI3K activity is highly dependent on PI3K δ isoform as observed in these cancers. PI3K δ selective inhibitor CAL101 blocks constitutive PI3K signaling that confers cancer cell survival, such as the phosphorylation of AKT and ERK1/2. Notably, CAL101 has also been demonstrated to reduce the levels of circulating chemokines CCL3, CCL4, and

CXCL13, which are associated with tumor metastasis and migration^[79,81]. Moreover, PI3K β -selective inhibitors are promising for the treatment of a group of solid tumors. PI3K β inhibitors were shown to inhibit tumor cell growth in PTEN-null advanced solid tumors. Nevertheless, prolonged treatment of PTEN-deficient cancers with PI3K β inhibitors may cause tumor cells to shift their dependency from the PI3K β to the PI3K α isoform^[82].

mTOR inhibitors

mTOR was originally identified to be a target for the anti-fungal agent rapamycin^[83]. The dysregulation of mTOR is shown to promote cancer growth and metastasis and mTOR thus serves as an important therapeutic target. mTOR regulates basic physiological processes and is frequently mutated in cancers. mTOR is the central component of both the mTORC1 and mTORC2 complexes. Both complexes differ in their components, substrate specificities, and subcellular localization. Aberrant mTOR signaling can be activated in various ways in cancer. First, mutations in the mTOR gene or in the genes that encode components of the mTORC1/2 complexes can lead to constitutive mTOR signaling. In addition, gain-of-function mutations in upstream oncogenes or loss-of-function mutations in upstream tumor suppressors contribute to unregulated mTOR signaling^[84]. Rapamycin, the compound from which mTOR gets its name, was the first agent that was discovered to bind mTOR. It was shown to bind and inhibit mTORC1, but due to its low solubility and poor pharmacokinetics, it is unsuitable for treating human cancers. As a result, several rapamycin analogs, “rapalogs”, have been discovered. The rapalogs everolimus and temsirolimus are prescribed to treat advanced renal cell carcinoma and breast cancer. It is evident that everolimus and temsirolimus exert tumor-suppressive effects *in vivo*, but the benefits of such agents in the clinic is only moderate. The limited clinical success of rapalogs is a result of the incomplete inhibition of mTOR and the development of acquired resistance in tumors. Nonetheless, clinical trials to determine the efficacy and breadth of rapalogs in different cancers is ongoing^[85].

In order to more completely suppress mTOR, ATP-competitive mTOR inhibitors have been developed to target both mTORC1 and mTORC2. Many of these ATP-competitive inhibitors target both mTOR and PI3K. In various cases, the dual inhibition of PI3K and mTOR is seen to be more effective than targeting either protein alone. For instance, in a recent study the effectiveness of the ATP-competitive PI3K/mTOR dual inhibitor BEZ235 in the treatment of drug-resistant gastric cancers was assessed. BEZ235 inhibits multiple class I PI3K isoforms and mTORC1/2 activity. Unlike mTOR inhibitors alone, BEZ235 was shown to decrease AKT activity *in vitro*, contributing to its potent anticancer effects. However, it is important to note that although BEZ235 has significant anti-tumor activity, it is shown to enhance ERK activity through mTORC2. The use of BEZ235 in combination with MEK inhibitors is seen to prevent ERK overactivation and to significantly inhibit cell growth^[86,87]. Similarly, another study reported the use of a newly developed PI3K/mTOR dual inhibitor, CMG002, in chemoresistant ovarian cancer. CMG002 was shown to suppress cancer cell proliferation and to induce apoptosis and G1 arrest in drug-resistant ovarian cancer cells^[88]. As such, both BEZ235 and CMG002 as single agents serve as promising strategies in cancer treatment. Further work is being done to study the effects of dual PI3K/mTOR inhibitors in combination with inhibitors that target the Ras/Raf/MEK/ERK pathway.

THE PAK FAMILY

The Pak family of protein kinases are important signaling proteins implicated in many cellular functions including cell proliferation, migration, and cytoskeletal organization^[89]. They can mediate a response to RTKs and other activators. They can lead to activation of MAP kinases and other signaling pathways. They were first identified as protein kinases that bind and are activated by the Rho GTPases Rac and Cdc42 (Fig. 4). The Pak family consists of 6 members. These include group A (Paks 1, 2, and 3) and group B (Paks 4, 5, and 6)^[89]. The Paks have several different domains, including a GTPase binding domain (GBD), a short sequence which can bind to the Rho GTPases Cdc42 and Rac, and a serine/threonine kinase domain.

The group A and group B Paks have approximately 50% amino acid identity in the GBD and kinase domains, but differ throughout their other domains^[89]. Among the Pak family members, the Pak1 and Pak4 genes are located on chromosomal regions that are frequently amplified in cancer^[90]. Increased Pak4 levels are associated with oncogenic transformation^[91,92] and occur in a number of cancers^[93-96]. The wild-type Pak4 gene was shown to be amplified in a panel of pancreatic cancer samples, in pancreatic ductal adenocarcinomas^[95,97,98], in squamous cell carcinomas^[99], and in high grade serous and endometrioid ovarian tumors^[100]. In ovarian cancer cell lines, siRNA studies have shown that Pak4 is one of the genes whose amplification is critical for cellular viability^[100]. High levels of Pak4 are found across all subtypes of breast cancer including ER+, Her2+, and triple negative^[92,101-105]. Thus, Pak4 could serve as a more specific target for breast cancer therapy.

Pak4 has several cellular functions that may explain why it is so tightly linked with cancer. For example, Pak4 leads to cytoskeletal changes. This may be directly linked with the control of cell shape, which is often disrupted in cancer, and with cell migration^[106], a critical function for cancer cells, particularly during metastasis [Figure 4]. Pak4 has also been shown to have key roles in promoting cell survival^[107-109]. It can promote cell survival and inhibit apoptosis in response to several different stimuli in both fibroblasts and epithelial cells, including serum withdrawal, or cytokine stimulation, and it can do so by a combination of different mechanisms, which involve both kinase dependent and kinase independent functions^[107-109]. Pak4 can also promote cell proliferation, another important aspect of tumorigenesis, and in some cells, this is associated with a prolonged activation of the ERK MAP kinase pathway^[101].

Due to the strong link of Pak4 to cancer, several anti-Pak4 compounds have been developed^[110-114]. One of these compounds is KPT-9274. Unlike other Pak4 inhibitors that block PAK4 catalytic activity, KPT-9274 reduces Pak4 protein levels. This may be important because Pak4 has been shown to have kinase independent functions^[96,108]. For example, a kinase dead Pak4 mutant can inhibit caspase 8 activity and consequently increase cell survival, as well as a wild-type Pak4. Pak4 can also regulate adhesion turnover and thereby promote migration, by a mechanism that is independent of its kinase activity^[92,113]. KPT-9274 could thus be more effective in antagonizing Pak4 functions in cancer compared with inhibitors that specifically block kinase activity. KPT-9274 has shown promise for the treatment of breast cancer, renal cancer, pancreatic cancer, and others both *in vitro* and *in vivo* and is now in a phase I clinical trial for patients with advanced solid malignancies and non-Hodgkin's lymphoma (NCT02702492)^[114-119]. Although KPT-9274 was identified as a Pak4 allosteric inhibitor, it also inhibits the metabolic enzyme nicotinamide phosphoribosyltransferase (NAMPT) by an undefined mechanism^[115]. NAMPT is the rate limiting enzyme for the production of nicotinamide adenine dinucleotide (NAD) via the salvage pathway^[120]. NAD is an important cofactor for redox reactions in metabolic pathways and its levels are tightly controlled by mechanisms that remain poorly understood. Whether Pak4 is linked to NAMPT, and how dual inhibition of Pak4 and NAMPT by KPT-9274 leads to growth inhibition of cancer cells warrant further investigation.

Recently, KPT-9274 was not only shown to lead to inhibition of Pak4 and NAMPT in triple negative breast cancer cells, but also inhibition of Rictor^[121]. Rictor is an important component of mTORC2. Conversely, mouse mammary epithelial cells overexpressing Pak4 had an increase in Rictor activity^[121,122]. AKT phosphorylation at Ser473 was dramatically decreased in response to KPT-9274 treatment, also a hallmark of mTORC2 activation. These findings reveal that prolonged inhibition of Pak4 by KPT-9274 inhibits mTORC2 activity, and mTORC1 activity was subsequently also found to be decreased. These results suggest that the anti-proliferative effects of KPT-9274 could be mediated by inhibition of mTOR signaling. They also raise the possibility that the PAK4 pathway is linked to the mTOR pathway, possibly via phosphorylation of a component of mTORC2. These results suggest that the simultaneous inhibition of both Pak4 and mTOR may be an effective way to block the growth of breast cancer cells, and this may have implications in other types of cancer as well.

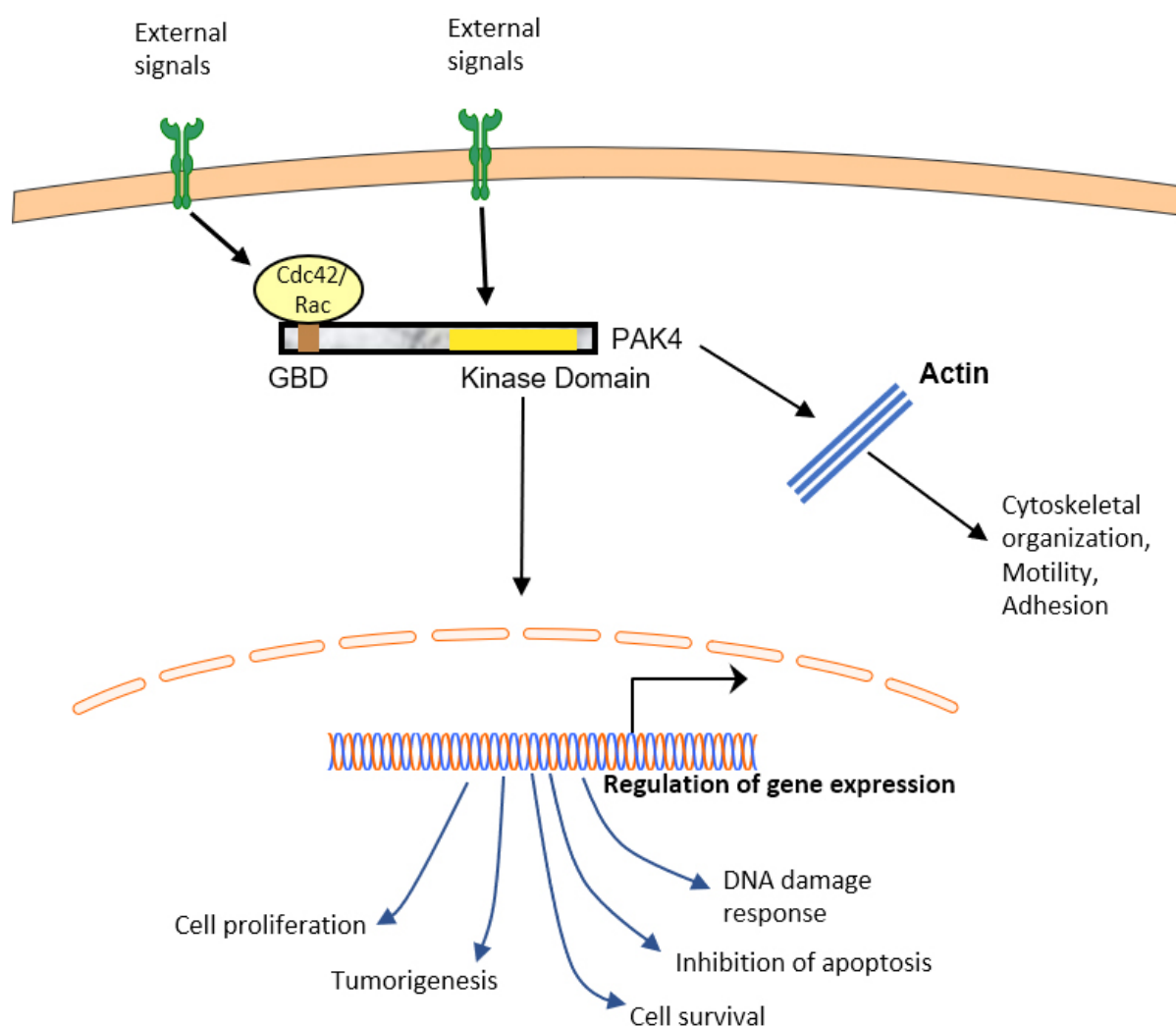


Figure 4. The role of PAK4 in cell signaling: external signals initiate the activation of small GTPases Cdc42 and Rac, which in turn bind to and activate PAK4. PAK4 has roles in cytoskeletal organization, motility, adhesion, and the regulation of gene expression. mRNA and protein levels of PAK4 are often seen to be overexpressed in several types of cancer, such as breast cancer and pancreatic cancer. GBD: GTPase binding domain

CONCLUSION

This review has highlighted some of the RTK regulated pathways that are important for cancer and for drug development. Several important RTK regulated pathways have been discussed. It is important to remember, however, that there are many more RTKs than we have discussed here. In fact there are at least 58 unique RTKs, which fall into at least 20 families^[123]. Several different drugs have been developed against proteins that are improperly regulated in RTK pathways. Even more are currently in development or under clinical trials. Some of these have had promising outcomes. It is important to remember, however, that serious challenges remain ahead. One issue is the development of resistance to drugs that were initially successful^[2]. To address this, current and future studies need to address the reason for this resistance, and the development of new drugs that will be effective for resistant cancers^[124]. Another challenge is the development of combination treatments. Single agent treatments are often not sufficient for effective treatments, but drug combinations can be more effective. One promising area of study is the combination of protein kinases with immune complex inhibitors, in order to combine immunomodulation with inhibition of RTK signaling pathways. Finally, identifying which treatments will be most effective in each

individual patient will contribute to a long-sought goal of developing individualized cancer treatments for each patient.

DECLARATIONS

Acknowledgments

The authors are grateful to Busch Biomedical Grant and the New Jersey Health Foundation for providing support for the lab.

Authors' contributions

Did the majority of the writing for this manuscript: Cordover E

Helped with the planning of the article, some of the writing, and editing: Minden A

Availability of data and materials

Not applicable.

Financial support and sponsorship

Financial support for the lab and for Emma Cordover's work was from the Busch Biomedical Grant, the New Jersey Health Foundation Grant, and the Aresty Research Center.

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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Phytochemicals and cancer chemoprevention

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How to cite this article: Dave A, Parande F, Park EJ, Pezzuto JM. Phytochemicals and Cancer Chemoprevention. *J Cancer Metastasis Treat* 2020;6:46. <http://dx.doi.org/10.20517/2394-4722.2020.106>

Received: 24 Sep 2020 **First Decision:** 12 Oct 2020 **Revised:** 05 Nov 2020 **Accepted:** 18 Nov 2020 **Published:** 30 Nov 2020

Academic Editor: Sanjay Gupta **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

The unending morbidity and mortality that results from cancer, as well as adverse reactions due to chemotherapy and the enormous economic burden of treatment and hospitalization, advocates for the necessity of chemopreventive measures. Cancer chemoprevention refers to the use of agents capable of reversing, reducing, or slowing down the pathology of cancer at various stages. Fortunately, a few therapeutic drugs with relatively low toxicity (e.g., tamoxifen, finasteride), and a sparse number of vaccines (hepatitis B, HPV), are used to prevent specific cancers. In the general population, however, therapeutic options for cancer prevention are not common. Nonetheless, it is generally agreed that diet affects the genesis of cancer, and phytochemicals have the capacity of functioning as cancer chemoprevention agents. This is supported by epidemiological studies and clearly documented with animal models designed to mimic human carcinogenesis. Additionally, some public health strategies, such as recommendations for greater consumption of fruits and vegetables, reflect the merits of cancer chemoprevention. Here, we focus on some well-established natural product cancer chemopreventive agents, including resveratrol (grapes), epigallocatechin-3-gallate (green tea), sulforaphane (cruciferous vegetables), anthocyanins (grapes and berries), curcumin (turmeric), silibinin (milk thistle), and lycopene (tomatoes). As aptly demonstrated by genomic analysis and other methods, the mechanistic underpinning is variable and complex. In addition, responses may be mediated through indirect mechanisms, such as interaction with the microbiome. Furthermore, ancillary applications of chemopreventive agents are worthy of consideration, such as management of sequelae induced by chemotherapy. Recognizing the loss of millions of cancer patients every year, it is obvious that negating malignant metastatic conditions remains of paramount importance. In meeting this objective, cancer chemoprevention offers great promise.



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Keywords: Chemoprevention, phytochemicals, resveratrol, curcumin, anthocyanins, silibinin, lycopene, epigallocatechin-3-gallate, anti-cancer activity

INTRODUCTION

Overall, cancer is the second leading cause of death in the US and a major public health issue throughout the world. Approximately 1,762,450 new cancer cases are estimated in the US in 2019^[1], and 18.1 million new cases worldwide. In 2018, 9.6 million deaths were attributed to this disease^[2]. Given these astounding demographics, and the associated pain, suffering and economic burden, the scientific and medical community continuously strive for better treatment options, improved palliative care, and effective preventative strategies. In this context, the role of diet in cancer has attracted considerable attention. This is especially compelling given that epidemiological studies have demonstrated regular consumption of phytochemicals from dietary sources like fruits, vegetables, herbs, and teas is associated with reduced risk of chronic diseases including cancer, cardiovascular disease and inflammatory disorders^[3,4].

Cancer chemoprevention can be classified into primary, secondary, and tertiary measures. Populations with no overt cancer risk factors, or those ostensibly at high risk due to factors such as successful surgical resection or family history, can be grouped under primary measures. Patients with pre-malignant lesions bearing risk of progressing to an invasive cancer (e.g., ductal carcinoma *in situ*) can be grouped under secondary measures. In these cases, standard protocols of chemopreventive practice would be highly desirable, but scarcely come into play^[5]. People in primary and secondary chemoprevention categories may be advised or decide on their own to increase dietary phytochemical consumption or to use over the counter products such as non-steroidal anti-inflammatory drugs. Finally, tertiary measures can be considered for patients with cancer relapses^[6]. A unique example of a tertiary chemopreventive measure is the administration of tamoxifen (or structural relatives), or aromatase inhibitors, for patients diagnosed with breast cancer^[7].

Around two and half thousand years ago, Hippocrates advised “let food be thy medicine and medicine be thy food”. This remains a powerful statement, as graphically illustrated in Figure 1. An inverse relationship between adequate fruit and vegetable consumption and cancer incidence has been established. In fact, it has been suggested that cancer incidence could be reduced by over 50% if people consume at least five servings of fruits and vegetables per day^[9]. On the other hand, obesity is generally associated with poor health and chronic illness, and there are certain foods that can act as carcinogens and initiate tumor formation.

Secondary metabolites (phytochemicals) are typically generated in plants to afford protection against external threats such as UV, fungal infection, and the generation of free radicals. The compounds so produced show a remarkable array of structural diversity. Notably, ingestion of these phytochemicals provide human beings with protective effects as well^[10], perhaps by reducing oxidative stress (ROS) and inflammation^[3,11]. However, the mechanisms by which phytochemicals function in a chemopreventive capacity are certainly intricate and multifaceted, as described to some extent in this review.

Interestingly, cancer and aging share several hallmarks in terms of the genetic pathways and biochemical processes. For example, DNA repair mechanisms are affected by ROS and this may result in the deregulation of signaling pathways such as p53 and nuclear factor- κ B (NF- κ B). In turn, such deregulation may accelerate aging and cancer development^[12]. In principal, antioxidants sourced from phytochemicals may neutralize ROS and attenuate oxidative stress^[13]. Further, as deregulation of signaling pathways is involved in progression of inflammatory diseases, modulation of these processes by phytochemicals may down-regulate proinflammatory factors^[14]. Considering the general safety of dietary phytochemicals,

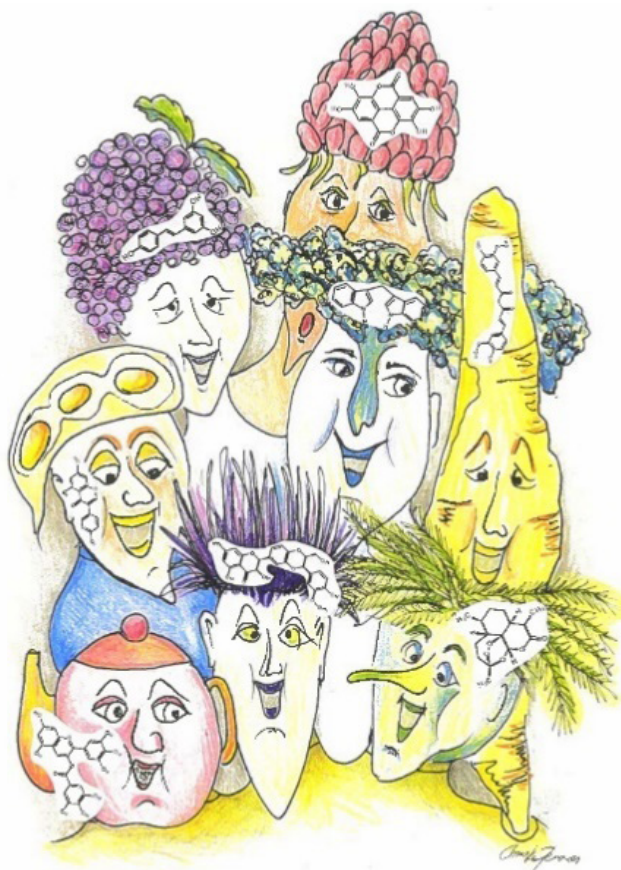


Figure 1. Phytochemicals and their chemical structures: anthocyanins in grapes, ellagic acid in raspberry, genistein in soy, 3,3'-diindolylmethane in broccoli, curcumin in turmeric, EGCG in tea, silibinin in milk thistle, and artemisinin in sweet wormwood. Reprinted/adapted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Natural Products for Cancer Chemoprevention. Single Compounds and Combinations, by Pezzuto and Vang^[8] ©2020

especially compared with narrow therapeutic index chemotherapeutic agents, the potential merit of chemoprevention is obvious.

This review encompasses studies involving the dietary role of phytochemicals including curcumin (turmeric), epigallocatechin gallate (green tea), resveratrol (grapes), anthocyanidin (grapes and berries), sulforaphane (cruciferous vegetables), silibinin (herb milk thistle), and lycopene (tomatoes). Mechanisms of alleviating multiple pathological conditions, such as oxidative stress, epigenetic alteration, angiogenesis, chronic inflammation, and effects on stem cell transformation are taken into account. Finally, some thoughts are provided in regard to future directions.

RESVERATROL

Chemical properties of resveratrol

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene, MW: 228.25 g/mol) is a naturally occurring stilbene with two phenolic rings connected by an ethylene group^[15]. It is a phytoalexin mainly synthesized as a protective mechanism in plants in response to environmental stress including fungal infection, UV radiation, and chemical exposure^[16]. The dominant dietary source is grapes and grape products^[17], but more recently, approximately 70 species of plants have been reported to produce resveratrol^[18]. As such, in an average diet, relatively small amounts of resveratrol can be found in peanuts (*Arachis hypogaea*), blackberries (*Morus* spp.) and blueberries (*Vaccinium* spp.)^[19,20]. Red wine is a major source of resveratrol in the Mediterranean diet,

as grape (*Vitis vinifera*) is a rich source of resveratrol. Specifically, resveratrol is found in seeds, skin, woody parts, and petioles. Therefore, red wine generally has a higher content of resveratrol than white wine, since, during the production of the wine, parts of grapes in which resveratrol is present are macerated for a longer period of time^[21]. During grape fermentation, the formation of alcohol facilitates the solubility of resveratrol which further leads to its extraction.

Polygonum cuspidatum is an extremely rich source of resveratrol and used as a therapeutic regimen for cardiovascular diseases in Chinese and Japanese traditional medicine practice^[22]. Similarly, the rhizome of *Veratrum formosanum*, containing abundant resveratrol, has been applied to treat hypertension in East Asia^[23].

Pharmacokinetic properties of resveratrol

Resveratrol is primarily metabolized by phase II enzymes in the liver. Through enterohepatic transport in bile, some of the compound returns to the small intestine^[24]. Moreover, resveratrol can stimulate its own metabolism by increasing the action of phase II hepatic detoxifying enzymes^[25]. Although *trans*-piceid, the naturally occurring glucoside, exhibits biological activities, glucuronide metabolites of resveratrol in humans seem to be less active.

The high rate of resveratrol metabolism produces conjugated sulfates and glucuronides which maintain some biological activity^[26]. Although metabolites can differ in their nature and quantity between subjects due to inter-individual variability, there are major five types of metabolites in the human urine: two isomeric forms of resveratrol monoglucuronide, resveratrol monosulfate, monoglucuronide dihydroresveratrol and monosulfate dihydroresveratrol^[27,28]. In human urine samples, *cis* metabolites have been found, mainly as *cis*-resveratrol-4'-sulfate, *cis*-resveratrol-4'-O-glucuronide and *cis*-resveratrol-3-O-glucuronide^[29,30]. Even though the *cis*-isomer displayed comparable activities to the *trans*-isomer in some experimental settings^[31,32], the *trans*-isomer is generally considered dominant and exerts greater activity. Other dietary components may influence metabolism. For example, quercetin has the potential to inhibit resveratrol glucuronidation and sulfation in the duodenal and liver, thus increasing bioavailability^[33].

Biological activities of resveratrol

Spearheaded by conceptualization of the “French Paradox”, the potential health benefits of phenolic compounds present in wine and grapes have been extensively studied. As a brief background, in the northern region of France, the dietary intake of saturated fat is relatively high. However, relative to other parts of the world where a similar amount of high saturated fat is consumed, the mortality due to coronary heart disease is reduced. This phenomenon, termed the “French Paradox”, was attributed to the relatively high wine consumption of the French^[34]. This possible benefit of wine was not ascribed to alcohol content, since alternative alcoholic beverages such as beer were not perceived to be effective in this regard. In turn, this led to speculation regarding the effectiveness of chemical constituents in wine other than alcohol. It is known that grapes, and consequently wine, contain scores of phytochemicals^[35], but a conundrum exists since these components are also found in other sectors of the diet. Alas, when the cancer chemopreventive potential of resveratrol was first described^[36], it was recognized this is a compound uniquely associated with the grape (and wine), and, in fact, grapes and wine are the dominant dietary source of this biologically active compound. Thus, resveratrol was perceived by some as a key to the “French Paradox” and many studies followed to explore broader biologic potential^[37].

Because of its physical and chemical properties, resveratrol can either interact with receptors present on the cell surface or move passively through cell membranes. Therefore, at the cellular level, action may be initiated by either triggering signaling pathways when binding to the cell membrane receptors, or by facilitating intracellular mechanisms^[38]. Accordingly, as discussed in previous reviews^[39,40], resveratrol

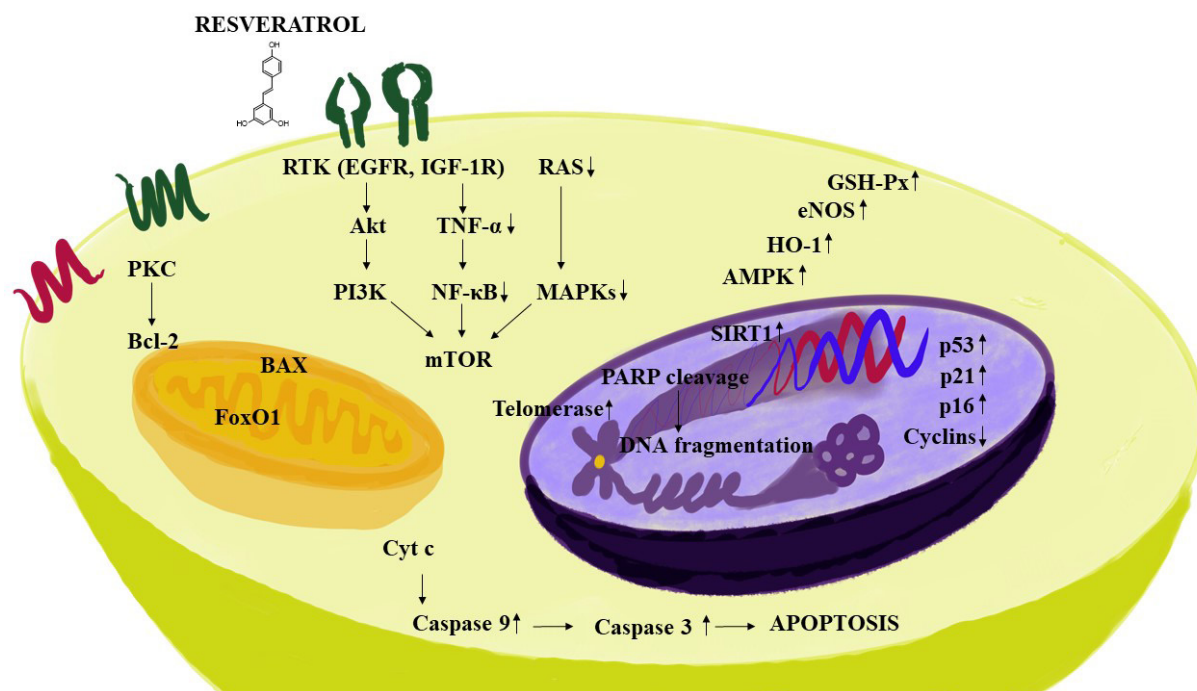


Figure 2. Some mechanisms of action mediated by resveratrol. Resveratrol exhibits an anti-inflammatory response by down-regulating pro-inflammatory factors. The compound decreases survival related proteins including phosphatidylinositol-3-kinase (PI3K) and Akt. Further, signaling cascades are impeded by down-regulating NF-κB, MAPKs and TNF-α, resulting in the inhibition of mTOR. In addition, resveratrol decreases the expression of anti-apoptotic marker BCL-2, and apoptotic pathways are controlled by up-regulating pro-apoptotic proteins which are responsible for the cell death, such as, BAX and caspase 3, with an increase in DNA fragmentation. The pleiotropic mechanisms of resveratrol are also accentuated by induction in the signaling pathways such as AMPK, SIRT1, HO-1, p53, p21, p16, eNOS and GSH-Px. NF-κB: nuclear factor-κB; MAPK: mitogen-activated protein kinase; TNF-α: tumor necrosis factor-α

is capable of mediating a myriad of responses. For example, resveratrol is known to down-regulate pro-inflammatory factors [Figure 2], thereby exhibiting an anti-inflammatory response^[41]. During the initial stage of an anti-inflammatory response, polymorphonuclear leukocytes play a key role in the process. Resveratrol abates the inflammatory responses initiated by calcium ionophore A23187, fMLP, or component fragment C5a^[42]. Inducible nitric oxide synthase (iNOS) activates macrophages, and resveratrol has been shown to decrease the production of iNOS^[43,44]. Resveratrol also impedes pro-inflammatory signaling which leads to inhibition of adenosine nucleotide secretion by activated platelets and reduces neutrophil functions through inhibition of P2 and PAP receptor signaling via mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK)^[45]. Resveratrol induces extracellular signaling regulated kinases (ERK), p38 MAPK and JNK in the mouse epidermal cells resulting in the phosphorylation of serine 15 of p53, a tumor suppressor gene^[46]. Further, resveratrol inhibits macrophage expression of EMMPRIN by inducing PPAR-γ^[47] and inhibits NF-κB^[48]. In addition, many other studies have confirmed that resveratrol inhibits activation by tumor necrosis factor-α (TNF-α)^[49-51].

Jang *et al.*^[36] demonstrated an anti-inflammatory response in a rat model of inflammation by treatment with resveratrol, and further demonstrated reduction of prostaglandin synthesis by inhibition of cyclooxygenase-1 (COX-1). Later, it was found that resveratrol could selectively reduce COX activity by suppressing the COX-1 pathway; however, not through the COX-2 pathway. Szewczuk *et al.*^[52] verified this observation. However, in yet another study, it was found that resveratrol inhibits the synthesis of prostaglandin E₂ by suppression of COX-2, but not by altering COX-1^[53]. One more study corroborates this result where resveratrol reduced colonic injury, neutrophil infiltration, and prostaglandin D₂ concentration by inhibiting COX-2 without affecting COX-1^[54]. It was also shown that resveratrol attenuates COX-2 expression^[53].

Chemopreventive mechanism of resveratrol

Many studies have been published describing the potential chemopreventive effect of resveratrol. In brief, resveratrol induces apoptosis by interacting with the $\alpha V\beta 3$ integrin receptor in the breast cancer cell line MCF-7^[55]. The compound inhibits aryl hydrocarbon receptor activity thereby suppressing tumor growth and exhibiting anti-cancer properties^[56]. Further, with MCF-7 cells, resveratrol inhibits NF- κ B and BCL-2^[57]. It antagonizes the aryl hydrocarbon receptor, which can relate to carcinogenic and immunosuppressive effects in cells^[58]. Some studies suggest that resveratrol shows antitumor effects at the level of initiation, promotion, and progression with prostate cancer cells^[59]. As noted above, resveratrol can inhibit COX-1 and COX-2, enzymes that are involved in tumor progression. With HL-60 cells, phenotypic markers indicative of reduced proliferation are induced. Also, in the initiation phase of carcinogenesis, resveratrol inhibits free radical generation^[36]. In human lymphoblast cell lines, resveratrol induces apoptosis through p53 activation^[60]. It also inhibits COX-2 activity^[61] and ribonuclease reductase^[62]. With osteosarcoma stem cells, resveratrol inhibits self-renewal, cell viability and tumorigenesis. Mechanically, resveratrol inhibited JAK2/STAT signaling and suppressed cytokine synthesis, which was consistent with the decline of CD133 cancer stem cell (CSC) markers^[63]. Thus, when evaluated with *in vitro* models, resveratrol mediates a variety of effects consistent with antitumor activity.

EPIGALLOCATECHIN-3-GALLATE

Chemical and pharmacological properties

Tea as a beverage is popularly consumed around the world, second only to water^[64]. Consumption of green tea has been advocated for several health benefits, such as ameliorating cardiovascular risk and prevention of cancer. It has exhibited various biological properties proven favorable for hepatic function and improved metabolic profiles^[65,66]. Pharmacologically, the polyphenols present in green tea demonstrate properties that can lead to anti-oxidative, anti-inflammatory, anti-atherosclerosis, anti-hypercholesterolemic and anti-carcinogenic activities^[67,68].

Active green tea polyphenols include catechins (members of the flavonoid family), like epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), and epigallocatechin-3-gallate (EGCG). ECG and EGCG are found in the high concentrations (50 to 80% of the catechins)^[64,69]. EGCG is the ester of epigallocatechin and gallic acid and known to be the most potent catechin in exhibiting anti-cancer and antioxidant effects. The hydroxyl group on the B-ring plays an important role in antioxidant reactions and this is increased by the trihydroxyl group on the D-ring^[70].

Inflammation is one dominant factor in the initiation of cancer. Increased oxidative stress promotes cell growth. Many *in vitro* studies and studies with animal models have conclusively demonstrated the potential of green tea to reduce tumorigenesis, although clinical trials are still not definitive. EGCG is hydrolyzed to EGC in the intestine by bacteria. Later, EGC and gallic acid undergo several conversions leading to metabolites such as 5-(3,5-dihydroxyphenyl)-4-hydroxyvaleric acid and 5-(3',5'-dihydroxyphenyl)- γ -valerolactone in glucuronide forms^[71]. In a clinical study, the plasma levels were 0.17 μ mol/L after having 2 cups of tea, while the *in vitro* concentrations used in many studies have been in the range of 10-100 μ mol/L^[66]. Epidemiological studies have suggested green tea has a positive effect on cancer prevention in certain types of cancer, namely breast, colon, and skin^[72-74]. Although there is lesser or no positive clinical effect seen with other types of gastrointestinal or oral cancers, there is bactericidal activity against *Escherichia coli*, *Streptococcus salivarius*, and *Streptococcus mutans*^[75-77].

Chemoprevention mechanism of EGCG

Since it has been established that cancer stem cells play a vital role in tumor progression, studies have been performed with lung CSCs such as A549 and H1299 illustrating the potential of EGCG to suppress tumor formation through the Wnt- β -catenin pathway^[78]. Other studies performed with chemotherapeutic

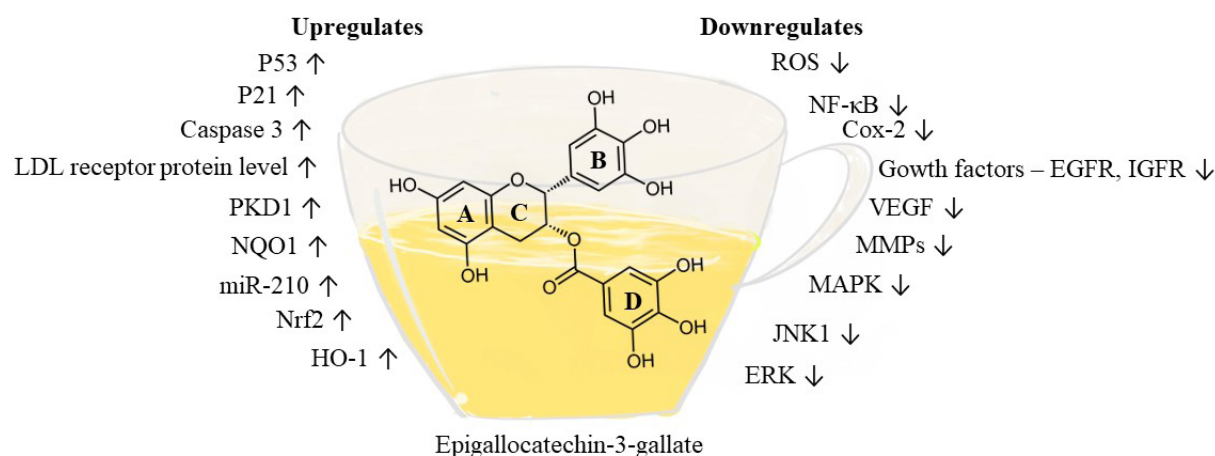


Figure 3. Pharmacological targets of epicatechin-3-gallate. EGCG affects various targets relevant to cancer prevention as shown in the figure. EGCG attenuates oxidative stress by down-regulating reactive oxygen species; inflammatory factors such as NF- κ B and COX-2 are down-regulated. In addition, EGCG down-regulates several kinases, such as MAPK, JNK1, and ERK, and targets several growth factors, such as IGF1R, HGFR, and VEGF, that abate tumor growth and progression. Apoptosis factors such as p53, p21 and caspase 3 are up-regulated by EGCG, and the expression of NAD(P)H:quinone oxidoreductase 1 (NQO1), HO-1, and Nrf2 are increased to reduce oxidative stress. EGCG: epigallocatechin-3-gallate; NF- κ B: nuclear factor- κ B; COX: cyclooxygenase; MAPK: mitogen-activated protein kinase; ERK: extracellular signaling regulated kinases; VEGF: vascular endothelial growth factor

agent-resistant cancer stem cells such as A549/CDDP (cisplatin-resistant cells) and 5-fluorouracil-resistant colorectal cancer cells have demonstrated the anti-cancer activity of EGCG^[79,80]. Mechanistic evaluations with various *in vitro* models have led to the recognition of MAPKs as an important molecular target for EGCG [Figure 3]. These factors are associated with cell proliferation, differentiation, migration, senescence, and apoptosis^[81]. EGCG inhibits signal regulated protein kinase and p38 phosphorylation. EGCG is also associated with inducing apoptosis, inhibition of transcription factors like NF- κ B and activator protein (AP-1), and reduction of receptor tyrosine kinase activity^[82].

ERK signaling

ERK is one of the major signaling cascades of the MAPK signaling pathway that is targeted by EGCG. EGCG inhibits ERK activation in a concentration-dependent manner, suggesting its effectiveness as an anti-cancer agent with several *in vitro* models. With MCF10A and MDA-MB-231 breast cancer cells, 5 μ mol/L EGCG inhibited hepatocyte growth factor-induced activation of ERK and AKT^[83]. In cervical tumor cell lines, like HeLa, Caski and SiHa, EGCG inhibited phosphorylated ERK1/2 by 83% and Akt by 50% at a concentration of 50 μ mol/L^[84].

Activation of nuclear factor- κ B and activator protein signaling pathways

Nuclear factor- κ B (NF- κ B) plays a vital role in the regulation of several genes central for cellular responses like inflammation, growth, and cell death. It is sequestered in cytoplasm in an inactive form and activated on phosphorylation. AP-1, a transcription factor, is known to be involved in tumor promotion and progression of cancer^[85]. EGCG exerts inhibitory effects on the binding of NF- κ B to DNA and thereby reduces inflammation and cell proliferation with *in vitro* models. However, concentrations required to mediate these effects were between 10-100 μ mol/L^[86,87]. EGCG evidently reduces binding of AP-1 along with NF- κ B to DNA; a process which promotes MMP-9 for tumor progression. This has been demonstrated with several *in vitro* studies using human breast cancer cells^[88], gastric AGS cells^[89], and bladder cancer cells^[90], in a dose-dependent manner, with concentrations ranging from 10 to 50 μ mol/L.

c-Jun N-terminal kinase 1/2

JNK is involved in cancer cell apoptosis, although recent studies have indicated that Janus signaling promotes cancer cell survival by acting synergistically with NF- κ B, JAK/STAT, and other signaling

molecules. The situation is complicated because JNK1 and JNK2 have opposite effects in relation to cancer cell survival. JNK1 promotes apoptosis while JNK 2 promotes cancer cell survival. The expression of p53 is negatively regulated by JNK1 and positively regulated by JNK2^[91]. EGCG attenuated reduced expression of JNK1 and oxidative damage and, at the same time, inhibited JNK2, thereby augmenting apoptotic signaling in cancer cells^[87,92-94].

p38/MAPK signaling pathway.

p38/MAPK is a third major signaling cascade in MAPK, playing a major role in controlling the process of apoptosis. Activation occurs by several environmental factors such as stress and inflammatory cytokines. p38 activates several downstream kinases that induce apoptosis^[95]. EGCG increases p38 levels and thereby inhibits growth in leukemic cells, HCC cells, and U373MG cells^[94,96,97].

SULFORAPHANE

Chemical and pharmacological properties

Sulforaphane (1-isothiocyanato-4-methylsulfinylbutane) is an aliphatic hydrocarbon that is the major by-product obtained during the hydrolysis of glucoraphanin^[98]. It was isolated in 1947 from radish. Later, glucoraphanin was found to be present in larger quantities in cruciferous vegetables such as cauliflower, broccoli, Brussel sprouts, and cabbage^[99]. It is generally found in broccoli and broccoli sprouts, yielding the highest concentrations found in any plant source^[100]. Broccoli and other cruciferous vegetables are widely consumed throughout the world for various health benefits.

Hydrolysis of glucoraphanin occurs due to disruption of the plant cell and the subsequent activity of the intrinsic enzyme myrosinase. Sulforaphane is not heat stable, but greater stability is retained when exposed to light and acidic pH levels, rendering the compound useful under gastric pH conditions^[99-101]. The presence of epithiospecifier protein (ESP) disrupts the process of glucoraphanin hydrolysis, reducing the bioavailability of sulforaphane and sulforaphane nitrile, with the nitrile form being less active in its binding to pharmacological targets^[102]. Since ESP is temperature insensitive, heating the broccoli at 60 °C decreases the formation of sulforaphane nitrile^[103]. Once absorbed, sulforaphane is conjugated with glutathione and metabolized by the mercaptopyruvate pathway; it is then excreted as *N*-acetylcysteine conjugates^[104]. Pharmacokinetic studies have demonstrated that the peak plasma levels of sulforaphane are relatively high after oral administration of broccoli, for 1.6 to 6 h. with 95% elimination after 12 h^[105]. In clinical studies, the plasma concentrations of sulforaphane following oral consumption of broccoli are in the range of 0.02-0.2 µmol/L^[106]. With animal models, the consumption of broccoli has been found to exhibit protection against cancer^[107].

Sulforaphane has also exhibited protective properties in the central nervous system by activating nuclear factor (erythroid derivative)-like 2 (Nrf2) and reducing oxidative stress and inflammation in nerve cells^[108]. Further, the compound shows insulin-sensitizing and hepatoprotective effects in rats fed a high fructose diet^[109]. Patients with type 2 diabetes treated with broccoli sprout powder (5 to 10 g for 4 weeks) showed reduced serum glucose levels and improved insulin levels^[110]. Patients with deregulated type 2 diabetes treated with 5 g of broccoli extract along with metformin (500 mg to 3000 mg) showed reduced HbA1c levels and there was a reduction in glucose production^[111]. In healthy male individuals, cholesterol and LDL cholesterol levels were reduced; in women, HDL cholesterol levels increased significantly after consumption of 100 g of fresh broccoli sprouts for 1 week^[112].

Sulforaphane reduced levels of iNOS with lipopolysaccharide (LPS) activated macrophages in a mouse model^[113]. Anti-inflammatory properties were further demonstrated in another study conducted with mice treated with sulforaphane; cytokine production was reduced in a concentration-dependent manner by activating the Nrf2 pathway. T-cell proliferation was also significantly inhibited^[114].

Sulforaphane demonstrates inhibition of phase I and phase II enzymes, induces cell-cycle arrest and inhibits angiogenesis. At 15 $\mu\text{mol/L}$, sulforaphane promotes apoptosis and cell-cycle arrest in prostate cancer cells (LNCaP and PC3) by decreasing histone deacetylase (HDAC) enzymes^[115].

Carcinogen-treated Wistar rats at 10 weeks of age were treated with 150 μmol of sulforaphane by oral gavage, mammary glands were extracted, and sulforaphane concentration after 12 h was 22 $\mu\text{mol/L}$. In addition, there was an increase of NQO1 and HO-1 levels observed in rat mammary gland. Subsequently, healthy women were placed on a cruciferous-free diet and administered 200 μmol of sulforaphane. The amount of sulforaphane distributed in breast tissue was found to be $0.92 \pm 0.72 \mu\text{mol/L}$ ^[116].

It is documented in various epidemiological studies that the consumption of isothiocyanates from cruciferous vegetables is inversely proportional to the incidence of lung cancer cases^[117]. This inverse correlation was even stronger in a study conducted in female patients who do not smoke cigarettes^[118]. There is also substantial evidence based on studies conducted with cisplatin-resistant cancer stem cells [such as human non-small cell lung cancer (NSCLC)] that up-regulation of miR-214 induced by sulforaphane may lead to anti-cancer activity^[119].

Chemopreventive mechanisms of sulforaphane

KEAP-nrf2 signaling

Genetic deletion of nrf2 can lead to detrimental effects on the survival of mice; they are more prone to brain injury and lung injury and other pathological conditions involving inflammation. To the contrary, the activation of KEAP1-nrf2 leads to protective effects in various animal models^[120,121]. Nrf2 is important for the regulation of antioxidant genes such as enzymes that produce glutathione (GSH) and NADPH^[122]. Sulforaphane, on entering cells, reacts with Kelch-like ECH associated protein, which functions as a sensor protein complex. Under basal conditions, KEAP1 binding to nrf2 leads to ubiquitination and proteasomal degradation. Sulforaphane protects nrf2 from degradation [Figure 4], allowing escape and the regulation of downstream target genes capable of mediating anti-inflammatory and antioxidant activities^[123]. NQO1 and GST levels are significantly elevated in sulforaphane-treated wild-type mice (nrf2^{+/+}) whereas nrf2^{-/-} deficient mice exhibited no changes in NQO1^[124].

Activity of cyclin dependent kinase and reduced cyclin D1

Cyclin D1 is a cell-cycle regulator and a transcriptional modulator for histone deacetylase 3. Overexpression of these factors has been linked to cancer progression. Therefore, reduction of cyclin D is considered as a potential strategy for chemoprevention^[125]. In this context, sulforaphane-treated A549 cells showed concentration-dependent reduction of cyclin D1 as well as increased expression of the p21^[126]. In DU-145 prostate cancer cells, sulforaphane reduced CDK4 activity and cyclin D1 levels when treated with 9 and 50 $\mu\text{mol/L}$, respectively. CDK4 activity was also affected by a concentration of less than 1 $\mu\text{mol/L}$, but not significantly^[127]. In an *in vivo* study, sulforaphane reduced tumor promotion and polyp formation in an ApcMin/+ mouse small intestine cancer model in a dose-dependent manner. However, biomarkers including cyclin D1 remained unaffected^[128].

Inhibition of HDAC activity in human prostate and colorectal cancer cells

Increased HDAC expression has been associated with the deregulation of cell-cycle and apoptotic processes. HDAC inhibitors have shown potential in clinical studies for chemoprevention. Sulforaphane has demonstrated potential to reduce HDAC activity in prostate and colorectal cancer cells^[129]. Sulforaphane (15 $\mu\text{mol/L}$) reduced HDAC activity by 30%, 40%, and 40% in LNCaP, BPH-1, and PC-3 cell lines, respectively. Expression of p21 associated with histone H4 was increased in all three cell lines leading to apoptosis^[130]. In 4-6 week-old NOD/SCID mice inoculated with A549 lung cancer cells, administration of 9 μmol sulforaphane by oral gavage for 4 weeks attenuated the increase of tumor volume, significantly

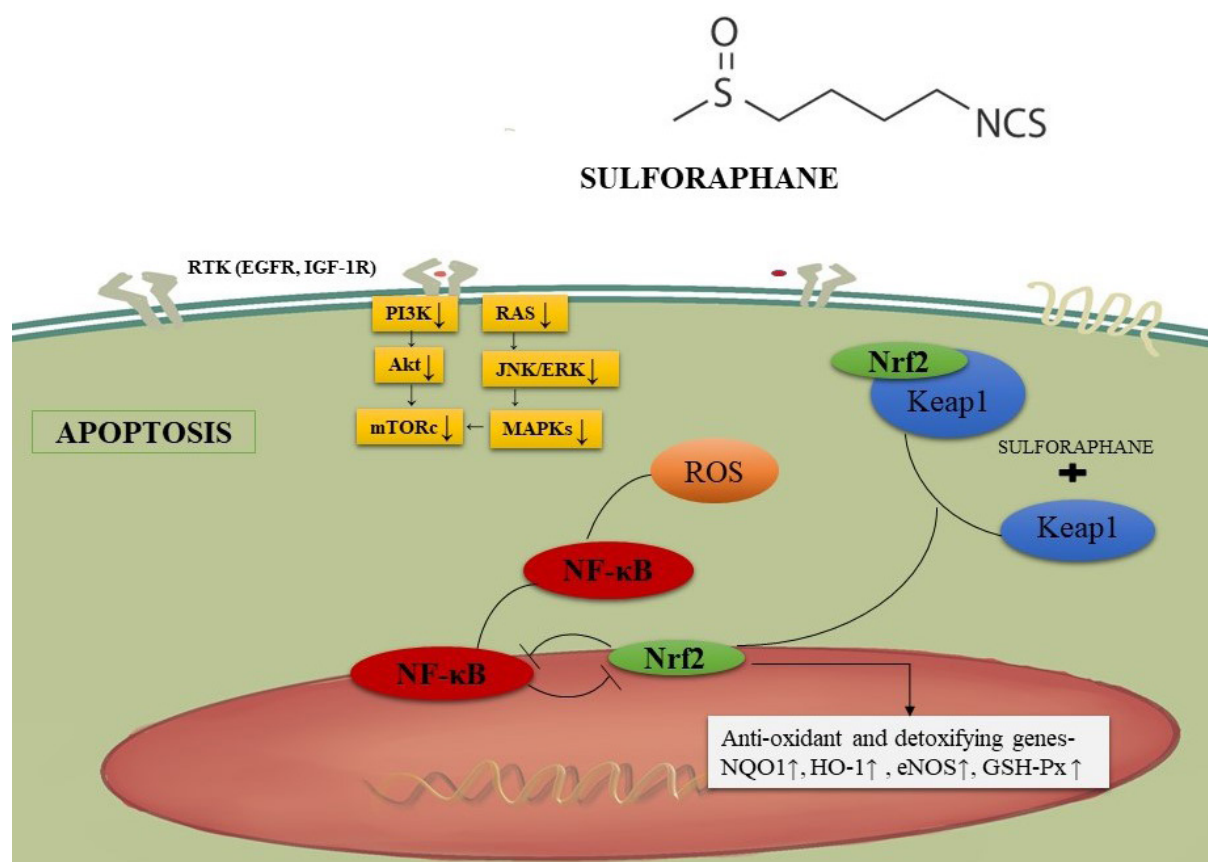


Figure 4. Sulforaphane and KEAP-nrf2 signaling. Sulforaphane enters cells and interacts with Kelch-like ECH associated protein. This prevents nrf2 degradation and leads to downstream anti-inflammatory and antioxidant effects. NF-κB: nuclear factor-κB

reduced HDAC activity, and increased acetylated histones 3 and 4^[131]. HDAC inhibitory effects of sulforaphane were also demonstrated with a mouse model bearing a colon cancer xenograft^[132]. The United States Food and Drug Administration approved the use of HDAC inhibitors for the treatment of cancer, so investigation of phytochemicals such as sulforaphane is reasonable^[133]. In a phase II clinical trial involving patients with recurrent prostate cancer, administration of 200 μmol/day of sulforaphane rich extracts for 20 weeks did not produce a reduction in prostate specific antigen (PSA) levels by at least 50%. However, PSA doubling time was increased^[134]. In a double-blinded study, there was a significant reduction in PSA levels in prostate cancer patients post-prostatectomy when given 60 mg sulforaphane orally with cancer therapy, followed by 2 months of sulforaphane with no other treatment^[135]. The promising results observed with sulforaphane in these clinical trials suggest the use of an HDAC inhibitor in combination with a chemopreventive agent for the treatment of prostate cancer.

ANTHOCYANINS

Chemical properties of anthocyanins

Anthocyanins are water-soluble secondary polyphenolic metabolites produced by plants^[136]. The substances are classified under the flavonoid group and provide blue, red, and purple pigmentation for plants. The basic chemical structure of an anthocyanin contains anthocyanidin without a sugar moiety. The anthocyanidins are comprised of an aromatic ring (A) which is bonded to a heterocyclic ring (C) that includes oxygen, and this is similarly attached to carbon-carbon bonds linking to a third aromatic ring (B)^[137]. There is a vast number of anthocyanins present in nature, the most common being petunidin, pelargonidin, delphinidin, peonidin, malvidin, and cyanidin. The only difference between these compounds is the nature and the

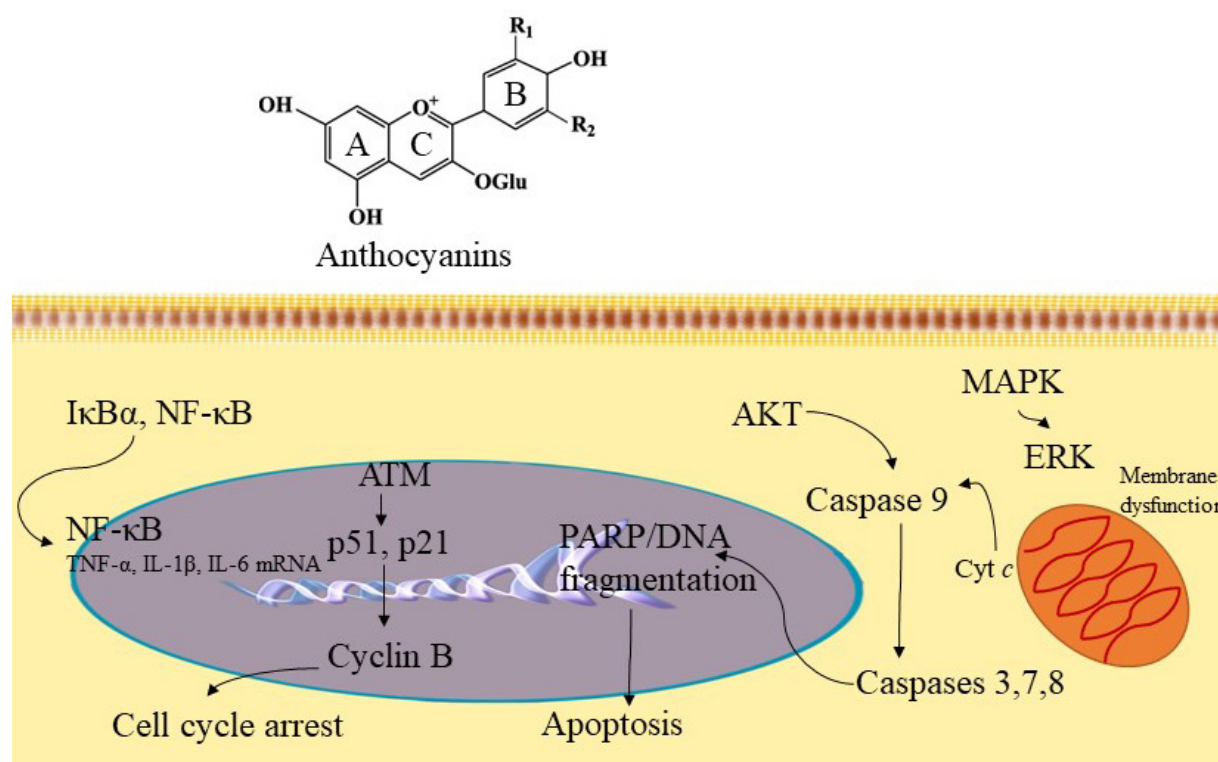


Figure 5. Anthocyanin mechanism of action. Based on structural-activity relationships (SAR), the *ortho*-dihydroxyphenyl functional group on the B-ring of anthocyanidins plays a key role in inhibiting the action of AP-1 and cell transformation. The compound inhibits MAPK/ERK and mitochondrial associated pathway caspases 3, 7, 8 to induce apoptosis. Further, anthocyanins have exhibited inhibition of cyclin-B by acting on p51 and p21 signaling pathways to induce cell cycle arrest. In addition, anthocyanins can act through the NF-κB signaling pathway to mediate anti-inflammatory functions. NF-κB: nuclear factor-κB; MAPK: mitogen-activated protein kinase; ERK: extracellular signaling regulated kinases

number of sugars attached to their structure, the number of hydroxylated groups in the molecule, the aromatic or aliphatic carboxylates attached to the sugar, and the position of these bonds^[138]. Studies have indicated the *ortho*-dihydroxyphenyl structure is the active moiety on the B-ring that suppresses tumor growth and metastasis^[139,140].

Chemopreventive mechanism of anthocyanins

MAPK pathway and AP-1

With mouse epidermal cells (JB6⁺), tumor promoters such as epidermal growth factor (EGF), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and TNF-α can stimulate AP-1 activity and neoplastic transformation by inducing MAPK comprising ERK, p38 kinase, or JNK^[141,142]. Cyanidin, petunidin, and delphinidin suppress TPA-stimulated AP-1 transcriptional activity and cell transformation with JB6⁺ cells^[139].

Evaluation of structure-activity relationships have shown that the *ortho*-dihydroxyphenyl functional group on the B-ring of anthocyanidins plays a key role in inhibitory action since malvidin, pelargonidin, and peonidin, devoid of the *ortho*-dihydroxyphenyl structure, fail to block AP-1 activity or cell transformation. Signal transduction analysis showed that delphinidin blocked ERK phosphorylation at early times and JNK phosphorylation at later times, but p38 was not blocked [Figure 5]^[139]. Furthermore, delphinidin inhibits the phosphorylation of c-Jun (a phosphorylation target of JNK and ERK), SAPK/ERK kinase (SEK, a JNK kinase), and MAPK/ERK kinase (MEK, an ERK kinase). Suppression of TPA-induced AP-1 activity and cell transformation by delphinidin involves inhibition of JNK and ERK signaling cascades. This effect is

increased in combination with superoxide dismutase (SOD) with any anthocyanidins bearing the *ortho*-dihydroxyphenyl arrangement on the B-ring. Multiplicative analysis demonstrated that inhibition between delphinidin and SOD is synergistic in nature^[139]. Therefore, even though the signaling pathways affected by SOD or delphinidin are different, both are believed to play a crucial role in the cancer preventive activity of anthocyanidins.

Suppression of inflammation through NF- κ B and COX-2

There are some antioxidants that block the expression of COX-2 by inhibiting the signaling mechanism that controls the COX-2 gene^[143]. In one study, the molecular mechanism of anthocyanins was evaluated using the mouse macrophage cell line RAW264. Bilberry and purified delphinidin derived anthocyanin extracts suppress LPS-induced COX-2 expression at the transcription and protein levels. Signal pathway analysis showed that delphinidin blocks LPS-induced I κ B degradation and inhibits NF- κ B stimulation and COX-2 gene expression. Thus, it appears anthocyanins act through the NF- κ B signaling pathway which is involved in the suppression of COX-2 gene expression^[144].

Apoptotic induction of cancer cells (by targeting ROS and JNK mediated caspase activation)

Petunidin, delphinidin, and cyanidin induced apoptosis with HL-60 cells, as demonstrated by DNA fragmentation and morphological changes, although peonidin, malvidin, and pelargonidin did not induce this response^[145]. The number of hydroxyl groups at the B-ring is proportional to the potency of apoptosis induced by anthocyanidins; the *ortho*-dihydroxyphenyl structure at the B-ring is vital for apoptosis induction^[145].

Mechanistic analysis indicated the induction of apoptosis by delphinidin may involve an oxidation/JNK-mediated caspase pathway. Delphinidin increases intracellular ROS which may trigger JNK. With delphinidin treated cells, JNK phosphorylation, caspase-3 activation, and c-jun gene expression were observed^[145]. Delphinidin-induced JNK phosphorylation, DNA fragmentation, and caspase-3 activation were effectively blocked by antioxidants such as *N*-acetyl-L-cysteine^[145]. Thus, delphinidin can potentiate the apoptotic death program in HL-60 cells through JNK signaling mediated through oxidative stress.

Chemopreventive mechanisms of anthocyanins

Anti-carcinogenic activities at the initial stage of tumorigenesis

Anthocyanins can prevent the occurrence of tumors by acting on antioxidant systems^[146]. These compounds scavenge free radicals reducing damage to the genome of the normal cells by oxidative stress and later malignant transformation fostered by gene mutations^[147,148]. Anthocyanins can induce the antioxidant effect by acting on the antioxidant response element through KEAP1-Nrf2. Furthermore, by regulating the expression of phase II antioxidant enzymes (quinone oxidoreductase, glutathione transferase, glutathione peroxidase, and glutathione reductase), anthocyanins can suppress the activity of caspase-3. The abnormal secretion and overexpression of inflammatory elements are important for tumorigenesis. It has been reported that anthocyanins can mediate anti-inflammatory functions by regulating the secretion and expression of inflammatory agents. This effect occurs due to the suppression of transcription factors NF- κ B through multiple pathways^[149,150]. In an *in vitro* study, anthocyanin containing purple-fleshed potato extracts elevated apoptosis and suppressed proliferation in a p53-independent manner in colon CSCs^[151]. *In vivo*, purple-fleshed potato decreased the number of crypts containing cells with nuclear β -catenin (which is an indicator of colon CSCs) by induction of apoptosis^[151]. These results provide evidence that anthocyanins may reduce the initial stage of tumorigenesis.

Anti-carcinogenic activities in the cancer formation stage

Anthocyanins can block tumorigenesis and induce terminal differentiation of tumor cells. It was found that differentiation of the acute promyelocytic leukemia cell line HL-60 could be induced by cyanidin-3-O- β -

glucopyranoside (Cy-g) in a dose-dependent manner by the activation of PKC and PI3K. Upon treating HL-60 cells with Cy-g (200 µg/mL), differentiation characteristics were observed such as enhanced activity of esterase, increased adhesion, and reduced expression of the oncogene c-Myc. When cells were treated with PKC or PI3K inhibitors, the effect of differentiation induced by Cy-g was substantially decreased^[152]. Since the degree of differentiation correlates with the degree of tumor malignancy, it may be suggested anthocyanins can act at the cancer formation stage by stimulating differentiation.

Anti-carcinogenic activities in the cancer development stage

Anthocyanins can induce apoptosis of cancer cells through the external death receptor pathway and the internal mitochondrial pathway. It was determined that delphinidin could activate p38-FasL and the Bid pathway, which is a pro-apoptosis protein that induces apoptosis with HL-60 cells, in a dose- and time-dependent manner^[153]. In vascular smooth muscle cells, delphinidin and cyanidin strongly inhibited the expression of vascular endothelial growth factor (VEGF) (stimulated by platelet derived growth factor) by repressing the JNK and p38-MAPK pathways^[154].

Pharmacological properties

Hydroxylation of nonreactive carbons is a major function of phase I cytochrome P450s and the monooxygenase system^[155]. Phase I hydroxylation of anthocyanins is noteworthy since hydroxyl groups structurally distinguish this group of compounds. Even though flavonoids are reported to have low bioavailability due to extensive metabolism, their metabolites may be present for a longer duration and result in significant bioactivity. This can be the case with anthocyanins, since metabolites have been found to retain basic structural characteristics, thereby preserving bioactivity^[156,157]. The majority of the flavonoids present in the urine and circulation as glycosylated, glucuronidated, sulfated, and methylated conjugates^[158,159]. Glucuronide conjugation is regarded as an important conjugation reaction in the metabolism of flavonoid^[158-162]. UDP-glucuronosyltransferases, which catalyzes the glucuronidation reaction, are observed in high concentrations in the intestine, kidney, and liver^[163,164]. However, in humans, following dietary consumption, evidence suggests that the initial site for flavonoid glucuronidation is the intestine^[159,165,166]. Methylation is observed as the second major conjugation reaction of flavonoids^[162]. O-Methylation is the most common such reaction. Catechol-O-methyltransferase catalyzes O-methylation utilizing the cofactor S-adenosyl methionine. The liver is a major organ responsible for methylation with the highest catechol-O-methyltransferase activity^[167]. The primary site of methylation is determined by the pattern of a flavonoid ring structure. Some studies have shown low oral doses of quercetin in animals and humans undergo extensive methylation^[168]. Glycation or sulfation are common conjugation reactions that predominate when low dose phenolic drugs are administered. Sulfotransferases are a small group of cytosolic enzymes that are widely distributed in the body. They use phosphoadenosine-5'-phosphosulfate as a cofactor and their recognized substrates include polyphenols (i.e., flavonoids), hydroxylamines, 4-nitrophenol, iodothyronines, and phenols^[168].

Studies suggest that anthocyanins have very low bioavailability (< 1% in plasma), although some amount has been found in the colonic tissues of patients, indicating the possibility of a local site of action^[169,170]. It is still unclear whether anthocyanins are effective against cancer in human beings and whether they can function as metabolites or as parent molecules.

CURCUMIN

Chemical and pharmacological properties

Curcumin [(1*E*,6*E*)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a yellow-colored phytochemical obtained from *Curcuma longa* (turmeric), a member of the ginger family (Zingiberaceae). It is commonly cultivated in the southwest Asia region and is popularly used as a spice for various cuisines^[171,172]. Curcumin is comprised of two *o*-methoxy phenolic rings which are connected by seven

carbon chains consisting of an α , β -unsaturated β -diketone component, and generally accounts for 60%-70% of curcuminoid extracts^[173,174]. The *o*-methoxyphenol rings are the primary domains for the antioxidant activity of curcumin and the β -diketone component forms chelation complexes with metals rendering them useful for the treatment of heavy metal poisoning^[175].

In a pre-clinical study, 500 mg/kg curcumin administered orally to rats gave a maximum plasma concentration of approximately 0.06 $\mu\text{g/mL}$, reaching the peak by approximately 41 min; the half-life was 28 min, giving an oral bioavailability of about 1%^[176]. As suggested by this poor bioavailability, the tolerance for curcumin is very high for humans. In a dose escalation study, minimal adverse events were observed which were unrelated to the dose after oral administration of 10,000 or 12,000 mg in healthy subjects. Still, only traces were detected in serum^[177]. At 10-12 g, the highest dose given to human subjects, the plasma concentration of curcumin and its metabolites were in the range of 0.075-10 $\mu\text{g/mL}$ ^[178]. Curcumin is metabolized by the process of glucuronidation in the intestine and by the liver yielding metabolites such as tetra-hydrocurcumin, and hexahydrocurcumin^[179].

Nevertheless, there are a vast number of studies conducted with curcumin to investigate anti-inflammatory, antioxidant, anti-carcinogenic, antiviral, and anti-infection activities. Owing to its antibacterial, antioxidant, anti-inflammatory, and antiseptic properties, curcumin has been proven useful in treating several skin diseases, such as psoriasis, infection, acne, skin inflammation, and skin cancer^[180]. Curcumin controlled the *in vitro* growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in an *in vivo* murine wound model, and topical administration accelerated healing^[181]. In a psoriasis keratin (K) 14-VEGF transgenic mouse model, after oral curcumin administration, symptoms like ear redness, weight, thickness, and lymph node weight were reduced significantly^[182].

There have been several conclusive *in vitro*, *in vivo*, and clinical studies conducted on the use and benefits of curcumin in treating inflammatory diseases. Curcumin exhibited anti-inflammatory effects in adipose tissue of C57BL/6 mice fed a high fat diet by down-regulating inflammatory transcription factors like NF- κ B and toll-like receptor-4^[183]. The clinical effectiveness of curcumin in treating inflammation was studied in patients diagnosed with osteoarthritis, and significant reductions in myeloperoxidase, collagen degradation activity, and C-reactive protein levels were observed; also, additional symptoms like pain and effusion were reduced^[184]. In 45 patients with active rheumatoid arthritis, curcumin (500 mg) was used in combination with diclofenac sodium (50 mg) and there was a significant reduction in their disease activity score compared with the group provided only with diclofenac sodium^[185]. In yet another clinical study, patients with acute rhinitis were administered 500 mg of curcumin orally. Symptoms like sneezing, itching, and obstruction rhinorrhea were reduced significantly in patients treated with curcumin compared to the placebo treatment, and inflammatory markers such as IL-4, TNF- α , IL-8, PEG₂ were significantly decreased^[186].

Curcumin as a chemopreventive and anticarcinogenic agent

Curcumin has been broadly studied for anti-cancer characteristics [Figure 6]. A pilot study conducted with patients diagnosed with benign prostatic hyperplasia (BPH) and administered 500 mg of curcumin twice a day along with the standard therapeutic regimen showed improved symptoms and improved quality of life relative to the group administered the standard treatment only^[187]. In another study, 85 patients with prostate cancer were administered curcumin and soy isoflavones along with the standard treatment, and elevated PSA levels were significantly decreased in patients treated with curcumin and soy isoflavones compared with the group given the standard treatment^[188].

In other forms of cancer, like breast and cervical, curcumin has proved effective. In patients with advanced metastatic breast cancer, curcumin reduced biomarkers like CEA with a maximum tolerated dose of 8000

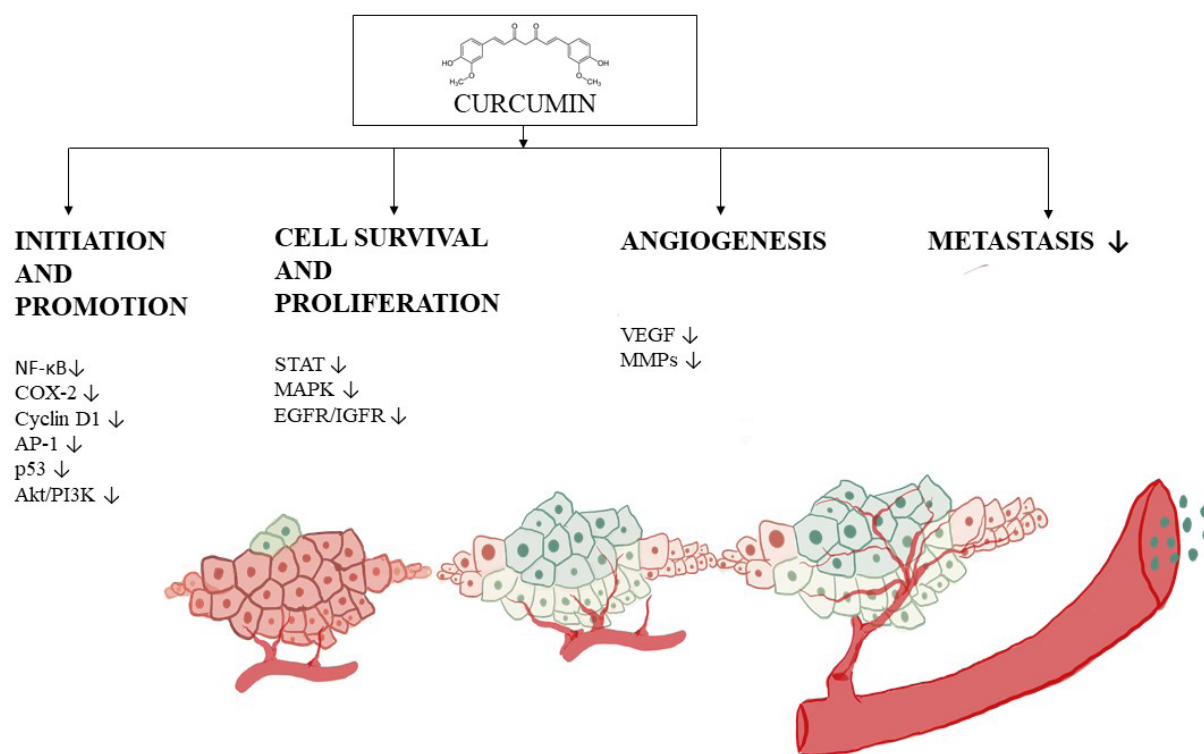


Figure 6. Progression of angiogenesis and the inhibitory mechanism of curcumin. Curcumin is capable of functioning through a myriad of mechanisms, but this figure illustrates down-regulation of factors involved in tumor initiation such as NF- κ B, COX-2, cyclin D1, p53 and Akt/PI3k, and proliferation factors such as STAT, MAPK EGFR. The progression phase of tumorigenesis is also inhibited, for example, by alteration of VEGF expression, leading to reduced angiogenesis and metastasis. NF- κ B: nuclear factor- κ B; COX: cyclooxygenase; PI3K: phosphatidylinositol-3-kinase; MAPK: mitogen-activated protein kinase

mg along with chemotherapy^[189]. In addition, topical application of curcumin along with other herbal preparations for 30 consecutive days resulted in significant clearance of HPV. However, vaginal curcumin capsules did not show any statistical difference^[190]. Oral administration of curcumin (8000 mg) is well tolerated in patients diagnosed with advanced pancreatic cancer^[191]. And, in a Phase II clinical trial, despite its limited absorption, curcumin exerted biological activity in a few patients, such as down-regulation of transcription factor NF- κ B and reduced COX-2 (8,000 mg/day dosage)^[192].

Mechanism of anti-carcinogenesis

VEGF

Since VEGF is a crucial regulator for angiogenesis, the use of potentially active phytochemicals including curcumin as an anti-angiogenic compound has been explored^[193]. With the H22 HCC cell line, curcumin inhibited the proliferation of cells *in vitro* and reduced VEGF expression and PI3K/AKT signaling *in vivo*^[194]. Curcumin reduced high mobility group box 1 and VEGF-D expression in gastric cancer AGC and SGC-7901 cell lines, and induced cell apoptosis through caspase-3 activation, in a dose-dependent manner^[195].

Role of curcumin in cell-cycle arrest

Curcumin inhibits the expression of cyclin E and cyclin D1 that enable cell-cycle arrest at the G1/S phase in LNCaP and PC-3 cell lines^[196]. In another *in vitro* study, with human osteosarcoma cells, cell-cycle arrest by curcumin at the G1/S phase was related to a reduction of cyclin D1^[197]. Human mantle cell lymphoma is characterized by overexpression of cyclin D1, which up-regulates NF- κ B and several genes such as, bcl-2, COX-2 and IL-6. However, on treatment with curcumin, cyclin D1 expression was down-regulated, along with AKT activation^[198]. Curcumin also improves the efficacy of chemotherapeutic agents such as cisplatin

by targeting cancer stem cells [such as A549 and H2170 (NSCLC cell lines)], down-regulating cyclin D1 and increasing the expression of p21^[199]. There is also evidence of involvement of curcumin in suppressing glioma growth and inducing apoptosis by AKT and signal regulated kinase pathway in U87-MG cells^[200]. In T-cell acute leukemia malignant cells, curcumin causes de-phosphorylation of active AKT, FOXO, and releases cytochrome c, which activates caspase-3. This leads to inhibition of cell proliferation^[201].

SILIBININ

Chemical properties of silibinin

Silibinin is a key constituent of silymarin, which is a polyphenolic flavonoid obtained from milk thistle seeds^[202]. It is constituted as two isomers, silybin A and silybin B, in the ratio of 1:1, functions as a weak acid in aqueous form, and is especially stable in the presence of acids and unstable in a basic environment^[203]. Silibinin is a major component of the silymarin complex, constituting 50%-60%, depending on the formulation^[204]. Many studies have demonstrated that silibinin has strong antioxidant properties, scavenging both reactive oxygen species and free radicals, which can lead to the enhancement of cellular antioxidant defense mechanisms^[205-209]. Notably, silibinin can affect cancer development by various modes of action, including modulation of oxidative stress, proliferation, inflammation, metastasis, and angiogenesis^[210-212]. Some studies have indicated a beneficial effect on toxicity due to short- and long-term exposure to radiation treatment and chemotherapy^[213].

Pharmacokinetics of silibinin

Silymarin is primarily conjugated and excreted into bile and urine and seems to undergo insignificant phase I metabolism; inadequate data exist concerning the role for phase II metabolism and transporters^[214,215]. Silymarin (where silibinin is an active constituent) pharmacokinetic analysis was done with healthy volunteers. There was rapid metabolism, forming conjugates such as glucuronides, that were detected in plasma^[214]. Also, it was found that conjugated silibinin metabolites are eliminated slowly as compared to free silibinin^[214]. Factors such as inefficient intestinal absorption, low water solubility, elevated metabolism, and rapid excretion, significantly decrease the serum concentration of silibinin, thus reducing its ability to reach target organs and consequently therapeutic efficiency is reduced^[216-218]. There have been many efforts to produce formulations to increase the bioavailability of silibinin^[219,220]. For example, silibinin that is complexed with phosphatidylcholine is known as "silipide". In pharmacokinetic studies conducted with healthy subjects, it was shown that silibinin derived from silipide has greater absorption in plasma and liver as compared with conventional silibinin^[221,222]. Silipide, when tested in cancer patients, demonstrated high plasma bioavailability^[223-225]. A comparative study revealed high bioavailability of silibinin in colon tissue but relatively poor levels in prostate tissue^[223-225]. This suggests organ specificity may be anticipated as a result of bioavailability following oral administration.

Chemoprevention mechanism of silibinin

Several studies suggest that silibinin may be effective against lung cancer. Silibinin reduced the production of matrix metalloproteinase-2 and urokinase-plasminogen activator when metastatic A549 lung cancer cells were treated with different concentrations, up to 100 $\mu\text{mol/L}$ ^[226]. Silibinin has been shown to reduce the development of human NSCLC, such as large cell carcinoma cells (H1299 and H460) and bronchioalveolar carcinoma cells (H322)^[227]. Silibinin treatment of cultured cells (10-75 $\mu\text{mol/L}$) has been shown to target cell-cycle progression leading to a G1 arrest and altered protein levels of cyclins (D1, D3, and E), CDKIs (p18/INK4C, p21/Cip1, and p27/Kip1) and cyclin-dependent kinases (CDKs)^[227]. Inhibitory effects of silibinin (75 $\mu\text{mol/L}$) were shown in both a human large-cell lung cancer cell line and human lung adenocarcinoma A549 cells^[228]. A combination of silibinin and indole-3-carbinol showed greater anti-proliferative effects than the single compounds alone. A549 cells, when treated with 100 $\mu\text{mol/L}$ of indole 3-carbinol and 75 $\mu\text{mol/L}$ of silibinin, or 200 $\mu\text{mol/L}$ of indole-3-carbinol plus 75 $\mu\text{mol/L}$ of silibinin, for 24 h, showed a reduction in proliferation by 40% and 62%, respectively. With H460 cells, the responses

were 31% and 69%, respectively. At the molecular level, the combination of indole-3-carbinol and silibinin inhibited Akt and ERK activation and induced apoptosis^[228]. Silibinin has also shown effects against cancer stem cells (CSC) in lung cancer^[229]. A model of acquired erlotinib resistance was established by growing NSCLC cells with a TKI-sensitizing EGFR exon 19 deletion in the constant presence of high doses of Erlotinib (Erlotinib-refractory PC-9/Erl-R cells). Treatment with silibinin (50-100 µg/mL) decreased the amount of lung cancer spheres in this model. These results suggest the possibility of using silibinin in combination with EGFR tyrosine kinase inhibitor Erlotinib to target CSC in EGFR-mutant NSCLC patients^[229].

The anti-prostate cancer properties of silibinin have been investigated in various studies. The substance has shown inhibition of androgen- and serum-stimulated PSA protein levels in LNCaP cells, and this is associated with cell growth inhibition through G1 arrest in cell cycle progression^[230]. Treatment with 25 and 75 µg/mL of silibinin for 24 h showed 45% and 59% decreases in PSA secretion in the medium, respectively. Silibinin has further shown up-regulation of insulin-like growth factor binding protein 3 expression and suppression of androgen-independent prostate cancer (PC-3) cell proliferation^[231]. With PC-3 cells in a medium supplemented with 10% FBS, treatment with silibinin (2 and 20 µmol/L) reduced cell growth by 17.3% and 54%, respectively^[231].

Silibinin has been shown to suppress the adhesion and migration of human prostate adenocarcinoma (PC-3) cells^[232], and the motility, migration, and invasion of ARCaPM prostate cancer cells^[233]. However, the response was weak with ARCaPM cells. When applied at a higher concentration (200 µmol/L), growth inhibition was only 18.5% in ARCaPM cells, but in the case of LNCaP, PC-3 and DU145 cells, the growth inhibition was found to be 48.7%, 60.0%, and 73.8%, respectively, after 48 hours of treatment. Moreover, silibinin induced morphological reversal to the epithelial phenotype from epithelial-mesenchymal transition (EMT), down-regulated MMP-2 and vimentin, and up-regulated cytokeratin-18. Also, silibinin inhibited NF-κB p50 translocation by up-regulating I kappa B alpha (IκBα) protein, and down-regulating the expression of two key EMT regulators, SLUG, and ZEB1 transcription factors^[234].

Several studies have demonstrated the activity of silibinin against colon cancer. Silibinin mediated apoptosis in cultured human colorectal carcinoma LoVo cells, which was associated with high levels of cleaved PARP and cleaved caspases (3 and 9). When LoVo cells were treated with silibinin (50-200 µmol/L) for 24 hours, growth was reduced by 30%-49%. The substance also induced strong cell cycle arrest at the G1 phase, and a minor although significant G2/M phase arrest, at the highest concentration tested. Moreover, silibinin reduced the levels of cyclins (A, B1, D1 and D3), CDK1, CDK2, CDK4, and CDK6, and elevated the level of CDKIs (p21 and p27) and phosphorylation of Rb protein^[235]. Anti-angiogenic effects, such as a decrease in inducible COX-1 and COX-2, NOS and NOS3, and VEGF and HIF-1α, were exhibited by silibinin^[236]. When administered along with TNF-related apoptosis-inducing agent (TRAIL), silibinin caused cell death in primary tumor cells (SW480), as well as TRAIL-resistant metastatic cells (SW620). Finally, silibinin induced up-regulation of death receptor 4 (DR4) and DR5, down-regulated the anti-apoptotic proteins XIAP and Mcl-1, and synergistically activated the mitochondrial apoptotic pathway^[237] [Figure 7].

LYCOPENE

Chemical properties lycopene

Lycopene is a lipophilic hydrocarbon carotenoid with red color due to a chromophore with 11 conjugated double bonds^[238]. The antioxidant properties of lycopene derive from this conjugated polyene structure^[239]. Unlike α- and β-carotenes, lycopene is not provitamin A due to the absence of a β-ionone ring and its acyclic structure^[239]. Having been studied for many decades, lycopene is found in tomatoes, watermelon, pink grapefruit, papaya, and other fruits^[240]. Unlike other widely distributed carotenoids, however, lycopene is mainly found in tomatoes and tomato products^[241]. Unique structural and chemical features may contribute to distinct biological properties^[239].

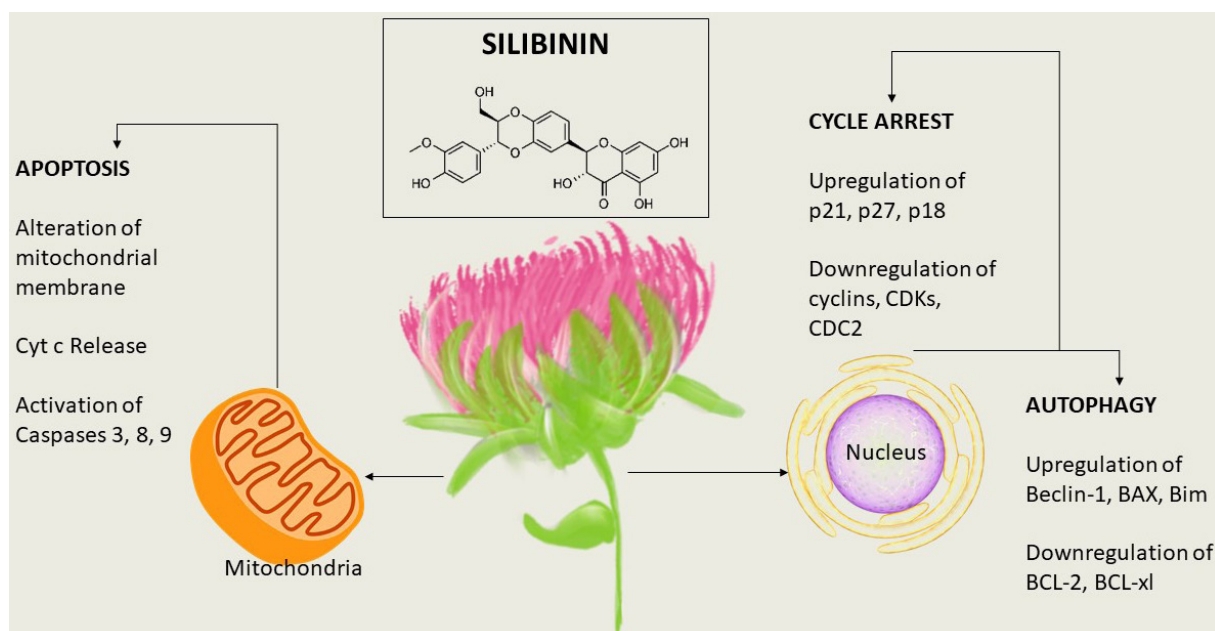


Figure 7. The molecular targets of silibinin. Silibinin exhibits a decrease in mitochondrial function and releases mitochondrial cytochrome c. The compound initiates cell death in primary tumor cells and causes up-regulation in p21, p27, and p18, leading to cell-cycle arrest. In addition, autophagy is induced by up-regulation of BAX, Bim, and Beclin-1

Pharmacological properties of lycopene

Lycopene has been shown to function through a variety of mechanisms [Figure 8], several of which are described below.

Antioxidant activity

Oxidative stress caused by the ROS is linked with aging, carcinogenesis, and cardiovascular diseases. Lycopene can function as an antioxidant by various mechanisms, with the best documented mechanism being through quenching singlet oxygen ($^1\text{O}_2$) due to the extended system of conjugated double bonds^[242].

In cell culture, V79 Chinese hamster lung fibroblasts were treated with peroxynitrite to cause DNA strand breakage and nitration of proteins. This was shown to be inhibited by lycopene in the concentration range of 0.31 to 10 $\mu\text{mol/L}$ ^[243]. In another preclinical study, lycopene at concentrations of 0.25 to 10 $\mu\text{mol/L}$, reduced the oxidative DNA damage caused by the redox-cycling of catechol estrogens in Chinese hamster lung fibroblasts and plasmid DNA^[244]. It was determined that 81% of the subcellular localization of lycopene in prostate cancer cells treated with lycopene was at the nucleus^[245]. Consistent with this, it is observed that the DNA protective effects exhibited by lycopene correlate with the localization of lycopene in the nucleus^[246].

Apoptosis

In one study, lycopene reduced mitochondrial transmembrane potential, decreased mitochondrial function, increased annexin V binding, and released mitochondrial cytochrome c^[247]. In another study, apoptosis was induced with LNCaP and PC3 cells with lycopene concentrations as low as 10 nmol/L^[248].

With human colon carcinoma (HuCC) cells, lycopene treatment at 2.0 or 4.0 $\mu\text{mol/L}$ induced apoptosis. However, the compound was not effective when incubated with a physiologically relevant concentration 1.0 $\mu\text{mol/L}$. The same group showed apoptosis in Raji cells, a prototype Burkitt lymphoma cell line, when exposed to 2.0 $\mu\text{mol/L}$ lycopene. There were no anti-apoptotic effects observed in human erythroleukemia

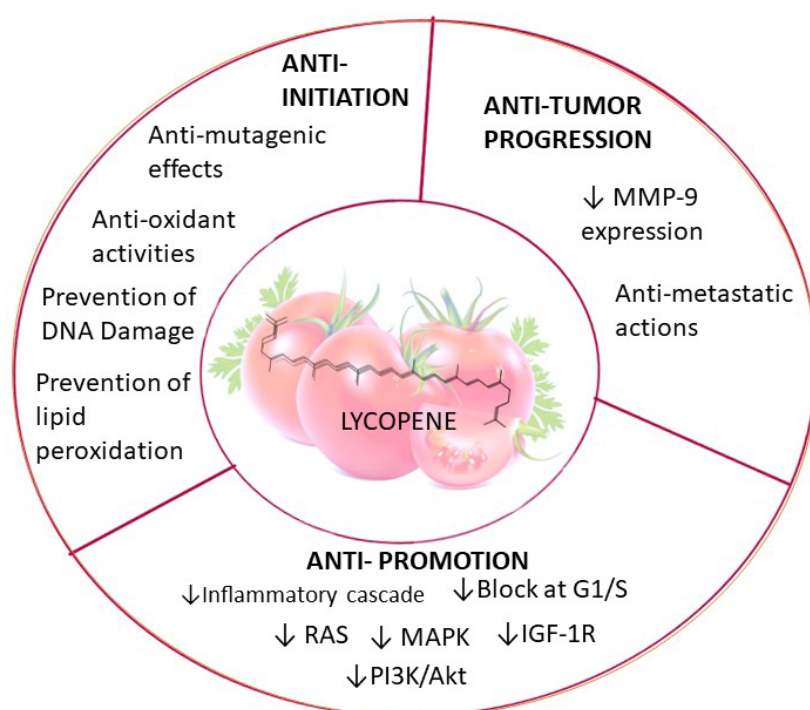


Figure 8. Pharmacological functions of lycopene. Lycopene induces factors leading to anti-initiation and anti-progression by down-regulating MMP-9 expression. The compound mediates anti-promotion effects by suppressing RAS, MAPK, IGF-1R, PI3K/AKT, and inflammatory cascades. MAPK: mitogen-activated protein kinase; PI3K: phosphatidylinositol-3-kinase

(K562) or B chronic lymphocytic leukemia (EHEB) cells treated with lycopene at a concentration of 4 $\mu\text{mol/L}$ [249]. These data indicate that lycopene shows greatest efficacy for inducing apoptosis in human prostate cancer cells.

Cell-cycle arrest

Studies have suggested that lycopene can induce cell-cycle arrest at the G1 phase. It was found that Hep3B human hepatoma cells were inhibited 20%-50% by lycopene at physiologically relevant concentrations as low as 0.2 $\mu\text{mol/L}$. Lycopene induced G0/G1 arrest [246]. Similarly, in a study with human prostate cancer cell lines PC3 and LNCaP, lycopene promoted mitotic arrest at the G0/G1 phase, facilitated by a decreased amount of cyclin dependent kinase 4 and cyclin D1 and E [248].

Pharmacokinetics and bioavailability

The pharmacokinetics of lycopene provides insight regarding relevant mechanisms by which intervention impacts disease. The ability to interpret epidemiologic findings, design clinical intervention trials, and plan physiologically relevant animal and *in vitro* mechanistic investigations hinges on overall bioavailability. Critical questions impacting interventions include absorption, distribution, tissue concentrations and metabolic breakdown.

One important parameter is plasma half-life. With patients given 20 mg of lycopene from tomato soup or a synthetic lycopene tablet for 8 sequential days, the systemic availability of lycopene in the form of a tablet was comparable to that of processed tomatoes (tomato paste available as a soup) and better than tomato juice. The plasma lycopene half-life was found to be 5.6 and 6.4 d for the tablets and the soup, respectively. Furthermore, the half-lives of 5-*cis*- vs. all-*trans*-lycopene were evaluated and found to be 7.4 and 5.4 d, respectively. These observations suggest differences in the pharmacokinetic parameter which may lead to distinctive tissue and plasma lycopene isomeric patterns [250]. The difference in the half-lives observed

in the geometrical isomers of lycopene may be due to factors such as the higher bioavailability of *cis* vs. *trans*^[251,252], greater thermodynamic stability of *cis* isomers at elevated temperature^[253], and endogenous isomerization, which may or may not be enzymatic^[254].

Cleavage of β -carotene gives homologous carbonyl products^[255,256]; the pathway was confirmed by the identification of BCO2 in zebra fish, ferrets, mice and humans^[257,258]. BCO2, an enzyme derived from mitochondria^[259], is mainly expressed in testis and liver, with a comparatively lower amount found in lung, heart, brain, colon, stomach, intestine, prostate, spleen, and kidney^[257,258]. In a study conducted with humans with lycopene, plasma of those who consumed tomato juice was found to contain apo-6-, apo-8'-, apo-10'-, apo-12'-, and apo-14'-lycopenal^[260]. Moreover, it was reported that non-provitamin A carotenoids, including zeaxanthin and lutein, are preferentially cleaved relative to provitamin A carotenoids, which indicates a major role of BCO2 in the metabolism of non-provitamin A carotenoid^[258,261]. In another investigation with humans given lycopene, a series of apo-lycopenals were identified in plasma, including apo-10'-lycopenal^[260]. However, it is not known whether these metabolites are enzymatically cleaved or products originating due to chemical oxidative cleavage^[260].

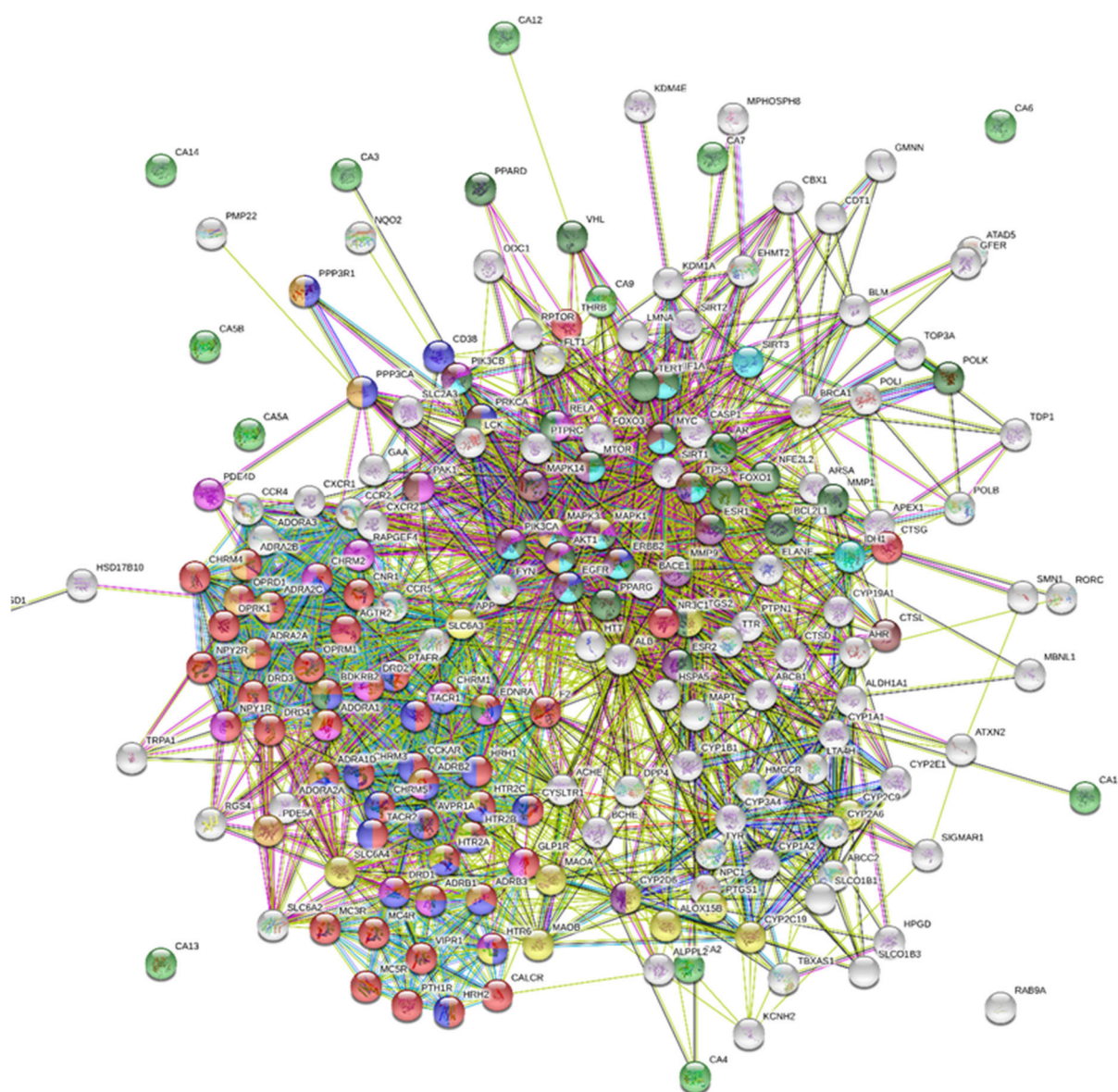
In a single blind, randomized, placebo-controlled study, lycopene supplements of 15 mg two times a day for 3 weeks (Lyc-O-Mato™, LycoRed, Beer-Sheva, Israel) were given to 15 of 26 men who were scheduled for radical prostatectomy due to organ confined malignancy. Over 3 weeks, in those taking lycopene supplements, a 22% increase in tissue and plasma levels and a statistically significant reduction in PSA was observed. Tumor volume was reduced. In addition, cellular proliferation biomarkers were decreased whereas cellular differentiation markers including connexin 43 and apoptosis were increased with intervention^[262]. In another study, it was reported that combined intake of tomato sauce, tomatoes, tomato juice, and pizza decreased the risk of prostate cancer^[263]. These findings suggest that tomato-based foods may be beneficial for prostate cancer.

CONCLUSIONS AND FUTURE DIRECTIONS

We currently review some well-studied phytochemicals with cancer chemopreventive potential. Of course, many other cancer chemopreventive agents are known, either naturally occurring or synthetic, such as genistein^[264], 6-gingerol^[265], diallyl disulfide^[266], lupeol^[267], honokiol^[268], plumbagin^[269], ellagic acid^[270], and quercetin^[271], but our arbitrary selection mainly focuses on agents found in the diet with strong evidence of bioactivity. Numerous studies have been completed or are ongoing with these compounds; copious amounts of data have been generated. As one example, more than 20,000 articles were disclosed when searching “resveratrol” as a topic on Web of Science (accessed on August 25, 2020), and there is no doubt a search involving other chemopreventive agents would reveal a multitude of publications as well. Given this huge corpus of data, and taking into account the current state of science, there is now an opportunity to further expand the field of chemoprevention. We currently suggest three such paradigm shifts, referred to as omics, indirectness, and sequelae.

Omics

The traditional dogma for the discovery of drug candidates is based on “one drug-one target”, where the goal is to find highly selective agents with one molecular target (or biomarker). The concept is to minimize adverse effects caused by off-target responses. However, this approach is disingenuous. First of all, an agent functioning in such a manner would be highly susceptible to failure due to the possibility of developing drug resistance. Moreover, signaling pathways are exceedingly linked via interconnected networks (cf. Figure 9). A small change in one target can result in multiple changes in other pathways, akin to the so-called Butterfly Effect (“Does the Flap of a Butterfly’s Wings in Brazil Set Off a Tornado in Texas?”)^[272]. Also, the genesis of cancer is comprised of multiple factors, not a single specific element. This is further complicated by a host of endogenous (e.g., aging, genetic susceptibility, DNA repair machinery, hormones,



KEGG Pathways			
pathway	description	count in gene set	false discovery rate
hsa04080	Neuroactive ligand-receptor interaction	49 of 272	2.36e-42
hsa04020	Calcium signaling pathway	29 of 179	3.22e-23
hsa00910	Nitrogen metabolism	12 of 17	1.74e-15
hsa04726	Serotonergic synapse	17 of 112	4.77e-13
hsa04024	cAMP signaling pathway	20 of 195	1.37e-12
hsa05200	Pathways in cancer	29 of 515	3.25e-12
hsa05230	Central carbon metabolism in cancer	13 of 65	1.93e-11
hsa04022	cGMP-PKG signaling pathway	17 of 160	4.70e-11
hsa01522	Endocrine resistance	14 of 95	7.66e-11
hsa05205	Proteoglycans in cancer	17 of 195	6.87e-10

Figure 9. Bubble map with target proteins of resveratrol using STRING software. Human target proteins of resveratrol that were selected from ChEMBL are visualized as a network of predicted associations. The network nodes represent proteins while the edges represent the protein-protein associations. The colored lines represent different types of evidence - Light blue line: curated database; purple line: experimentally determined; green line: neighborhood; red line: gene fusion; blue line: gene co-occurrence; yellow line: text mining; black line: co-expression. The colored nodes demonstrate the pathways. The KEGG pathway table demonstrates the top ten pathways enriched by resveratrol based on a gene list of human target proteins. Neuroactive ligand-receptor interaction appears at the top of the list with the lowest false discovery rate (2.36×10^{-42})

growth factors, inflammation) and exogenous (e.g., radiation, chemical carcinogens, viruses, smoking, lack of exercise, nutrient imbalance) risk factors, and typically long latency periods^[273]. Further, cause-effect relationships are often nebulous.

In the context of chemoprevention, it is likely that dietary phytochemicals currently play an important role in cancer reduction or delay, but that role is difficult to quantify in a direct manner. Daily consumption varies, as does the phytochemical content of the foods consumed. For a more predictable response, especially for individuals at high risk for developing malignancies, a cocktail of chemopreventive agents would be preferred^[8]. Development of such a cocktail needs to take into account the pleiotropic activities of typical chemopreventive agents, as demonstrated by those described in this review and elsewhere. On one hand, pleiotropic responses are considered a distinct advantage. On the other hand, the creation of a proper preparation becomes a daunting task as a result of complexity. However, unique tools are now available that can be put to use. For example, with agents known to function in a chemopreventive capacity, we can take into account novel pathways uncovered utilizing primary -omics data (e.g., genomics, proteomics, metabolomics) or data mining with publicly accessible biological data repositories (e.g., ChEMBL, PubChem).

Consider resveratrol as an example. Based on literature reports describing individual actions of this compound, we input human target proteins listed in ChEMBL (accessed on August 25 2020)^[274] on STRING (accessed on August 25 2020)^[275]. As shown in Figure 9, the gene list with network edges is visualized with enriched pathways (e.g., neuroactive ligand-receptor interaction, calcium signaling pathway, nitrogen metabolism, serotonergic synapse, cAMP signaling pathway). Clearly, this is a more realistic view of the action of resveratrol, relative to thinking of it as simply inhibiting or stimulating factor x, y or z. Now, if we consider a second chemopreventive agent, and the network of factors modulated by that agent, and overlay the two individual networks, it is easy to perceive the complexity of the actual response leading to a chemopreventive response.

There is little doubt that the intrinsic response of a mammal exposed to a chemopreventive agent correlates to some extent with the theoretical response shown in Figure 9. As an example, an area of great interest for our group is the potential effect of grapes on health^[276], and the corresponding mechanisms. In a recent study (unpublished) in which mice were provided diets with or without whole grape supplementation, RNA expression data were examined. As shown in Figure 10, remarkable alterations were observed that would likely relate to some of the beneficial properties. There is little doubt this modulation of genetic expression results from the action of phytochemicals contained within the grape. Grapes have received great notoriety for being a primary dietary source of resveratrol but, in actual fact, whole grapes contain over 1,600 phytochemicals^[34]. Thus, it is perhaps not surprising that such a profound effect on genetic expression was observed. This provides a good illustration of the complexity of a real-life response and accentuates the naivety of one drug-one target philosophy.

Indirectness

As noted above, the low bioavailability of phytochemicals may lead to discrepancies in effective doses observed with *in vitro* models but required for *in vivo* responses. For example, the oral bioavailability of resveratrol is reported as under 1% due to rapid metabolism in the intestine and liver^[277]. As exemplified in Figure 11, this has led to the exploration of technology designed to enhance bioavailability. Here, a map was created based on bibliographic data (co-occurrence) of “resveratrol” from Web of Science, and words such as “drug-delivery” and “encapsulation”, and highlighting using VOSviewer^[278].

Of course, metabolism of chemopreventive agents may have a major influence on efficacy. Several major metabolites derived from the chemopreventive agents described herein are shown in Figure 12. In some

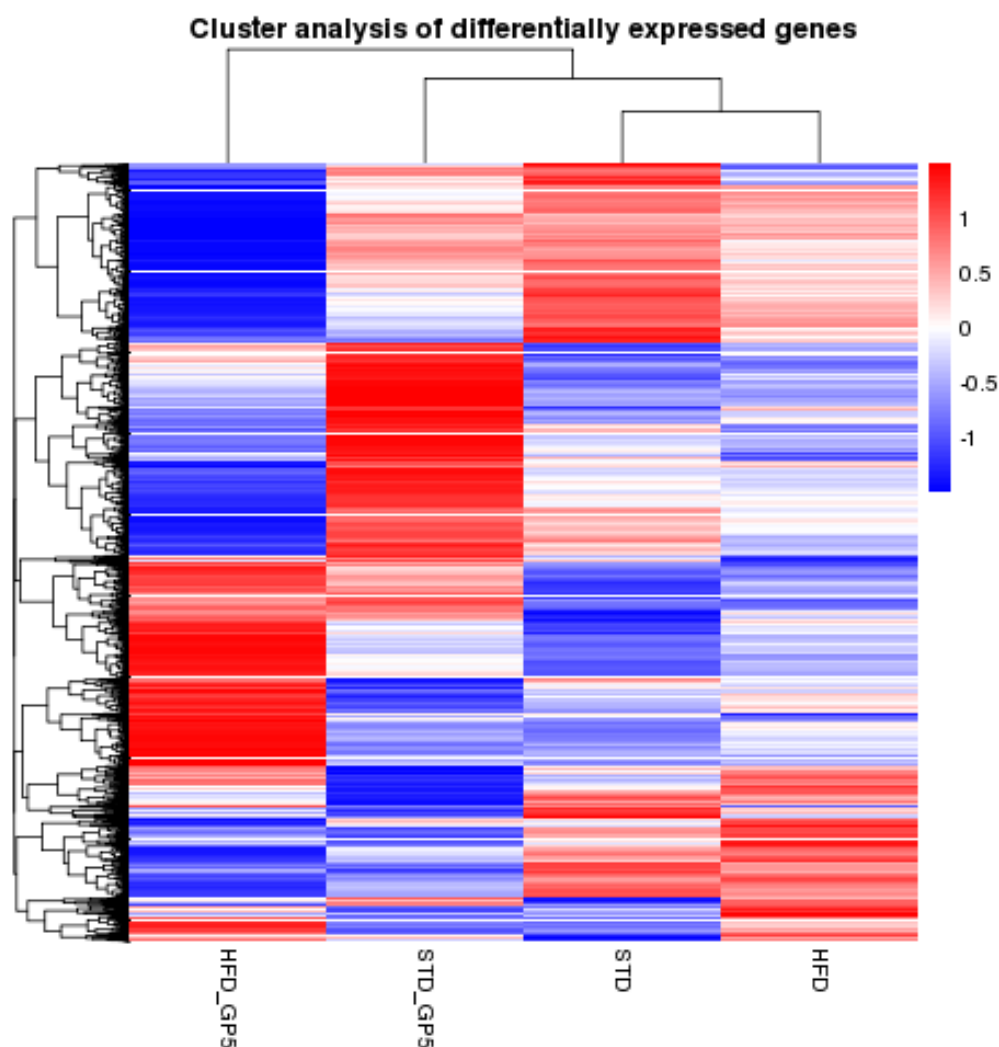


Figure 10. Hierarchical clustering heatmap. A study was conducted with female C57BL/6J mice. Starting at age 10 weeks, animals were provided the following diets *ad libitum*: standard synthetic diet (STD); STD supplemented with 5% whole grape powder (STD5GP); a high-fat (Western) diet (HFD); HFD supplemented with 5% whole grape powder (HFD5GP). Cluster analysis was produced using the $\log_{10}(\text{FPKM})+1$ value that shows the influence of grape on the expression levels of genes. Red color indicates up-regulated gene expression; blue color indicates down-regulated gene expression. The interrelationships between groups is presented as hierarchical clustering. The order of the rows and columns is based on resemblance correlations that can be interpreted based on the hierarchical clustering shown in the heatmap. The data indicate dietary supplementation with whole grape powder influences the expression levels of genes. The heatmap was produced by analyzing a total of 11,047 genes

cases, these metabolites may be less active than the parent molecule. In other cases, such as with resveratrol 3'-sulfate^[279], the activity may be similar or even greater.

In addition to well-known phase 1 and phase 2 mammalian enzymes that participate in drug metabolism, clearly, the overall metabolic profile of chemopreventive agents is influenced by the gut microbiome. Even further, however, health benefits mediated by phytochemicals may result from indirect effects such as influencing products produced by the gut microbiome itself. For example, recent studies have shown enrichment of short-chain fatty acids (SCFAs)-producing bacteria (e.g., Bacteroidetes and Parabacteroides) in mice treated with resveratrol^[280]. SCFAs, microbial-derived metabolites, are reported to affect CNS functions in the microbiota-brain-axis^[281].

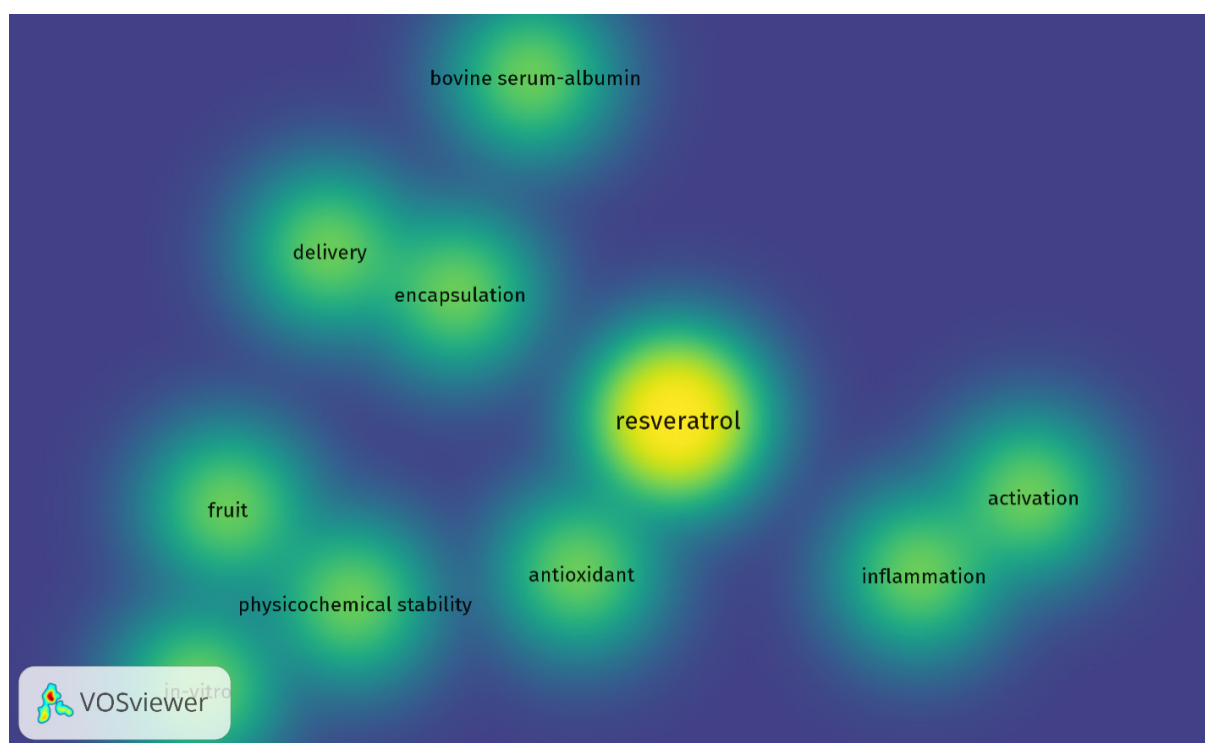


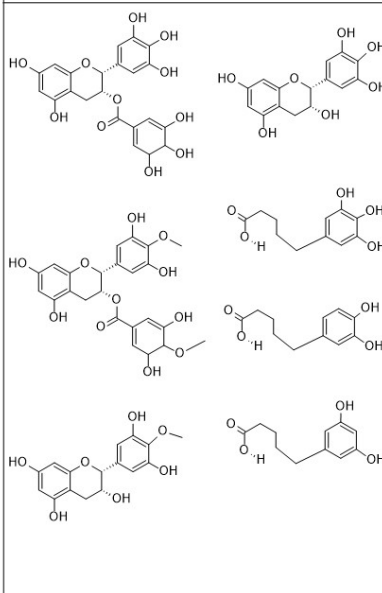
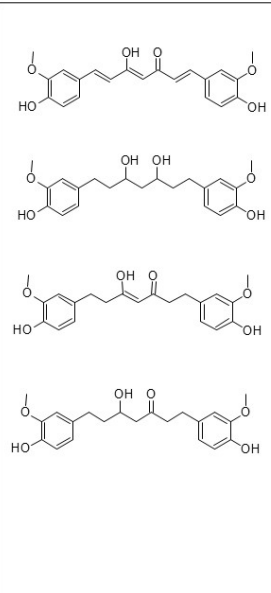
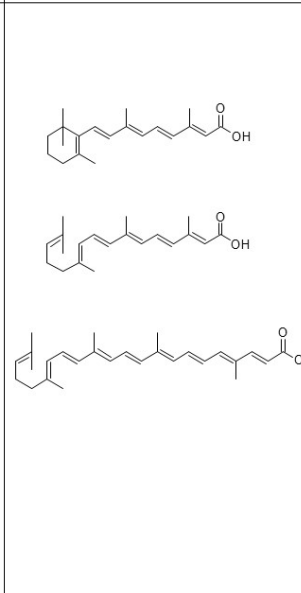
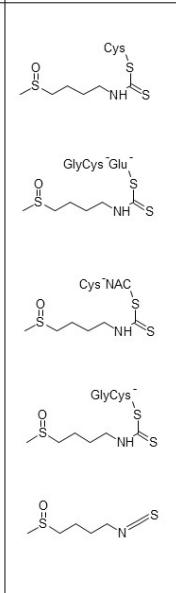
Figure 11. Density visualization of “resveratrol” and co-occurring words by VOSviewer. Co-occurrence words with resveratrol are displayed based on text data from Web of Science. The color illustrated in the figure changes from blue to yellow based on the frequency with which a word is linked to resveratrol

Conversely, dietary chemopreventive agents may influence the actual composition, and therefore the metabolic capacity, of the gut microbiome. For example, in studies we have conducted with mice in which the diet was supplemented with whole grapes (unpublished), the overall organization of the gut microbiome was clearly altered [Figure 13]. This is yet another factor that needs to be considered in defining the overall action of chemopreventive agents.

Sequelae

Over the past decades, there have been significant advancements in understanding cancers and in improving early detection, treatment, and prevention. As a result, the survival rate of cancer patients has increased. And, as the number of cancer survivors increases, the quality of life for these individuals must receive greater attention, given that adverse effects (sequelae) are frequently encountered during or after chemotherapy. One common sequela is cancer-related cognitive impairment (CRCI), or chemotherapy-induced cognitive impairments, colloquially called “chemobrain” or “chemofog”, a decline in cognitive function following cancer treatment. Central neurotoxicity can remain as long-term sequelae following the termination of treatment. For example, it has been reported that breast cancer and childhood cancer survivors may experience cognitive dysfunction^[282]. Notably, some chemopreventive agents are at least touted to improve this condition^[283,284].

Given the mode of action of chemopreventive agents, it seems logical that acute or chronic side-effects resulting from some forms of cancer chemotherapy could be ameliorated by concomitant administration. However, since chemotherapy is often designed to be overtly toxic, and therapeutic indices may be low, an effective chemopreventive agent could actually have an adverse effect on outcome by negating anti-cancer drug action. An appropriate balance is necessary, but some promising results have been described. For

EGCG and its metabolites	Curcumin and its metabolites	Lycopene and its metabolites	Sulforaphane and its metabolites
			

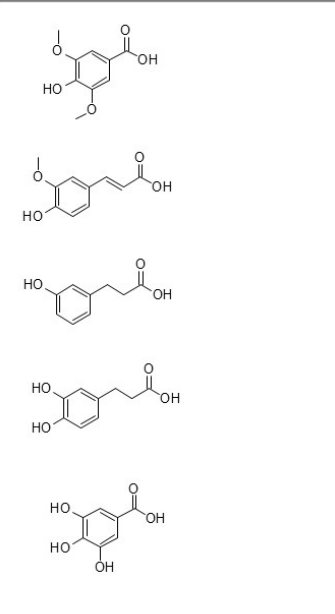
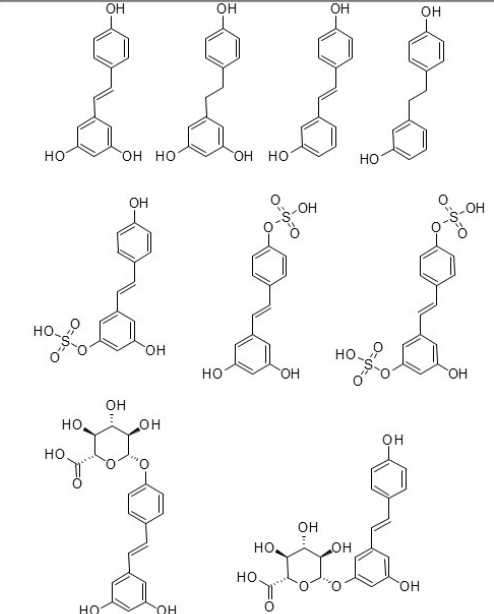
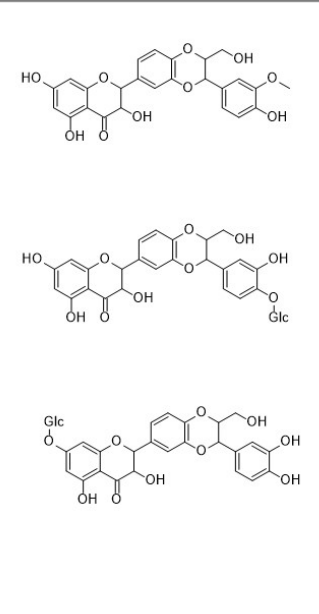
Anthocyanins and its metabolites	Resveratrol and its metabolites	Silibinin and its metabolites
		

Figure 12. Chemical structures of phytochemicals and their metabolites. EGCG and metabolites - EGC, 4'-MeEGC; 4',4'-diMeEGCG, 5-(3', 4'-dihydroxyphenyl)-γ-valerolactone (M6); 5-(3', 4', 5'- trihydroxyphenyl)-γ-valerolactone (M4); 5-(3', 5'- dihydroxyphenyl)-γ-valerolactone (M6'). Curcumin and metabolites - tetrahydrocurcumin, hexahydrocurcumin, hexahydrocurcuminol. Lycopene and metabolites - apo-10'a-lycopenoic acid, acylclo-retinoic acid, *all-trans*-retinoic acid. Sulforaphane and metabolites - sulforaphane-GSH, sulforaphane cysteinylglycine. Anthocyanin and metabolites - syringic acid, ferulic acid 3,4-dihydroxyphenylpropionic acid, *m*-hydroxyphenylpropionic acid, gallic acid. Resveratrol and metabolites - *trans*-resveratrol (RSV), *trans*-resveratrol-3-O-sulfate, *trans*-resveratrol-4'-O-sulfate, *trans*-resveratrol-3,4'-O-disulfate, *trans*-resveratrol-3-O-glucuronide, *trans*-resveratrol-4'-O-glucuronide, dihydroresveratrol (DHR), 3,4'-O-dihydroxy-*trans*-stilbene, lunularin. Silibinin and metabolites - 20-O-β-D-Glu, 7-O-β-D-Glu, O-demethylated metabolite

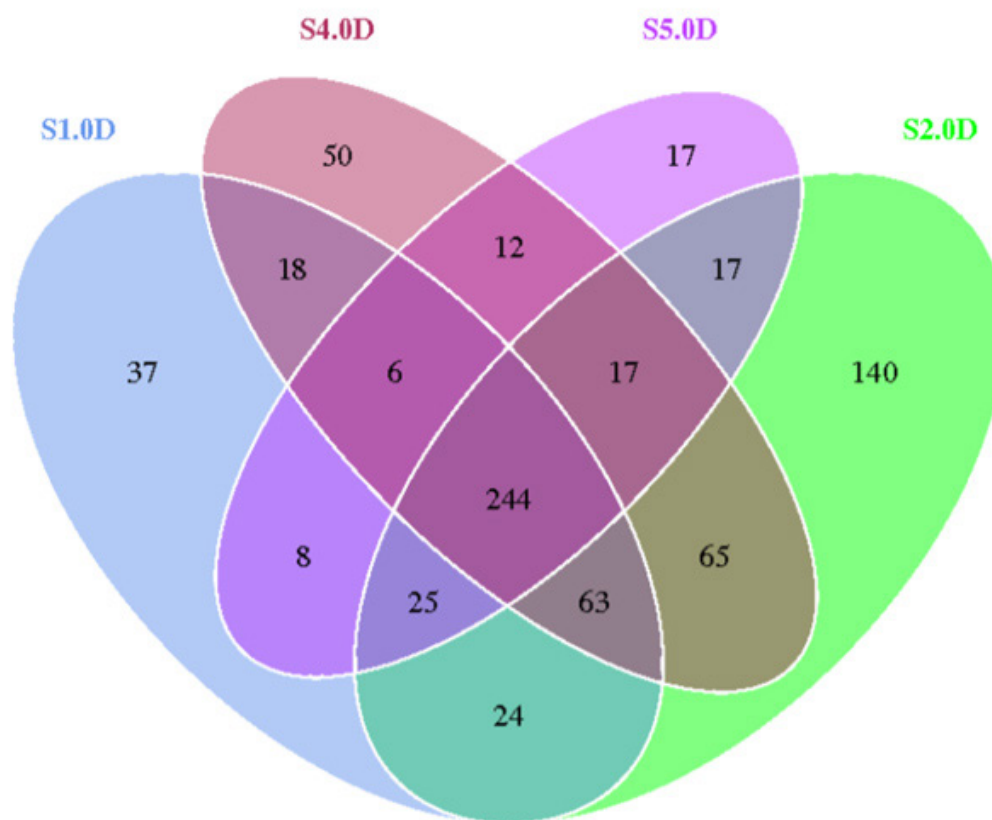


Figure 13. The figure shows a Venn diagram where each circle represents one group of microorganisms. The values in overlapping parts represent common Operational Taxonomic Units (OTUs) while others are OTUs specific to each group. Female HSD: ICR (CD-1[®]) mice at the age of 4 weeks were divided into four groups and given the following diets *ad libitum* for 20 weeks: S1.0D, standard synthetic diet; S2.0D; standard diet supplemented with 5% whole grape powder; S4.0D, high-fat (Western) diet; S5.0D, high-fat diet supplemented with 5% whole grape powder. The gut microbiota was analyzed using fecal samples. The data illustrate dietary grape supplementation influenced the composition of the gut microbiome. The overall organization of the gut microbiome is altered by grape and a higher number of microbiome groups can be observed in the grape fed diet groups

example, recent studies have demonstrated the neuroprotective potential of phytochemicals by reducing chemotherapy-induced CRCI in animal models. In addition, orally administered curcumin, at a dose of 100 mg/kg 1 h prior to cisplatin treatment, attenuated cisplatin-induced autophagy in the murine hippocampus accompanied by the activation of AMPK-JNK signaling^[283]. Further, piceid, a resveratrol derivative, given orally at a dose of 50 mg/kg/d for 4 weeks, protected against doxorubicin-induced cognitive impairment concomitant with the up-regulation of Nrf2, inhibition of the NF-κB pathway, and reduction of apoptosis in the rat hippocampus^[284]. It seems the ameliorative potential of chemoprevention is worthy of serious consideration.

In summary, over the past 50 years or so, many phytochemicals have been discovered and characterized that can delay, prevent, or reverse the process of carcinogenesis, and thereby modulate the incidence of the end stage cancer. Given the consequences of malignant metastatic disease, the promise of this therapeutic approach is evident yet remains largely untapped. As we continue to strive for primary intervention strategies that can be offered to all of those in need, additional potential benefits have become apparent, some of which are described herein and depicted in Figure 14. Not only does chemoprevention offer significant promise for disease prevention, but given the enormous economic burden imposed by contemporary biologic and drug therapies^[285], the approach would be cost-effective.

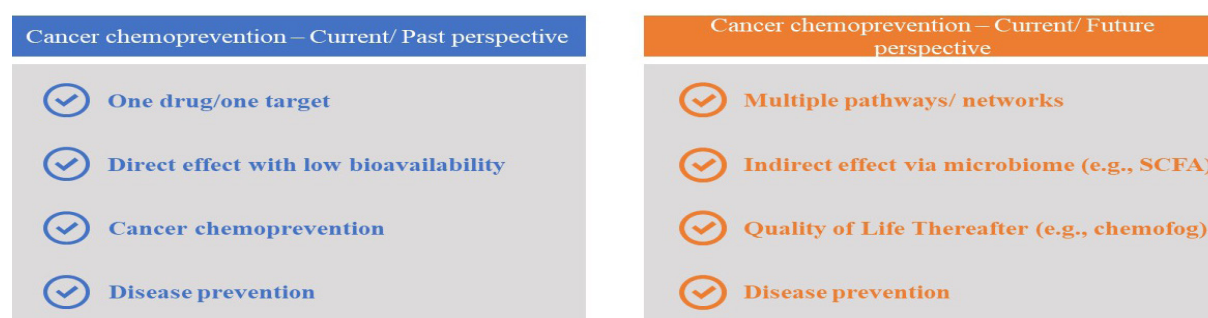
Dietary phytochemicals in cancer chemoprevention

Figure 14. Possible paradigm augmentation in cancer chemoprevention. As we realize shifts in the perspective of handling the dilemma of cancer, changing roles for chemopreventive agents can be envisioned. Emphasis is placed on sustaining “holistic health” and wellbeing

DECLARATIONS**Acknowledgments**

The authors are grateful to the California Table Grape Commission for providing support for this work.

Authors’ contributions

Participated in the conception and preparation of this review: Dave A, Parande F, Park EJ, Pezzuto JM

Availability of data and materials

Not applicable.

Financial support and sponsorship

This work was supported in part by the California Table Grape Commission.

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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ROR2 regulates the survival of murine osteosarcoma cells in lung capillaries

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How to cite this article: Tran DTP, Kuchimaru T, Pongsuchart M, Nguyen KT, Co Soriano JC, Kadonosono T, Kizaka-Kondoh S. ROR2 regulates the survival of murine osteosarcoma cells in lung capillaries. *J Cancer Metastasis Treat* 2020;6:47. <http://dx.doi.org/10.20517/2394-4722.2020.104>

Received: 22 Sep 2020 **First Decision:** 10 Nov 2020 **Revised:** 10 Nov 2020 **Accepted:** 24 Nov 2020 **Published:** 10 Dec 2020

Academic Editor: Ian Judson **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Aim: Lung metastasis is a leading cause of death in patients with osteosarcoma (OS). No effective therapy exists that improves the five-year overall survival rate of OS patients with metastasis. Therefore, finding novel therapeutic targets will help develop new treatment strategies for OS patients with lung metastasis.

Methods: Based on analysis of gene expression profiles between sublines of the Dunn OS LM8 cell line with high (LM8-H) and low (LM8-L) metastatic ability, we have identified Wnt signal-related genes that play an important role in lung metastasis of OS. Function of the genes was investigated by establishing sublines of gene knockout and assessing their metastatic ability using a mouse lung metastasis model. The molecular mechanism underlying the function of the genes was further investigated by *in vitro* experiments.

Results: We have identified that receptor tyrosine kinase-like orphan receptor 2 (ROR2), a receptor of the non-canonical Wnt signaling pathway, was involved in OS cell survival in lung capillaries during metastasis. LM8-H knocked out of *Ror2* (H/*Ror2*-KO) significantly reduced lung metastasis by decreasing the viability in lung capillaries 48 h after intravenous injection. *In vitro* study revealed that ROR2 increased anoikis resistance through AKT activation. Reconstitution of ROR2 expression in H/*Ror2*-KO cells restored their metastatic ability and viability in lung capillaries.



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Conclusion: The results demonstrate a novel ROR2 function in OS lung metastasis and may inform new treatment strategies for OS patients.

Keywords: Osteosarcoma, lung metastasis, cell survival, lung capillary, ROR2

INTRODUCTION

Osteosarcoma (OS) is a primary malignant bone tumor that typically occurs in the long bones, with peak incidence in adolescents and young adults^[1]. Distant metastases of OS, such as lung metastases, are hard to control and are usually associated with poor prognosis. Recent treatment advances involving combinations of chemotherapy, surgery, and other clinical applications have increased the survival rate of OS patients without lung metastasis to 60%. However, the survival rate of OS patients with lung metastasis has not improved over the past 30 years^[2]. A better understanding of the molecular mechanism behind the OS metastatic process will facilitate the development of new therapeutic strategies for improving the outcomes of patients with OS.

Wnt signaling plays essential roles in the malignant process of OS. The Wnt signaling transduction cascades are classified into two pathways: canonical (β -catenin-dependent) and noncanonical (β -catenin-independent) pathways^[3]. The factors of canonical Wnt signaling, such as Wnt3a, Wnt10, Lef1, and β -catenin, are involved in the development and malignant progression of OS^[4]. In contrast, noncanonical Wnt signaling is known to be transduced by binding Wnt5a to Frizzled or receptor tyrosine kinase-like orphan receptors (RORs), and promotes OS malignancies through phosphatidylinositol-3 kinase (PI3K)/AKT signaling^[5] and c-Jun N-terminal kinase (JNK) pathway^[6].

Receptor tyrosine kinase-like orphan receptor 2 (ROR2) is characterized by three main domains: the intracellular tyrosine kinase domain, extracellular Frizzled-like cysteine-rich domain, and membrane-proximal Kringle domain^[7-9]. The extracellular domain of ROR2 is assumed to mediate protein-protein interactions, and ROR2 functions as an alternative or coreceptor for Wnt5a, a representative noncanonical Wnt ligand^[10]. ROR2 activates JNK pathway^[11,12] and inhibits the β -catenin-dependent Wnt pathway^[10-12]. ROR2 is overexpressed in 73.8% of OS samples and is correlated with tumor metastasis^[13], suggesting a pivotal role in OS progression. However, the molecular mechanism of ROR2 function in OS lung metastasis has not been completely elucidated.

The process of OS metastasis to the lungs includes migration, intravasation into the circulation, survival in the circulation and lung capillaries, and extravasation into the lungs. In the lung capillaries, most of OS cells die for various reasons such as mechanical stress and lack of attachment to proper extracellular matrix (ECM). Anoikis is a type of apoptosis induced by the lack of proper ECM attachment necessary for cell maintenance in tissues^[14]. Tumor cells gain the ability to resist anoikis and undergo the metastatic process, prolonging their survival time and promoting migration and colonization at secondary sites^[15]. Several mechanisms of anoikis resistance in cells of epithelial origin have been described^[14]. However, there are few studies on anoikis resistance in cells of non-epithelial origin, such as OS cells.

We have recently established LM8 murine OS sublines with different abilities to metastasize to the lung using an *in vivo* image-guided screening system^[16]. Based on the microarray data of the LM8 sublines, we recently identified a novel lymphoid enhancer-binding factor 1 (LEF1)-cytoglobin (CYGB) axis as a part of noncanonical Wnt signaling pathway that promotes OS cell extravasation into the lungs^[16]. In the present study, we aimed to search for new therapeutic targets using the same techniques to develop treatment strategies for OS lung metastases.

METHODS

Gene-set enrichment analysis

Differentially expressed genes were selected from the microarray data of LM8 sublines and subjected to gene set enrichment analysis of the Kyoto Encyclopedia of Genes and Genomes. The analysis was performed with the clusterProfile package using R/Bioconductor. The reference gene set used was the Wnt signaling pathway (mm0431) set.

Cell culture

The LM8 cell subline was provided by Dr. Hideki Yoshikawa (Osaka University). LM8 cell line with high (LM8-H) and low (LM8-L) was established from LM8 in a previous study through the *in vivo* image-guided screening system^[16]. Murine vascular endothelium cells, bEnd3, were obtained from the American Type Culture Collection. All cells used in this study were cultured in 5% fetal bovine serum (FBS) Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL).

Reverse transcription PCR and quantitative PCR

Total RNA was extracted using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. One microgram of total RNA was reverse-transcribed using the Oligo(dT)20 primer (Toyobo, Osaka, Japan) and ReverTra Ace (Toyobo). Quantitative PCR (qPCR) and reverse transcription PCR (RT-PCR) were carried out using the Thunderbird® SYBR qPCR Mix (Toyobo) and EmeraldAmp® GT PCR Master Mix (Takara Bio, Shiga Japan), respectively.

Western blotting

Cells were lysed in RIPA buffer (50 mmol/L Tris HCl, pH 8.0; 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing a protease cocktail inhibitor (Nacalai Tesque, Kyoto, Japan), and protein concentration was determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA). Proteins were separated by electrophoresis on a 10% acrylamide gel, transferred to a hydrophilic polyvinylidene fluoride membrane (Merck, Kenilworth, NJ) and blocked with 5% skim milk or 5% bovine serum albumin in TBST (20 mmol/L Tris, pH 7.5; 150 mmol/L NaCl, 0.1% Tween 20). The membrane was then probed with relevant primary antibodies [anti-AKT (#2920S, 1:5000), anti-phospho-AKT (Ser473; #4060S, 1:5000), anti-ROR2 (#88639S, 1:5000), and anti-GAPDH (#2118S; Cell Signaling Technology, 1:5000)], and secondary antibodies [anti-mouse IgG HRP-linked antibody (#7076, 1:5000) and anti-rabbit IgG HRP-linked antibody (#7074, 1:5000, Cell Signaling Technology)]. The resultant membranes were washed with TBST and detected by ImageQuant LAS 4000 (GE Healthcare Life Science, Marlborough, MA) after processing with Chemi-Lumi One (Nacalai Tesque). To investigate the effect of AKT inhibition, cells were treated with 50 nmol/L MK2206 in 5% FBS DMEM for 24 h.

Gene knockout using the CRISPR-Cas9 system

Ror2 gene knockout (KO) was performed in LM8-H cells to establish the cell line H/*Ror2*-KO by using the CRISPR-Cas9 system. The sequence of the guide RNA (5'-caccgTCGTGGCTCTTGCACAACCG-3') was used for targeting *Ror2*. The *Ror2* guide RNA was inserted into a unique BbsI site of the pX330 plasmid (42230; Addgene). The cells whose genomes were correctly edited by the CRISPR-Cas9 system were selected by using a fluorescence indicator system with the pCAG/EGFP plasmid^[17], provided by Dr. Masahito Ikawa (Osaka University). GFP-positive cells were picked up, and ROR2 protein expression level was validated by western blotting.

Establishment of cell lines with ROR2-overexpression

The cDNA of *Ror2* (NM_013846.4) was amplified using the KOD® FX Kit (Toyobo) using the following primer set: 5'-TGGAATTCTGCAGATATGGCTCGGGGCTGGGTG-3' and 5'-GCCACTGTGCTGGA TTCAGGCTTCAAGCTGGACATG-3'. The *Ror2* cDNA fragment was cloned into the pcDNA3.1-myc-

His vector (Invitrogen, Carlsbad, CA) at the *EcoRV* site using the Infusion Cloning Enhancer kit (Takara Bio). LM8-L and H/*Ror2*-KO cells were transfected with the plasmid by the NEPA21 type II electroporator (NEPAGENE, Chiba, Japan), and appropriate transfectants were cloned to establish L/ROR2 and KO/ROR2 after culturing with G418 (2 mg/mL) selection medium.

Proliferation assay

Cell proliferation was evaluated with the water-soluble tetrazolium salt 1 (WST-1) reagent (Sigma Aldrich, St. Louis, MO) according to the manufacturer's instructions. Cells (1×10^3 cells/100 μ L culture medium) were seeded in 96-well plates. After culturing for 24, 48, or 72 h, the medium was removed and 100 μ L of WST-1-containing medium (10-fold dilution) was added to each well. The cells were further incubated for 3 h, and then the absorbance of each well was measured at 450 nm with a reference wavelength of 750 nm after shaking the plates for 1 min with the microplate reader Model 680XR (Bio-Rad, Hercules CA).

Mice

Male C3H mice were obtained from the Charles River Laboratory (Yokohama, Japan). All mice used were 6-8 weeks of age and were housed in the animal facilities at the Tokyo Institute of Technology. Animal experiments were performed with the approval of the Animal Ethics Committees of the Tokyo Institute of Technology (no. D20170004-2) and in accordance with the Ethical Guidelines for Animal Experimentation of the Tokyo Institute of Technology.

Analysis of OS lung metastasis model

The mice were injected intravenously with 1×10^6 LM8 cells in 100 μ L of PBS. Twenty days after inoculation, isolated lungs were embedded in an optimal cutting temperature compound (Sakura Finetechnical Co., Ltd, Tokyo, Japan) and stored at -80 °C overnight. Lungs were divided into cryosections of 10 μ m in thickness and then fixed in 4% paraformaldehyde. Fixed lung cryosections were then stained with hematoxylin and eosin (HE) and observed under a microscope BZ-X710 (Keyence, Osaka, Japan). A whole lung image was obtained by stitching together partial lung images using BZ-X analyzer software (Keyence).

Analysis of tumor cells in lungs

Cells were labeled with 100 μ mol/L CellTracker® Green (Thermo Fisher Scientific) and intravenously injected into C3H mice (1×10^6 cells/100 μ L PBS). DyLight® 594-labeled isolectin B4 (6 mg/kg; Vector Laboratories, Burlingame, CA) was injected intravenously to stain endothelial cells 5 min before dissecting the mice. The lungs were collected and observed under confocal microscopy (Carl Zeiss, Oberkochen, Germany). The number of fluorescently labeled cells in the three microscope fields of each lung was quantified using ImageJ software (<http://rsb.info.nih.gov/ij/>)^[18]. Results are shown as the average number of cells per field. Each group was analyzed in triplicates. For measuring the fluorescence intensity of the lungs, lung lysate was prepared with RIPA buffer and well-homogenized. The supernatant was then collected, and the fluorescence intensity was measured using Infinite F500 (Tecan, Männedorf, Switzerland) with a filter set for CellTracker® Green (Ex/Em = 480 nm/517 nm).

Anoikis assay

Poly(2-hydroxyethyl methacrylate) (poly-HEMA; Thermo Fisher Scientific) was completely dissolved in 95% ethanol (20 mg/mL). Plates were coated with poly-HEMA solution and dried on a clean bench overnight. Cells (5×10^4) in serum-free medium were seeded in a poly-HEMA-coated 24-well plate. After 24 h, calcein AM (Thermo Fisher Scientific) and ethidium homodimer-1 (Thermo Fisher Scientific) were added at final concentrations of 2 μ mol/L and 4 μ mol/L, respectively, and the cells were further incubated for 30 min. The fluorescent signal was observed under fluorescence microscopy and quantified with the BZ-X Analyzer (Keyence). To investigate the effect of AKT on LM8 anoikis, the AKT inhibitor MK2206

(Nacalai Tesque) was prepared with dimethyl sulfoxide (DMSO), and the cells were cultured for 24 h in poly-HEMA-treated plates containing 50 nmol/L MK2206 in 5% FBS DMEM.

Statistical analysis

Data were statistically analyzed with a two-sided Student's *t*-test. *P*-values of less than 0.05 were considered statistically significant.

RESULTS

ROR2 function was required for OS lung metastasis

Our recent study using the LM8 sublines (LM8-H and LM8-L) and their gene expression microarray data revealed that noncanonical Wnt signaling is associated with LM8 extravasation into lung tissue via the LEF1-CYGB axis^[16]. The microarray data of LM8-H and LM8-L was used to search for genes associated with the noncanonical Wnt pathway. Gene set enrichment analysis^[19] identified several Wnt signaling-related genes that showed higher expression in LM8-H than in LM8-L [Figure 1A]. Mapping of these genes using Kyoto Encyclopedia of Genes and Genomes^[20] and clusterProfile package using R/Bioconductor indicated that ROR1/2 were receptors for the noncanonical Wnt signaling that is likely involved in the lung metastasis of LM8 [Supplementary Figure 1]. Expression levels of noncanonical Wnt signaling receptor genes *Ror1*, *Ror2*, and *Frizzled class receptor 1* (*Fzd1*) were examined by RT-PCR. *Ror2*, but not *Ror1* or *Fzd1*, was expressed at a significantly higher level in LM8-H than in LM8-L [Figure 1B]. Furthermore, the mRNA level of *Ror2* was well-correlated with the metastasis-free survival of OS patients [Supplementary Figure 2]. Therefore, we focused on ROR2 for further analysis. The effect of deletion of *Ror2* [Figure 1C] on the proliferation and metastatic potential of LM8-H was first investigated. The proliferation rate of H/*Ror2*-KO was similar to that of LM8-H and LM8-L [Figure 1D], indicating that ROR2 function is not involved in proliferation. The size and number of foci in the lungs injected with H/*Ror2*-KO were significantly smaller than those in the lungs injected with LM8-H [Figure 1E]. These data demonstrated the critical role of ROR2 function in LM8 lung metastasis.

ROR2 was necessary for LM8 survival in lung capillaries

To determine how ROR2 was involved in the extravasation process *in vivo*, the LM8 sublines in the lungs were examined at 30 min and 48 h post-injection because the tumor cells had reached the lungs by 30 min and began extravasation into lung tissues by 48 h post-injection. The LM8 sublines were labeled with a green fluorescent dye before injection, and removed lungs were observed under an inverted confocal fluorescence microscope. Similar numbers of fluorescently labeled cells were observed in the lungs injected with LM8-H and H/*Ror2*-KO 30 min after injection [Figure 2A], indicating that lack of ROR2 function did not affect the survival of circulating LM8 cells before reaching the lungs. On the other hand, 48 h after injection, the number of fluorescently labeled cells in lungs injected with H/*Ror2*-KO was significantly reduced compared to that in lungs injected with LM8-H [Figure 2A]. The reduction reflects that more H/*Ror2*-KO die in lung capillaries than LM8-H, suggesting that ROR2 function was involved in the survival of LM8 cell in lung capillaries prior to extravasation. To confirm ROR2's function, LM8-L, LM8-H, H/*Ror2*-KO, ROR2-overexpressing LM8-L (L/ROR2), and ROR2-overexpressing H/*Ror2*-KO (KO/ROR2) [Figure 2B] were examined at 48 h post-injection in terms of their survival in lung capillaries. Significantly higher fluorescence intensity was detected in lung lysates from mice injected with the LM8 sublines expressing high ROR2 (LM8-H, L/ROR2, and KO/ROR2) compared to that in the lung lysates from mice injected with the LM8 sublines expressing low ROR2 (LM8-L and H/*Ror2*-KO) [Figure 2C]. These results confirmed a correlation between ROR2 expression and LM8 survival in lung capillaries.

The AKT signaling pathway was involved in ROR2-induced anoikis resistance in LM8

Tumor cells that have reached the lung capillaries are stressed mechanically because the blood vessels are smaller in diameter and are less compliant^[21]. This mechanical stress causes cell death in up to more

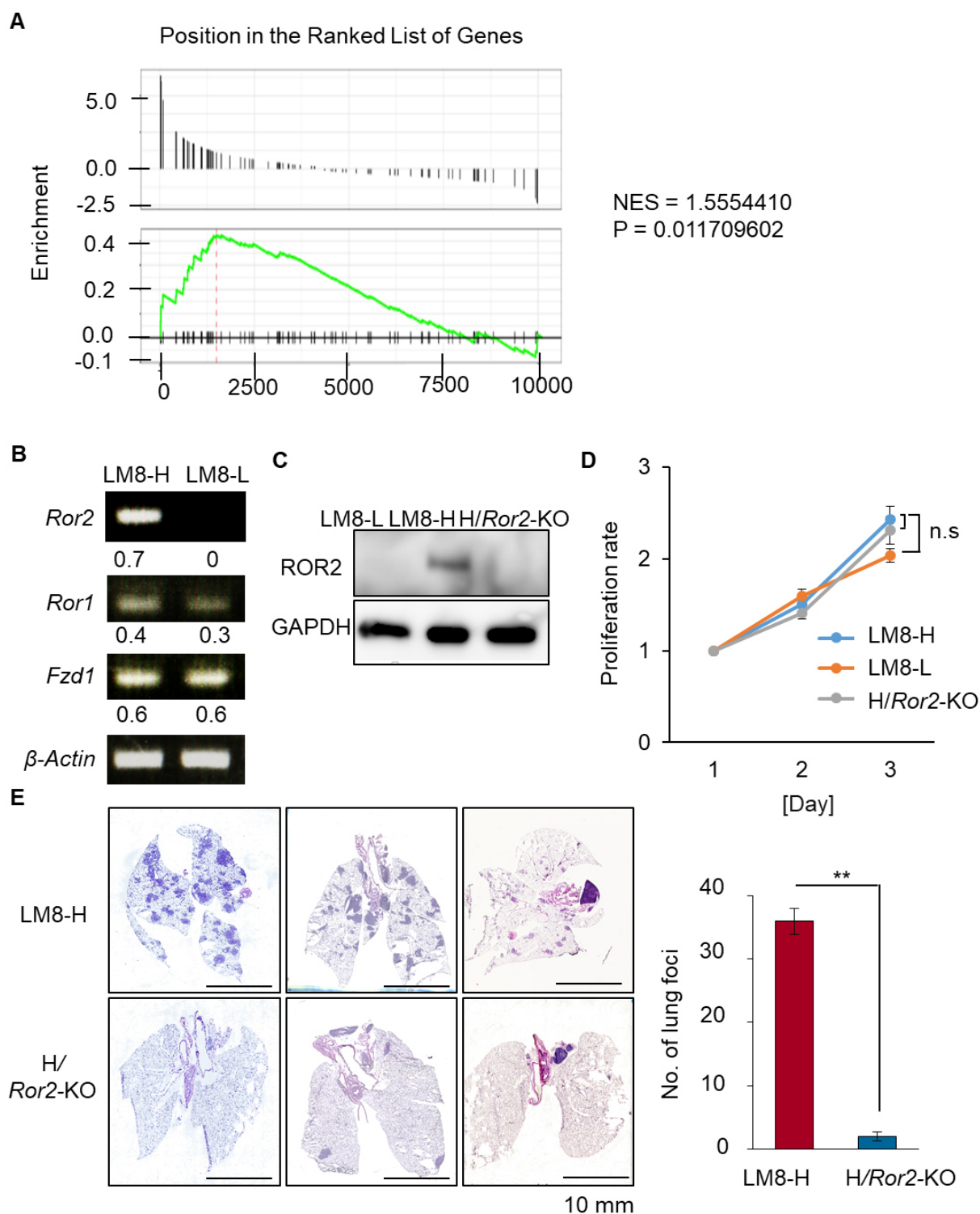


Figure 1. ROR2 regulated lung metastasis of LM8. A: gene set enrichment analysis. Microarray data for LM8-H and LM8-L were analyzed using Kyoto Encyclopedia of Genes and Genomes software to identify significant gene sets. The enrichment plot shows the distribution of genes in the set that are correlated with the Wnt signaling pathway (mm0431); B: mRNA expression levels of *Ror2*, *Ror1*, and *Fzd1* in LM8-H and LM8-L analyzed by RT-PCR. The numbers below the bands indicate the corresponding expression levels relative to β -actin; C: ROR2 protein expression levels in LM8-L, LM8-H, and H/Ror2-KO examined by western blotting; D: proliferation rates of LM8-H, LM8-L, and H/Ror2-KO cultured under adhesion conditions for 3 days; E: representative lung images stained with hematoxylin-eosin (left) and the number of lung foci bigger than 1 mm in diameter (right) at day 20 after intravenous injection of LM8-H and H/Ror2-KO. Scale bars: 10 mm, $n = 5$, $^{**}P < 0.01$. ROR2: receptor tyrosine kinase-like orphan receptor 2; LM8-H: LM8 cell line with high metastatic ability; LM8-L: LM8 cell line with low metastatic ability; *Fzd1*: Frizzled class receptor 1; H/Ror2-KO: LM8-H knocked out of *Ror2*; n.s: not significant

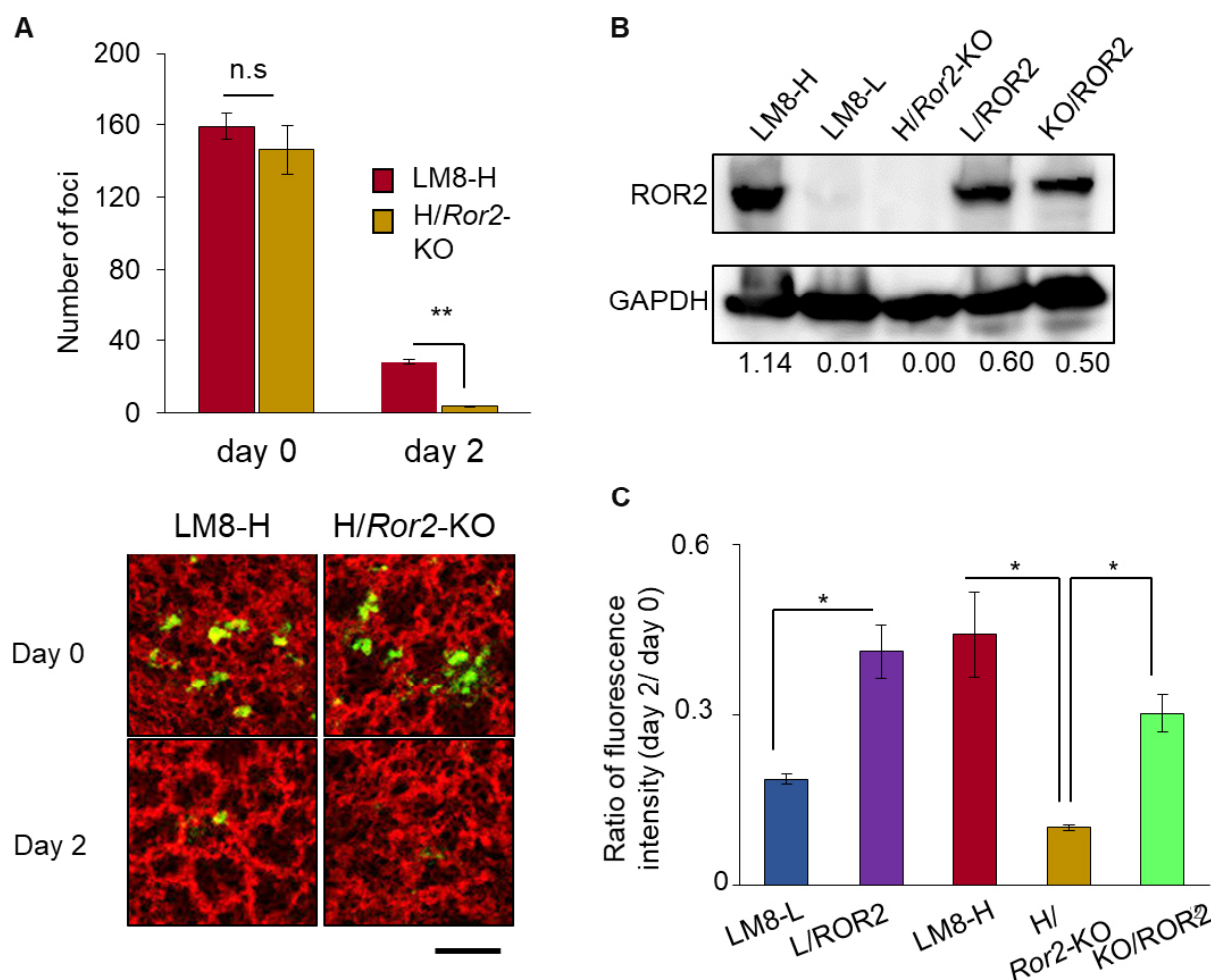


Figure 2. ROR2 regulated the survival of LM8 in lung capillaries. A: fluorescently labeled LM8 cells in the lungs were quantified using an inverted confocal fluorescence microscope (top). Data are shown as the mean \pm SD of three fields of fluorescently labeled cells in each lung on the indicated days. $n = 3$, $**P < 0.01$. Representative image of LM8 cells in lungs on the indicated days (bottom). Red: blood vessel, Green: tumor cell, Black: lung tissue. Scale: 50 μ m; B: ROR2 protein level in the indicated LM8 sublines. The numbers below the bands indicate the corresponding ROR2 expression levels relative to GAPDH; C: fluorescence intensity in lung lysates prepared from mice injected with the indicated LM8 sublines was quantified on day 0 (30 min) and day 2 (48 h). Data are shown as the ratio of day 2 fluorescence intensity normalized to day 0. $n = 3$, $*P < 0.05$. ROR2: receptor tyrosine kinase-like orphan receptor 2; LM8-H: LM8 cell line with high metastatic ability; LM8-L: LM8 cell line with low metastatic ability; H/Ror2-KO: LM8-H knocked out of Ror2; L/ROR2: LM8-L expressing ROR2; KO/ROR2: H/Ror2-KO expressing ROR2; n.s: not significant

than 90% of tumor cells entering the capillaries^[22]. The LM8 sublines were examined for sensitivity to mechanical stress *in vitro* using a hypotonic buffer that causes hypotonic cell swelling, an established perturbation method that examines the strength against mechanical stress by inducing elongation of the plasma membrane in a well-controlled manner^[23]. Incubation of LM8-H and H/Ror2-KO in hypotonic buffer for 30 min reduced viability by less than half in both sublines compared to incubation in isotonic buffer [Supplementary Figure 3], indicating that ROR2 was not involved in the resistance to mechanical stress in the lung capillaries. The differences in anoikis resistance among the LM8 sublines were examined in terms of viability after culturing for 24 h on poly-HEMA-coated dishes (under low adhesion conditions) using fluorescent dyes that differentially label live and dead cells. The LM8 sublines with low ROR2 expression (LM8-L and H/Ror2-KO) significantly increased anoikis, and those with high ROR2 expression (L/ROR2 and KO/ROR2) significantly decreased anoikis [Figure 3A]. It has been reported that the activity of Akt was upregulated in anoikis resistant human OS cells^[24]. Therefore, we investigated the involvement of AKT signaling in LM8 ROR2-induced anoikis resistance. First, AKT activation (Ser473 phospho-

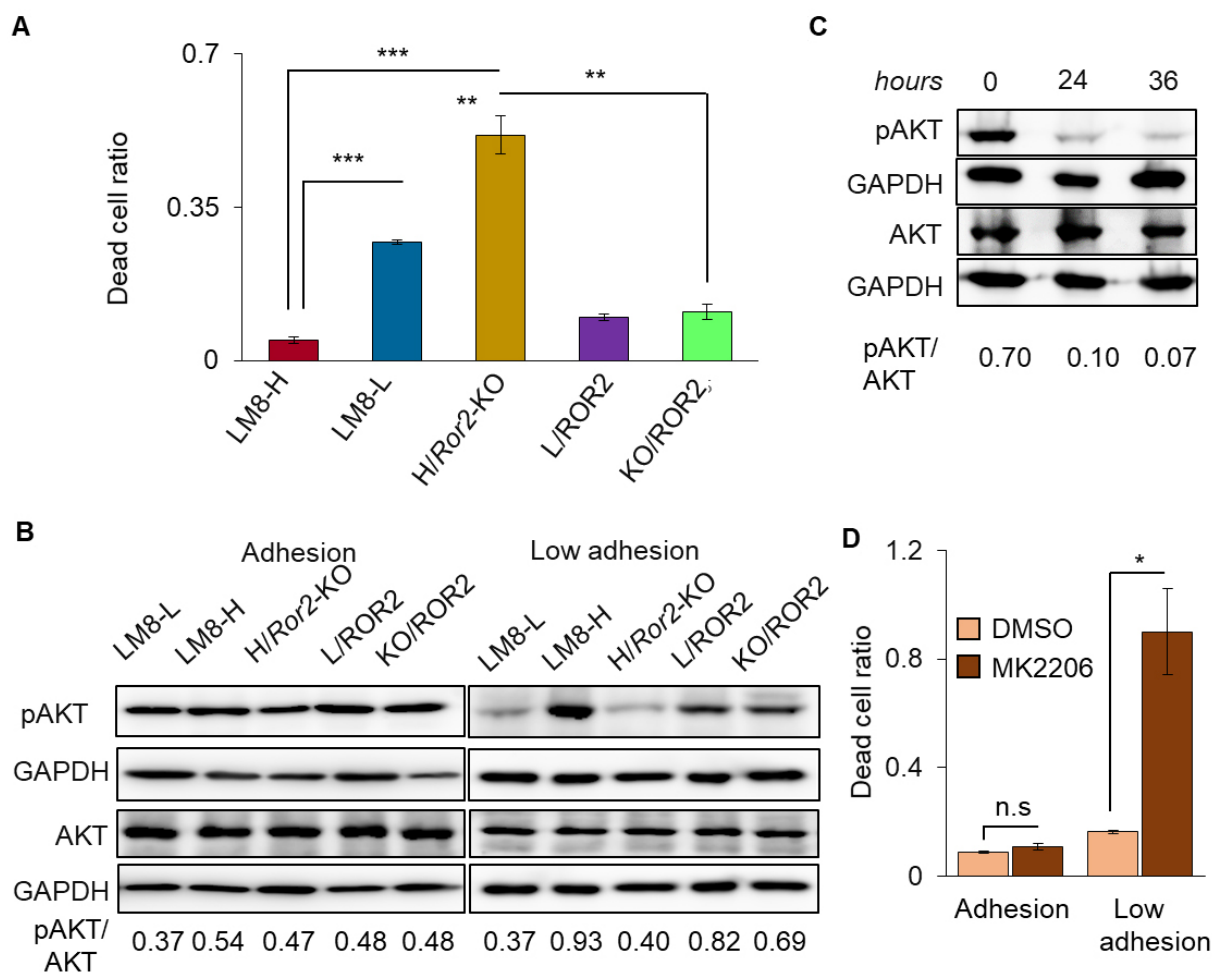


Figure 3. AKT signaling was involved in anoikis regulation by ROR2 in LM8. A: fluorescence intensities of live and dead cells labeled with different dyes were measured using appropriate filters after 24 h of culture under low adhesion conditions as described in the Methods. The dead cell ratio was calculated by dividing the fluorescence intensity of the dead cells by the total fluorescence intensity. $n=3$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; B: pAKT and AKT levels in LM8 sublines cultured under adhesion (left) and low adhesion (right) conditions analyzed by western blotting. The pAKT/AKT at the bottom of the figure shows the ratio of pAKT to AKT levels normalized by GAPDH levels; C: pAKT and AKT levels in LM8-H treated with MK2206 for indicated time under low adhesion conditions. The western blotting experiments were repeated three times, and representative data are shown; D: dead cell ratio of LM8-H cells cultured with MK2206 or solvent only (DMSO) for 24 h under adhesion or low adhesion conditions. Dead cell ratios are shown as normalized values for cells treated with MK2206 vs. those for untreated cells. $n=3$, $*P < 0.05$. ROR2: receptor tyrosine kinase-like orphan receptor 2; LM8-H: LM8 cell line with high metastatic ability; LM8-L: LM8 cell line with low metastatic ability; H/Ror2-KO: LM8-H knocked out of Ror2; n.s: not significant; DMSO: dimethyl sulfoxide; L/ROR2: LM8-L expressing ROR2; KO/ROR2: H/Ror2-KO expressing ROR2

AKT/AKT ratio) was examined in LM8 sublines with different ROR2 expression levels. Under adhesion conditions, AKT activation was not related to ROR2 expression levels. However, higher AKT activation was observed in the LM8 sublines with high ROR2 expression (LM8-H, L/ROR2, and KO/ROR2) compared to the LM8 sublines with low ROR2 expression (LM8-L and H/Ror2-KO) under low adhesion conditions [Figure 3B]. Second, the correlation between AKT activation and anoikis was examined using MK2206, an AKT inhibitor. AKT activation in LM8-H under low adhesion conditions was significantly suppressed by MK2206 [Figure 3C], resulting in a significant increase in anoikis in these cells [Figure 3D]. These results suggest a novel ROR2 function involved in LM8 anoikis resistance through AKT activation.

DISCUSSION

In the present study, we identified a novel function of ROR2 in lung metastases of the mouse OS cell line LM8; ROR2 may contribute to OS cell survival in lung capillaries by increasing anoikis resistance through

AKT activation. Previous studies of various tumor cells have reported important functions of ROR2 in migration and invasion^[25-29], which are essential metastasis initiation steps in the pre-circulatory processes. The lung metastasis model used in this study was designed to focus on the post-circulatory processes of metastasis, allowing us to discover another important function of ROR2 in the lung metastatic process.

Previous study using the same lung metastasis model revealed that CYGB function is crucial for LM8 extravasation ability, and *Cygb* was previously identified as a LEF1-regulated gene^[16]. Although *Ror2* is one of the Wnt signaling-related genes, the ROR2 expression level did not correlate with the LEF1 expression level [Supplementary Figure 4A]. In contrast, *Cygb* mRNA levels were significantly reduced in LM8-L and H/*Ror2*-KO [Supplementary Figure 4B], suggesting that ROR2 signaling may be an alternative CYGB regulator independent of LEF-1 signaling in LM8. In fact, overexpression of ROR2 in LM8-L resulted in significantly increased endothelial transmigration abilities while knocking out of *Ror2* in LM8-H resulted in significantly decreased endothelial transmigration abilities [Supplementary Figure 4C]. These results are similar to those observed in the previous study using CYGB-overexpressing LM8-L and *Cygb*-KO LM8-H^[16]. Together with our previous study of the LEF1-CYGB axis, this study of ROR2-CYGB axis provided new insight into the mechanism of Wnt-signaling in OS lung metastasis. Although the Wnt5a-ROR2 axis has been reported to induce the migration of human OS cells^[25,28,30,31], we found that recombinant Wnt5a did not promote transmigration of LM8-H [Supplementary Figure 5], suggesting that Wnt5a-ROR2 signaling was not involved in the post-circulatory process of lung metastasis in LM8 sublines.

We demonstrated that the anoikis resistance regulated by ROR2 was dependent on AKT activation and that AKT inhibition reduced ROR2-induced anoikis resistance. Since *Ror2* expression level did not affect LM8 cell proliferation under adhesion conditions [Figure 1D], the increased viability of LM8 sublines with high ROR2 expression is not due to increased proliferation. The AKT inhibitor used here, MK2206, is currently in phase II clinical trials for recurrent and advanced endometrial cancer. In addition to inhibiting tumor growth in neuroblastoma and colorectal cancer^[32], MK2206 also impairs the proliferation of human OS cells such as U2OS and HOS^[33]. MK2206 was found to reduce LM8-H viability only under low adhesion conditions, and no cell death was observed with MK2206 treatment under adhesion conditions. These results indicate that MK2206 might be an ideal drug to specifically kill OS cells that have lost anchorage dependency, such as cells that have acquired the ability to invade and metastasize.

In conclusion, our findings suggested that the ROR2-AKT axis enhanced OS lung metastasis through anoikis resistance and that MK2206 enhances the induction of apoptosis in OS cells under low adhesion conditions. These discoveries may shed light on specific stages of the metastatic process and provide clues for the development of effective OS treatment.

DECLARATIONS

Acknowledgments

We thank Sakiko Yonezawa and Ngoc Thi Hong Hoang for technical advice of Western blotting, qPCR and RT-PCR. We also thank the Open Research Facilities for Life Science and Technology, Tokyo Institute of Technology for technical assistance. We would like to thank Editage (www.editage.com) for English language editing.

Authors' contributions

Designed overall projects: Tran DTP, Kuchimaru T, Kizaka-Kondoh S

Performed experiments: Tran DTP, Kuchimaru T

Analyzed and interpreted data: Tran DTP, Kuchimaru T, Pongsuchart M, Nguyen TK, Co Soriano JC, Kadonosono T, Kizaka-Kondoh S

Wrote the manuscript: Tran DTP, Kizaka-Kondoh S.

Availability of data and materials

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

Financial support and sponsorship

This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas “Integrative Research on Cancer Microenvironment Networks from the Ministry of Education”, Culture, Sports, Science and Technology of Japan” (Kizaka-Kondoh S), and Grant-in-Aid for Young Scientist (B) (Kuchimaru T)

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Male C3H mice were obtained from the Charles River Laboratory, Japan (Yokohama, Japan). All mice used were 6-8 weeks of age and were housed in the animal facilities at the Tokyo Institute of Technology. Animal experiments were performed with the approval of the Animal Ethics Committees of the Tokyo Institute of Technology (No. D20170004-2) and in accordance with the Ethical Guidelines for Animal Experimentation of the Tokyo Institute of Technology.

Consent for publication

Not applicable.

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Commentary

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Isoflavone research towards healthcare applications

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How to cite this article: Gryniewicz G. Isoflavone research towards healthcare applications. *J Cancer Metastasis Treat* 2020;6:48. <http://dx.doi.org/10.20517/2394-4722.2020.112>

Received: 10 Oct 2020 **First Decision:** 20 Nov 2020 **Revised:** 23 Nov 2020 **Accepted:** 30 Nov 2020 **Published:** 10 Dec 2020

Academic Editor: Sanjay Gupta **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

A survey of the current literature on natural isoflavones and their biological activity is presented. This subcategory of a large group of plant polyphenolics has particular characteristics, structural as well as pharmacological, which makes it suitable for discussion of pleiotropic activities of phytochemicals and their exploitation in healthcare, beyond the concept of selectively targeted new drugs for narrow therapeutic indication.

Keywords: Isoflavones, phytoestrogens, genistein, novel drugs, nutraceuticals, functional food

INTRODUCTION

Aging populations inevitably suffer from chronic pathologic conditions recognized as metabolic syndrome, cancers, and neurodegenerations, with projections of ever-increasing burden on future healthcare budgets and service providers^[1,2]. Modern medicine is constantly gaining efficient new tools based on advances in molecular biology and genetic engineering, but they are used more for cancer biology research, diagnostics and experimental procedures than in routine clinical therapeutic interventions. These typically rely on the administration of pharmacologically active preparations provided by the pharmaceutical industry, via a lengthy and exorbitantly costly multistage process of drug discovery and development. One of the most outstanding problems facing contemporary human healthcare, even in most affluent societies, is cancer treatment, since oncological morbidity and mortality continue to be leading causes of death, projecting a gloomy epidemiological forecast for the future^[1,2]. Despite cancer pharmacology and the corresponding drug category started from synthetic alkylating agents, the overall pool of contemporary



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oncological therapeutics contains a very high proportion of natural products (NP) - native or slightly modified phytochemicals (notable examples include: anthracyclines, bis-indolyl alkaloids of *Vinca* and *Catharanthus*, diterpene taxanes, marine nucleoside analogs, podophyllotoxins, topotecans, *etc.*)^[3-6]. Through pharmacognosy, every newly explored class of secondary metabolites was at first perceived as a collection of drug leads, based on the rich tradition of ethnopharmacology, which used to secure a natural remedy for every ailment. After a period of high hopes connected with high throughput chemical syntheses as source of new pharmaceuticals, NPs appear to offer better new drug leads, as well as better chances for successful chemopreventive interventions^[7-10], provided support from cheminformatics and bioinformatics is properly applied. Presently, concerning very large but limited resources of NPs, we know what we do not know. With the number of species estimated at ca. 350 thousand, the number of secondary metabolites probably tops one million chemical entities; however, present lists of identified compounds from biological sources barely exceed 250 thousand in total^[11-13], and knowledge of their biological activities is scant and fragmentary. The structural diversity of naturally derived chemicals is of particular value because of their intrinsic biocompatibility, indicating structure - biological activity relationships, essential for medicinal chemistry and novel drug design. We can imagine chemical space as a collection accommodating not only compounds from all databases but in fact all possible chemical structures (estimated at ca. 10^{60} in total), which can be navigated in search of structure clusters, featuring compounds of desired biological activities and acceptable physicochemical properties. Currently available cheminformatics tools make such searches possible, and new artificial intelligence (AI) and machine learning (ML) methodologies help to turn any collection of chemical structures (such as a class of phytochemicals) into a big data resource, through an extensive parametrization of its elements. Such an operation allows the substitution of some expensive biological activity testing with the property assessment by *in silico* modeling. Thus, a large pool of phytochemical metabolites can be conveniently segmented into subcategories of compounds that are privileged by featuring some desirable parameters, such as affinity to selected molecular targets^[14,15]. It is generally believed that plant polyphenolics constitute a collection of metabolites with relatively high chemical affinity to peptides and proteins, representing a rich pool of prospective drug leads. The group is large and very heterogeneous, biogenetically and structurally. Although it has provided many contemporary drugs via traditional pharmacology efforts comprising target-based and structure-based searches^[4-6], we prefer to select a smaller and more structurally consistent NP group for our assessment of prospective phytochemicals valued for therapy and prevention. Flavonoids can be chosen as a representative group of plant phenolics, having medium size (approx. 10 thousand), well-defined biogenesis, and structural similarity, which nevertheless contains considerable amount of native diversity and ample room for its expansion, via chemical or biotechnological derivatization^[16-18]. Flavonoid subcategories [Figure 1] feature very interesting pharmacological activities, exemplified in a small and structurally distinct group of isoflavones [Figure 2], important for human nutrition as constituents of the leading agricultural crop - soybeans^[19-21]. In our opinion, focus on isoflavone research serves well to illustrate changing trends in the role of phytochemicals in the interface between official academic medicine, less regulated segments of healthcare, and professional nutrition sciences. However, for a closer look and better perception of isoflavones, their placement within a wider context of the biogenetic family of flavonoids seems advisable.

FLAVONOID PHARMACOLOGY OUTLOOK

The name flavones (from Latin word for yellow; later expanded to flavonoids to accommodate more structural variety) was coined by S. Kostanecki for a group of yellow plant dyes containing a chromane nucleus with aromatic (phenolic) substituents, towards the end of the XIXth century^[22,23]. Early stages of their biological activity studies were expertly summarized in numerous monographic works^[24-26]. Roles of flavonoids in plant physiology and ecology are now better understood and can be related to the biology of other organisms, including humans^[27-30]. Historically, interest in flavonoids was limited to a narrow field of natural pigments and their occurrence and chemistry. In the 1930s, amid the race for vitamin C resources, Albert Szent-Györgyi noticed that citrus and green pepper flavonoids (then called "citrin" and proposed to

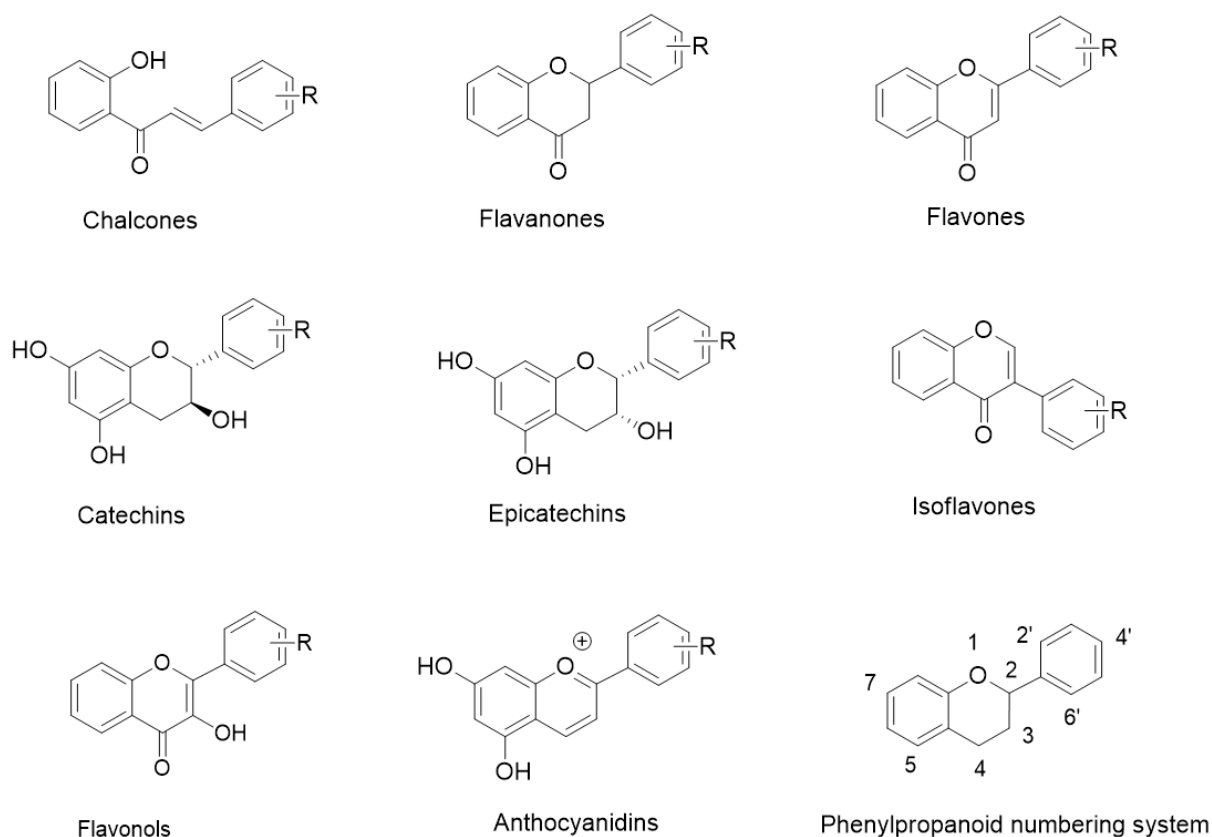


Figure 1. Principal categories of natural flavonoids

be included in a vitamin category) greatly enhanced the antiscorbutic activity of L-ascorbic acid^[31], which commenced lasting interest in their pharmacological activity. Flavonoids constitute a class of ubiquitous plant metabolites, which stem from common biogenetic transformation, combining shikimic acid - phenylalanine, and acetyl CoA pathways, resulting in the assembly of a phenylpropanoid scaffold ending up as 2- or 3-phenylchromane derivatives, categorized as: flavones, flavanones, catechins, anthocyanidins, and isoflavones^[26,27]. These structures tend to be further diversified biocatalytically by oxidation, hydroxyl group alkylation (methylation, isoprenylation), and/or glycosylation, but also by C-C coupling reactions, which lead to oligomerization and polymerization, producing in turn a wide array of structural variety, and consequently a very complex matrix of biological activity (Figure 1 shows low-molecular-weight flavonoids only). Presently, it is clear that the biogenesis of flavonoids, which is common throughout the plant kingdom, can be considered one of the most significant evolutionary developments, occurring first as a protective environmental adaptation and further adopted for many other functions^[32].

In the last decades, hundreds of new papers devoted to the biological activity of plant flavonoids (including review papers) have appeared in the scientific literature. The majority have dealt with preclinical pharmacology, studied using molecular and subcellular models, as well as cell lines and experimental animals^[33-40]. Antioxidant activity and anti-inflammatory pathways were among the favorite study subjects^[28]. Cancer prevention and anticancer activity evoked continuous interest^[20,30] and the proportion of papers heralding the intimate connection between flavonoids and cancer is remarkably high^[37-41]. Comments on flavonoid systemic activity point out their limited bioavailability^[42,43], which offers poor prognosis for clinical efficacy. Despite encouraging results in many preclinical studies, no flavonoid drug candidate could be seen advancing beyond early stages of clinical trials until very recently. A breakthrough came from the side of dihydrochalcone - phloretin glycoside, (phlorizin, known since the mid-XIXth

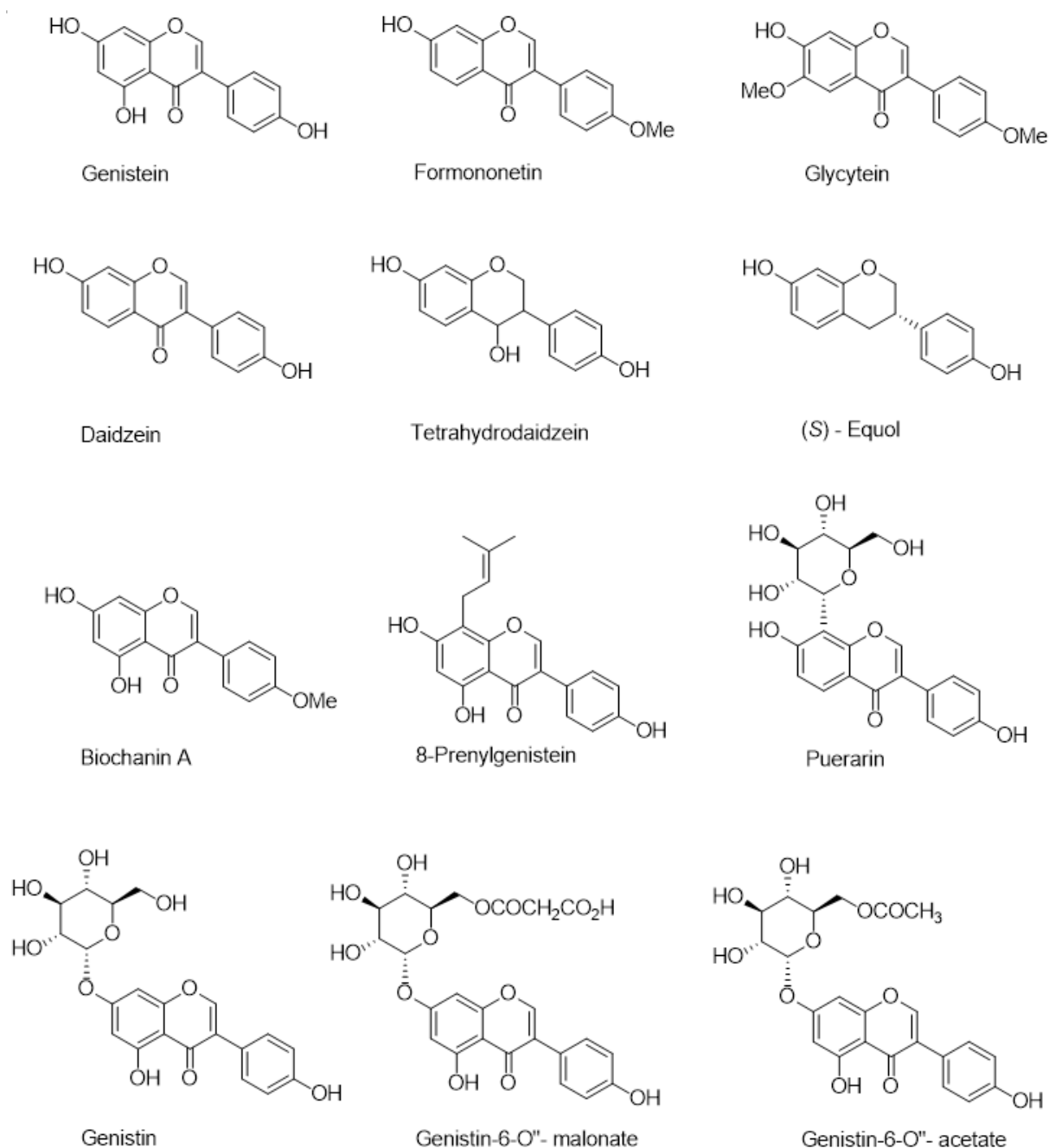


Figure 2. Representative structures of natural isoflavones

century), which surfaced as an antidiabetic drug candidate, featuring effective inhibition of GLUT family D-glucose transporters. Phlorizin turned out to be metabolically unstable, because susceptibility to hydrolases; this ultimately led to the synthesis of C-glycosyl analogs, which turned into a new generation of antidiabetics, started by the launch of dapagliflozin in 2012^[44-46].

ISOFLAVONE ESTROGENS AND FOOD CONNECTION

One immediate reflection on the massive amount of research accumulated in recent decades on flavonoid biological activity, is that of the large discrepancy in adopted quality standards, which even became a subject of separate study^[47,48]. Strict adherence to accuracy in measurement and good laboratory practices

should certainly help in interdisciplinary discussions of somewhat fuzzy notions such as research material specification, molecular similarity, antioxidant properties, estrogenicity, chemoprevention, *etc.* Despite the constant refinement of scientific information through the exchange of peer opinion, some statements that lack factual support can persist in literature for decades. Nearly all papers tackling the subject of isoflavone activity underline genistein similarity to 17- β -estradiol (E2), offering only a single parameter - distance of ca. 12Å between two hydroxyl groups in both ER ligands for support of that opinion. Such superficial statements disregard the fact that molecular similarity is an important issue in current cheminformatics as well as medicinal chemistry, which calls for quantification in reference to a selected model^[49-51]. It should not be overlooked that molecular parametrization systems, which are compatible with AI, ML, and neural networks, must take over to be able to cope with big data. Coping with expanding databases, which store astronomical quantities of information on the structures of chemical and biological objects and their parameters and interrelations, quickly become essential for *in silico* drug design and initial evaluation^[52-54].

Natural isoflavones belong to the phenylpropanoid (flavonoid) category through a common biogenetic pathway and share a considerable part of chemical and biochemical characteristics with the large group of plant polyphenols, also these being from other branches of aromatic secondary metabolite origin. Their fundamental structural difference from other subcategories of flavonoids (which are 2-aryl chromones) resides in the phenylpropanoid ABC ring system connectivity [Figure 2], which stems from isoflavone synthase action, transferring the aromatic B ring to the C-3 atom of the AC chromenone system. Unlike ubiquitous flavones, flavonols and anthocyanidins which are widespread throughout the plant kingdom, isoflavones occur mainly in the family *Fabaceae*, which is particularly important for animal feed and human nutrition^[55].

An interest in biological activity of isoflavones initiated when seasonal intake of *Trifolium subterraneum*, containing an isoflavone fraction rich in formononetin, was identified as the cause of sheep fertility problems (called “clover disease”) in Western Australia around 1940^[56,57]. After thorough veterinarian investigation which followed, isoflavones were classified as phytoestrogens and occasionally even included in a category of endocrine disruptors (negative classification of environmental industrial pollutants with phenolic characteristics). Later, this seemingly local problem, seriously affected the perception of soy as principal agricultural crop and important source of animal feed and human food^[58,59]. Soybeans contain on average more than one mg/g of isoflavones (genistein, daidzein, and glycitein and their glycosides; Figure 2) which during regular oil separation-oriented industrial process end up in the protein fraction and further in soy flour-derived products^[60,61]. Phytoestrogenic food components may be considered beneficial for some consumer segments (such as women in their post-menopausal period of life) but may cause serious concerns for others (infants fed with soy-based formula, prepubertal youth, cancer patients, *etc.*)^[62-64]. This warranted basic pharmacological research which started soon after the discovery of estrogen receptors (ERs), and its results are a matter of continuous reassessment, critical review and constant debate. Isoflavones such as the soy constituent genistein are proven ER ligand subtypes, although with considerably lower affinity than the natural substrate: 17- β -estradiol. Because they can attain much higher concentrations than natural estrogens, competition for the ER binding site is possible, as proven by radioisotope-labeling experiments. However, the ligand-receptor affinity issue is only a minute part of the estrogenic effect complexity. Bioavailability, pharmacokinetics and metabolism (including microbiome biotransformation) can make a dramatic difference on a systemic level. Pharmacodynamically, phytoestrogens can exert their effects via different mechanisms and pathways. Ligands that can directly enter cells and internalize into the nucleus bind to ER and initiate ERE (estrogen response elements) responses effects via interactions with DNA (genomic mechanism). Alternatively, non-genomic signaling takes place when ligands bind to membrane receptors and start intracellular kinase cascades. Genistein (4', 5, 7-trihydroxyisoflavone), one of the most studied NPs, is a good example of a partially agonistic ligand of both ERs, which are differentially expressed in various tissues and can regulate antagonistically such basic processes as cell cycle, proliferation

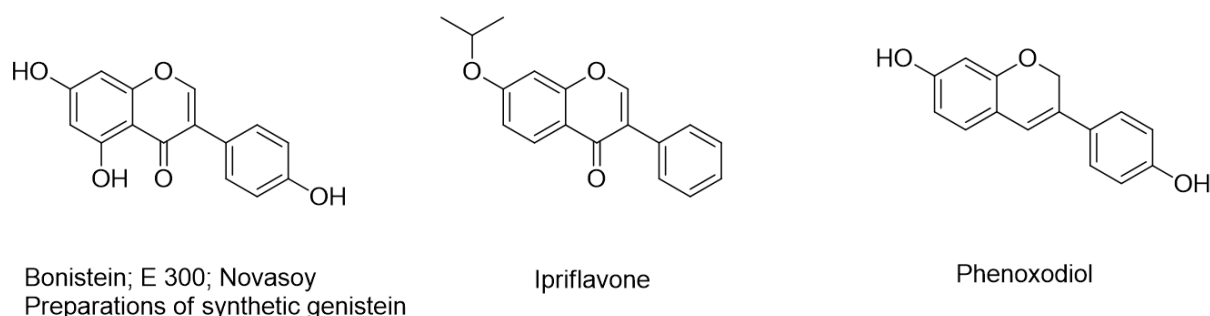
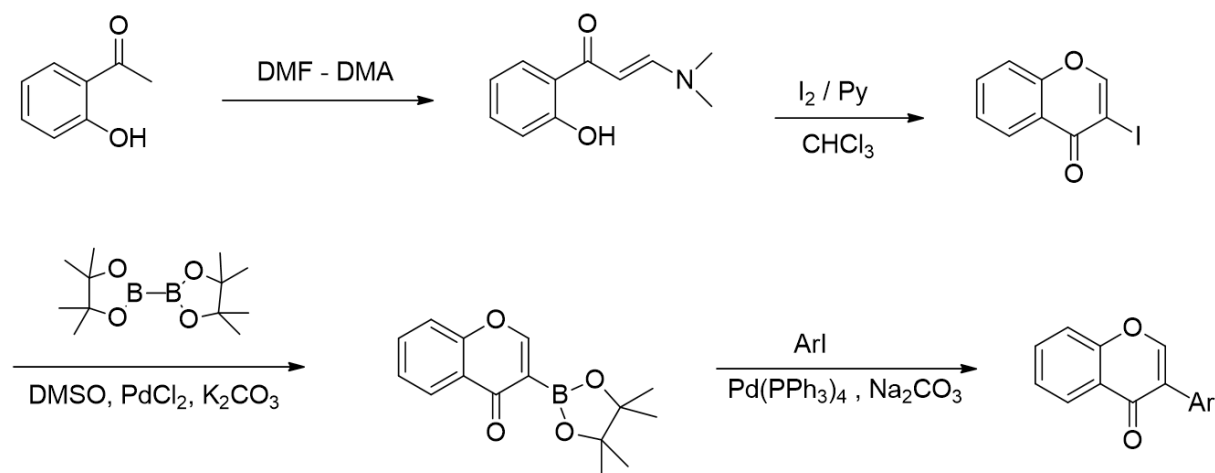


Figure 3. Isoflavones in advanced clinical trials

or apoptosis, which makes it very difficult to predict its chemopreventive or therapeutic intervention effect^[65]. Additionally, adequate analytical techniques such as HPLC/MS/MS for proper quantification of phytoestrogen in a plant/food matrix, assessment of its pharmacokinetic parameters from serum analysis and also systemic metabolite determination, have become available relatively recently. When the major FDA decision concerning soy nutrient was undertaken, recommending everyday 25 g soy protein administration for cholesterol level control, neither proteins nor isoflavones which it contained, were specified^[66]. Currently, when any plant-derived material can be analyzed with great accuracy, soy protein isolates and concentrates, with varying isoflavone content declared, remain in the segment of nutraceuticals with countless estrogenic preparations based on herbal extracts. Soy is grown in huge amounts of 300 million tons harvested annually, which contains at least 1 g of isoflavone fraction per kilogram of crop. Numerous technical processes allowing for isoflavone isolation exist, practically ready for implementation^[25-27]. Nevertheless, medicinal research materials, which are on a drug candidate evaluation path towards clinical trials (aiming at validation of isoflavones as preventive or therapeutic agents in bone health, oncological problems or neurodegeneration) are recently more often synthetic than of natural origin. Thus, synthetic genistein (BonisteinTM) was registered for assistance of bone health and to stop bone loss in postmenopausal women^[67]. As a phytoestrogen, genistein have been validated for a number of targets other than ER, such as NF- κ B, EGFR, CDK, KIF20A, PLK1, and AR, in molecular and cellular models^[68]. Even at that preclinical level, there are inconsistencies in results of cell cycle arrest, kinase inhibition data and proliferation of cells with various ER status^[65]. Genistein has been the subject of many randomized clinical trials in connection with postmenopausal health, various cancers^[69] and neurodegenerating San Filippo disease, which is genetically conditioned faulty storage of glycosaminoglycans^[70] [Figure 3].

New hurdles appear when it comes to clinical trials, plagued by problems of low isoflavone solubility and bioavailability, and high genetic as well as metabolic variety among clinical trial participants. Generally, there seems to be a problem of design and crisis of credibility in clinical trials, which affects many drug candidates from the phytochemical pool, especially in the anticancer field^[64,65,68]. Soy products for human food use are generally considered beneficial for health, although some controversies concerning its estrogenic effects persist. Technically, soy isoflavone-free products could be obtainable by relatively minor soybean process modifications but GRAS status and good market standing of soy nutraceuticals provide arguments against such decision. Reflection on advancement of evidence-based medicine requirements seem to indicate that the chances of further progress of soy isoflavone phytoestrogens towards registered medicine status are very slim; on the other hand, their existence among nutraceuticals and on the complementary and alternative medicine market is unquestionable. Also, rational nutrition is a large forum for innovation aiming at improvement of healthcare by prevention rather than therapeutic intervention.

Nutrition habits are already considerably influenced by the availability of high-quality scientific knowledge obtainable through the internet and open sources. Future food choices undoubtedly are going to reflect



Scheme 1. Novel synthesis of isoflavone skeleton

availability of both scientifically validated food and health data and relevant commercially available materials for personalized diet.

NEW DEVELOPMENTS IN GENISTEIN RESEARCH

An example of turning a XIXth century phytochemical discovery (apple tree glycoside - phlorizin) into XXth century medicinal and pharmaceutical hit: a new generation of antidiabetic drugs - gliflozins^[44-46] cited above, indicates clearly that the potential of combined chemical and biological sciences for significant industrial applications is far from being exhausted. Consequently, research on genistein, which is a good drug lead but poor drug candidate (low solubility and bioavailability, far from optimal metabolism) is going strong^[71], not only in academic environment. The main genistein shortcoming - low bioavailability, is being addressed in two ways: by chemical derivatization, and by the preparation of a suitable availability-enhancing formulation, preferably nanotechnological. Two ways of genistein structure modification are being energetically pursued: one uses phenolic groups for pharmacophore extension via alkylation followed by further functionalization^[72]; the second aims at glycoconjugation through chemical glycosylation, which offers ample room for modulating molecular polarity balance^[17,21]. Both directions in principle exploit natural isoflavone structure as a starting point, but new synthetic development based on *de novo* assembly of the isoflavone skeleton significantly expands the scope of structural modifications [Scheme 1].

Newly synthesized genistein derivatives revealed improved bioavailability and very promising pharmacodynamic profiles, but their investigation stopped at early preclinical evaluation. Meanwhile, a project that investigated the radiation-protecting activity of genistein, which obtained state support for research on nanoformulation in the USA, achieved radical improvement in isoflavone bioavailability by preparing nanosuspensions of its particles of ca. 200 nm. The synthetic genistein preparation BIO 300 is presently the subject of two investigational new drug (IND) applications at the FDA^[73-75].

CONCLUSION

Isoflavones, which constitute a well-defined subcategory of medicinally important large collection of plant phenolics, remain of interest as subjects of medicinal chemistry, biologically active food components, and nutraceuticals of increasing interest for functional food, complementary medicine and self-medication. Application of novel research tools (e.g., in bioinformatics, synthesis, nanotechnology) open new opportunities in healthcare R&D. Recent advancement of genistein to IND level as a protective agent against acute radiation syndrome heralded a breakthrough in overcoming the low bioavailability of active

pharmaceutical ingredients, by the application of a suitable nanosuspension. This new pre-formulation solution is likely to revive some older clinical trial projects.

DECLARATIONS

Authors' contributions

The author contributed solely to the article.

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

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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Primary malignant tumors of bone surface: a review with emphasis in differential diagnosis

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How to cite this article: Pacheco M, Righi A. Primary malignant tumors of bone surface: a review with emphasis in differential diagnosis. *J Cancer Metastasis Treat* 2020;6:49. <http://dx.doi.org/10.20517/2394-4722.2020.85>

Received: 27 Aug 2020 **First Decision:** 09 Nov 2020 **Revised:** 16 Nov 2020 **Accepted:** 07 Dec 2020 **Published:** 18 Dec 2020

Academic Editor: Ian Judson, Lucio Miele **Copy Editor:** Whitney Xu **Production Editor:** Jing Yu

Abstract

Surface tumors of the bone are broadly defined as a diverse group of osteogenic and chondrogenic benign and malignant neoplasms that arise adjacent to the outer surface of cortical bone. They may be a cause of diagnostic difficulty due to a degree of histological overlap, rarity, and nomenclature. In this review we summarize the different histological types of primary malignant tumors of bone surface, namely, secondary peripheral chondrosarcoma, periosteal chondrosarcoma, parosteal osteosarcoma, dedifferentiated parosteal osteosarcoma, periosteal osteosarcoma, and high-grade surface osteosarcoma. We provide a comprehensive updated review of their pathogenesis and highlight radiological, macroscopic, and histopathological features and recently available ancillary diagnostic tools that may aid in the differential diagnosis.

Keywords: Secondary peripheral chondrosarcoma, periosteal chondrosarcoma, parosteal osteosarcoma, periosteal osteosarcoma, high-grade surface osteosarcoma

INTRODUCTION

Primary malignant tumors of the bone surface are a group of osteogenic and chondrogenic neoplasms with different clinical behaviors, which depend greatly on their histologic type. Their overall incidence is very low, constituting approximately 3.5% of all primary bone tumors^[1]. Their rarity, overlapping morphology, and the therapeutic implications of their correct identification make their diagnosis better rendered by expert hands in an integrated, multidisciplinary setting. The aims of this review are to outline the



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main clinicopathologic and radiologic features of malignant tumors of bone surface, to summarize their pathogenesis and to offer a useful framework for their differential diagnosis with emphasis on potential diagnostic difficulties.

CHONDROGENIC TUMORS

Secondary peripheral chondrosarcoma

Definition and clinical features

Secondary peripheral chondrosarcomas are cartilaginous malignant neoplasms arising from the chondroid cap of pre-existent osteochondromas. They are graded into grades 1 to 3, according to the World Health Organization (WHO) histological grading system used for central chondrosarcomas. Grade 1 neoplasms receive the name of secondary peripheral atypical cartilaginous tumor if they arise in the appendicular skeleton, and secondary peripheral chondrosarcoma grade 1 if they arise in axial locations^[2].

They constitute approximately 12%-18% of all chondrosarcomas^[1,3], and occur after puberty, with a peak incidence between 20 and 40 years. Males are affected more than females^[1,4]. Secondary peripheral chondrosarcomas most commonly arise in the bones of the pelvis (40% of cases). Other common locations are the proximal and distal femur (19.5%), scapula (10%), vertebral column (9%), and ribs (5%)^[1].

Rapid growth or onset of pain on a pre-existing osteochondroma after puberty, should raise suspicion of malignant transformation, which occurs in 1% of solitary osteochondroma and in up to 5% of patients with hereditary multiple osteochondromas. Depending on the location of the neoplasm, neurologic (e.g., numbness, weakness, radiating pain, paraplegia), urinary, or colonic symptoms may be present.

Imaging

Radiographically, secondary peripheral chondrosarcoma has the typical aspect of an osteochondroma with lytic areas and fuzzy margins. On CT, large, noncalcified tumoral lobules, ring-like or popcorn-like radiopacities, and a thick cartilaginous cap are evident features^[1] [Figure 1]. T1MRI images show a lobulated, ill-defined, inhomogeneous muscular signal intensity mass; whereas on T2MRI, heterogeneous signal intensity with foci of signal void due to the calcified areas and a thick peripheral layer of white signal due to the cap of the lesion are evident^[1].

Macroscopic features

Secondary peripheral chondrosarcomas are large, cauliflower-like masses covered by a thin pseudo-capsule and beneath it, a lobulated and chalky cartilaginous cap measuring more than 2 cm in its thickest portion (thickness mean 3.9 cm)^[4] [Figure 1].

Microscopic features

The overall appearance of the cap is that of disorganized cartilage arranged in lobules divided by fibrous trabecula. Grade 1 tumors (65% of the Rizzoli series of cases) are hyaline and mildly hypercellular, contain disorganized chondrocytes, and have areas of coarse calcifications. Grade 2 and 3 neoplasms (30% and 5% of Rizzoli series of cases, respectively) are more hypercellular, exhibit nuclear atypia and pleomorphism, and have a mostly myxoid cartilaginous matrix. Necrosis can be seen. Mitosis and spindling of peripheral chondrocytes are more easily found in grade 3 neoplasms^[1].

Secondary peripheral chondrosarcoma rarely grows in a truly infiltrative pattern. Instead, lobules of cartilage push into the soft tissues, sometimes in the form of satellite nodules. Soft tissue infiltration is a *bona fide* sign of malignancy.

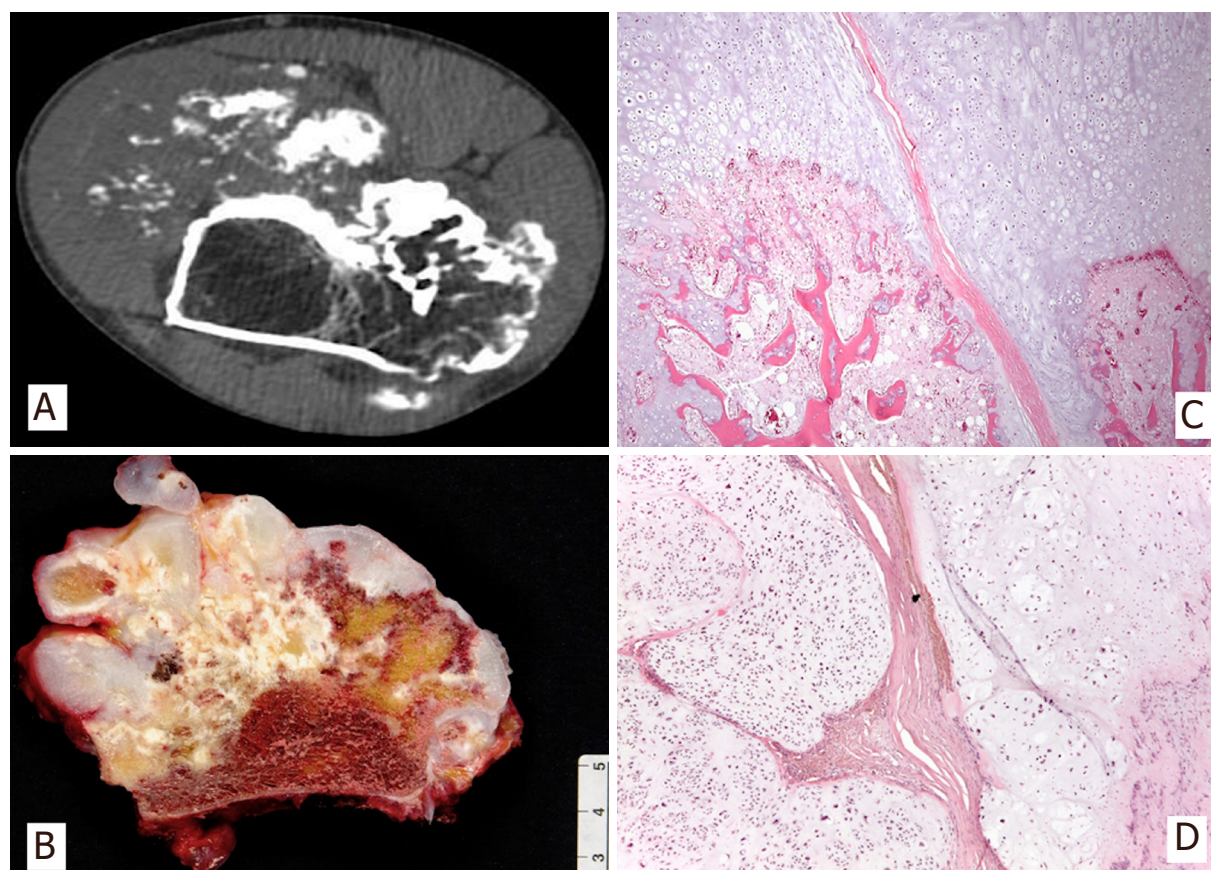


Figure 1. CT scan image of femoral peripheral secondary chondrosarcoma in patient with hereditary multiple osteochondromas with characteristic cortical flaring at the junction of the host bone with the stalk of the neoplasia (A); Macroscopic image of exophytic osteochondromatous neoplasm containing a glistening cartilaginous cap more than 2 cm thick (B); Cartilaginous cap at the interphase with bone stalk, which is continuous with the host medullary canal (H&E; 25X) (C); Disorganized, hypercellular and atypical hyaline cartilage arranged in lobules separated by fibrous bands (H&E; 50X) (D)

There is continuity between the cortex and the medulla of the host bone and the bony stalk of the neoplasm [Figure 1].

Pathogenesis

The cartilaginous cap in osteochondromas is mosaic (genetically), and contains a subpopulation of chondrocytes harboring biallelic mutations of tumor-suppressor genes *Exostosin-1* or *2* (*EXT1* or *EXT2*), admixed with chondrocytes that are either wild-type or have monoallelic mutations^[5-7]. *EXT1* and *EXT2* genes encode transmembrane glycosyltransferases that act in the polymerization of heparan sulfate chains^[5].

The functional outcome of *EXT1* and *EXT2* mutations is the production of truncated proteins with loss of enzymatic activity^[8] and impaired synthesis of heparan sulphate chains on cell surface and matrix proteoglycans. In the growth plates, heparan sulfate plays an important role in establishing and maintaining tissue polarity^[6,9], in the diffusion of ligands, and in the binding of signaling molecules to receptors of intracellular signal transduction pathways^[6,9,10] including Ihh, BMP, Wnt, and FGF^[10,11]. The impaired elongation of heparan sulfate glycosaminoglycans disrupt cell polarity and the activity of these signaling pathways, which may result in ectopic growth of mutated cells and osteochondromagenesis.

Progression to secondary peripheral chondrosarcoma, a neoplasia with chromosomal instability and complex karyotypes^[12], is hypothesized to happen upon acquisition of additional genetic changes by the

chondrocytes within the cartilaginous cap that retain one or both functional copies of *EXT* genes^[9,13]. The proliferative advantage and clonal outgrowth are likely driven by loss of cell cycle regulators *CDKN2A* and *TP53*^[9,13,14].

Diagnostic ancillary techniques

Immunohistochemical or molecular testing are not relevant for diagnosis.

Main differential diagnosis

Osteochondroma develops in skeletally immature patients during stature growth. Peak incidence is in the first and second decades of life^[1]. As opposed to secondary peripheral chondrosarcomas, the great majority of cases of osteochondromas occur in the growth plate area in the juxta-epiphyseal region of long tubular bones and less frequently involve flat bones. The most objective parameters to raise suspicion of a secondary peripheral atypical cartilaginous tumor/secondary peripheral chondrosarcoma grade 1 arising in an osteochondroma is the presence in the latter, of a cartilaginous cap greater than 2 cm (measured perpendicularly to the osteocartilaginous interphase). Nevertheless, this feature is in and of itself not enough to make a diagnosis of secondary peripheral chondrosarcoma in the absence of other signs of malignancy, such soft tissue invasion, as a very small percentage of osteochondromas may exhibits a cartilaginous cap greater than 2 cm^[2,15]. Microscopic features can greatly overlap, thus a multidisciplinary approach to diagnosis is mandatory.

Periosteal chondrosarcoma lies directly over the cortex of the host bone and does not present corticomedullary continuity of the tumor with the bone of origin. A peripheral layer of metaplastic bone (i.e., pseudo-cortex) is present in nearly 50% of periosteal chondrosarcomas^[16].

Periosteal osteosarcoma contains osteoid-producing primitive mesenchymal cells between the cartilage lobules, which are absent in secondary peripheral chondrosarcoma. Central chondrosarcoma is separated from secondary peripheral chondrosarcoma by its location in the medullary cavity. The former is molecularly characterized by *IDH* mutations, which are absent in secondary peripheral chondrosarcoma^[17].

Treatment and prognosis

The treatment is surgery with wide margins, and will depend on the severity of symptoms, the size of the lesion, and its location^[18,19]. Amputation is necessary when the neoplasm is otherwise inoperable. Radiotherapy and chemotherapy are not effective. Local recurrences can occur and are related to incomplete excision. Prognosis after treatment is excellent and metastasis is very rare.

Periosteal chondrosarcoma

Definition and clinical features

Periosteal chondrosarcoma is a low grade malignant cartilaginous neoplasm of the bone surface that, by definition, invades the underlying cortex and/or measures more than 5 cm^[2]. It is approximately three times less frequent than periosteal chondroma^[1,20] and represents 2.5% of all chondrosarcomas.

It occurs in adults, predominantly in males, with the peak incidence in the fourth decade^[1]. It typically involves the metaphysis of long tubular bones, the distal femur being the most frequent location, followed by the proximal humerus.

Imaging

Radiographic findings of periosteal chondrosarcoma include a round, lobulated mass on the surface of bone with chondroid matrix mineralization in the form of “popcorn” opacities and sometimes metaplastic ossification. Occasionally, the neoplasm can exhibit the same radiopacity as soft tissues. The underlying

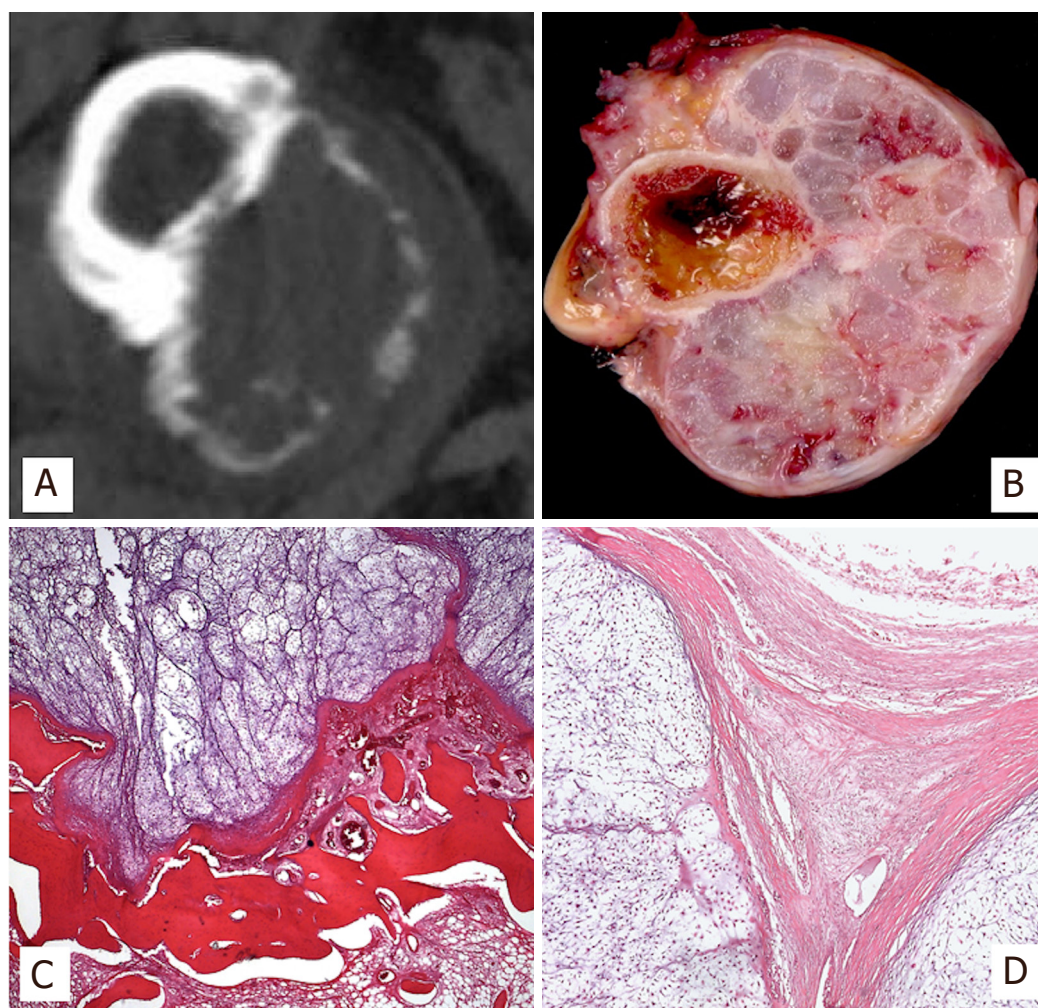


Figure 2. CT scan image of periosteal chondrosarcoma arising in the femur with cortex erosion in a well-limited way (A); Macroscopic image of periosteal chondrosarcoma showing a large, lobulated mass lying in direct apposition with the underlying outer cortex (B); Microscopic interphase of the neoplasm with the host bone cortex, showing erosion and focal thinning (H&E; 25X) (C); Outer surface of the tumor showing bosselated margins and hypercellular, myxoid neoplastic lobules (H&E; 50X) (D)

cortex often appears thickened and sclerotic, slightly saucer-shaped and with a sharply defined border [Figure 2], although it may be thin and radiographically indistinct beneath tumors arising in infrequent sites, such as spongy bones^[21].

Macroscopic features

Periosteal chondrosarcoma presents as a large mass, usually measuring over 5 cm, located directly on the bone surface and associated with erosion or invasion of the subjacent cortical bone [Figure 2]. Grossly, it is lobulated, and its cut surface is greyish and glistening, frequently with gritty areas of calcification.

Microscopic features

Periosteal chondrosarcoma is characterized by lobular growth of most often moderately cellular cartilage exhibiting myxoid matrix, frequent calcifications, and endochondral ossification. The neoplastic chondrocytes show variable degrees of cytological atypia, although marked nuclear pleomorphism is not a feature of periosteal chondrosarcoma. Osteoid or bone directly formed by the neoplastic chondrocytes is absent. Mitosis figures are very rare.

Invasion of the underlying cortex is present, even in tumors less than 5 cm. Also, involvement of medullary canal is seen in a proportion of cases, as is invasion of soft tissues. In a subset of cases (50%, in our experience) the periphery of the tumor is encased by a complete or incomplete layer of mature cortical bone^[16,22].

Pathogenesis

A subset of periosteal chondrosarcomas harbors heterozygous, somatic *IDH1* R132C mutations^[17,22,23]. *IDH1* is a metabolic enzyme of the tricarboxylic acid cycle that catalyzes the conversion of isocitrate to α -ketoglutarate.

Mutated *IDH1* has reduced catalytic activity for the production of α -ketoglutarate and increased catalytic activity for the production of 2-hydroxyglutarate, due to an acquired neomorphic activity^[24].

The elevated level of the oncometabolite 2-hydroxyglutarate epigenetically inhibits osteogenic differentiation of mesenchymal stem cells through histone hypermethylation in the promoter regions of alkaline phosphatase. It also promotes chondrogenic differentiation through histone modifications of promoter regions of master transcription factors for chondrogenesis, *SOX9* and *COL2A1*^[25,26].

Loss of protein expression of cell cycle regulator *CDKN2A/P16/INK4A* is found in 50% of cases of periosteal chondrosarcomas suggesting that the pRB pathway is involved in tumoral progression^[22].

Diagnostic ancillary techniques

The commercially available *IDH1* R132H antibody is a highly specific and sensitive marker for the detection of the R132H mutant allele, but does not detect other *IDH* mutants, including the R132C found in periosteal chondrosarcomas^[17].

IDH mutational analysis can be useful in differentiating periosteal chondrosarcoma from the more aggressive periosteal osteosarcoma, but it does not discriminate between periosteal chondroma and periosteal chondrosarcoma.

Main differential diagnosis

The main differential diagnosis is periosteal chondroma, which occurs predominantly in younger adults and children (peak incidence in the second decade) in the proximal humerus and the short tubular bones of the hands^[27]. Radiographically, it shows solid periosteal bone buttressing, which is absent in periosteal chondrosarcoma. Although some overlap in size might exist, periosteal chondromas are, on average, smaller than periosteal chondrosarcomas, the former only rarely exceeding 3 cm. Periosteal chondroma can erode and scallop the cortical bone, thus tumor nodules within the sclerotic cortex can be seen sometimes. They do not represent true invasion, but an artifact due to tangential sectioning. Real invasion of cortical bone and soft tissues is absent. Histologically, periosteal chondromas are usually hypocellular and devoid of cytological atypia.

Periosteal osteosarcoma is diaphyseal and has a pathognomonic radiological appearance. The cartilaginous areas in periosteal osteosarcoma show marked pleomorphism compared to the cartilage of periosteal chondrosarcoma. Also, the intervening bands of bone-producing primitive mesenchymal cells present in periosteal osteosarcoma are, by definition, absent in periosteal chondrosarcoma.

Secondary peripheral chondrosarcoma exhibits the characteristic corticomedullary continuity of the stalk of the tumor and the bone of origin, which is absent in periosteal chondrosarcoma [Figure 2].

Treatment and prognosis

Conservative surgery with wide margins is usually effective. The prognosis is very good, with local recurrence of 13%-28% and metastatic rates of 512.2%^[20]. Metastasis primarily involve the lungs and, rarely, the lymph nodes^[28]. In the Rizzoli case series of periosteal chondrosarcomas, no patient died of tumor^[1,21].

OSTEOGENIC TUMORS

Parosteal osteosarcoma

Definition and clinical features

Parosteal osteosarcoma is a low-grade osteosarcoma with low-metastatic potential that arises on the cortical surface of bone. It constitutes 4.3% of all osteosarcomas^[1] and, although infrequent, is the most common osteosarcoma of the bone surface. Its peak of incidence is in young adults between 20 and 40 years old (mean age of 31 years) with a slight female predominance^[29]. It almost exclusively affects the metaphysis of long bones of the extremities, with a marked predilection for the distal metaphysis of the femur (60% cases). Only exceptionally it occurs in flat bones, small bones of hands and feet and vertebrae. Parosteal osteosarcoma most commonly debuts as a long standing, painless and indurated mass.

Tumoral progression into a high-grade dedifferentiated parosteal osteosarcoma is seen in a percentage of cases and can occur either at presentation (primary/synchronous type) or at the time of recurrence (metachronous type). In primary dedifferentiated parosteal osteosarcomas the clinical history is sometimes more abrupt.

Imaging

Due to its indolent growth, parosteal osteosarcoma is usually discovered when it has achieved large dimensions. It is a circumferential or mushroom shaped mass of osseous radiodensity fused to the cortex [Figure 3]. Radiodensity is maximal near the implant base, while the outer margin tends to be blurred. Detailed radiographies show a mesh of trabecula ("steel-wool" pattern), ranging from ground glass to ivory density^[1]. Areas of radiolucency on plain films or CT scans and hyperintense on fluid-sensitive MRI can be present in approximately 60% of dedifferentiated parosteal osteosarcomas^[30].

The combination of MRI and CT allows for high accuracy in the determination of the extent of intramedullary infiltration of the tumor, thus assisting in the planning of conservative surgeries that avoid surgical morbidity^[31].

Macroscopic features

Grossly, parosteal osteosarcoma presents as a firm, exophytic and lobulated mass with a broad base attachment to the cortex of the host bone. It can grow either longitudinally along the long axis of the bone or encircle it [Figure 3]. Elevation of periosteum and reactive periosteal bone formation are typically absent. The cut surface is whitish, hard, or gritty and may contain areas of cartilage. When dedifferentiation is present, its occurrence is characterized by a tan and fleshy component not infrequently accompanied by areas of hemorrhage and fluid cavities that may correlate well with the lucent areas in imaging studies^[2].

Microscopic features

It is composed of long fascicles of deceptively bland spindle cells embedded in a collagenous stroma and intervening, well-formed bone trabecula. The fibrous fascicles are hypocellular and the spindle cells exhibit minimal nuclear atypia. Mitotic figures are inconspicuous. The bone trabecula are typically arranged in parallel streamers but can also be more curvilinear and arranged in an anastomosing fashion [Figure 3]. Osteoblastic riming is inconsistently present.

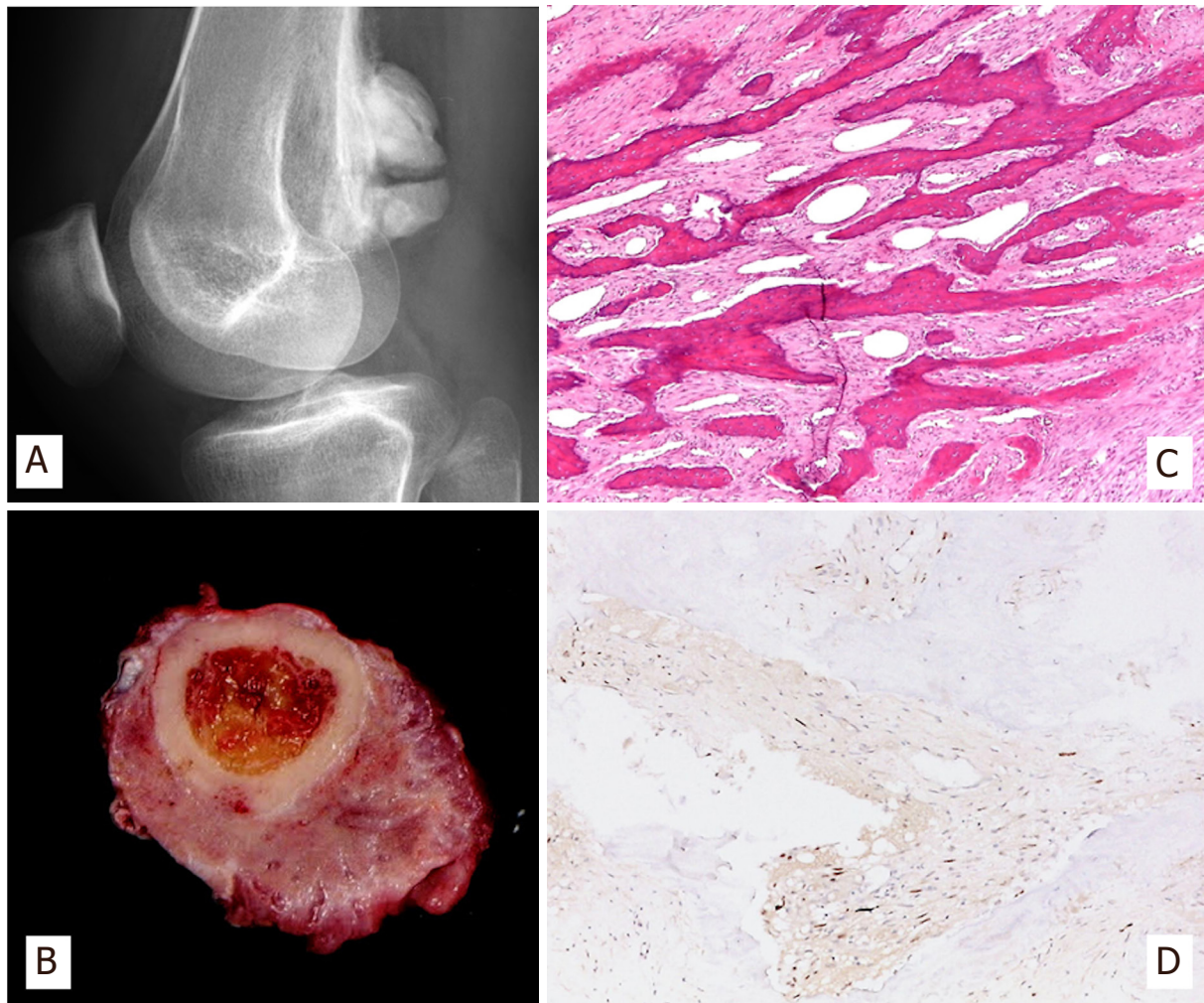


Figure 3. X-rays of distal femur exhibiting an exophytic and sclerotic mass projecting from the cortex in its popliteal fossa (A); Gross specimen of parosteal osteosarcoma, encircling the femur with a gritty cut surface (B); Characteristic microscopic arrangement of parallel bone trabecula and the intervening low-grade fibroblastic component (H&E; 50X) (C); MDM2 nuclear positivity (IHC; 50X) (D)

A peripheral cartilaginous component is seen in 25-50% of cases^[1,2] in the form of chondroid nodules. The presence of areas of higher histological grade, frank anaplasia and brisk mitotic activity within the tumor indicates dedifferentiation. The high-grade dedifferentiated component most commonly exhibits features of conventional osteosarcoma or undifferentiated pleomorphic sarcoma^[30], although rhabdomyosarcomatous dedifferentiation has also been reported^[32]. Infiltration of the soft tissue and medullary canal can be seen.

Pathogenesis

Supernumerary ring chromosomes containing high-level amplification of chromosomal regions 12q13-15, including *MDM2* and *CDK4* loci, is a consistent finding in parosteal osteosarcoma^[33,34]. As a result, two major pathways of growth regulation may be inhibited: *MDM2* may downregulate the p53-mediated growth control and *CDK4* may affect the function of retinoblastoma tumor suppressor protein (pRb)^[35], leading to deregulation of the cell cycle.

Diagnostic ancillary techniques

MDM2 gene amplification by *in situ* hybridization techniques, or surrogate immunohistochemical expression of *MDM2* are useful and routinely used to confirm diagnosis^[36].

Main differential diagnosis

Juxtacortical myositis ossificans is composed by a spindle-cell proliferation admixed with woven bone that exhibits a zonation pattern of progressive maturation toward the periphery of the lesion. The spindle cell component is similar to that seen in nodular fasciitis, characterized by non-cohesive, plump, spindle-shaped cells reminiscent of tissue culture; instead of the long fascicles present in parosteal osteosarcoma. *MDM2* amplification is absent in myositis ossificans, while rearrangement of *USP6* can be detected^[37].

Desmoplastic fibroma and fibrous dysplasia are also characterized by hypocellular fascicles of bland spindle cells in a collagenous background but lack the bone trabecula seen in parosteal osteosarcoma. In ambiguous situations, they can be confidently separated from parosteal osteosarcoma by the absence of *MDM2* amplification or *MDM2* nuclear immunostaining.

Osteoma affects almost exclusively bones formed by membranous ossification, thus locations outside the craniofacial bones and jaws, where this entity might constitute a diagnostic differential, are extremely rare. Osteoma contains cortical-type bone and lacks the fascicles of spindle cells seen in parosteal osteosarcoma.

Up to a fourth of bizarre parosteal osteochondromatous proliferation (BPOP) may arise in long tubular bones^[38-40], a location where it must be distinguished from parosteal osteosarcoma. The latter is exceedingly rare in small tubular bones of hands and feet, which are the sites of predilection of BPOP.

BPOP are smaller lesions measuring from a few millimeters up to 3 cm. BPOP has a zonal architecture, absent in parosteal osteosarcoma, comprised by an admixture of cancellous bone, aggregates of cartilage and fibrous tissue. Characteristically in BPOP, an irregular zone of endochondral ossification and calcification, characterized by the presence of so-called “blue bone” is present in the interface between cartilage and bone.

Dedifferentiated parosteal osteosarcoma can be distinguished from periosteal and high-grade surface osteosarcoma by the presence of a low-grade spindle-cell component and *MDM2* amplification in dedifferentiated parosteal osteosarcoma.

Treatment and prognosis

Parosteal osteosarcoma is a locally aggressive tumor with limited potential for distant spread. Hence, surgical resection with adequate margins is the treatment of choice. Chemotherapy is reserved for dedifferentiated parosteal osteosarcomas. The incidence of local recurrences has been demonstrated to be strictly related to the adequacy of surgical margins^[41].

The prognosis of parosteal osteosarcoma is excellent. Long-term survival rate for this entity is at least 90%. Nevertheless, it possesses a risk of transformation into an intermediate or high-grade sarcoma (dedifferentiation), either at first diagnosis or after recurrences, estimated to occur in between 14%^[42] and 24% of cases^[29,30]. Dedifferentiation confers a significantly worse prognosis to parosteal osteosarcomas, diminishing overall survival and equating it to that of conventional high-grade osteosarcomas^[29,43].

Periosteal osteosarcoma

Definition and clinical features

Periosteal osteosarcoma is an intermediate grade, mainly chondroblastic, bone-producing sarcoma that arises on the surface of bone beneath the periosteal layer^[2]. It is rare, accounting for less than 2% of all osteosarcomas^[1,27,44,45]. It mostly affects children, adolescents and young adults with a peak incidence in the second decade of life^[1]. The most common sites of involvement are the diaphysis of the tibia and the femur^[1,27,44,46], but other long bones of extremities and much less frequently, flat bones, can be involved^[1,44].

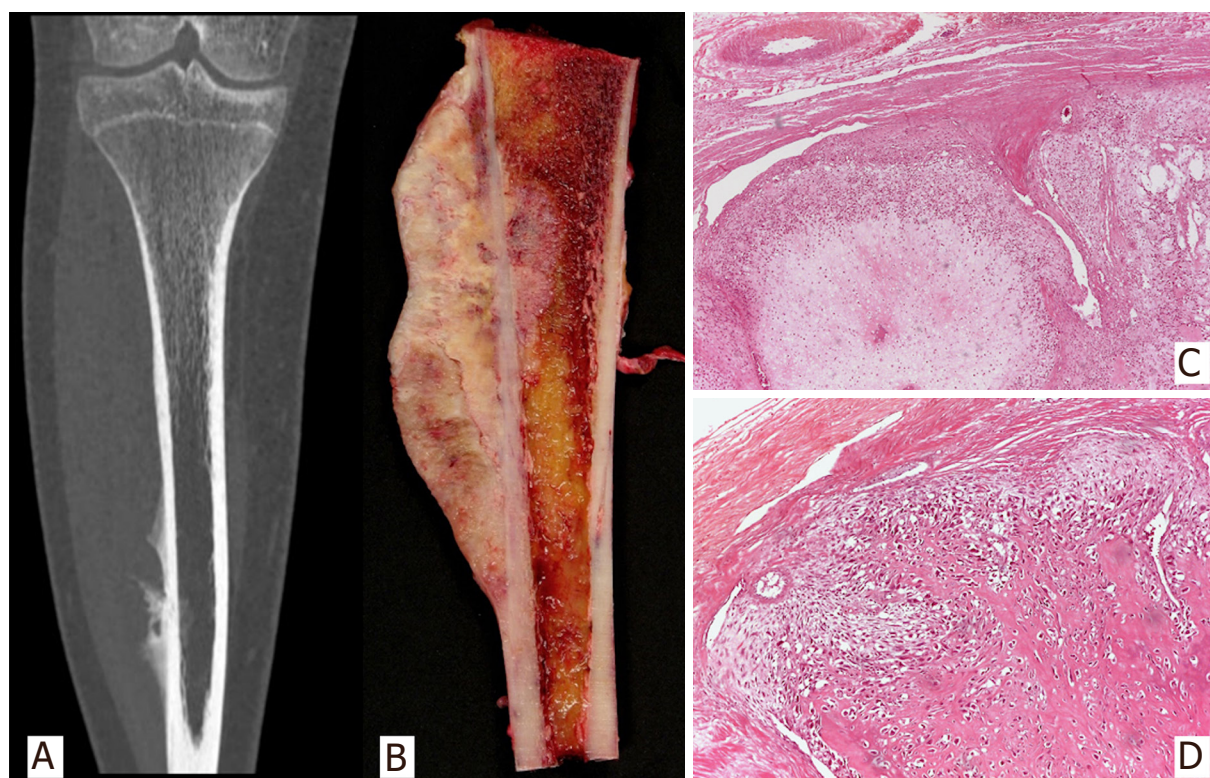


Figure 4. CT scan image of periosteal osteosarcoma originating in tibial diaphysis, forming bone, with thick periosteal bone formation. The neoplasm is elongated, mainly lucent (A); Subperiosteal, fusiform mass with broad base of attachment to outer cortex of host bone. The external surface is covered by fibrous tissue. The cut surface is mostly cartilaginous, but also contains perpendicular bands of ossification (B); Chondroblastic lobules separated by bands of undifferentiated spindle cells (H&E; 25X) (C); Osteoid-producing undifferentiated neoplastic cells (H&E, 50X) (D)

Periosteal osteosarcomas present as swellings of relatively short duration, with or without accompanying pain.

Imaging

Periosteal osteosarcoma is a fusiform, mainly lucent mass with well-defined borders [Figure 4]. It usually contains perpendicular linear striae that radiate from the underlying host bone. Sometimes a Codman triangle can be seen^[1,3]. CT and MRI are helpful in ruling out neoplastic infiltration to the bone marrow, which is rarely present.

Macroscopic features

Periosteal osteosarcoma is a subperiosteal, fusiform mass with a broad base attached to the outer cortex. Periosteal elevation and reactive bone formation are present. The external surface is covered by a layer of fibrous tissue derived from periosteum. Scalloping and thickening of outer cortex is characteristic. Intramedullary extension may be present. The cut surface consists of lobules of glistening cartilaginous areas, separated by bands of ossification perpendicular to the cortical surface of the host bone [Figure 4].

Microscopic features

Ill-defined lobules of atypical cartilage dominate the histological picture. The cartilaginous areas are separated by sarcomatous bands of undifferentiated spindle cells with primitive appearance. Within these bands, osteoid and immature bone produced by neoplastic cells are seen [Figure 4]. Perpendicular streaks of reactive bone with osteoblastic rimming traverse the neoplasm.

Pathogenesis

No consistent genetic abnormality has been reported for periosteal osteosarcoma^[2].

Diagnostic ancillary techniques

Compared to low-grade central osteosarcoma and parosteal osteosarcomas, MDM2 and CDK4 are very rarely expressed in periosteal osteosarcoma and therefore they do not seem to be molecules central to the control of cancer development, growth, and progression in this rare subtype of osteosarcoma^[47].

Main differential diagnosis

BPOP arising in long bones may be mistaken for a periosteal osteosarcoma, due to its rapid growth, high rate of local recurrences and atypical histology^[38,39]. BPOP does not exhibit the brisk mitotic activity nor the level of cytological atypia observed in periosteal osteosarcomas. In addition, blue bone is a feature of BPOP absent in periosteal osteosarcoma.

Parosteal osteosarcoma arises in metaphysis of long bones in slightly older population. Their radiological growth patterns are dissimilar. Microscopically their recognition is usually straightforward due to the fact that parosteal osteosarcoma is predominantly fibroblastic, is typically low-grade, and contains abundant tumor bone; whereas periosteal osteosarcoma is predominantly chondroblastic and of intermediate to high grade.

Differentiating a periosteal osteosarcoma from a high-grade surface osteosarcoma is of prognostic and therapeutic importance, as high-grade surface osteosarcoma behaves in a much more aggressive manner. High-grade surface osteosarcomas most frequently arise in metaphysis of long bones of extremities. Histologically, periosteal osteosarcoma shows less anaplasia and more prominent chondroid differentiation than high-grade surface osteosarcoma. High-grade surface osteosarcoma is a high-grade tumor (grades 3 or 4), whereas periosteal osteosarcoma is an intermediate grade tumor (grades 2 or 3)^[2].

Periosteal chondrosarcoma arises predominantly in metaphyseal locations and in older patients. The neoplastic cartilage lacks marked nuclear pleomorphism, mitotic figures are rare, and osteoid or bone formed by the neoplastic chondrocytes are absent.

Treatment and prognosis

Treatment consists of *en bloc* resection with wide margins without chemotherapy. Periosteal osteosarcoma has less aggressive behavior compared to high-grade osteosarcoma, including a lower propensity for systemic recurrence which is reported to be around 15% in periosteal osteosarcoma^[21,48]. The 10-year overall survival rate is 77.1%-84%^[44-46].

High-grade surface osteosarcoma

Definition and clinical features

High-grade surface osteosarcoma is a high-grade, malignant, bone-forming neoplasm arising in the bony surface with minimal or no involvement of the underlying cortical bone. It is the least common type of surface osteosarcoma, representing approximately 1% of all osteosarcoma types. It mostly affects adolescents and young adults with a peak incidence in the second decade of life and a striking male predominance^[1]. The skeletal distribution is similar to that of conventional osteosarcoma, most frequently involving the metaphysis of the distal femur, proximal tibia, and proximal humerus.

Imaging

X-rays show mixed non-mineralized and mineralized matrix with fine cloud-like areas of opacity^[1]. Periosteal reaction is uncommon, and the margins are indistinct [Figure 5]. Up to one third of the tumors

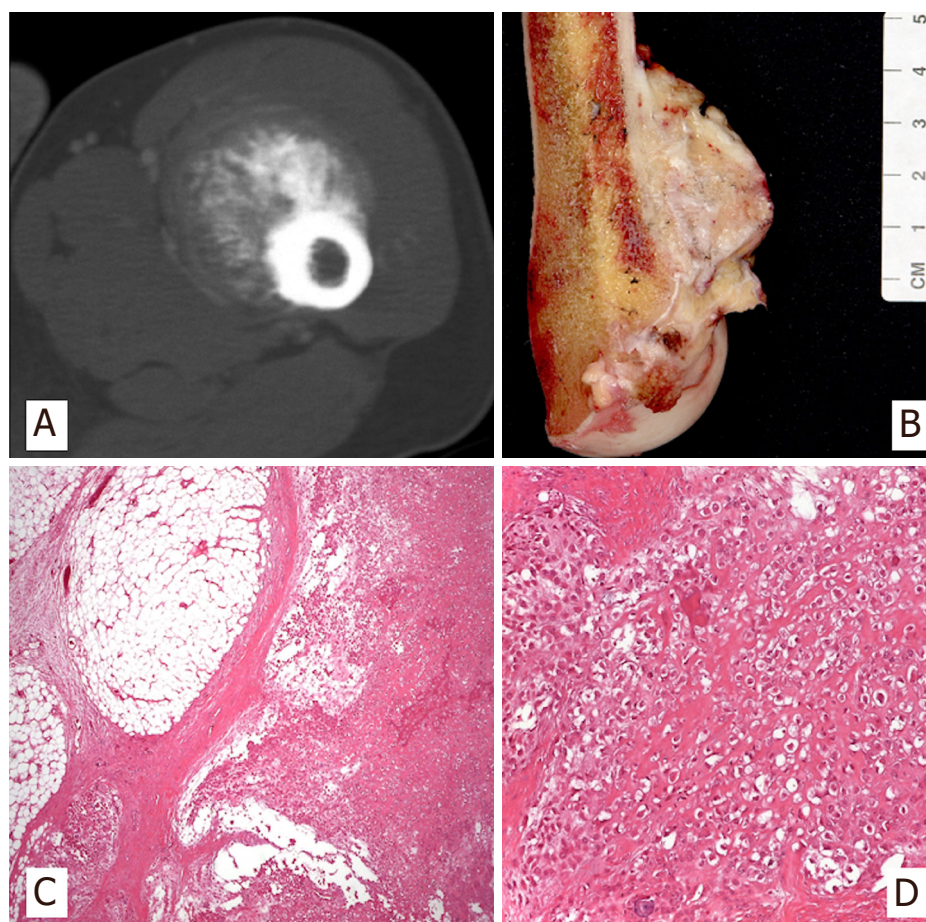


Figure 5. CT scan shows a tumor that develops in the soft tissue but starts from the cortex (A); High-grade surface osteosarcoma of distal femur with cortical erosion (B); Infiltrating neoplasm in the surrounding soft tissues (H&E;25X) (C); Highly atypical features and osteoid production (H&E; 50X) (D)

show minimal medullary involvement in CT and MRI images^[3]. MRI facilitates the identification of cartilage that is scattered throughout the mass.

Macroscopic features

Lobulated, relatively well-circumscribed, broad-base mass on the periosteal surface of the bone. It frequently extends to the periosteum and can erode the cortex, but the vast majority of the tumor remains outside the bone [Figure 5]. Any significant penetration beyond the endosteum exclude the diagnosis of high- grade surface osteosarcoma. The cut surface may be dominated by cartilaginous tissue or composed of hard, tan-white areas admixed with others with fish flesh consistency.

Microscopic features

The microscopic features are identical to high-grade conventional osteosarcoma. They exhibit anaplasia, necrosis, osteoid, and immature bone and/or cartilage formation [Figure 5]. Mitotic activity is brisk, and atypical mitotic figures are easily found. Microscopic evidence of medullary extension is either absent or minimal.

Pathogenesis

The pathogenesis remains unknown.

Diagnostic ancillary techniques

The diagnostic ancillary techniques are not clinically relevant for high-grade surface osteosarcoma.

Main differential diagnosis

The distinction between a high-grade surface osteosarcoma and dedifferentiated parosteal osteosarcoma or extramedullary extension from a conventional central osteosarcoma does not carry prognostic or therapeutic implications. Areas of low-grade fibroblastic osteosarcoma are found in dedifferentiated parosteal osteosarcoma and absent in high-grade surface osteosarcoma. A considerable intramedullary component argues in favor of a central conventional osteosarcoma.

Due to therapeutic and prognostic implications, the most important differential diagnosis is with periosteal osteosarcoma as indicated in the paragraph on periosteal osteosarcoma.

Treatment and prognosis

Treatment and prognosis do not differ from those of central conventional osteosarcoma. Treatment is preoperative chemotherapy followed by surgical resection. The overall 5-year survival rate ranges between 46% to 62%^[49,50]. A poor response to neoadjuvant chemotherapy is associated with a poor outcome^[3].

CONCLUSION

There are a variety of bone and, to a lesser extent, soft tissue entities that should be considered in the differential diagnosis of primary tumors of the bone surface. These comprise a wide clinical spectrum of neoplasms that can impose diagnostic challenges outside the setting of large referral centers, due to their extremely low incidence and unawareness of their clinical, radiological, and often subtle microscopic differences. A multidisciplinary and expert approach to diagnosis is mandatory.

DECLARATIONS

Acknowledgments

We would like to thank Fundación Martha Stella C. de Vallarino for financial support through a medical educational scholarship to one of the authors (MP).

Authors' contributions

Preparing the manuscript draft and capturing the microscopic images: Pacheco M

Reviewing the manuscript: Righi A

Writing, producing the illustrations, reading and approving the final manuscript: Pacheco M, Righi A

Availability of data and materials

Not applicable.

Financial support and sponsorship

Not applicable.

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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Current updates in management of relapsed/refractory small cell lung cancer

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How to cite this article: Abughanimeh O, Ernani V, Marr A, Ganti AK. Current updates in management of relapsed/refractory small cell lung cancer. *J Cancer Metastasis Treat* 2020;6:50. <http://dx.doi.org/10.20517/2394-4722.2020.110>

Received: 4 Oct 2020 **First Decision:** 12 Nov 2020 **Revised:** 23 Nov 2020 **Accepted:** 3 Dec 2020 **Published:** 18 Dec 2020

Academic Editor: Robert Kratzke **Copy Editor:** Monica Wang **Production Editor:** Jing Yu

Abstract

Small cell lung cancer (SCLC) is an aggressive subtype of neuroendocrine tumor. It is characterized by a rapid doubling time and early development of metastatic disease. Despite being responsive to initial chemotherapy, most of the patients will have relapse of the disease within a few months. The prognosis of SCLC is dismal with a 5-year survival rate of less than 5%. For that reason, management of SCLC has been an active area of research. The utilization of immunotherapy has provided promising results in treatment of SCLC in the front-line setting. Therefore, utilization of immunotherapy and targeted therapy is being studied in the setting of relapsed/refractory disease, and currently, different clinical trials are exploring new drugs and further options. In this review, we will explore the latest updates in management of relapsed/refractory SCLC.

Keywords: Small cell lung cancer, relapsed small cell lung cancer, chemotherapy, immunotherapy, targeted therapy

INTRODUCTION

Lung cancer is a major public health concern. In 2020, it is estimated that the United States (US) will have more than 200,000 new cases, making lung cancer the second most common malignancy and leading in cancer-related mortality in both genders^[1]. Small cell lung cancer (SCLC) accounts for \approx 20% of the total lung cancer cases globally^[2]. In the US, SCLC accounts for 16% of new lung cancer cases^[3]. SCLC is divided



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into limited-stage disease (LS-SCLC), which shows confined growth, or extensive-stage disease (ES-SCLC) which is associated with metastasis. It is estimated that approximately 60% of SCLC patients present with extensive-stage disease at the time of initial diagnosis. The most common sites of spread include contralateral lung, adrenal glands, brain, liver, bones, and bone marrow^[4,5].

SCLC is highly responsive to chemotherapy^[3,5]. The standard first-line treatment of LS-SCLC includes concurrent chemotherapy (cisplatin-etoposide) and radiation, while ES-SCLC is treated with a combination chemotherapy (platinum-etoposide) and an immune checkpoint inhibitor. For long time, the treatment of ES-SCLC consisted of platinum agents and etoposide^[5]. This was changed recently due to results from IMpower-133 and CASPIAN studies, both of which demonstrated improved survival by adding atezolizumab and durvalumab, respectively, to platinum and etoposide^[6,7].

Despite being responsive to chemotherapy, most SCLC patients will experience tumor relapse within a few months, making management of these patients challenging^[8,9]. If the relapse occurs within 3 months of treatment, the disease is called refractory or resistant, and the response to further treatment is < 10%^[9]. If the relapse occurs after 3 months, the expected response to further treatment is 25%. Options for patients with refractory or relapsed disease are limited and patients with relapsed or refractory disease have a median survival of 8-9 months^[9,10].

The treatment of refractory/relapsed SCLC has been an active area in research given the dismal prognosis and the poor outcome. Chemotherapy remains the cornerstone of treatment, but recently newer agents including immunotherapy are being studied, with promising results. In this review, we will discuss the current agents that are used in relapsed or refractory SCLC.

TREATMENT OPTIONS OF RELAPSED/REFRACTORY SMALL CELL LUNG CANCER

Chemotherapy

Topotecan

Topotecan is a semisynthetic water-soluble analog of camptothecin which acts as an inhibitor of the nuclear enzyme topoisomerase I, leading to DNA damage^[11,12]. For a long time, it was the only drug that was approved by the Food and Drug Administration (FDA) for relapsed small cell lung cancer.

One of the earliest studies of topotecan in SCLC was conducted by the European Organization for Research and Treatment of Cancer^[11]. This study was a phase II trial which included 92 patients (47 patients who were refractory to first-line treatment, and 45 patients had disease relapse after 3 months of stopping chemotherapy). In both arms, patients received intravenous (IV) topotecan at 1.5 mg/m² for five consecutive days every 3 weeks. Topotecan demonstrated an overall response rate (ORR) of 6.4% (95%CI: 1.3%-17.6%) in patients who failed first-line treatment, and 37.8% (95%CI: 23.8%-53.5%) in patients who had disease relapse after 3 months of finishing chemotherapy treatment. The overall response in both groups was 21.7%. The overall median duration of response was 7.6 months (95%CI: 5.1-12.2 months), the median time to progression was 2.8 months (95%CI: 2.2-3.9 months), and the overall survival (OS) was 5.4 months (95%CI: 4.8-6.3 months). This study showed that topotecan had good activity in SCLC, specifically in patients who responded to initial chemotherapy^[11].

von Pawel *et al.*^[12] conducted a randomized phase III trial comparing topotecan to Cyclophosphamide, Doxorubicin, and Vincristine (CAV). In this study a total of 211 patients were recruited (107 treated with topotecan and 104 treated with CAV). Patients in the topotecan arm received IV topotecan 1.5 mg/m² daily for five consecutive days every 3 weeks. The ORR of topotecan was 24.3% compared to 18.3% in the CAV arm. The median time to progression and median survival were similar in both arms (13.3 weeks *vs.* 12.3 weeks, and 25 weeks *vs.* 24.7 weeks, respectively). Despite the similarity in outcomes, this study showed that

patients who received topotecan had better improvement in symptoms including dyspnea ($P = 0.002$), anorexia ($P = 0.042$), fatigue ($P = 0.032$), and hoarseness ($P = 0.043$).

Later in the early 2000s, a phase III clinical trial was conducted by O'Brien *et al.*^[13] to compare oral (PO) topotecan to supportive care alone. This study demonstrated prolonged survival with topotecan compared to supportive care. Moreover, it showed that patients who received topotecan had greater symptoms control and slower quality of life deterioration.

Eckardt *et al.*^[14] performed a randomized phase III clinical trial to compare PO topotecan with IV topotecan in relapsed SCLC. In this study, 309 patients were included, 153 patients received oral topotecan 2.3 mg/m^2 daily for five consecutive days every three weeks whereas 151 received IV topotecan 1.5 mg/m^2 daily for five consecutive days every three weeks. The study showed similar ORR in both arms of 18.3% vs. 21.9% respectively. There was no difference in median time to response (6.1 weeks for both), median duration of response (18.3 weeks vs. 25.4 weeks), and median time to progression (11.9 weeks vs. 14.6 weeks).

Hematologic complications have been commonly reported with topotecan. Despite having lower risk of grade 4 neutropenia compared to CAV, topotecan has higher risk of grade 4 thrombocytopenia and anemia^[12]. Non-hematologic toxicities were also reported such as fatigue, alopecia, nausea, and other gastrointestinal complications^[11,12,14].

Another study from Germany showed that a lower dose of topotecan 1.25 mg/m^2 had a similar efficacy to the traditional dose of 1.5 mg/m^2 , but most importantly it was associated with reduced toxicity^[15]. It is worth mentioning that there have been some studies which evaluated the usage of weekly topotecan instead of the standard regimen, however the results were not conclusive and weekly topotecan is no longer routinely used in clinical practice^[9].

Lurbinectedin

Lurbinectedin is a synthetic analog of trabectedin, which acts through inhibition of the active transcription protein-coding genes. This drug binds to CG-rich regions in the DNA causing irreversible arrest of elongating RNA polymerase on the DNA template, leading to accumulation of DNA breaks and apoptosis^[10,16].

This drug was first studied in humans in 2009 when Elez *et al.*^[17] conducted a phase I clinical trial in patients with advanced solid tumors and showed both safety and anti-tumor effect. Later, lurbinectedin was studied in combination with doxorubicin in patients with relapsed SCLC. The study showed tolerability and an overall response rate of 57.7%^[18].

Recently, a single arm, multicenter, phase II clinical trial was conducted on 105 patients who were treated with 3.2 mg/m^2 of IV lurbinectedin every 3 weeks. Among them, 45 patients were considered to have resistant disease (defined as chemotherapy-free interval < 90 days) and 60 patients with sensitive disease (defined as chemotherapy-free interval ≥ 90 days). The study showed an ORR of 35.2% (95%CI: 26.2-45.2). Patients with sensitive disease had better ORR, [45% (95%CI: 32.1-58.4)] compared to the resistant disease group [22.2% (95%CI: 11.2-37.1)]. The overall median duration of response was 5.3 months (95%CI: 4.1-6.4), which was also higher in the sensitive disease group 6.2 (95%CI: 3.5-7.3) vs. 4.7 (95%CI: 2.6-5.6). In this study, the median progression free survival (PFS) was 3.5 months (95%CI: 2.6-4.3) and the median OS was 9.3 months (95%CI: 6.3-11.8), both were also better in the sensitive disease group^[16]. These results led to accelerated approval by the FDA to be used in patients with metastatic SCLC after progression on platinum-based chemotherapy. The National Comprehensive Cancer Network (NCCN) guidelines included lurbinectedin as a “preferred” agent in the second line treatment options along with topotecan or clinical trials^[9].

Hematologic toxicities were reported with lurbinectedin treatment. Grade 4 neutropenia was recorded in 25% of the patients, whereas grade 4 thrombocytopenia was recorded in 4%. None of the patients developed grade 4 anemia, however 9% of them developed grade 3. Other non-hematologic toxicities included fatigue, decreased appetite and different gastrointestinal symptoms^[16].

Currently, a phase III clinical trial, the ATLANTIS study (NCT02566993) is being conducted to compare the activity of lurbinectedin combined with doxorubicin, with either topotecan or CAV as second line treatment for SCLC^[10].

Irinotecan

Irinotecan is a water-soluble derivative of camptothecin that acts through inhibition of DNA topoisomerase I, leading to antitumor effects^[19]. The use of irinotecan in SCLC has been established in the last century. In 1990, a phase II study was conducted in Japan by Masuda *et al.*^[19]. This study enrolled 16 patients with refractory or relapsed SCLC. All patients received IV irinotecan 100 mg/m² every week. In this study, irinotecan led to an ORR of 47% (95%CI: 21.4%-71.9%). The median duration of response was 58 days (28-156 days). These findings were supported by another study that was done in Japan which demonstrated an ORR of 50% (95%CI: 25%-75%)^[20]. A newer study was conducted in Japan which evaluated 30 patients with previously treated SCLC who received irinotecan 100 mg/m² on days 1, 8, every 3 weeks. The study showed an ORR of 41.3% (95%CI: 25.5-59.3) and a disease control rate of 69%. The same study showed a median PFS and OS of 4.1 months and 10.4 months, respectively^[21].

The major toxicities associated with irinotecan treatment were hematologic, mostly leukopenia, followed by nausea and pulmonary toxicity^[19].

Taxanes

Paclitaxel was evaluated in multiple studies in patients with relapsed/refractory SCLC. A phase II trial was performed by Smit *et al.*^[22] in Netherlands where patients received IV paclitaxel 175mg/m² every 3 weeks. Paclitaxel led to an ORR of 29% (95%CI: 12%-51%). Furthermore, it was associated with a median duration of response of 108 days (64-243 days), median time to progression of 65 days (33-243days), and median survival of 100 days (23-262 days).

Paclitaxel was assessed in another phase II trial that was conducted by Yamamoto *et al.*^[23] who studied 21 patients with refractory SCLC. The study showed that single agent IV paclitaxel at a dose of 80 mg/m² weekly had an ORR of 23.8% (95%CI: 5.59-42.03), with a median survival of 5.8 months. The most common toxicity associated with paclitaxel was grade 3-4 neutropenia (66.6%), other reported side effects included neuropathy, infections, and other gastrointestinal symptoms^[23].

Docetaxel is another taxane that was studied in relapsed/refractory SCLC. In the mid-1990s, docetaxel was studied in a phase II trial in patients with previously treated SCLC^[24]. The study showed that IV docetaxel 100 mg/m² once every 3 weeks was associated with an ORR of 25%, with a median duration of response ranging between 3.5 and 12.6 months. The main toxicities reported in this trial were neutropenia, alopecia, and fatigue^[24].

It is worth to mention that Cabazitaxel was also studied in the setting of relapsed SCLC, but a study conducted by Evans *et al.*^[25] showed inferior PFS and OS when compared to topotecan.

Temozolomide

Temozolomide (TMZ) is an oral alkylating agent, which acts through production of O⁶-alkyl-guanine lesions on DNA. These lesions are removed by O⁶-methylguanine-DNA methyltransferase (MGMT).

However, if these lesions remain unrepaired, they can lead to cytotoxicity and ultimately apoptosis^[26,27]. At its earliest stages, temozolomide was used in treating refractory astrocytoma and glioblastoma multiforme^[26].

In SCLC, several studies suggested that TMZ can be useful. Pietanza *et al.*^[26] performed a phase II study on 64 patients where 48 had sensitive disease (defined as having relapse or progression ≥ 60 days after first line chemotherapy) and 16 had refractory disease. Patients received oral TMZ 75 mg/m² daily for 21 days during a 28-day cycle. The study reported an ORR of 23% (95%CI: 12%-37%) in the sensitive group and 13% (95%CI: 2%-38%) in the refractory group. Patients who had methylated MGMT experienced a higher response compared to patients with unmethylated MGMT (38% *vs.* 7%, $P = 0.08$). Interestingly, patients with brain metastasis had an ORR of 38% (95%CI: 14%-68%)^[26]. Another study showed that TMZ can also be effective and tolerable using a regime of 200 mg/m² daily for 5 days in 28-day cycles for patients with relapsed SCLC^[28]. The most common toxicities reported with temozolomide were fatigue, gastrointestinal symptoms, and hematologic toxicities (most commonly lymphopenia)^[26].

Etoposide

Etoposide has been used in treatment of SCLC for a long time. The use of etoposide in the second line setting has also been studied in patients who had received IV etoposide. A phase II trial showed that oral etoposide 50 mg/m² daily for 21 days can lead to an ORR of 45.5% (95%CI: 27%-65%), median duration of response of 4 months (1.5-9.5 months), and median survival of 3.5 months (1-15 months)^[29]. Another phase II trial showed a response rate of 23%^[30]. The most common observed toxicities were myelosuppression and alopecia^[29].

Vinorelbine

Vinorelbine is a semisynthetic vinca alkaloid which acts through binding to microtubular proteins, preventing tubulin polymerization^[31]. There are data that suggest efficacy of vinorelbine in the setting of SCLC. A phase II study was conducted on 26 patients with history of recurrent SCLC, who received vinorelbine 30 mg/m² weekly, having shown a partial response rate of 16% (95%CI: 4%-36%) whereas 28% of the patients had stable disease^[31]. Leukopenia was the major associated toxicity with vinorelbine as it occurred in 80% of the patients. Other common toxicities included anemia, gastrointestinal symptoms, and drug related fever^[31]. Recently, a study in Poland showed that combining vinorelbine and cisplatin with electroporation (EP) was associated with increased anticancer activity due to the exposure of the cells to high intensity electric pulses, allowing the usage of lower doses of drugs^[32].

Bendamustine

Bendamustine is an alkylating agent that has been commonly used in different lymphoproliferative disorders. The clinical benefit of bendamustine in SCLC was demonstrated initially in a phase II clinical trial that was conducted in Europe. In this study, Schmittel and his colleagues^[33] enrolled 21 patients with SCLC who had a relapse ≥ 2 months after completion of first line therapy. Twenty-one patients received bendamustine at a dose of 120 mg/m² in the first two days every 3 weeks. The study showed a response rate of 29% with a median survival of 7 months.

Subsequently, another phase II study was conducted in North America for patients with relapsed SCLC where patients received 120 mg/m² on the first 2 days of a 21-day cycle. This study subdivided the population to a sensitive disease group; defined as stable or responsive disease to a platinum containing therapy for at least 90 days, or resistant disease group. A total of 50 patients participated with a response rate of 26% (95%CI: 13.3%-39.5%). The response rate was higher in the sensitive disease group compared to the resistant disease group (33% *vs.* 17%). The overall clinical benefit (complete response + partial response + stable disease) rate was 67%. The median OS was 4.8 months (95%CI: 3.8-6.3 months) which was also

better in the sensitive group (5.7 months vs. 4.1 months)^[34]. The most common toxicities were fatigue, anemia, thrombocytopenia, and different gastrointestinal symptoms^[34].

Gemcitabine

A phase II study showed that treatment with gemcitabine at a dose of 1000 mg/m² on day 1, day 8 and day 15 of a four-week cycle, resulted in an overall response rate of 13% (95%CI: 6%-27%), with a median survival of 17 weeks (4-84 weeks)^[35]. Interestingly, a different study tried gemcitabine 1,250 mg/m² on day 1 and 8 every 3 weeks as a second line treatment of SCLC. But the results were not encouraging, as none of the 27 patients had a response^[36].

Amrubicin

Amrubicin is a 3rd generation anthracycline and a topoisomerase II inhibitor that has a chemical structure similar to doxorubicin^[37,38]. The first data about amrubicin in SCLC was obtained from Japan after a phase II trial studied it in patients with untreated ES-SCLC. This study showed an overall response rate of 75.8% (95%CI: 57.7%-88.9%) and a median survival of 11.7 months (95%CI: 9.9-15.3 months)^[37]. Later, several studies were conducted, but the most notable one was a phase III clinical trial which compared amrubicin to topotecan in the second line setting. This trial evaluated 637 patients who were randomized 2:1 to amrubicin 40 mg/m² daily for three days or topotecan 1.5 mg/m² for 5 days every 3 weeks. Amrubicin was associated with a better overall response rate (31.1% vs. 16.9%, $P = 0.001$) and median PFS (4.1 months vs. 3.5 months, $P = 0.01$). However, it failed to show improvement in OS which was the primary endpoint^[38]. This drug is not recommended in the NCCN guidelines for treatment of SCLC^[9].

Vinflunine

Vinflunine is a microtubule inhibitor that has been used in different malignancies including non-small cell lung cancer. Spigel and colleagues^[39] performed a phase II study on 51 patients with relapsed SCLC. Patients received IV vinflunine at 320 mg/m² every 3 weeks. The study showed an ORR of 19.6% (95%CI: 10-33%). The median PFS and OS were 1.6 months (95%CI: 1.3-3.9 months) and 4.9 months (95%CI: 3.2-6.5 months) respectively. Despite being well tolerated, 5% of the patients had grade 3/4 toxicities with neutropenia being the most common (32%). Other side effects included fatigue (16%), arthralgia (16%), and different gastrointestinal symptoms^[39].

Combined chemotherapy

Multiagent chemotherapy have been the standard treatment for extensive-disease SCLC for long time^[40,41]. Chemotherapy regimens like etoposide with platinum, CAV, and cyclophosphamide-doxorubicin-etoposide (CDE) all showed reasonable response rate when used in the first line treatment. Nevertheless, the use of combined chemotherapy is limited in the setting of recurrent/relapsed SCLC, mainly due to intolerable toxicity at that stage^[40]. The NCCN guidelines suggest only CAV in the 2nd line setting^[9].

One of the few studies to evaluate combined chemotherapy agents in the 2nd line setting was a phase III trial from Japan which compared a combination of cisplatin-irinotecan-etoposide (CIE) to topotecan in the setting of relapsed SCLC. This study revealed that CIE had a better OS (18.2 months vs. 12.5 months; $P = 0.0079$). However, the toxicity was significantly higher in the CIE arm and therefore it is not commonly used in practice^[42].

Table 1 summarizes some of the clinical trials that evaluated different chemotherapy agents in relapsed/refractory small cell lung cancer.

Immunotherapy

The utilization of the immune system in treating cancers has been an exciting field that is being developed over the last years. The immune system recognizes cancer cells but, in most situations, it is not able to

Table 1. Clinical trials of chemotherapy agents in the setting of recurrent/relapsed SCLC

Agent	Clinical trial design	Median response rate	Median progression free survival	Median overall survival	Median duration of response
Topotecan	Phase III, IV topotecan vs. CAV ^[12]	24.3%	13.3 weeks	25 weeks	14.4 weeks
	Phase III PO topotecan vs. supportive care ^[13]	7%	16.3 weeks	25.9 weeks	Not reported
	Phase III, IV vs. PO topotecan ^[14]	18.3% - PO; 21.9% - IV	11.9 weeks -PO; 14.6 weeks - IV	33 weeks - PO; 35 weeks - IV	18.3 weeks - PO 25.4 weeks - IV
Lurbinectedin	Phase II, single arm 2nd line ^[16]	35.2%	3.5 months	9.3 months	5.3 months
Irinotecan	Phase II, single arm 2nd line ^[19]	47%	Not reported	187 days	58 days
	Phase II, single arm 2nd line ^[21]	41.3%	4.1 months	10.4 months	Not reported
Paclitaxel	Phase II, single arm ≥ 2nd line ^[22]	29%	65 days	100 days	108 days
	Phase II, single arm ≥ 2nd line ^[23]	23.8%	Not reported	5.8 months	Not reported
Docetaxel	Phase II, single arm ^[24]	25%	4.7 months	Not reported	4.7 months
Temozolomide	Phase II, single arm 2nd or 3rd line ^[26]	23% - sensitive 13% - refractory	1.6 months	5.8 months	3.5 months
Etoposide	Phase II, PO etoposide ≥ 2nd line ^[29]	45.5%	4 months	3.5 + months	4 months
	Phase II, single arm PO etoposide ≥ 2nd line ^[30]	23%	Not reported	18 weeks	CR = 16 weeks PR = 9 weeks
Vinorelbine	Phase II, single arm 2nd line ^[31]	PR = 16% SD = 28%	Not reported	Not reported	Not reported
Bendamustine	Phase II, single arm 2nd and 3rd line ^[34]	26%	4 months	4.8 months	Not reported
Gemcitabine	Phase II, single arm ≥ 2nd line ^[35]	13%	8 weeks	17 weeks	10-20 weeks
Amrubicin	Phase III, Amrubicin vs. topotecan 2nd line ^[38]	31.1%	4.1 months	7.5 months	4.8 months
Vinflunine	Phase II, single arm 2nd line ^[39]	19.6%	1.6 months	4.9 months	2.7 months
CIE	Phase III, CIE vs. topotecan 2nd line ^[42]	84%	5.7 months	18.2 months	Not reported
CAV	Phase III, IV topotecan vs. CAV 2nd line ^[12]	18.3%	12.3 weeks	24.7 weeks	15.3 weeks

SCLC: small cell lung cancer; CAV: Cyclophosphamide, Doxorubicin, and Vincristine; PO: compare oral; CIE: cisplatin-irinotecan-etoposide; PR: partial response; SD: stable disease; CR: complete response

eliminate the cancer cells due to inhibitory receptors and signals (checkpoints). Programmed death-1 (PD-1) and cytotoxic lymphocyte antigen 4 (CTLA-4) are the most common checkpoints that have been studied in solid malignancies^[8]. While immunotherapy is now recommended in the front-line setting, there have been trials in immunotherapy naïve patients with relapsed SCLC, that were conducted prior to availability of IMPower 133 and CASPIAN results.

Nivolumab

The CheckMate 032 trial^[43] evaluated nivolumab in the setting of recurrent SCLC. In this study, patients were randomized to three groups where they received either nivolumab 3 mg/kg alone every 2 weeks until disease progression, nivolumab 1 mg/kg + ipilimumab 3 mg/kg every 3 weeks for 4 cycles followed by maintenance nivolumab every 2 weeks, and nivolumab 3 mg/kg plus ipilimumab 1 mg/kg every 3 weeks for 4 cycles followed by maintenance nivolumab every 2 weeks. The number of patients in each group was 98, 61, and 54 respectively. A fourth group included only three patients who received nivolumab 1 mg/kg + ipilimumab 1 mg/kg. The study showed a response rate of 10% for nivolumab alone, 23% for the nivolumab 1 mg/kg + ipilimumab 3 mg/kg, 19% for the nivolumab 3 mg/kg + ipilimumab 1 mg/kg, and 33% for the nivolumab 1 mg/kg plus ipilimumab 1 mg/kg. Interestingly the expression of programmed death-1 ligand (PD-L1) did not correlate with the response to therapy. Grade 3-4 treatment related toxicities were most common in the nivolumab 1 mg/kg + ipilimumab 3 mg/kg group (30%) with diarrhea being the most common^[43]. An updated analysis of the Checkmate 032 trial showed a higher response rate in the combination of nivolumab 1 mg/kg plus ipilimumab 3 mg/kg compared to nivolumab alone (21.9% vs. 11.6%; odds ratio 2.12; 95% CI: 1.06-4.26, *P*-value = 0.03)^[44]. However, it demonstrated similar OS between the 2 groups. The median OS in the nivolumab group was 5.7 months (95%CI: 3.8-7.6 months) compared to 4.7 months in the combination arm (95%CI: 3.1-8.3 months). Furthermore, toxicities were higher in the combination arm. The last 2 findings led the NCCN panel to recommend nivolumab alone instead of the combination^[9].

The CheckMate 331 trial (NCT02481830)^[45] is an ongoing phase III clinical trial that is comparing nivolumab to topotecan and amrubicin. The trial estimated complete date is in mid-2021, however, preliminary data showed no significant difference in overall survival between nivolumab (median of 7.5 months) and chemotherapy (median of 8.4 months) with a hazard ratio of 0.86 (95%CI: 0.72-1.04).

Pembrolizumab

The KEYNOTE-028 trial (NCT02054806)^[46], is a phase Ib study that evaluated the safety of pembrolizumab 10 mg/kg every 2 weeks in patients with advanced PD-L1 positive ES-SCLC. This study revealed a promising efficacy of pembrolizumab in SCLC with an ORR of 33% (95%CI: 16%-55%)^[46]. The KEYNOTE-158 trial (NCT02628067)^[47], is an ongoing phase II trial, to evaluate the benefit of pembrolizumab in advanced SCLC. The preliminary results showed an ORR of 18.7% (95%CI: 11.8%-27.4%). The response was higher in patients who had PD-L1 positive tumor compared to PD-L1 negative tumor (35.7% vs. 6%)^[47]. A recent paper was published by Chung *et al.*^[48] who performed a combined analysis of both KEYNOTE-028 and KEYNOTE-158. The results demonstrated an ORR of 19.3% (95%CI: 11.4%-29.4%) with a median OS of 7.7 months (95%CI: 5.2-10.1 months). As revealed by the KEYNOTE-158 trial, patients who had PD-L1 positive tumor had better ORR and OS. Nevertheless, the NCCN panel added pembrolizumab as a second line therapy regardless of the PD-L1 results^[9].

Durvalumab

Durvalumab is another immunotherapy agent that was approved by the FDA in 2020 to be used with combined chemotherapy in the first line setting based on the CASPIAN trial^[7]. A phase I study evaluated the use of durvalumab and tremelimumab in patients who had disease progression on at least one treatment. The results of this study showed an ORR of 13.3%, PFS of 1.8 months and an OS of 7.9 months^[49]. However, a phase II study did not show sufficient response of durvalumab and tremelimumab when it was used with or without radiation^[50].

Atezolizumab

Atezolizumab was approved by the FDA to be used in the front line setting based on the IMpower-133 trial which demonstrated significant improvement in the PFS and OS by adding atezolizumab to chemotherapy^[2]. However, the use of it in the second line setting is still under study. A recent phase I trial on 17 patients showed that atezolizumab was tolerated and it had some efficacy with a median OS of 5.9 months^[51].

There are no doubts that the field of immunotherapy will continue to expand, with many different clinical trials currently ongoing. Table 2 summarizes some of the trials that investigated immunotherapy in relapsed small cell lung cancer based on reported studies in literature.

Targeted therapy

Targeted therapy has been an exciting field for different malignancies. Until recently, its use in SCLC has not been successful^[8]. Several trials were done to assess targeted therapies as a single agent or in combination with chemotherapy but many of them did not reach their primary endpoint^[8]. These therapies included bevacizumab, vandetanib, aflibercept, vismodegib, cixutumumab, panobinostat, oblimersen, and obatoclax^[52-58]. However, there are some targeted therapies that have shown some promising results.

Alisertib

Alisertib is an oral aurora kinase A inhibitor^[8]. Melichar *et al.*^[59] performed a study to evaluate alisertib use in different relapsed solid malignancies including SCLC. A total of 48 patients with SCLC were enrolled, with a total overall response rate of 21%. However, the time to progression was only 2.6 months.

Table 2. Clinical trials of immunotherapy in the setting of recurrent/relapsed SCLC

Agent	Study design	Overall response	Progression free survival	Overall survival	Median duration of response
Nivolumab	CheckMate-032: phase I/II Nivolumab 3mg/kg (G1) vs. Nivolumab 1mg/kg + Ipilimumab 3mg/kg (G2) vs. Nivolumab 3mg/kg + Ipilimumab 1mg/kg (G3) as ≥ 2nd line ^[43]	G1: 10% G2: 23% G3: 19%	G1: 1.4 months G2: 2.6 months G3: 1.4 months	G1: 4.4 months G2: 7.7 months G3: 6 months	G1 = not reached G2 = 7.7 months G3 = 4.4 months
	CheckMate 331: phase III Nivolumab vs. chemotherapy as 2nd line ^[45]	14%	1.4 months	7.5 months	8.3 months
Pembrolizumab	KEYNOTE-028: phase Ib, ≥ 2nd line ^[46]	33.3%	1.9 months	9.7 months	19.4 months
	KEYNOTE-158: Phase II, 2 nd line ^[47]	18.7%	2 months	9.1 months	Not reached
Durvalumab	Phase I, durvalumab + tremelimumab as ≥ 2nd line ^[49]	13.3%	1.8 months	7.9 months	18.9 months

SCLC: small cell lung cancer

Veliparib

Veliparib is an inhibitor of poly (ADP-ribose) polymerase (PARP)^[60]. A study compared the combination of veliparib with TMZ to TMZ and placebo in patients with relapsed/refractory SCLC. The study failed to show difference in PFS or OS, but it showed that the combination of TMZ and veliparib was associated with better ORR compared to the other group (39% vs. 14% respectively, $P = 0.016$)^[27].

Pazopanib

Pazopanib is a tyrosine kinase inhibitor (TKI) of vascular endothelial growth factor receptors (VEGFR-1, VEGFR-2, and VEGFR-3), platelet-derived growth factor receptors (PDGFR), and c-kit. It showed promising results when used in the second line setting in refractory/relapsed SCLC^[61]. In a phase II study, 39 patients with platinum sensitive disease and 19 patients with refractory disease received pazopanib 800 mg daily. The partial response rate was 13.8% (95%CI: 5-22.7), with 34.5% achieving stable disease. The median PFS was 2.5 months (95%CI: 1.9-3.1 months) and OS was 6 months (95%CI: 3.8-8.2 months). Interestingly, the study showed that one cycle of pazopanib resulted in significant decrease in number of patients with ≥ 5 circulating tumor cells (CTCs)/7.5ml blood. That led the authors to suggest consideration of CTCs enumeration as biomarker of response^[61].

Anlotinib

Anlotinib is another TKI that targets VEGFR-2, VEGFR-3, PDGFR-b, and c-Kit. The “ALTER 1202” trial, is a phase II, double-blinded, randomized, placebo-controlled study that enrolled 120 patients with SCLC who had disease progression after at least 2 lines of treatment. Eighty-two patients received anlotinib 12 mg daily for 2 weeks on and one week off cycle while the rest got placebo. The study demonstrated a significant improvement in PFS in the anlotinib arm [4.1 months (95%CI: 2.8 to 4.2 months)] compared to placebo [0.7 months (95%CI: 0.7 to 0.8 months)] (P -value < 0.0001). The disease control rate (DCR) was significantly higher in the anlotinib group compared to placebo (71.6% vs. 13.2%, P -value < 0.0001)^[62]. Later, an update was published which also showed an improvement in OS in the anlotinib arm (7.3 months vs. 4.9 months)^[63].

Table 3 summarizes some of the clinical trials that involved targeted therapy in recurrent/relapsed SCLC.

FUTURE CONSIDERATIONS AND CHALLENGES

The treatment of relapsed/refractory SCLC has been challenging over the last several years given the lack of effective therapies. Till 2020, topotecan was the only FDA approved drug for relapsed SCLC before the FDA granted accelerated approval for lurbinectedin. There is no doubt that the management of SCLC is actively developing. Currently, there has been a focus on immunotherapy and targeted therapies in the relapsed/refractory disease setting especially after the results of the IMpower133 and CASPIAN trials.

Table 3. Clinical trials of targeted therapies in the setting of recurrent/relapsed SCLC

Agent	Study design	Overall response	Progression free survival	Overall survival	Median duration of response
Alisertib	Phase II, single arm 2nd or 3rd line in different solid malignancies including SCLC ^[59]	21%	2.1 months	Not reported	4.1 months
Veliparib	Phase II, veliparib + TMZ to TMZ + placebo 2nd line ^[27]	39% vs. 14 (P-value = 0.016)	3.8 months vs. 2 months (P = 0.39)	8.2 months vs. 7 months (P = 0.5)	4.61 months vs. 3.68 months (P = 0.0507)
Pazopanib	Phase II, single arm 2nd line ^[61]	13.8% PR	2.5 months	6 months	Not reported
Anlotinib	Phase II anlotinib vs. placebo as ≥ 3rd line ^[62,63]	DCR: 71.6% vs. 13.2% (P-value < 0.0001)	4.1 months vs. 0.7 months	7.3 months vs. 4.9 months	Not reported

SCLC: small cell lung cancer; TMZ: Temozolomide; DCR: disease control rate; PR: partial response

Table 4. Active clinical trials evaluating new treatments for recurrent/relapsed SCLC

Study name/ClinicalTrials.gov ID	Study design	Treatment arms	Primary outcome
ATLANTIS/NCT02566993	Phase III	Lurbinectedin + doxorubicin vs. CAV vs. topotecan	Overall survival
CheckMate 331/NCT02481830	Phase III	Nivolumab vs. topotecan vs. amrubicin as 2nd line	Overall survival
TAHOE/NCT03061812	Phase III	Rovalpituzumab vs. topotecan as 2nd line	Overall survival
KEYNOTE-158/NCT02628067	Phase II	Pembrolizumab as 2nd line	Overall response rate
MCC-19163/NCT03406715	Phase II	Single arm: Ipilimumab + Nivolumab + Dendritic Cell Based p53 Vaccine as a 2nd line and beyond	Disease control rate
AFT-17/NCT02963090	Phase II	Topotecan IV vs. Pembrolizumab as 2nd line	Progression free survival
CA001-030/NCT02247349	Phase I/II	BMS-986012 + nivolumab vs. BMS-986012 alone as 2nd line	Safety as measured by frequency of adverse events.
KEYNOTE-028/NCT02054806	Phase I	Pembrolizumab as 2nd line in different solid tumors	Overall response rate
MEDIOLA/NCT02734004	Phase I/II	Olaparib + bevacizumab + durvalumab vs. Olaparib + durvalumab as 2nd line and beyond	Disease control rate, safety and tolerability of the drugs, overall response rate.

SCLC: small cell lung cancer; CAV: Cyclophosphamide, Doxorubicin, and Vincristine

Table 4 summarizes some of the ongoing clinical trials to investigate new approaches for relapsed/refractory SCLC.

EFFECT OF COVID-19 ON SCLC TREATMENT

In late 2019, multiple cases of atypical pneumonia had been reported in Wuhan, China, caused by a novel type of coronavirus named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), that led to the COVID-19 disease^[64]. So far, its impact on lung cancer diagnosis and treatment is not well reported. However, a recent study from Spain did show that patients with lung cancer and COVID-19 infection have a higher mortality rate compared to the general population with COVID-19 alone^[61]. While there is a concern that treating these patients may increase the risk of complications associated with the SARS-CoV-2, relapsed SCLC has a very aggressive course. We recommend continuing treatment of these patients while monitoring for the development of COVID-19 disease.

CONCLUSION

In conclusion, relapsed SCLC remains a difficult disease with a dismal prognosis. Most of the patients will have disease relapse after a few months of first-line treatment. Till date, there are only 2 drugs approved by the FDA, topotecan and lurbinectedin both with modest efficacy. However, the recent advances in immunotherapy and targeted therapy are exciting, and the results of ongoing trials may help find a strategy that will improve outcomes for these patients.

DECLARATIONS

Authors' contributions

Wrote the manuscript: Abughanimeh O

Reviewed, edited, and approved the final manuscript: Ernani V, Marr A, Ganti AK

Contributed to the manuscript: Abughanimeh O, Ernani V, Marr A, Ganti AK

Availability of data and materials

Not applicable.

Financial support and sponsorship

All authors have declared that they have no financial relationships at present that is related to this paper.

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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Glycogen synthase kinase 3 β biology in bone and soft tissue sarcomas

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How to cite this article: Abe K, Shimozaki S, Domoto T, Yamamoto N, Tsuchiya H, Minamoto T. Glycogen synthase kinase 3 β biology in bone and soft tissue sarcomas. *J Cancer Metastasis Treat* 2020;6:51. <http://dx.doi.org/10.20517/2394-4722.2020.117>

Received: 28 Oct 2020 **Accepted:** 27 Nov 2020 **Published:** 17 Dec 2020

Academic Editor: Ivory Ma, Ian Judson **Copy Editor:** Whitney Xu **Production Editor:** Jing Yu

Abstract

Bone and soft tissue sarcomas are malignant neoplasms probably originating from musculoskeletal and mesenchymal progenitor cells. More than 80 different histopathological subtypes are encountered in orthopedics. The standard of care for sarcoma patients involves a multidisciplinary combination of surgery, anthracycline-based multiagent chemotherapy and radiation. Unfortunately, these are associated with adverse events and occasionally disappointing outcomes. Various genomic-, biologically-, and immunologically-based therapies are still under evaluation in early-phase clinical trials. However, there are strong barriers to the development and clinical translation of new therapeutic modalities. This is due to the rarity of these diseases, the broad spectrum of tumor subtypes with genetic and biological heterogeneity, and the wide variability in clinical manifestation, response to treatment and prognosis. A potential approach toward overcoming this barrier is to identify therapeutic targets that cover multiple sarcoma types. Glycogen synthase kinase 3 β (GSK3 β) has emerged as a common therapeutic target in more than 25 different cancer types. Here we review the evidence for tumor-promoting roles of GSK3 β in the major types of bone and soft tissue sarcomas including osteosarcoma, rhabdomyosarcoma, synovial sarcoma, and fibrosarcoma. In this review, we describe the therapeutic effects of inhibiting GSK3 β in these sarcoma types, while also protecting healthy cells and tissues from detrimental effects associated with conventional therapies, such as doxorubicin-induced cardiotoxicity. Consequently, we highlight GSK3 β as a potential therapeutic target spanning multiple sarcoma types.

Keywords: Osteosarcoma, rhabdomyosarcoma, synovial sarcoma, fibrosarcoma, glycogen synthase kinase 3 β



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INTRODUCTION

Sarcomas are rare malignant neoplasms of putative mesenchymal resident (progenitor) cell origin that comprise 20% of all bone sarcomas and 80% of all soft tissue sarcomas^[1,2]. They account for almost 1% of newly diagnosed malignancies and deaths from the disease^[3]. Sarcomas can arise at almost any anatomical site and occur most often in children, adolescents, and young adults. More than 80 histopathological subtypes of sarcomas are defined by the updated WHO classification^[4], with wide variability in their clinical manifestation, response to treatment and prognosis. Accurate and differential diagnosis of the respective sarcoma types is challenging because of their similarity and overlapping morphological features. Several new approaches for cytogenetic, molecular, and immunohistochemical testing methods have been combined with clinical and histopathological evaluation^[5,6]. The wide variety of tumor subtypes with a difficult histopathologic diagnosis and the occurrence of tumors at many possible anatomical sites complicate the overall biological and clinical understanding of bone and soft tissue sarcomas. Hence, the current clinical practice guidelines for bone and soft tissue sarcomas^[7,8] do not adequately cover patient management for all sarcoma types.

Surgery remains the mainstay of treatment for most sarcoma patients with localized tumor. This is often combined with chemotherapy and/or radiation in neoadjuvant and adjuvant settings. Patients with metastasis, at initial diagnosis or after curative surgery, undergo chemotherapy and radiation, either alone or in combination^[7,8]. Currently, anthracycline-based chemotherapy is widely accepted as the first-line therapy for most patients with advanced sarcoma. Doxorubicin remains a pivotal agent and is prescribed in various combinations with other chemotherapeutics including ifosfamide, dacarbazine, gemcitabine, and docetaxel. However, the empirical cytotoxic chemotherapies are associated with disappointing patient outcomes and inevitable adverse effects, even when combined with new generation anticancer agents such as eribulin and trabectedin^[9,10].

Recently, major efforts to decipher the genomic, epigenomic, and other biological properties of various sarcoma types have identified several actionable molecular targets with potential therapeutic application. Some of the molecular alterations found in sarcomas include activation of mutations in the c-kit and B-raf genes, gene translocation involving growth factors such as platelet-derived growth factor (PDGF) receptor (PDGFR) and colony stimulating factor 1 receptor, gene translocation involving transcription factors such as vascular endothelial growth factor receptor (VEGFR), inactivation of tumor suppressor genes (TSC1/2 and PTEN) leading to activation of mechanistic target of rapamycin (mTOR), and overexpression of PDGFR and VEGFR^[11-15]. Several agents developed against these actionable targets have been tested in clinical trials of advanced sarcoma patients and preliminary results shown improved survival. However, most clinical trials for sarcoma remain in the early stages (phase I or phase II)^[11,16,17]. The rarity of sarcoma, the wide variety of histological subtypes and the lack of predictive biomarkers are major hurdles in the clinical evaluation of available targeted agents. Consequently, ongoing clinical trials have yet to show a significant survival benefit of the targeted agents over conventional chemotherapy. Identification of therapeutic targets that span multiple sarcoma subtypes is therefore required to break the current deadlock in developing innovative sarcoma therapies. This review focuses on glycogen synthase kinase 3 β (GSK3 β) as an emerging and common therapeutic target in major sarcoma types including osteosarcoma, rhabdomyosarcoma, synovial sarcoma, and fibrosarcoma that are frequently encountered in orthopedics.

OVERVIEW OF GSK3 β BIOLOGY AND DISEASES

GSK3 β was initially identified as an isoform of the GSK3 family of protein kinases. In addition to its primary function of phosphorylating and thus inactivating glycogen synthase, GSK3 β phosphorylates serine and threonine residues in various functional and structural proteins, thereby serving multipurpose roles in pivotal cellular pathways. GSK3 β is constitutively active in cells upon tyrosine 216 phosphorylation (pGSK3 β ^{Y216}). Negative regulation of its activity via serine 9 phosphorylation (pGSK3 β ^{S9}) occurs to control

vital activities and homeostasis in normal cells in response to endogenous and exogenous stimuli^[18,19]. Aberrant expression and activation of GSK3 β contribute to the pathogenesis and progression of common diseases including glucose intolerance, neurodegenerative disorders with cognitive disturbance, and chronic inflammatory diseases^[20,21]. Such differential functions in healthy and diseased cells have highlighted GSK3 β as a potential drug target in various diseases and have stimulated the development of pharmacological GSK3 β inhibitors^[22-24].

In the field of oncology, GSK3 β has long been hypothesized to suppress tumorigenesis. This is based on its inactivation, as indicated by pGSK3 β ^{S9}, in major pro-oncogenic pathways mediated by Wnt/ β -catenin, hedgehog (Hh), notch, and c-Myc signaling, as well as in the process of epithelial-to-mesenchymal transition (EMT)^[25,26]. However, few studies have shown that active GSK3 β suppresses tumor development and progression by disrupting these pro-oncogenic pathways. In contrast to the hypothesis that GSK3 β is a tumor suppressor, a growing number of experimental studies over the past 15 years have demonstrated that deregulated expression and activity of GSK3 β contributes to the pathogenesis and progression of various cancer types. The notion that GSK3 β has pro-tumorigenic properties is supported by observations that tumor cells depend mechanistically on GSK3 β for their survival, proliferation, and invasion, and that GSK3 β renders them unresponsive to chemotherapy, radiation, and some molecular targeted agents in refractory cancer types. A tumor-promoting role for GSK3 β is also supported by evidence of specific and strong therapeutic effects of various GSK3 β inhibitors against at least 25 different cancer types, while sparing the normal cells and tissues^[27-30]. This increasing experimental evidence supports the notion of GSK3 β as a promising therapeutic target in cancer, thereby encouraging the screening and identification of GSK3 β -specific inhibitors for treatment of cancer^[24,31,32].

GSK3 β INVOLVEMENT IN BONE AND SOFT TISSUE SARCOMAS

Among the many bone and soft tissue sarcoma types, the tumor-promoting role of GSK3 β has been reported in osteosarcoma, rhabdomyosarcoma (alveolar and embryonal types), synovial sarcoma, and fibrosarcoma [Table 1].

Osteosarcoma

Although rare, osteosarcoma is the most prevalent primary malignant bone tumor, followed by chondrosarcoma and Ewing sarcoma. It typically affects the long bone of the limbs in children, adolescents, and young adults^[33]. The anatomical site of the primary tumor, clinical characteristics, treatment response, and patient prognosis distinguish high-grade osteosarcoma (accounting for 80% to 90% of cases) from low/moderate-grade osteosarcoma (10% to 20%)^[34]. The current standard of care for the treatment of patients with no detectable metastasis at initial diagnosis (accounting for 80% to 85% of cases) sequentially combines surgery with pre-operative (neoadjuvant) and post-operative (adjuvant) chemotherapy. The remaining 15% to 20% of patients have metastasis at diagnosis and undergo multi-agent chemotherapy. The most effective chemotherapy regimen combines high-dose methotrexate, doxorubicin, and cisplatin^[7,33,35,36]. Beginning in the 1970s, the use of multi-agent chemotherapy in patients with localized disease increased their survival rate from less than 20% to almost 70%^[33]. However, no further substantial improvement has been achieved over the past 25 years. In contrast to the survival benefits obtained with chemotherapy for localized primary tumors, little improvement has been achieved in the 5-year survival rate for patients with concurrent metastasis or post-operative recurrence. This highlights the need for new therapeutic approaches against metastatic progression in osteosarcoma^[37,38].

During the last decade, comprehensive genomics studies have revealed the highly heterogeneous nature of genetic alterations in high-grade osteosarcoma. Although several studies suggested candidate genetic biomarkers for future clinical translation, no actionable genes for targeted therapy have yet been identified^[13]. Based on studies of the biological and immunological characteristics of osteosarcoma, nearly 30 clinical trials involving osteosarcoma patients have tested several agents that target receptor-

Table 1. Tumor-promoting properties of GSK3 β reported in bone and soft tissue sarcomas

Tumor type	Tumor-promoting properties and underlying mechanisms	Ref. No.*
Osteosarcoma	GSK3 β promotes tumor cell survival, proliferation, and low responsiveness to chemotherapy via the NF- κ B-mediated pathway.	[41,45]
	Deregulated GSK3 β sustains tumor cell survival and proliferation via suppression of the Wnt/ β -catenin osteosarcoma suppressor pathway.	[46]
	The therapeutic effect of degalactotigonin (a natural compound from <i>Solanum nigrum</i> L.) against osteosarcoma depends on GSK3 β inactivation-mediated repression of the Hh/Gli1 pathway.	[50]
Rhabdomyosarcoma		
Alveolar-type	GSK3 β phosphorylates and sustains the transcriptional activity of PAX3-FOXO1 fusion proto-oncoprotein in tumor cells.	[62]
Embryonal-type	GSK3 β sustains proliferation and inhibits differentiation of self-renewing, tumor-propagating cells via suppression of the canonical Wnt/ β -catenin pathway.	[64]
Synovial sarcoma and fibrosarcoma	Aberrant expression and activity of GSK3 β sustains survival, proliferation, and invasion of tumor cells through the cyclin D1/CDK4-mediated pathway and enhanced extracellular matrix degradation machinery.	[79]

*Respective reference numbers correspond to the references cited in the text. GSK3 β : glycogen synthase kinase 3 β ; NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cells; Hh: hedgehog; PAX3: paired box 3; FOXO1: forkhead box O-subfamily 1; CDK: cyclin-dependent kinase

type tyrosine kinases (e.g., VEGFR, PDGFR, and c-Kit), cyclin-dependent kinases (CDKs; e.g., CDK4 and CDK6), pro-oncogenic signaling pathways (e.g., Hh and mTOR), the bone microenvironment (e.g., osteoclasts), and immune checkpoint systems. Most trials are in early phases (I and/or II) and none of the targeted agents have so far been approved for the treatment of osteosarcoma^[13,16,36,39,40].

During the past decade, GSK3 β has been proposed as a potential therapeutic target in bone and soft tissue sarcomas including osteosarcoma [Table 1]. An earlier study showed an inverse association between the level of pGSK3 β ^{S9} (inactive form) and the capacity of tumor formation in human osteosarcoma cells^[41]. This study also demonstrated the role of a constitutively active form of GSK3 β (artificial transversion of S9 to alanine) in promoting tumor proliferation. Moreover, it was shown that lithium chloride, an ATP-non-competitive and non-specific GSK3 β inhibitor^[42], attenuated the proliferation of osteosarcoma cells, induced their apoptosis and enhanced the efficacy of doxorubicin and methotrexate against sarcoma cells. The therapeutic effect of lithium against tumor cells was shown to be associated with reduced activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B)^[41], consistent with studies showing that GSK3 β is indispensable for the NF- κ B-mediated pro-survival pathway^[43,44]. Subsequently, another study showed overexpression of GSK3 β in primary osteosarcomas, and induction of apoptosis in sarcoma cells following treatment with a pharmacological GSK3 β inhibitor that reduced Bcl-2 expression^[45]. Our work has shown that the expression of GSK3 β and level of pGSK3 β ^{Y216} (active form) in osteosarcoma cells was higher than in normal osteoblasts, but the level of pGSK3 β ^{S9} was lower. We also demonstrated that GSK3 β -specific inhibitors and RNA interference attenuated the survival and proliferation of tumor cells and induced apoptosis, while sparing normal osteoblasts. The effect of GSK3 β inhibition against tumor cells was coincident with reduced phosphorylation of GSK3 β -phospho-acceptor sites in β -catenin and with increased β -catenin expression, nuclear translocation, and co-transcriptional activity^[46]. Our results suggest the therapeutic effects of GSK3 β inhibition are associated with the activation of β -catenin, a putative tumor suppressor in osteosarcoma^[47,48]. However, the role of the Wnt/ β -catenin pathway in the development of osteosarcoma remains controversial^[49]. A recent study reported that the therapeutic effect of degalactotigonin (a natural compound from *Solanum nigrum* L.) against osteosarcoma occurred via GSK3 β inactivation-mediated repression of the Hh/Gli1 pathway, thus indirectly suggesting a pro-tumorigenic role for GSK3 β ^[50]. As discussed in FUTURE PERSPECTIVES FOR GSK3 β IN SARCOMA BIOLOGY AND THERAPY, the GSK3 β / β -catenin axis has opposing roles in normal osteogenesis and in the osteoclastic process. A series of studies described here have helped to understand the biology of GSK3 β and identified it as a promising target for the treatment of osteosarcoma [Figure 1]. These advances should facilitate the development of new GSK3 β inhibitors for this refractory disease^[51].

Rhabdomyosarcoma

Rhabdomyosarcoma is the most prevalent pediatric sarcoma and is characterized by tumor cells with a skeletal myoblast-like phenotype, possibly arising from primitive mesenchymal cells^[52,53]. The two major subtypes are embryonal (approximately 60% of cases) and alveolar (20%) rhabdomyosarcoma, with the less prevalent subtypes being pleomorphic (10%) and spindle/sclerosing (10%)^[54]. While rhabdomyosarcomas can arise at any anatomical site, the embryonal subtype preferentially arises in the head and neck region and in the genitourinary tract of children and young adolescents. This tumor subtype frequently shows loss of heterozygosity at the 11p15 locus that includes the insulin-like growth factor-II gene. The alveolar subtype is notoriously aggressive and affects the trunk (in particular, the perineal and paraspinal areas) and extremities in adolescents and young adults. This subtype is characterized genetically by gene rearrangement of the forkhead box O-subfamily 1 (FOXO1) resulting in t(1;13)(p36;q14) translocation generating the paired box (PAX)3-FOXO1 fusion or t(2;13)(q35;q14) translocation generating the PAX7-FOXO1 fusion proto-oncogene^[52-55].

The treatment strategy for rhabdomyosarcoma is based on a risk stratification (low-, intermediate-, and high-risk) of the disease that consists of tumor histological subtype, the tumor stage (equivalent to TNM classification) prior to treatment, and the post-surgery clinical grouping (e.g., extent of residual tumor, presence of lymph node metastasis and of distant metastasis)^[52,53,55]. Most rhabdomyosarcoma patients require a multimodal combination of chemotherapy, surgery, and/or radiation therapy. The two standard chemotherapy regimens include the combination of vincristine and actinomycin D with either cyclophosphamide or ifosfamide. Implementation of combined multi-agent chemotherapy has significantly improved patient outcomes. However, the efficacy of treatment for patients with high-risk rhabdomyosarcoma (defined as the presence of distant metastasis^[52,55]) has not improved for the past three decades^[53,55]. Several clinical trials for rhabdomyosarcoma conducted over the last decade have evaluated molecular targeted agents, immune checkpoints-blocking agents, and cellular immunotherapeutics. Only pazopanib, a multi-kinase inhibitor that targets PDGFR- α , VEGFRs, and c-Kit^[56], has been tested in a phase III clinical trial for patients with metastatic soft tissue sarcomas including rhabdomyosarcoma. All the remaining trials have been either phase I or II^[57]. Comprehensive whole genome analyses for embryonal and alveolar rhabdomyosarcomas has failed to identify any actionable therapeutic targets^[55,58,59], hence the urgent need to identify new therapeutic targets.

A previous study showed that a liposome-protamine-siRNA (LPR) nanoparticle that targets the PAX3-FOXO1 fusion proto-oncogene transcript inhibited the proliferation of alveolar rhabdomyosarcoma cells and their xenograft tumors^[60]. Another study demonstrated that entinostat, a class-I histone deacetylase inhibitor, reduced the expression of PAX3-FOXO1 in alveolar rhabdomyosarcoma cells, thereby sensitizing them to chemotherapeutic agents^[61]. These studies suggest that PAX3-FOXO1 fusion proto-oncogene and its product are potentially actionable targets in the treatment of alveolar rhabdomyosarcoma. Consistent with this suggestion is an earlier study^[62] that screened 160 different kinase inhibitors against alveolar rhabdomyosarcoma cell lines and identified GSK3 β inhibitors including TWS119^[63] as tumor type-selective inhibitors. This study found that GSK3 β phosphorylated the PAX3-FOXO1 fusion protein in tumor cells and that inhibition of GSK3 β attenuated the transcriptional activity of this oncoprotein, suggesting a role for GSK3 β in sustaining alveolar rhabdomyosarcoma^[62] [Figure 1]. Subsequently, a large chemical screen directed against self-renewing, tumor-propagating cells (TPCs) in embryonal rhabdomyosarcoma identified GSK3(β) inhibitors (e.g., BIO, CHIR 98014, and CHIR 99021) as potent suppressors of this tumor type via the inhibition of proliferation and the induction of terminal myogenic differentiation of TPCs^[64]. The tumor-suppressive effect of GSK3(β) inhibitors was associated with induction of the canonical Wnt/ β -catenin pathway, which was underpinned by the finding that recombinant Wnt3A and stabilized β -catenin enhanced the terminal differentiation of rhabdomyosarcoma TPCs [Figure 1]. Collectively, these studies^[62,64] suggest that GSK3 β is a potential therapeutic target that covers the major subtypes (embryonal

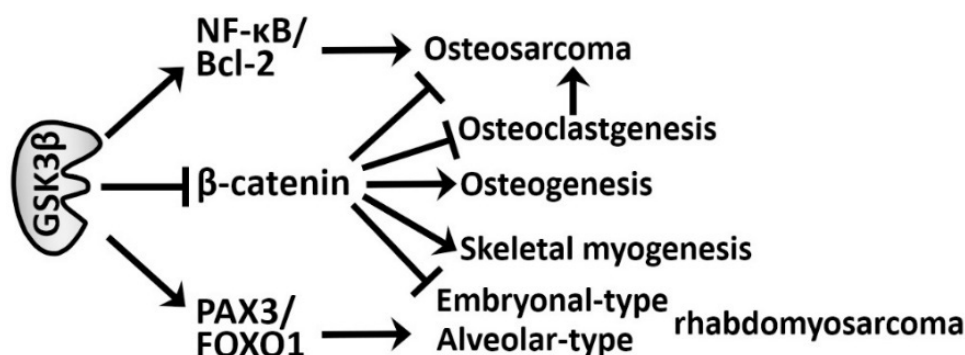


Figure 1. Reported mechanisms by which GSK3β promotes (→) the development of osteosarcoma and rhabdomyosarcoma, while suppressing (—|) osteogenesis and skeletal musculogenesis. →: promoting; —|: suppressive; GSK3β: glycogen synthase kinase 3β; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; FOXO1: forkhead box O-subfamily 1; PAX3: paired box 3

and alveolar; in total nearly 80%) of rhabdomyosarcoma.

Synovial sarcoma and fibrosarcoma

In addition to rhabdomyosarcoma, the major soft tissue sarcoma types include synovial sarcoma, fibrosarcoma, and undifferentiated pleomorphic sarcoma^[1,2,65]. Synovial sarcoma accounts for 5%-10% of all soft tissue sarcomas. Although it can arise at almost any anatomical site and at any age, synovial sarcoma frequently affects the extremities, particularly the popliteal fossa, in adolescents and young adults^[66]. Histologically, this sarcoma is characterized by biphasic tumor cells comprising epithelioid and spindle-shaped cellular components, thereby mimicking synovial tissue. However, unlike its nomenclature, this sarcoma does not arise from synovial tissue and does not express synovial cell markers^[67]. Genetically, more than 90% of synovial sarcoma tumors show a pathognomonic t(X;18)(p11.2;q11.2) translocation that generates a fusion of the synovial sarcoma 18 (SS18) and SSX genes, encoding a pro-oncogenic transcription factor^[68]. Despite the standard approach of wide radical surgery combined with radiation of the primary tumor^[69], local recurrence and distant metastasis are frequently encountered^[70], resulting in poor patient outcome^[71]. Although synovial sarcoma is relatively chemosensitive compared to other soft tissue sarcomas^[72], there is only limited survival benefit from anthracycline-based adjuvant chemotherapy for high-risk patients with metastatic and/or residual tumor^[73].

Fibrosarcoma is defined as a malignant neoplasm of fibroblast origin and characterized histologically by a “herringbone” architecture formed by the tumor cells and stromal deposition of collagen within the tumor^[4,74]. Fibrosarcomas are divided into the congenital-type that rarely metastasizes, and the adult-type that is highly malignant^[75]. The incidence of adult-type fibrosarcoma has declined over the years as the diagnostic criteria has become more strict and other mesenchymal tumors that mimic fibrosarcoma are more accurately defined. Most fibrosarcomas arise from the fascia and tendon of soft tissue, with rare occurrences in the medullary canal and periosteum of bones. Adult-type fibrosarcoma affects the deep soft tissues of the extremities, trunk, head, and neck in middle- and older-aged adults. The mainstay of treatment is surgical removal of the tumor, occasionally followed by radiation for high-grade tumors and cases with insufficient surgical margin. Although chemotherapy is not recommended for the management of fibrosarcoma patients, anthracycline-based chemotherapy is the first-line regimen. However, fibrosarcoma is characterized by low sensitivity to chemotherapy and frequent tumor recurrence. This results in poor overall prognosis, with a 10-year survival rate of 60% and 30% for patients with low- and high-grade tumors, respectively^[76].

During the past decade, several targeted agents have been developed for soft tissue sarcomas^[14-17], together with a new generation of chemotherapeutic agents (e.g., trabectedin and eribulin)^[9,10]. Recently, the multi-target kinase inhibitors pazopanib^[56] and anlotinib^[77] were approved for multiple soft tissue sarcoma types

including synovial sarcoma and fibrosarcoma, followed by the approval of tazemetostat, an inhibitor of enhancer of zeste homolog 2 (EZH2)^[78], for advanced or metastatic epithelioid sarcoma^[15]. These agents improved the progression-free and overall survival of soft tissue sarcoma patients but showed little improvement over conventional anticancer agents. Therefore, identification of new therapeutic targets is imperative to allow the development of efficient, biologically-based treatments for both sarcoma types.

Recently, we showed the level of pGSK3 β ^{Y216} (active form) was higher in human synovial sarcoma and fibrosarcoma cell lines than in untransformed fibroblast cells, considered to be the normal mesenchymal counterpart cells. Inhibition of the activity or expression of GSK3 β suppressed the survival and proliferation of sarcoma cells, attenuated their invasion into collagen gel, and induced their apoptosis. These effects of GSK3 β inhibition against sarcoma cells were associated with G0/G1-phase cell cycle arrest and reduced expression of cyclin D1, CDK4, and matrix metalloproteinase 2. Intraperitoneal administration of GSK3 β -specific inhibitors attenuated the growth of synovial sarcoma SYO-1 and fibrosarcoma HT1080 cell xenografts in athymic mice, with no obvious side effects. This treatment also suppressed cell proliferation and induced apoptosis in the xenograft tumors. These results indicate that synovial sarcoma and fibrosarcoma depend on deregulated activity of GSK3 β to enhance the cyclin D1/CDK4-mediated pathway for cell proliferation and degradation of extracellular matrix for tumor invasion [Table 1]. Our study therefore provides a biological basis for GSK3 β as a new and common therapeutic target for these sarcoma types^[79] as well as for osteosarcoma^[41,45,46] and embryonal/alveolar rhabdomyosarcomas^[62,64].

FUTURE PERSPECTIVES FOR GSK3 β IN SARCOMA BIOLOGY AND THERAPY

In order to confirm GSK3 β as a relevant and potentially valuable therapeutic target in bone and soft tissue sarcomas, it is important to broaden the spectrum of targetable tumor types. Moreover, it is important to explore the mechanistic influence of GSK3 β on emerging sarcoma therapies and to clarify the properties of this kinase in normal cells and tissues affected by sarcoma therapy.

Potential involvement of GSK3 β in undifferentiated pleomorphic sarcoma (malignant fibrous histiocytoma)

Undifferentiated pleomorphic (UP) sarcoma is currently defined as a subset of the sarcoma type previously designated as malignant fibrous histiocytoma (MFH) that encompassed myxofibrosarcoma, pleiomorphic liposarcoma/rhabdomyosarcoma, and UP sarcoma^[4]. UP sarcoma is one of the most prevalent soft tissue sarcomas, accounting for 10% of cases in adults. It frequently affects deep soft tissues in the extremities and trunk, but rarely occurs in superficial regions such as subcutaneous tissue^[80,81]. As with most soft tissue sarcomas, the mainstay of curative treatment for UP sarcoma is surgical excision of the tumor and post-surgery irradiation. Optional adjuvant chemotherapy is reported to increase the overall survival of patients^[82]. The first-line treatment for metastatic UP sarcoma is doxorubicin-based chemotherapy, occasionally combined with ifosfamide or olaratumab, an anti-PDGF antibody. Although other anti-tumor agents such as trabectedin and pazopanib have shown some efficacy, the outcome of patients with advanced UP sarcoma is worse than for other soft tissue sarcoma types^[83].

The genetic profile of UP sarcoma has not been fully elucidated^[84], although the inactivation of Rb and loss of p53 function are frequently observed in MFH^[85]. A previous study showed the side population cells (hypothetically corresponding to stem-like cells) of UP sarcoma display activation of both Hh- and notch-mediated pathways responsible for sarcoma cell self-renewal. This study suggested that UP sarcoma cells share the same molecular pathways as mesenchymal stem cells (MSCs)^[86]. Another study demonstrated that human MSCs could be transformed via inhibition of Wnt/ β -catenin signaling to form UP sarcoma-like tumors in athymic mice, thus suggesting MSCs as the origin of UP sarcoma^[87]. GSK3 β is a negative regulator of the canonical Wnt/ β -catenin pathway^[18-20] and of the maintenance of MSCs, as described below. Therefore, GSK3 β may potentially play a role in the tumorigenesis and progression of UP sarcoma and

could be a therapeutic target in this sarcoma in addition to osteosarcoma^[41,45,46,50], rhabdomyosarcoma^[62,64], synovial sarcoma, and fibrosarcoma^[79], as discussed above.

GSK3 β and upfront therapies in bone and soft tissue sarcomas

Immunotherapy

Immunotherapy has recently attracted considerable attention for the treatment of many cancer types^[88,89]. It has also emerged as a possible upfront therapy for bone and soft tissue sarcomas^[17,90-92]. Currently available cancer immunotherapies are based on innate immune reactions represented by natural killer T (NKT)-cells against cancer cells, adoptive anti-tumor immunity exerted by CD8⁺ memory T cells and genetically engineered chimeric antigen receptor (CAR)-T cells, vaccination with tumor-specific antigens, and finally on the blockade of immune checkpoints with therapeutic antibodies to programmed cell death 1 (PD-1), programmed cell death-ligand 1 (PD-L1), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)^[88,89]. Various regimens consisting of many of these immunotherapeutic cells and agents either alone or in combination with chemotherapy, radiation or other targeted agents have been evaluated in many early phase (I or II) clinical trials. Unfortunately, most have so far resulted in disappointing outcomes^[17,90-92].

As reviewed recently by ourselves and others^[30,32], inhibition of GSK3 β in normal peripheral NKT cells facilitates their maturation and enhances their cytotoxic effects against acute myeloid leukemia cells^[93,94]. In adoptive tumor immunity, CD8⁺ memory T-cells differentiate into pluripotent memory stem T-cells that are capable of self-renewal and have anti-tumor properties via activation of the Wnt/ β -catenin pathway^[95]. Consistently, GSK3 β inhibition enhances the cytotoxic effect of CD8⁺ memory stem T-cells against gastric cancer cells through the induction of effector T-cell-derived Fas-ligand^[96]. Similar to CD8⁺ memory T-cells, inhibition of GSK3 β in mouse glioblastoma-specific CAR-T cells increased their survival, proliferation, and generation of memory phenotype, thereby enhancing their cytotoxic capacity^[97]. During the blockade of immune checkpoints between tumor cells and CD8⁺ memory T-cells, inactivation of GSK3 β suppresses PD-1 expression via upregulation of the transcription factor Tbx21, thereby enhancing CD8⁺ cytotoxic T-cell responses^[98,99]. Moreover, GSK3 β inhibition reverses the blockade of CD28 by CTLA-4^[100] that is required to rescue exhausted CD8⁺ T-cells^[101]. These preliminary findings suggest broader roles for GSK3 β within the cancer immunosuppressive environment by negatively regulating innate and adoptive anti-tumor immune reactions and by sustaining the immune checkpoints mediated by the PD-1/PD-L1 axis and by CTLA-4^[102]. Consequently, these early studies hold considerable promise for targeting GSK3 β during immunotherapy for various cancer types^[102] including bone and soft tissue sarcomas [Figure 2].

MSC therapy in bone sarcomas

MSCs are a rare population of non-hematopoietic stromal (stem) cells in the bone marrow and other connective tissues such as adipose tissue. They are capable of self-renewal and of undergoing differentiation into the specific mesenchymal cell types. MSCs have attracted widespread interest in sarcoma research and management as a plausible origin of tumorigenesis and as a component of the tumor-promoting microenvironment^[103-105]. Paradoxically, MSCs could also be potential cellular weapons in therapeutic applications^[106,107]. Studies have shown that MSCs contribute to osteosarcoma progression through their ability to home into the tumor and induce neovascularization and elicit an immunosuppressive tumor environment, thereby sustaining tumor cell survival and proliferation^[105,108]. Conversely, other studies reported that MSCs suppress proliferation and induce apoptosis in tumor cells while altering the properties of stromal cells to induce anti-inflammatory effects, inhibit tumor angiogenesis, and ultimately prevent metastasis^[107,108].

Based on the tumor site tropism of MSCs, several recent laboratory studies have genetically engineered MSCs to function as vehicles for the delivery of various anti-tumor agents. These agents include interferons (e.g., IFN- α), interleukins (e.g., IL-12), oncolytic viruses (e.g., coxsackie and adenovirus), tumor necrosis factor (TNF)- α , TNF-related apoptosis-inducing ligand (TRAIL), therapeutic antibodies, and enzyme/

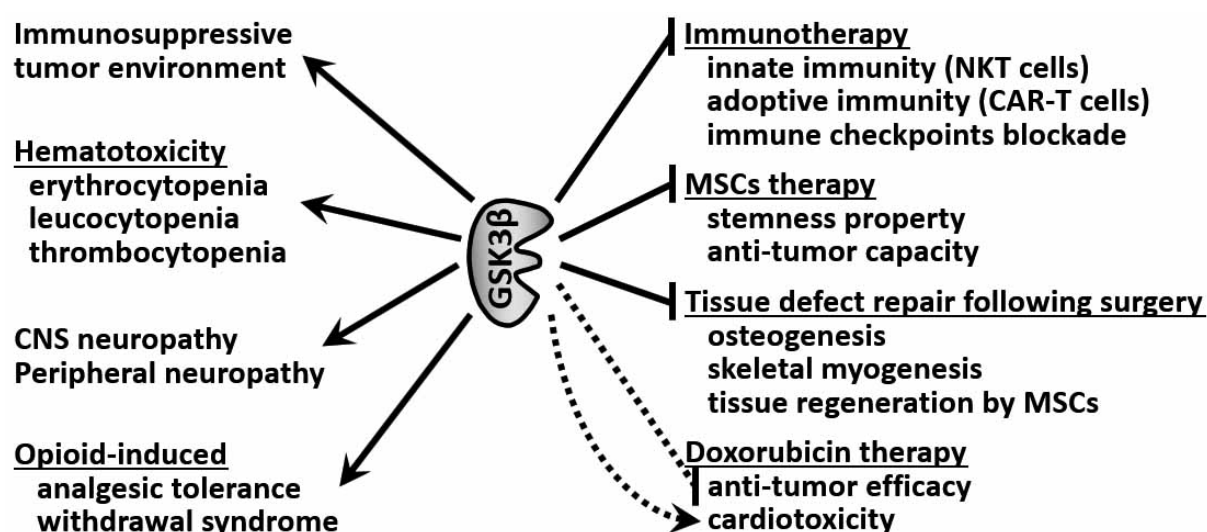


Figure 2. Causative involvement of GSK3β in upfront sarcoma therapies and in the adverse events associated with therapy. →: promoting; —|: suppressive; dotted line: hypothetical effect; NKT: natural killer T; MSCs, mesenchymal stem cells; CAR: chimeric antigen receptor; CNS: central nervous system; GSK3β: glycogen synthase kinase 3β

prodrug combinations [e.g. cytosine deaminase (CD) combined with 5-fluorocytosine (FC)]^[108,109]. Recent preclinical studies^[109] have evaluated the safety and therapeutic efficacy of transduced MSCs loaded with TRAIL, combined CD/5-FC, IL-12, and osteoprotegerin (OPG)^[110]. OPG is a soluble proteoglycan and member of the TNF receptor superfamily that inhibits tumor-promoting osteoclastogenesis and bone resorption by acting as a decoy receptor for the receptor activator of NF-κB ligand (RANKL)-mediated pathway^[110]. Previous studies on tissue regeneration and repair have demonstrated the effects of GSK3β inhibitors on sustaining the stemness phenotype and proliferation of MSCs from different origins, as well as inducing their transdifferentiation into mature mesenchymal cells^[111-115]. These preliminary observations warrant further investigations to clarify whether GSK3β inhibition enhances the therapeutic efficacy of MSCs against bone sarcomas [Figure 2].

GSK3β and normal tissue damage associated with sarcoma treatment

Although the mainstay treatments for bone and soft tissue sarcomas remain to be surgery and chemotherapy^[7,8], they are inevitably associated with post-surgery tissue defects and adverse events related to the chemotherapeutics, respectively. This section focuses on the beneficial effects that targeting GSK3β has on the undesirable events associated with sarcoma treatment.

Normal tissue defect and repair following surgery

Defects in the constitutive normal tissue adjacent to the tumor are an unavoidable consequence of surgery and are particularly serious for patients with musculoskeletal tumors. As discussed earlier, adjuvant chemotherapy, radiation, and targeted therapies are usually combined with surgery to optimize resection of the tumor and to minimize the resulting defect in tumor-adjacent, healthy tissues. There is strong evidence for a critical role of the Wnt/β-catenin pathway in bone formation and homeostasis through induction of osteoblastogenesis and differentiation of the osteogenic cell lineage^[116-120] while also suppressing osteoclastogenesis and the resultant bone resorption^[121,122] [Figure 1]. Osteoclasts within bone sarcoma lesions have been shown to facilitate the progression of osteosarcoma^[40], thereby partially supporting the tumor-suppressive function of the Wnt/β-catenin pathway^[47,48]. Moreover, GSK3β inhibition protects skeletal muscle cells from apoptosis, promotes their maturation^[123,124], and sustains proliferation and the stemness phenotype (both self-renewal and transdifferentiation capacity) of MSCs from various tissues^[111-115] as discussed above. Considering its therapeutic effects against the major sarcoma types [Table

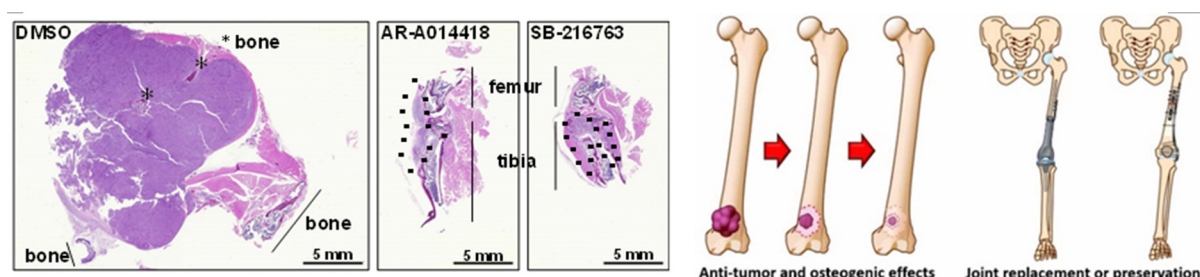


Figure 3. Therapeutic efficacy of GSK3 β inhibitors (AR-A014418 and SB-216763) against human osteosarcoma cell orthograft tumors in the knee joints of mice^[46] (left panels) and schematic representation of the hypothetical triple benefits of GSK3 β inhibitors: anti-tumor effect, reduction of post-surgery tissue defect, and bone preservation (right panels). Dotted lines in the middle two panels indicate the area of orthograft tumors. GSK3 β : glycogen synthase kinase 3 β ; DMSO: dimethyl sulfoxide (diluent for GSK3 β inhibitors)

1], the targeting of GSK3 β in musculoskeletal sarcomas may have three therapeutic advantages: a direct therapeutic effect against the tumor, reduction of the defect in unaffected tissue following surgical resection of the tumor, and preservation and repair of adjacent healthy tissues [Figures 2 and 3].

Doxorubicin-induced cardiotoxicity

Doxorubicin is an anthracycline derivative that comprises the amino sugar daunosamine linked to a hydroxy anthraquinone aglycone^[125]. It is the backbone of first line chemotherapy regimens for most bone and soft tissue sarcomas^[7,8]. Like all anthracyclines^[125], its antitumor effects are mediated by interaction with DNA, generation of oxidative stress, and inhibiting the functions of topoisomerase II in maintaining DNA tangles and supercoils. Resistance to treatment and drug-induced cardiotoxicity are the major concerns with doxorubicin treatment of patients with advanced bone and soft tissue sarcomas. High cumulative dosage of doxorubicin frequently leads to cardiac toxicity and occasionally to irreversible congestive cardiac failure, with younger patients being the most susceptible^[126]. This devastating adverse event is associated with disruption of mitochondrial fusion and mitochondrial fragmentation in cardiomyocytes, resulting in impaired mitochondrial function^[127]. While the exact molecular mechanism of cardiotoxicity has yet to be clarified, the targeting of impaired mitochondrial dynamics and function is a potential strategy for the prevention and treatment of this adverse cardiac event^[127,128]. Many studies have investigated the cardiomyoprotective effect of various compounds derived from phytochemicals such as phenols, terpenoids, quinones, alkaloids, polysaccharides, carotenoids, lignans, and others. Although these phytochemicals are expected to serve as templates for drug development, to date none has yet proven clinically effective in the prevention of doxorubicin-induced cardiotoxicity^[128].

The possibility of using GSK3 β as a therapeutic target for cardiomyocyte protection has attracted considerable attention^[129-132]. Earlier studies reported a causative role for GSK3 β in the necrosis and apoptosis of cardiomyocytes^[129] and a protective role against cardiac fibrosis^[130]. GSK3 β inhibition protects cardiomyocytes from necrosis via opening the mitochondrial permeability transition pore. It also protects cardiomyocytes from apoptosis induced by pressure overload or by repeat ischemia and perfusion. This is associated with reduced phosphorylation of p53, heat shock factor-1, and myeloid cell leukemia sequence-1, and inhibition of Bax translocation^[129]. Subsequent studies showed the effect of targeting GSK3 β on the maintenance of myocardial homeostasis, as well as the therapeutic effects of GSK3 β inhibition against diabetes-associated myocardial injury and experimentally induced myocardial infarction^[131,132]. A recent study demonstrated that GSK3 β inhibition ameliorates triptolide-induced acute cardiac injury in rodents by desensitizing the mitochondrial permeability transition^[133]. Another study showed that phosphorylation-mediated inactivation of GSK3 β (pGSK3 β ^{S9}) is associated with the alleviation of doxorubicin-induced inflammation, oxidative stress, and apoptosis in H9c2 rat cardiomyocytes^[134]. This was achieved by treatment with Yangxin granules, a Chinese herbal medicine confirmed to possess clinical

benefits for the treatment of heart failure. These preliminary studies support the causative involvement of GSK3 β in doxorubicin-induced cardiotoxicity and occurrence of congestive heart failure. They also provide new insights into the underlying mechanisms of this fatal cardiac complication and suggest a possible therapy [Figure 2].

Recently, we reviewed the benefits of targeting GSK3 β for various cancer therapy-induced adverse events including immunosuppression, hematotoxicity, central, and peripheral neuropathy, and opioid-induced analgesic tolerance and withdrawal syndrome^[30] [Figure 2]. Increasing evidence has indicated new roles for GSK3 β in the repair of DNA base excision and double-strand breaks and in the inhibition of apoptosis via NF- κ B activation, thus highlighting the potential of GSK3 β inhibitors for inducing chemo- and radio-sensitization in various cancer types^[135]. In summary, targeting of GSK3 β during standard chemotherapy for bone and soft tissue sarcomas is expected to provide the dual benefits of enhancing cytocidal efficacy while reducing the cardiotoxicity of doxorubicin [Figure 2].

CONCLUSION

GSK3 β sustains the progression of aggressive bone and soft tissue sarcomas including osteosarcoma, embryonal and alveolar rhabdomyosarcomas, synovial sarcoma, and fibrosarcoma, and potentially also UP sarcoma. Laboratory studies have demonstrated therapeutic effects of GSK3 β inhibition against these sarcoma types, as well as against therapy-associated adverse effects including defects in healthy tissues following surgery and doxorubicin-induced cardiotoxicity. The accumulated evidence has provided new insights into the causative role of GSK3 β in bone and soft tissue sarcomas, thus reinforcing GSK3 β as a potential therapeutic target.

DECLARATIONS

Acknowledgments

We acknowledge Dr. Barry Iacopetta (University of Western Australia) for critical review and editing of the manuscript.

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Made substantial contributions to conception and design of this review: Minamoto T

Original draft preparation: Abe K, Shimozaki S

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Performed literature research: Abe K, Shimozaki S, Domoto T

Availability of data and materials

Not applicable.

Financial support and sponsorship

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology-Japan and from the Japan Society for the Promotion of Science (to Abe K, Yamamoto N, Tsuchiya H, and Minamoto T).

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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Negative effects of tumor cell nitric oxide on anti-glioblastoma photodynamic therapy

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How to cite this article: Girotti AW, Fahey JM, Korytowski W. Negative effects of tumor cell nitric oxide on anti-glioblastoma photodynamic therapy. *J Cancer Metastasis Treat* 2020;6:52. <http://dx.doi.org/10.20517/2394-4722.2020.107>

Received: 25 Sep 2020 **Accepted:** 1 Dec 2020 **Published:** 24 Dec 2020

Academic Editor: Lombardi Giuseppe **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Glioblastomas are highly aggressive brain tumors that can persist after exposure to conventional chemotherapy or radiotherapy. Nitric oxide (NO) produced by inducible NO synthase (iNOS/NOS2) in these tumors is known to foster malignant cell proliferation, migration, and invasion as well as resistance to chemo- and radiotherapy. Minimally invasive photodynamic therapy (PDT) sensitized by 5-aminolevulinic acid (ALA)-induced protoporphyrin IX (PpIX) is a highly effective anti-glioblastoma modality, but it is also subject to NO-mediated resistance. Studies by the authors have revealed that glioblastoma U87 and U251 cells use endogenous iNOS/NO to not only resist photokilling after an ALA/light challenge, but also to promote proliferation and migration/invasion of surviving cells. Stress-upregulated iNOS/NO was found to play a major role in these negative responses to PDT-like treatment. Our studies have revealed a tight network of upstream signaling events leading to iNOS induction in photostressed cells and transition to a more aggressive phenotype. These events include activation or upregulation of pro-survival/pro-expansion effector proteins such as NF- κ B, phosphoinositide-3-kinase (PI3K), protein kinase-B (Akt), p300, Survivin, and Brd4. In addition to this upstream signaling and its regulation, pharmacologic approaches for directly suppressing iNOS at its activity vs. transcriptional level are discussed. One highly effective agent in the latter category is bromodomain and extra-terminal (BET) inhibitor, JQ1, which was found to minimize iNOS upregulation in photostressed U87 cells. By acting similarly at the clinical level, a BET inhibitor such as JQ1 should markedly improve the efficacy of anti-glioblastoma PDT.

Keywords: Glioblastoma, photodynamic therapy, nitric oxide, inducible NO synthase



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INTRODUCTION

Glioblastoma, also known as glioblastoma multiforme (GBM), is classified as a grade IV glioma by the World Health Organization and is one of the most aggressive and persistent of all known human tumors^[1-3]. The yearly incidence of glioblastoma in the United States is ~3 per 100,000 individuals. Difficulties in distinguishing highly invasive malignant zones from normal brain tissue make tumor resection very challenging^[2,3]. Glioblastomas are known to be resistant to most conventional interventions, including ionizing radiation or chemotherapy with drugs such as cisplatin and temozolomide^[4-6]. Drug resistance can either be inherent or acquired during treatment^[7]. Photodynamic therapy (PDT), which employs non-ionizing radiation, has several advantages over radiotherapy or chemotherapy, including the ability to often overcome resistance associated with these treatments^[8-10]. Nevertheless, various forms of pre-existing or treatment-induced resistance also apply for PDT^[11,12]. One important example pertains to nitric oxide (NO) generated by inducible nitric oxide synthase (iNOS) in PDT-challenged tumor cells. There is solid evidence for this mode of resistance in glioblastoma cells as well as several other human cancer lines, including breast, prostate, and melanoma^[13,14]. In addition to this anti-PDT effect, iNOS/NO has been shown to stimulate proliferation, migration, and invasion of cells that survive a photodynamic challenge. In this review, we discuss findings such as these and their implications on anti-glioma PDT at the clinical level. Relevant topics include: (1) NO and its underlying role in tumor promotion/persistence; (2) basic principles of PDT and how it suppresses solid tumors; (3) iNOS/NO-mediated hyper-resistance to PDT and hyper-aggressiveness of surviving cells; (4) mechanism of iNOS/NO induction by PDT; (5) tumor expansion via PDT-induced bystander effects; and (6) pharmacologic approaches for limiting the negative effects of iNOS/NO after PDT. Much of this discussion is based on studies carried out in the authors' laboratories.

Two key aspects of these studies distinguish them from most others dealing with the pro-tumor effects of iNOS/NO: (1) Rather than simply using unchallenged tumor cells, we applied an oxidative stress-based challenge, *viz.* PDT, and assessed how it was affected by endogenous iNOS/NO; and (2) We discovered that, in most cases, it was PDT-upregulated iNOS rather than pre-existing (constitutive) enzyme that generated sufficient NO to stimulate resistance and surviving cell aggressiveness. Better recognition of these negative responses to PDT is needed in advance of developing approaches for mitigating them and improving PDT efficacy. What we discuss here may also provide new insights into how iNOS/NO could impact anti-tumor chemotherapy or radiotherapy.

NITRIC OXIDE: TUMOR-PROMOTING VERSUS TUMOR-SUPPRESSING EFFECTS

NO is a short-lived free radical molecule ($\tau < 2$ s in H₂O) that diffuses freely on its own in aqueous media and, similar to O₂, can partition into hydrophobic environments such as cell membranes^[15,16]. Naturally occurring NO is generated by three enzyme isoforms in the nitric oxide synthase family: neuronal (nNOS/NOS1), inducible (iNOS/NOS2), and endothelial (eNOS/NOS3)^[17,18]. Whereas nNOS and eNOS operate at low constitutive levels and require Ca²⁺ and calmodulin for optimal activity, iNOS can be induced to relatively high levels and does not require stimulatory Ca²⁺ or calmodulin^[18]. All three enzymes catalyze the five-electron oxidation of L-arginine to L-citrulline and NO at the expense of NADPH and O₂. NO is involved in many different normo- and pathophysiologic processes. For example, eNOS-derived NO at low steady state levels (1-10 nM) stimulates cyclic-GMP formation, leading to blood vessel relaxation and lowering of blood pressure. In contrast, iNOS-derived NO at much higher levels (≥ 1 μ M), as produced by vascular macrophages in response to infection, is cytotoxic and potentially carcinogenic, e.g., by inducing DNA mutations^[19,20]. NO itself may act thusly by binding to iron in iron-sulfur or heme proteins, but often does so after reacting with superoxide radical (O₂⁻) to give peroxynitrite (ONOO⁻), a strong indiscriminate oxidant^[21]. If generated chronically, ONOO⁻ may be carcinogenic, e.g., by causing tyrosine nitration or initiating lipid peroxidation^[21]. On the other hand, for established tumors, ONOO⁻ can be cytotoxic, since

such tumors are typically more sensitive to oxidative pressure than normal counterparts^[19,20]. In many tumor cells, including glioma cells, NO can activate pro-survival signaling pathways by modifying effector proteins such as soluble guanylyl cyclase (sGC), hypoxia-inducible factor-1 α (HIF-1 α), extracellular signal-regulated kinases-1 and -2 (ERK-1/2), epidermal growth factor receptor (EGFR), or protein kinase-B (Akt) via phosphoinositide-3-kinase (PI3K)^[22-25]. Such modification may occur via S-nitrosation of thiol groups on specific cysteine residues^[26]. In this case, NO itself does not react usually, but rather some oxidized form of NO such as nitrosyl anhydride (N₂O₃) or a trans-nitrosating species such as S-nitroso-glutathione (GSNO)^[26-28]. iNOS-derived NO from myeloid-derived suppressor cells (MDSCs) may also benefit malignant tumors by inactivating anti-tumor cytotoxic T-cells^[29]. In this case, the cytotoxic agent is a strong NO-derived oxidant such as ONOO⁻. There is increasing evidence that endogenous NO at low levels (e.g., from tumor cells themselves or proximal vascular cells) can also increase tumor resistance to ionizing radiation or chemotherapeutic agents such as cisplatin and docetaxel^[30]. This has been amply demonstrated for malignant gliomas, glioma stem cells (GSCs) in these tumors exhibiting much of this resistance^[31]. Significant resistance to non-ionizing photodynamic therapy can also develop, which is discussed after basic principles of this treatment are described.

ANTI-TUMOR PHOTODYNAMIC THERAPY: SOME BASIC PRINCIPLES

Photodynamic therapy (PDT) was introduced about 45 years ago as a novel means of selectively eradicating a variety of solid malignancies, many of which are refractory to conventional chemotherapy or radiotherapy^[32-34]. PDT is a minimally invasive modality which typically exhibits little, if any, off-target cytotoxicity. Classical PDT consists of three operating components: (1) an administered photosensitizing agent (PS); (2) PS photoexcitation by non-ionizing radiation, typically in the far visible to near-infrared wavelength range; and (3) molecular oxygen^[32-34]. For many tumors, including glioblastomas, light can be delivered interstitially via fiber optic networks, making this approach highly selective for the tumor target^[33,34]. Without photoactivation, most PS are innocuous to tumor cells as well as normal cells, which distinguishes these PS from many chemotherapeutic agents, e.g., platinum-based drugs. In a common photodynamic reaction (Type II process), ground state PS is excited to a meta-stable singlet state, which crosses over to a longer-lived triplet excited state. The latter then transfers energy to ground-state O₂, giving singlet molecular oxygen (¹O₂), a cytotoxic reactive oxygen species (ROS)^[33,34]. For some PS, more complex electron or hydrogen transfer may occur (Type I process), resulting in formation of free radical or free radical-derived ROS, e.g., superoxide (O₂^{-•}), hydroxyl radical (HO[•]), and hydrogen peroxide (H₂O₂). Similar to ¹O₂, these ROS can kill tumor cells by oxidizing vital molecules (proteins, lipids, and nucleic acids) and activating death signaling pathways^[34]. In 1995, Photofrin[®], a hematoporphyrin oligomer, became the first PS to be FDA-approved for anti-tumor PDT, esophageal malignancies being treated initially^[32]. Since then, PDT with Photofrin[®] and other PSs has been used to combat numerous other malignancies, including prostate, breast, cervical, head and neck, and brain (gliomas)^[33,34]. PDT is now considered one of the most promising alternatives to radiotherapy and chemotherapy for treating highly aggressive brain malignancies such as glioblastoma^[8-10,35,36]. One explanation for this pertains to distinct subcellular targets. PDT usually damages cytoplasmic organelles (mitochondria, lysosomes, and endoplasmic reticulum), whereas radiotherapy (X-rays and γ -rays) and chemotherapy (e.g., with platinum-based drugs) damage nuclear DNA^[37]. As a result, any constitutive or acquired resistance to chemo- or radiotherapy may not apply when PDT is used. Moreover, PDT elicits a robust anti-tumor immune response, and this provides an additional advantage by eliminating cells that might withstand a PDT challenge^[38].

Unlike Photofrin[®] and other PSs that are administered as such, pro-sensitizers have been developed which are converted to active PS after being administered. One important example is 5-aminolevulinic acid (ALA), which enters tumor cells via an amino acid transporter and is metabolized to active PS, protoporphyrin IX (PpIX), via the heme biosynthetic pathway [Figure 1], the PpIX accumulating initially in mitochondria^[39-41]. This pathway is typically more active in malignant cells (e.g., glioblastomas) than normal counterparts,

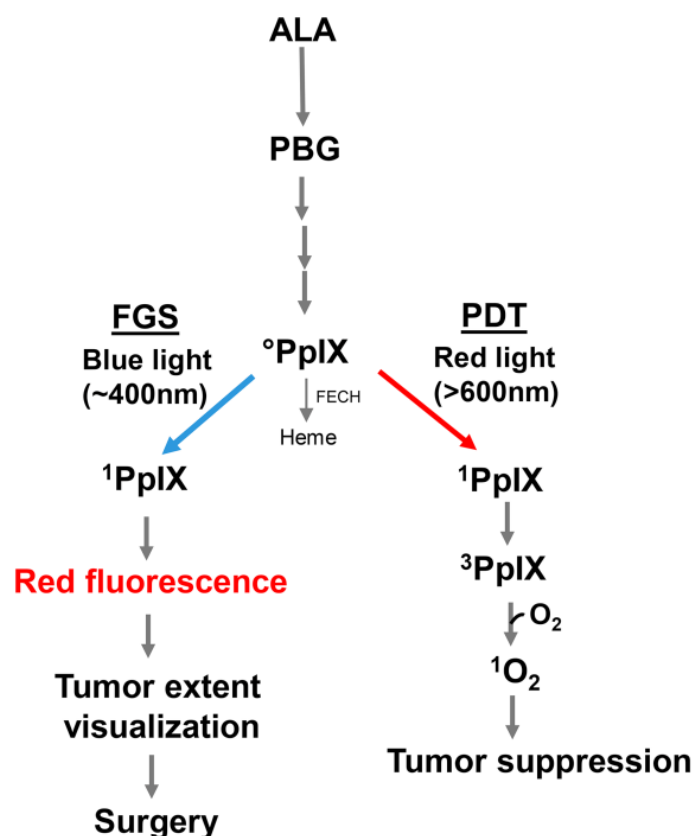


Figure 1. 5-Aminolevulinic acid (ALA)-induced protoporphyrin IX (PpIX) via the heme biosynthetic pathway. Utilization of PpIX for photodynamic therapy (PDT) or fluorescence-guided surgery (FGS) is illustrated. PBG: porphobilinogen; FECH: ferrochelatase; $^{\circ}\text{PpIX}$: ground state porphyrin; $^1\text{PpIX}$: singlet excited state porphyrin; $^3\text{PpIX}$: triplet excited state porphyrin; $^1\text{O}_2$: photogenerated singlet oxygen

which accounts, at least in part, for the greater PDT susceptibility of the former. Moreover, unlike normal brain, malignant brain typically loses most of its blood-brain barrier function, allowing ALA access via the blood stream. In some tumors, including glioblastomas, this PpIX buildup is augmented by partial downregulation of ferrochelatase (FECH), the enzyme that inserts ferrous iron into PpIX to give heme^[40,41]. In ALA-based PDT [Figure 1], relatively intense red light elevates ground state PpIX to a singlet excited state ($^1\text{PpIX}$), much of which undergoes intersystem crossing to a long-lived triplet ($^3\text{PpIX}$). Energy transfer from the latter to O_2 gives $^1\text{O}_2$, a non-radical ROS, whereas indirect reaction could give free radical ROS, as indicated above. In addition to sensitizing cytotoxic PDT reactions, ALA-induced PpIX can be used diagnostically to define tumor boundaries. In this case, low intensity blue light (~400 nm) generates significant $^1\text{PpIX}$, which, upon decay to ground state, releases red fluorescent light. Many oncologists, particularly those treating difficult glioblastomas, have exploited this property for fluorescence-guided surgery (FGS), i.e., for clear demarcation of tumor boundaries before surgical resection [Figure 1]^[42]. When applied carefully, using a surgical fluorescence microscope, FGS can greatly improve procedural accuracy by limiting inadvertent removal of non-tumor tissue^[42-44]. Thus, ALA-induced PpIX has the advantage of serving as a surgical guide on the one hand and PDT sensitizer on the other hand. In addition to being used individually, FGS and PDT are often run sequentially, the latter to eradicate any residual tumor cells after the former is carried out^[44]. Various pharmacologic approaches have been used for improving both FGS and PDT efficacy, e.g., FECH inhibitors or iron chelators^[44] to further elevate ALA-induced PpIX levels. ALA-based FGS and PDT are rapidly becoming the new standards of care for the management of malignant brain tumors.

ANTAGONISTIC EFFECTS OF ENDOGENOUS NO IN GLIOBLASTOMA PDT MODELS

About 20 years ago, Henderson *et al.*^[45] and Korbélik *et al.*^[46], using various mouse syngeneic tumor models (e.g., RIF, SCCVII, and EMT6) and Photofrin® as PS, were the first to determine how endogenous NO might affect PDT efficacy *in vivo*. They showed that PDT cure rate could be significantly improved when NG-nitro-L-arginine (L-NAME), a non-specific inhibitor of NOS activity, was administered immediately after irradiation. A striking correlation was made between NO output and extent of improvement with L-NAME: tumors with the highest output responded best and those with the lowest output worst^[46]. It was concluded that endogenous NO signaled for increased tumor resistance to PDT repression and did so in a NO dose-dependent manner. The L-NAME effects were principally attributed to NO's vasodilatory effects acting in opposition to PDT's known constrictive effects on the tumor microvasculature^[45,46]. Follow-up studies by Reeves *et al.*^[47], using ALA-induced PpIX as PDT sensitizer for mouse RIF and EMT6 tumors, confirmed the above findings and again concluded that endogenous NO, by opposing vascular damage, can significantly increase tumor resistance to PDT. Although these studies^[45-47] and more recent ones by Rapozzi *et al.*^[48] clearly established that NO can antagonize PDT, several key questions were left largely unsettled, which include: (1) whether this NO is generated by tumor cells per se, proximal endothelial cells, macrophages, fibroblasts, or possibly all of these; (2) which NOS isoform plays a dominant role in any given tumor; (3) whether the NOS/NO in question acts at a pre-existing level or is upregulated in response to PDT stress; and (4) the signaling mechanisms involved in NOS expression and NO-induced resistance. Over the past ten years, the authors and lab colleagues have focused on these questions using various cancer cell lines, including glioblastoma lines. Key findings from this work are discussed below.

Hyper-resistance imposed by photostress-upregulated iNOS/NO

As indicated above, PDT can often circumvent any innate or acquired tumor resistance to conventional chemotherapy or radiotherapy. It is now clear, however, that resistance mechanisms also exist for PDT, some of which are acquired during treatment. For example, there is evidence that activity of cytosolic ROS scavenging enzymes such as type-1 glutathione peroxidase and catalase are increased in lymphocytes subjected to a modest photodynamic challenge^[49]. In addition, many cancer cell types, including glioblastomas, can export PpIX and other PS via the ABCG2 transporter, inhibition of which increases photosensitivity^[44,50]. Another PDT resistance mechanism, which was discovered in the authors' laboratory, involves NO generated specifically by tumor cell iNOS, particularly that which is upregulated in response to PDT stress^[51-55]. This was demonstrated in recent experiments carried out on human glioblastoma U87-MG and U251-MG cells (henceforth referred to as U87 and U251)^[56]. As shown in Figure 2A, U87 cells sensitized in mitochondria with ALA-induced PpIX were progressively inactivated after exposure to increasing fluences of broad-band visible light, 4 J/cm², reducing the viable fraction by ~45% 20 h after irradiation^[56]. ALA alone or light alone was completely innocuous. When added before ALA/light treatment, 1400W (an enzyme inhibitor with a high specificity for iNOS) increased the extent of cell photokilling throughout, as did the NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) [Figure 2A]. Similar results were obtained with U251 cells [Figure 2B]. ALA/light-induced U87 or U251 cell death occurred primarily via intrinsic (mitochondria-initiated) apoptosis, as assessed with Annexin V-fluorescein isothiocyanate (V-FITC), and this was substantially enhanced by 1400W or cPTIO, again consistent with iNOS/NO-imposed resistance^[56]. Thus, it appeared that NOS-derived NO in U87 and U251 cells was acting cytoprotectively after a PDT-like challenge. When immunoblot analysis was used to assess iNOS status in these cells, it was found that the enzyme level increased progressively during post-irradiation incubation. After 6 h, it reached ~4-times the basal level in U87 cells [Figure 2A] as well as U251 cells [Figure 2B]. As expected for this phenotype, U87 cells also expressed nNOS^[56], but, unlike iNOS, it was not upregulated after ALA/light treatment [Figure 2A]. Therefore, both glioblastoma cell types studied added significantly to their expressed iNOS the after a photodynamic challenge, and the resulting NO clearly enhanced their resistance to photokilling. Evidence for a large boost in NO steady state level was obtained by using the fluorescent probe diaminofluorescein-

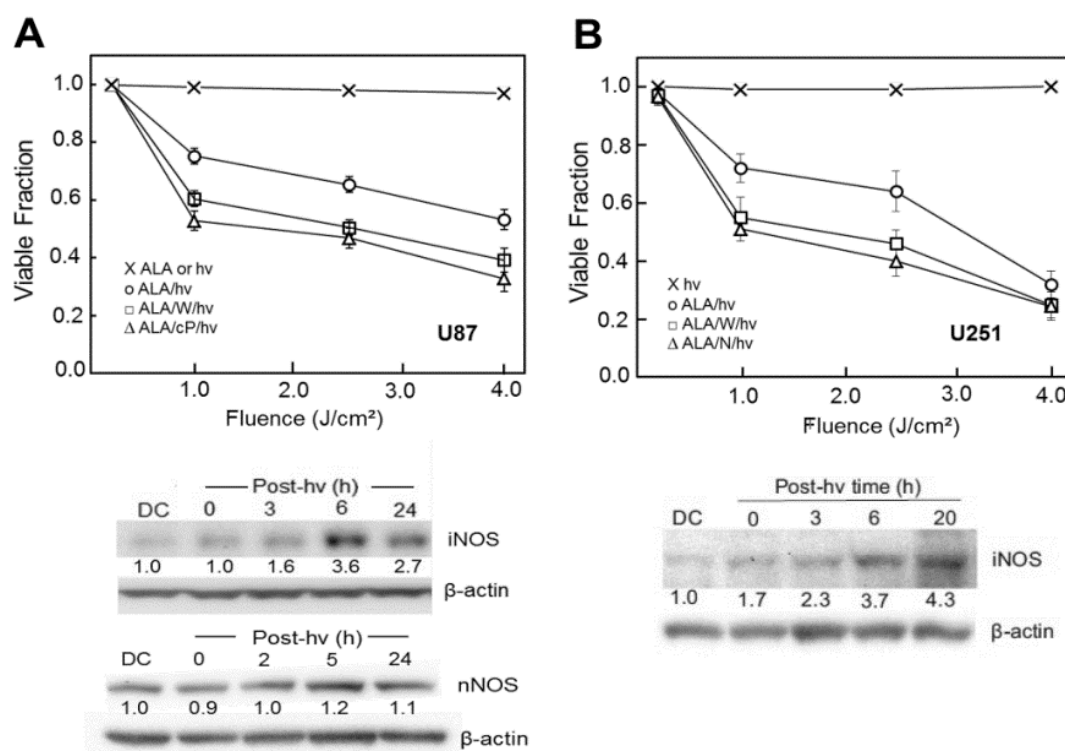


Figure 2. Viability loss of ALA/light-challenged glioblastoma cells and inhibition thereof by stress-induced iNOS/NO. Cells ~ 60% confluency in serum-free medium were dark-incubated with 1 mM ALA for 30 min, switched to ALA-free medium, then irradiated with increasing fluences of broad-band visible light in the absence or presence of 25 μ M 1400W (W), 1mM L-NAME (N), or 25 μ M cPTIO (cP). ALA-only and light-only controls were run alongside. After treatment, cells were switched to serum-containing medium and after 20 h of dark incubation, assessed for viability by MTT assay or iNOS and nNOS status by Western blot analysis. A: U87 cells; B: U251 cells. Plotted values in (A) and (B) are means \pm SEM ($n = 3$). Number below each NOS band is integrated band Intensity relative to β -actin and normalized to the dark control (DC). ALA: 5-Aminolevulinic acid; PpIX: protoporphyrin IX; NO: nitric oxide; iNOS: inducible NO synthase; nNOS: neuronal NO synthase; post-hv: post-irradiation (Reproduced from Ref. 56, with permission)

2-diacetate (DAF-2DA) which, after cell uptake and hydrolysis, detects NO via a byproduct such as N_2O_3 ^[57]. Photostress-upregulated iNOS/NO has also been observed in human melanoma, breast, and prostate cancer lines, some of which, e.g., prostate PC3, boosted iNOS to much higher levels (8-10 folds) than evidenced in U87 or U251 cells^[53-55].

Fahey and Girotti^[58] recently extended the above *in vitro* findings to the *in vivo* level, using female immunodeficient (SCID) mice engrafted with breast MDA-MB-231 tumors. After intraperitoneal ALA administration, mouse tumors were irradiated, using a 633-nm Omnilux-Revive® LED source. Tumor growth in irradiated animals was significantly reduced compared with that in light-only controls over a 1-2-day period post-irradiation. However, an iNOS activity inhibitor (1400W or GW274150) in multiple doses (once daily over nine days) reduced growth much further, implying that iNOS/NO was stimulating tumor resistance to PDT^[58]. For control animals irradiated without prior ALA treatment, 1400W had little (if any) effect on tumor growth, suggesting that pre-existing iNOS/NO had no significant protective effect^[58]. Analysis of tumor samples after ALA-PDT revealed a striking ~5-fold upregulation of iNOS protein over a low basal level, as well as a 1400W-inhibitable increase in NO-derived nitrite^[58]. This was the first published *in vivo* evidence for iNOS upregulation by PDT and for increased resistance imposed by iNOS-derived NO. It should be emphasized that the bulk of this resistance was due to stress-upregulated iNOS/NO. This possibility has not been well recognized heretofore, either for PDT or other cancer therapies. Given that iNOS-generated NO is known to antagonize *in vivo* chemo/radiotherapy for glioblastoma^[24,25], it is likely that when evidence becomes available, it will also apply to *in vivo* PDT for glioblastoma, at least in an animal model.

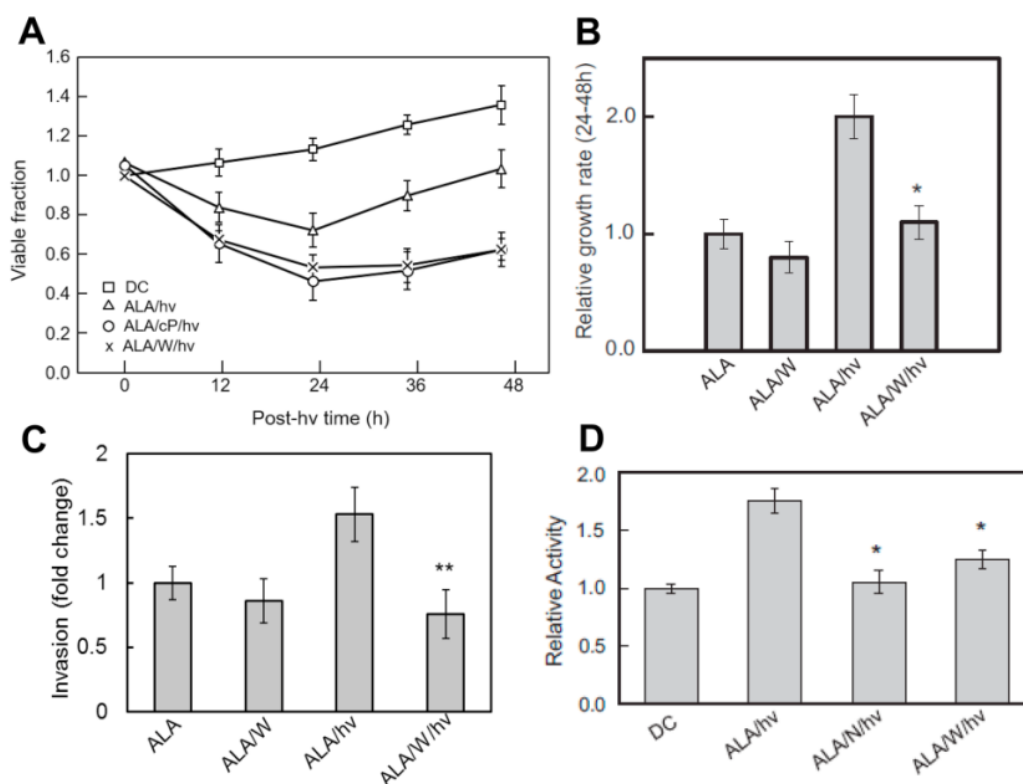


Figure 3. Enhanced proliferation and invasion rate of glioblastoma cells that survive ALA/light treatment: iNOS/NO dependency. U87 cells were sensitized with ALA-induced PpIX as described in Figure 2, exposed to a light fluence of $\sim 1 \text{ J/cm}^2$, and then assessed for various post-irradiation parameters. 1400W(W), L-NAME (N), or cPTIO (cP) was either absent or present throughout. A: loss of viability (0-24 h post-hv) and subsequent proliferation of surviving cells (24-48 h post post-hv); DC: ALA-only dark control; B: surviving cell proliferation rate for selected conditions represented in panel (A); C: surviving cell invasiveness measured with a trans-well device; D: matrix metalloprotein-9 (MMP-9) activity measured by gelatin zymography 24 h after cells were ALA-light-treated. Plotted values are means \pm SEM ($n = 3$); * $P < 0.01$ vs. ALA/hv (B); ** $P < 0.01$ vs. ALA/hv (C); * $P < 0.01$ vs. ALA/hv (D). ALA: 5-Aminolevulinic acid; PpIX: protoporphyrin IX; NO: nitric oxide; iNOS: inducible NO synthase; post-hv: post-irradiation (Reproduced from Ref. 56 and 62, with permission)

Hyper-aggressiveness of cells that survive PDT

When ALA/light-treated U87 cells *in vitro* were tracked beyond a 24 h post-irradiation point [cf. Figure 2], a striking observation was made, *viz.* that surviving cells (still attached) were proliferating more rapidly than controls over at least two additional days [Figure 3A]^[56,59]. Similar to the viability loss over the first 24 h, the increase in proliferation rate was strongly attenuated by 1400W or cPTIO [Figure 3A]. In additional experiments, Fahey *et al.*^[56] showed that 1400W nearly nullified the ~ 2 -fold spurt in surviving cell proliferation, but had essentially no effect on proliferation of a dark (ALA-only) control [Figure 3B]. This suggested that preexisting iNOS/NO, unlike the upregulated forms, had little (if any) effect on cell division rate. Two additional manifestations of U87 hyper-aggressiveness were observed after an ALA/light challenge: (1) accelerated migration, as assessed by a gap-closure (wound-healing) assay; and (2) accelerated invasion, as assessed by a trans-well assay involving cell traversal through an extracellular matrix (ECM)-like interface^[56]. The large increase in migration rate (not shown) and invasion rate [Figure 3C] of photostressed cells was strongly blunted by 1400W, which, once again, had no significant effect on a dark control. As observed for proliferation, therefore, the more aggressive migratory and invasive properties of U87 cells that could withstand photostress were strongly dependent on upregulated iNOS/NO. Matrix metalloproteinases (MMPs) such as zinc-containing MMP-9 catalyze the degradation of collagen and other ECM components, and thus play a key role in cancer cell invasiveness and metastasis^[56]. Innate migration and invasion of glioma cells is known to be promoted by MMP-9, which becomes activated by proteolytic

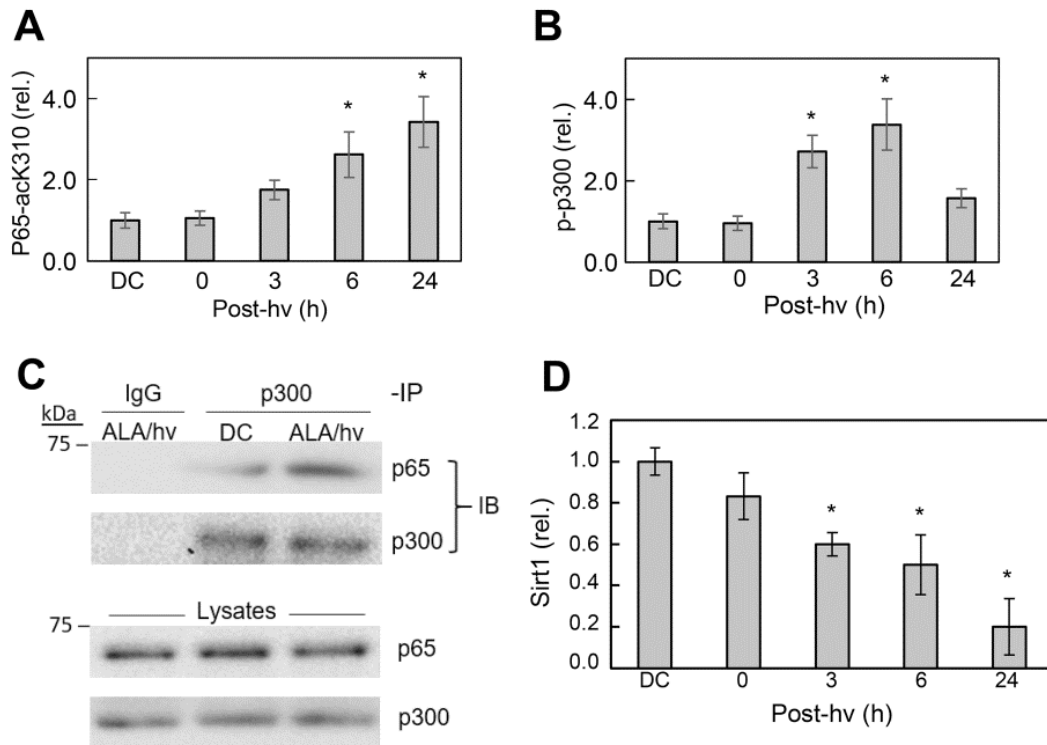


Figure 4. Some upstream events that signal for greater iNOS transcription in ALA/light-treated glioblastoma U87 cells. A: post-irradiation upregulation of p65-ack310; DC: ALA-only dark control; B: post-irradiation upregulation of phosphorylation-activated p300 (p-p300); C: stimulation of p300 and p65 association in photostressed cells, as assessed by immunoprecipitation assay, using monoclonal p300 and p65 antibodies; D: time-dependent down-regulation of Sirt1 in photostressed cells, as assessed by immunoblot analysis. ALA: 5-Aminolevulinic acid; NO: nitric oxide; iNOS: inducible NO synthase; post-hv: post-irradiation; Sirt1: sirtuin 1 (Reproduced from Ref. 59, with permission)

cleavage of its exported precursor, pro-MMP-9^[60]. Using in-gel zymography to measure the activity of externalized MMP-9 in ALA/light-stressed U87 cells, Fahey *et al.*^[56] found it to be ~80% higher than that of dark controls [Figure 3D]. As shown, L-NAME and 1400W strongly inhibited this activation, pointing again to substantial iNOS/NO dependency. Immunoblot-assessed expression of three other proteins known to play important roles in glioblastoma aggressiveness was also 1400W-inhibitable in photostressed U87 cells: (1) downregulation of tissue-inhibitor of metalloproteinase-1 (TIMP-1); (2) upregulation of anti-apoptotic Survivin; and (3) upregulation of pro-metastatic S100A4^[56]. Strong induction of S100A4 was the most remarkable of these because this protein was barely detectable in a dark control. Much remains to be learned about how NO modulated the expression of these effector proteins; however, the observed modulations are all consistent with the photostress responses shown in Figure 3.

MECHANISMS OF iNOS UPREGULATION AND NO-MEDIATED RESISTANCE IN PHOTOSTRESSED CELLS

Regarding underlying mechanisms, most research to date has focused on how iNOS is upregulated by photodynamic stress rather than how the resulting NO signals for greater cell resistance to photokilling, although some headway has been made on the latter issue. Early studies on human breast COH-BR1 cells^[51-53] and more recent ones on glioblastoma U87 and U251 cells^[56,59] revealed that activation of transcription factor NF- κ B is necessary for iNOS transcription in response to an ALA/light challenge. NF- κ B activation may have been due to engagement of stress signaling elements IRE1 or PERK^[61]. Our evidence indicated that NF- κ B subunit p65/Rel A of ALA/light-treated U87 cells translocated from the cytosol to nucleus for initiation of iNOS transcription. Based on non-glioma studies by Huang *et al.*^[62], we

postulated that acetylation of specific lysine residues in p65 was necessary for stimulating transcription. As supporting evidence, Fahey *et al.*^[63] showed that acetylation of lysine-310 (p65-acK310) increased progressively during post-irradiation incubation of U87 cells, reaching > 3-times the control level after 24 h [Figure 4A]. The rise in acK310 level was blocked by C646, an inhibitor of activated p300, confirming that the latter had catalyzed this acetylation^[63]. The acetyltransferase p300 and its paralog CREB-binding protein (CBP) act as transcriptional co-activators for several tumor-promoting transcription factors^[64,65]. p300 stimulates gene expression at promoter sites by catalyzing acetylation of specific lysine residues on histones or transcription factors such as NF- κ B^[64]. Therefore, we determined whether p300 is involved in p65-K310 acetylation and, if so, how photodynamic stress might affect p300 expression/activity. Immunoblot analysis revealed that photostress had no effect on overall p300 level relative to a dark control. As with p65-acK310 build-up, however [Figure 3A], there was a progressive increase in activated p300, i.e., Ser-1834-phosphorylated enzyme (p-p300), over at least a 6-h post-hv period [Figure 4B]. Moreover, immunoprecipitation (pull-down) analysis revealed a striking photostress-enhanced interaction of activated p300 with NF- κ B-p65, thus favoring acetylation of the latter [Figure 4C]^[59]. Another striking finding of this study is that Sirtuin-1 (Sirt1), a Class-III deacetylase that modulates gene expression by catalyzing acetyl group removal^[66], was strongly downregulated in photostressed U87 cells [Figure 4D], whereas a homolog, Sirt2, was unaffected^[59]. Along with these effects, there was a striking post-hv upregulation of type-4 bromodomain and extra-terminal domain (BET) protein (Brd4), an epigenetic “reader” and transcriptional co-activator for various stress-responding genes^[63]. In contrast, Brd2 (a paralog of Brd4) was unaffected, providing another example of signaling specificity in this system. Brd4-regulated expression of stress proteins such as E-selectin and IL-8 was first demonstrated for lung cancer cells^[67], but our studies were the first to link Brd4 to iNOS expression in glioblastoma cells^[59,63]. Looking at other events upstream of iNOS transcription, Fahey *et al.*^[59] found that p65-acK310 formation in photostressed U87 cells was dependent on phosphorylation-activation of PI3K. This stimulated phosphorylation-activation of protein kinase B (Akt) which, in turn, depended on activation of phosphoinositide-dependent kinase-1 (PDK1). PI3K/Akt-mediated signaling is known to play a central role in cancer cell survival and proliferation^[67]. A specific PI3K inhibitor (LY294002) prevented p300 activation as well as iNOS upregulation after an ALA/light challenge, thereby linking the iNOS response to upstream events set in motion by photodynamic stress. Evidence for another key upstream event was also obtained, *viz.* oxidative inactivation of tumor suppressor PTEN, which would have fostered PI3K/Akt activation via elevation of phosphatidylinositol triphosphate (PIP₃) level^[59]. Taken together, the above findings, which are depicted schematically in Figure 5, reveal a well-coordinated stress signaling network leading ultimately to iNOS/NO induction and a pro-survival/expansion outcome. Other pro-survival effectors, e.g., COX-2, Survivin, and S100A4, are upregulated by photostress similar to iNOS/NO^[56], but it is not yet clear whether this occurs independently of NO or results from downstream signaling by NO^[19,20].

How ALA/light-induced NO can elicit photokilling resistance or greater aggressiveness of surviving cells is a question of ongoing interest. Since NO does not scavenge ¹O₂^[68], this has been ruled out as a possible cytoprotective mechanism, leaving open the possibility of downstream species scavenging. Studies by Niziolek *et al.*^[68,69] revealed that NO from the chemical donor spermine-NONOate (SPNO) could suppress PpIX-sensitized (¹O₂-initiated) lipid peroxidation in model membranes and also breast cancer cells. In the latter case, ALA-induced PpIX was allowed to diffuse from mitochondria to plasma membrane before cell irradiation in the absence *vs.* presence of SPNO. Irradiated cells died mainly by membrane-breaching necrosis and NO protected against this by acting as a chain-breaking antioxidant, as was observed previously by Rubbo *et al.*^[70], using a non-photodynamic model system. Niziolek *et al.*^[68,69] deduced that, in their system, NO acted by intercepting chain-carrying lipid-derived radicals, i.e., LOO[•]/LO[•], thereby protecting cells against necrosis due to free radical-mediated membrane damage. There is no evidence yet as to whether endogenous NO can act similarly on peroxidation of mitochondrial membrane lipids sensitized by ALA-induced PpIX. Such peroxidation is highly likely, given that PpIX accumulates initially in mitochondrial membranes^[39,40]. Whereas chain breaking by NO occurs via an irreversible chemical

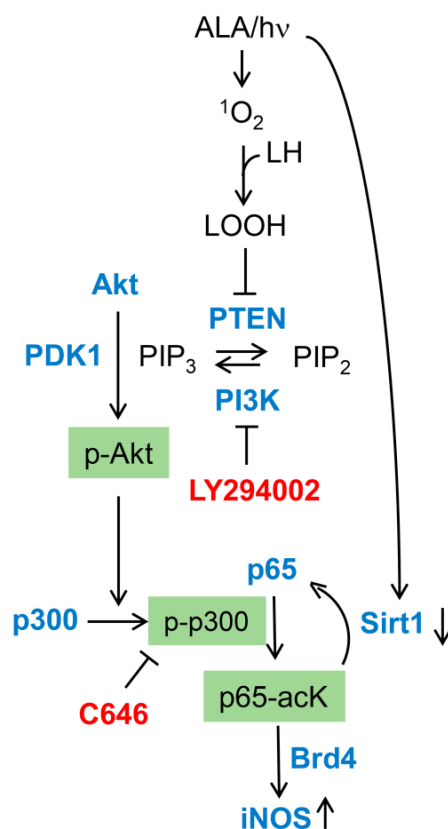


Figure 5. Photostress-induced upstream signaling events leading ultimately to iNOS transcriptional upregulation. Key effectors (LY294002, C646) and their protein targets. ALA: 5-Aminolevulinic acid; NO: nitric oxide; iNOS: inducible NO synthase; hv: irradiation; 1O_2 : photogenerated singlet oxygen; LOOH: hydroperoxide of mitochondrial membrane lipids; PTEN: phosphatase and tensin homologue; PIP₃: phosphatidylinositol-3,4,5-triphosphate; PIP₂: phosphatidylinositol 4, 5-bisphosphate; PI3K: phosphoinositide 3-kinase; PDK1: 3-Phosphoinositide-dependent protein kinase-1; p-Akt: phosphorylated Akt; p-p300: phosphorylation-activated p300; Sirt1: sirtuin 1; Brd4: bromodomain-containing protein 4; NO: nitric oxide; iNOS: inducible NO synthase (Reproduced from Ref. 59, with permission)

reaction, reversible NO reactions can also take place and fall into the signaling category. A well-known example is protein S-nitrosation, i.e., reaction of specialized cysteine thiol groups with NO (or more likely NO-derived N_2O_3) to give S-nitroso (SNO) adducts^[57]. Except for a conference report in 2002^[71], no solid evidence for SNO formation in the context of PDT has been reported thus far. In contrast, several effector proteins have been reported to undergo S-nitrosation in non-photodynamic systems, including: (1) mitogen-activated protein kinases (MAPKs) such as ASK-1 and Jun-N-terminal kinase (JNK), whose pro-apoptotic activities are inhibited^[72]; (2) caspase-9, whose pro-apoptotic activation or activity is inhibited^[73]; (3) anti-apoptotic Bcl-2, whose ubiquitination and proteosomal degradation are inhibited^[74]; and (4) anti-apoptotic MAPK phosphatase-1 (MKP-1), whose proteosomal degradation is also inhibited^[75]. Protein S-nitrosation can be monitored by mass spectrometry, but analysis is complex and the modification is often transient due to thioredoxin-mediated denitrosation^[76]. In the case of PDT, the latter could occur at some point after photostress is incurred, so optimal timing of cell or tissue analysis after irradiation poses a challenge.

BYSTANDER EFFECTS OF PDT-UPREGULATED INOS/NO

Most advanced tumors, including glioblastomas, have a limited vascular supply, and, because of this, not all tumor cells will be uniformly accessed by an active PS or pro-PS such as ALA. Moreover, during subsequent irradiation, some cells will be less exposed than others due to light field limits, variable tumor geometry, and other complex factors. Thus, it is conceivable that cells experiencing the greatest photodynamic stress might respond to it by sending signals to non- or weakly-stressed neighboring cells,

i.e., bystanders. Such a phenomenon is well documented for cancer cells exposed to ionizing radiation (e.g., X-rays and γ -rays), and various signaling mediators have been described, including NO^[77,78]. To determine whether bystander effects might also apply to PDT, Bazak *et al.*^[79,80] developed a novel approach involving impermeable silicone rings to initially separate targeted cells (ALA/light-treated, outside rings) from non-targeted bystanders (light-only, inside rings) on a large culture dish. At some interval (e.g., 2 h) after a given light fluence (e.g., 1 J/cm²) from an LED source, rings are removed and responses in both cell compartments are monitored during subsequent dark incubation, e.g., iNOS/NO levels and proliferation/migration rates. Initial experiments with human prostate carcinoma PC3 cells revealed not only an expected boost in iNOS/NO level and growth/migration rate of targeted cells, but similar responses in non-stressed bystander cells^[79]. Although the latter responses were more moderate, they were inhibited by 1400W, cPTIO, or knockdown of targeted cell iNOS, implying that NO produced by targeted cell iNOS was responsible for the bystander effects. Use of a NO fluorescence probe (DAF-FM-DA) provided more direct evidence for this^[79]. Conditioned medium from targeted cells did not induce bystander effects, suggesting that short-lived, continuously generated NO was solely responsible. In addition to iNOS, several other pro-tumor effectors were upregulated in PC3 bystanders, including Akt, ERK1/2, and COX-2^[79]. In more recent studies, similar NO-mediated bystander effects were observed using glioblastoma U87 cells, and they were compared with those obtained with prostate PC3, breast MDA-MB-231, and melanoma BLM cells. After ALA treatment, irradiation conditions were adjusted to produce the same cell kill for all four types (~25%), thus allowing clear conclusions to be made about NO-elicited resistance. Under these conditions, bystander proliferation and migration rates increased with extent of iNOS upregulation in surviving targeted cells in the following order: BLM < U87 < MDA-MB-231 < PC3^[80]. Thus, targeted cells with the greatest iNOS/NO induction after an ALA/hv challenge elicited the greatest increases in bystander aggressiveness. These findings suggest that a NO-based “relay” process is set in motion by photodynamic stress. In this process, NO overproduced by targeted cells (e.g., U87 or U251) diffuses to non-stressed bystanders and induces iNOS/NO there, thus beginning a NO “feed-forward” process that propagates through the bystander population. Whereas photodynamic stress activates NF- κ B and thence iNOS transcription in targeted cells, the transcription factor responsible for NO-initiated iNOS induction in bystander cells has not yet been defined. If occurring in an actual tumor, e.g., GBM, after a PDT challenge, NO-mediated bystander effects might stimulate tumor growth and metastatic expansion. While this unfortunate possibility is well recognized in connection with therapeutic ionizing radiation^[78], it is still not so with regard to PDT for any solid malignancies, including glioblastomas. As discussed in the next section, these negative effects of NO from targeted cells could be attenuated by pharmacologic interventions aimed at either inhibiting iNOS enzymatic activity or iNOS transcription. This would be expected to increase the overall anti-tumor efficacy of PDT at the clinical level.

PHARMACOLOGIC MITIGATION OF NITRIC OXIDE'S ANTI-PDT EFFECTS

Although not yet tested in the clinic, it is likely, based on evidence presented above, that inhibiting iNOS activity or expression would significantly improve PDT outcomes against glioblastoma and other solid tumors. At least two iNOS activity inhibitors, L-NIL and GW274150, have already been tested in clinical trials, but these were unrelated to cancer or PDT^[81,82]. Instead, both agents were tested for relieving asthmatic inflammation and, importantly, neither one had any negative side effects. As indicated above, GW274140 significantly improved PDT efficacy in a human breast tumor xenograft model^[58], suggesting that this inhibitor would be a good test adjuvant for clinical PDT against gliomas and other solid tumors. As already discussed, iNOS transcription in glioblastoma cells is regulated by NF- κ B subunit p65, which is activated by p300-catalyzed acetylation of lysine-310^[59]. Knowing this and that (1) Brd4 is a necessary co-activator of iNOS transcription; (2) Brd4 is increasingly upregulated by photostress; (3) p65 is increasingly K310-acetylated by photostress; and (4) that the latter promotes Brd4 interaction with p65^[59,63], we asked how the latter response might be suppressed in order to reduce iNOS upregulation in ALA/light-challenged glioblastoma cells. Bromo- and extra-terminal domain (BET) proteins act as epigenetic “readers” of acetylated lysine residues on histones and transcription factors, thereby co-regulating gene transcription at

promoter sites^[83]. BET protein inhibitors such as JQ1 and OTX015 were recently introduced as powerful new means of suppressing tumor development and progression at the transcriptional level^[84,85]. These inhibitors function by binding to BET domains on Brd4 and other BET proteins, thereby preventing interaction with acK groups on transcription factors (e.g., p65-acK310) or on histones^[85]. When tested on ALA/light-treated U87 cells, JQ1 at a minimally cytotoxic concentration: (1) increased cell killing synergistically compared with photostress alone; (2) strongly inhibited Brd4 binding to p65-acK310; (3) greatly reduced iNOS/NO upregulation after irradiation; and (4) nearly abolished the hyper-aggressiveness of cells that could withstand the ALA/light challenge^[63]. One other striking observation in this study is that the concentration of JQ1 used (~0.3 μ M) was far below that of 1400W capable of producing similar effects. Another glioblastoma line, U251 cells, responded similarly to JQ1 after being photostressed^[63]. In addition to iNOS, several other NF- κ B-regulated proteins were affected by photostress in U87 cells, including pro-survival Bcl-xL and Survivin, which were upregulated, and tumor suppressor p21, which was downregulated^[63]. Each of these photostress responses, similar to iNOS upregulation, was strongly suppressed by JQ1, thereby promoting cell photokilling^[63]. Although Bcl-xL and Survivin transcription may have been directly affected by JQ1, an indirect iNOS/NO-mediated effect was also possible, since NO is known to modulate expression of these effector proteins^[19,20]. Thus, in at least these two cases, JQ1 could have acted directly by preventing Brd4 binding at promoter sites and/or indirectly by inhibiting iNOS expression. In any event, JQ1 inhibition of iNOS transcription appeared to play the major role in improving the efficacy of glioblastoma cell photokilling. It is clear, therefore, that JQ1 would make a highly promising PDT adjuvant, particularly since it has already been used successfully with other anti-cancer therapies. In the case of glioblastoma, for example, JQ1 has been reported to synergize with temozolomide in cytotoxicity at the *in vitro* as well as *in vivo* level^[86]. We anticipate that JQ1 or some other BET inhibitor will act similarly when used in combination with PDT in glioma animal models and eventually glioma patients.

CONCLUSIONS

The many attractive features of ALA-PDT, including tumor site specificity, non-toxicity of components individually (ALA-induced PpIX, light, and O₂), and its demonstrated efficacy on difficult tumors such as glioblastomas, make it an appealing therapy for these malignancies^[8-10,41]. An added advantage of using ALA is that tumor-localized PpIX can be employed for fluorescence-guided surgery (FGS), which is often followed up by PDT to eradicate any residual cells^[43]. It is now well established that cells in many tumors, including gliomas, exploit low-level NO to avoid apoptosis, stimulate proliferation and migration, and resist radio- or chemotherapy^[19-21]. As pointed out above, such NO can also impose a strong resistance to PDT. The NO can derive from tumor cells themselves, although proximal vascular cells (macrophages, fibroblasts, and endothelial cells) may contribute. The *in vitro* and *in vivo* studies described in this review are unique in demonstrating that endogenous iNOS/NO in many tumor cells, including glioblastomas, plays a major role not only in PDT resistance, but also enhanced aggressiveness of surviving cells and non-targeted bystanders. Although both basal and photostress-induced iNOS might be implicated in these responses, there is now solid evidence that induced enzyme plays a preponderant role in several cancer types^[13]. This evidence is unprecedented because most therapy-based studies up to now have considered only pre-existing iNOS/NO and not the possibility of overexpression due to the treatment itself. Concerns about a more aggressive (proliferative and migratory/invasive) phenotype of PDT-surviving cells could be mitigated by turning to pharmacologic inhibitors of iNOS enzymatic activity or iNOS transcription. We suggest possible candidates in each of these categories, emphasizing the greater advantages of those in the latter category, i.e., BET inhibitors.

DECLARATIONS

Acknowledgements

Reshma Bhowmick, Magda Niziolek, Mariusz Zareba, Anna Pilat, and Jerzy Bazak are thanked for their valuable contributions to some of the studies described. Lisa Litzenberger is thanked for helping to format and finalize the five figures.

Authors' contributions

Wrote the manuscript and secured funding for the ALA-PDT/iNOS/NO studies carried out in his laboratory: Girotti AW

Planned and carried out most of the experiments represented in [Figures 2-5](#), and made helpful suggestions about the manuscript: Fahey JM

Secured funding for the PDT-bystander studies and offered helpful suggestions about the manuscript: Korytowski W.

Financial support and sponsorship

The research of two authors (Albert W. Girotti and Witold Korytowski) was supported by the following grants: USPHS Grant CA70823 from the National Cancer Institute, BSC Grant FP12605, and Rock River Grant FP14869 from the MCW Cancer Center (to Albert W. Girotti); NCN Grant 2017/27/B/NZ5/02620 (to Witold Korytowski).

Conflicts of interest

The authors have no conflicts of interest to declare.

Consent for publication

Not applicable

Ethics approval

Not applicable

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Review

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Angiogenesis in acute myeloid leukemia

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How to cite this article: Testa U, Castelli G, Pelosi E. Angiogenesis in acute myeloid leukemia. *J Cancer Metastasis Treat* 2020;6:53. <http://dx.doi.org/10.20517/2394-4722.2020.111>

Received: 9 Oct 2020 **First Decision:** 30 Oct 2020 **Revised:** 16 Nov 2020 **Accepted:** 30 Nov 2020 **Published:** 24 Dec 2020

Academic Editor: Domenico Ribatti **Copy Editor:** Miao Zhang **Production Editor:** Jing Yu

Abstract

Angiogenesis is a word that refers to new blood vessel formation, and this process is of fundamental importance for physiological development and tissue homeostasis, as well as the genesis of several diseases, including tumors. Thus, studies carried out in the last years have shown that angiogenesis is essential for the growth of many solid tumors. Angiogenesis is also important for the growth of many hematological malignancies, including acute myeloid leukemia (AML). Endothelial cells are essential constituents of the bone marrow vascular niches, structures essential for the survival and maintenance of normal hematopoietic stem/progenitor cells. Bone marrow endothelial cells play an essential role in leukemia development and there is growing evidence that a targeting of both leukemic and endothelial cells of the leukemic vascular niche may improve the efficacy of antileukemic therapies. Bone marrow angiogenesis is frequently increased in AML, is morphologically evidenced as increased microvascular density, and is typically associated with some AML subtypes. The molecular mechanisms underlying the increased angiogenesis in some AML subtypes have been defined. In conclusion, a better understanding of angiogenesis as well as the fundamental interactions between bone marrow endothelial cells and leukemic stem cells may contribute to improve antileukemia treatments.

Keywords: Acute myeloid leukemia, angiogenesis, microvascular density, vascular niche, endothelial cells, endothelial growth factors

INTRODUCTION

Angiogenesis is a fundamental and vital process required for the generation of a functional vasculature essential for tissue and whole organism survival^[1]. Blood vessels are not only simple conduits required to



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supply oxygen and nutrients to tissues, but are also involved in specialized functions to support tissue-specific homeostasis, through tissue-specific endothelial cells^[1].

A deregulation of angiogenetic mechanisms is involved in many diseases, characterized by excessive angiogenetic processes, as observed in many malignant tumors or dysfunctional angiogenetic processes, such as in diabetic microvasculopathies^[1]. Historically, the occurrence of a vascular phase of tumor development was first demonstrated for solid tumors, characterized by an angiogenic capability required to promote new vessel formation to sustain tumor growth^[2,3]. Only more recently the occurrence of tumor-related angiogenic mechanisms was reported also for hematological malignancies, although some doubts were raised about the role of angiogenesis in the progression of hematological malignancies. However, numerous studies have shown that the degree of angiogenesis or the level of endothelial growth factors correlate with stage of disease, prognosis, or response to therapy, thus suggesting that angiogenesis induction in hematological malignancies has a physiological role in the mechanisms responsible for disease progression^[4,5].

The terms angiogenesis and vasculogenesis underline two different biologic processes involving new blood vessel formation: the first one describes the formation of new blood vessels starting from pre-existing vessels, while the second one identifies the *de novo* formation of blood vessels and mostly occurs during embryonic and fetal life.

Blood vasculature forms a closed circulatory system involved in the circulation of blood from heart to all peripheral tissues; this complex function requires a highly articulated network composed of different types of vessels, including arteries, veins, and capillaries; the inner layer of vessels is constituted by endothelial cells, a unique and typical component of vessels. There is growing evidence that endothelial cells exert a number of complex biological functions not limited to the generation of a barrier between blood and tissues, but also consisting in specialized functions, such as the creation of a stem cell niche, a function particularly important at the level of bone marrow^[6].

Endothelial cells originate from the mesoderm layer of the early embryo and subsequently a subset of endothelial cells, the hemogenic endothelium, gives rise to hematopoietic stem cells (HSCs)^[7]. At the level of hemogenic endothelium, the hemangioblast, a mesoderm-derived stem cell, gives rise to both the angioblast, the progenitor of endothelial cells, and hematopoietic stem cells, the progenitors of hematopoietic cells. During embryogenesis, endothelial cells undergo a process of specification, necessary to generate the peculiar features of distinct vessel subtypes (arteries, veins, capillaries, and lymphatic vessels) to provide essential support to tissue morphogenesis^[7]. During tissue and organ formation, endothelial cells undergo an additional process of tissue- and organ-characteristic specification.

However, recent studies indicate that the relationship between endothelial cells and hematopoietic lineage during embryonic life is more complex. During embryonic life, some mesoderm cells located in the extraembryonic organ called the yolk sac generate a population of HSCs, called erythro-myeloid progenitors (EMPs); EMPs migrate from the yolk sac into embryo and generate a population of primitive hematopoietic cells, but they also differentiate into endothelial cells, contributing to the vasculature of several organs^[8]. Interestingly, the percentage of endothelial cells that originate from EMPs ranges from 30% in the brain to 60% in the liver^[8]. Further studies will be required to demonstrate to which extent these observations made in mice apply also to humans; however, these studies strongly support the existence of a strong link between endothelial and hematopoietic cell lineages.

Recent studies support the existence of endothelial stem/progenitor cells located at the level of the endothelium of vessels of various tissues; under steady-state conditions, the endothelial stem cells are in a state of quiescence, but they can be activated by stimuli requiring a tissue regenerative response^[9,10].

The mechanisms through which tumors stimulate angiogenesis are complex and heterogeneous in the various tumors implying different molecular and cellular processes; however, it is evident that in all tumors a major determinant of angiogenesis is related to the tumor microenvironment^[11,12]. Tumor angiogenesis is also a key determinant of tumor heterogeneity in that the level of proximity of cancer cells to blood vessels in a tumor greatly influences phenotype and functional and metabolic properties of tumor cells^[13].

The present paper analyzes the angiogenic mechanisms occurring in acute myeloid leukemia focusing on the analysis of changes in vessel density, architecture, and functional properties and on the direct contribution of bone marrow endothelial cells to the development of leukemia progression, promoting leukemic cell homing, survival and proliferation of leukemic cells, and resistance to therapy. As discussed below, a better understanding of the complex interactions occurring between leukemic cells and bone marrow vascular niches may contribute to the development of new therapeutic approaches, including the targeting of leukemic endothelium.

ROLE OF ENDOTHELIAL CELLS IN LEUKEMIA DEVELOPMENT

Role of bone marrow endothelial cells in the control of hematopoiesis

The bone marrow capillary network exhibits a complex structure and shows a linear columnar organization at the level of the metaphysis and endosteum and a sinusoidal organization forming a network of fenestrated, highly branched sinusoidal vessels at the level of the bone cavity; columnar and sinusoidal vessels are interconnected, thus generating a single capillary network, providing a unique and fundamental structure for supporting hematopoiesis^[14]. The columnar and sinusoidal vessels can be distinguished according to the pattern of expression of some endothelial cell surface markers, such as endomucin and CD31: due to the high expression of these markers, columnar vessels are defined as type H vessels, whereas sinusoidal vessels are defined as type L vessels for the low expression of these markers^[15]. These phenotypic differences between type H and type L vessels correspond also to important functional differences in that columnar vessels exhibit a higher oxygen pressure and blood flow than sinusoidal vessels^[16]. The lower endothelial permeability of columnar vessels induces the generation of a microenvironment characterized by low reactive oxygen species (ROS)^[17]. These differences between type H and type L vessels have important consequences at the tissue level, contributing to the generation of different microenvironments; thus, type H vessels connect to arterioles, are surrounded by osteoprogenitors, release factors that promote osteogenesis, and create a local microenvironment promoting the survival and quiescence of HSCs, while type L vessels lack arteriolar connections and association with osteo-progenitors and generate a local microenvironment more permissive of the differentiation of HSCs and Hemopoietic Progenitor Cells (HPCs)^[14] [Figure 1].

Type H endothelial cells display high proliferation rate and mediate vascular growth in bone; regulators of vessel growth are highly expressed in type H endothelial cells, such as neuropilin1, plexin D1, and vascular endothelial growth factor-receptor 3 (VEGF-R3), compared to the low levels of these regulators observed in sinusoid type L endothelial cells. The generation of type H bone marrow vessels is promoted by NOTCH activation in endothelial cells, a surprising finding given the inhibitory effects on angiogenesis of NOTCH signaling exerted in other tissues^[18].

Blood flow was shown to be crucial for the formation of type H capillaries and angiogenic growth of the bone marrow vasculature; a pharmacologically-induced reduction of blood flow resulted in the inhibition of angiogenesis, osteogenesis, and NOTCH activity in the endothelium^[19]. With aging, there is a decline of both blood flow and NOTCH endothelial activity^[19].

Type H endothelial cells are essential for maintaining HSC number and vitality. A major determinant of this effect is represented by the production of the cytokine stem cell factor (SCF) selectively produced by

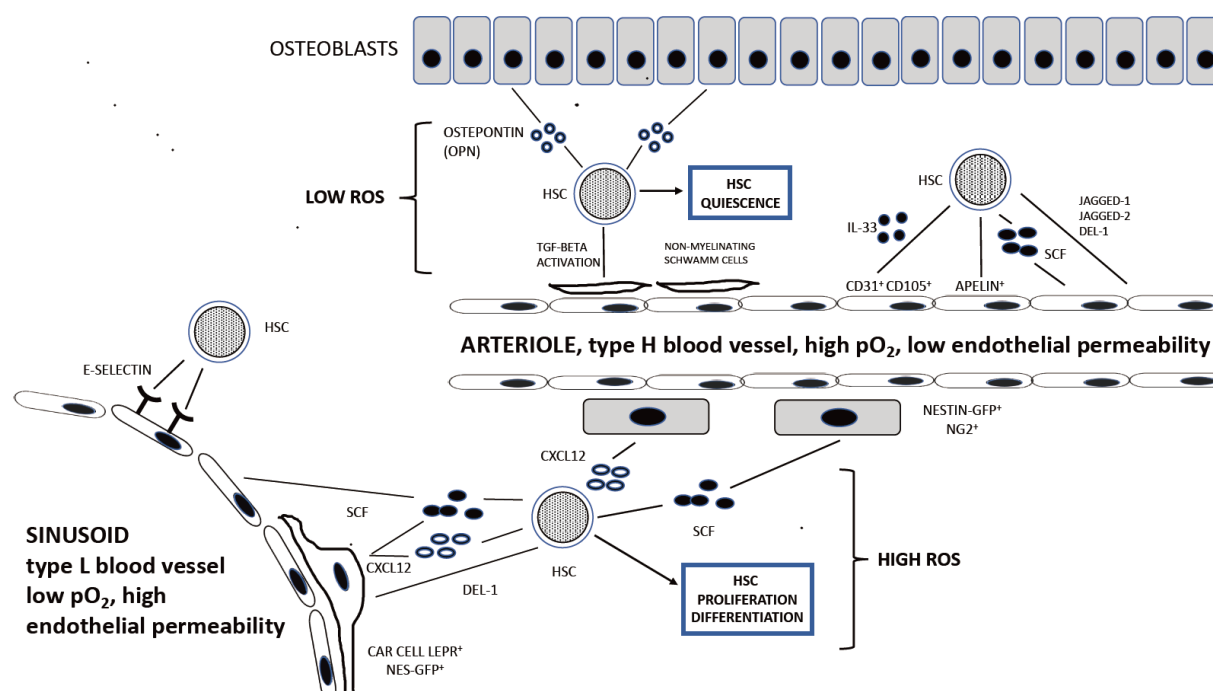


Figure 1. The HSC niche. Under normal conditions, HSCs reside near the bone marrow vessels, either endosteal arterioles or sinusoids. Endothelial cells, as well as mesenchymal stem cell populations (Nestin-GFP NG2⁺ cells and CAR cells) promote the maintenance of HSCs. The endosteal niche contributes to the creation of a microenvironment with higher pO₂ levels; the type H vessels constituting the endosteal niche display low permeability and create an environment low in reactive oxygen species (ROS), promoting HSC quiescence. The sinusoidal niche contributes to the creation of a microenvironment with lower pO₂ levels; the L vessels constituting the sinusoidal niche have a high permeability and create a microenvironment high in ROS, promoting HSC proliferation and differentiation. HSC: hematopoietic stem cell

type H endothelial cells but not by type L endothelial cells^[6] [Figure 1]. Genetic deletion of SCF in type H endothelial cells elicited a significant reduction of the number and activity of HSCs^[20]. Importantly, lineage-tracing experiments have shown that type H and type L endothelial cells self-generate independently after a genotoxic insult, such as a radiation exposure; this finding suggests the existence of radioresistant endothelial progenitors separate for H type and L type endothelial cells^[20].

Other cytokines or chemokines released by bone marrow endothelial cells, such as Chemokine (C-X-C motif) Ligand 12 (CXCL12)^[21], Interleukin 33 (IL33)^[22], and pleiotrophin^[23], play an important role in the survival of HSCs. Particularly, endothelial cell-specific deletion of CXCL12 determines a decrease in the number and repopulating activity of HSCs^[21]. Interestingly, in human bone marrow, IL33 is released from a subtype of endothelial cells expressing CD105 [Transforming Growth Factor β 1 (TGF- β 1) co-receptor], involved in regeneration of endothelial cell after chemotherapy injury and displaying several similarities with murine type H endothelial cells^[22].

Recent studies have reported the identification of an endothelial cell subpopulation, characterized by the production of the peptide Apelin: Apelin⁺ endothelial cells are distinct from other sinusoidal endothelial cells, express NOTCH ligands and pleiotrophin, and play a key role in HSC maintenance, hematopoiesis, and hematopoietic regeneration after irradiation^[24]. Apelin receptor allows the identification in human fetal and adult bone marrow of a mesodermal-derived cell population with hemogenic potential^[25].

Other studies have identified the secreted developmental endothelial locus-1 (Del-1) as a regulator of myelopoiesis in the HSC niche: this HSC niche factor interacts with β 3 integrin on HSCs and is produced

by bone marrow arteriolar endothelial cells and mesenchymal stromal cells (CAR cells)^[26]. Del-1 regulates HSC proliferation and differentiation toward the myeloid lineage^[26].

Endothelial permeability in acute myeloid leukemia

Vascular permeability is a peculiar property of blood vessels wall to be permissive to the flow of small molecules or even of whole cells in and out of the vessel. This flow occurs at the level of the cell junctions between endothelial cells that form microscopic gaps. Vascular permeability is a highly controlled process related to the intrinsic property of each single vessel and to the physiologic conditions of the tissue of residence of vessels.

A recent study by Passaro *et al.*^[27] provided strong evidence that leukemic cells exert a disruptive effect on bone marrow permeability and vascular architecture; these events are essential for the development of the malignant phenotype.

To better understand these studies, it is important to analyze the studies on vascular permeability of vessels of normal bone marrow. In a fundamental study, Itkin and coworkers provided evidence that distinct bone marrow blood vessels, with different permeability properties, play a key role in the control of homeostasis of hematopoiesis through a control of hematopoietic stem cell/hematopoietic progenitor cell quiescence and differentiation. Thus, less-permeable arterial blood vessels maintain HSC in a state of metabolic quiescence, characterized by a low ROS state, whereas the more permeable sinusoids promote a state of metabolic activation of stem/progenitor cells, with high ROS production, triggering differentiation^[28]. Sinusoids represent the site for immature and mature leukocyte trafficking to and from the bone marrow^[28].

It is well known that there is a deterioration of bone and hematopoiesis with aging, and there is evidence for the progressive degeneration of arterial endothelial cells from endosteal regions of bone. Aging determines a reduction in HSC survival and an increase in bone marrow permeability^[29].

The bone endothelial cell permeability is a physiologically very relevant process because it regulates the microenvironment and the hematopoietic stem and progenitor cell transmigration. Stem/progenitor cell transmigration is an essential and fundamental event in the process of homing of transfused HSCs in stem cell transplantation. This process implies first the firm adhesion of HSC/HPC to endothelial cells and subsequent transmigration across the endothelial lining.

The fundamental importance of vascular bone marrow permeabilization is highlighted by the procedure of mobilization of HSCs and HPCs from bone marrow into blood, utilized in clinical practice for the treatment of patients with hematological malignancies who undergo a treatment with HSC transplantation. In clinical studies, granulocyte-colony stimulating factor (G-CSF) is the preferred mobilizing agent. Several studies have in part clarified the mechanisms responsible for G-CSF-mediated HSC/HPC mobilization. The available evidence indicates that G-CSF-induced trafficking is mediated by bone marrow endothelial cells mainly through a mechanism involving a remodulation of the CXCL12-CXCR4 axis: (1) under steady-state conditions, the chemokine receptor CXCR4 expressed on bone marrow endothelial cells actively binds and internalizes CXCL12, its ligand, resulting in the translocation of this chemokine in the bone marrow with consequent activation of a homing signal for HSCs and HPCs in this tissue^[30]; (2) G-CSF administration determines a decrease of both CXCL12 and CXCR4, determined by serine proteases able to cleave these molecules^[31,32]; (3) among the various proteases, the dipeptidyl peptidase CD26 seems to play an essential role in the process of G-CSF-mediated mobilization, mediating CXCL12 cleavage^[33]; and (4) G-CSF induces an increase of the expression of CD26 on the surface of bone marrow endothelial cells, promoting the cleavage of the neuropeptide Y (NPY) to its truncated form, which in turn binds with higher affinity to NPY receptors expressed on sinusoidal endothelial cells, triggering VE-cadherin internalization and degradation, an event that consistently enhances bone marrow vascular permeability^[34].

Vascular bone marrow permeability is also affected by inflammatory and infectious processes. In fact, studies carried out in the context of infectious experimental models have shown a key role of endothelial bone marrow cells as mediators of stimuli enhancing granulopoiesis during acute infection^[35]. Using a model of inflammatory response induced by interferon α (IFN α), a cytokine rapidly produced in response to infection, the induction of a rapid stimulation of endothelial cells bone marrow cells *in vivo* was shown, resulting in an increase of endothelium activation, vascular permeability, and vascularity^[36]. This IFN α -mediated activation of bone marrow endothelial cells is in part dependent on an increased production of VEGF by bone marrow cells^[36].

Passaro *et al.*^[27] explored the bone marrow vasculature using intravital two-photon microscopy in acute myeloid leukemia (AML) patient-derived xenografts; this approach allowed defining changes in bone marrow vascularity since the first stages of leukemic cells engraftment in bone marrow of recipient animals. Using this approach, it was shown that: (1) AML engraftment induced a leukemic-specific expansion of the endothelial compartment among the non-hematopoietic stroma, associated with an increase of microvessel density (MVD) and an alteration of the architecture of bone marrow vasculature with loss of sinusoidal structures, reduction of the mean diameter of vessels, and an increase of tissue hypoxia; (2) increased vascular leakiness in bone marrow; (3) induction of remission with chemotherapy failing to induce a recovery of vascular architecture and permeability in bone marrow; (4) analysis of molecular signatures in vascular endothelial cells showed a consistent deregulation of various pathways involved in permeability regulation and cell adhesion; (5) importantly, among the genes hyperexpressed in vascular endothelial cells, there are the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (NOX4) gene, encoding A NADPH, which, in response to hypoxia, increases ROS levels in the vascular cells and the endothelial nitric oxide synthetase (eNOS), responsible for the production of nitric oxide (NO) molecules; (6) as a consequence of the deregulated Nox4 and eNOS expression, all leukemic vascular niches express high ROS and NO levels; and (7) combined treatment with chemotherapy and genetic or pharmacological inhibitors of eNOS resulted in an improvement of the antileukemic effects and a restoration of bone marrow vasculature^[27].

Duarte *et al.*^[37] analyzed by intravital microscopy (IVM) the changes in bone marrow vasculature induced by the engraftment of primary AML cells; the consistent advantage of this technique is related to its minimal invasiveness and its compatibility with longitudinal observations of cellular dynamics. These studies showed that following AML engraftment in bone marrow blood vessels appeared damaged: most vessels were narrower than those in control mice; limited and abnormal sprouting of bone marrow vessels; progressive decrease of vessels in the endosteum and metaphysis; endosteal vessels are progressively lost at intermediate and advanced disease stages; and the number of CD31^{high}/Endomucin^{high} was significantly reduced^[37]. These changes in bone marrow (BM) vasculature are accompanied by a concomitant progressive depletion of bone marrow stroma. This endosteal remodeling correlated with a loss of normal hematopoiesis, further supporting the evidence that the vascular endosteal system is structurally and functionally damaged by the leukemic process^[37]. Importantly, endosteal areas represent the major site for initiation of AML relapse. Finally, studies in genetically engineered mice provided evidence that rescue of endosteal vessels improves the efficacy of standard antileukemic chemotherapy^[37].

Other components of the endothelial niche play a key role in the control of normal hematopoiesis and in AML development. Osteoblasts are an important component of the endosteal niche and regulate hematopoiesis through the secretion of various factors, such as osteopontin (OPN)^[38]. OPN is a cytokine involved in many physiological processes, including angiogenesis. In adult bone marrow, OPN production is restricted to endosteal region, where this cytokine is required for HSC homing and quiescence^[38]. In the endosteal niche, a truncated form of OPN, trOPN, interacts with $\alpha_4\beta_1$ and $\alpha_9\beta_1$ integrins expressed on HSCs and inhibits their proliferation and differentiation^[39]. Interestingly, OPN expression is increased both

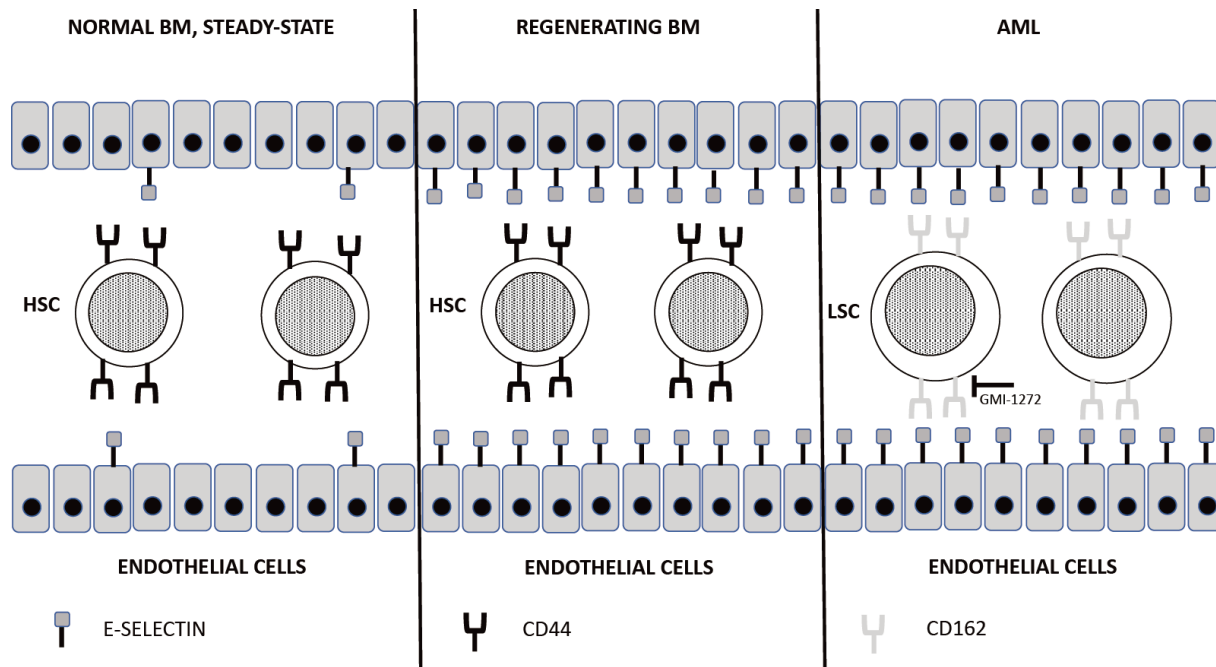


Figure 2. Role of endothelial E-selectin in the control of HSC and LSC. (Left) under steady-state conditions, few bone marrow endothelial cells express the adhesion molecule E-selectin on their surface, mediating in few instances the interaction with the CD44 receptor expressed on HSCs; (middle) under conditions of bone marrow regeneration following damage caused by radiotherapy or chemotherapy, E-selectin expression on bone marrow endothelial cells markedly increases and promotes HSC proliferation; (right) in AML patients, the inflammatory microenvironment promotes a marked increase of E-selectin expression on bone marrow endothelial cells, mediating its interaction with the CD162 receptor overexpressed on the surface of LSCs. GMI-1271, a E-selectin inhibitor, blocks the interaction between E-selectin and CD162, reducing the binding of LSCs to endothelium and their chemoresistance. HSC: hematopoietic stem cell; LSC: leukemic stem cell

in bone marrow leukemic blasts and in bone marrow serum; AML patients exhibiting high levels of bone marrow OPN showed a reduced overall survival^[40]. The prognostic role of OPN was particularly evident at the level of the intermediate risk AMLs^[40].

TARGETING LEUKEMIC ENDOTHELIUM

Another set of recent studies has shown the key role of some adhesion molecules expressed on endothelial cells of the vascular niche in homing, survival, and chemoresistance of AML cells. E-selectin, also known as ELAM-1 (endothelial-leukocyte adhesion molecule-1) or CD62E (CD62 antigen-like family member E), is a selectin cell adhesion molecule selectively expressed on activated endothelial cells. E-selectin binds to different ligands expressed on various types of hematopoietic cells. E-selectin mediates the adhesion of some tumor cell types to endothelium through the interaction with ligands expressed on tumor cells.

E-selectin is constitutively expressed on bone marrow endothelium, where it plays a key role in allowing the homing and engraftment of HSCs/HPCs that express the correspondent ligands. Winkler *et al.*^[41] showed that an increase of E-selectin at the vascular HSC niche in the bone marrow corresponds to a stimulation of proliferation and differentiation of dormant HSCs: following antileukemic chemotherapy or radiation treatment, E-selectin expression increases at the level of bone marrow by about 10-20-fold during the recovery phase of hematopoiesis in correspondence with the reparative proliferation and differentiation of HSCs; a genetic or pharmacologic inactivation of E-selectin reduces this response and increases the proportion of HSCs returning to quiescence [Figure 2]. Interestingly, deletion or blockade of E-selectin significantly enhanced the survival of normal HSCs after treatment of mice with chemotherapeutic drugs or irradiation^[41]. These effects do not seem to be mediated by canonical E-selectin ligands, such as CD44 and CD162^[41].

Importantly, E-selectin expression was observed at the level of bone marrow areas where leukemia cells home at the moment of their engraftment^[42]. Xenograft models of immune-deficient mice transplanted with AML blasts showed that a small number of CD34⁺ leukemic stem cells that survived antileukemia chemotherapy was detected under form of clusters located around endosteal vascular endothelium, a region where E-selectin is expressed on endothelial cells^[43,44].

Recent studies have shown a direct role of bone marrow vascular E-selectin expression in homing and chemoresistance of leukemic cells. Thus, Winkler *et al.*^[45] initially reported a remarkable up-modulation of E-selectin expression on the bone marrow vasculature in mice with AML; studies in the murine AML model generated by retroviral transduction of the MLL-AF9 fusion oncogene into HSCs showed that leukemic blasts rapidly upregulate E-selectin expression following oncogenic transformation. Experiments based on genetic or pharmacological inhibition of E-selectin expression provided evidence that E-selectin expression is important for retention of leukemic stem cells in bone marrow and protects leukemic stem cells from the cytotoxic effects of chemotherapy^[45]. In a more recent study, the same authors showed that MLL-AF9 AML cells surviving cytarabine therapy display an increased E-selectin binding potential^[46]. This is due to the capacity of these leukemic cells to interact with E-selectin-positive endothelial cells present in the vascular niche. This conclusion is supported by two lines of observations: (1) vascular niche E-selectin blockade by GMI-1271, a specific inhibitor of E-selectin binding, inhibits malignant AML reconstitution/survival potential *in vivo*; and (2) these effects occur through the inhibition of several pro-survival signals induced in leukemic cells^[46]. According to these observations, it was suggested that E-selectin blockade may synergize with other pathway inhibitors to improve the therapeutic response of AML patients^[46].

In a more recent study, the same investigators showed that AML cells generate an inflammatory state in the bone marrow at the level of the sites where they home, driving increased E-selectin expression on bone marrow endothelial cells^[47] [Figure 2]. This condition creates the basis for the interaction between leukemic cells and E-selectin-expressing endothelial cells, triggering the generation of pro-survival signaling (PI3K/AKT and mTOR pathways) that stimulates leukemic cell proliferation and promotes leukemic chemoresistance^[47]. These observations support the clinical use of E-selectin inhibitors in combination with standard induction chemotherapy for AML patients. The preliminary results of a phase I clinical trial involving the administration of GMI-1271 in combination with induction chemotherapy to a group of 19 relapsed/refractory AML patients showed a high response rate (42% of complete responses), with a sufficient duration of response to allow five patients to proceed to salvage stem cell transplant. Phase II of this study is ongoing and implies two arms of treatment, one corresponding to an expansion cohort of phase I and the other involving treatment of newly diagnosed AML patients ≥ 60 years.

Very recently, the membrane E-selectin receptor expressed on HSCs and responsible for induction of chemoresistance was identified^[48]. E-selectin may interact with two different membrane receptors, CD44 and CD162, expressed on hematopoietic cells. To explore this issue, Erbani and coworkers used the CD34⁺ human myeloid cell line KG1a. This cell line express CD44 and CD162, and both of these receptors are functional in terms of E-selectin binding capacity. Only the silencing of both receptors abrogates the capacity of KG1a cells to bind E-selectin; however, only CD162 is critical for E-selectin-mediated chemoresistance *in vitro*. Importantly, CD162 expression on AML cells *in vivo* is a major determinant for E-selectin binding, bone marrow vascular niche retention, and leukemic progression^[48] [Figure 2]. Deletion of CD162 in AML cells induces a clear increase of the sensitivity of leukemia stem cells to therapy^[48]. According to these findings, it was suggested that the binding of CD162 to E-selectin represents a potential therapeutic target to improve therapeutic outcomes through a potentiation of the efficacy of antileukemic chemotherapy.

Interestingly, recent studies carried out on chronic myeloid leukemia (CML) led to evidence similar to that observed in AML about a key role of interactions with E-selectin mediating leukemic homing and

survival^[49]. In fact, it was shown that binding of BCR-ABL1⁺ CML cells to E-selectin in the vascular niche stimulates cell cycle progression and response to imatinib treatment^[49]. For CML cells, the E-selectin CD44 receptor and not CD162 seems to be involved in the mediation of E-selectin induced effects^[50].

ANGIOGENESIS IN AML BONE MARROW: MICROVESSEL DENSITY

The technique currently used to evaluate the extent of angiogenesis at the level of bone marrow biopsies consists in staining of the histological sections with specific endothelial cell markers and then evaluation with a microscope of the density of microvessels present in these tissue sections (the number of vessels per millimeter length of bone marrow core biopsy is evaluated). This technique provides a score of MVD. Using this approach, two different groups of investigators in 2000 provided evidence that the MVD was increased (approximately doubled) in AML specimens compared to normal bone marrow^[51,52]. Examination of AML bone marrow specimens after induction of disease remission was associated with a clear decrease of bone marrow MVD; in patients not achieving remission following chemotherapy treatment, no decrease of bone marrow MVD was observed^[51,52]. Kini *et al.*^[53] reported that the MVD and hot spot density were particularly increased in bone marrow biopsies of acute promyelocytic leukemia patients compared with normal bone marrow specimens; treatment with retinoic acid induced disease remission and a clear decrease of MVD. Other studies have evaluated whether the increased MVD observed in AML bone marrow was associated with an increased production of VEGF. Padrò *et al.*^[54] showed, through the immunohistochemical analysis of 32 AML bone marrow samples, higher VEGF and VEGF-R2 expression than in control normal bone marrow: expression of VEGF and VEGF-R2 was clearly higher in patients with a high degree of MVD, compared to those observed with lower MVD. A direct correlation was observed between VEGF expression and MVD^[54]. Ghannadan and coworkers explored VEGF expression in the bone marrow of 41 AMLs and observed positive expression in all AML subtypes classified according to the FAB classification, with the expression of most immature FAB M0 AMLs, expressing undetectable or only low levels of VEGF^[55]. VEGF was detectable in immature elements, whereas it was undetectable or expressed at very low levels in mature hematopoietic elements^[55].

Other studies confirmed the increased MVD in AML bone marrow and showed also a correlation between the increase of MVD and the proliferation index of leukemic blasts^[56] and the association between increased MVD and increased bone marrow VEGF levels, both parameters decreasing in patients who achieved disease remission following induction therapy^[57].

Kuzu *et al.*^[58] showed a higher MVD in AML patients compared to controls, independent of cellularity or blast percentage; higher baseline MVD values in AML patients were associated with a shorter overall survival and thus are a negative prognostic factor.

Interestingly, Weidenaar and coworkers explored the vascular morphology within AML bone marrow biopsies^[59]. The analysis of a pericyte marker (smooth muscle actin) provided evidence that in AML bone marrow at diagnosis only 35% of vessels were pericyte-coated, compared to 73% in the normal bone marrow and 55% in AML patients in remission. Furthermore, the percentage of pericyte-coated vessels was significantly higher in the group of AML patients with “low vessel count” compared with that observed in AML patients with “high vessel count”^[59]. Two different patterns of vascular morphology were observed in AML bone marrow biopsies: high number of vessels with a large lumen and thin walls and a high vessel count with a network of small vessels with thin walls, narrow lumen, and branching. The first vessel pattern was associated with high secreted vascular endothelial growth factor A (VEGFA) protein levels^[59].

Other investigators have reported a comparative analysis of bone marrow MVD in various hematological malignancies, confirming the higher values of MVD in AML compared to normal bone marrow. These studies also showed an increase of MVD in Acute Lymphoblastic Leukemia (ALL) and CML, at a level

higher than that observed in AML^[60,61]. These studies failed to demonstrate a significant increase in serum/plasma VEGF levels in AML patients^[60,61].

As reported above, the analysis of plasma/serum VEGF levels in AML patients showed conflicting results as not all studies showed an increase of VEGF levels in these patients^[59]. These discrepancies may be related to the variable sensitivities of the immunodetection assays used to quantify VEGF levels and the heterogeneity of AML patients (*de novo* or relapsed AMLs) included in these studies. However, the majority of these studies showed increased VEGF levels in plasma/serum of AML patients, a conclusion supported by a recent large meta-analysis performed by Song *et al.*^[62]. A meta-analysis carried out by Guo *et al.*^[63] showed that high VEGF expression in AML was associated with worse event-free survival and poor overall survival. In this context, two studies are particularly interesting. Aguayo *et al.*^[64] showed that VEGF levels were similarly elevated in AML and MDS patients: in AMLs, but not in MDSs, elevated plasmatic VEGF levels were associated with reduced survival and reduced remission rate. De Bont *et al.*^[65] evaluated the release of VEGF by leukemic blasts of pediatric AMLs, and the levels of this endothelial growth factor release by leukemic cells are an independent prognostic factor for relapse-free survival.

As mentioned above, some studies^[59] have shown that the increased MVD in AML is a negative prognostic factor. This conclusion was confirmed by other studies. Rabitsch *et al.*^[66] analyzed bone marrow MVD in 38 younger AML patients undergoing standard chemotherapy treatment and consecutive allogeneic bone marrow transplantation: at diagnosis, the MVD was markedly higher in AML patients than in normal controls (30/mm² vs. 7/mm²); in patients who failed to achieve a complete response following induction chemotherapy, the MVD was higher at diagnosis than in those achieving complete remission (41.5/mm² vs. 28.5/mm²); and patients with high MVD displayed a shorter overall survival and a higher risk of relapse than patients with lower MVD. Similarly, studies carried out in myelodysplastic syndromes, conditions frequently preceding AMLs, have shown that increased MVD was associated with a shorter survival time^[67].

Myeloid sarcoma, also known as granulocytic sarcoma or chloroma, is a tissue extramedullary mass form of AML composed of myeloid blasts^[68,69]. Myeloid sarcoma may occur *de novo*, may precede or coincide with AML, or may correspond to a blastic transformation of a preceding myeloproliferative neoplasm or myelodysplastic syndrome^[68,69]. Myeloid sarcoma may occur as a manifestation of relapse in an AML patient, even after allogeneic stem cell transplantation^[70]. Piccaluga *et al.*^[71] explored the bone marrow MVD in 60 myeloid sarcomas and showed that these tumors have an increased MVD compared to normal bone marrow. The MVD observed in myeloid sarcomas was similar to that observed in AMLs. Among myeloid sarcomas, those with a monocytic morphology displayed a significantly higher MVD than those with blastic appearance^[71]. In these patients, higher MVD was associated with a reduced overall survival in multivariate analysis^[71].

MOLECULAR MECHANISMS RESPONSIBLE FOR STIMULATION OF ANGIOGENESIS

Recent studies have explored the molecular mechanisms involved in the stimulation of angiogenesis in some AML subtypes and have defined a link between leukemia-specific genetic abnormalities and enhanced angiogenesis.

Initial studies by Hiramatsu and coworkers showed that, among the various AML subtypes, AML M3, corresponding to APL, having the translocation t(15;17) generating the fusion protein Promyelocytic/Retinoic Acid Receptor Alfa (PML/RARA), showed the highest expression of VEGF and VEGF-R1^[72]. The AML subtype characterized by the specific t(8;21) translocation, generating the fusion protein AML1/ETO (Eight-Twenty-One), displayed high expression of VEGF and VEGF-R2^[72].

In line with this initial study, Imai *et al.*^[73] showed that t(8;21) AML cells are responsive to exogenous VEGF stimulation with activation of AKT pathway. Furthermore, t(8;21)-positive AML cell lines are inhibited in their proliferation by VEGF-R2 kinase inhibitors. The gene AML1/RUNX1 located on chromosome 21 is frequently involved in genetic alterations, such as chromosomal translocation events (AML1/ETO or AML1/EVI1) resulting in the formation of fusion proteins, associated with a loss of function of AML1/RUNX1. The analysis of a large dataset of gene expression arrays relative to AML samples showed the existence of an inverse correlation between expression of VEGF and AML1/RUNX1, with the highest VEGF levels being observed in leukemic blasts bearing t(8;21)^[73]. Gene expression and transfection experiments provided evidence that AML1/RUNX1 acts as a repressor of VEGFA expression, through its direct binding at the level of three sites present on the promoter of the VEGF gene; AML1/ETO fails to exert this repressive effect on VEGF expression and results in a stimulation of its expression^[72]. In line with this interpretation, silencing of AML1/ETO expression in t(8;21) leukemic cell lines resulted in an inhibition of VEGF expression^[74].

Studies carried out in APLs have led to similar conclusions concerning the mechanisms through which the fusion protein observed in t(15;17) AMLs stimulates angiogenesis. Saulle *et al.*^[75] through the analysis of a large TCGA dataset on gene expression in AMLs, confirmed that the highest expression of VEGF-A mRNA was observed in M3 t(15;17) AMLs. The molecular mechanism through which PML/RARA stimulates VEGF expression is related to its capacity to induce a significant downmodulation of Hematopoietically Expressed Homeobox (HHEX) expression, a homeobox transcription factor exerting a repressive effect on VEGF gene expression. The downmodulation of HHEX was also responsible for the stimulation of the expression of other genes involved in the control of angiogenesis^[75].

EXPRESSION OF ENDOTHELIAL GROWTH FACTOR RECEPTORS IN AMLS

The receptors for various endothelial growth factors are frequently expressed on leukemic blasts and several studies have characterized the properties of AMLs expressing these receptors.

Padrò and coworkers explored VEGF-R1 and VEGF-R2 expression in AML bone marrow biopsies and observed that VEGF-R2 but not VEGF-R1 expression in AMLs was increased compared to normal bone marrow; VEGF-R2 expression was mostly increased in AMLs with an immature myeloid phenotype, classified as M1 and M2 in the FAB classification system or those with a monocytic phenotype (M5 AMLs)^[54]. Studies in a model of mice xenotransplanted with human AML cells showed that inhibition of paracrine (dependent on endothelial cells present in the bone marrow microenvironment) and autocrine (dependent on leukemic cells) VEGF/VEGF-R2 signaling pathway was essential to induce long-term remission of these mice^[76]. This study provided a rationale to investigate in humans the antileukemic potential of VEGF-R2 inhibitors. Other preclinical studies supported an inhibitory effect of anti-VEGF-R2 monoclonal antibodies on human AML development in leukemia animal models^[77].

Zahiragic *et al.*^[78] treated nine AML patients with refractory/relapsing disease with the anti-VEGF monoclonal antibody bevacizumab. Bevacizumab treatment reduced VEGF expression at the level of bone marrow but failed to show any significant clinical antileukemic activity^[78]. It is interesting to note that, while bevacizumab reduced VEGF expression at the level of bone marrow, it was unable to modify bone marrow MVD^[66]. Other clinical trials incorporating anti-VEGF or anti-VEGF-R2 antibodies have not produced results supporting a significant clinical benefit^[79,80].

More recent studies have reevaluated the role of VEGF-R2 in leukemia development and its targeting with new VEGF-R2 inhibitors. Nobrega-Pereira provided evidence that VEGF-R2 signaling is involved in the mechanism of AML chemoresistance^[81]. Treatment of chemoresistant AML cells with a VEGF-R2 inhibitor

sensitized AML cells to chemotherapy^[81]. These observations suggest the potential use of VEGF-R2 inhibitors in association with antileukemia chemotherapy.

Other recent studies have explored the effects of apatinib, a new VEGF-R2 inhibitor. Apatinib is an oral small-molecule tyrosine kinase inhibitor of VEGF-R2, showing in various experimental systems a marked inhibitory activity on angiogenesis. This inhibitor showed promising effects in several solid tumors^[82]. *In vitro* studies have shown that apatinib exerts a consistent cytotoxicity toward AML by targeting VEGF-R2-mediated prosurvival signaling and angiogenetic effects. The sensitivity of AML blasts to apatinib was correlated with some molecular features, including presence of Nucleophosmin 1 (NPM1) mutations and FAB M2 and M5 subtypes; importantly, AML blasts of relapsed/refractory patients displayed sensitivity to apatinib^[83,84].

Sorafenib is a multi-kinase inhibitor exerting its effects by reducing the activity of various kinase receptors, including VEGF-R2, Fms Like Tyrosine Kinase 3 (FLT3), and Kinase Insert Tyrosine kinase (KIT). Sorafenib when used in monotherapy failed to exert a pronounced antileukemic activity in AML patients with refractory/relapsing disease^[85]. However, a phase II study in untreated AML patients (aged \leq 60 years) explored the antileukemic activity of sorafenib added to standard induction chemotherapy compared to placebo: sorafenib induced a significant prolongation of both event-free survival (EFS) and relapse-free survival (RFS) compared to placebo^[86]. In an exploratory subgroup analysis, it was observed that no EFS prolongation was observed among AML patients with FLT3-ITD mutation, while AML patient FLT3-WT had significantly improved EFS and RFS^[86]. This finding suggests that an anti-angiogenesis effect of sorafenib through inhibition of VEGF-R and platelet-derived growth factor receptors (PDGF-R) could mediate the effect of this drug on significant prolongation of EFS and RFS^[86].

In addition to VEGF and its receptors, the endothelial growth factors angiopoietin1 (Ang1) and angiopoietin2 (Ang2) and their receptors Tie1 and Tie2 are also important regulators of physiologic and pathologic angiogenesis^[87]. Peculiar is the function of the constitutively expressed Ang1 that acts as a stabilizer of blood vessels. In addition, Ang1 through the binding to Tie2 promotes endothelial cell survival and endothelial barrier function^[88]. Ang2 acts as a context-dependent agonist or antagonist of the Ang1-Tie2 signaling axis^[87]. Ang2 is expressed by endothelial cells. Its levels are increased by hypoxia and proinflammatory signals, and they are also increased in many types of cancers^[69]. In several tumor models, there is evidence that Ang2 protects stressed endothelial cells from apoptosis^[89] and limits the effects induced by VEGF inhibition^[90].

The Ang-Tie system was explored in AMLs. In an initial study, Watarai *et al.*^[91] showed that an AML subset, characterized by the inappropriate expression of the lymphoid membrane marker CD7, displays expression of Ang2, in association with an elevated expression of integrin-family adhesion molecules. The expression of Tie2 in these CD7⁺ AMLs was low^[91].

Schliemann *et al.*^[92] explored Ang1, Ang2, and Tie2 expression in 64 adult patients with newly diagnosed AML. They observed that: (1) expression of Ang2 was significantly higher in the bone marrow of AML patients than in healthy controls; (2) the expression of Ang1 in AML was similar to that observed in normal controls; and (3) Tie2 expression was often increased in AML samples compared to the levels observed in normal bone marrow^[92]. Ang2 levels but not Ang1 or Tie2 levels had a prognostic impact: patients with high Ang2 levels exhibited a better overall survival compared to those with low Ang2 levels^[92]. In a subsequent study, the same investigators analyzed the prognostic impact of plasmatic Ang1, Ang2, and soluble Tie2 (sTie2) in 68 AML patients^[93]. Circulating levels of Ang2 and sTie2, but not of Ang1, were significantly elevated in AML patients as compared to controls^[75]. Higher levels of Ang2 and sTie2 were predictive of poor survival in these patients; particularly, patients with elevated plasmatic Ang2

displayed a significantly reduced overall survival compared to that observed in patients with low plasmatic Ang2 levels^[93]. The discrepancy observed in these two studies may be tentatively related to some relevant differences: the first study evaluated bone marrow expression of Ang2, while the second study evaluated plasmatic Ang2 levels and the source of circulating Ang2 is not only related to leukemic blasts, but also reflects the production by other cell types, including endothelial cells^[93].

Loges et al.^[94] explored VEGF-A, VEGF-C, Ang1, Ang2, and Tie2 mRNA levels in a cohort of 90 patients with *de novo* AML. This study showed that high Ang2 levels have a good prognostic impact on patient's survival: sub-analysis according to the levels of other endothelial growth factors showed that the prognostic impact of Ang2 mRNA expression was most evident in AML subgroups with low VEGF-C and Ang1 levels^[94].

Riccioni and coworkers reported a detailed immunophenotypic analysis of AMLs expressing high levels of Tie2^[92]. In this study carried out in 111 *de novo* adult AML patients, 35% displayed high levels and 20% moderate levels of Tie2. Tie2 expression on leukemic blasts was associated with the expression of monocytic markers. Furthermore, Tie2 expression was associated with concomitant expression of other endothelial growth factors such as VEGF-R1, VEGF-R2, and VEGF-R3^[95]. Highly-expressing Tie2 AMLs were characterized by high blast cell counts at diagnosis and frequent FLT3 mutations^[95]. According to these findings, it was suggested that AMLs exhibiting high Tie2 expression resemble Tie2-expressing monocytes, a subpopulation of monocytes playing a role in promotion of tumor angiogenesis^[96].

About 30% of adult AMLs display FLT3 mutations; in addition, 10%-15% of AMLs exhibit high FLT3 expression, in the absence of FLT3 mutations. This last group of AMLs is characterized by recurrent expression of receptors for endothelial growth factors, including Tie2^[97].

The possible clinical efficacy of Tie2 inhibitors was not yet explored in AML patients. Pexmetinib, a dual inhibitor of Tie2 and p38 MAPK, showed antileukemic activity in preclinical models of AML^[98].

Recent studies support a role for epidermal growth factor ligand 7 (EGFL7) as a pro-angiogenic factor promoting angiogenesis in AMLs. This secreted angiogenic factor possesses the unique property to be almost exclusively expressed by endothelial cells. EGFL7 is maximally expressed in proliferating endothelial cells and acts on endothelial cells^[99]. Interestingly, microRNA-126, an endothelial cell-specific miRNA, is located in intron 7 of the EGFL7 gene. EGFL7 interacts with the extracellular domain of NOTCH, resulting in an antagonistic effect on NOTCH activation^[99]. EGFL7 is a potent angiogenic factor, playing a key role in the control of vascular angiogenesis during embryogenesis^[100]. EGFL7 is aberrantly overexpressed in solid tumors. A recent study showed that EGFL7 mRNA and EGFL7 protein levels are increased in blasts of AMLs compared to normal bone marrow cells: in AML patients with cytogenetically normal AMLs, high EGFL7 mRNA levels associate with decreased overall survival rates^[101]. *In vitro* studies showed that EGFL7 stimulates the proliferation of leukemic blasts, whereas high EGFL7 expression predicts poor prognosis in AML patients undergoing allogeneic stem cell transplantation^[102]. Functional studies have shown that EGFL7 inhibits NOTCH signaling in AML blasts antagonizing canonical NOTCH ligand binding; anti-EGFL7 treatment resulted in reactivation of NOTCH signaling in AML cells, increased differentiation, and apoptosis, thus suggesting that it may represent a therapeutic strategy^[103].

CONCLUSION

Endothelial cells are intimately associated with HSCs throughout the life of the stem cell, from peculiar endothelial cells (hemogenic endothelium) that give rise to HSCs, to the perivascular niche endothelial cells that regulate HSC homeostasis. Endothelial cells as constituents of bone marrow vascular niches play a key role in the control of HSC homing, migration, and maintenance. Bone marrow endothelial cells

also play an essential role in the development of leukemia and growing evidence shows that targeting of both leukemic endothelium and leukemic stem cells represents a more effective antileukemic therapeutic strategy.

Bone marrow angiogenesis is enhanced in many patients with AML and several studies have shown a link between some leukemic-specific genetic alterations and stimulation of bone marrow angiogenesis. A better understanding of angiogenesis in AMLs may contribute both to a better understanding of the leukemogenic process and to define an improved strategy for the treatment of these leukemias.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception, design of the study and analysis and interpretation of the literature data: Testa U, Castelli G, Pelosi E

Contributed to the preparation of the manuscript and to the supervision of all data included in this manuscript: Testa U

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

Not applicable.

Consent for publication

Not applicable.

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Review

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Lung cancer management during the COVID-19 pandemic: experience of a medical oncology unit at a tertiary hospital in Singapore

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How to cite this article: Vong EKY, Chia PL, Chang AY. Lung cancer management during the COVID-19 pandemic: experience of a medical oncology unit at a tertiary hospital in Singapore. *J Cancer Metastasis Treat* 2020;6:54.
<http://dx.doi.org/10.20517/2394-4722.2020.116>

Received: 22 Oct 2020 **First Decision:** 16 Nov 2020 **Revised:** 30 Nov 2020 **Accepted:** 2 Dec 2020 **Published:** 24 Dec 2020

Academic Editor: Robert Kratzke **Copy Editor:** Monica Wang **Production Editor:** Jing Yu

Abstract

Coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was declared a pandemic by the World Health Organisation (WHO) on 11 March 2020. The pandemic has had profound effects on healthcare systems across the world, and also poses unique challenges for oncology services. Singapore saw its first imported case of COVID-19 on 23 January 2020, and there has since been 52,000 confirmed cases and 27 deaths as of early August 2020 locally. Oncologists have a special duty to our patients to ensure patient safety and provide optimum care without undue disruption which may compromise long-term cancer-specific outcomes. We herein examine the impact that the COVID-19 pandemic has had on our clinical services, and share our experience with regards to manpower reconfiguration, infection control measures, diagnostic evaluation of patients with suspected COVID-19, oncological management of lung cancer patients, as well as changes in the education and training of juniors, from our unique position as a Medical Oncology department in Tan Tock Seng Hospital, a tertiary hospital affiliated with the National Centre of Infectious Diseases in Singapore.

Keywords: COVID-19, lung cancer, thoracic cancer



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INTRODUCTION

Since the identification of a cluster of novel viral pneumonia cases caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that was first reported in Wuhan, China, in December 2019, coronavirus disease 2019 (COVID-19) has spread rapidly across the world. As of early August 2020, global cases of COVID-19 have exceeded 18 million, with close to 700,000 deaths and case fatality ratios (CFRs) ranging from 0.1% to 15% across different countries^[1]. Singapore saw its first imported case on 23 January 2020, and had one of the highest numbers of reported COVID-19 cases outside of China in February 2020. There has since been 52,000 confirmed cases and 27 deaths as of early August 2020 locally. Early supportive care and monitoring are the cornerstones of clinical management for the majority of patients with mild illness. In patients with severe illness, dexamethasone and remdesivir are 2 drugs that have been shown in randomised phase 3 trials thus far to reduce mortality^[2] and improve time to recovery^[3], respectively.

Building on past experience with the severe acute respiratory syndrome (SARS) outbreak in 2003, Singapore has systemically strengthened its ability to manage emerging infectious diseases outbreaks. Since 7 February 2020, Singapore has been in Disease Outbreak Response System Condition (DORSCON) Orange, the second highest national alert level signifying that SARS-CoV-2 has not spread widely in Singapore and is still being contained^[4]. A multi-agency taskforce was formed to coordinate the national response to the outbreak. National public health measures implemented include aggressive contact tracing, mandatory stay home notices for returning travellers, travel restrictions, telecommuting, mandatory mask-wearing and safe distancing measures at public spaces, workplaces and schools.

The National Centre for Infectious Diseases (NCID) is a 330-bed purpose-built facility officially opened in September 2019, designed to augment Singapore's capabilities in infectious disease management and prevention^[5]. It has been the epicentre of the national response to COVID-19. The provision of clinical services at NCID is supported by the National Healthcare Group (NHG) and Tan Tock Seng Hospital (TTSH), which is situated next to NCID within the Novena campus located in central Singapore. TTSH is Singapore's second largest tertiary care hospital with over 1,700 beds. The medical oncology department at TTSH is the third largest oncology provider amongst tertiary public hospitals in Singapore, and also works closely with other clinical departments and services in TTSH.

The COVID-19 pandemic poses several unique challenges for oncology services, and in particular the management of patients with thoracic malignancies. A key challenge faced by oncologists is the balance of the risk of increased severity of COVID-19 in lung cancer patients versus the consequences of delay in diagnosis and time-sensitive oncological treatment which may occur due to reasons such as changes in health-seeking behaviour and reallocation of healthcare resources to COVID-19. A global survey of medical oncologists has demonstrated that COVID-19 has significantly influenced decision-making by oncologists and significant reductions in cancer-related patient encounters have also been reported^[6-8]. We herein describe several key experiences relevant to the field of thoracic oncology during the COVID-19 pandemic from the perspective of our department and as guided by available data in the literature.

FACILITY AND MANPOWER SEPARATION AND CROSS-DEPARTMENTAL COLLABORATION

COVID-19 is spread primarily via droplets and is highly infectious with a basic reproduction number (R_0) of 2.0-2.5, with established community transmission that has threatened to overwhelm healthcare systems globally^[9]. Disease severity ranges from mild illness in the majority (80%) of patients to severe illness requiring mechanical ventilation and intensive care^[9]. Patients with advanced age and comorbidities including cancer, chronic respiratory disease (COPD), uncontrolled hypertension and diabetes are at increased risk of severe illness and mortality from COVID-19^[10]. Infection with SARS-CoV-2 can cause considerable disruption to oncological treatment plans. These factors underscore the critical need for

a coordinated effort to ensure operational continuity with minimal disruption to cancer care while maintaining patient and staff safety.

Infection control measures and general patient management algorithms

Infection control measures were ramped up rapidly at NCID and TTSH to ensure that all staff are adequately protected from infection risk. NCID and TTSH wards were segregated into wards for non-COVID-19, suspected and confirmed COVID-19 patients. Patients presenting with acute respiratory infection (ARI) to the NCID Screening Centre (SC) or TTSH Emergency Department (ED) were identified and separated from the general patient pool at the point of triage in order to reduce the risk of exposure to other patients and healthcare staff. At subsequent medical assessment, SARS-CoV-2 reverse transcription-polymerase chain reaction (RT-PCR) testing as well as laboratory and chest radiography were conducted according to hospital protocols.

Suspect and confirmed COVID-19 patients were admitted to negative-pressure isolation rooms in NCID for further management. Patients diagnosed with community-acquired pneumonia were admitted to neutral pressure isolation rooms in TTSH under an enhanced pneumonia surveillance program and were required to undergo SARS-CoV-2 testing before they could be de-isolated. The result of SARS-CoV-2 RT-PCR testing is generally available in less than 6 hours when performed in NCID or TTSH, and this quick turnaround time has been an important factor in the success and efficiency of the local screening and containment measures. Suspect case definitions for COVID-19 were updated regularly based on evolving epidemiological factors and disseminated by the Ministry of Health (MOH) to all doctors via email, and patient management algorithms were updated accordingly by hospital management.

Other key measures undertaken include compulsory personal protective equipment (PPE) training and N95 mask-fitting for all healthcare workers. Strict adherence to hospital PPE protocols is mandated when reviewing patients who are suspect or confirmed cases of COVID-19. For routine patient care, surgical mask and meticulous hand hygiene is practiced. All TTSH staff were provided with personal thermometers for twice-daily online temperature recording. Staff with ARI were advised to seek medical attention promptly at NCID or the TTSH Occupational Health Clinic for assessment and COVID-19 diagnostic testing, and were provided at least 5 days of medical leave.

TTSH also put in place temperature screening and health declaration measures for patients and visitors at hospital entrances. Measures for physical distancing were implemented in our outpatient oncology clinics and chemotherapy units. Patients attending outpatient appointments were limited to one accompanying person at any one time. Designated consult rooms and PPE are employed for the assessment of patients with ARI before they are appropriately redirected to NCID or the TTSH ED [Figure 1]. For patients who are on quarantine orders due to close contact with confirmed COVID-19 cases or those on stay home notices after return from abroad, non-urgent outpatient appointments were postponed until after completion of the quarantine orders.

Clinical service reconfiguration

The COVID-19 pandemic has also led to service reconfiguration of our medical oncology department. One of the first measures undertaken by our department during the start of the outbreak in Singapore was team segregation, in order to reduce the loss of workforce and avoid quarantine of the whole department in the event of COVID-19 exposure or infection. We segregated our department into 2 independently functioning teams each comprising junior doctors, fellows and attendings geographically confined to the inpatient and outpatient sectors. A similar approach has been reported by other cancer centres in Singapore as well^[11,12]. Large group gatherings were minimised; department meetings and education activities were conducted in small groups and via secure video-conferencing platforms.

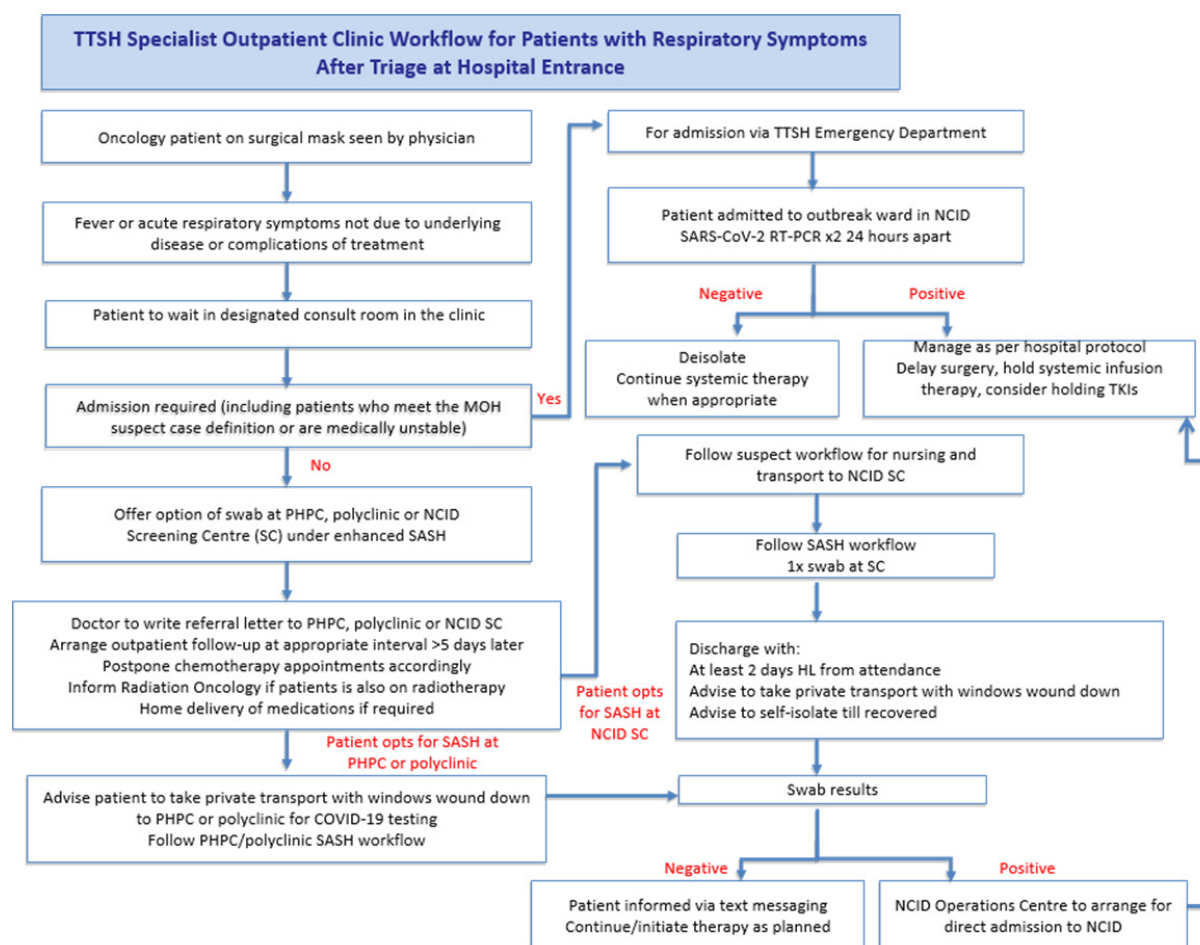


Figure 1. TTSH specialist outpatient clinic workflow for patients with respiratory symptoms after triage at hospital entrance. TTSH: Tan Tock Seng Hospital; MOH: Ministry of Health; NCID: The National Centre for Infectious Diseases; SASH: Swab-and-Send-Home; PHPC: Public Health Preparedness Clinic; SC: screening Centre; COVID-19: Coronavirus disease 2019; RT-PCR: reverse transcription-polymerase chain reaction; TKIs: tyrosine kinase inhibitors; SARS-CoV-2: severe acute respiratory syndrome virus 2

Physicians' non-essential leave was also cancelled in order to maximise existing manpower resources. The Singapore MOH also prohibited cross-hospital movement of physicians, thus necessitating clinic closures and redistribution of patients under the care of our visiting medical oncology consultants. Similarly, inter-hospital transfers of inpatients were discouraged unless there is a clinical need for specialised medical services not available at the parent hospital. Medical oncologists have also had to take up different duties in the management of the COVID-19 pandemic, with clinical redeployment to NCID on a regular rotational basis to provide acute care for confirmed and suspect COVID-19 patients as part of pandemic coverage in a broader hospital and national effort.

COVID-19 SCREENING IN LUNG CANCER PATIENTS

A recent meta-analysis has reported a pooled prevalence of cancer in patients with COVID-19 of 2.0%, an increased incidence compared to the general population^[13]. Multicentre registries and observational studies have demonstrated an association between increased COVID-19 mortality of 13%-28% in patients with cancer and COVID-19^[14,15]. Common independent factors include active cancer (as opposed to cancer in remission), underlying comorbidities such as COPD, smoking status, advanced age, and male gender^[14,15]. Patients with Eastern Cooperative Oncology Group (ECOG) performance status of 2 or higher are at increased risk of worse outcomes from COVID-19^[14]. One study has demonstrated lower rates of SARS-

CoV-2 antibody production (seroconversion) in cancer versus non-cancer patients after symptomatic COVID-19^[16].

Patients with lung cancers are thought to be at disproportionately increased risk of death from COVID-19, with mortality ranging from 25%-33%^[17]. However, the increase in mortality is thought to be related to patient-specific features as outlined above, rather than cancer-specific features or specific anti-cancer treatments such as tyrosine kinase inhibitors (TKIs), cytotoxic chemotherapy, or immune checkpoint inhibitors (ICIs), albeit compared to cancer instead of non-cancer controls. The ongoing TERA-VOLT study is the largest cohort study of patients with thoracic malignancies including non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), mesothelioma, thymic epithelial tumours and other pulmonary neuroendocrine neoplasms^[17]. Preliminary results have been reported from the first 200 patients enrolled, of whom 151 (76%) had NSCLC, 29 (15%) had SCLC, 36 (18%) and 147 (74%) had stage III and stage IV disease respectively, and 147 (74%) were on active anticancer therapy. Forty-eight (33%), 34 (23%), and 28 (19%) were on chemotherapy, ICIs or TKIs alone respectively, and 20 (14%) were on chemotherapy in combination with ICIs. The majority of patients received therapy administered for a median of 7 days (interquartile range 0-17) before COVID-19 diagnosis. Eighty percent of patients developed pneumonia or pneumonitis, and 27% of patients developed acute respiratory distress syndrome. The mortality rate was 33%. In multivariable analysis, only smoking was significantly associated with an increased risk of death. Although the findings above are highly relevant and have allowed quick dissemination of real-time data and guidance in the midst of a global pandemic, observational studies are associated with inherent limitations such as confounders and biases such as selection and recall bias. Larger patient cohorts and longer-term follow-up are required to confirm the findings as well as ascertain long-term effects on lung cancer mortality.

Another key consideration in managing patients with lung cancers during the COVID-19 pandemic lies in their overlapping clinical and radiological features, which poses unique challenges in the identification of suspect cases of COVID-19 and calls for a high index of suspicion. Overlapping symptoms include cough, dyspnoea, fever, arthralgia/myalgia and fatigue or malaise^[9,10,14,15,17,18]. Furthermore, immunocompromised lung cancer patients, especially those on myelosuppressive chemotherapy, may present atypically without fever. In addition to clinical symptoms, a detailed history of possible exposure to suspected or confirmed cases of COVID-19 is critical. Patients with thoracic malignancies often have thoracic imaging performed routinely as part of follow-up and response assessment. Chest radiography (CXR) and computed tomography (CT) appearances overlap amongst patients with primary thoracic malignancies, pulmonary metastases, treatment-related pneumonitis (from TKIs, ICIs, or radiotherapy), and COVID-19, with common features such as the presence of unilateral or multifocal ground glass opacities. In COVID-19, ground glass opacities are usually peripheral and/or bibasal. Pulmonary nodules, pleural effusions and mediastinal lymphadenopathy which are seen in thoracic malignancies are less commonly observed in patients with COVID-19^[19]. SARS-CoV-2 RT-PCR from nasopharyngeal or respiratory tract (e.g., sputum) samples remains the gold standard for COVID-19 diagnosis.

The unique clinical challenges relating to clinical risk and overlapping presenting features as described above require the use of distinct surveillance protocols from non-cancer patients. On 8 May 2020, the Singapore MOH amended its enhanced Swab-and-Send-Home (SASH) criteria to mandate SARS-CoV2 RT-PCR testing for all cancer patients on chemotherapy presenting with ARI with or without fever. Patients who are not medically unstable nor assessed to require inpatient admission can be managed under the enhanced SASH protocol when they are seen at primary care, instead of being referred to NCID or hospital EDs. Patients with positive results will be recalled and conveyed to NCID. The capacity for diagnostic testing of COVID-19 has been gradually increased nationwide, with expansion from hospitals to Public Health Preparedness Clinics (PHPCs) and polyclinics in the community. From 1 July 2020, the SASH

criteria were subsequently expanded to include all persons with ARI aged 13 and above in view of increase in community transmission of COVID-19.

Figure 1 illustrates a management algorithm for cancer patients in TTSH presenting with ARI or radiological findings of pneumonia. For patients on cytotoxic chemotherapy, it is important to exclude secondary infections and neutropenic sepsis which is a life-threatening oncological emergency. Patients with incidental infective changes (not attributable to cancer) noted on routine CXR or CT scans done for response assessment are advised to proceed with COVID-19 testing under the SASH protocol, with non-urgent outpatient appointments postponed to at least 5 days later. Cases where the need for SARS-CoV-2 testing and disposition are unclear are discussed with the COVID-19 consultant on-call. Currently, routine testing of lung cancer patients without fever or ARI before initiation of systemic therapy has not been implemented in our practice.

CHANGE OF PRACTICE IN THE TREATMENT OF LUNG CANCER PATIENTS

Advances in the pace and scope of research, as well as collaboration across hospitals and oncology centres regionally and worldwide in the creation of multicentre COVID-19 registries, have been critical in providing up-to-date knowledge about COVID-19 and its impact on cancer care and delivery^[20]. Oncology societies such as the European Society for Medical Oncology (ESMO), National Comprehensive Cancer Network (NCCN) and the International Association for the Study of Lung Cancer (IASLC) have also issued guidelines on lung cancer management during the COVID-19 pandemic^[21-23]. While providing a relevant overarching framework, it is also important to recognise that variations in healthcare resources and severity of the COVID-19 outbreak across countries affect generalisability of these guidelines. Individualisation of treatment options remains critical.

The massive rise of COVID-19 infections in Singapore has caused an overload on the healthcare system and resources have to be reprioritised and distributed between patients with COVID-19 infections as well as to continue ongoing medical care to the rest of the patient population. Healthcare workers are also under the pump as clinic staff, nurses and doctors get out-posted to the COVID-19 frontline, leaving less team members available to continue with the routine clinical workload. During the pandemic, many elective surgeries and non-urgent procedures are deferred; however, disciplines such as the medical oncology and radiation oncology departments often could not do the same. It is crucial for patients with lung cancer to continue treatments in a timely fashion despite the pandemic. The number of patients seen at the oncology outpatient clinic at TTSH in Singapore has also remained relatively stable during the DORSCON Orange pandemic period despite a likely reduction in the number of patients undergoing investigations for new cancer diagnoses and less elective surgeries. The average monthly number of specialist outpatient clinic attendances in our hospital for patients with newly diagnosed thoracic malignancies increased slightly by 10% from 2019 to 2020, suggesting the absence of treatment delays despite the pandemic for patients with newly diagnosed thoracic malignancies requiring systemic therapy. The number of follow-up visits decreased slightly by approximately 8%, likely due to the planned postponement of non-urgent appointments such as for patients on survivorship follow-up [Table 1].

There were a total of 1,356 outpatient visits for patients with thoracic malignancies in TTSH from January to August 2020. There were only 2 cases of COVID-19 diagnosed amongst these patients, both community acquired without evidence of nosocomial transmission, and zero cases in medical and nursing staff within the medical oncology department.

Despite the potential increased risk of inadvertent COVID-19 exposure, it is important to weigh the competing risks of disease progression and treat the patients urgently especially in the cases where there is an opportunity for cure. The ESMO guidelines regarding treatment of cancer patients during the

Table 1. Specialist outpatient clinic attendances for thoracic cancers in TTSH in 2019 and 2020, prior to and during the COVID-19 pandemic

		2019 (Jan-Dec)	2020 (Jan-Aug)	Percentage change
No. of first visits	Total	135	100	
	Monthly average	11.25	12.5	+10%
No. of review visits	Total	2,048	1,256	
	Monthly average	170	157	-8%

TTSH: Tan Tock Seng Hospital; COVID-19:Coronavirus disease 2019; No.: number

pandemic also stated that high priority should be given for outpatient lung cancer patient with a new diagnosis or suspicion of invasive lung cancer (clinical stage II/IIIA/IIIB or metastatic NSCLC or SCLC) and patients with disease-related symptoms (dyspnoea, pain, haemoptysis, *etc.*) and the need for visits for treatment administration^[24]. Recently, many guidelines and consensus statements have been published related to cancer management during the COVID-19 pandemic to provide guidance to oncologists during this difficult period^[21,23,25]. The guidelines recommend that the clinical situation and quality of care for a patient with lung cancer should not be changed, treatment plans should continue to be discussed with a multidisciplinary team and implementation of the recommendations by the multidisciplinary team should not be considered negotiable in light of the COVID-19 pandemic and its challenges^[26]. The ESMO guidelines are defined by 3 levels of therapeutic intervention. Firstly, the high priority tier focuses on immediately life-threatening cases, clinically unstable cases, and/or cases where the magnitude of benefit appears to be substantial leading to a significant improvement in overall survival or quality of life (QOL). Medium priority cases include non-critical situations where any more than a 6- to 8-week delay could impact the patient's overall outcome or magnitude of benefit. The lowest priority tier is defined as stable conditions that allow services to be delayed for the duration of the pandemic with no survival benefit or a change/reduction in QOL^[25,26]. Lung cancer treatment at TTSH follows the guidelines as specified by ESMO in a similar fashion and adapts a "business as usual" model for patients requiring high priority treatment [Table 2].

For patients with stage III lung cancer who require definitive chemoradiation and small cell lung cancer patients who require urgent systemic chemotherapy, the medical oncology department at TTSH continues to prioritise timely interventions and strives to provide the same quality of care as in pre-pandemic situations. Lung multidisciplinary meetings have continued to function on a regular basis discussing complex cases with provisions for the setup of adequate social distancing and ensuring only attendance of key personnel during this period of time to reduce exposure risk and to segregate teams as mentioned previously.

For patients with small cell lung cancer where systemic chemotherapy is known to be effective and time-sensitive, medical oncologists often still opt to institute chemotherapy urgently and the first cycle can continue to be given as an inpatient. Patients who are diagnosed in the peripheral hospitals who require urgent treatment and medical oncology management would be reviewed on a case-by-case basis and transfer to TTSH can be arranged upon approval by the Head of Department, Division Head and the Chairman of the Medical Board of both hospitals.

The medical oncology department at TTSH has instituted certain measures to review the outpatient clinic list with aims to postpone appointments for patients deemed feasible for a longer interval review (those with low/intermediate risks of relapse) or who are on mainly survivorship follow-ups. The primary oncologist in charge of the clinic is asked to review the list of patients scheduled for outpatient clinics during the height of the pandemic period and prescriptions may be topped up for patients on targeted therapies if they are deemed to be stable and may be able to delay their follow up appointments. A

Table 2. Priorities of treatment for lung cancer patients at TTSH (Adapted from ESMO guidelines of management of lung cancer patients during the COVID-19 pandemic)

	Prioritised without delay	Medium priority	Lower priority/ Delay appointment
Small cell lung cancer treatment	*		
Neoadjuvant chemotherapy in clinical stage III NSCLC	*		
Concomitant or sequential chemoradiotherapy for inoperable stage III NSCLC	*		
Starting consolidation durvalumab (within 42 days)	*		
Delivery of adjuvant chemotherapy in stage II/III as recommended by lung tumour board	*		
Newly diagnosed lung cancer patients with targetable mutations	*		
Newly diagnosed lung cancer patients who are candidates for 1st-line treatment including chemotherapy, chemotherapy plus IO, IO alone or TKIs to improve prognosis, cancer-related symptoms and QOL	*		
Start 2nd-line chemotherapy or IO in symptomatic and progressive disease patients	*		
Start 2nd-line TKI in progressive disease patients	*		
Oncological emergencies (management of hypercalcaemia, cord compression, SVC obstruction, serious immune mediated adverse effects etc.)	*		
Start 2nd and beyond line chemotherapy or IO in asymptomatic patients, in absence of threatening disease (volume/location)		*	
Discussion of adjuvant chemotherapy for Stage IB		*	
Anti-PD-(L)1 scheduled cycles may be modified/delayed to reduce clinical visits (for instance, using 4-weekly or 6-weekly dosing instead of 2- or 3-weekly for selected agents when appropriate)		*	
Postpone antiresorptive therapy (zoledronic acid, denosumab) that is not needed urgently			*
Follow-up for patients at low/intermediate risk of relapse			*
Survivorship visits			*

TTSH: Tan Tock Seng Hospital; ESMO: European Society for Medical Oncology; COVID-19: Coronavirus disease 2019; NSCLC: non-small cell lung cancer; TKIs: tyrosine kinase inhibitors; IO: ImmunoOncology drug; QOL: quality of life; SVC: superior vena cava; PD-(L)1: Programmed death-(ligand)1

medication delivery service has been set up by the hospital pharmacy which provided convenience to patients and carers during the pandemic period in avoiding hospital visits and reducing long wait times at the outpatient pharmacies. Without compromising patient safety and efficacy, systemic treatment regimens should be adjusted to reduce hospital visits. Immunotherapy treatments may be given with a longer interval via dose adjustments such as Nivolumab at 480 mg 4-weekly instead of 240 mg fortnightly; and 400 mg Pembrolizumab 6-weekly instead of 200 mg 3-weekly. Efforts are also currently underway for pilot and subsequent larger scale implementation of telemedicine consults.

Important clinical trials that are deemed to impact the patient's overall outcome and have a significant magnitude of benefit were able to continue treatment throughout the entire DORSCON Orange period. Trials that may not have a significant impact on patient's outcomes, in particular, qualitative research and survey studies were placed on hold for recruitment during the height of the pandemic period as well.

EDUCATION AND TRAINING OF RESIDENTS AND JUNIORS DURING COVID-19 PANDEMIC

The COVID-19 pandemic has led to a significant impact on post-graduate education in Singapore and educators now need to consider alternative novel methods of providing education to minimise disruption to training of the junior medical staff^[27]. Almost universally across all academic centres, there has been a transition to teaching via videoconferencing technologies which has been recognized as an effective teaching modality in situations where distant learning is required^[28,29]. However, online videoconferencing teaching had posed several issues including the need for additional information technology resources and funding for extra laptops, projectors and speakerphones. There is also a need to ensure that the students have access to a stable internet connection throughout the different sites during the online teaching

sessions. Educators also need to adapt to these new practices and come up with effective teaching strategies for web conferencing, which can be challenging for physicians who are used to conventional bedside teaching of students and trainees^[29].

To reduce the risks of inter- and intra-departmental infection transmission, different meeting rooms are also utilized to segregate the inpatient and outpatient service teams during the videoconferencing teaching sessions or multidisciplinary case discussions. The weekly scheduled medical oncology teaching sessions at TTSH utilized pre-recorded lectures on various subspecialty topics supervised by a consultant in charge. Bedside teaching during ward rounds to the inpatient junior team would be led by the ward consultant in charge. Case discussions and clinical handover to the weekend on-call team using third party software and online platforms also posed concerns in ensuring security of information in terms of the Personal Data Protection Act (PDPA), which came into full effect in July 2014 in Singapore.

During the DORSCON Orange alert period, there is a need to minimise cross cluster transfer of medical staff, hence rotations of residents and registrars could only be limited to the current institution that they are based in with no external rotations to other hospitals allowed. Although this may lead to a certain level of disruption to the training exposure, measures are in place to ensure that they are allowed to be rotated to a different department of choice within the same academic institution.

For staff deployed to the NCID to manage outbreak wards, timely training is conducted to refresh their knowledge on Personal Protective Equipment (PPE) and to orientate them to the facilities and equipment at NCID. Communications on the latest developments of the COVID-19 situation and updated protocols on screening and management for COVID-19 cases are available on the hospital's intranet and distributed electronically to staff via emails.

It is critical to provide emotional support to clinical staff and trainees during this difficult period given the high risk of burnout when faced with such a prolonged crisis. A recent survey was conducted by the NHG residency office pertaining to the challenges faced by residents as a result of the interruptions to training posed by the ongoing COVID-19 outbreak. They reported an increase in the level of stress and burnout, citing an average of 4.7 on a scale of 0 (no stress at all) to 10 (extreme level of stress) and 61% of the respondents either agree or strongly agree when asked whether the current outbreak had adversely affected their training and or career^[30]. TTSH has set up the 3S (Staff Support Staff) hotline with the ICU(Intensive Care Unit) teams providing an emotional debrief session and also a technical debrief to iron out any problems faced at each shift. The Staff Wellbeing Committee is also set up to ensure that staff feels supported and to ensure meals and snacks are distributed to the staff on duty in the outbreak wards.

VACCINE DEVELOPMENT IN CLINICAL TRIAL EVALUATION

It is a race against time and a global effort is made towards rapid SARS-CoV-2 vaccine development with the aim of inducing protective immunity following vaccination. There are now phase II clinical trials with a trajectory towards phase III efficacy evaluation, however there are added challenges with the dynamic changes seen with this virus^[31-33].

Patients with advanced cancer have historically been under-represented in vaccine clinical trials. As targeted therapy and immunotherapy have significantly improved the life expectancy for patients with metastatic NSCLC, these patients should not be reflexively excluded from vaccine trials on the basis of old preconceptions limiting care for these patients. We advocate that vaccine trials should include patients with malignancies including thoracic cancers, so as to ascertain safety and efficacy in this group of patients.

CONCLUSION

Compared to prior pandemics, we now have far greater scientific, medical and technological capabilities to manage COVID-19, but also increased vulnerabilities and transmission risks in a globalised world and economy. COVID-19 has had wide-ranging implications globally, ranging from its impact on healthcare systems to economic and long-term psychological impact. Infection control and outbreak mitigation measures, and the availability of effective therapeutics and vaccines will shape the eventual outcome of the pandemic.

Oncologists have a special duty to our patients to ensure patient safety and provide optimum care without undue disruption which may compromise long-term cancer-specific outcomes. We must carefully weigh the management of an established threat (lung cancer) where treatment delays can lead to worse outcomes, versus the uncertain additional risk of COVID-19 infection. Treatment decisions should be individualised based on best available evidence, along with multidisciplinary input and shared decision-making with patients. Important areas for future research include long term COVID-19 and cancer-specific outcomes, late complications and survivorship issues, and psychological impact on patients and healthcare providers.

The COVID-19 pandemic has significantly changed day-to-day practices within our institution. Singapore has been relatively fortunate in having a structured and well-prepared healthcare system to cope with the COVID-19 pandemic, with ready accessibility to SARS-CoV-2 testing, good population compliance to public health measures and appropriate reallocation of healthcare resources without excessive disruption to the care of oncology patients. With better control of the outbreak locally, there has also been a corresponding gradual increase in the provision of routine oncology services back to pre-COVID levels. We must continue to adapt to the evolving situation to ensure preservation of healthcare capacity, protection of vulnerable populations and care of oncology patients in the form of a sustainable new normal for the future.

DECLARATIONS

Authors' contributions

Conception, manuscript writing, final approval of manuscript: Vong EKY, Chia PL, Chang AY

Availability of data and materials

Not applicable.

Financial support and sponsorship

All authors have declared that they have no financial relationships at present that is related to this paper.

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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Private somatic mutations identified with liquid biopsy lead tumor progression in solid cancers

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How to cite this article: Palmieri M, Baldassarri M, Iuso N, Fava F, Fabbiani A, Cetta F, Fallerini C, Tita R, Mencarelli MA, Renieri A, Frullanti E. Private somatic mutations identified with liquid biopsy lead tumor progression in solid cancers. *J Cancer Metastasis Treat* 2020;6:55. <http://dx.doi.org/10.20517/2394-4722.2020.105>

Received: 23 Sep 2020 **First Decision:** 23 Nov 2020 **Revised:** 4 Dec 2020 **Accepted:** 21 Dec 2020 **Published:** 30 Dec 2020

Academic Editor: PRAVIN POTDAR **Copy Editor:** Monica Wang **Production Editor:** Jing Yu

Abstract

Aim: Primary tumors can be divided into oncogene-addicted (e.g., lung) and non-oncogene addicted (e.g., breast). Only the former group has an Achilles-heel single gene for successful target therapy, whereas the latter has mutations of multiple causative genes. Currently, tissue biopsy used for genetic surveys do not give a complete picture of the molecular profile and clonal evolution, but only provide static information over time.

Methods: A series of 133 patients with 16 different solid tumors were enrolled. Blood samples were collected and cell-free DNA (cfDNA) was extracted. cfDNA libraries were analyzed using AVENIO circulating tumor DNA (ctDNA) Expanded Kit and Illumina NextSeq 550 for sequencing was used. In order to evaluate the clinical evolution over time, a second cfDNA analysis was performed after a mean interval of 2 months.

Results: Through the cfDNA liquid biopsy, we found 89 pathogenic variants in 54 genes. Breast, lung, and prostate cancers showed the largest number of mutated genes. *TP53*, *PIK3CA*, *FGFR3*, *KRAS*, and *ERBB2* were the most frequently mutated genes among 16 different tumors. Gene distribution didn't show any type of prevalence. In particular, every patient with disease progression seems to have a "private" combination of gene pair mutations, with *TP53* as the most frequently mutated gene.



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Conclusion: We showed that the clonal evolution of tumors includes a private combination of genes, regardless of tumor type. In the future, the cancer treatment can be the targeted therapy against specific tumor mutation(s). The present approach seems promising to both identify key cancer genes and follow clonal evolution over time.

Keywords: Cell-free DNA, liquid biopsy, solid tumors, advanced tumors, private mutations, targeted-therapy

INTRODUCTION

Daily experience shows that different malignancies, such as breast or lung cancers, can be controlled using standard protocols for many years before tumor relapse. This is possible thanks to the detection of the so-called “Achilles’ heel” that represents the molecular target for different tumor groups named “oncogene addict”^[1]. However, only a few tumors have a unique or a small number of mutated genes that could be susceptible to target therapy, whereas the vast majority of cancers are “non-oncogene addicted”^[1]. Tissue biopsies from primary tumors usually do not show details of molecular heterogeneity and are even less informative when obtained from metastases. Additionally, biopsies from metastases are not always easy to perform^[2].

Cancer is an evolving microcosm driven by selective pressures due to the environment and drug therapy. Malignant cells are in competition and/or cooperation with each other and with the surrounding environment^[3]. Cancer therapy must cope with the cellular complexity of the disease and face its dynamic evolutionary aspect^[4]. In order to facilitate treatment choice, it is crucial to distinguish between germline and somatic mutations. New technologies such as the liquid biopsy of cell-free DNA (cfDNA) facilitate proper detection of somatic tumor mutations, which are the key mutation of specific cancer driver genes. The technique is non-invasive, is a valuable alternative to physical biopsies, and opens new avenues for personalized medicine^[5].

In this study, we show that cfDNA analysis is able to follow, over time, the clonal evolution of multiple solid tumors. In particular, patients belonging to the same tumor type exhibit different private pairs of mutations. The most frequently mutated gene is *TP53* that we found in combination with *PIK3CA*, *KRAS*, *EGFR* with a frequency of 71%, or in combination with *BRCA1*, and *ERBB2* with a frequency of 42%, or finally with *PTEN* and *MYC* with a frequency of 28.6%.

The second liquid biopsy proves to be a powerful tool to understand which pair of mutated genes is specific for the patient and unique to him, thus leading to a fully personalized treatment.

METHODS

Patients and sampling

Our cohort has been enrolled at the Medical Genetics Unit of the Azienda Ospedaliera Universitaria Senese, Siena, Italy for diagnostic purposes. The cohort consisted of 133 patients with different solid tumors who experienced disease progression after standard therapy. Patients were previously treated in advanced/metastatic settings and most of them were not eligible for curative treatment. Written informed consent and assent for genetic analysis was obtained from all patients.

Inclusion criteria provided patients with either locally advanced or metastatic solid tumors independent from the primary tumor site. Patients were excluded if they had early-stage solid tumors and still have to experience all possible pharmacological treatments of standard guidelines. The main information collected for each patient includes oncological data, family tree, and cancer history in a genetic consultation setting.

A first peripheral blood sample for cfDNA analysis was taken during the genetic counseling visit at the stage of disease progression. Plasma was used for cfDNA extraction. A second sample for cfDNA analysis was taken after a mean time interval of 2 months (range 1-6 months).

cfDNA extraction and sequencing

Peripheral blood samples (10 mL) were collected from each patient and placed into a Cell-Free DNA BCT tube (Streck, La Vista, NE, USA). cfDNA was extracted from 4 mL of plasma using AVENIO ctDNA Expanded Kit according to the manufacturer's instructions. cfDNA quality and quantity were verified as described in Palmieri *et al.*^[5]. cfDNA sequencing was performed using AVENIO circulating tumor DNA (ctDNA) Expanded Kit (Roche, Basel, Switzerland) on Illumina NextSeq 550 (Illumina, San Diego, CA, USA). This technology is able to identify various types of alterations, including single nucleotide variants, insertions/deletions, gene fusions, and copy number variations present in genes linked to cancer (clinical actionable mutations) with a reportable range up to 0.05%. The sequencing analysis was performed using AVENIO Oncology Analysis Software (Roche, Basel, Switzerland).

RESULTS

Patient characteristics

From March 2018 to July 2020, a total of 133 patients with locally advanced or metastatic solid tumors were enrolled at Azienda Ospedaliera Universitaria Senese, Siena, Italy, and included in the study. The mean age of patients at the first cfDNA analysis was 56 years (range 2-83 yrs); 48% of patients were females and 52% were males. Out of 56 patients who did at least a second liquid biopsy, 22% had cancer from breast, 14% lung, 14% ovarian cancer, 5% colorectal, 6% pancreas, 6% prostate, whereas uterine cancer, retinoblastoma, cholangiocarcinoma, and gastric cancer accounted for 2% each, and soft tissue sarcoma (including right infratemporal fossa, oral, pharynx, and larynx), Wilms' tumor, and glioblastoma accounted for 1% each of the entire series. The median follow-up time for all patients was 2 months (range 1-6 months).

Mutated gene and tumor type association

Next-generation sequencing (NGS) analysis in 133 patients at the first liquid biopsy identified 86 clinically meaningful pathogenic variants in 54 genes allowing to pick up key mutations in 67.6% ($n = 90$) of cases [Supplementary Table 1]. After a mean of 2 months, a second liquid biopsy was performed and 87.5% of patients remained/became positive. Table 1 summarizes all the mutated genes resulting from the second liquid biopsy associated with different tumor types. Mainly, the breast, lung, and prostate cancers were the tumors types that showed the largest number of mutated genes. The tumor type, grade, and stage as well as the drug treatments for the entire cohort of second liquid biopsy patients are summarized in the Supplementary Table 1.

Most frequently mutated genes

At the second liquid biopsy time, the mutated genes decreased from 54 to 38 in 16 different tumor types. Among these 38 mutated genes, those most frequently represented were the following: *TP53* (30%), *PIK3CA* (10%), *EGFR* (10%), *KRAS* (8%), *ERBB2* (8%), *FGFR3* (6%), and *BRCA2* (6%). Interestingly, these genes were mutated in 16 different tumor types without a specific prevalence among them [Figure 1].

However, clonal mutation was not confirmed in the entire cohort at the second liquid biopsy. Indeed, 9 patients resulted as negativized, 13 patients had only one mutated clone, and 18 had two clones. One patient had 5 mutated clones [Figure 2].

The most frequent mutations in our cohort were in the *TP53* gene, regardless of the primary tumor type. *TP53* was usually mutated in association with another gene [Figure 3]. The most mutated genes in

Table 1. Mutated genes identified at second liquid biopsy time

Tumor types (n° of pts)	ABL1	NF2	IDH2	NFE2L2	KEAP1	AR	JAK2	APC	CTNNB1	MYC	MAP2K1	MET	ERBB2	FGFR1	FGFR2	EGFR	FGFR3	BRAF	NRAS	KRAS	AKT1	PIK3CA	TP53	CDKN2A	CCND1	CCND3	PTEN	ESR1	BRCA2	SMAD4	GNAS	GNAQ	MTOR	MLH1	MSH6	PMS2	SF3B1	ROS1	Tot. mutated gene
breast (11)	x						x		x					x						x	x	x	x	x	x	x	x						x	x		x	x	17	
lung (7)		x					x		x						x		x		x				x																11
ovarian (7)									x								x			x																			6
colorectal (4)								x										x		x																			6
pancreatic (3)			x																x																				3
prostatic (3)		x				x											x			x																			9
uterine (2)									x																														3
retinoblastoma (2)																																							3
colangiocarcinoma (2)																																							3
gastric (2)																																							5
																																							3
sarcoma (1)																																							5
																																							1
oral (1)																																							4
pharinx (1)																																							3
larinx (1)																																							3
wilms (1)																																							3
glioblastoma (1)																																							2

combination with *TP53* were *EGFR* and *PIK3CA* in 20% of cases, *KRAS* and *ERBB2* in 16% of cases, *FGFR3* and *BRCA2* in 15% of cases, or less frequently with other genes for a total number of 14 genes.

DISCUSSION

Early dissemination of resistant tumor cells is the major cause of metastatic recurrence in cancer patients^[6]. Currently, tissue biopsy remains the gold standard. However, it is jeopardized by the wide heterogeneity that is frequently found between different regions of the same tumor (spatial heterogeneity), as well as between the primary tumor and local or distant recurrences in the same patient (temporal heterogeneity)^[7]. Intratumor heterogeneity can lead to underestimation of the tumor genomics landscape portrayed from single tumor-biopsy samples and may represent a major challenge towards personalized-medicine and biomarker development^[8]. For this reason, liquid biopsy could be of primary importance to obtain a complete scenario of the molecular profile of all tumor metastases. Indeed, the cfDNA is the results of fragmented genomic DNA coming from the apoptosis or necrosis process of normal and tumoral cells. Performing the NGS-liquid biopsy on the cfDNA guarantees the total representations of the molecular information coming from the totality of metastasis.

The higher number of mutated genes occurred in breast, lung, and prostate cancer, irrespective of the number of these tumor types in the present series. Most patients had breast tumor; whereas only three patients had prostatic cancer; number of patients with lung cancer were the same as those with ovarian cancer;

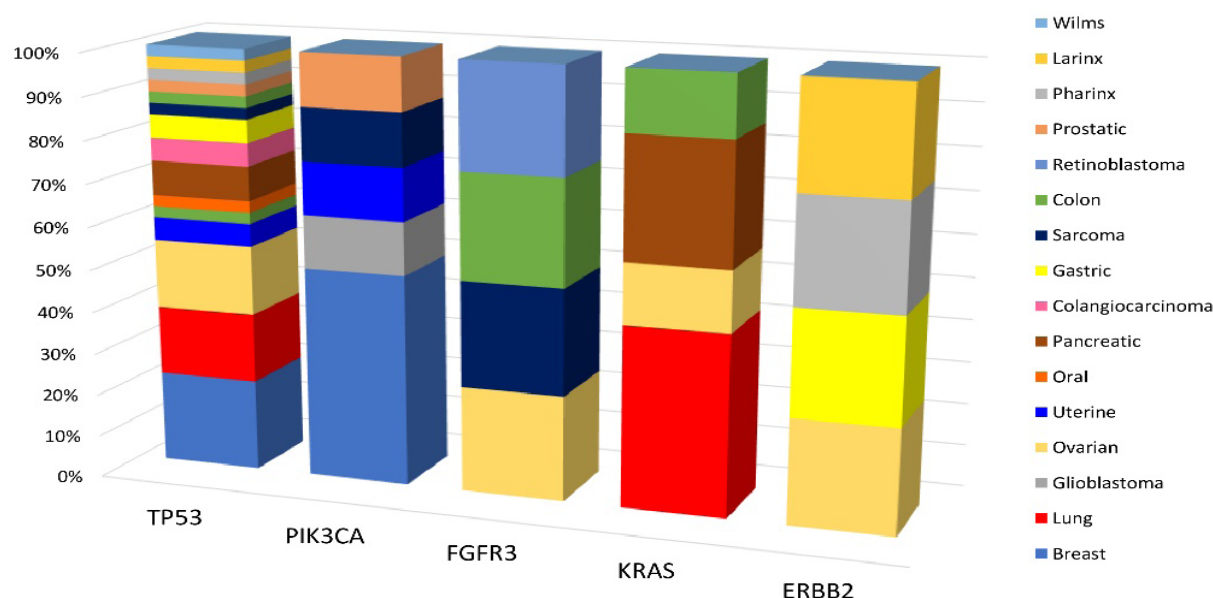


Figure 1. Distribution of the most frequently mutated genes. Among the 38 mutated genes, point mutations in *TP53*, *PIK3CA*, *KRAS*, and *ERBB2* and copy number variation in *FGFR3* were the most commonly observed alterations in all 16 tumor types

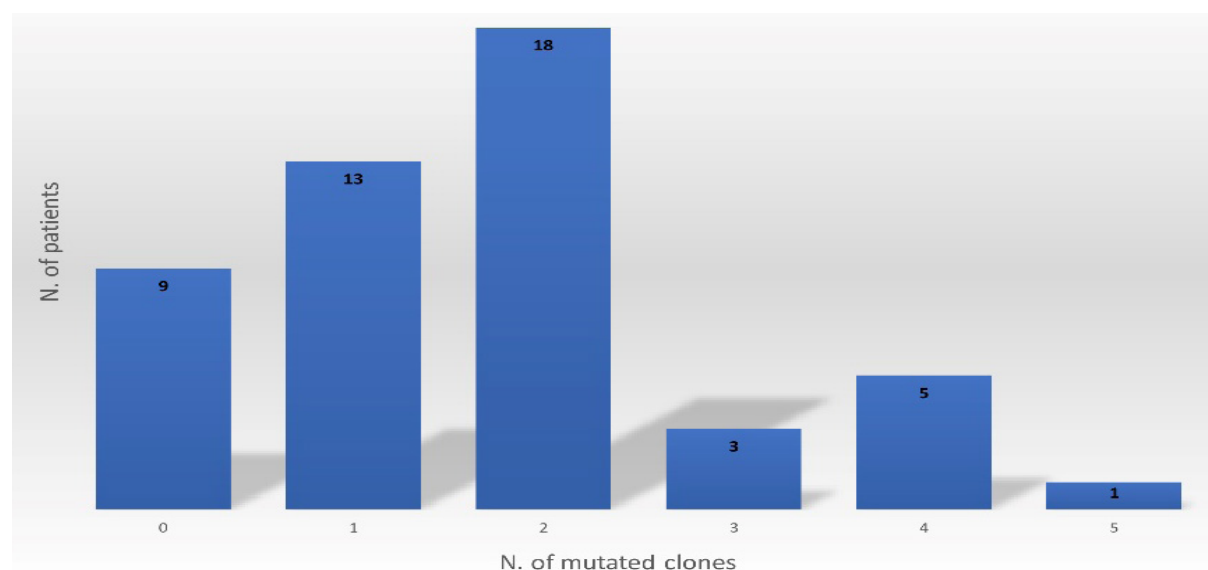


Figure 2. Second liquid biopsy: number of mutated clones. The histogram shows on the absciss axis the number of mutated clones and on the ordinate the number of patients. Most patients have two mutated clones at the time of the second liquid biopsy

but only patients with lung cancer had a great number of mutated genes [Table 1].

In accordance with previous data in the literature, our study confirmed that SNVs (small nucleotide variants) in *TP53*, *PIK3CA*, and *KRAS*, and CNVs (copy number variations) in *FGFR3* and *ERBB2* are the most commonly observed mutated genes in breast and lung cancer^[9-10] [Figure 1]. However, these mutations are not only confined to breast and lung cancer but are also found in other types of cancer without a specific prevalence in distribution [Figure 1].

In comparison with data from our previous study^[5], showing that at the beginning of tumor expansion there was a consistent, although variable, mutational burden from tens to hundreds, disease relapse

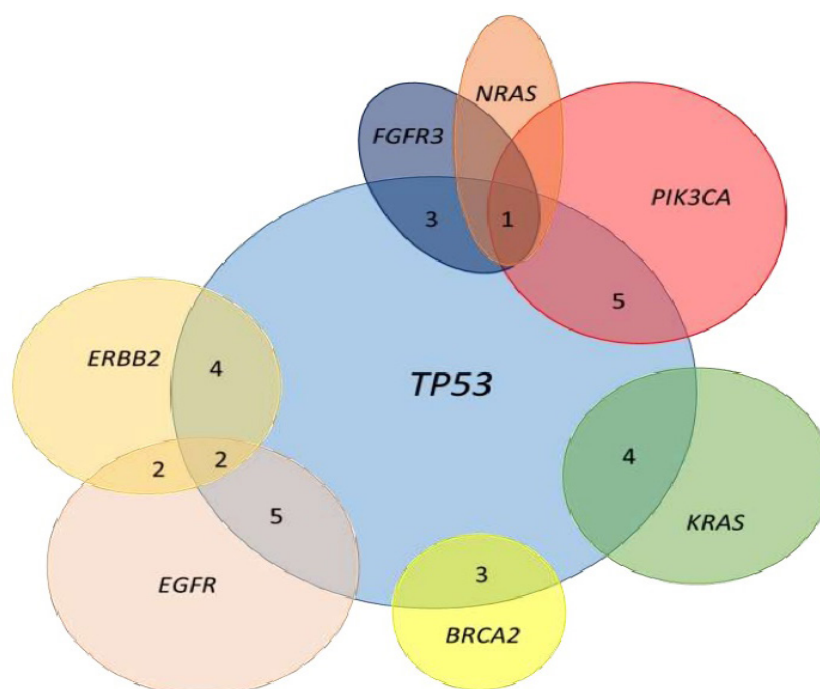


Figure 3. Eulero Venn diagram. Eulero Venn diagram shows the higher correlation between *TP53* with the *PIK3CA* and *EGFR* (5 folds each), with *KRAS* and *ERBB2* (4 folds each), or with *BRCA2* and *FGFR3* (3 folds each). The figure also shows correlations of other genes with each other, e.g., *ERBB2* and *EGFR*

is generally characterized by a “driver” clone that is responsible for metastases. The curve in Figure 2, simulating a Gaussian bell shape (similar to the typical population curve), underlines this concept. Indeed, it shows that in metastatic patients, a negative selection occurs in the initial number of mutated clones, reducing them to a maximum of two in comparison to the considerable number of tumor clones in the early stages of the disease.

In particular, in relapsing patients, the second liquid biopsy showed that 37% had two mutated clones, 26% only one clone, and 18% more than two clones [Figure 2].

Notably, one of the two mutated genes was often *TP53* associated with *PIK3CA* (20%) or *EGFR* (20%), *KRAS* (16%) or *ERBB2* (16%), *FGFR3* (15%) or *BRCA2* (15%), and other genes with a lower frequency [Figure 3]. We hypothesize that tumor survival does not depend only on the expansion of the *TP53* clone, but also requires a mutation in other genes for tumor progression. In fact, we have repeatedly found that in breast cancer, the *TP53* mutation is associated with *PIK3CA*. Indeed, *PIK3CA* mutations generally arise in advanced stages of breast carcinogenesis from dysplasia to carcinoma *in situ*^[11] resulting in a greater potential to migrate and invade *in vitro*, as well as to metastasize^[12]. Moreover, in lung cancer, *TP53* was frequently associated with *KRAS* mutation.

A striking finding deriving from the comparison between the first and the second liquid biopsy in the same patient has been the constant reduction in the number of mutated genes. Present data suggest that after the early phase of tumor diffusion, that is characterized by a great number of new somatic mutations, a selection takes place among the various mutated genes to determine which are the most effective clones for tumor progression.

In a small subset of patients, we were able to perform a third liquid biopsy, but numbers for the moment were too small. Data from the third biopsy, in case of further tumor relapse, will better elucidate the trend

in tumor progression, showing further reduction of the number of clones, or a new increase of “random” mutations.

In conclusion, additional study in larger series is required for further confirmation of these preliminary data and suggestions. A greater use of liquid biopsy is recommended for proper detection of genetic mutations and for “the right treatment in the right patient at the right time”.

DECLARATIONS

Acknowledgments

Authors thank the support from the Regione Toscana - Istituto Toscano Tumori (ITT) (Project “Identification of genetic bases of individual predisposition to lung cancer in non-smokers”) and the ASSO (Associazione per lo Sviluppo della Scienza Oncologica) Onlus. This work was, in part, supported by the MIUR project “Dipartimenti di Eccellenza 2018-2022” to the Department of Medical Biotechnologies, University of Siena”. This work is generated within the ERN-EURACAN (European network for Rare Adult Aolid Cancer).

Authors' contributions

Performed the experiments, analyzed the data and wrote the paper: Palmieri M

Performed the experiments: Iuso N

Wrote the paper: Cetta F

Analyzed the data: Fallerini C, Tita R

Took care of the clinical part of the study: Fava F, Fabbiani A

Performed genetic counseling and provided patient samples: Baldassarri M, Mencarelli MA

Designed the research strategy, analyzed the data, and wrote the paper: Renieri A, Frullanti E.

Availability of data and materials

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

The consent to participate was obtained from patients included in the study.

Consent for publication

The consent for publication was obtained from patients included in the study.

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Editorial	An Editorial is a short article describing news about the journal or opinions of senior editors or the publisher.	None required	None required	/
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Perspective	A Perspective provides personal points of view on the state-of-the-art of a specific area of knowledge and its future prospects. Links to areas of intense current research focus can also be made. The emphasis should be on a personal assessment rather than a comprehensive, critical review. However, comments should be put into the context of existing literature. Perspectives are usually invited by the Editors.	Unstructured abstract. No more than 150 words.	3-8 keywords	/

2.3 Manuscript Structure

2.3.1 Front Matter

2.3.1.1 Title

The title of the manuscript should be concise, specific and relevant, with no more than 16 words if possible. When gene or protein names are included, the abbreviated name rather than full name should be used.

2.3.1.2 Authors and Affiliations

Authors' full names should be listed. The initials of middle names can be provided. Institutional addresses and email addresses for all authors should be listed. At least one author should be designated as corresponding author. In addition, corresponding authors are suggested to provide their Open Researcher and Contributor ID upon submission. Please note that any change to authorship is not allowed after manuscript acceptance.

2.3.1.3 Abstract

The abstract should be a single paragraph with word limitation and specific structure requirements (for more details please refer to Types of Manuscripts). It usually describes the main objective(s) of the study, explains how the study was done, including any model organisms used, without methodological detail, and summarizes the most important results and their significance. The abstract must be an objective representation of the study: it is not allowed to contain results which are not presented and substantiated in the manuscript, or exaggerate the main conclusions. Citations should not be included in the abstract.

2.3.1.4 Keywords

Three to eight keywords should be provided, which are specific to the article, yet reasonably common within the subject discipline.

2.3.2 Main Text

Manuscripts of different types are structured with different sections of content. Please refer to Types of Manuscripts to make sure which sections should be included in the manuscripts.

2.3.2.1 Introduction

The introduction should contain background that puts the manuscript into context, allow readers to understand why the study is important, include a brief review of key literature, and conclude with a brief statement of the overall aim of the work and a comment about whether that aim was achieved. Relevant controversies or disagreements in the field should be introduced as well.

2.3.2.2 Methods

Methods should contain sufficient details to allow others to fully replicate the study. New methods and protocols should be described in detail while well-established methods can be briefly described or appropriately cited. Experimental participants selected, the drugs and chemicals used, the statistical methods taken, and the computer software used should be identified precisely. Statistical terms, abbreviations, and all symbols used should be defined clearly. Protocol documents for clinical trials, observational studies, and other non-laboratory investigations may be uploaded as supplementary materials.

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This section should discuss the implications of the findings in context of existing research and highlight limitations of the study. Future research directions may also be mentioned.

2.3.2.5 Conclusion

It should state clearly the main conclusions and include the explanation of their relevance or importance to the field.

2.3.3 Back Matter

2.3.3.1 Acknowledgments

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Each author is expected to have made substantial contributions to the conception or design of the work, or the acquisition, analysis, or interpretation of data, or the creation of new software used in the work, or have drafted the work or substantively revised it.

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References should be numbered in order of appearance at the end of manuscripts. In the text, reference numbers should be placed in square brackets and the corresponding references are cited thereafter. Only the first five authors' names are required to be listed in the references, other authors' names should be omitted and replaced with "et al.". Abbreviations of the journals should be provided on the basis of Index Medicus. Information from manuscripts accepted but not published should be cited in the text as "Unpublished material" with written permission from the source.

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Journal articles ahead of print	Odibo AO. Falling stillbirth and neonatal mortality rates in twin gestation: not a reason for complacency. <i>BJOG</i> 2018; Epub ahead of print [PMID: 30461178 DOI: 10.1111/1471-0528.15541]
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. The genetic basis of human cancer. 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. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. pp. 182-91.
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Additional data and information can be uploaded as Supplementary Material to accompany the manuscripts. The supplementary materials will also be available to the referees as part of the peer-review process. Any file format is acceptable, such as data sheet (word, excel, csv, cdx, fasta, pdf or zip files), presentation (powerpoint, pdf or zip files), image (cdx, eps, jpeg, pdf, png or tiff), table (word, excel, csv or pdf), audio (mp3, wav or wma) or video (avi, divx, flv, mov, mp4, mpeg, mpg or wmv). All information should be clearly presented. Supplementary materials should be cited in the main text in numeric order (e.g., Supplementary Figure 1, Supplementary Figure 2, Supplementary Table 1, Supplementary Table 2, *etc.*). The style of supplementary figures or tables complies with the same requirements on figures or tables in main text. Videos and audios should be prepared in English, and limited to a size of 500 MB or a duration of 3 minutes.

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