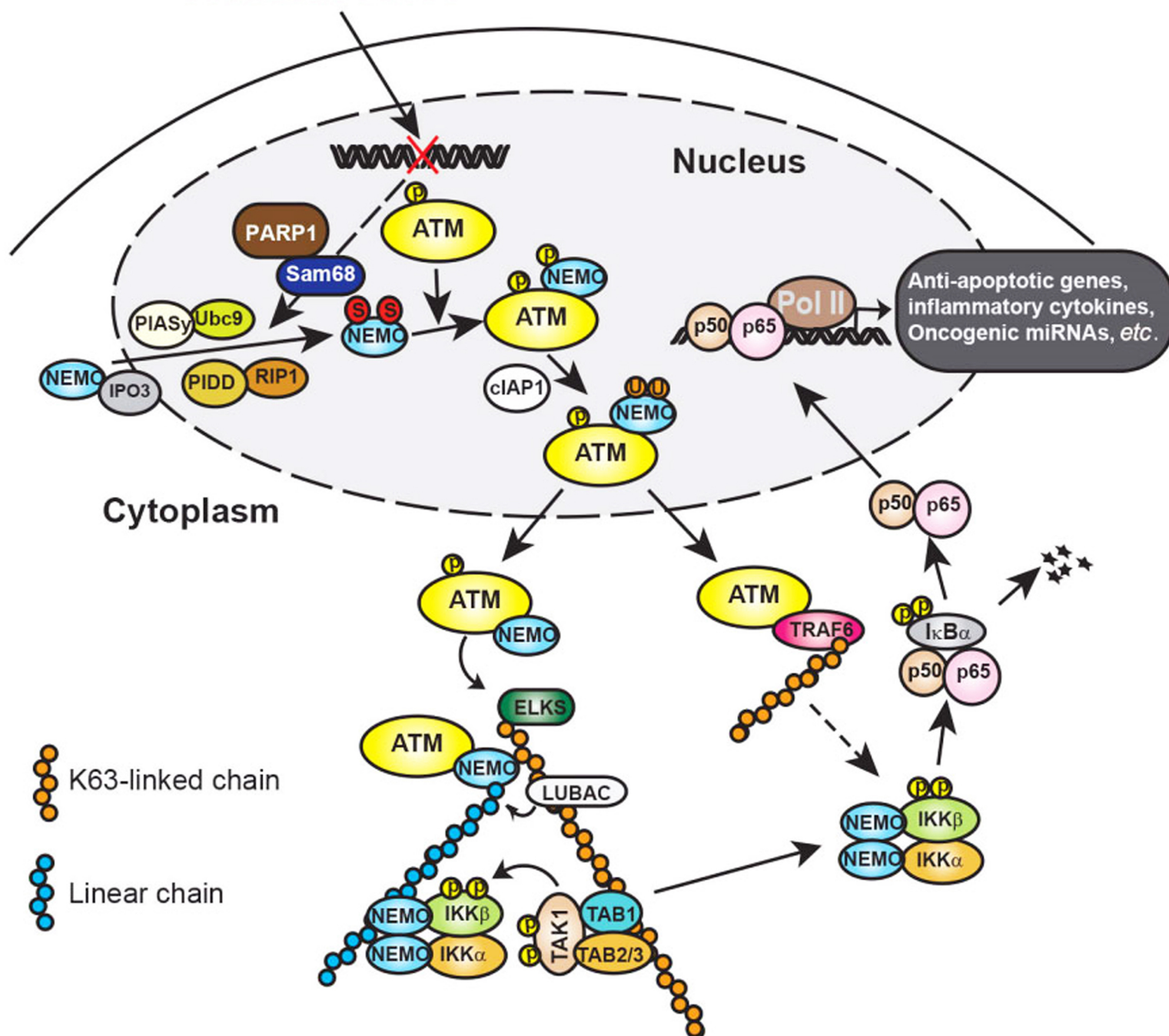


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Circulating tumor cells: hope to diagnose and treat metastatic cancer

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Dr. Pravin D. Potdar's present interest is to study molecular profiling of circulating tumor cells (CTC), circulating tumor DNA, cancer associated fibroblasts and cancer stem cells involved in metastatic process of cancers, and to see how this process can be reverted back to normal by using innovated technologies which include nanotechnology and nanomedicine.

CHARACTERISTICS OF CIRCULATING TUMOR CELLS

In 1869, Thomas Ashworth, an Australian physician, for the first time observed the cells which are morphologically identical to cancer cells in the blood circulation of metastatic cancer patients. Today, these cells are known as circulating tumor cells (CTCs).^[1] Since most cancer deaths are associated with metastases, there is a need to study the exact mechanism of this cancer spread.

Until recently, primary biopsy has been the basis of cancer diagnosis, as it has been difficult to develop a research or diagnostic test based on Thomas Ashworth's finding. The major reason for this is the extremely small number (6-10) of CTCs in the

bloodstream, and their heterogeneity affecting their cell surface markers in isolation. However, tissue biopsies have limited effectiveness as a diagnostic tool because they are invasive and not suited to repeated sampling; biopsies also cannot monitor the treatment of metastatic cancer patients during therapy. Analysis of CTCs analysis is non-invasive and can be carried out easily in combination with available liquid biopsies such as blood and body fluids of metastatic cancer patients. Multiple samples can also be made available to monitor treatment protocols of metastatic cancer patients during therapy. In addition, CTCs analysis allows clinicians to monitor disease progression over a period of time and provide appropriate treatment modification in a patient's therapy, thus improving the patient's prognosis and quality of life.



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The CTCs are simply shredded tumor cells from the original tumor which enter blood circulation and form secondary tumor growth at several sites, featuring the suitable environment shown in Figure 1. The CTCs are large cells with large nuclei and a granular cytoplasm with a very specific spike-like appearance on the surface of each cell.^[2] It is presumed that these spike-like processes help these cells to adhere to blood vessels and promote the metastatic, invasive progress of cancer.

The CTCs are confirmed to be cancer cells with expressed cytokeratin (CK) as a marker for the epithelial origin of tumors, but these cells do not express a CD45 marker indicating that they do not have a hematopoietic origin.^[3] During the cancer metastatic process, CTCs undergo several epithelial mesenchymal transitions (EMT) and lose their CK and epithelial cell adhesion molecule (EpCAM) molecule on their surface. These CTCs may be considered resistant to chemo- and radiotherapies and are suggestive of highly metastatic tumors. Often, CTCs are found in clusters. These clusters have cancer-specific biomarkers indicative of increased metastatic risk and poor prognosis.^[4]

CTCS AND SOLID TUMOR MALIGNANCIES

As circulating tumor cell analysis is a non-invasive process, it is very useful in diagnosis and monitoring of solid tumor malignancies. Most solid tumors such as breast, ovarian, lung, prostate, pancreatic, and colon cancers are mainly developed inside the body and many times are only diagnosed at the last stage of cancer development. Such tumor analysis can presently be done by tissue biopsies which are invasive

and cannot be repeated at intervals for monitoring cancer therapies. Liquid biopsies can be available at any time during chemo- or radiotherapy treatment and can help clinicians in understanding the response of drug therapies, which can be altered in individual cancer patients as per their response to treatment. Therefore, CTCs analysis is useful in diagnosis and management of therapies of solid tumor malignancies, as shown in Figure 2.

ENUMERATION OF CTCS IN CANCER DIAGNOSIS AND THERAPIES

Enumeration of CTCs has great importance in prognostic and therapeutic cancer treatment; however, due to the very small number of CTCs in blood circulation, these cells are difficult to isolate and quantitate accurately. So far it has been reported that only 0.01% cells are present in metastatic cancer patients and the frequencies of circulating tumor cell vary from 1 to 10 cells/mL of whole blood in patients with metastatic cancer. So far, CTCs have been detected in several epithelial cancers including breast, prostate, lung, and colon cancer, as shown in Figure 2. Similarly, enumeration of CTCs can describe the level of metastatic potential in malignant tumors and suggest the appropriate treatment to manage this disease.

Another major problem in isolation of CTCs is the change in their surface markers during the EMT phase. Many CTCs lose their surface CK19 and EpCAP proteins during EMT and thus it is very difficult to use even the FDA-approved “Cell Search Kit” in this situation.^[5] It has been generally observed that most CTCs enter into the EMT phase in highly metastatic

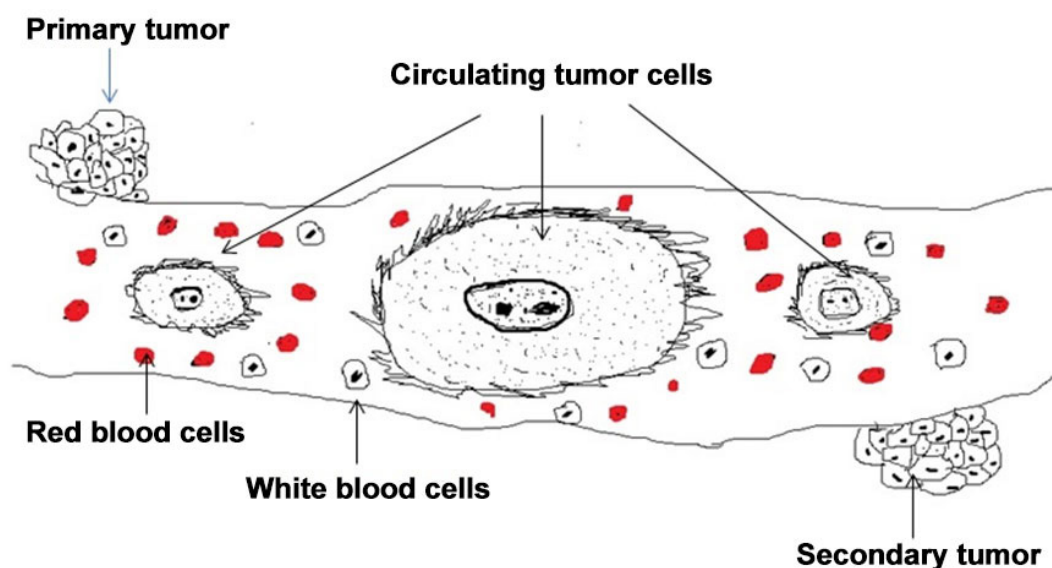


Figure 1: Large oval-shaped CTCs with spikes on their surface in the blood circulation of a metastatic cancer patient; these CTCs are shredded from the original tumor and subsequently form a secondary tumor at a different site. CTCs: circulating tumor cells

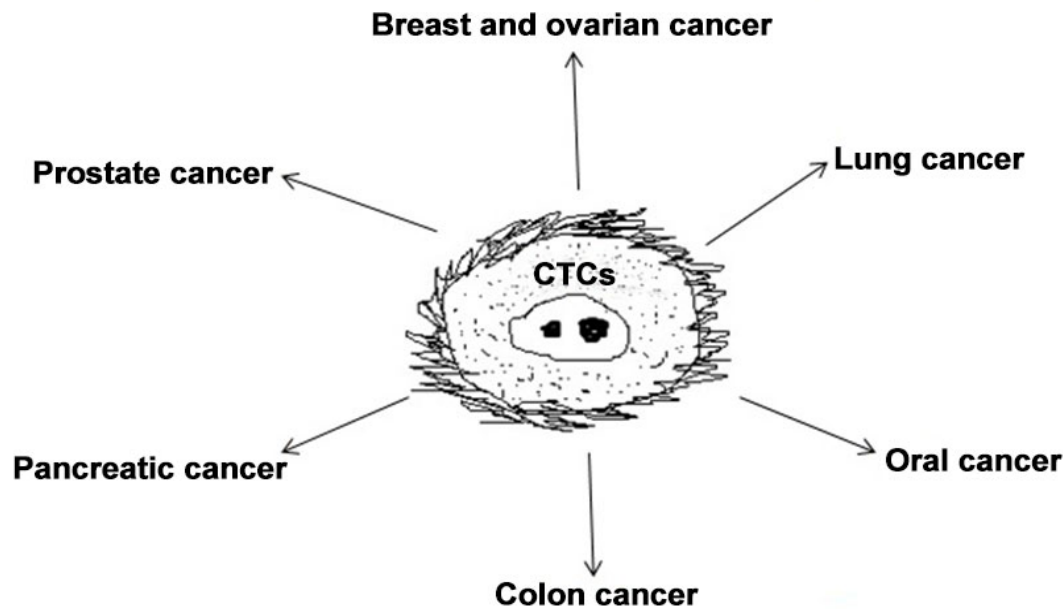


Figure 2: Circulating tumor cells analysis in several epithelial cancers including breast, prostate, lung and colon cancer. CTCs: circulating tumor cells

tumors and thus cannot be separated using the Cell Search Kit. Besides this kit, various CTC isolation methods are being established to isolate CTCs from liquid biopsies, including the Ficoll Gradient Method, the Islet Method, the Microfluidic Method and others. It is a very important task for cancer scientists to establish a precise method for isolation of CTCs from liquid biopsies, taking into consideration their EMT phase and surface marker alterations during metastatic process.

MOLECULAR PROFILING OF CTCs

The CTCs can undergo a variety of changes during the metastatic process and show heterogeneous characteristics, causing difficulties in giving proper treatment to cancer patients. Therefore, there is a need to well characterize these isolated cells by gene expression profiling. Molecular profiling of CTCs is therefore an integral part of CTCs analysis and provides accurate phenotypes of these cancer cells.^[6] So far it is well established that certain genes are upregulated, downregulated or mutated in several solid tumor malignancies and their analysis can help in proposing targeted therapies to treat many of these cancers. A major phenomenon seen in CTCs in metastatic cancer is their transformation during EMT transition. During the EMT process, CTC cells go through several modifications at both cellular and molecular levels.^[7] There is downregulation of E-cadherin genes and upregulation of TWIST1 and TWIST1 genes. In addition, there is an upregulation of extracellular matrix

proteins such as vimentin and fibronectin which are responsible for invasive processes.^[8]

The CTCs are extensively studied in the diagnosis and treatment of breast cancer. Molecular profiling of CTCs shows the progression of breast cancer and its response to therapy.^[9] Swaby *et al.*^[10] have described HER2- breast cancer patients with HER2+ CTCs who were given a trastuzumab-based therapy. This led to a considerable revision in their treatment protocol and proved that CTCs profiling is very important in identification of the most useful therapy in HER2- breast cancer patients. The CK19 and TP53 gene mutation can also reveal the metastatic potential of triple-negative breast cancer patients. In prostate cancer, expression of the fusion of TMPRSS2 and ERG genes and downregulation of PTEN have been shown to be responsible for cancer causation.^[11] Schölch *et al.*^[12] have discovered a KRAS mutation in the CTCs of CRC patients whose primary tumor was KRAS wild-type, suggesting that the sequence analysis of CTCs can better discover the presence of KRAS mutation.

Treatment of other cancers can also benefit from CTCs analysis. This analysis is especially useful in lung cancer because repeated biopsies are not possible in these patients. Evaluating mutations on exon 19 and 21 of the EGFR gene are the prime determinant for drug-based therapies in lung cancer. In addition, mutations such as T790M, EML4-ALK rearrangement, BRAF, KRAS, HER2, PIK3CA/AKT1, ROS, FGFR1, and MET can all be studied for lung cancer treatment.^[13] Overexpression of H-RAS oncogene and

mutations in FGR-R genes in CTCs can be considered as a diagnostic marker in bladder cancer. Similarly, TWIST and vimentin are considered as diagnostic markers for hepatocellular carcinoma. Häfner *et al.*^[14] have isolated CTCs from metastatic cervical cancer patients and evaluated levels of HPV16-E6 mRNA by real-time PCR, as a more sensitive molecular marker than CK19 mRNA. At the same time, Kuhlmann *et al.*^[15] have shown that ERCC1+ CTCs can predict platinum resistance therapy in ovarian cancer. Overall, it seems that molecular profiling of CTCs is becoming a popular tool for diagnosis and therapies of solid tumor malignancies.

CLINICAL APPLICATIONS OF CTCs ANALYSIS

Recently, analysis of CTCs in the blood of metastatic cancer patients has received enormous attention because of its very important clinical applications in personalized medicine.^[16] Several investigators have studied CTCs derived from breast cancer patients for diagnosis and treatment of breast cancer. They have shown direct correlation of CTCs with disease prognosis and survival, and shown that the greater the number of CTCs, the lower the chances of survival. The CellSearch® system, FDA-approved, is most commonly used for enumeration of CTCs in these experiments, which are mainly based on positive expression of EpCAM and CK protein on the surface of these cells.^[17] However, this may limit the selection due to the EMT transition process undergoing in CTCs during the metastatic process of cancer, in which there is a downregulation of EpCAM surface protein. The CTCs values are measured before and after chemo- or radiotherapies and treatment can be determined by greater or lesser CTCs counts in patients. Peeters *et al.*^[18] have shown that patients with more than 80 CTCs in 7.5 mL blood died within one year following diagnosis of metastases.^[18] CTCs analysis is very much useful in understanding stages of various cancers. Overall this suggests that CTCs enumeration and molecular profiling with proper follow-up can determine the aggressiveness of cancers, which could help clinicians decide more efficacious and targeted treatments for management of metastatic cancer patients.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

There is no patient involved.

Ethics approval

This article does not contain any studies with human participants or animals.

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Profiling of circulating tumor cells in liquid biopsies from metastatic cancer patients

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Dr. Pravin D. Potdar's present interest is to study molecular profiling of circulating tumor cells (CTC), circulating tumor DNA, cancer associated fibroblasts and cancer stem cells involved in metastatic process of cancers, and to see how this process can be reverted back to normal by using innovated technologies which include nanotechnology and nanomedicine.

ABSTRACT

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Circulating tumor cells,
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Aim: Circulating tumor cells (CTCs) are crucial to tumor metastasis and valuable for prediction of clinical outcome in patients with solid tumors. Here, the authors aimed to establish a method for enumeration and characterization of CTCs from liquid biopsies. **Methods:** Peripheral blood mononuclear cells (PBMCs) were separated from blood samples from patients with metastatic cancer using Ficoll-Hypaque gradients and cultured to isolate and enumerate CTCs. Cultured CTCs were morphologically characterized by light and phase contrast microscopy. The tumorigenicity of Ficoll-Hypaque-separated PBMCs was examined, in addition to their expression of mRNA metastasis markers. **Results:** CTCs were isolated in culture and enumerated by counting under phase contrast microscopy, demonstrating that 0.01-0.04% of total PBMCs were CTCs. CTCs were dormant, with large, oval-shaped, spiky morphology. PBMCs obtained from liquid biopsies exhibited anchorage-independent growth, forming numerous colonies in soft agar assays. Molecular profiling demonstrated expression of several metastatic genes, but not of cadherin 1 (encoding the adhesion protein), in all patients. **Conclusion:** The authors successfully isolated, enumerated, and characterized CTCs from liquid biopsies of metastatic cancer patients. This study has potential to facilitate the development of new diagnostic and therapeutic methods using liquid biopsies, for application in metastatic cancers.



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INTRODUCTION

Cancer cells are invasive and can metastasize; once metastasis has occurred, disease is usually incurable. Recent technological developments have enabled evaluation of the metastatic potential of cells using “liquid biopsies”,^[1,2] noninvasive tests for circulating tumor cells (CTCs), which are present even at early stages of disease.^[3] Additionally, molecular profiling and enumeration of CTCs can predict their metastatic potential, thereby facilitating improved treatment and prognosis.^[3] Such testing has potential to revolutionize personalized cancer medicine by avoiding biopsies, since it captures cancer cells that tumors shed into the circulation, rather than sampling the tumor itself. Liquid biopsies permit repeated, noninvasive sample collection from cancer patients that can be profiled for gene expression, allowing clinicians to choose specific therapies targeting particular mutations. Ultimately, liquid biopsies could become a routine cancer screening method, alongside current methods, such as mammograms and colonoscopies.^[4,5] Liquid biopsies could be useful in the majority of invasive solid tumors, including breast, colon, lung, prostate, and pancreatic cancers.

Isolation and enumeration of CTCs is difficult because of their low numbers, size, and heterogeneity. Several methods have been established for isolation and enumeration of CTCs from liquid biopsies, including the Ficoll-Hypaque gradient method,^[6] flow cytometry,^[7,8] the CELLSEARCH® system,^[9] isolation by size of epithelial tumor cells (ISET),^[10,11] and microfluidic systems.^[12] The CELLSEARCH® system is primarily based on selection of EpCAM- and cytokeratin-positive CTCs, and has been approved by the Food and Drug Administration (FDA) and used in several clinical studies;^[13,14] however, one study reported that CTCs which have undergone epithelial mesenchymal transition (EMT) exhibit down-regulation of epithelial markers, including EpCAM and keratin,^[15] and such CTCs would be missed using this technology. Similarly, in some cases, CTCs from HER2-positive metastatic breast cancer patients include EpCAM-negative cells.^[15] Hence, EpCAM-independent methods may enable improved detection of CTCs in certain cancer patients. Advances in microfluidic technologies, biomaterials, and molecular profiling have led to rapid growth and interest in achieving liquid biopsies for cancer diagnosis and treatment. CTCs are primarily characterized and identified by their morphology and immunostaining pattern with specific antibodies; however, the heterogeneity of CTC's is a major obstacle to their isolation, identification, and characterization from liquid biopsies.

Chemotherapy is a standard mode of treatment for all cancers. CTC levels are determined before and after rounds of chemotherapy. In the majority of cases of non-metastatic breast cancer, reduction in the number of CTCs is observed after the first round of chemotherapy; however, CTCs can be resistant to therapy, leading to administration of increasing doses in subsequent rounds of chemotherapy. In one study, patients with > 80 CTCs in 7.5 mL of blood died within one year of diagnosis due to metastases.^[16] In another study of patients with metastatic breast cancer, when CTCs continued to be detected after the first round of chemotherapy, there was rapid progression of the disease; it was suggested that, in such cases, it would be preferable to opt for alternative treatment, rather than continuing with the same chemotherapy.^[17] A review of CTCs in patients with breast cancer in 2013 considered the clinical application of CTCs in breast cancer therapy and assessed the use of HER2 treatment in HER2-negative breast cancer patients, selected on the basis of CTC detection.^[13,14] Rack *et al.*^[18] concluded that there is an independent prognostic relevance of CTC determination both before and after adjuvant chemotherapy in a study of > 2,000 patients with primary breast cancer. Bidard *et al.*^[13] also studied the clinical utility of CTCs in metastatic breast cancer, mainly focusing on first and second line treatments.

Completion of the human genome project and the advent of molecular profiling has led to an understanding of the genetic profiles of cancer cells, including CTCs.^[19] *MMP1*, *MMP2*, *MMP9*, *VEGFA*, *MT-CO2*, *ICAM1*, *CD44*, and *PROM1* are major genes involved in the processes of invasion and metastasis. Moreover, cadherin-1, N-cadherin, fibronectin, and integrin β are adhesion molecules involved in the metastatic process. During invasion and metastasis, CTCs frequently undergo the process of EMT, which involves several genes including *TWIST1* and *TWIST2*,^[20] in combination with *TGFB1*, *WNT*, and *NOTCH2*. The majority of CTCs isolated from breast cancer patients express EMT markers, including *ETV5*, *NOTCH1*, *SNAIL*, *TGFB1*, *ZEB1*, and *ZEB2*.^[21] Keratin analysis is also a major molecular test in cancer, while *CK19* and *TP53* mutations are frequently found in CTCs from triple-negative breast cancer patients,^[22] and are a factor in the progression of the disease to the triple-negative stage. Patients with breast cancer expressing *CK19*, *SCGB2A2*, and *ERBB2* showed poor survival rates.^[22] Riethdrof *et al.*^[23] demonstrated that HER2+ CTCs can be identified in HER2- breast cancer patients, leading to consideration of revision of ongoing treatment, with trastuzumab-based therapy applied to patients with HER2+ CTCs and HER2-primary tumors, demonstrating that CTCs can assist in

determining the changing course of disease in a timely manner and have potential to determine the metastatic state of breast cancer.

CTCs are present in very low numbers in whole blood and are difficult to identify and characterize. The primary aims of the present study were to establish a simple protocol for the isolation, identification, and enumeration of CTCs from various metastatic cancer patients and to molecularly profile genes involved in the processes of invasion and metastasis, and to evaluate the potential clinical utility of liquid biopsies in the treatment of advanced stage cancer.

METHODS

Materials

Low-glucose Dulbecco's modified Eagle's Medium (DMEM), penicillin/streptomycin (PenStrep), phosphate-buffered saline (PBS), trypsin EDTA, erythrosin B, and colchicine, were purchased from HiMedia (Mumbai, India); fetal bovine serum (FBS) from GIBCO BRL (Carboside, MA); Trizol reagent, cDNA preparation kits, and agarose from Invitrogen (Carlsbad, CA, USA); and histopaque and primers for *KRT18*, *KRT19*, *PROM1*, *CD44*, *CXCR4*, *NOTCH2*, *VEGFA*, *MMP1*, *MMP2*, *MMP9*, *ICAM1*, *CDH1*, *KCNH2*, and *ACTB* from Sigma Chemicals, USA.

Sample collection

A total of eight metastatic cancer patients recruited by oncology clinic of Jaslok Hospital and Research Centre, Mumbai India (three breasts, two ovarian, two prostate, and one nasopharyngeal cancer) and five healthy individuals were included in this study. All patients had stage IV disease, with invasive and metastatic cancer. All tumors were histopathologically proven to have metastatic potential. Fresh blood samples (10 mL) were collected from each metastatic cancer patient and healthy control individual in sterile EDTA vacutainers with proper consent from the patients, according to the ethical committee guidelines of Jaslok Hospital and Research Center, Mumbai, India, and sent to the tissue culture laboratory of the Molecular Medicine and Biology Department.

Culture of peripheral blood mononuclear cells for isolation of CTCs

Plasma rich in peripheral blood mononuclear cells (PBMCs) was separated from blood samples from cancer patients and healthy individuals by allowing blood to stand for approximately 1 h. We obtained approximately 2 mL of PBMC-rich plasma from 10 mL of blood. Next, PBMCs were isolated by Ficoll-Hypaque gradient centrifugation as follows. Briefly, 300 μ L

aliquots of PBMC-rich plasma were mixed with 300 μ L of 1 \times PBS and this mixture was layered onto 300 μ L of Ficoll-Hypaque solution. We prepared two tubes each for cancer patients and healthy individuals for our culture study. Tubes were centrifuged at 3,000 g for 30 min to obtain a middle layer containing PBMCs. The PBMCs isolated from the two tubes were pooled together, washed with 1 \times PBS, and suspended in 4 mL of RPMI growth media, supplemented with 10% FBS, 1% PenStrep, L-glutamine, and vitamin C. Tissue culture dishes (65 mm) containing PBMCs were incubated in a 5% CO₂ incubator at 37 °C. After 24 h, non-adherent cells were removed and the remaining adherent cells fed with 4 mL of DMEM growth medium supplemented with 10% FBS + PenStrep + L-glutamine, which were changed twice a week. Cultures were observed and photographed every day for 30 days under a phase contrast microscope (model AXiovert 40CFL from Carl Zeiss), equipped with TS view software (Tucson Imaging, Fuzhou, and PR China) and images were captured and analyzed to determine and record the morphology of adherent cells. Adherent cells that appeared CTC-like by phase contrast microscopy were counted manually under a phase contrast microscope to calculate the number of cells present in each metastatic cancer patient and were then fixed in 50% methanol, stained with Giemsa, and examined by light microscopy (AXiovert 40CFL, Carl Zeiss) to determine their general morphological features.

Anchorage-independent soft agar assay

PBMCs were isolated from metastatic cancer patients and healthy individuals and their tumorigenic potential determined using soft agar assays. A total of 3 \times 10³ PBMCs per individual were layered on 0.4% soft agar in DMEM growth medium in 65 mm dishes. The plates were then incubated at 37 °C with 5% CO₂ for 2 weeks. Emerging colonies were observed under a phase contrast microscope and photographed.

Molecular markers in PBMCs (liquid biopsies) and cultured CTCs

Total RNA was extracted from all patient's PBMCs and isolated cultured CTCs from Ovarian, prostate and CNS cancer patients using the Trizol method. RNA was then reverse transcribed to cDNA using the Applied Biosystems High Capacity cDNA Kit (Applied Biosystems, USA). PCR was carried out using specific forward and reverse primers with defined annealing temperatures [Table 1]. For all genes, PCR reactions were performed at 95 °C for 5 min; followed by 40 cycles at 95 °C for 30 s, at the respective annealing temperatures [Table 1] for 30 s, and at 72 °C for 30 s; with a final extension at 72 °C for 7 min. Amplicon sizes were checked by 2% agarose gel electrophoresis,

Table 1: Forward and reverse primer sequences used for respective molecular markers with their annealing temperature and size

Serial No.	Name		Primer sequence (5'-3')	Annealing (°C)	Size (bp)
1	ACTIN	F	GACTACCTCATGAAGATC	55	512
2	ACTIN	R	GATCCACATCTGCTGCAA	55	512
3	KERATIN	F	GAGATCGAGGCTCTCAAGGA	55	357
4	KERATIN	R	CAAGCTGGCCTTCAGATTTT	55	357
5	CD44	F	CAACCCTACTGATGATGACG	60	302
6	CD44	R	GGATGCCAAGATGATCAGCC	60	302
7	CXCR4	F	GGACCTGTGGCCAAGTTCTAGTT	60	273
8	CXCR4	R	ACTGTAGGTGCTGAAATCAACCCA	60	273
9	NOTCH-2	F	ACTTCCTGCCAAGCATTCC	60	278
10	NOTCH-2	R	GTCCATGTCTTCAGTGAGAAC	60	278
11	CD133	F	ACCTGCGTAATCCCATCT	60	340
12	CD133	R	TTGTCCGACCAGTTCTTC	60	340
15	VEGFR	F	GAAGTGGTGAAGTTCATGGATGTC	62	422
16	VEGFR	R	CGATCGTTCTGTATCAGTCTTTCC	62	422
17	MMP1	F	CTGAAGGTGATGAAGCAGCC	55	427
18	MMP1	R	AGTCCAAGAGAATGGCCGAG	55	427
19	MMP2	F	GCGACAAGAAGTATGGCTTC	58	390
20	MMP2	R	TGCCAAGGTCAATGTCAGGA	58	390
21	MMP9	F	CGCAGACATCGTCATCCAGT	64	405
22	MMP9	R	GGATTGGCCTTGAAGATGA	64	405
23	E -Cadherin	F	TGCTCTTGCTGTTTCTTCGG	60	422
24	E -Cadherin	R	TGCCCCATTGTTCAAGTAG	60	422
25	I -CAM1	F	AGGCCACCCAGAGGACAAC	58	405
26	I -CAM1	R	CCCATTATGACTGCGGCTGCTA	58	405

followed by using Ethidium Bromide dye and visualization using a gel documentation system (Alpha Imager HP from Cell Bioscience) and photography.

RESULTS

Isolation of CTCs from PBMCs from metastatic patients

PBMCs were isolated from metastatic cancer patients, counted using a hemocytometer, and 3×10^5 cells were cultured for 24 h, followed by culture only of adherent cells. After 15-20 days of incubation, we observed circular cells with spikes on their circumference [Figure 1], which were metastatic tumor cells that had been circulating in the blood of the cancer patients. We considered these cells to be CTCs and examined their morphology and other characteristics daily by phase contrast microscopy. CTCs were large spherical cells, with spikes, single nuclei, and granular cytoplasm, which differed from other cell types [Figure 1], clearly indicating that the cells observed in samples from metastatic patient were CTCs involved in cancer metastasis. CTCs isolated from all metastatic cancer patients were morphologically similar, with no differences in samples from patients with different types of cancer [Figure 1]. No such cells were observed in PBMCs from healthy individuals cultured simultaneously.

To determine the general morphological feature of CTCs, we stained them with Giemsa, revealing that these large cells had distinct nuclei, with granulated

cytoplasm at the periphery of the nucleus [Figure 1]. CTCs were dormant, and did not multiply for several months.

Enumeration of CTCs in metastatic cancer patients

Enumeration of CTCs in metastatic cancer patients was a major aim of this study. After 30 days of culture of adherent cells emerging from PBMCs, CTCs were clearly visible in culture dishes [Figure 1] and were counted manually under phase contrast microscopy. In samples from the three breast cancer patients, CTC counts ranged from 120 to 160 cells (average = 145 cells; percentage of total cells plated = 0.045%); from the two patients with prostate cancer, the count ranged from 120 to 160 (average 140 cells; percentage of total cells plated, 0.042%); samples from the two ovarian cancer patients yielded 90-120 CTCs (average = 105; percentage of total cells plated = 0.032%); and from the single nasopharyngeal cancer sample we obtained 50 CTCs (percentage of total cells = 0.015%). All experiments were performed in duplicate. This cell counting process was very consistent and a successful straightforward method to enumerate CTCs.

Anchorage-independent soft agar assays

Anchorage-independent soft agar assays were performed to determine the tumorigenic potential of PBMCs isolated from metastatic cancer patients and healthy individuals. No colonies were observed in samples from healthy individuals [Figure 2A]. In contrast, several large colonies grew in agar plates

containing PBMCs isolated from all metastatic cancer patients [Figure 2B]. Hence PBMC samples from metastatic cancer patients clearly contained a tumorigenic population, whereas those from healthy individuals did not. We also observed the formation of circular rings around each colony, indicating that the cells secreted proteolytic enzymes [Figure 2B]. This assay only indicates the basic cancer phenotype of cells; tumorigenic potential can be confirmed by “*in vivo* transformation assays” using nude mice, or by PCR determining their molecular phenotypes. We confirmed the tumorigenicity of the cells by molecular analysis.

Molecular markers in isolated cultured CTCs

To determine the molecular cancer phenotypes of isolated CTCs, we harvested cells after 30 days of culture of PBMCs from metastatic cancer patients and determined the expression of specific mRNAs involved in metastasis in these cells by RT-PCR. We studied the expression of only five specific genes: *KRT18*, *PROM1*, *CD44*, *CXCR4*, and *NOTCH2*, in addition to the *ACTB* housekeeping gene in these samples, due to the limited quantities of mRNA available. We obtained CTCs from ovarian, prostate, and central nervous system (CNS) cancers for this analysis. We found that ovarian cancer CTCs expressed *KRT18*, *PROM1*, and *CD44*; however, *CXCR4* and *NOTCH2* were not expressed in CTCs obtained from this cancer type

[Figure 3]. CTCs from CNS cancer exhibited expression of *KRT18* and *NOTCH2* and absence of expression of *PROM1*, *CD44*, and *CXCR4*, whereas prostate cancer CTCs expressed all five genes [Figure 3]. Overall our results indicated that the molecular profiles of CTCs varied according to the type of cancer; therefore, we suggest that individual profiling of metastatic cancer patients will be essential for management of therapy in these patients.

Molecular markers in PBMCs from metastatic cancer patients (liquid biopsies)

We also examined molecular markers present in whole plasma PBMCs (liquid biopsies), from patients with metastatic breast ($n = 3$), ovarian ($n = 1$), nasopharyngeal ($n = 1$), and prostate ($n = 1$) cancer. We analyzed expression of 11 genes related to metastasis: *KRT18*, *CD44*, *PROM1*, *CXCR4*, *NOTCH2*, *MMP1*, *MMP2*, *MMP9*, *KCNH2*, *ICAM1* and *CADH1*. *KRT18*, *CD44*, *PROM1*, *CXCR4*, and *NOTCH2* were expressed by PBMCs from all patients [Figure 4] indicating that they were present in all cancer patients and have roles in the metastatic process.

The expression of *KRT19*, *MMP1*, *MMP2*, *MMP9*, *ICAM*, and *CDH1* was studied in samples from three breast cancer patients, one ovarian cancer patient, one nasopharyngeal cancer patient, and one prostate cancer patient, and the results are shown in Figure 5.

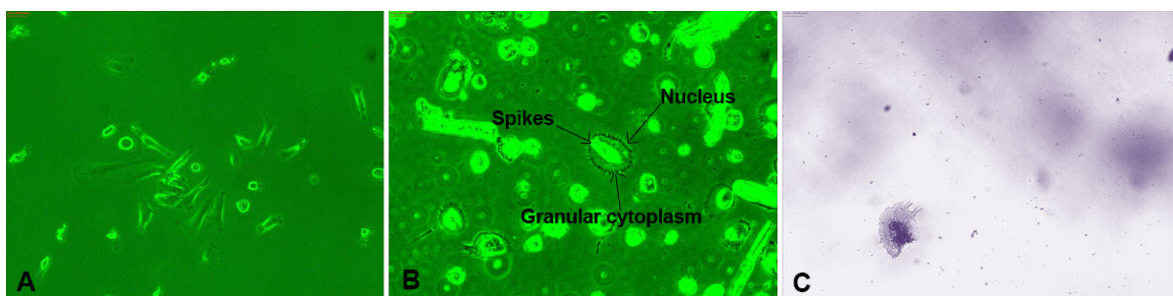


Figure 1: Morphology of circulating tumor cells (x20). A and B are phase contrast microscopy and C is Giemsa stained

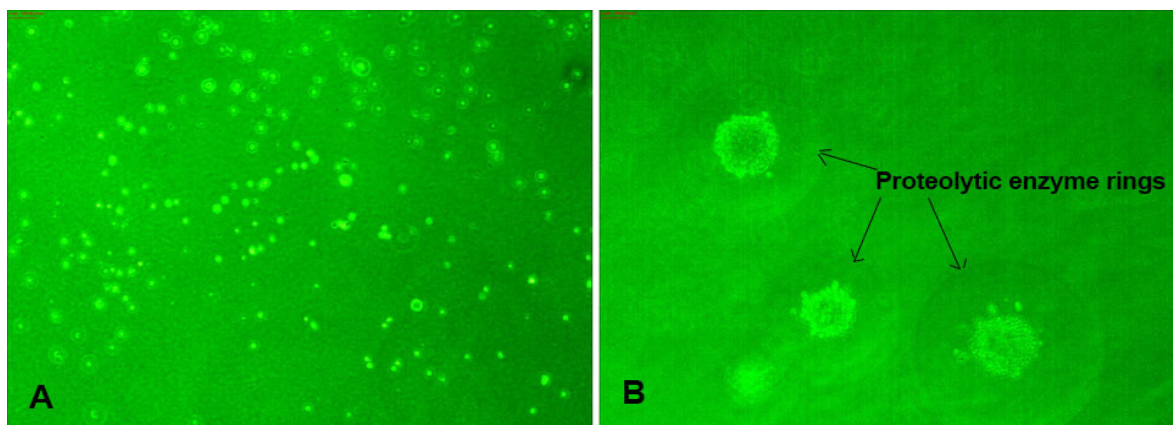


Figure 2: Anchorage independent growth assay (x40). (A) Normal PBMCs; and (B) metastatic cancer PBMCs. Each colony shows proteolytic enzyme ring (arrows). PBMCs: peripheral blood mononuclear cells

KRT19 was expressed in the ovarian, nasopharyngeal, and prostate cancer samples, whereas its expression was completely absent in samples from all three breast cancer patients; and the metastatic genes, *MMP1*, *MMP2*, and *MMP9*, were expressed in all patients studied, confirming the metastatic potential of these cells. The *MMP2* gene was highly expressed in breast and nasopharyngeal cancer, whereas *KCNH2*, which is specific for prostate cancer, was faintly expressed only in the sample from the prostate cancer patient and its expression was absent from all other samples. The *ICAM* gene, which encodes a molecule involved in cellular adhesion, was expressed in breast and prostate cancer samples, but expression was absent from ovarian and nasopharyngeal cancer samples; in contrast, the expression of another gene, *CADH1*, encoding the adhesion molecule (cadherin 1), was completely absent from the samples from all four types of cancer patients, indicating that cells in liquid biopsies from metastatic cancer patients were in the EMT phase, in which they lose their adhesive properties and become free to move in the blood stream, which may also explain the high metastatic potential of these cells.

DISCUSSION

In their early stages, the majority of cancers are asymptomatic, whereas they exhibit rapid growth rates later in disease progression; therefore, it is difficult to treat advanced-stage cancer patients, due to spread of the disease to various organs and tissues.^[24] CTCs are shed by primary tumors into the vasculature and circulate in the blood of cancer patients;^[25] however,

they are not easily identified, due to their very limited numbers. Pantel *et al.*^[26] reviewed the importance of CTCs, including the biological properties of metastatic cells involved in cancer progression, and stated that CTC-derived cell lines could be used to develop new therapeutic targets, and for drug screening. Several investigators have described the development of *in vivo* and *in vitro* methods for the isolation of CTCs in larger numbers from metastatic cancer patients, for use in research into the role of CTCs in cancer progression,^[27-29] and with the aim of targeting such cells to cure cancer; hence CTCs are a topic of intense discussion among oncologists.^[25]

Many techniques have been developed and are under continuous improvement to enhance the efficacy of

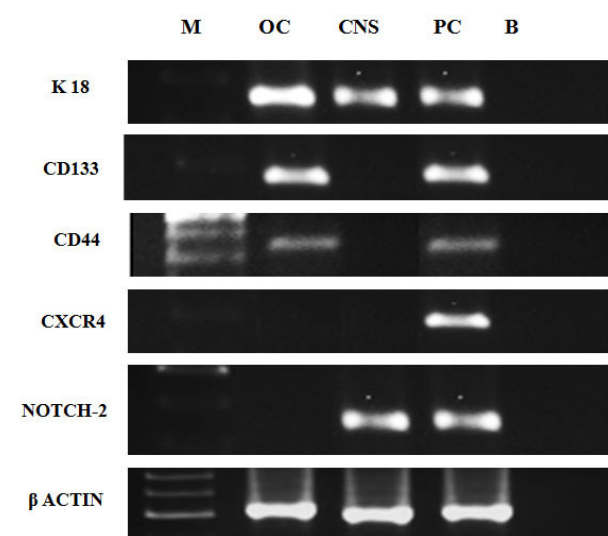


Figure 3: Expression of *K18*, *CD33*, *CD44*, *CXCR4* and *NOTCH2* genes in circulating tumor cells isolated from culture peripheral blood mononuclear cells of metastatic patients. B indicates black sample and B is a blank lane

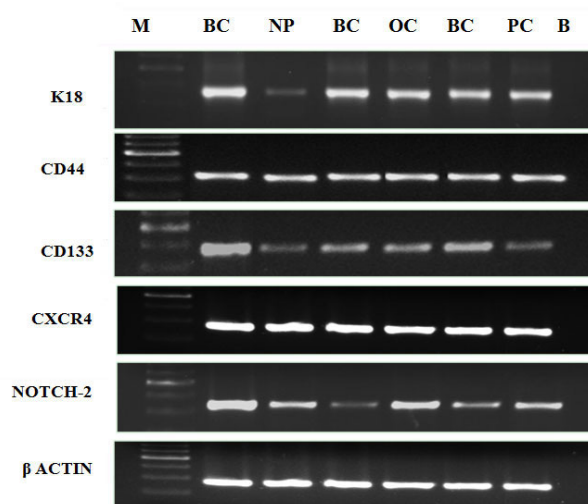


Figure 4: Molecular profiling of liquid biopsies (PBMC) of metastatic cancer patients. PBMC: peripheral blood mononuclear cell

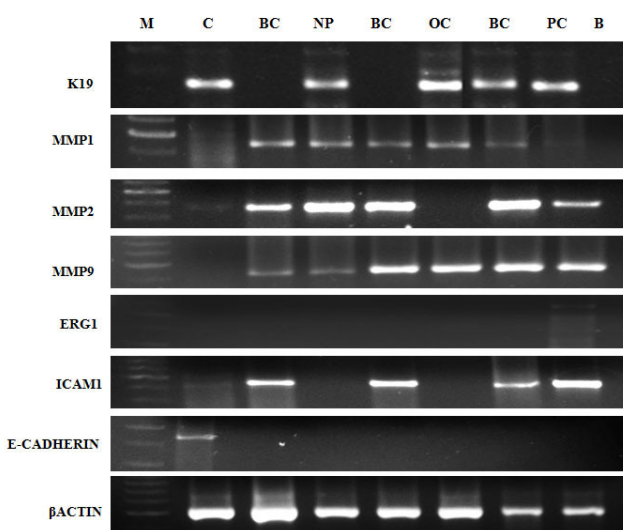


Figure 5: Molecular profiling of liquid biopsies (PBMC) of metastatic cancer patients. C indicates positive control for respective gene. PBMC: peripheral blood mononuclear cell

CTC isolation and enumeration.^[16] Comparison of the OncoQuick density gradient method with Ficoll-Hypaque density gradients for separation of tumor cells from bone marrow and peripheral blood aspirates indicated that CTCs can be easily aspirated by both methods and analyzed further to determine their presence and quantity.^[30] In the present study, we used the simple and cost-effective Ficoll-Hypaque density gradient method to separate PBMCs from whole plasma of cancer patients. These cells were then cultured to isolate CTCs, which were identified by their morphology (i.e. large oval shape, with large nuclei and peripheral spikes) [Figure 1]. Similar morphology was first reported by Gascoyne *et al.*^[31] It is likely that the spike-like processes allow CTCs to adhere to the surface of basement membranes during the process of invasion. We confirmed the phenotype of these cells by studying cancer-associated genes in isolated CTCs and from liquid biopsies [Figures 3 and 4].

The presence of CTCs in an advanced metastatic cancer patient was first reported in 1869 by Ashworth;^[32] however, limited information is available regarding the numbers of CTCs in the blood of patients with different stages and types of cancer,^[33] making proficient isolation of CTCs in a viable and intact state challenging. The CELLSEARCH® system^[34] is the only method approved by the US FDA for clinical use in metastatic breast, colorectal, and prostate cancer.^[13,17,35] Patients with > 80 CTCs in 7.5 mL of blood were reported to die within one year of diagnosis from metastatic disease,^[16] and CTCs continue to be detected after the first round of chemotherapy in some breast cancer patients with rapid progression of the disease to metastasis;^[17] it has been suggested that, in such cases, it may be preferable to opt for an alternative treatment with novel therapeutic agents, rather than continuing with the same chemotherapy.

In the present study, we established a simple method of isolation and enumeration of CTCs from metastatic cancer patients. PBMCs were cultured in 65-mm dishes and numbers of CTC-like cells were counted manually under a phase contrast microscope. We observed that there were more CTCs in patients with final-stage, compared with early-stage, metastatic cancer. We found approximately 50-160 CTCs in the majority of cancer patients, representing 0.01-0.04% of overall PBMCs. All three breast cancer patients and one prostate cancer patient were determined to be in the late stages of metastatic disease, according to CTC enumeration, which was confirmed by molecular profiling. Hence, our results indicate that enumeration and molecular profiling of CTCs can distinguish early- and later-stage disease in metastatic cancer patients.

Moreover, the values obtained by enumeration of CTCs correlated well with those from previous reports.^[13,14]

The CELLSEARCH® system, is primarily selective for EpCAM- and cytokeratin-positive CTCs;^[34] however, Lianidou *et al.*^[15] demonstrated that CTCs which have undergone EMT exhibit down-regulation of epithelial markers, including EpCAM and K19 and hence such cells will be missed by this technology. Similarly, some cases of HER2-positive metastatic breast cancer have EpCAM-negative CTCs. Hence, additional markers should be considered in the development of methods to isolate and enumerate CTCs in metastatic cancer. Our study also demonstrated that KRT19 and CADH1 were not expressed in liquid biopsies from any breast cancer patients [Figure 5]. Moreover, the observed absence of CADH1 expression indicated that CTCs obtained from all cancer patients were in EMT. As the method we used was based on counting intact cultured CTCs by phase contrast microscopy, the possibility of missing CTCs was small.

The soft agar colony formation assay is a common method to monitor anchorage-independent growth in semi-solid agar during 3-4 week incubation by manual counting of colonies under phase contrast microscopy. This method is considered one of the most accurate and sensitive *in vitro* assays for detection of malignant transformation of cells and confirmation of the phenotype of tumor cells with metastatic potential.^[36] In this study, we found that all PBMCs isolated from metastatic cancer patients resulted in significant numbers of colonies on soft agar, indicating their high tumorigenic potential. There were rings around each colony [Figure 2], indicating the activity of secretory proteases from these cells, which are primarily responsible for the degradation of basement membranes during invasion and metastasis.^[37]

Molecular profiling of tumor cells has become important for understanding genes involved in cancer development,^[38] and provides insights into the selection of tumor cells and resistance mechanisms in patients undergoing systemic therapies; therefore, molecular profiling of liquid biopsies or isolated CTCs, to determine the major genes involved in the processes of invasion and metastasis, was a primary aim of this study. We performed molecular profiling of isolated CTCs as well as liquid biopsies obtained from metastatic cancer patients. The results indicated that CTCs and liquid biopsies from all cancer patients expressed *KRT18*, *PROM1*, *CD44*, *CXCR4*, and *NOTCH2* genes [Figures 3 and 4], demonstrating that the malignant phenotypes of CTCs and liquid biopsies can be evaluated by molecular profiling. We

also observed that *KRT19* was not expressed down-regulated in all breast cancer patients, possibly due to EMT of these cells during the invasive process, and suggesting a need for aggressive treatment for these patients. *MMP1*, *MMP2*, and *MMP9* are metastatic genes involved in the invasion of tumor cells into underlying epithelium.^[39] This is the first report that liquid biopsies from all cancer patients tested express high levels of *MMP1*, *MMP2*, and *MMP9*, providing a potential new avenue for treatment of metastatic cancers by suppression of these genes; however, these results require confirmation and further investigation. PBMC from five normal individual were tested and found to be negative for expression of cancer-related genes.

Adhesion molecules play important roles in the migration of tumor cells to distant organs.^[40] Cadherin 1 is primarily responsible for adherence of cells to surfaces and reduced mobility of tumor cells; however, due to down-regulation of cadherin 1, metastatic cells are highly mobile and can easily travel to distant organs in short periods of time.^[41] Our results indicated that the gene encoding cadherin 1 (*CADH1*) was undetected in all cancers studied, indicating that all CTCs obtained from metastatic cancer patients were in EMT.

Overall, this is the first report of molecular profiling of several cancer genes in isolated CTCs and liquid biopsies from metastatic cancer patients. We suggest that liquid biopsies can be profiled for the metastatic genes identified in this study as potentially useful for the monitoring and treatment of metastatic cancer patients, with the aim of achieving improved outcomes. We realize that additional tests, including immunofluorescence, will be required to fully characterize the cells we have observed in metastatic patients' blood. We plan to perform these studies in the near future.

In conclusion, the present study describes a simple protocol for the isolation and enumeration of CTCs from liquid biopsies and the morphological and molecular characterization of CTCs from metastatic cancer patients. Molecular profiling of CTCs and whole liquid biopsies clearly indicated the expression of metastatic genes and adhesion molecules involved in the metastatic process; thus molecular profiling for these genes could enable the establishment of improved diagnosis and treatment protocols for last stage cancer using liquid biopsies.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Patient consent was obtained from the patients.

Ethics approval

The study has been approved by ethical committee guidelines of Jaslok Hospital and Research Center.

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Lung cancer biopsy dislodges tumor cells into circulating blood

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ABSTRACT

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Key words:

Lung cancer,
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Aim: A “seed” of lung cancer metastasis is circulating tumor cells (CTCs), which may be dislodged from a tumor during biopsy. This possibility was assessed among patients who underwent lung tumor biopsy using flexible fiber-topic bronchoscopy (FFB). **Methods:** The study involved six patients with non-small cell lung cancer who underwent FFB biopsy to diagnose a lesion pathologically (5 males and 1 female, median age 63 years, 6 adenocarcinomas, of 4 clinical-stage IA, 1 stage IB, and 1 stage IIIA), CTCs were extracted from the peripheral vein blood at pre-FFB and at post-FFB using a size selection method. **Results:** No tumor cell was detected at pre- and post-FFB was in three cases (50%); no tumor cells were detected pre-FFB while CTCs were detected at post-FFB in two cases (33.3%); and CTCs were detected at pre-FFB with numerous CTCs detected at post-FFB in one case (17.7%). In addition, similar tendencies were observed in each analysis of single-cell and clustered-cell categories. **Conclusion:** These results suggest that a FFB biopsy of lung cancer may potentially dislodge CTCs from a tumor into the circulating peripheral blood.

INTRODUCTION

There are three principal methods to diagnose a pulmonary lung cancer region pathologically. One is trans-bronchial biopsy (TBB) using flexible fiber-topic bronchoscopy (FFB) and others are computed tomography (CT), guided fine needle aspiration biopsy (FNAC), and surgical resection.^[1] Each method is effective but has some weak points.

First, FNAC has the potential to disseminate cancer

cells through the needle tract.^[2] According to a study using extracted human lung lobe with cancer lesions, cancer cells were detected at a rate of 10% in the saline used to wash the pulmonary lobe surface. This rate increased to 60% after FNAC.^[2] In a clinical setting, it has been reported that the rate of relapse (pleural recurrence) was higher in an FNAC group than in a control group among surgical lung cancer cases.^[3,4] In addition, there has been a recommendation use caution in employing FNAC as a method of lung cancer diagnosis based on evidence.^[5]



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Pulmonary wedge resection is also a crucial diagnostic technique for pulmonary nodules that may indicate lung cancer. This procedure also may potentially dislodge tumor cells from the surgical margin to the body, even when a specimen of pulmonary wedge resection contents histologically malignant negative surgical margin.^[6,7] Malignant-positive results in the surgical margin have been reported to be an indicator of poor prognosis in a retrospective study.^[8] Furthermore, it has been reported that malignant surgical margins of pulmonary wedge resection for lung cancer was a negative prognostic predictor in a subset analysis included in a multicenter prospective study of limited pulmonary resection for compromised lung cancer patients.^[9]

TBB is also an important method in diagnosing lung cancer. However, there have been reports demonstrating a prognostic disadvantage for biopsy using FFB.^[10,11] An observational study revealed that patients who were diagnosed with lung cancer using a trans-pleural technique had a statistically ($P = 0.04$) better 5-year survival rate than patients diagnosed using TBB.^[10] This phenomenon was also reported in a study using propensity score matched analysis.^[11] These results may mean that an intervention in a cancer lesion using TBB may dislodge cancer cells from the lesion to the circulating blood.

There are also studies revealing that manipulation during lung cancer surgery has the potential to dislodge cancer cells into the circulating blood, which is reported to be a prognostic indicator of poor outcomes.^[12-15] Above all, detecting cluster circulating tumor cell (CTC) is speculated the best a strong predictor of early recurrence.^[13] FFB also manipulates the area of lung cancer during biopsy; thus, CTCs might be dislodged from the lesion to the circulating blood as the same manner as surgical manipulation. For that reason we assessed the status of CTC before and after FFB biopsy to diagnose lung cancer.

METHODS

This investigation was approved by the institutional review board of the Hoshigaoka Medical Center and all patients provided their informed consent to participate in this study.

Among 6 patients with non-small cell lung cancer (NSCLC) who underwent FFB to diagnose a lesion pathologically, CTCs were extracted from a peripheral vein at pre-FFB and at post-FFB using a size selection method [ScreenCell® Cyto (ScreenCell, Westford, MA)]: using a micro-pore film that extracts formalin-

fixed tumor cells.^[16] ScreenCell® Cyto is designed for cytological studies and the filter allows a fast and regular filtration, preserving the CTC morphology and microcluster structures. Blood samples were diluted with the (LC/CC) ScreenCell® (ScreenCell, Westford, MA) dilution buffers for fixed cells. At the end of filtration, the ScreenCell® Cyto filter was released onto a standard microscopy glass slide; a 7 mm circular coverslip was then laid down on the filter with the appropriate mounting medium.

Peripheral blood (3 mL) was collected into an EDTA tube pre- and post-FFB. Tumor cells in two blood samples from each patient were simultaneously extracted using the method. These extracted cells were stained using a hematoxylin and eosin method and observed with a conventional microscope. Tumor cells were classified using three categories: no tumor cells detected (N), single cell or less than four cells (S), and clustered cells (C).

RESULTS

FFB-TBB was carried out under localized anesthesia using radiography to confirm that a sampling device reaches hits at a lesion. Samples for cytology and pathology were collected from a lesion. Cytological diagnosis of malignancy was achieved in only four cases, while all lesions revealed a pathological diagnosis, which is the result of manipulation that FFB-TBB made while contacting a cancer lesion. There was no complication during and after FFB-TBB and all patients were discharged without event.

Patient/tumor characteristics and status of CTC in each patient are shown in Table 1. There were five males and one female with a median age of 63 years, (range 59-78 years). According to CT findings, all lesions were solid and tumor size on CT findings was a median 2.5 cm (range 2.1-3.5 cm). Tumor invasiveness status in pathological diagnosis was "invasive" in all cases. Clinical stage was clinical -- stage IA in four cases, stage IB in one case and stage IIIA in one case. Singular or cluster CTCs were detectable as shown in Figure 1. The CTC counts at pre- and post-FFB procedures are shown in Table 1. In one case CTC was detectable before FFB. This case was stage IIIA with mediastinal lymphadenopathy. In an analysis of all cell categories, no tumor cell was detected at pre-FFB; at post-FFB a tumor cell was detected in three cases (50.0%). No tumor cell was detected at pre-FFB, while CTCs were detected at post-FFB in two cases (33.3%) and CTCs were detected at pre-FFB, while numerous CTCs were detected at post-FFB in one case (17.7%). In analysis of singular cells, no tumor

cell was detected at pre-FFB, while in three cases cells were detected at post-FFB (50.0%); no tumor cell was detected at pre-FFB while some CTCs were detected at post-FFB in two cases (33.3%) while some CTCs were detected at pre-FFB. Numerous CTCs were detected at post-FFB in one case (17.7%). In addition, in the analysis of cluster CTC alone ($n = 6$), no tumor cells were detected at pre-FFB and at post-FFB in five cases (63.3%), with some CTCs being detected at pre-FFB and many CTCs detected at post-FFB in one case (17.7%). The alterations of CTC counts are graphically demonstrated in Figure 2.

DISCUSSION

In our assessment of CTCs at pre-FFB and at post-FFB biopsy, the amount of CTC is not decreased after an FFB procedure, and in cases involving CTCs the count of CTCs increased at post-FFB. These results suggest that a tumor biopsy of a lung cancer lesion has the potential to dislodge tumor cells into

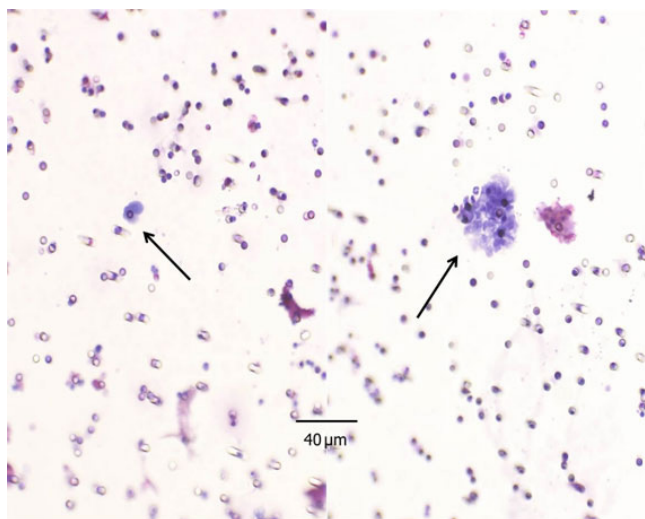


Figure 1: CTC detected around lung tumor biopsy. Left: singular CTC; right: cluster CTC. CTC: circulating tumor cell

the peripheral circulating blood, as can occur with a surgical procedure.^[12-15]

Initially, tumor cell dislodgement to the peripheral circulating blood by surgical manipulation was demonstrated using a cytological technique, but the sensitivity was very low.^[17] Later, polymerase chain reaction^[18,19] and flow-cytometry^[20] methods were introduced to improve sensitivity and specificity, but results were not predictable. Morphological detection of CTC dislodgement was shown using the EpCAM positive selection method [CellSearch[®] system (Janssen Diagnostics, Raritan, NJ)], but the sensitivity was still low.^[15] As such, isolated tumor cells (ITC), surrogates of CTC, were extracted from pulmonary vein blood, revealing that detecting ITC/CTC was an indicator of early recurrence; the presence of cluster ITC/CTC indicated wrong prognosis, using the EpCAM positive selection method^[21] or the CD45 negative-depression gravity method [RosettSep[®] (Stemcell Technologies, Vancouver, Canada)].^[22-24] As the sensitivity of such CTC detecting methods was not greater, the sample used pulmonary vein blood, because it contains more ITC/CTC than the peripheral circulating blood.^[24]

Size selection methods [ISET[®] (Rarecells Diagnostics, Paris, France) and ScreenCell[®] Cyto] are highly sensitive for cluster CTC, and therefore the sampling of CTC extraction using size selection methods can use peripheral blood.^[16,25] Recently, CTC dislodgement during surgery for lung cancer was proven by detecting CTCs in the peripheral circulating blood using a size selection method, and the presence of cluster CTC has been an indicator of early recurrence among surgical lung cancer patients.^[13]

A CTC assessment during FFB procedures needs a method that can extract CTCs from the peripheral blood sensitively, and for this reason we chose the sensitive

Table 1: Patient/tumor characteristics and status of CTC

No.	Age	Gender	CT findings			Stage			Status of CTC					
			Type	Size (cm)	Tumor histology	C-stage	C-T	C-N	All CTC categories		Singular CTC		Cluster CTC	
									Pre	Post	Pre	Post	Pre	Post
1	65	M	Pure solid	2.1	Invasive AD	IA	1b	0	0	0	0	0	0	0
2	64	M	Pure solid	2.5	Invasive AD	IA	1b	0	0	2	0	2	0	0
3	78	M	Pure solid	2.9	Invasive AD	IA	1b	0	0	0	0	0	0	0
4	63	M	Pure solid	2.4	Invasive AD	IIIA	1b	2	17	37	9	21	8	16
5	59	F	Pure solid	3.5	Invasive AD	IB	2a	0	0	0	0	0	0	0
6	62	M	Pure solid	2.8	Invasive AD	IA	1b	0	0	5	0	5	0	0

CT: computed tomography; CTC: circulating tumor cell; M: male; F: female; AD: adenocarcinoma; C: clinical; T: tumor; N: node

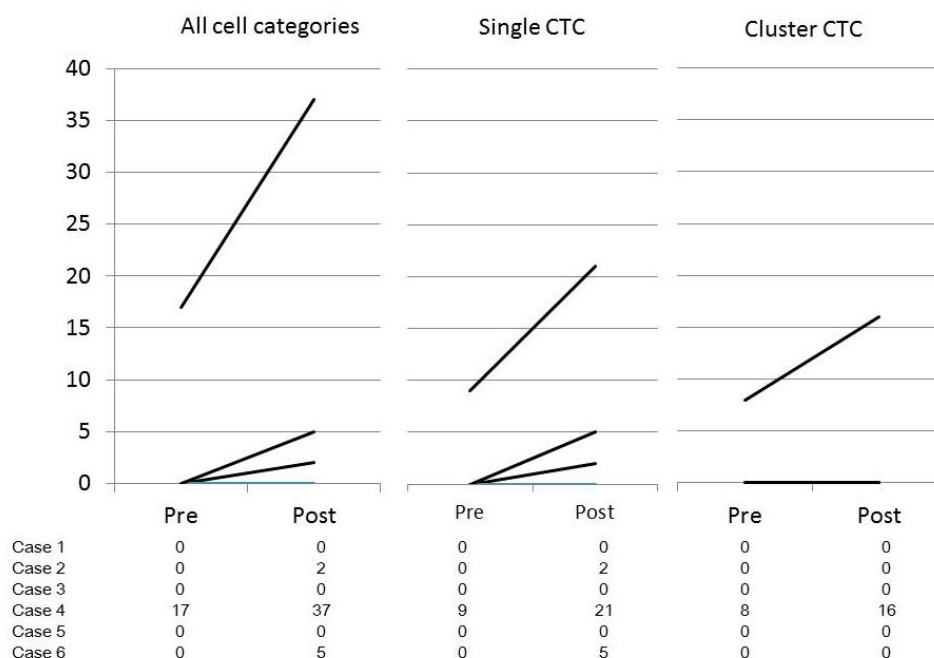


Figure 2: Alteration of circulating tumor cell count at pre- and at post-flexible fiber-topic bronchoscopy biopsy of lung cancer. CTC: circulating tumor cell

method of micropore membrane size selection. This let us that the FFB procedure causes CTCs dislodgement into the circulating blood, with distinguishing cluster CTC that may be a crucial indicator of tumorigenesis.^[26]

In addition to tumor cell dissemination into the circulating blood by surgical manipulation and TBB, the pleural cavity and the surgical margin are other areas where cancer cells can be disseminated by interventions such as FNAC and pulmonary wedge resection. Sawabata *et al.*^[2] demonstrated cancer cell dissemination from a lung cancer lesion through the needle tract at the visceral pleura. This phenomenon may support the high rates of relapse as pleural carcinomatosis in surgical patients with lung cancer who undergo FNAC.^[3,4] In cases of pulmonary wedge resection of lung cancer, malignant positive margin detected by cytology is an indicator of recurrence and poor survival,^[6] while attaining a malignant-negative margin of pulmonary wedge lung cancer resection is speculated to be an indicator of good prognosis. Altorki *et al.*^[27] reported a prognosis of pulmonary wedge lung cancer resection similar to that of segmentectomy when a wedge resection was carried out with sufficient margin distance and malignant-negative surgical margin.

In addition to surgical margin dissemination, residual lung lobe is another area where tumor cells are disseminated during pulmonary wedge lung cancer resection.^[28] The possibility of tumor cells in the residual lung lobe parenchyma is related to the margin distance

from a cancer to the margin of the pulmonary wedge resection, and a detection of clustered tumor cells is an indicator of early recurrence.^[28] These observations in FNAC and surgery suggest that the importance of controlling cancer cell dissemination is as great in those settings as in TBB.

Although this study has the limitation of a relatively small patient population, results using the size selection method revealed an increased number of both singular and cluster CTC post-FFB biopsy, therefore, further investigation into the implications of such CTCs is recommended.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

All patients provided their informed consent to participate in this study.

Ethics approval

This investigation was approved by the institutional review board of the Hoshigaoka Medical Center (No. 1412) in April 4, 2014.

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Use of a dedicated day care unit in a modern radiation oncology facility -- a short audit

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Our radiotherapy department specializes in all types of conformal therapy including stereotactic body radiotherapy, stereotactic radiosurgery and brachytherapy including interstitial and prostate seed implant. The department also includes a dedicated four-bed day care unit attached to the integrated brachytherapy suite. It is managed by a resident doctor, along with four staff nurses under the supervision of a radiation oncologist. We performed an audit of our day care unit to gain insight into its utilization and needs.

We decided to consider each encounter (admission to the unit) as one event. We collected data from one year, from April 2014 to March 2015. The relevant information was collected and tabulated using the hospital's electronic recording system. We then performed an analysis of the frequency of various events within the unit. Results were summarized in one composite bar diagram.

A total of 504 events occurred in the day care unit during the one-year period. The results are summarised in Figure 1. The mean length of day

care stay was six hours out of the 504 encounters, 3 resulted in patients being shifted to the inpatient department for further care.

This is the first time in medical literature that the use and utility of a radiation oncology day care unit has been recorded and presented. Most of the insurance companies require patient admission for initiation of medical coverage,^[1,2] therefore many of our patients used the day care facility to satisfy this insurance requirement and also save money and time. The added advantage of the day care unit for the patient is that the patient becomes acquainted with the department and most of the departmental staff on the first day, making the remainder of treatment more comfortable.

In the department besides these four beds, we also have four other beds where admissions were not recorded. They are meant for use by our patients during initiation and continuation of treatment for the same purposes as the four beds we have described above.



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The summary of encounters at our day care from April 2014 to March 2015 (n = 504)

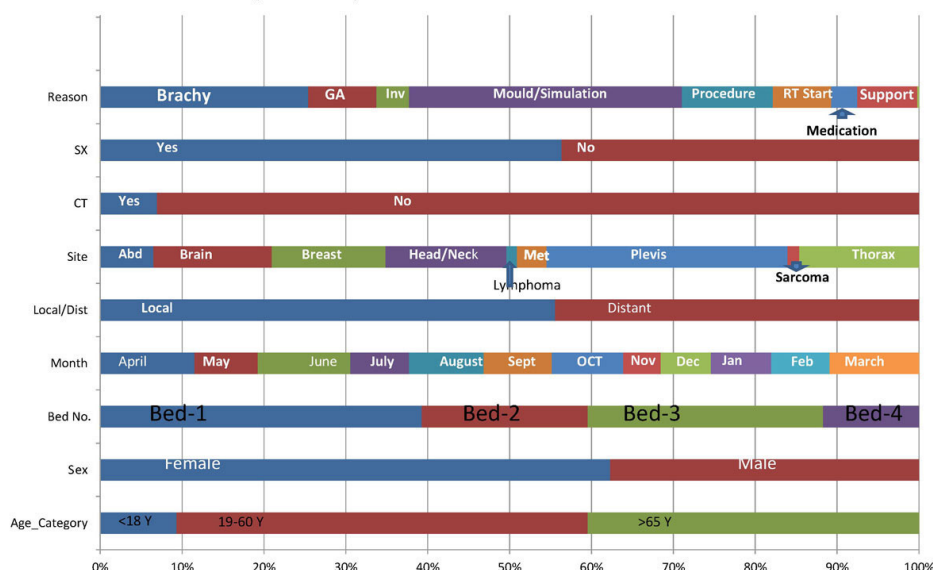


Figure 1: Reason-Reason for admission, Sx-Post surgery, GA-General anaesthesia, support-Supportive care, CT-Patient on concurrent Chemotherapy by medical oncology, Met-Treated for metastasis, Local/Distant-Measure of daily travel by patient; RT start-starting of radiation therapy. Procedure: minor invasive procedures

The day care unit remains the heart of our on-treatment patient coordination activities for several reasons. The unit helps provide personalized care to each patient in each visit, and offers suggestions for patient health problems. It also provides supportive care to patients in the form of IV fluids and injectable analgesics, under supervision. The day care unit also provides specialized procedures like brachytherapy and recovery from short-term anaesthesia.

Studies of the needs and patterns of admissions of oncology and radiotherapy patients are increasing in number. Serious needs can lead to unplanned admission for many radiation patients.^[3] The admissions in oncology are also patients who deserve serious attention.^[4] Patients who come for elective brachytherapy, radiotherapy, or simply to satisfy insurance requirements, can receive mild to moderate supportive care, which can be well managed in an fully-equipped day care unit. If needed, the patients can be shifted to an inpatient ward for further care or intervention. This approach will effectively save resources for the institution and will also be less taxing for the patients and the insurance companies.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Patient consent was obtained from the patients.

Ethics approval

This article does not contain any intervention with human participants or animals.

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A versatile method for enumeration and characterization of circulating tumor cells from patients with breast cancer

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ABSTRACT

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Key words:

Circulating tumor cells,
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circulating tumor cell enumeration,
quantitative reverse transcription
polymerase chain reaction,
EpCAM

Aim: To establish a standardized protocol for the isolation and enumeration of circulating tumor cells (CTCs) from peripheral blood of patients with metastatic breast cancer. **Methods:** The protocol used tumor cells spiked in a lymphoid cell line with detection by flow cytometry and quantitative reverse transcription polymerase chain reaction (QRT-PCR). Cells of the human mammary cancer subtypes were spiked into Jurkat cells, which served as the lymphocyte designate in numbers from 10 to 500 per 10⁵ Jurkat cells. This mixed population was probed for CD45, EpCAM, and pancytokeratin acquired from flow cytometry and characterized by microscopy. QRT-PCR was done for CK-19, MUC-1, EpCAM, and GAPDH. Validation was attained with blood samples from 22 patients with metastatic breast cancer and 20 healthy individuals. **Results:** Flow cytometry could detect 1 breast cancer cell per 100,000 Jurkat cells, with similar detection levels in the breast cancer subtypes. Samples from patients with breast cancer showed a range of CTCs from 1-85 per 10 mL of blood. Quantitation of expression for EpCAM, CK-19, Muc-1, and Her2neu confirmed the presence of CTCs in 76% of samples. **Conclusion:** Density gradient and immunomagnetic enrichment accomplished isolation of CTCs and quantitation was achieved using flow cytometry. Combined QRT-PCR and imaging further validated these findings, rendering a robust methodology.

INTRODUCTION

Circulating tumor cells (CTCs) in peripheral blood has emerged as an important surrogate marker for prognosis of cancer.^[1-4] Various studies have demonstrated the presence of CTCs in peripheral blood of patients

and their association with tumor progression and metastatic development.^[5-7] Reports have also shown that a change in CTCs number predicts response to therapy and can evaluate residual disease.^[8-11] Hence, to establish CTC number and molecular characteristics, a necessary requirement is a feasible



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and suitable approach.

There are several technologies which have been developed and are available for CTC enrichment and detection as cited in literature.^[12,13] The current standard method that has been widely used in large multicenter clinical trials world-wide and which continues to be preferred is the FDA-approved quantitative, semi-automated, Cell Search system,^[14,15] which assesses 7.5 mL of blood. It is based on the epithelial cell adhesion molecule (EpCAM/CD326) conjugated immunomagnetic enrichment followed by microscopic imaging using positive immunostaining of cytokeratin (CK), negative immunostaining of leucocyte common antigen CD45, and DNA staining with DAPI.

The literature shows that almost 60% of studies on detection and enumeration of CTCs have preferred methods using reverse transcription polymerase chain reaction (RT-PCR) because of its sensitivity and low cost as compared to using the Cell Search technology. One such RT-PCR kit-based technology is the Adnagen assay.^[16,17] This entails enrichment on the basis of epithelial and tumour-specific antigens, isolation of RNA, conversion to cDNA and subsequent amplification of tumour-associated genes and a control gene for normalization. Amplified cDNA samples are then analyzed on a chip system (Bioanalyzer) and simultaneously compared with positive controls of each gene provided in the kit.

Strategies using cell-imaging, quantitative RT-PCR (QRT-PCR)^[18] and flow cytometric detection^[19-21] for enumeration and characterization of CTC have also been employed.

The purpose of this study was to devise a feasible, relatively low-cost methodology which would enumerate CTCs as well as validate their assessment. The protocol for enumeration was developed using tumor cells from cell lines of breast cancer subtypes spiked in a lymphoid cell line and its quantitation by flow cytometry. Cells of human mammary cancer subtypes were spiked into a Jurkat cell line which served as the lymphocyte designate in numbers from 10 to 500 per 10^5 Jurkat cells. This mixed population was then probed for CD45, EpCAM, and pancytokeratin acquired on a flowcytometer and quantitated. Similar spiking experiments were done for QRT-PCR and expression of CK-19, MUC-1, EpCAM, and GAPDH. The methods described by others^[22,23] have been adapted for quantitation of CK 19, EpCAM, MUC-1, and Her2neu. Data pertaining to these methods have been generated and any one of these methods can be used. Our method will overcome the differences

in expression of the selected genes across clinical samples, estimate copies/mL in blood samples and enable direct comparison of samples across time points of collection. Thus, besides characterization, a quantitative measure of gene expression translating to numbers of CTCs can be obtained.

This methodology was substantiated with blood samples obtained from patients and healthy normal individuals. Blood from 22 patients with metastatic breast cancer and 20 healthy individuals was separated on a Ficoll gradient to obtain the peripheral blood mononuclear cells (PBMCs) fraction, positively enriched for EpCAM, quantitated by flow cytometry and validated with QRT-PCR for the presence of CTCs.

METHODS

Materials

DMEM, RPMI 1640, Fetal Calf Serum (FCS), Trizol, High capacity cDNA kit, TaqMan assays for CK-19, EpCAM, MUC1, Her2neu and GAPDH, 2 X TMM, and PBS were procured from Invitrogen Life Technologies (Carlsbad, CA, USA). RNeasy plus micro kit was from Qiagen, GmbH, D-40724, Hilden, Germany. FcR blocking reagent, CD326 (EpCAM) Microbeads, MACS BSA Stock Solution, rinsing buffer, and washing buffer were purchased from Miltenyi Biotec GmbH, Germany. Ficoll-Paque PLUS was procured from GE Healthcare, Bio-Sciences AB, Uppsala, Sweden. EDTA vacutainer tubes were from Greiner Bio-One, Austria. All other chemicals were obtained locally and were of analytical grade.

Antibodies

CD326 (EpCAM) antibody conjugated to APC (clone HEA-125), pan-Cytokeratin antibody conjugated to FITC (clone CK3-6H5), CD45 antibody conjugated to PerCP (clone 30F11.1), mouse IgG1 isotype control antibodies (clone-LS5-21F5) conjugated to FITC, APC, and PerCP were purchased from Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany.

Buffers

Separation buffer composition: phosphate-buffered saline (PBS; pH 7.2), 5% bovine serum albumin (BSA), 2 mmol/L EDTA prepared by diluting MACS BSA Stock Solution with auto MACS™ rinsing solution (1:20); FACS buffer contains 0.02% NaN_3 , 1% FCS in PBS (pH 7.4); Saponin buffer for permeabilization contained 0.1% saponin in FACS buffer.

Cell lines

Human mammary carcinoma cell lines: MCF-7,

T-47D, ZR75-1, BT-474, and MDA-MB-468 and the lymphoid cell line, Jurkat, were used for cell spiking and standardization of the flow cytometry assay, and qPCR. MCF-7, BT-474, and MDA MB-468 were maintained in DMEM containing 10% FCS; T-47-D and ZR75-1 in RPMI-1640 with L-glutamine (2 mmol/L) and 10% FCS; Jurkat in DMEM containing 10% FCS and antibiotic mixture containing gentamycin, streptomycin and anti-fungal, Forcan. Cell line ZR75-1 was purchased from the National Centre for Cell Science, Pune, India. All other cell lines were available in the institute or laboratory and procured for ongoing studies.

Patients and controls

A total of 22 breast cancer patients with metastatic disease were included in this study. In addition, blood samples from 20 healthy volunteers were accrued. After collection, blood samples were immediately processed for isolation and detection of CTCs.

Blood collection and PBMCs isolation

Peripheral blood (25 mL) was drawn by phlebotomy in EDTA vacutainer tubes. The initial 5 mL was routed for other routine tests to avoid contamination with cells from the skin and blood vessels. Tumor cells were isolated along with the PBMCs on a Ficoll Hypaque gradient. Blood diluted with PBS was layered over Ficoll Hypaque and centrifuged at 400 *g* for 30 min at 25 °C. The interphase was collected, washed twice, with separation buffer, spun at 400 *g* for 15 min at 4 °C to obtain a cell pellet, which was suspended in separation buffer and the total yield and viability was assessed.

Immunomagnetic enrichment and CTCs isolation

CTCs were enriched from the total cell pellet. Depending on the total cell count, initially 50-100 µL of FcR blocking reagent (Miltenyi Biotec) was added to block Fc receptors and eliminate non-specific binding. Subsequently, 50-100 µL of micro beads conjugated to monoclonal antibody for EpCAM (Miltenyi Biotec) was added and this mixture was incubated for 30 min at 4 °C. Subsequently, cells were washed and re-suspended in 700 µL of separation buffer. This labeled cell suspension was then acquired on autoMACS Pro® Separator (Miltenyi Biotec) with a double-positive selection. The positive fraction constituted cells that expressed EpCAM and thus was an enriched fraction of EpCAM-expressing cells. The negative fraction constituted lymphocytes, cells that do not express EpCAM and flow through the column. The positive and negative fractions were divided into two aliquots: one for multi-parameter flow cytometry, the other for

quantitative RT-PCR.

Cell spiking experiments

Human mammary cancer cell lines representing the molecular subtypes were grown as adherent cultures and harvested using sterile trypsin-EDTA solution at 80% confluency. The Harvested cells were collected, washed, and re-suspended in a fixed volume of culture medium. An average of 3 cell counts and viability were determined by the trypan blue dye exclusion method. This cell suspension was used for serial dilution in cell spiking experiments and cells were spiked into the Jurkat cell line, which served as the WBC designate of the PBMC fraction. Jurkat cell number was selected on the basis of the average cell count obtained in the positive fraction following immunomagnetic enrichment of 20 mL of peripheral blood, which is approximately 1×10^5 cells counted with a hemocytometer. Human mammary tumor cells were spiked into 1×10^5 Jurkat cells at levels ranging from 0.5% to 0.001%. These cell suspensions were labeled with antibodies as described below, acquired on the flowcytometer and the percentage expected to the percentage recovered was plotted as shown below.^[24] For each sub-type, experiments were repeated at least thrice.

Antibody labeling and acquisition on flow cytometer

Serially diluted samples of tumor cells spiked into Jurkat cells were surface-stained with monoclonal antibodies that target epithelial cell antigens EpCAM (CD326) and CD45 or the corresponding isotype control antibody by incubating the cells in 50 µL of FACS buffer for 30 min in the dark at room temperature. Cells were then washed with FACS buffer and fixed with 1% paraformaldehyde for 15 min at 4 °C before permeabilization for intracellular staining. To permeabilize cells, the pellet was resuspended in 0.1% saponin buffer. Cells were subsequently stained with anti-pan CK antibody or the relevant isotype control and incubated for 45 min in the dark at room temperature. After staining, cells were washed with saponin buffer, resuspended in 300 µL of FACS buffer, and immediately acquired on a FACS Aria™ flowcytometer (Becton Dickinson, USA), which is equipped with a 488 nm blue laser (for excitation of FITC and PerCP) and a 633 nm red laser (for excitation of APC) and the following filters: FITC: 530 nm band pass; PerCP: 670 nm long pass; APC: 660 nm band pass. Setup and automatic compensation were performed using cells stained for each marker. PMT voltages used for recording fluorescence signals were as follows: FITC = 429, PerCP = 599, APC = 396. The absolute number of spiked tumor cells was estimated by acquiring a minimum of 10,000 events in the analyzed sample. Data were analyzed with BD

FACSDiva™ software v6.1.3 (BD Biosciences).

RNA isolation and cDNA synthesis

A Trizol/RNeasy hybrid RNA extraction protocol was used for isolation of total RNA under RNase-free conditions. The aqueous phase obtained was passed through a gDNA eliminator column to remove genomic DNA contamination. Total RNA isolated from each sample was suspended in RNase-free water. Concentration and purity were determined on Nanodrop ND-1000 and stored at -80 °C until further use. cDNA synthesis was carried out with high capacity reverse transcriptase kit (Applied Biosystems) according to manufacturer's instructions.

Preparation of qRT-PCR calibrators

QRT-PCR calibrators were prepared adapting the Aerts *et al.*^[22] method with selection of the average cell number obtained in the positive fraction post-immunomagnetic enrichment from patient samples. Serial dilutions corresponding to five log steps of cell number by diluting RNAzol lysates of MCF-7 in the WBC cell line (Jurkat) with ratios of 1:1 to 1:10⁴ of CK19+ and EpCAM+ cells per CK19- and EpCAM-cells were made. Dilution of lysates was performed to avoid the need for permanent maintenance of cell cultures for preparation of cell-cell dilutions. RNA extraction and cDNA synthesis were performed as described. Each sample was measured in triplicate.

We also prepared qRT-PCR calibrators by adapting the Strati *et al.*^[23] method as a qRT-PCR requires analysis of samples across time frames. Individual PCR amplicons corresponding to gene-targets CK-19, EpCAM, MUC-1, and Her2Neu that could serve as quantification calibrators were generated. For this purpose, total RNA was extracted from MCF-7 and BT474 cells, DNase-treated, and quantified. cDNA was synthesized from 1 µg of DNase-treated RNA using a high capacity cDNA kit according to manufacturer's instructions. PCR for each target gene was carried out with TaqMan primers on a Bio-Rad Peltier Thermal cycler to ensure production of calibrators from the same amplicon that would be synthesized during the qRT-PCR of clinical samples. PCR amplicons were separated on a 3% Agarose gel, excised under UV, purified using QIAquickGel Extraction Kit (Qiagen), and quantified on Nanodrop. Copy number was calculated from the concentrations using Avogadro constant and molecular weight of each amplicon number of bases of the PCR product multiplied by the mean molecular weight of a pair of nucleic acids, which is 660. Serial dilutions of these stock amplicons were made in TE buffer (1 × 10⁶ copies to 1 × 10⁰ copies) and used as quantification calibrators throughout

the study. For quantification of a gene transcript, a TaqMan quantitative PCR assay was done for the gene transcript for every dilution in duplicate and an external calibration curve was obtained by plotting the concentration as copy number vs. the corresponding threshold cycle.^[25] Each of these calibration curves were repeated at least thrice.

Quantitative PCR

TaqMan assays were done for cytokeratin-19 (CK19), epithelial cell adhesion molecule (EpCAM), Mucin1 (MUC1), Her2-neu, and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Reactions for all the above genes were performed with 10 µL volume with cDNA corresponding to 20 ng. All the samples were analyzed in duplicates and the average value of the two was used as the quantitative value. A non-template control, also in duplicate, was used for all genes.

Statistical analysis

For all groups studied, values were represented as mean ± SD. A simple linear regression model was used to assess the fit for the recovery of expected cells.

RESULTS

Flow cytometry for CTCs analysis

A tricolor setup was configured to detect the CD45 PerCP signal in 670 nm long pass, EpCAM-APC signal in 660 nm bandpass, and Cytokeratin-FITC signal in 530 nm band pass filter. Events that fell within the region P2, i.e. EpCAM, and CK dual-positive were counted as meeting criteria for human tumor cells [Figure 1A and B]. Thresholds for specific EpCAM APC and CK FITC signals were determined using the sample stained with isotype control antibodies. The same gating strategy was then applied for detecting EpCAM+CK+CD45- cells in the clinical sample stained with the specific antibodies.

Specificity (ability to differentiate between epithelial tumour cells and WBC designate)

EpCAM-APC (clone HEA-125) and Pan-Cytokeratin-FITC (clone CK3-6H5) Abs were found to be specific for human mammary cancer cells, with no non-specific binding to Jurkat cells [Figure 1C-E]. Similarly, human CD45 PerCP antibody (clone 30F11.1) was found to be highly specific for leukocytes and did not stain human mammary tumour cells [Figure 1F-H]. To determine applicability of the method to various human mammary cancer cell lines, the ability to detect MCF-7, T-47D, ZR-75-1, BT-474, and MDA-MB-468 spiked in Jurkat cells was tested. In all cases, tumor cells exhibited similar staining to EpCAM and CK antibodies and could be clearly differentiated from Jurkat cells.

Sensitivity (ability to detect lowest number of epithelial tumor cells)

The assay of serial dilutions (0.001-0.5%) of human breast tumor cells in Jurkat cells established that the lower detection limit for sensitivity of the method was 0.001% or 10^{-5} , corresponding to 1 human cell per 100,000 Jurkat cells. Recovery and linearity were highly reproducible across separate experiments [Figure 2] and the number of tumor cell events could be positively correlated. Linear correlation and regression analysis showed $R^2 > 0.98$ for ZR-75-1, BT-474, and MDA-MB-468 cell lines, whereas in the case of MCF-7 and T-47D, R^2 was 0.9465 and 0.9589, respectively [Supplementary Figure 1]. The percentage of tumor cells recovered was not significantly different from the percentage of tumor cells expected, based on serial dilutions.

Recovery

Recovery of the flow cytometry protocol was determined by spiking 1-500 tumor cells in 1×10^5 Jurkat cells. The gating strategy for detection of EpCAM+CK+CD45-cells is as shown in Figure 1A and B. Recovery of spiked cells was highly linear and revealed a mean recovery of 75% of spiked tumor cells (range: 43-

100%), as shown in Table 1.

Analysis of CTCs in patients

To validate the use of this technique in a clinical setting, 17 patient samples and 13 normal controls were assessed. As described in Methods, the PBMC fraction was separated from peripheral blood sample with an average PBMC cell count of $20.2 \times 10^6 \pm 9 \times 10^6$ cells for patient samples and $23.7 \times 10^6 \pm 8.6 \times 10^6$ cells in normal controls [Supplementary Figure 2]. CTCs were enriched from the PBMC fraction using immunomagnetic double-positive selection for EpCAM. The number of cells in the positive fraction represented cells that were enriched for EpCAM and, as the number was very low, the hemocytometer count would not be an appropriate representation. CTCs would be detected in this fraction. Average cell number obtained in the negative fraction (as described in Methods) for the patient sample was $17.3 \times 10^6 \pm 5.7 \times 10^6$ cells and in the normal control was $15.6 \times 10^6 \pm 8.03 \times 10^6$ [Supplementary Figure 2 and Supplementary Table 2]. The pre-enrichment fraction, positive and negative fractions, and controls were acquired and analyzed by flow cytometry. CTCs were selected on the basis of cell size and presence of EpCAM and CK. Figure 3

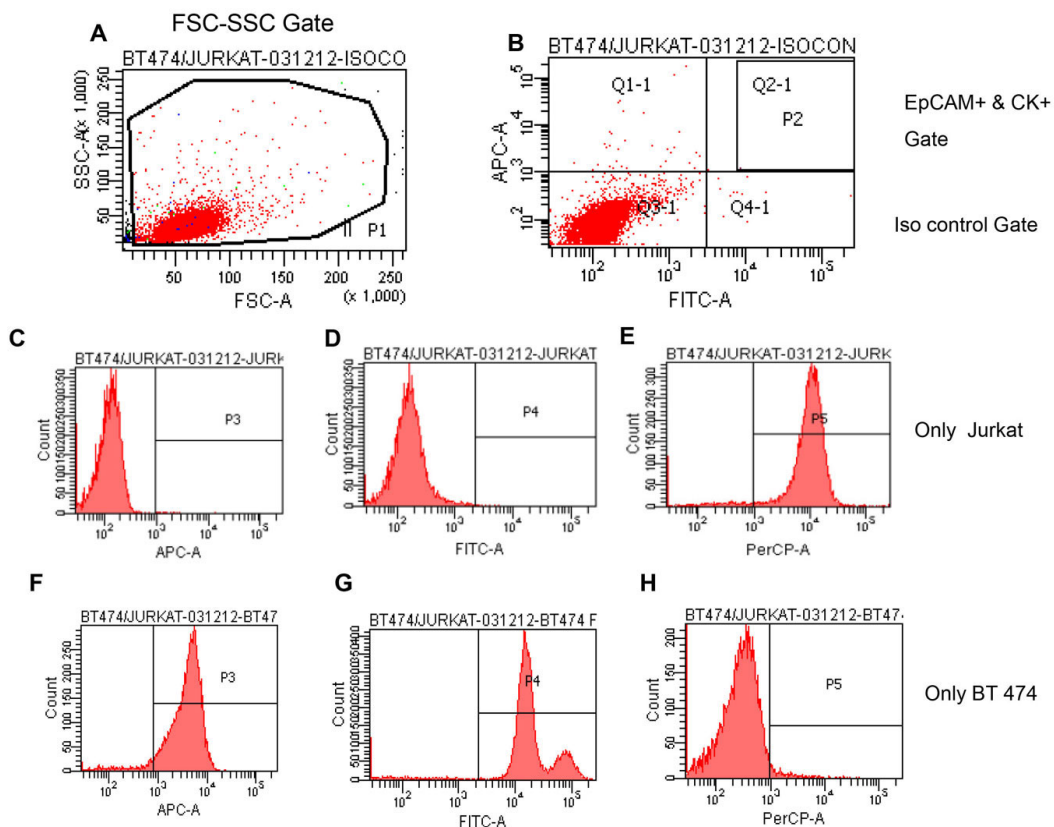


Figure 1: EpCAM+CK+CD45- tumor cells detected by flow cytometry by first gating out cell debris and cell clumps in forward/side scatter plot (A); threshold for specific EpCAM (APC) and CK (FITC) signals determined using sample stained with isotype control antibodies. Gates set to have no positive events above these thresholds in control sample (B); representative histograms shown of individual criteria gated on (C-E) Jurkat cells and (F-H) BT-474 human tumor cells

Table 1: Recovery of spiked cancer cells as determined by flow cytometry analysis (%)

Tumor Cells Expected	MCF-7			T47 D			ZR 75-1			BT 474			MDA MB 468		
	Mean tumor cells recovered	Average recovery	Mean tumor cells recovered	Mean tumor cells recovered	Average recovery	Mean tumor cells recovered	Mean tumor cells recovered	Average recovery	Mean tumor cells recovered	Mean tumor cells recovered	Average recovery	Mean tumor cells recovered	Mean tumor cells recovered	Average recovery	Average recovery
0.001	0.00575	> 100	0.006	0.006	> 100	0.0078	0.0078	> 100	0.006	0.006	> 100	0.00575	0.00575	> 100	> 100
0.005	0.0065	> 100	0.015	0.015	> 100	0.016	0.016	> 100	0.008	0.008	> 100	0.00825	0.00825	> 100	> 100
0.01	0.00975	97.5	0.01433	0.01433	> 100	0.0178	0.0178	> 100	0.01	0.01	> 100	0.01025	0.01025	≈ 100	≈ 100
0.015	0.01475	98.3	0.015	0.015	100	0.0262	0.0262	100	0.014	0.014	> 100	0.01225	0.01225	81.6	81.6
0.02	0.01575	78.75	0.014	0.014	70	0.0328	0.0328	70	0.0198	0.0198	> 100	0.01825	0.01825	91.25	91.25
0.025	0.01875	75	0.019	0.019	76	0.0402	0.0402	76	0.0173	0.0173	> 100	0.0205	0.0205	82	82
0.05	0.02571	51.4	0.049	0.049	99	0.0726	0.0726	99	0.04	0.04	> 100	0.023	0.023	46	46
0.1	0.034125	34.13	0.0596	0.0596	60	0.085	0.085	60	0.072	0.072	85	0.06725	0.06725	67.25	67.25
0.5	0.137	27.4	0.216	0.216	43	0.415	0.415	43	0.415	0.415	83	0.3135	0.3135	62.7	62.7

and Table 2 show the number of CTCs identified in patient samples and normal controls. Double-positive cells (CK+, EpCAM+) were CTCs obtained from the positive-enriched fraction from patient blood samples and normal controls. The positive enriched fraction was split equally to be analyzed by flow cytometry and quantitative RT-PCR. Patient blood samples showed a range of 1-85 per 10 mL of blood, with an average of 23.35 ± 22.85 , and for normal controls the range was 0-14 per 10 mL of blood, with an average of 5 ± 4 .

Clinical characteristics of patients are as described in [Supplementary Table 1](#). Hormone receptor status showed that 10 (45%) were ER-positive, 6 (27%) ER-negative an equal number of 9 (41%) were PR-positive and PR-negative, 3 (14%) were Her2-positive, and 13 (60%) were Her2-negative.

Quantitative PCR

Quantification using cell line dilution series

Adapting the Aerts *et al.*^[22] method, a standard curve of ΔCt values was generated from the dilution series, as described in Methods, to determine the number of circulating tumor cells in a clinical blood sample by interpolation from this standard curve. This curve displayed a linear relationship between ΔCt values and the logarithm of marker-positive. As seen in [Figure 4](#) and [Supplementary Figure 3A and B](#), standard curves were generated for CK-19, EpCAM, and MUC1 using breast cancer cell lines T47D, MCF-7, and ZR-75-1. All Ct values were normalized to the values obtained from Taqman PCR for GAPDH as a “house-keeping” gene to overcome differences of efficiency of cDNA synthesis by formulae $\Delta Ct = Ct (CK-19/EpCAM/MUC1) - Ct (GAPDH)$. [Table 2](#) shows the cell numbers obtained by this method in patients with EpCAM, CK-19, and MUC-1.

Quantification using concentration (copy number) of the marker transcript

Based on the Strati *et al.*^[23] method, we developed a CTC gene expression qRT-PCR assay by using quantification calibrators for four gene transcripts (CK-19, EpCAM, MUC1, Her2) containing a known number of copies, prepared as described in Methods. These were synthesized as described and aliquoted to eliminate experimental variation. The basis for adopting this method was to enable comparisons of CTC analysis as copy number in clinical samples collected and analyzed at different time points, and also to allow for the possibility of a low detection limit such as 1 copy. [Figure 5](#) shows the calibration curve for the four selected transcripts in the range from 10^6 copy number to 1 copy, with a linearity observed from 10^6 copies to 10 copies with correlation coefficient close

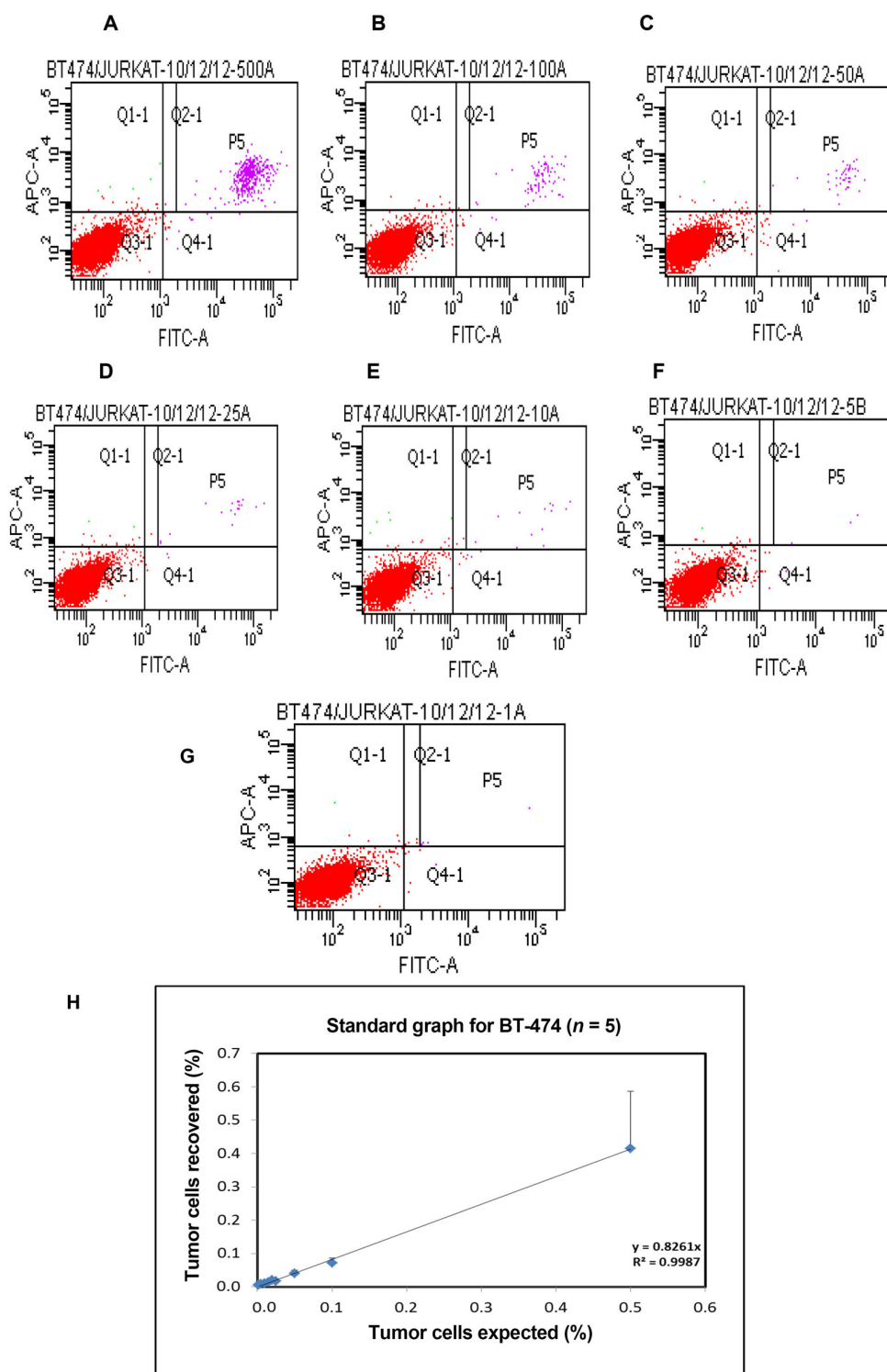


Figure 2: Enumeration of circulating tumor cell in spiking experiments. Representative BT-474 cells shown for (A) 0.5%, (B) 0.1%, (C) 0.05%, (D) 0.025%, (E) 0.01%, (F) 0.005%, (G) 0.001%; (H) Correlation and regression analysis of recovered versus expected number of positive tumor events at different dilutions

to 1 (0.99), indicating a precise log-linear relationship.

CTCs in patients and controls

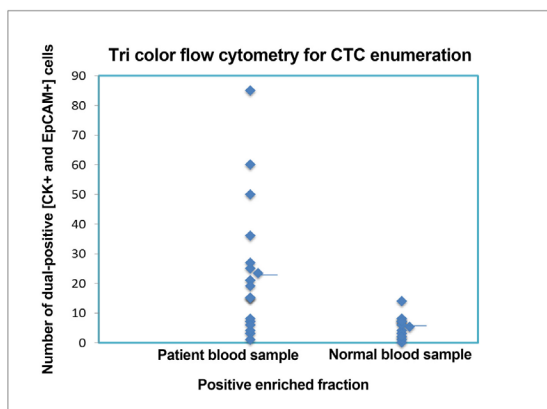
Quantitative PCR was done with 17 patient samples

and 15 controls. These were probed for at least three genes: *EpCAM*, *CK-19*, *GAPDH*. Her2 and MUC 1 were assessed in a few samples subject to the IHC report and availability of cDNA [Table 2]. All patients

Table 2: Tricolor flow cytometry for enumeration of CTC done as described in Methods (complements Figure 3)

Patient number	Number of dual positive cells [EpCAM+ CK+] CTCs
1	25
2	36
3	85
4	60
5	15
6	15
7	27
8	21
9	50
11	7
13	1
14	3
15	4
16	8
17	15
20	19
22	6
For 5 patients only RT-PCR no flow	
Normal individual number	
1	8
2	4
3	14
4	7
5	7
6	3
7	8
9	0
10	1
17	2
18	6
19	7
20	2
For 6 normal individuals only RT-PCR done	
Flow cytometry not done	
One sample lost during enrichment	

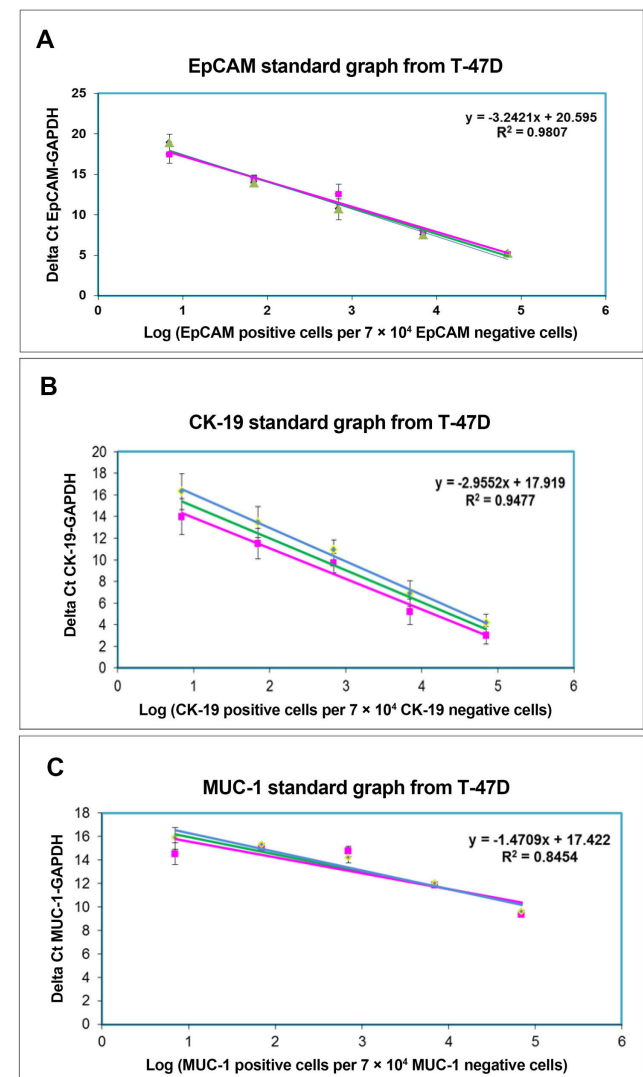
CTC: circulating tumor cell; RT-PCR: reverse transcription polymerase chain reaction; CK: cytokeratin

**Figure 3:** Tri color flow cytometry for CTC enumeration. CK+/EpCAM+ dual-positive cells represent CTCs seen in positive enriched fraction. The number of dual-positive cells in patient and normal healthy individuals is shown. CTC: circulating tumor cell

as well as control samples showed Ct values lower than 30 for GAPDH. From the 17 patient samples, 14 (82%) gave Ct values below 36. Of the 14, 9 (64%) were found to be positive for two marker genes, 10 (71.4%) for EpCAM, 9 (64%) for CK19, and 6 (43%) for Muc-1. There were two samples that were positive for all the four markers, with a high copy number recorded for Her2. Her2 expression levels were comparable to protein levels obtained by IHC, which was reported as 3+. In the 15 control samples, 4 recorded Ct values that could be converted to copies/mL and these values were below 1.

Imaging CTCs

In blood samples from patients, where CTCs were higher, captured cells were subjected to morphological characterization and examined for presence of a

**Figure 4:** Standard curve for Aerts *et al.*^[22] method for T47-D cell line. (A) Log number of EpCAM-positive cells versus Delta Ct (EpCAM-GAPDH); (B) log number of CK-19-positive cells versus Delta Ct (CK-19-GAPDH); (C) log number of MUC-1-positive cells versus Delta Ct (MUC-1-GAPDH)

nucleus. **Figure 6** shows representative images of two CTCs characterized as EpCAM and CK positive, with a well-defined nucleus and were negative for the leucocyte antigen, CD45. Imaging further confirmed

the presence of CTCs in samples.

DISCUSSION

We propose a workable method for the isolation and enumeration of CTCs wherein a two-tier protocol of cell isolation with an initial separation based on density gradient centrifugation, followed by EpCAM immunomagnetic-positive double enrichment has been described and adopted. The enriched fraction of tumour cells is further divided, one analyzed for enumeration of CTCs using flow cytometry based on large size of tumor cells, with the presence of CK, EpCAM, and the absence of CD45. Dissimilar expression levels of EpCAM could compromise the detection of CTCs,^[26,27] hence, the initial standardization for flow and QRT-PCR analysis was done with cell spiking of cancer cells from different breast cancer subtypes to assess possible differences. Tumor cells from the different subtypes exhibited similar staining to EpCAM and CK antibodies and could be clearly distinguished from Jurkat cells. As described for serial dilutions **[Figure 2]** recovery and linearity showed reproducibility and were highly consistent across independent experiments. A positive correlation was observed between recovered tumor events and expected tumor events. Based on the serial dilution assay, the percentage of tumor cells recovered was not significantly different from the percentage of tumor cells expected.

The flow cytometry protocol was validated with blood samples obtained from patients with metastatic breast cancer. Eighteen of these patients were with tumor grade II-III and 17 were diagnosed with verified metastasis. Clinical characteristics are as described in **Supplementary Table 1**. Median age was 50 years (range: 25-76 years). All, except two, were diagnosed as invasive ductal carcinoma, the most common type of breast cancer. Hormone receptor status showed 10 (45%) were ER-positive, 6 (27%), ER-negative, equal number 9 (41%) PR-positive and PR-negative, 3 (14%) Her2-positive, and 13 (60%) Her2-negative. CTCs showed a range of 1-85 per 10 mL of blood, with an average of 23.35 ± 22.85 . Twenty healthy women volunteers were also included in this validation, with values ranging from 0-14 per 10 mL of blood, with an average of 5 ± 4 . A cut-off of 10 and above has been selected, based on these results.

For clinical samples where CTCs were higher, captured cells were subjected to morphological characterization and examined for presence of a nucleus. **Figure 6** shows representative images of two CTCs characterized as EpCAM and CK positive, with a well-defined nucleus and negative for the

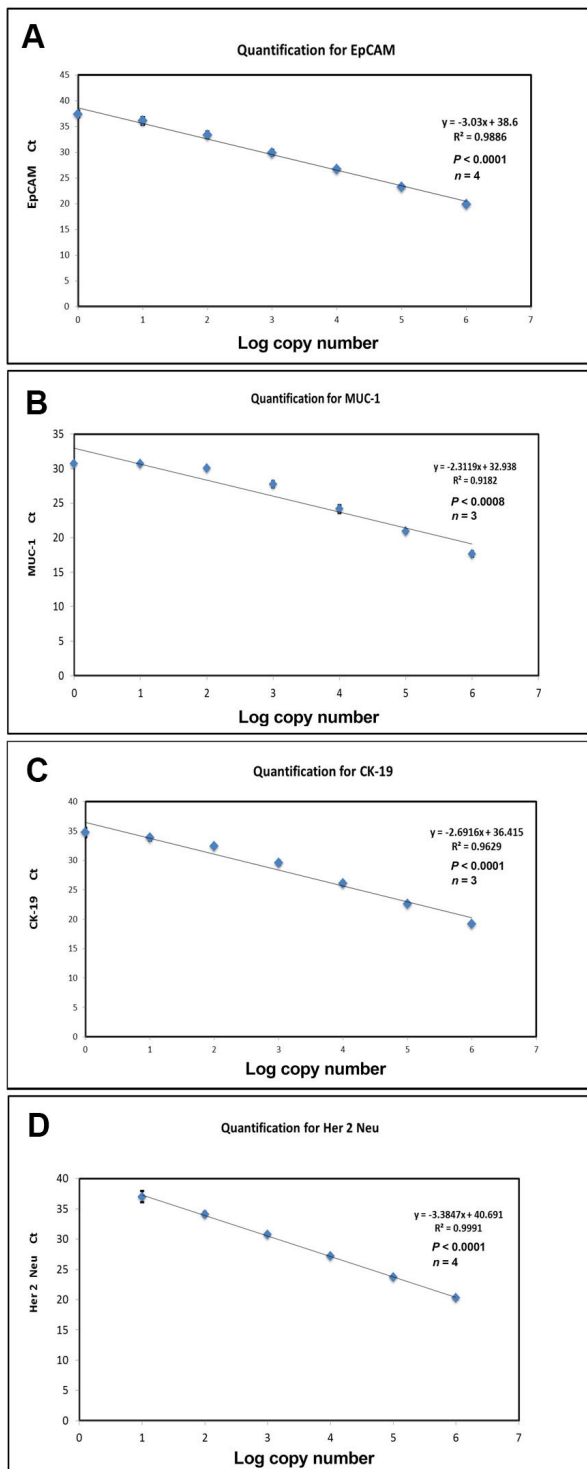


Figure 5: Standard curve with Strati *et al.*^[23] method done as described in the text. (A) Log number of EpCAM copies versus EpCAM Ct values; (B) log number of CK19 copies versus CK19 Ct values; (C) log number of Muc-1 copies versus Muc-1 Ct values; (D) log number of Her-2 copies versus Her-2 Ct values

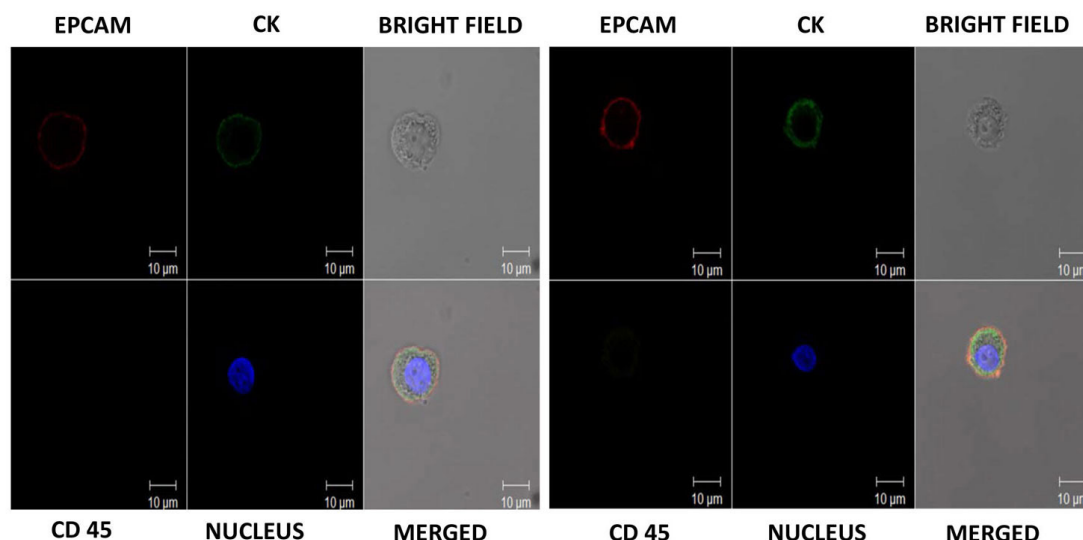


Figure 6: Images of captured CTCs from blood sample of a patient with breast cancer. CTCs are positive for EpCAM and CK with the presence of a well-defined nucleus and negative for the common leucocyte antigen CD45. CTCs: circulating tumor cells; CK: cytokeratin

leucocyte antigen, CD45. Immunophenotyping and imaging further confirmed the presence of CTCs in samples.

Determination by quantitative RT-PCR for CK 19, EpCAM, and MUC-1 in the enriched fraction was also a specific approach which was applied in validating CTCs numbers obtained by flow cytometry. Further, to overcome representation of results as expression in relative fold and to show absolute quantitation, the Strati *et al.*^[23] and Aerts *et al.*^[22] methods were adapted. These methods can overcome differences in expression of the selected genes across samples, estimate the quantities as copies/mL in blood samples, and enable direct comparison of samples across time points of collection.

Sixty-four percent of patient samples showed concordance with flow and RT-PCR evaluation of EpCAM and CK. The lower number was because all samples were not analyzed by both methods and five for each group were analyzed by only one method.

We have developed a protocol that is technically feasible and economically viable in the laboratory settings for the study of CTCs. In the literature presence of CTCs has been correlated with poor prognosis as well as progression-free survival, and CTCs have been identified as indicators of treatment efficacy in different tumours.^[6,28-30] Their role in the metastatic process is endorsed by several studies, yet their clinical use for tumour staging, disease monitoring, and choice of treatment seems a distant reality. Never the less, monitoring CTCs has the potential to gauge the extent of disease and serve as a liquid biopsy. Attempts to formulate economically

viable and feasible protocols become an essential requisite.

Financial support and sponsorship

None.

Conflicts of interest

There are no conflicts of interest.

Patient consent

The patient consent was obtained for all patients participating in this study.

Ethics approval

Blood samples collected were from an approved study and all the human subjects participating in the study have given the required informed consent.

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Alkaline phosphatase flare with hyperostosis of bone metastases in lung adenocarcinoma treated with gefitinib

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ABSTRACT

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Alkaline phosphatase,
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Alkaline phosphatase (ALP) flare has been reported to occur during cancer treatment as a favorable event, particularly in the presence of bone metastasis. There have been only a few reports in lung cancer and associated radiographic findings have seldom been described. The authors observed ALP flare in a female patient with lung adenocarcinoma soon after the initiation of gefitinib. Moreover, on computed tomography, metastatic lesions of the rib and thoracic spine showed marked hyperostosis, with sizes larger than the original bone structure, suggesting efficacy of gefitinib. The significance of such hyperostosis should be elucidated.

INTRODUCTION

Alkaline phosphatase (ALP) flare is known as a transient elevation of serum ALP value in cancer patients with bone metastasis, particularly in breast or prostatic cancer.^[1] It is generally accepted that ALP flare emerges when systemic treatment is effective, since osteosclerotic change of the osteolytic lesion is seen radiographically.^[2,3] Although osteosclerosis without ALP flare has been well documented,^[4,5] ALP flare is reported to occur in only 5% of non-small cell lung cancer (NSCLC) patients treated with epidermal growth

factor tyrosine kinase inhibitor (EGFR-TKI).^[6] Since the frequency is relatively low, precise characteristics of ALP flare in NSCLC have not yet been elucidated, but it seems important not to misinterpret ALP flare as a progression of bone lesion.^[4] We report a patient who presented ALP flare and unusual hyperostosis of metastatic bone lesions shortly after initiation of EGFR-TKI for lung adenocarcinoma.

CASE REPORT

A 66-year-old woman visited our department



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complaining of backache that had developed two weeks before. She had no history of smoking. Physical examination revealed no significant findings. However, chest X-ray showed a mass shadow at the left upper lung field, and computed tomography (CT) confirmed a tumor of 4.5 cm in diameter with irregular margins at the left upper lobe. The tumor was accompanied by ground glass-like consolidation [Figure 1A]. Osteolytic lesions of the fourth thoracic spine [Figure 2A] and the fourth left rib [Figure 2B] were also shown. Laboratory data demonstrated elevation of serum carcinoembryonic antigen (CEA) [Table 1]. ALP was also elevated at 379 IU/L (normal range from 104 to 338). CT-guided needle biopsy was carried out and the acquired specimen was pathologically diagnosed as adenocarcinoma. The tumor was also found to carry a mutation of EGFR (L858R). Treatment with gefitinib began and her subjective symptom was relieved quickly within several days.

On treatment day 13, ALP increased to 952 IU/L, about 2.5 times the pretreatment level. Serum transaminases were also simultaneously elevated (AST 101 and ALT 168), suggesting gefitinib-induced liver dysfunction. Serum Ca remained within normal limits. Electrophoretic analysis showed that ALP-isozyme 1 accounted for 8.6%, ALP-2 56.3%, and ALP-3 35.1%, respectively. Although bone-derived ALP-3 was seen to increase, ALP-2 liver-derived isozyme had the larger increase, probably because of simultaneous drug-induced liver injury.

Since the significance of elevated ALP was unclear, another CT was carried out on treatment day 38. Results showed the primary pulmonary tumor was reduced to 3 cm in diameter [Figure 1B]. Previously osteolytic lesions had become osteosclerotic. Lesion sizes are evidenced excessive growth, larger than the size of the original bones [Figure 2C and D]. ALP gradually decreased and liver dysfunction regressed [Figure 3]. CEA also decreased to 34.7 ng/mL, about one fourth of the maximum value [Figure 3].

DISCUSSION

A phenomenon, so called osteoblastic flare, has originally been recognized as a transient increased uptake of radiotracer of bone scintigraphy.^[1,7] However, ALP appeared to replace later because of infrequent use of bone scan, rapid and easy application of ALP, and coincident fluctuation of both.^[6] Our patient demonstrated a rapid improvement of bone pain and tumor regression by gefitinib. This study might support the previous report by Arai *et al.*^[3] suggesting a favorable response to EGFR-TKI in case of ALP flare. It is of interest that Shimazaki *et al.*^[8] first observed ALP flare-like phenomenon in a patient with multiple myeloma who received bortezomib for recurrent massive bone lesions. Their patient showed a transient ALP-3 increase without disease progression. Recent extreme efficacy of novel therapeutic agents might

Table 1: Laboratory data on admission

Inspection item	Value
White blood cells	10,790 μ L
Red blood cells	456 $\times 10^4$ μ L
Hemoglobin	13.6 g/dL
Hematocrit	40.5%
Platelets	38.5 $\times 10^4$ μ L
Carcinoembryonic antigen	137.9 ng/mL (0-5.0)*
Sialyl Lewis-X antigen	110 ng/mL (< 38.0)*
Alkaline phosphatase	379 IU/L (104-338)*
Lactate dehydrogenase	468 IU/L (108-221)*
Aspartate aminotransferase	25 IU/L
Alanine aminotransferase	14 IU/L
Total bilirubin	0.7 mg/dL
Leucine aminopeptidase	59 IU/L
Gamma glutamyl transpeptidase	37 IU/L
Albumin	3.9 g/dL
Blood urea nitrogen	11.5 mg/dL
Creatinine	0.76 mg/dL
Uric acid	4.8 mg/dL
Calcium	10.2 mg/dL
Sodium	142 mEq/L
Potassium	4.2 mEq/L
Chloride	103 mEq/L
C-reactive protein	0.10 mg/dL
HBsAg	negative
HBsAb	negative
HBcAb	negative
HCVAb	negative

*Abnormal data are in bold. Their normal ranges are indicated in the following parentheses

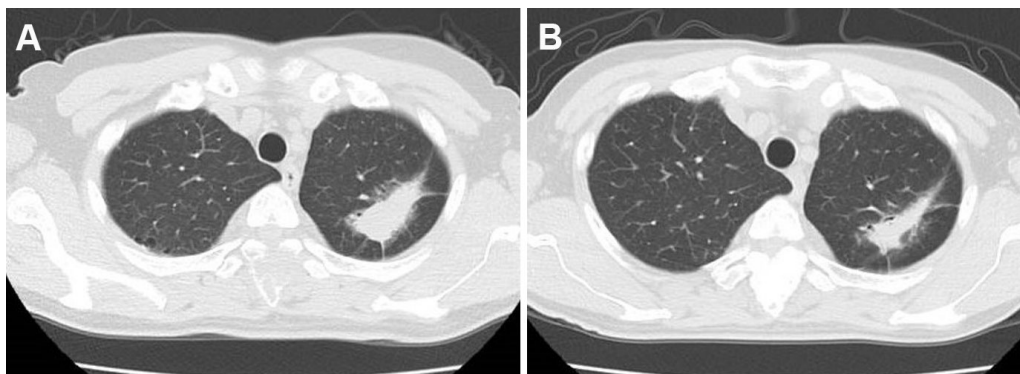


Figure 1: (A) Irregular shaped tumor at left upper lobe; (B) the tumor reduced in size after the initiation of gefitinib

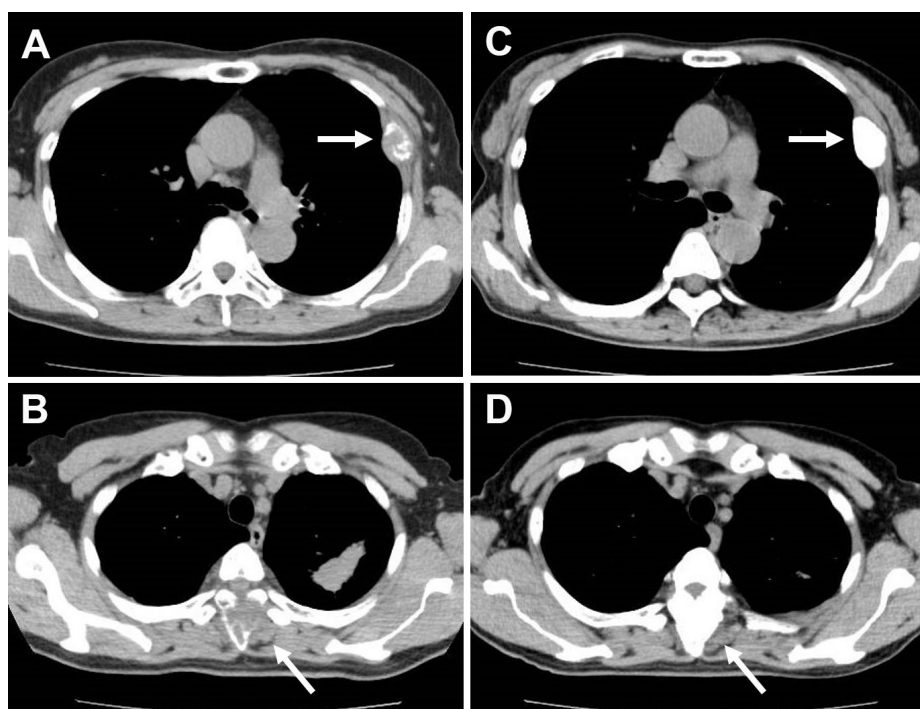


Figure 2: Osteolytic metastatic lesions of the left fourth rib (A) and the fourth thoracic spine (B); hyperostosis of the lesions was seen by gefitinib treatment (C, D)

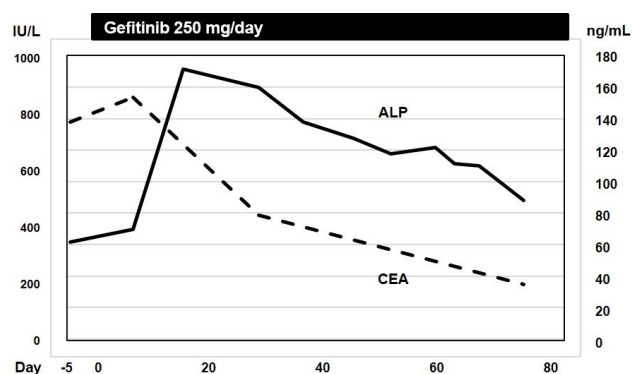


Figure 3: Transient ALP flare after the initiation of gefitinib is shown. CEA continued to decrease. ALP: alkaline phosphatase; CEA: carcinoembryonic antigen

induce ALP flare in various malignancies with bone lesions. It is also intriguing that NSCLC patients even without definite bone metastasis who experienced ALP flare showed better survival.^[6] Bone-derived ALP flare that might represent osteoblastic reaction is supposed to predict a response to EGFR-TKI, although a precise mechanism is to be elucidated. A previous investigation referred to the reduction of osteoclast differentiation in the bone marrow caused by gefitinib,^[9] suggesting a relation between therapeutic response and osteoblastic reaction. It is notable that ALP flare has mainly been found in Japanese patients. There might be racial differences among biological responses to EGFR-TKI.

To our knowledge, CT findings of improvement of bone metastatic lesions accompanied by ALP flare

have seldom been demonstrated previously. Although osteosclerotic changes within the metastatic lesions were depicted in these studies,^[4,5] our patient showed excessive calcification including metastatic lesions as shown in Figure 2. There has been no similar description of such hyperostosis in English literature. We hypothesized that radiographically latent tumor tissue around the bone metastasis might also be calcified by EGFR-TKI therapy, resulting in hyperostosis. On the other hand, radiographic osteoblastic change of metastasis before treatment was also regarded as a favorable prognostic marker for NSCLC treated with EGFR-TKI.^[10] It is suggested that osteoblastic reaction regardless before or after the initiation of treatment might influence tumor reduction, as well as ALP flare phenomenon.

Moreover, drug-induced liver injury might cover ALP flare if liver transaminases or biliary tract markers also markedly elevate as well as ALP. Drug-induced liver damage was reported to be seen in 5 of 41 (12.5%) patients with NSCLC who were treated with EGFR-TKI.^[11] Thus, the frequency of ALP flare is supposed to be much higher than practically observed. Negativity of hepatitis viral markers or outstandingly elevated ALP among liver function markers might help detecting ALP flare. Physicians should pay more attention to ALP flare as well as therapeutic response of radiographic findings of bone metastasis to elucidate the significance of osteoblastic reactions in the outcome of lung cancer. At least, ALP flare strongly suggests that EGFR-TKI

should not be discontinued even if adverse effects of other organs might emerge.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

All involved patients give their consent forms.

Ethics approval

Ethics approval was obtained from Institute Review Board for case reports.

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Cytogenetic and molecular basis of BCR-ABL myelodysplastic syndrome: diagnosis and prognostic approach

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ABSTRACT

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Myelodysplastic syndromes (MDS) include a heterogeneous group of blood disorders generally afflicting older people. Several genetic factors have been reported from these patients that have an important role in the diagnosis, prognosis, and treatment of this disease. BCR-ABL1 is a genetic factor that has occasionally been reported in some studies. This review attempts to characterize MDS patients reported to harbor this fusion and to assess the diagnostic, therapeutic, and prognostic potential of BCR-ABL1 fusion in MDS patients. This review showed that BCR-ABL fusion has been reported in 22 MDS patients whose condition generally transformed to acute myeloblastic leukemia and was not responsive to conventional therapies. However, these patients showed a good response to treatment with tyrosine kinase inhibitors. Therefore, even though incidence of BCR-ABL fusion appears to be low in MDS patients, its detection is essential in assessing disease prognosis and choosing appropriate treatment.

INTRODUCTION

Myelodysplastic syndromes (MDS) are a group of clonal myeloid disorders with morphological characteristics such as hypercellular bone marrow (BM), single- or multilineage dysplasia, and cytopenia in peripheral blood (PB).^[1,2] Mortality associated with cytopenia and risk of transformation to acute myeloblastic leukemia (AML) are important problems for MDS patients. In fact, one-third of MDS patients become

AML patients, and the remaining two-thirds succumb to progressive BM failure, which leads to bleeding, frequent infections, and severe anemia.^[3] MDS is generally an adult disease with an average age upon diagnosis of 65-70 years; less than 10% of patients are younger than 50 years. The annual incidence rate of MDS is approximately 5 cases per 100,000 population; incidence increases to 22-45 cases per 100,000 in people over 70 years of age.^[4] MDS is generally diagnosed by accurate assessment of PB followed by



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morphological BM examination. According to the 2016 WHO revision, MDS patients are divided into lower- and higher-risk MDS. Lower-risk MDS conditions that have below 5% of blasts include: MDS with single-lineage dysplasia, MDS with single-lineage dysplasia and ring sideroblasts (RS), MDS with multilineage dysplasia without RS and with RS, MDS with isolated del (5q), and MDS unclassifiable (MDS-U). Higher-risk MDS conditions (5-19 blasts) include: MDS-EB1 (5-9% blast and/or 2-4% in PBS) and MDS-EB2 (10-19% blasts; Auer rods, or 5-19% in PBS).^[5]

t(9;22) (q34;q11.2) translocation and its variants give rise to Philadelphia chromosome (Ph), which results in juxtaposition of DNA sequence of BCR and ABL1 genes, mRNA translation of this chimeric gene, and eventual dysregulated expression of oncogenic tyrosine kinase of BCR-ABL1 fusion, which seems to be sufficient to initiate the leukemogenesis process.^[6] Three different forms of BCR-ABL1 fusion protein are produced based on the breakpoint site in the BCR gene: p190, p210, and p230. Although they are all associated with development of leukemia, these three forms have different clinical outcomes.^[7] Although BCR-ABL1 chromosomal abnormality is pathognomic for chronic myeloid leukemia (CML), it is observed de novo in B-cell precursor acute lymphoblastic leukemia (ALL), especially in adults, as well as in 0.48-3% of patients with AML.^[8,9] In contrast, Ph is extremely rare in MDS patients and shows up in the last stages of disease, so it is associated with leukemic transformation in most cases.^[10] Although few cases of Ph⁺ MDS have been reported, diagnosis of this disorder is especially important, since these patients show a poor response to conventional therapeutic approaches.^[11]

The presence of common traits in MDS and myeloproliferative disease (MPD) suggests that some genetic abnormalities associated with MPD are most likely involved in the development or progression of MDS. Lack of knowledge about the importance of this abnormality in MDS patients may lead to inaccurate assessment of BCR-ABL fusion and choice of an inappropriate therapeutic protocol. Therefore, besides studying the reported cases, this review aims to investigate the typical features of Ph⁺ MDS patients and will assess the role of genetic abnormalities, especially the impact of BCR-ABL fusion, on response to treatment in MDS patients.

CYTOGENETIC AND MOLECULAR MARKERS

All classification and prognosis systems of MDS in recent decades have been based on cytomorphological findings in PB and BM, including May-Grünwald-

Giemsa (MGG) staining, myeloperoxidase staining, nonspecific esterases (especially for CMML), as well as iron staining and assessment of cytopenia.^[12] MDS diagnosis is often challenging for several reasons, such as varying clinical manifestations in different patients and the absence of dysplasia in some cases. For this reason, cytogenetic tests have been introduced for correct diagnosis of some MDS subtypes; for example, in the fourth classification of WHO, del 5q is considered as a separate subgroup. In patients whose diagnosis is controversial, cytogenetic analysis seems to be a helpful addition to clinical and hematological findings when seeking a definitive diagnosis.^[13]

Genetic abnormalities in MDS patients include deletions, gains, and chromosomal rearrangements, as well as molecular changes such as point mutations, epigenetic changes, and dysregulated miRNAs.^[13] Conventional cytogenetics and fluorescent in situ hybridization (FISH) analysis are commonly used methods for detection of karyotype abnormalities; both methods have advantages and disadvantages. Karyotype commonly evaluates 20 metaphase cells. FISH analysis can detect chromosomal abnormalities with a higher resolution, but it is limited to regions with predefined probes.^[14] Therefore, it seems prudent to perform initial assessment by conventional karyotyping and to use FISH analysis for further investigations. Several studies have shown that FISH analysis in conjunction with karyotyping can provide further information, especially in cases where the karyotype appears normal.^[15,16] Chromosomal abnormalities have been detected in approximately 50% of patients with de novo MDS and in more than 80% of MDS cases secondary to chemotherapy and toxic agents. In a large-scale study on 2124 MDS patients, 48% had normal karyotype and 52% showed abnormal karyotype. The most common cytogenetic abnormality was del 5q in 30% of patients, followed by -7/del 7q in 21%, and +8 in 16% of cases.^[17] Detection of cytogenetic abnormalities plays a significant role in disease prognosis, so it has been recognized as a marker in all the prognostic systems, including international prognostic scoring system (IPSS), revised-international prognostic scoring system (IPSS-R), and WPSS. IPSS-R is one of the most widely used prognostic systems for MDS patients.^[18] In this classification system, -Y and del (11q) have a very good prognosis; normal karyotype, del (5q), del (12p), del (20q), and double including del (5q) have good prognosis; del (7q), +8, +19, and i (17q) a moderate prognosis; -7, inv (3)/t (3q), double including -7/del (7q), complex 3 abnormalities have poor prognosis; and finally patients with karyotype of complex with > 3 abnormalities have a very poor prognosis.^[19]

Technological advances in the field of genetic analysis, including high-throughput next-generation sequencing (HT-NGS), led to the discovery of several genetic mutations in MDS patients.^[20] Studies have shown that approximately 83% of MDS patients show genetic mutations.^[21] In Table 1, some of the most common mutant genes in MDS patients are summarized.

Although these mutations involve a range of genes, their use as a diagnostic marker for MDS patients is difficult. A good diagnostic marker must have a high incidence in patients as well as an acceptable level of specificity, but none of these genes has a high prevalence in MDS patients (low frequency), and no mutant gene has been specifically reported for MDS.^[34] Mutations have been partially assessed as prognostic markers and have generally been associated with poor prognosis.^[14] Therefore, although these mutations seem to be good prognostic factors, prognostic systems have not yet taken advantage of them in their classifications.^[18]

DIAGNOSIS AND PROGNOSIS

According to search of MEDLINE database, there have been 22 cases of MDS patients harboring BCR-ABL1 chromosome abnormality. There were 15 male and 7 female patients that were classified into two groups: adults with an average age of 64.5 years and children with an average age of 25 months. Mean hemoglobin concentration was 8.4 g/dL (94.7% had hemoglobin levels less than 11.5 g/dL, i.e. were anemic). Mean white blood cell count was $6.7 \times 10^6/\text{mL}$ and mean platelet count was $135 \times 10^3/\text{mL}$ (61.1% had platelet counts lower than 100×10^3). Karyotype analysis in 20 cases revealed t (9;22) translocation, but in two other cases, FISH test indicated the presence of Phfusion despite normal karyotype.^[11,35] Molecular analysis was done in only 10 cases; of these 5 represented Ph P190 variant, 4 cases had Ph210, and 1 case had both variants [Table 2]. According to these findings, Ph

fusion was most prevalent in RAEB subgroup; 54.6% of cases (including 27.3% RAEB, 9.1% RAEB2, and 18.2% of RAEBt) were classified in this subgroup, followed by RA in 13.6% of cases. This finding was in contrast to some extensive studies of the epidemiology of different subtypes of MDS, which indicate that RA, RARS, RAEB, and RAEBt are the most common subtypes, respectively.^[17,36] There was a relatively poor prognosis in these patients. Only 5 patients responded to treatment, among which 2 cases were treated with imatinib.^[11,13] Forty-five percent ($n = 10$) of patients progressed to AML, among whom 3 patients showed P190 variant, 3 patients showed P210, and 1 patient showed both variants [Table 2]. Only one patient showing P190 variant progressed to ALL. Three patients progressed to CML for whom unfortunately no molecular study was conducted.^[4,9,13]

DISCUSSION AND FUTURE PROSPECTIVE

Using current advances in molecular diagnosis, several genetic factors have been identified in MDS patients with occasional diagnostic, prognostic, and therapeutic value. Ph chromosome is a factor intermittently reported in some cases of MDS. Given the pathognomic role of Ph in other hematologic neoplasms, it is assumed that in case of high incidence of Ph in MDS patients, an MDS subgroup known as Ph⁺ MDS can be introduced. However, the importance of this genetic abnormality in MDS patients has not been extensively studied in MDS patients up to the present time.

The fact that only 22 cases of Ph⁺ MDS have been reported to date is not conclusive evidence of low prevalence of this fusion in MDS patients. We state this for two reasons: (1) retrospective studies are inefficient for these patients because of the lack of careful examination of BCR-ABL fusion, and (2) no study up to the present time has specifically examined this fusion in MDS patients. Given that in some cases

Table 1: The most common mutations in myelodysplastic syndromes

	Mutated gene	Prevalence (%)	Prognosis	Ref.
RNA splicing	<i>SF3B1</i>	16	Favorable	[22,23]
	<i>SRSF2</i>	13	Poor	
	<i>U2AF1</i>	10	Poor	
	<i>TET2</i>	23	Favorable	
DNA methylation	<i>DNMT3A</i>	9	Poor	[24]
	<i>IDH1/2</i>	7.5	Poor	[25]
	<i>ASXL1</i>	20	Poor	[26]
Chromatin modification	<i>EZH2</i>	6	Poor	[27]
	<i>Tp53</i>	9.4	Poor	[28]
	<i>Ras</i>	15	Poor	[29]
Oncogenes	<i>EVI1</i>	1-2	Poor	[30]
	<i>RUNX1</i>	12	Poor	[31]
Others	<i>JAK2</i>	53 in RARS-T	Not studied	[32]
				[33]

Table 2: Characteristics of MDS patients with BCR-ABL fusion

No.	Age/gender	MDS subtype	Ph+ phase/type	Cytogenetic findings	Hematological findings	Outcome	Ref.
1	69/M	RAEBt	At diagnosis/P190	46, XY[3]/45, X, -y[2]/50, XY, +Y, -3, del5 (q12q34), +8, +14, add(18)(p11), +22, +min[11]/idem, t(9;22) (q34;q11)	Hb = 8.1 WBC = 5.3 Plt = 77	Progressed to AML/died	[37]
2	64/M	RAEB	At diagnosis/P190	46, XY[7]/47, XY, +8, t(9;22) (q34;q11)[6]	Hb = 7.8 WBC = 6.9 Plt = 98	Progressed to AML/died	[37]
3	3/M	RAEBt	AML late stage transformation/P210	46, XY, t(9;22)(q34;q11)	Hb = 6.2 WBC = 4.7 Hb = 47	Progressed to AML	[38]
4	54/M	RA	ALL transformation stage/P190	46, XY, t (9;22) (q34;q11).20q- (18/20)/46, XY, 20q-	Hb = 8.6 WBC = 3.2 Plt = 142	Progressed to ALL/died	[39]
5	78/M	RAEBt	At diagnosis/P190	46, XY, der (3) t(1;3) (p22;p14), del (5) (q13q33)/ FISH revealed fusion signal of BCR and ABL probes on an apparently normal chromosome 22	Hb = 9.8 WBC = 13.5 Plt = 29	Died in 5 months	[35]
6	67/F	RAEB-2	At diagnosis/P210(b2a2)	Ph+ [29/30], normal [1/30]	Hb = 11.5 WBC = 3.4 Plt = 111	Complete remission with imatinibmesylate	[11]
7	39/M	RAEB	AML transformation/early stage p210 and late stage p210 and p190	46, XY, t (3;3)(y21:q26)[50] 46, XY, del (1)(p22). t(3;3) (q21: y26). -16[6] 46, XY, t(3;3)(q21:q26), t (9;22) (q34;q11)[3]	Hb = 7.1 WBC = 7.1 Plt = 547	Progressed to AML/died	[40]
8	25 months/F	unclassified	At diagnosis/-	46, XX, t (9;22) (q34;q11) [15]	Hb = 8.7 WBC = 7.9 Plt = 39	Died in 28 months	[10]
9	20 months/F	unclassified	24 months after diagnosis/-	37-45, XX, -18[7]/46, XX[4]. nuc fish 9q34 (ablx2), 22q11 (bcx2) (ablxcon bcrx1) [4/200]	Hb = 5.9 WBC = 26.3 Plt = 71	Treated with low dose chemotherapy	[10]
10	73/M	CMMoL	7 months after diagnosis/-	46, XY, t(4;6) (p15;p12), t(9;22) (q34;q11) [10%]	Hb = 15.4 WBC = 18.1 Plt = 31	CML/died in 10 months	[41]
11	63/M	RA	During myeloproliferative phase/-	46, XY, t(9;22) (q34;q11) [100%]	Hb = 10.2 WBC = 1.4 Plt = 165	CML/died in 3 months	[41]
12	66/M	RAEB-2	AML transformation/P190	Karyotype was neg for Ph but FISH indicate a fusion signal in 60%	Hb = 6.2 WBC = 1.7 Plt = 33	Progressed to AML/died	[42]
13	73/M	RAEB	In CML transformation/P210	46, XY, t (9;22)/fish indicated single Ph 98.0%	-	Progressed to CML then all died	[43]
14	66/F	RAEB	At diagnosis/-	47, XX, +8, t(9;22;16) (q34;q11.2;q23) [4]/46, XX, idem, der (12) t(12;17) (p11.2;q11.2) [7]/46, XX[9]	Hb = 4.4 WBC = 0.9 Plt = 52	Progressed to granulocytic sarcoma skin in 9 months and died 1 month later	[44]
15	71/M	RAEB	At diagnosis/-	46, XY, t(9;22) (q34;q11) [20]	Hb = 9 WBC = 4000 Plt = 55	Progressed to RAEBt in 5 months and died 9 months after diagnosis	[44]
16	59/M	RAEB	At diagnosis/P210	46, XY, t(9;22) (q34;q11) [20]	Hb = 9.2 WBC = 1.3 Plt = 78	Progressed to AML/treated with allogeneic transplant	[44]

Continued...

No.	Age/gender	MDS subtype	Ph+ phase/type	Cytogenetic findings	Hematological findings	Outcome	Ref.
17	78/F	RCMD	At CML transformation/-	46, XX, t(9;22) (q34;q11)	Hb = 10.2 WBC = 2.6 Plt = 152	Progressed to CML accelerated phase/response to imatinib with significant cytopenia	[45]
18	56/M	RA	At diagnosis/-	Complex karyotype with PH1 chromosome	Hb = 4.8 WBC = 2.4 Plt = 350	Progressed to AML/died	[46]
19	49/F	-	At diagnosis/-	t(9;22) (q34;q11) [38%]	Hb = 8.2 WBC = 6.5 Plt = 425	Progressed to AML/died	[47]
20	62/M	RAEB	AML transformation	t(9;22) (q34;q11) [100%]	Hb = 9.8 WBC = 3.2 Plt = 120	Progressed to AML/died	[48]
21	70/F	RARS	At diagnosis/-	46, XX[3]/46, XX, t(9q;22q) [12]	Hb = 9.5 WBC = 6.4 Plt = 316	Stable/alive	[49]
22	69/M	t-MDS	AML transformation	46, XY, t(9;22)(q34;q11) [35]	Hb (no data) WBC = 1.3 Plt = 129	Progressed to AML	[50]

MDS: myelodysplastic syndromes; AML: acute myeloblastic leukemia; CML: chronic myeloid leukemia; ALL: acute lymphoblastic leukemia

only FISH analysis has managed to detect BCR-ABL fusion in MDS patients, lack of detection in normal karyotype analysis does not indicate definitive absence of this fusion.^[5,11] Assessment of reported cases shows that MDS patients harboring this chromosomal abnormality typically do not respond well to conventional treatments but do show a good response to imatinib therapy.^[11,13] Since imatinib is not routinely used in treatment of MDS patients, lack of Ph detection in these patients may lead to incorrect treatment and thus put the patient's life at risk.

In general, although the findings of this study indicate the importance of Ph detection in MDS patients, they are not sufficient to clarify the precise role of Ph in MDS patients. Therefore, specific assessment of this chromosomal abnormality in MDS patients is recommended in future studies.

Authors' contributions

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Patient consent was obtained.

Ethical approval

This article does not contain any studies involving human or animal subjects.

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DNA damage-induced nuclear factor-kappa B activation and its roles in cancer progression

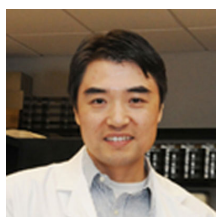
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Dr. Zhao-Hui Wu is an Associate Professor in the Department of Pathology and Laboratory Medicine at the University of Tennessee Health Science Center. The research in his lab focuses on exploring the mechanisms involved in the acquired and innate therapeutic resistance of cancer cells. Through delineating the molecular and cellular signaling pathway exploited by tumor cells to evade the elimination by anti-cancer drugs, they are aiming to screen potential novel drug targeting these pathways to improve the therapeutic efficacy of cancer treatments.

ABSTRACT

DNA damage is a vital challenge to cell homeostasis. Cellular responses to DNA damage (DDR) play essential roles in maintaining genomic stability and survival, whose failure could lead to detrimental consequences such as cancer development and aging. Nuclear factor-kappa B (NF- κ B) is a family of transcription factors that plays critical roles in cellular stress response. Along with p53, NF- κ B modulates transactivation of a large number of genes which participate in various cellular processes involved in DDR. Here the authors summarize the recent progress in understanding DNA damage response and NF- κ B signaling pathways. This study particularly focuses on DNA damage-induced NF- κ B signaling cascade and its physiological and pathological significance in B cell development and cancer therapeutic resistance. The authors also discuss promising strategies for selectively targeting this genotoxic NF- κ B signaling aiming to antagonize acquired resistance and resensitize refractory cancer cells to cytotoxic treatments.

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INTRODUCTION

The genome of all living organisms is constantly

threatened by a variety of agents which cause DNA damage. DNA lesions may occur by altering DNA bases (i.e. O⁶-methylguanine and thymine glycols),



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creating breaks on DNA backbone, and forming cross-links between DNA strands and proteins. Failure to repair these lesions can lead to genomic instability and detrimental consequences.^[1] Breaks on both strands of DNA (double-stranded break, DSB) represent one of the most lethal types of genomic lesion, which has been associated with pathogenesis of a variety of human diseases and aging.^[2] DSB can be induced by environmental exposure such as ultraviolet (UV) or ionizing radiation (IR), as well as by endogenous agents like reactive oxygen species generated by cell metabolism.^[3] Genomic lesions can be recognized and labeled by recruitment of sensor proteins, which activates a complex network of cellular responses known as DNA damage response (DDR) and mobilizes DNA repair machinery in order to maintain genomic integrity.^[4] Low levels of DNA damage cause cell cycle arrest and promote repair of DNA lesions, whereas severe DNA damage leads to apoptosis or permanent cell cycle arrest (senescence) to avoid neoplastic transformation.^[5] DDR is often deregulated in malignant cells, which allows them to escape apoptosis or senescence. These cells could proliferate while harboring DNA lesions, which significantly increases the chance of genetic mutation. A number of anti-apoptotic signaling pathways, such as nuclear factor kappa B (NF- κ B), have been shown to also play critical roles in modulating cancer cell response to DNA damage.^[6]

NF- κ B is a family of transcription factors that play critical roles in inflammation, immunity, cell proliferation, development, survival and apoptosis.^[7-9] The inactive NF- κ B is present in the cytoplasm in most cell types and it can be activated by a variety of extra-cellular stimuli such as pro-inflammatory cytokines, bacterial lipopolysaccharides, and viral RNA and DNA, via the activation of membrane and cytosolic receptors.^[10,11] NF- κ B was also shown to be activated by DNA damaging drugs in a membrane receptor-independent manner, which involves a retrograde signaling cascade from nucleus to cytoplasm.^[6,12-14] It has been reported that NF- κ B was activated in response to a variety of DNA lesions, such as temozolomide-induced S_N1-methylation,^[15] cisplatin-induced DNA cross-linking,^[16] and IR-induced DSB. Recent studies have revealed a variety of roles of DNA damage-activated NF- κ B in cancer cell responses to radiation and chemotherapies as well as in cancer progression and metastasis. This review will focus on the recent progress in understanding DNA damage-induced signaling, DDR, and genotoxic DSB agent-induced NF- κ B signaling cascade, as well as their physiological functions and pathological significance in cancer progression, therapeutic resistance and metastasis.

DSB AND DNA DAMAGE SIGNALING

DSB is the most severe form of genomic lesion due to the potential errors incurred during DSB repair. Cells are equipped with several repair mechanisms including homologous recombination (HR), classical non-homologous end joining (cNHEJ), back-up alternative NHEJ (aNHEJ) and single-strand annealing.^[4,17-19] Successful HR is generally error-free while NHEJ and other alternative forms of DNA repair are more likely to introduce DNA lesions. Most DSBs are repaired quickly, but those DSBs which repaired incorrectly or escaped repair mechanisms could cause chromosomal aberrations, loss of heterozygosity, oncogenic mutation, or cell death.

Endogenous and exogenous causes of DSBs

DSB can be induced by endogenous molecules such as reactive oxygen species, lipid peroxides, endogenous reactive chemicals (e.g. aldehydes and S-adenosylmethionine), telomere attrition and depurination mechanism.^[3] Physiological DSB can also be generated during V(D)J recombination of immunoglobulin chains in lymphocytes.^[20] Moreover, DSBs are also formed indirectly from collapse of stalled transcription forks or arrested replication forks. These replication fork arrests could occur during normal replication at sequences which are prone to form secondary structures such as tRNA genes and chromosomal fragile sites.^[21,22]

Genotoxic agents are present in the environment at a very low level, whereas higher levels can be found in diagnostic tools and tumor therapies. The exogenous causes of DSBs are mostly either accidental exposure or medical procedures. A harmful dose of IR is normally not present in the environment, but such a dose could be received from accidental exposure to radioactive materials or, theoretically, a nuclear attack. IR at a dose of 1 Gy leads to approximately 1,000 SSBs and 20-40 DSBs per cell, among which DSBs are more cytotoxic although less in the number of breaks.^[4] Keep in mind that diagnostic imaging techniques such as X-rays and mammograms use a very low level of radiation which could induce DSB directly and indirectly via oxidative stress. In addition, radiation therapy and cytotoxic chemotherapeutic drugs, such as camptothecin, doxorubicin and daunorubicin, induce DSBs in cancer cells through directly damaging DNA or interfering DNA topoisomerase function, leading to apoptosis and elimination of malignant cells.

DNA damage response

Damage to DNA can elicit a complex cellular response by activating multiple signaling cascades, which are

generally termed DNA damage response.^[4] DNA double-strand breaks could be recognized and bound by a protein complex called MRE11/RAD50/NBS1 (MRN) within a few seconds of their formation. As a DSB lesion sensor, MRN complex binds to the break extremities, stabilizes them close to each other and initiates DDR via NBS1-dependent recruitment of ATM kinase.^[23-25] In resting cells, inactive ATM dimer associates with the Tip60 histone acetyltransferase and protein phosphatase 2A.^[26,27] Upon DNA damage, MRN complexes bound to DNA breaks recruit Tip60 on histone 3 trimethylated on K9 (H3K9me3). This interaction activates Tip60 which in turn acetylates ATM kinase on K3016.^[28,29] ATM acetylation induces its conformation change and auto phosphorylation on S367, S1893, S1981 and S2996 as well as dimer-to-monomer transition.^[30-32] The dissociation of protein phosphatase 2A, which targets phosphorylated S1981, from ATM is also required for full activation of ATM.^[27]

Around 10% of activated ATM by irradiation or neocarzinostatin treatment associates with chromatin in the form of ionizing radiation induced foci (IRIF), whereas the majority of active ATM remains free in nucleoplasm.^[33,34] As a master regulator of the DSB-induced DDR, ATM phosphorylates various substrates at the consensus target sequence, (S/T)Q, so as to orchestrate the activation of multiple signaling pathways regulating cell cycle arrest, DNA repair, and apoptosis as well as other pathophysiological processes.^[35] ATM substrates can be divided into subsets based on their subcellular localization, such as chromatin-associated (H2AX, KAP1), integrated in the IRIF (MDC1, 53BP1, BRCA1, NBS, MRE11, RNF20-RNF40), IRIF-adjacent and phosphorylated by IRIF-bound ATM (Chk2), or phosphorylated by a free-floating pool of ATM (p53, NEMO) in nucleoplasm. Beyond those nuclear substrates, a subset of ATM substrates localized in the cytoplasm (4EBP1, TAB2) has also been reported to play critical roles in cellular response to DSBs.^[2,4]

ATM belongs to a family of PI3K-related protein kinases which includes ATM, ATR, DNA-PKcs, mTOR, SMG-1 and TRRAP.^[4] Although they all share the similar kinase domain as that in lipid kinase PI3K, they are protein kinases except for TRRAP whose kinase activity remains to be validated. Along with ATM, DNA-PK and ATR also play essential roles in mediating DNA damage response. In human cells, most breaks are rapidly repaired by cNHEJ throughout the entire cell cycle.^[17,36] DNA-PKcs is indispensable for repairing DNA double-strand breaks by NHEJ. DNA-PKcs can be recruited to DSBs by the Ku70/Ku80 heterodimer and form the active DNA-PK complex, which promotes synapsis of the broken DNA ends.^[37] Like ATM, DNA-

PKcs is constitutively associated with Tip60, which controls its activity. Knockdown of Tip60 by siRNA reduces the phosphorylation and activation of DNA-PKcs in response to bleomycin.^[38] Most DNA-PK substrates are implicated in DNA repair (DNA-PK itself, Artemis, polynucleotide kinase, XLF, excision repair cross complementing 4), whereas DNA-PK-dependent phosphorylation of H2AX, KAP-1, p53 leads to activation of cell death machinery.^[39,40] As a replication stress sensor binding to single-strand DNA, heterotrimeric Replication Protein A (RPA) accumulates at stalled replication forks and recruits ATR interacting protein (ATRIP) in association with ATR kinase. Activation of ATR also requires Rad9/Rad1/Hus1 heterotrimer (9-1-1 complex) and the DNA topoisomerase binding protein 1 (TopBP1).^[41] ATR kinase activity is necessary for stabilization and restart of stalled replication forks, and for signaling to cell cycle checkpoint activation.^[42,43] Therefore, ATR is essential for cell replication and viability as well as maintaining genomic stability.^[44,45]

NF- κ B ACTIVATION IN DNA DAMAGE RESPONSE

Besides the prompt cellular responses (e.g. cell cycle arrest, DNA repair) to counteract DNA lesions, transcription/expression of a large number of genes can also be altered in response to DNA damage. Two transcription regulators, p53 and NF- κ B, have been identified as the major players for reprogramming the transcription of these genes in response to IR.^[46-48] DNA damage-dependent regulation of p53 signaling has been well studied and comprehensively reviewed.^[2,49,50] Here we focus on the recent progress in understanding genotoxic stress-induced NF- κ B signaling.

NF- κ B family

NF- κ B is a family of transcription factors composed of five members, p65 (RelA), c-Rel, RelB, p105/p50 and p100/p52, which form hetero- or homo-dimers and regulate a variety of physiological and pathological processes. In resting cells, NF- κ B localizes in the cytoplasm in association with a family of inhibitor proteins called I κ Bs (inhibitor of NF- κ B), such as I κ B α .^[11,51] Upon stimulation, NF- κ B is released from I κ Bs and translocates into the nucleus, where it binds to the promoter and/or enhancer regions of its target genes and regulates their transcription. In addition to nuclear translocation, posttranslational modification of NF- κ B, such as phosphorylation, acetylation and methylation of p65, also plays a significant role in modulating transcriptional activity.^[11,52] A large number of NF- κ B-target genes have been identified (see list

at www.nf-kb.org), which participate in a wide range of physiological and pathological processes, such as cell proliferation, innate and adaptive immune responses, inflammation, cell migration, and regulation of apoptosis, among others.^[11,53]

Classical and alternative NF- κ B signaling pathways

Previous studies have established two well-defined NF- κ B activation signaling pathways initiated from membrane-bound receptors, the so-called “classical” and “alternative” pathways.^[54] The classical NF- κ B pathway depends on activity of the IKK (I κ B kinase) kinase complex, which is composed of IKK α , IKK β and IKK γ /NEMO. Upon activation of the IKK complex, the IKK β subunit directly phosphorylates NF- κ B-associated I κ B α , leading to its proteasomal degradation and release of p65/p50 heterodimer. Free NF- κ B then translocates into the nucleus and regulate gene transcription. The

alternative pathway of NF- κ B activation relies on the IKK α homodimer activation in a manner dependent on NF- κ B inducing kinase (NIK). Activated IKK α then phosphorylates p100 and promotes partial processing of p100 and yielding of p52. Consequent p52: RelB dimer then translocates into nucleus and regulate the transcription of its target genes.

DNA damage-induced NF- κ B signaling pathway

DNA-damaging agents also activate NF- κ B in a canonical IKK complex-dependent fashion. However, in contrast to classical or alternative NF- κ B signaling pathways, this genotoxic signaling cascade is initiated in the nucleus instead of via membrane-bound receptors. In the following section, we will discuss the detailed molecular signaling events mediating this retrograde signaling pathway [Figure 1].^[6,14]

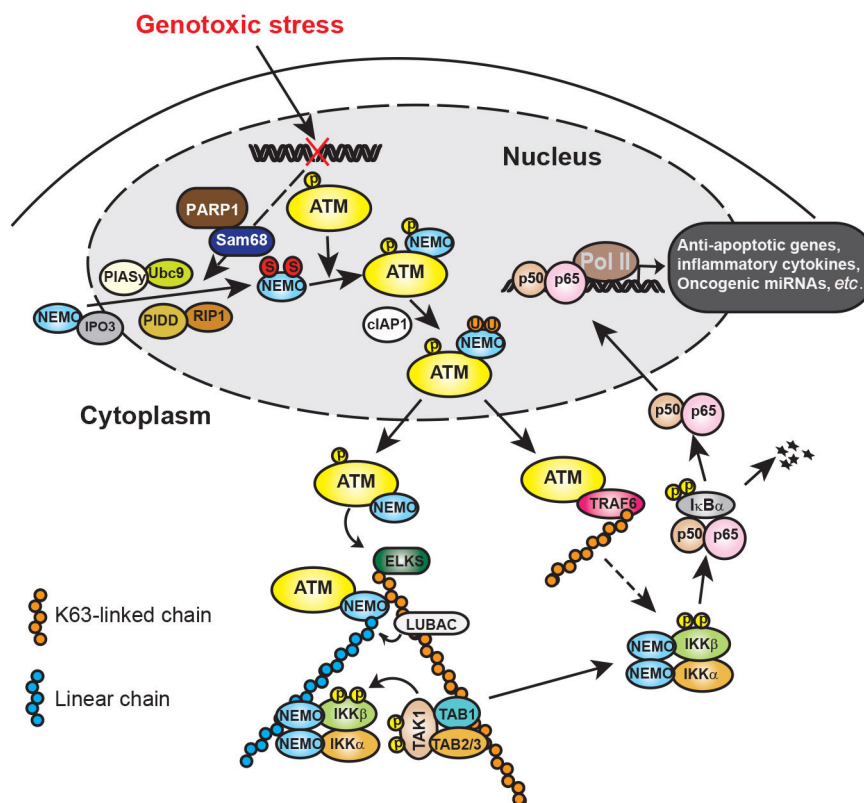


Figure 1: Illustration of genotoxic stress-induced NF- κ B signaling cascades. In response to genotoxic treatments, NEMO translocated into nucleus could be SUMOylated by PIASy, which enhances the nuclear accumulation of NEMO. The SUMOylation of NEMO may be facilitated by PARP-1/Sam68 and/or PIDD/RIP complex. Nuclear accumulated NEMO can further form a complex with ATM that phosphorylates NEMO and promotes NEMO monoubiquitination. Monoubiquitinated NEMO then exports into cytoplasm along with ATM, where they form a complex with ELKS. ATM-promoted ELKS ubiquitination with K63 chains recruits LUBAC complex, which facilitates the assembly of linear ubiquitin chain attached on NEMO. The ELKS/NEMO anchored ubiquitin chains stabilize binding of TAK1 and IKK complex thereby promoting their activation. ATM may also export into cytoplasm and form a complex with TRAF6, which leads to TRAF6 polyubiquitination. The polyubiquitin chains attached on TRAF6 could also enhance IKK activation. Activated IKK then phosphorylates I κ B α and frees NF- κ B for nuclear translocation. In the nucleus, NF- κ B could drive transactivation of anti-apoptotic genes (e.g. Bcl-xL, XIAP and Survivin), inflammatory cytokines (e.g. IL-6 and IL-8) and oncogenic miRNAs (e.g. miR-21 and miR-181a), resulting in therapeutic resistance and aggressive metastasis in cancer cells. NF- κ B: nuclear factor kappa B; PIASy: protein inhibitor of activated; PARP-1: poly (ADP-ribose) polymerase 1; PIDD: p53-induced death domain protein; RIP: receptor interacting protein; TAK1: TGF-beta activated kinase; IKK: I κ B kinase 1; TRAF6: TNF receptor-associated factor 6

Nuclear steps: ATM phosphorylates SUMOylated NEMO

ATM is the pivotal kinase involved in NF- κ B activation following DNA damage. In 1998, Lee *et al.*^[55] first observed that NF- κ B activation by IR was reduced in human cells with ATM deficiency (A-T cells). Later Piret *et al.*^[56] reported that decreased NF- κ B activation by the chemotherapeutic drug CPT in A-T cells could be restored by complementation with ATM. Moreover, both IR and NCS treatment induced ATM-dependent IKK β activation in HEK293 cells.^[46] Therefore, the activation of ATM by DNA damage likely serves as a cornerstone to bridge nuclear DNA damage response to cytoplasmic activation of NF- κ B signaling cascade.

The IKK kinase complex is the core component of the classical NF- κ B cascade which is also required for DNA damage induced NF- κ B activation. The non-catalytic subunit of the IKK complex, NEMO, was found to play unique roles in mediating genotoxic NF- κ B activation which may be dispensable in classical NF- κ B signaling. For example, the C-terminal zinc finger (ZF) domain of NEMO was shown to be essential for NF- κ B activation following treatment with DNA-damaging agents. In contrast, NEMO ZF domain deletion minimally affected NF- κ B activation following treatment with canonical stimuli (e.g. lipopolysaccharide). Importantly, a small fraction of NEMO was found to disassociate from IKK α/β upon DNA damage and translocate into nucleus by association with IPO3 (importin 3, transportin 2).^[57] Subsequently, nuclear NEMO can be modified by a small protein called SUMO (small ubiquitin-like modifier) in the nucleus in response to DNA damage.^[58] Similar to ubiquitin, SUMO can be covalently conjugated onto lysine residue of its target proteins and thereby altering the function and activity of the SUMOylated proteins. SUMO E3 ligase PIASy (protein inhibitor of activated STATy)-mediated SUMOylation on K277 and 309 of NEMO enhanced its nuclear accumulation which is essential for subsequent signaling events activating NF- κ B upon DNA damage.^[58,59] Nevertheless, the mechanism through which SUMOylated NEMO is localized to nucleus is still unclear.

Two different protein complexes have been shown to regulate the NEMO SUMOylation. Subsequent to DNA damage, p53-induced death domain protein (PIDD) and receptor interacting protein 1 (RIP1) associate with nuclear NEMO as a heterotrimer and accumulate in the nucleus. This PIDD/RIP1/NEMO complex may promote NEMO SUMOylation following chemotherapeutic drug treatment in HEK293 cells.^[60] The second modulator of NEMO SUMOylation is poly (ADP-ribose) polymerase 1 (PARP-1). PARP-1 is an abundant chromatin-associated enzyme that can be quickly recruited

to sites of SSB and DSB as a DNA damage sensor. After recruitment to the breaks, PARP-1 is activated by post-translational modifications by adding poly (ADP-ribose) to acceptor proteins such as itself and histones. PARylation alters the steric properties of the PARylated proteins, leading to change of their interacting partners. PARP-1 is essential for maintaining genomic integrity and involved in base excision repair, SSB and DSB repair, DNA methylation, transcription regulation, and also signal transduction.^[61-63] DNA-bound PARylated PARP-1, or free PARylated PARP-1 in the nucleoplasm, serves as a docking platform for several proteins, such as PIASy.^[64,65] Upon DNA damage, PARylated PARP-1 was found to form a transient nuclear signalsome along with ATM, NEMO and PIASy, and PIASy binding to active PARylated PARP1 is essential for DNA damage-induced NEMO SUMOylation and nuclear accumulation.^[65] A recent study also identified Src-associated-substrate-during-mitosis-of-68 kDa/KH domain containing RNA binding, signal transduction-associated 1 (Sam68/KHDRBS1) as a key NF- κ B regulator in the genotoxic stress-initiated NF- κ B signaling pathway.^[66] Sam68 deficiency abolished DNA damage-induced PARylation and the PARP1-dependent NF- κ B-mediated transactivation of anti-apoptotic genes. Consistently, Sam68 deficient cells are hypersensitive to genotoxic treatment while overexpression of Sam68 elevated PAR production and NF- κ B-mediated anti-apoptotic transcription in colon cancer cells. Another study suggested that cell membrane protein MUC13 may also participate in regulation of genotoxic NF- κ B signaling. Although the detailed mechanism is still unclear, it may involve stabilization of PARP1, enhanced ATM phosphorylation and NEMO SUMOylation.^[67]

In cells exposed to genotoxic treatments, increased nuclear localization of NEMO substantially enhances its association with ATM.^[68] Furthermore, PARP-1 may also stabilize the interaction between NEMO and ATM through PARylation of ATM and formation of the aforementioned nuclear signalsome.^[65] The association between NEMO and activated ATM leads to ATM-dependent phosphorylation of NEMO on Ser 85.^[68] The precise function of NEMO phosphorylation remains to be determined, but ATM activity and intact NEMO-Ser85 are prerequisites for subsequent NEMO monoubiquitination, suggesting NEMO phosphorylation on Ser85 may serve as a cue for its subsequent ubiquitination, such as cIAP1 recruitment. E3 ubiquitin ligase cIAP1, was shown to mediate NEMO mono-ubiquitination at K277 and 309 in the nucleus upon DNA damage in an ATM-dependent manner.^[69] cIAP1 may compete with SUMO ligase PIASy for NEMO association, as both bind to the same

region of NEMO and target the same residues. When overexpressed, cIAP1 inhibits NEMO SUMOylation. Although the function of NEMO monoubiquitination is not fully elucidated, various studies suggested that ubiquitination also regulates subcellular localization of NEMO. In contrast to SUMOylation, monoubiquitination appears to promote nuclear export of NEMO so as to transduce a nuclear signaling into cytoplasm and relay to downstream signaling events.

The nuclear export of NEMO is essential to convey the signal from nucleus to cytoplasm and the underlying mechanisms have been only partially elucidated. Huang *et al.*^[58,68] demonstrated that NEMO monoubiquitination is required for its Ca²⁺- and Ran-GTP-dependent nuclear export. ATM and NEMO are found to be exported together. Another study showed that NEMO may be monoubiquitinated in the cytoplasm after the export of SUMOylated NEMO from the nucleus.^[70] ATM may be also exported in a Ca²⁺-dependent but PARP-1/NEMO/PIASy-independent manner. Nevertheless, the presence of monoubiquitinated NEMO in cytoplasm delivers the nuclear DNA damage signal to cytoplasmic compartment and leads to a cytoplasmic NEMO: ATM: IKK complex, which further promotes NF- κ B activation.^[21]

Cytoplasmic steps: ATM mediates TAK1-IKK activation

In the cytoplasm, ATM still plays important roles to activate the IKK complex. NEMO and ATM were found to form a complex with the IKK-associated protein ELKS (a protein rich in glutamate, leucine, lysine and serine, also called ERC1).^[68] ELKS has been shown to play a role in synaptic plasticity, intracellular transport, and exocytosis by regulating release of neurotransmitters at presynaptic active zones.^[71-73] ELKS has been found as a putative IKK complex component regulating IKK-dependent I κ B α phosphorylation in TNF α -induced NF- κ B activation.^[74] ELKS also forms a complex with NEMO, ATM and IKK, which is required for activation of IKK-upstream kinase TGF-beta activated kinase 1 (TAK1).^[68,75] Further investigation revealed that ELKS is conjugated with K63-linked polyubiquitin chains which depends on ATM and ubiquitin ligase XIAP. Moreover, ATM may also directly bind and promote the ubiquitin ligase activity of TNF receptor-associated factor 6 (TRAF6), leading to TRAF6 auto-ubiquitination with K63-chains.^[70] The K63-linked polyubiquitin chains conjugated on ELKS and TRAF6 could then serve as a docking platform of TAK1/TAB1/TAB2 complex and lead to its activation.

Besides K63-linked polyubiquitination, linear ubiquitin

chains also contribute to NF- κ B activation by DNA damage.^[76] Linear ubiquitin chains are connected by a head-to-tail peptide bond between C-terminal Gly76 of one ubiquitin and N-terminal α -amino group on Met1 of another ubiquitin molecule.^[77] The LUBAC protein complex comprised of hemeoxidized IRP2 ubiquitin ligase-1 (HOIL1), HOIL1-interacting protein (HOIP) and shank-associated RH domain interactor (SHARPIN), was identified as the only E3 ligase specifically to promote linear ubiquitin chain formation.^[78-81] Upon TNF α stimulation, LUBAC was found in a TNFR-supercomplex where it facilitates linear ubiquitination of RIP1 and NEMO.^[82,83] The UBAN domain of NEMO has high affinity for interaction with the linear ubiquitin chain,^[84,85] suggesting the linear ubiquitin chains attached on NEMO or RIP1 may be stabilizing the NEMO/IKK complex within the TNFR-supercomplex, leading to effective activation of IKK. Similarly, NEMO could be modified by linear ubiquitin chains in the cytoplasm of cells exposed to DNA damage. LUBAC is required for DNA damage-induced NEMO linear ubiquitination.^[76]

The linear chain-conjugated lysine residues in NEMO have been identified as Lys285 and Lys309. Lys309 could be modified by monoubiquitination and Lys285 was shown to be conjugated with a single ubiquitin moiety upon DNA damage in another study.^[70] Therefore, it is likely the mono-ubiquitin attached on Lys285 and 309 may serve as a cornerstone for further extension of linear ubiquitin chains. The K63 chains attached on ELKS and TRAF6, along with linear chains anchored on NEMO, may form an intertwined network which provides an optimal binding platform for recruiting and stabilizing association of TAK1/TAB1/TAB2 and IKK complexes. The clustering of TAK1 and IKK complexes leads to effective auto-phosphorylation and activation of TAK1 and subsequent TAK1-dependent IKK activation upon DNA damage. After IKK activation, the downstream signaling events are similar to that in the classical NF- κ B signaling cascade, which involves IKK-dependent I κ B α phosphorylation and degradation, free NF- κ B nuclear translocation and target gene transcription alteration.

In addition to the well-described mechanistic connection linking DNA damage signals to the canonical IKK-NF- κ B pathway, DNA damage may also lead to activation of alternative NF- κ B pathways. RelB was found to be enriched in the nuclei following ionizing radiation in prostate cancer cells, which correlated with poor prognosis in prostate cancer patients.^[86-88] In osteosarcoma cell lines, p100 phosphorylation and subsequent processing to p52 observed following multiple forms of DNA damage.

However, how DNA damage activates alternative NF- κ B pathway, and the mechanistic roles of IKK α , NEMO or ATM in this genotoxic signaling cascade, still remain to be elucidated.

RESOLUTION OF DNA DAMAGE-INDUCED NF- κ B ACTIVATION

As NF- κ B activation plays critical roles in both physiological (e.g. immunity, cell proliferation and survival) and pathological (e.g. inflammation, auto-immune response and cancer progression) processes, tight control of NF- κ B activation is essential for maintaining homeostasis of cell functions. Negative feedback regulation assumes an important part in the control of NF- κ B activity.^[89,90] A classic example is NF- κ B-dependent induction of I κ B α synthesis after stimulation, which specifically antagonizes NF- κ B activity and prevents prolonged NF- κ B activation.^[91,92] Cells deficient in I κ B α present basal, and more sustained signal-inducible, NF- κ B activities.^[93]

Another shared negative feedback mechanism relies on an induced inhibitory mechanism targeting NF- κ B-activating signaling events. Recent studies have shown that expression of deubiquitinases (DUBs), such as A20, are prompted by TNF α stimulation in an NF- κ B-dependent manner. These DUBs then cleave polyubiquitin chains to limit IKK activation.^[94-97] Thus, a deficiency in DUBs A20 or CYLD (cylindromatosis) can prompt augmented and sustained NF- κ B activity in response to inflammatory stimuli and lead to inflammatory disorders as well as oncogenesis. In the following section, we focus on the negative feedback mechanisms induced by DNA damage, which limit the genotoxic NF- κ B activation [Figure 2].

SEN2-dependent inhibition of NF- κ B activation by DNA damage

In DNA damage signaling, SUMOylation of NEMO is a critical signaling event in mediating IKK and NF- κ B activation.^[58] SUMOylation is the posttranslational modification of lysine residues in target proteins by covalent connection of a SUMO moiety, and is biochemically analogous to, but functionally distinct from, ubiquitination. Like DUBs restricting ubiquitination, individuals from the Sentrin/SUMO-specific protease (SEN) family remove SUMO conjugates from their substrates.^[98,99] Due to the reversible nature of SUMOylation, it has been tempting to speculate that desumoylation by an inducible SENP may negatively regulate genotoxic NF- κ B activation. Indeed, it was found the SENP2 and SENP1 are major and minor negative regulators, respectively, of NF- κ B signaling induced by genotoxic stimuli.^[100] Among the six human SENPs, SENP2 interacted most efficiently with NEMO and robustly attenuated NF- κ B activation by genotoxic stress. SENP2 overexpression decreased the level of NEMO SUMOylation and NF- κ B activation initiated by DNA damage. While wild-type MEFs demonstrated transient NF- κ B activation, Senp2^{-/-} MEFs indicated increased genotoxic stress-instigated NEMO SUMOylation and NF- κ B activation.^[100] More interestingly, SENP2 and SENP1 genes are direct targets of NF- κ B whose transcription was substantially increased upon DNA damage. Chromatin IP analysis indicated that treatment with the genotoxic drug etoposide, but not TNF α , leads to increased H3K4me2 at the SENP2 promoter, an epigenetic marker associated with active transcription.^[101,102] The induced histone methylation was ATM-dependent, which is consistent with previous findings that ATM regulates telomere elongation through the H3K4 methyltransferase SpSet1p.^[103]

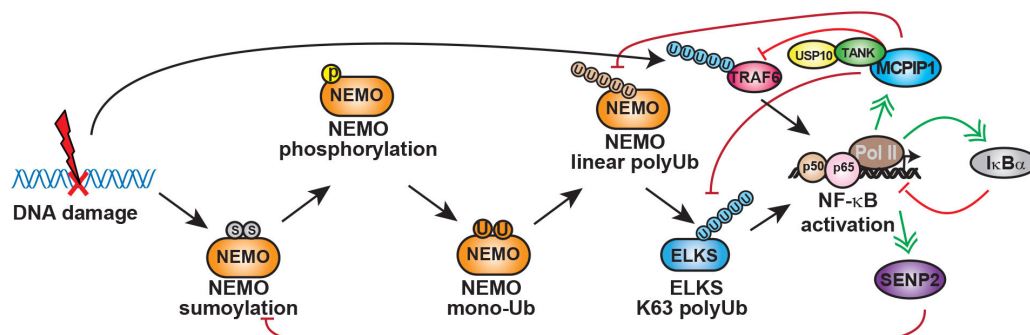


Figure 2: Negative feedback mechanisms modulating genotoxic NF- κ B activation. Upon genotoxic NF- κ B activation, desumoylation enzyme SENP2 can be transcriptionally upregulated, which in turn decreases NEMO sumoylation and suppresses genotoxic NF- κ B signaling. Similarly, MCPIP1 can be upregulated by NF- κ B in response to genotoxic treatment. MCPIP1 may decrease NEMO linear ubiquitination and ELKS K63 polyubiquitination by facilitating their interaction with USP10. Meanwhile, MCPIP1/USP10 forms a complex with TANK, which bridges the association of the deubiquitinase complex with TRAF6 and suppresses TRAF6 ubiquitination. All these deubiquitination events could contribute to the abrogation of genotoxic NF- κ B activation. In addition, as a canonical NF- κ B target gene, I κ B α induction could also diminish NF- κ B activation by DNA damage. NF- κ B: nuclear factor kappa B; SENP2: Sentrin/SUMO-specific protease 2; MCPIP1: monocyte chemotactic protein-1-induced protein-1; TANK: TRAF family member-associated NF- κ B activator; TRAF6: TNF receptor-associated factor 6

Moreover, ATM was shown to promote DNA repair by regulating RNF20 phosphorylation and histone methylation, including H3K4me2.^[104,105] These results also indicate that ATM not only mediates activation of NF- κ B signaling in response to DNA damage, it also facilitates NF- κ B-dependent transcription by promoting epigenetic modification to synergistically enhance NF- κ B-target gene induction. As in the situation with DUBs in the cytokine signaling,^[106] the relative significance of NF- κ B-dependent feedback regulation mediated by SENPs in genotoxic signaling probably depends on cell types and the nature of DNA damage stimuli. Nevertheless, SENP2/1 induction upon genotoxic stress provided the first negative-feedback mechanism to uniquely regulate DNA damage-induced NF- κ B activation through modulating SUMOylation/desumoylation of NEMO.

Suppression of genotoxic NF- κ B activation by MCPIP1/USP10

Monocyte chemotactic protein-1-induced protein-1 (MCPIP1, also called ZC3H12A) was initially recognized as a potential transcription factor in cardiac myocytes regulating apoptosis and chronic inflammatory response.^[107] Further studies demonstrated that MCPIP1 can be induced in macrophages, which depend on down-regulation of NF- κ B signaling, to modulate inflammatory gene expression. MCPIP1-knockout mice displayed severe immune disorders, growth retardation and premature death.^[108-110] It was found that MCPIP1 could bind to the 3'-untranslated regions (UTRs) of a subset of inflammatory cytokine genes including IL6 and IL12, and destabilize the bound mRNAs through its RNase activity.^[110] Intriguingly, MCPIP1 was found to limit LPS-induced NF- κ B activation in macrophages through expulsion of polyubiquitin chains from TRAF proteins.^[109,111] The transcription of MCPIP1 was also upregulated, in a NF- κ B-dependent fashion, in cells exposed to genotoxic stimulation.^[112] Further investigation revealed that MCPIP1, although itself lacks DUB activity, could serve as an adaptor protein to enhance interaction of deubiquitinase USP10 with polyubiquitinated NEMO, which in turn removes the linear ubiquitin chains from NEMO and suppresses NF- κ B signaling upon DNA damage. Two N-terminal domains of MCPIP1 are essential for MCPIP1 to direct USP10-dependent deubiquitination. The RNase-CCCH domain is required for interaction between MCPIP1/NEMO/USP10 and the UBA domain is required for MCPIP1 binding to ubiquitin chains, which may present the ubiquitinated substrates to USP10 for cleavage.^[112] Consistently, NF- κ B-dependent gene transcription upon DNA damage was significantly enhanced in MCPIP1-deficient cells. Therefore, induction of MCPIP1 serves as a negative

feedback response to attenuate NF- κ B activation by DNA damage.

TANK-dependent inhibition of NF- κ B signaling

TRAF family member-associated NF- κ B activator (TANK, also known as I-TRAF) was originally identified as a protein associated with the TRAF2 and TRAF3, and involved in TRAF-mediated NF- κ B signaling pathways.^[113-115] In response to viral infection-induced retinoic acid-inducible gene 1 (RIG-I) activation, TANK serves as an adaptor bridging TRAF3 association with TBK1 and IKK ϵ , which promotes phosphorylation and activation of Interferon Regulatory Factor 3 (IRF3)/IRF7 as well as NF- κ B signaling.^[116-119] However, TANK was also shown to negatively regulate NF- κ B activation.^[119,120] NF- κ B activation upon TLR or BCR (B-cell receptor) stimulation was enhanced in macrophages and B cells isolated from Tank^{-/-} mice compared with their wild-type counterparts. Interestingly, TANK deficiency increased TRAF6 ubiquitination in response to TLR stimulation in macrophages, which may account for the increased NF- κ B activation. However, no canonical deubiquitination enzyme domain can be found in TANK. Also, neither A20 nor CYLD, two common DUBs involved in negative regulation of NF- κ B signaling, was found as TANK-binding partners.^[119] Therefore, the mechanism by which TANK inhibited TRAF6 ubiquitination has been elusive.

We recently reported that TANK represses genotoxic NF- κ B activation, which may rely on suppression of TRAF6 ubiquitination.^[121] TRAF6 polyubiquitination has been shown to play an important role in mediating IKK activation upon DNA damage.^[70] DNA damage-induced NF- κ B activation and TRAF6 ubiquitination were substantially increased in TANK-deficient cells which was reduced by reconstitution of TANK. TRAF6 was found to interact with TANK through its TRAF-C domain and this interaction is required for TANK-mediated deubiquitination of TRAF6. Intriguingly, TANK was identified as a MCPIP1-interacting protein in a proteomic screen and we confirmed that TANK forms a complex with MCPIP1 and USP10. The TANK-associated USP10 is required for the decrease of TRAF6 polyubiquitination, which in turn diminishes the NF- κ B activation by DNA damage. Therefore, USP10 is able to efficiently terminate DNA damage-induced NF- κ B activation through attenuating two critical ubiquitin events, linear ubiquitination of NEMO and K63-ubiquitination of TRAF6, in genotoxic NF- κ B signaling. In addition to genotoxic stress, the TANK-MCPIP1-USP10 complex is also responsible for restraining TRAF6 ubiquitination in cells treated with LPS or IL-1 β . Altogether, these data support that TANK may also serve as a negative regulator of genotoxic

NF- κ B activation by directing USP10-MCPIP1 complex to polyubiquitinated TRAF6.^[121]

Inhibiting genotoxic NF- κ B activation by targeting PARP-1

In cells undergoing apoptosis upon severe DNA damage, PARP-1 is cleaved by caspases which likely restrict any further activation of NF- κ B. The PARP-1 cleavage not only diminishes the DNA repair capacity of the cells, but also blocks anti-apoptotic NF- κ B activation in response to DNA damage, which ensures the elimination of cells with unreparable DNA lesions. In signaling pathways leading to NF- κ B activation, PARP-1 plays a unique role in mediating DNA damage-induced signaling cascade. DNA damage-induced NF- κ B activation is believed to play important roles in mediating acquired resistance in cancer cells treated with genotoxic agents.^[122,123] As NF- κ B also has critical physiological functions, selective inhibition of NF- κ B activated by genotoxic treatments is expected to effectively reduce therapeutic resistance to radiation and chemotherapies with minimal toxicity. Targeting PARP-1 may provide an attractive opportunity for selectively inhibiting genotoxic NF- κ B activation while sparing the canonical and alternative NF- κ B pathways.^[124]

PHYSIOLOGICAL AND PATHOLOGICAL FUNCTIONS OF GENOTOXIC NF- κ B ACTIVATION

DNA damage-induced NF- κ B activation and B-cell differentiation

Endogenous DSBs occurrence is an obligate consequence of B-lymphocyte development because of somatic rearrangement of immunoglobulin loci that is important for generation of antibody diversity and isotype class switching. These physiological DSBs mobilize the DDR machinery in a way reminiscent of the cell reaction to exogenous DNA damage.^[125] As a result, mice deficient in DDR components, particularly in genes required for detecting and repair of DSBs (e.g. ATM), demonstrate defects in normal B-lymphocyte function and reactions to pathogens.^[126] Also, in human, a number of essential immune deficiencies occur, owing to monogenic defects in DDR signaling.^[127,128] Interestingly, NF- κ B activation by endogenous DSBs formed during lymphocyte differentiation has been detected in mice.^[129] Similar to the response to exogenous DSBs, NF- κ B activation by endogenous DSBs requires NEMO and ATM signaling. In this scenario, NF- κ B activation by endogenous DSBs up-regulates a cohort of genes including Pim2 and CD40, whose expression is required for preventing apoptosis and B-cell development. Consistently, patients with

mutations in the gene encoding NEMO show defects in B-lymphocyte differentiation. Several mutations in NEMO map to the C-terminal ZF area that is vital for DNA damage-induced NF- κ B activation. All tested NEMO ZF alleles have proven specifically defective for the genotoxic NF- κ B pathway, although NF- κ B activation by LPS stimulation remains largely intact. A prominent feature of B lymphocytes from patients with NEMO ZF mutations is the inability to carry out class switch recombination and almost complete absence of memory B cells.^[130,131] Notably, microarray examination indicated that those genes required for class switch recombination and proliferation failed to be induced in patient B cells undergoing class switching *in vitro*.^[131] This phenotype may be explained, to some degree, by the modest defects in CD40 signaling that are also observed in these samples. However, it is also speculated that the defect in B-cell functions observed in patients with NEMO ZF mutations may specifically require DNA damage-induced NF- κ B activation.^[132]

Constitutive ATM-NEMO-NF- κ B activity in AML and MDS

Besides DNA damage-induced ATM-NEMO-dependent NF- κ B activation, a recent study showed that ATM-NEMO-NF- κ B signaling is constitutively activated in certain acute myeloid leukemia (AML) cell lines and in a high percentage of primary myelodysplastic syndrome (MDS) and AML patient samples.^[133] Utilizing the P39 AML cell line, it was found that ATM is constitutively active with NEMO and PIDD in the nucleus, and an ATM-NEMO nuclear complex is clearly detectable without genotoxic treatments. Inhibition of ATM by KU55933 or ATM knockdown results in the loss of nuclear NEMO and PIDD, dissociation of ATM-NEMO complex, restraint of NF- κ B activation, and induction of cell death. Also, active ATM was detected in CD34⁺ bone marrow mononuclear cells acquired from all high-risk MDS or AML patient samples, indicating constitutive ATM activation. In line with this notion, pharmacological inhibition of ATM induced cytoplasmic redistribution of NEMO and PIDD (which were found constitutively in the nucleus of these cells), inhibition of constitutive NF- κ B activation and cell death.^[133] Although constitutive activation of NF- κ B is frequently found in various types of tumor specimens,^[134-136] in many cases, the mechanisms that maintain NF- κ B activity are unclear. Considering the genomic instability as a hallmark for human cancers,^[137] it is tempting to speculate that endogenous DNA damage-induced signaling may account for, at least in part, maintaining constitutive NF- κ B activation in various human malignancies, in addition to high-risk MDS and AML.

Genotoxic NF- κ B activation in acquired cancer therapeutic resistance

Substantial evidence indicates that NF- κ B regulates oncogenesis and tumor progression. NF- κ B activation (i.e. nuclear localization) has been observed in a variety of solid tumors.^[138,139] In general, NF- κ B is believed to promote cancer development by contributing to all intrinsic hallmarks of cancer, including therapeutic resistance and metastasis.^[137,138] Ionizing radiation and chemotherapeutic drugs such as doxorubicin, 5-fluorouracil and cisplatin have been found to activate NF- κ B signaling in various cancer cells.^[140-142] The genotoxic agent-induced NF- κ B activation has been considered as a major mechanism through which various cancers acquire therapeutic resistance.^[143]

A number of NF- κ B target genes which prevent apoptosis and promote proliferation, such as cyclin D1, bcl-2, bcl-xL, survivin, and XIAP, were upregulated in cancer cells upon genotoxic treatments.^[144-146] Furthermore, chemotherapeutic resistance of cancer cells highly correlates with their ability to metastasize, which indicates that genotoxic treatment may induce pro-metastatic responses in refractory cancer cells.^[147]

Recent studies have suggested that DNA damage response could enhance the expression of pro-inflammatory cytokines, such as Interleukin-6 (IL-6) and Interleukin-8 (IL-8), favoring tumor growth, angiogenesis and malignant cell invasion.^[148] Inflammation has been shown to play a significant role in promoting cancer metastasis.^[149] As NF- κ B is a key modulator of inflammation, it is plausible that chemotherapy-induced NF- κ B activation may facilitate tumor metastasis by promoting an inflammatory response. Along with pro-inflammatory cytokines, accumulating evidence also suggests that certain cancer-related microRNAs may also play important roles in breast cancer metastasis.^[150-152] MicroRNAs are a class of small non-coding RNAs (~20-24 nucleotides), which primarily bind to the 3'-untranslated region (3'-UTR) of target mRNA and negatively regulate gene expression at the post-transcriptional level.^[153] Most miRNA genes are transcribed into primary miRNAs by RNA polymerase II, which can be sequentially processed into precursor, then mature miRNAs. The transcription of miRNA can be regulated by both upstream DNA transcription regulatory elements, such as conserved transcription factor binding sites, and epigenetic modifications.^[154] Genotoxic stimulation was shown to alter miRNAs expression in cancer cells at transcriptional and post-transcriptional levels.^[155-157] NF- κ B has also emerged as an important transcription regulator of miRNA genes.^[158-161] A recent report showed that genotoxic NF- κ B activation regulates the expression of both pro-

inflammatory cytokine IL-6 and oncogenic miR-21 in TNBC cells, which may promote TNBC cell survival and invasion upon Dox treatment.^[162] Moreover, IL-6-dependent STAT3 activation further enhanced miR-181a transcription, whose upregulation suppressed pro-apoptotic gene BAX level and reduced cancer cell apoptosis upon chemotherapy.^[163] It is plausible that NF- κ B plays a critical role in regulating therapeutic resistance and subsequent metastasis in genotoxic drug-treated breast cancer cells, through coordinating expression of anti-apoptotic genes, pro-inflammatory cytokines and oncogenic miRNAs. Thus, inhibiting genotoxic drug-induced NF- κ B activation may serve as a promising strategy to reduce chemotherapeutic resistance and subsequent metastasis.

TARGETING NF- κ B TO SUPPRESS THERAPEUTIC RESISTANCE

A number of NF- κ B blocking agents, such as IKK inhibitors, inhibitory peptides, antisense RNA, proteasome inhibitors and dietary supplements, are currently being tested in combination with chemotherapy and radiotherapy. These studies aim to sensitize cancer cells to the tumoricidal effects of chemotherapeutic drugs and radiation by blocking NF- κ B activation, thereby preventing acquired resistance. It is noteworthy that NF- κ B also plays critical roles in regulating physiological process, such as immune response. Non-selectively inhibiting NF- κ B may lead to severe adverse effects such as immunodeficiency. Whether such a NF- κ B inhibitor will do more harm than good with respect to the immune system in cancer patients is likely to depend on the particular target or combination of targets on which it acts.

In this regard, agents selectively targeting NF- κ B signaling activated specifically by DNA damage are expected to show much greater promise in antagonizing therapeutic resistance without compromising the immune system. The recent development of PARP-1 inhibitors may provide an extraordinary opportunity for clinical application of such a genotoxic NF- κ B selective inhibitor. It has been shown that PARP inhibitors significantly augmented cell death in BRCA1/2-deficient cancer cells, while no overt toxicity was observed in normal cells expressing functional BRCA1/2.^[164,165] This effect was termed "synthetic lethality", which is believed to be a promising "targeted" strategy to selectively eliminate cancer cells harboring BRCA1/2 mutation while sparing normal cells.^[166,167] Currently, FDA has approved the PARP inhibitors olaparib and rucaparib for treating ovarian cancer patients with BRCA mutation. It has been shown that DNA damage-induced NF- κ B activation in human

cancer cells was significantly attenuated by PARP-1 inhibitors.^[168] Moreover, a PARP1 inhibitor, AG-014699 was shown to sensitize glioma cells to radiation via inhibiting NF- κ B activation.^[169] Thus, inhibiting PARP1 may not only further diminish the DNA repair capacity of BRCA-deficient breast cancer cells, but also abolish genotoxic drug-induced NF- κ B activation and sensitize TNBC patients to chemotherapy.

CONCLUSION AND PERSPECTIVE

The studies on DNA damage response and NF- κ B have significantly improved our understanding of molecular signaling leading to genotoxic NF- κ B activation in the last two decades. These studies have also provided promising drug targets, such as PARP-1, for selectively inhibiting NF- κ B activation by radiation and chemotherapeutics in cancer cells, which may be able to resensitize treatment-refractory cancer cells to conventional chemotherapy. The extension of the period that cancer patients can benefit from these cost-effective “old” chemotherapeutic drugs will also substantially alleviate the financial burden the patients bear due to the high cost of newly developed therapeutic agents. Nevertheless, further investigation is still much needed to explore the critical downstream NF- κ B-target genes which are specifically induced by DNA damage. Better understanding of how these induced genes, protein genes or non-coding RNAs, modulate cell response to DNA damage, will help to develop novel therapeutic agents selectively targeting those pro-survival/metastatic targets and counteract acquired therapeutic resistance. Furthermore, these gene signatures may also serve as predictive biomarkers for evaluating the potential benefit and effectiveness in patients who receive cytotoxic chemotherapies and radiation.

Authors' contributions

The study's conception and design: W. Wang, A.M. Mani, Z.H. Wu

Paper's writing: W. Wang, A.M. Mani, Z.H. Wu

Manuscripts review and edition: Z.H. Wu

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Conflicts of interest

There are no conflicts of interest.

Patient consent

There is no patient involved.

Ethics approval

This article does not contain any studies with human

participants or animals.

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Percutaneous, computed tomography guided neurolysis using continuous radiofrequency for pain reduction in oncologic patients

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ABSTRACT

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Aim: This study evaluates the efficacy and safety of percutaneous computed tomography (CT)-guided neurolysis using continuous radiofrequency for pain reduction in oncologic patients. **Methods:** Over the course of 16 months, 22 patients underwent radiofrequency neurolysis as palliative therapy for pain reduction in celiac and splachnic plexus ($n = 9$), thoracic ($n = 1$), lumbar ($n = 2$) and superior hypogastric plexus ($n = 5$), as well as stellate ganglion ($n = 5$). Pain levels before treatment, one week after treatment, and at the last follow-up (average follow-up 6 months) were compared by means of a Numeric Visual Scale (NVS) questionnaire and a Brief Pain Inventory (Short Form) questionnaire. **Results:** Median procedure time was 44 min. Median number of CT scans, performed to control correct positioning of the cannula and precise electrode placement, was 8. Pain scores of questionnaires prior to treatment (mean value 9.50 NVS units, range 8-10 NVS units) and post treatment (mean value 3.27 NVS units, range 2-6 NVS units) showed a mean decrease of 6.23 NVS units in terms of pain reduction and life quality improvement ($P < 0.05$). Overall mobility improved in 18/18 (100%) patients. No complication was observed. **Conclusion:** This study concludes that CT-guided neurolysis by means of continuous radiofrequency ablation is a safe and efficient technique for pain palliation in oncologic patients.

INTRODUCTION

Approximately half of cancer patients report pain at the

time of diagnosis, and nearly 80% of advanced stage cancer patients report moderate to severe pain.^[1]

Cancer pain can be classified as nociceptive (caused



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by ongoing tissue damage) or neuropathic (caused by damage or dysfunction in the nervous system), or mixed pain.^[2,3] The World Health Organization (WHO) analgesic ladder was introduced in 1986 as a simplified model of analgesics escalation.^[4] The three-step analgesic ladder proposed by WHO provides satisfactory pain management in a significant proportion of cancer patients. However, nearly one third of oncologic patients complain of refractory (nonresponsive) pain.^[5] Despite its value, opioid administration can be costly; additionally, dose and continuous use relate directly to risk of harm.^[6,7] Recently, a fourth step was proposed for refractory pain, which includes minimally invasive percutaneous techniques.

Radiofrequency ablation has been used since the 1970s for chronic pain therapy in cases of refractory pain. Ablation with continuous radiofrequency results in high temperatures (60-80 °C) that promote neurolysis and destroy the target nerves.^[8,9] In contrast, application of pulsed radiofrequency maintains the temperature below 42 °C, promoting neuromodulation of the target nerves.^[10,11] Both continuous and pulsed radiofrequency modes have been applied for pain reduction in symptomatic cancer patients with refractory pain. Percutaneous neurolysis can be either chemical (phenol or alcohol injection) or thermal (radiofrequency or cryoablation). During continuous radiofrequency (RF) ablation an electrode is placed close to a nerve fiber, applying energy which is transformed to high temperature causing protein denaturation and destruction of the axons, resulting in transmission blockage of nociceptive signals from the periphery.^[12]

The purpose of this study is to evaluate efficacy and safety of percutaneous computed tomography (CT)-guided neurolysis using continuous radiofrequency for pain reduction in oncologic patients with pain refractory to standard treatments proposed in the WHO three-

step analgesic ladder.

METHODS

All patients were informed about the technique itself as well as about possible benefits and complications. All patients signed a written consent form for the procedure. Authors have no conflict of interest to declare. No industry support was received for this study.

Patient selection

This is a retrospective study evaluating a consecutive series of patients undergoing CT-guided neurolysis using continuous radiofrequency ablation. During the last 16 months, 22 patients (10 males/12 females) suffering from cancer pain refractory to systemic therapy with opioids and adjuvant drugs were referred for percutaneous CT-guided neurolysis as palliative therapy for pain reduction. All patients treated had no contraindications for regional blockade. Malignant background included pancreatic carcinoma ($n = 8$), pancoast tumor ($n = 5$), lymphoma ($n = 1$), renal cell ($n = 1$), endometrial ($n = 2$), colon ($n = 2$) and ovarian ($n = 3$) carcinoma. Patients underwent neurolysis by means of radiofrequency (Diros Technology Inc, Ontario Canada) in celiac and splanchnic plexus ($n = 9$), in thoracic ($n = 1$), in lumbar ($n = 2$), in superior hypogastric plexus ($n = 5$) and in stellate ganglion ($n = 5$) [Figure 1].

Technique

All procedures were performed in the CT room, under anesthetic monitoring and strict aseptic technique by two interventional radiologists with cooperation of an anesthesiologist. Unilateral or bilateral approach was used, depending on the blockage that was performed. The procedural route and site of the skin puncture were determined with CT guidance. Once the puncture site was located, local anesthetic (5-10 mL of Lidocaine Hydrochloric 2%) was injected into the subcutaneous soft tissue. Under continuous CT scans at the level



Figure 1: Cannulae are percutaneously placed in the desired location, most commonly under computed tomography guidance. Coaxially 10 mm active tip radiofrequency electrodes are inserted and connected to the generator. Motor and sensory tests are performed prior to the neurolysis session

of interest, 20 G trocar(s) was (were) percutaneously inserted and advanced. The final trocar position was verified with CT scan post contrast medium injection.

For the celiac plexus neurolysis, a posterior transcrural approach was used, with needles passing through the diaphragmatic crura in route to the celiac plexus anterolateral to the aorta. For the neurolysis of the splanchnic nerves, a retrocrural approach was performed with the needles remaining posterior to the diaphragmatic crura and placed at the level of L1 vertebral body (cephalad half) and at midportion of T12 vertebral body. For the lumbar plexus neurolysis, two or three needles were placed over the transverse process of L2, L3 and L4 vertebrae, respectively, with the needle tip at the anteromedial vertebral body surface where the lumbar sympathetic block lies. For the superior hypogastric neurolysis, the needle was placed at the anterolateral surface of L5-S1 intervertebral disc, either via posterolateral access through the sacral ala and

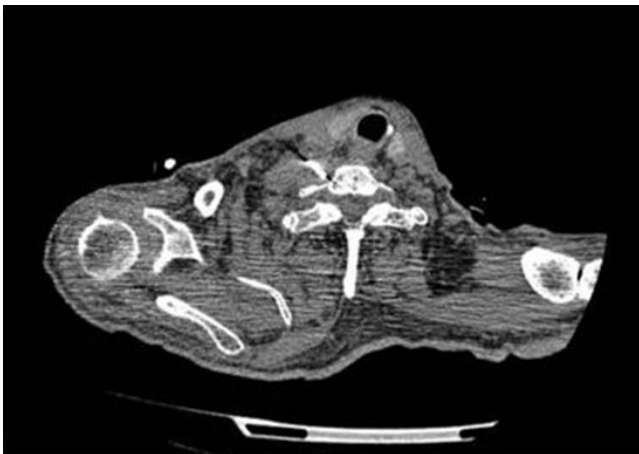


Figure 2: Stellate ganglion neurolysis: the ganglion is usually located lateral to the outer border of longus colli muscle anterior to the neck of the first rib and the transverse process of the 7th cervical vertebra

the superior articular process of S1 or via transdiscal access. Thoracic neurolysis was performed with the needles placed at the space between vertebral body (lateral aspect) and pleura at T2 and T3 levels.

Coaxially, the RF electrode was then inserted [Figures 2-4]. Motor and sensory tests were performed to verify the electrode's correct position near the sensory nerve segment and away from the motor root. Upon satisfactory test results, two CRF ablation sessions were performed at 80 °C, with total duration time of 90 s each. All patients were closely observed postoperatively for pain, sensory and motor deficits, as well as for vital signs. Patients remained in the hospital overnight for hydration and observation and exited the morning after the procedure.

Outcome measures

Pain assessment was performed using the Numeric Rating Scale (NRS, 0-10) questionnaire and Brief Pain Inventory (Short Form) questionnaire for reviewing quality of life.^[19] The questionnaires were recorded before the treatment, one week after treatment, and at the last follow-up (average follow-up 6 months).

RESULTS

Twenty-two patients were studied, all suffering from cancer pain refractory to systemic therapy with opioids and adjuvant drugs. All patients completed the follow-up of six months.

Median procedure time was 44 min. Median number of CT scans, performed to control correct positioning of the cannula and precise electrode placement, was 8. No complications occurred during the procedure, and all patients tolerated the procedure well.

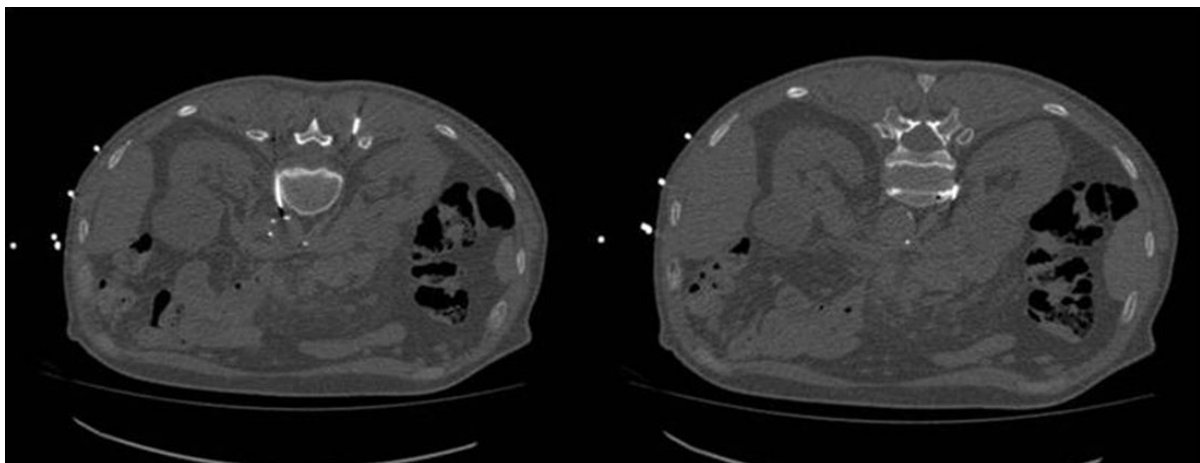


Figure 3: Splanchnic neurolysis is a modification of the classic retrocrural approach for celiac plexus. Needles are placed at the midportion of T12 vertebral body

Comparing the self-reported pain scores of questionnaires prior to treatment (mean value 9.50 NRS units, range 8-10 NRS units) and at 6 months post treatment (mean value 3.27 NRS units range 2-6 NRS units), there was a mean decrease of 6.23 NRS units in terms of pain reduction and life quality improvement [Figure 5]. Overall mobility improved in 18/18 (100%) patients.

DISCUSSION

Cancer pain has direct implications for patients' quality of life. Cancer pain can be classified as nociceptive (described as somatic or visceral and caused by



Figure 4: Neurolysis of lumbar sympathetic chain: three needles are placed over the anterolateral surface of L2, L3 and L4 vertebral bodies

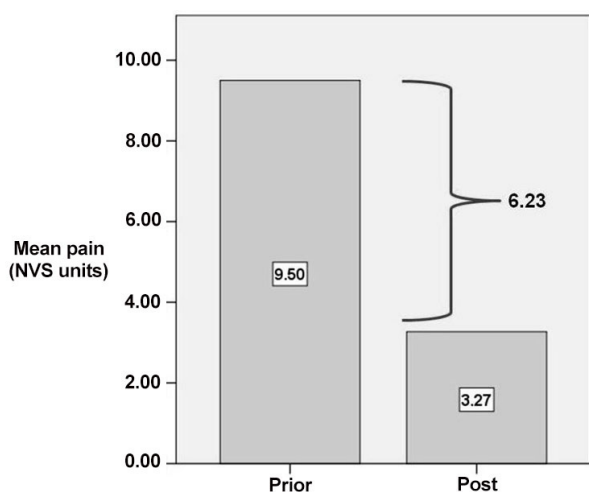


Figure 5: Chart illustrating mean pain scores and pain reduction prior to and after the neurolysis session. NVS: numeric visual scale

ongoing tissue damage) or neuropathic (caused by damage or dysfunction in the nervous system).^[2,3] The WHO analgesic ladder has three steps for acute pain, chronic pain without control, or acute crises of chronic pain. In step 1, nonopioids, analgesics, and NSAIDs are administered to the patient. In step 2, weak opioids can be added to the treatment regime. In step 3, methadone or strong opioids can be administered orally or by means of a transdermal patch.

Unfortunately, conservative therapy does not adequately reduce pain in the vast majority of oncologic patients (56% to 82.3%).^[2,3] On the other hand, numerous studies in the literature report significant pain reduction post chemical or thermal neurolysis.^[13-16] Papadopoulos *et al.*,^[17] who conducted treatment with radiofrequency ablation of splanchnic nerves on 35 patients with end-stage pancreatic abdominal cancer pain refractory to conservative treatment, reported significant decrease in pain scores and consumption of opioids and significant improvement in the patient quality of life during a follow-up period of 6 months.

Our study included patients with a diversity of malignant substrate, evaluating the efficacy of RF neurolysis in celiac and splanchnic plexus ($n = 9$), in the thorax ($n = 1$), in the lumbar region ($n = 2$), in superior hypogastric plexus ($n = 5$), and in the stellate ganglion ($n = 5$). The results of our study (statistically significant mean decrease of 6.23 NVS units on terms of pain reduction and life quality improvement) are in agreement with the previously mentioned success rates.

Percutaneous neurolysis has been reported as a safe procedure with a low complications rate. The most commonly reported complications include transient diarrhea (10-25%), orthostatic hypotension (20-42%), and local pain. Rarer complications include paresis, pneumothorax, shoulder pain (1%), hemorrhagic gastritis, duodenitis, and death.^[18-20] In our study we performed continuous RF neurolysis in all our patients, and we did not experience any complications. We believe that continuous RF neurolysis has a shorter risk-benefit ratio than alcohol neurolysis, since it is a more sophisticated and targeted interventional technique. When compared to medical management by opioids, percutaneous neurolysis superior in terms of fewer burdensome side effects.^[5,21,22]

Correct cannula positioning should always be verified with electrical stimulation prior to ablation. Two stimulation types are performed: sensory and motor. Successful electrical sensory stimulation triggers pain that aligns with the patient's usual distribution of pain. When motor stimulus is performed, there should be no motor response in a threshold below 2.0 volts or below

twice the threshold value of the sensory test. Sensory testing increases the technique's efficacy, and motor stimulation ensures safety from motor impairment.

Limitations of our study include its retrospective nature as well as the diversity of the malignant substrate. Additionally, there was no randomized comparison between continuous RF neurolysis and placebo therapy, chemical neurolysis with phenol or alcohol, or medical management.

Radiofrequency neurolysis under CT guidance is feasible and reproducible, efficient (70-80% success rate), and safe (> 0.5% mean complications rate) as palliative therapy for pain reduction in oncologic patients with refractory pain. Thorough knowledge of nervous system anatomy and pain transmission pathways is essential for proper patient and technique selection.

Authors' contributions

Text writing: P. Zavridis, A. Mazioti

Statistic analysis: M. Tsitskari

Submission editing: D. Filippiadis

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Conflicts of interest

There are no conflicts of interests.

Patient consent

Patient consent was obtained.

Ethics approval

Ethics approval was obtained.

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Expression of *PANDA*, *LincRNA-p21*, *PUMA* in lung tissues of lung cancer patients in the Xuanwei and non-Xuanwei areas of Yunnan Province

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ABSTRACT

Aim: To study the expression of *PANDA*, *LincRNA-p21*, and *PUMA* in lung tissue of patients with lung cancer from Xuanwei of Yunnan Province. **Methods:** Forty-five cases of lung cancer patients from Xuanwei and 42 lung cancer cases from non-Xuanwei were enrolled. Extraction of RNA was done using the Trizol kit. Real-time fluorescence quantitative PCR assay was done to obtain the relative expression. **Results:** Expressions of *PANDA*, *LincRNA-p21*, and *PUMA* in male and female patients or in squamous cell carcinoma and adenocarcinoma were not significantly different ($P > 0.05$). However, expression of *LincRNA-p21* in Xuanwei patients was higher than non-Xuanwei patients ($P < 0.05$). Expression of *PUMA* in tumor tissue was lower than that in normal lung tissue ($P < 0.05$), and in Xuanwei patients was lower than non-Xuanwei patients ($P < 0.05$). In patients from non-Xuanwei regions, expression of *LincRNA-p21* in patients with smoking index > 400 was higher than in those < 400 and non-smokers. **Conclusion:** Expressions of *PANDA*, *LincRNA-p21*, and *PUMA* in lung tissues have no gender differences or tissue specificity. High expression of *LincRNA-p21* in Xuanwei patients may have relationship with cell damage caused by coal burning pollution in Xuanwei.

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Xuanwei area

INTRODUCTION

Xuanwei is located in the northeast of Yunnan, China. In the east it is bordered by Panxian County of Guizhou,

in the south by Zhanyi and Fuyuan Counties. Xuanwei has a population of 1,518,500. It has an estimated 801,100 males and 717,400 females. The primary source of income in Xuanwei is from agriculture, coal



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mining, and coal-related industries.

Xuanwei is an area with high deposits of coal and also has high incidence of lung cancer as well as high mortality rate from lung cancer. From 2004 to 2005, the mortality rate of lung cancer in Xuanwei was 91.30/10⁵, 2.26 times higher than that of Yunnan Province and 2.96 times higher than that of all of China.^[1] Current research suggests that the high incidence of lung cancer in Xuanwei is related to air pollution caused by bituminous coal, with environmental factors perhaps accounting for 70% and 30% depending on susceptibility factors.^[2]

Development of lung cancer is a complex biological, multistep process, which not only activates oncogenes and inactivates the tumor suppressor genes, but also has a close relation with imbalance of apoptosis.^[3] PANDA (DNA damage activates p21 related non-coding RNA), LincRNA-p21 (Cyclin-dependent kinase inhibitor 1a, CDKN1A/p21), and PUMA (p53 upregulated modulator of apoptosis) play an important role in p53-dependent mitochondrial apoptotic pathway when DNA is damaged. When DNA damage occurs, it activates expression of p53 and LincRNA-p21. In one aspect, the combination of p53 and LincRNA-p21 activates expression of PUMA, which then interacts with antiapoptotic members of the family Bcl-2, Bcl-XL, Bcl-2L1, Bcl-2L2, Bcl-2L3, Bcl-2L4, Bcl-2L5, Bcl-2L6, Bcl-2L7, Bcl-2L8, Bcl-2L9, Bcl-2L10, Bcl-2L11, Bcl-2L12, Bcl-2L13, Bcl-2L14, Bcl-2L15, Bcl-2L16, Bcl-2L17, Bcl-2L18, Bcl-2L19, Bcl-2L20, Bcl-2L21, Bcl-2L22, Bcl-2L23, Bcl-2L24, Bcl-2L25, Bcl-2L26, Bcl-2L27, Bcl-2L28, Bcl-2L29, Bcl-2L30, Bcl-2L31, Bcl-2L32, Bcl-2L33, Bcl-2L34, Bcl-2L35, Bcl-2L36, Bcl-2L37, Bcl-2L38, Bcl-2L39, Bcl-2L40, Bcl-2L41, Bcl-2L42, Bcl-2L43, Bcl-2L44, Bcl-2L45, Bcl-2L46, Bcl-2L47, Bcl-2L48, Bcl-2L49, Bcl-2L50, Bcl-2L51, Bcl-2L52, Bcl-2L53, Bcl-2L54, Bcl-2L55, Bcl-2L56, Bcl-2L57, Bcl-2L58, Bcl-2L59, Bcl-2L60, Bcl-2L61, Bcl-2L62, Bcl-2L63, Bcl-2L64, Bcl-2L65, Bcl-2L66, Bcl-2L67, Bcl-2L68, Bcl-2L69, Bcl-2L70, Bcl-2L71, Bcl-2L72, Bcl-2L73, Bcl-2L74, Bcl-2L75, Bcl-2L76, Bcl-2L77, Bcl-2L78, Bcl-2L79, Bcl-2L80, Bcl-2L81, Bcl-2L82, Bcl-2L83, Bcl-2L84, Bcl-2L85, Bcl-2L86, Bcl-2L87, Bcl-2L88, Bcl-2L89, Bcl-2L90, Bcl-2L91, Bcl-2L92, Bcl-2L93, Bcl-2L94, Bcl-2L95, Bcl-2L96, Bcl-2L97, Bcl-2L98, Bcl-2L99, Bcl-2L100, Bcl-2L101, Bcl-2L102, Bcl-2L103, Bcl-2L104, Bcl-2L105, Bcl-2L106, Bcl-2L107, Bcl-2L108, Bcl-2L109, Bcl-2L110, Bcl-2L111, Bcl-2L112, Bcl-2L113, Bcl-2L114, Bcl-2L115, Bcl-2L116, Bcl-2L117, Bcl-2L118, Bcl-2L119, Bcl-2L120, Bcl-2L121, Bcl-2L122, Bcl-2L123, Bcl-2L124, Bcl-2L125, Bcl-2L126, Bcl-2L127, Bcl-2L128, Bcl-2L129, Bcl-2L130, Bcl-2L131, Bcl-2L132, Bcl-2L133, Bcl-2L134, Bcl-2L135, Bcl-2L136, Bcl-2L137, Bcl-2L138, Bcl-2L139, Bcl-2L140, Bcl-2L141, Bcl-2L142, Bcl-2L143, Bcl-2L144, Bcl-2L145, Bcl-2L146, Bcl-2L147, Bcl-2L148, Bcl-2L149, Bcl-2L150, Bcl-2L151, Bcl-2L152, Bcl-2L153, Bcl-2L154, Bcl-2L155, Bcl-2L156, Bcl-2L157, Bcl-2L158, Bcl-2L159, Bcl-2L160, Bcl-2L161, Bcl-2L162, Bcl-2L163, Bcl-2L164, Bcl-2L165, Bcl-2L166, Bcl-2L167, Bcl-2L168, Bcl-2L169, Bcl-2L170, Bcl-2L171, Bcl-2L172, Bcl-2L173, Bcl-2L174, Bcl-2L175, Bcl-2L176, 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Bcl-2L359, Bcl-2L360, Bcl-2L361, Bcl-2L362, Bcl-2L363, Bcl-2L364, Bcl-2L365, Bcl-2L366, Bcl-2L367, Bcl-2L368, Bcl-2L369, Bcl-2L370, Bcl-2L371, Bcl-2L372, Bcl-2L373, Bcl-2L374, Bcl-2L375, Bcl-2L376, Bcl-2L377, Bcl-2L378, Bcl-2L379, Bcl-2L380, Bcl-2L381, Bcl-2L382, Bcl-2L383, Bcl-2L384, Bcl-2L385, Bcl-2L386, Bcl-2L387, Bcl-2L388, Bcl-2L389, Bcl-2L390, Bcl-2L391, Bcl-2L392, Bcl-2L393, Bcl-2L394, Bcl-2L395, Bcl-2L396, Bcl-2L397, Bcl-2L398, Bcl-2L399, Bcl-2L400, Bcl-2L401, Bcl-2L402, Bcl-2L403, Bcl-2L404, Bcl-2L405, Bcl-2L406, Bcl-2L407, Bcl-2L408, Bcl-2L409, Bcl-2L410, Bcl-2L411, Bcl-2L412, Bcl-2L413, Bcl-2L414, Bcl-2L415, Bcl-2L416, Bcl-2L417, Bcl-2L418, Bcl-2L419, Bcl-2L420, Bcl-2L421, Bcl-2L422, Bcl-2L423, Bcl-2L424, Bcl-2L425, Bcl-2L426, Bcl-2L427, Bcl-2L428, Bcl-2L429, Bcl-2L430, Bcl-2L431, Bcl-2L432, Bcl-2L433, Bcl-2L434, Bcl-2L435, Bcl-2L436, Bcl-2L437, Bcl-2L438, Bcl-2L439, Bcl-2L440, Bcl-2L441, Bcl-2L442, Bcl-2L443, Bcl-2L444, Bcl-2L445, Bcl-2L446, Bcl-2L447, Bcl-2L448, Bcl-2L449, 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Bcl-2L541, Bcl-2L542, Bcl-2L543, Bcl-2L544, Bcl-2L545, Bcl-2L546, Bcl-2L547, Bcl-2L548, Bcl-2L549, Bcl-2L550, Bcl-2L551, Bcl-2L552, Bcl-2L553, Bcl-2L554, Bcl-2L555, Bcl-2L556, Bcl-2L557, Bcl-2L558, Bcl-2L559, Bcl-2L560, Bcl-2L561, Bcl-2L562, Bcl-2L563, Bcl-2L564, Bcl-2L565, Bcl-2L566, Bcl-2L567, Bcl-2L568, Bcl-2L569, Bcl-2L570, Bcl-2L571, Bcl-2L572, Bcl-2L573, Bcl-2L574, Bcl-2L575, Bcl-2L576, Bcl-2L577, Bcl-2L578, Bcl-2L579, Bcl-2L580, Bcl-2L581, Bcl-2L582, Bcl-2L583, Bcl-2L584, Bcl-2L585, Bcl-2L586, Bcl-2L587, Bcl-2L588, Bcl-2L589, Bcl-2L590, Bcl-2L591, Bcl-2L592, Bcl-2L593, Bcl-2L594, Bcl-2L595, Bcl-2L596, Bcl-2L597, Bcl-2L598, Bcl-2L599, Bcl-2L600, Bcl-2L601, Bcl-2L602, Bcl-2L603, Bcl-2L604, Bcl-2L605, Bcl-2L606, Bcl-2L607, Bcl-2L608, Bcl-2L609, Bcl-2L610, Bcl-2L611, Bcl-2L612, Bcl-2L613, Bcl-2L614, Bcl-2L615, Bcl-2L616, Bcl-2L617, Bcl-2L618, Bcl-2L619, Bcl-2L620, Bcl-2L621, Bcl-2L622, Bcl-2L623, Bcl-2L624, Bcl-2L625, Bcl-2L626, Bcl-2L627, Bcl-2L628, Bcl-2L629, Bcl-2L630, Bcl-2L631, 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Bcl-2L723, Bcl-2L724, Bcl-2L725, Bcl-2L726, Bcl-2L727, Bcl-2L728, Bcl-2L729, Bcl-2L730, Bcl-2L731, Bcl-2L732, Bcl-2L733, Bcl-2L734, Bcl-2L735, Bcl-2L736, Bcl-2L737, Bcl-2L738, Bcl-2L739, Bcl-2L740, Bcl-2L741, Bcl-2L742, Bcl-2L743, Bcl-2L744, Bcl-2L745, Bcl-2L746, Bcl-2L747, Bcl-2L748, Bcl-2L749, Bcl-2L750, Bcl-2L751, Bcl-2L752, Bcl-2L753, Bcl-2L754, Bcl-2L755, Bcl-2L756, Bcl-2L757, Bcl-2L758, Bcl-2L759, Bcl-2L760, Bcl-2L761, Bcl-2L762, Bcl-2L763, Bcl-2L764, Bcl-2L765, Bcl-2L766, Bcl-2L767, Bcl-2L768, Bcl-2L769, Bcl-2L770, Bcl-2L771, Bcl-2L772, Bcl-2L773, Bcl-2L774, Bcl-2L775, Bcl-2L776, Bcl-2L777, Bcl-2L778, Bcl-2L779, Bcl-2L780, Bcl-2L781, Bcl-2L782, Bcl-2L783, Bcl-2L784, Bcl-2L785, Bcl-2L786, Bcl-2L787, Bcl-2L788, Bcl-2L789, Bcl-2L790, Bcl-2L791, Bcl-2L792, Bcl-2L793, Bcl-2L794, Bcl-2L795, Bcl-2L796, Bcl-2L797, Bcl-2L798, Bcl-2L799, Bcl-2L800, Bcl-2L801, Bcl-2L802, Bcl-2L803, Bcl-2L804, Bcl-2L805, Bcl-2L806, Bcl-2L807, Bcl-2L808, Bcl-2L809, Bcl-2L810, Bcl-2L811, Bcl-2L812, Bcl-2L813, 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Bcl-2L996, Bcl-2L997, Bcl-2L998, Bcl-2L999, Bcl-2L1000, Bcl-2L1001, Bcl-2L1002, Bcl-2L1003, Bcl-2L1004, Bcl-2L1005, Bcl-2L1006, Bcl-2L1007, Bcl-2L1008, Bcl-2L1009, Bcl-2L1010, Bcl-2L1011, Bcl-2L1012, Bcl-2L1013, Bcl-2L1014, Bcl-2L1015, Bcl-2L1016, Bcl-2L1017, Bcl-2L1018, Bcl-2L1019, Bcl-2L1020, Bcl-2L1021, Bcl-2L1022, Bcl-2L1023, Bcl-2L1024, Bcl-2L1025, Bcl-2L1026, Bcl-2L1027, Bcl-2L1028, Bcl-2L1029, Bcl-2L1030, Bcl-2L1031, Bcl-2L1032, Bcl-2L1033, Bcl-2L1034, Bcl-2L1035, Bcl-2L1036, Bcl-2L1037, Bcl-2L1038, Bcl-2L1039, Bcl-2L1040, Bcl-2L1041, Bcl-2L1042, Bcl-2L1043, Bcl-2L1044, Bcl-2L1045, Bcl-2L1046, Bcl-2L1047, Bcl-2L1048, Bcl-2L1049, Bcl-2L1050, Bcl-2L1051, Bcl-2L1052, Bcl-2L1053, Bcl-2L1054, Bcl-2L1055, Bcl-2L1056, Bcl-2L1057, Bcl-2L1058, Bcl-2L1059, Bcl-2L1060, Bcl-2L1061, Bcl-2L1062, Bcl-2L1063, Bcl-2L1064, Bcl-2L1065, Bcl-2L1066, Bcl-2L1067, Bcl-2L1068, Bcl-2L1069, Bcl-2L1070, Bcl-2L1071, Bcl-2L1072, Bcl-2L1073, Bcl-2L1074, Bcl-2L1075, Bcl-2L1076, Bcl-2L1077, Bcl-2L1078, 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from Xuanwei and 42 patients with lung cancer were from non-Xuanwei regions. All patients were ethnic Han Chinese. Non-Xuanwei regions included Kunming, Yuxi, Baoshan, Dali, Chuxiong, Honghe, and other areas. Eighteen cases of squamous cell carcinoma and 27 cases of adenocarcinoma were from Xuanwei. Seventeen cases of squamous cell carcinoma and 25 cases of adenocarcinoma were from non-Xuanwei. There were 24 males and 21 females from Xuanwei and 24 males and 18 females from non-Xuanwei regions. Mean age of patients from Xuanwei was 54.91 ± 9.62 years while in the non-Xuanwei group, the mean age was 58.33 ± 10.13 years. There were 21 patients from Xuanwei and 22 from non-Xuanwei who were smokers. Eighteen patients from Xuanwei consumed alcohol whereas 16 from non-Xuanwei did [Table 2].

Expressions of LincRNA-p21, PANDA, and PUMA in Xuanwei and non-Xuanwei patients

Expression of LincRNA-p21 in lung tissues of Xuanwei patients was increased ($P < 0.05$). There was no significant difference in expression of it between 2 groups of patients in lung cancer tissues and normal

lung tissues ($P > 0.05$). There was no significant difference in the expression of PANDA in lung cancer tissue and normal lung tissue between the 2 groups ($P > 0.05$). Expression of PUMA in lung cancer tissue was lower than normal tissue ($P < 0.05$). Expression of PUMA in patients from Xuanwei was lower than that of non-Xuanwei patients, not only in cancer tissue, but also in normal lung ($P < 0.05$) [Tables 3 and 4].

Expression of LincRNA-p21, PANDA, and PUMA in squamous cell carcinoma and adenocarcinoma

There was no significant difference in the expression of three genes in squamous cell carcinoma and adenocarcinoma ($P > 0.05$) [Table 5].

Expression of LincRNA-p21, PANDA, PUMA in smokers and non-smokers

The expression of LincRNA-p21, PANDA, PUMA in smokers and non-smokers was showed in Table 6.

Analysis of correlation in expression of LincRNA-p21, PANDA and PUMA in tissues of lung cancer patients from Xuanwei and non-Xuanwei areas

LincRNA-p21 and PANDA in lung cancer tissues and normal lung tissues were characterized by positive correlation. PANDA with PUMA in lung cancer and normal lung tissue showed no corresponding correlation [Table 7].

Expression of LincRNA-p21, PANDA and PUMA in lung cancer patients from non-Xuanwei areas: LincRNA-p21 was positively correlated with PUMA in normal tissue, but not in lung cancer tissue. LincRNA-p21 and PANDA in lung cancer and normal lung tissue all showed positive correlation. PANDA was positively correlated with PUMA in normal lung tissue but not in lung cancers [Table 8].

Table 2: Comparison between the two groups

	Xuanwei	Non-Xuanwei
<i>n</i>	45	42
Squamous cell carcinoma	18	17
Adenocarcinoma	27	25
Male	24	24
Non-smoking	3	2
Smoking* < 400	7	9
Smoking > 400	14	13
Female	21	18
Non-smoking	21	18
Smoking < 400	0	0
Smoking > 400	0	0
Age	54.91 ± 9.62	58.33 ± 10.13
Alcohol	18	16

*Smoking index: the number of cigarettes smoked per day x number of years of smoking, high-risk groups > 400

Table 3: Expression of LincRNA-p21, PANDA, PUMA in males and females

Gene		Male	Female	<i>P</i>
LincRNA-p21	Xuanwei	15.18 ± 1.44	15.19 ± 1.65	0.63
	Non-Xuanwei	13.33 ± 1.85	13.01 ± 1.05	0.51
PANDA	Xuanwei	14.36 ± 1.65	14.38 ± 2.79	0.88
	Non-Xuanwei	14.14 ± 1.23	14.03 ± 1.67	0.85
PUMA	Xuanwei	5.32 ± 1.85	5.23 ± 1.43	0.74
	Non-Xuanwei	7.45 ± 2.18	7.34 ± 2.42	0.77

Expression of the 3 genes in lung tissues of both males and females showed no statistical difference ($P > 0.05$)

Table 4: Expression of LincRNA-p21, PANDA, PUMA in Xuanwei and non-Xuanwei patients

Gene		Xuanwei	Non-Xuanwei	<i>P</i>
LincRNA-p21	Cancer tissue	15.12 ± 1.27	12.21 ± 1.51	0.03
	Normal tissue	15.07 ± 1.42	12.19 ± 1.62	0.04
PANDA	Cancer tissue	14.36 ± 1.46	14.08 ± 1.27	0.52
	Normal tissue	14.44 ± 1.55	14.11 ± 2.32	0.47
PUMA	Cancer tissue	5.14 ± 1.37	7.38 ± 2.74	0.01
	Normal tissue	$6.19 \pm 1.43^*$	$8.82 \pm 2.89^{**}$	0.01

* $P = 0.04$ (cancer tissue vs. normal tissue); ** $P = 0.03$ (cancer tissue vs. normal tissue)

Table 5: Expression of *LincRNA-p21*, PANDA and PUMA in squamous cell carcinoma and adenocarcinoma

Gene		Adenocarcinoma	Squamous cell carcinoma	P
<i>LincRNA-p21</i>	Xuanwei	15.23 ± 1.31	15.09 ± 1.53	0.56
	Non-Xuanwei	12.31 ± 1.73	12.18 ± 2.05	0.58
PANDA	Xuanwei	14.38 ± 1.64	14.22 ± 1.88	0.74
	Non-Xuanwei	14.11 ± 1.31	14.01 ± 2.15	0.82
PUMA	Xuanwei	5.09 ± 1.15	5.33 ± 1.84	0.64
	Non-Xuanwei	7.39 ± 1.98	7.36 ± 2.76	0.86

Table 6: Expression of *LincRNA-p21*, PANDA, PUMA in smokers and non-smokers

Gene		Non-Smoker	Smoking < 400	Smoking > 400
<i>LincRNA-p21</i>	Xuanwei	15.19 ± 1.01	15.14 ± 1.21	15.16 ± 2.02
	Non Xuanwei	12.95 ± 2.13	13.16 ± 1.73	14.74 ± 2.11*
PANDA	Xuanwei	14.37 ± 1.21	14.37 ± 1.53	14.38 ± 1.74
	Non Xuanwei	14.08 ± 0.88	14.15 ± 1.29	14.14 ± 1.29
PUMA	Xuanwei	5.31 ± 1.47	5.33 ± 1.91	5.32 ± 1.12
	Non Xuanwei	7.31 ± 1.82	7.33 ± 1.46	7.34 ± 2.05

*P < 0.05 (comparing non-smokers with smokers with smoking index < 400 and > 400)

DISCUSSION

The Xuanwei region of Yunnan, China, has a high incidence of lung cancer.^[2] The characteristics of lung cancer in Xuanwei are: (1) the mortality rate of lung cancer in Xuanwei was 28.20/100,000 in 1973-1975, in 1990-1992 it was 40.29/100,000, and in 2004-2005 it was 83.28/100,000, which were higher than the average of the entire Yunnan and the national average; (2) lung cancer caused death 10-15 years earlier than the national average age; (3) lung cancer incidence is high among female population whereas the sex ratio (male:female) is low in other places like Netherland (16.32), Sweden (4.15), China (2.01), whereas in it is 1.09. In the small town of Laibin in Xuanwei the sex ratio for the incidence of lung cancer is 0.87, with females being affected more than males; (4) the incidence of lung cancer in Xuanwei area may be familial.

Indoor burning of bituminouscoal, leading to indoor air pollution, is considered to be the main reason for the high incidence of lung cancer in Xuanwei. Polyaromatic hydrocarbons (PAHs), nano silica particles and other substances in bituminouscoal dust and smoke are thought to be the causes of lung cancer.^[6,7] In 1970, the Chinese government implemented a policy at all levels to improve coal burning stoves which could lead to improvement of indoor air quality. Despite these efforts, lung cancer incidence is still high and with an increasing trend.

The development of lung cancer is a complex biological process which is multistep and multifactorial. It not only includes the activation and inactivation of oncogenes and tumor suppressor gene respectively, but also has close relationship with apoptosis. There are two pathways of apoptosis of which one is the activation of the apoptotic caspase through extracellular signaling while the other is release of cytochrome C and activation of apoptotic enzyme

Table 7: Expression of *LincRNA-p21*, PANDA and PUMA in lung cancer patients from Xuanwei

Gene	Tissue type	Spearman correlation	
		R	P
<i>LincRNA-p21</i> and PUMA	Cancer tissue	0.08	0.62
	Normal tissue	0.12	0.33
PANDA and PUMA	Cancer tissue	0.07	0.58
	Normal tissue	0.14	0.28
<i>LincRNA-p21</i> and PANDA	Cancer tissue	0.81	0.00
	Normal tissue	0.77	0.00

Table 8: Expression of *LincRNA-p21*, PANDA and PUMA in lung cancer patients from non-Xuanwei areas

Gene	Tissue type	Spearman correlation	
		R	P
<i>LincRNA-p21</i> and PUMA	Cancer tissue	0.06	0.67
	Normal tissue	0.35	0.04
PANDA and PUMA	Cancer tissue	0.08	0.62
	Normal tissue	0.42	0.00
<i>LincRNA-p21</i> and PANDA	Cancer tissue	0.84	0.00
	Normal tissue	0.71	0.00

through mitochondrial caspase.^[8,9] It is well known that repeated DNA damage can be carcinogenic, and tumor suppressor p53 increases as a response to DNA damage. Burning of coal produces numerous harmful pollutants and carcinogens which causes damage to DNA. At the molecular level, DNA damage is sensed by human protein kinase ATM (ataxia-telangiectasia, mutated), leading to phosphorylation and activation of ATM. Downstream phosphorylation and activation of TP53, together with RUNX3, activate transcription of downstream mediators as a response to injury to DNA.^[10]

In our previous study with 25 patients of lung cancer (adenocarcinoma and squamous cell cancer) from Xuanwei, we found differences in 33 genes, of which *LincRNA-p21* was one of them. As a result, we chose to study *LincRNA-p21*. In this study, we focused on three selected mediators of TP53 response: PUMA, *LincRNA-p21* and PANDA. PUMA is a member of the BCL2 family and is an important mediator of apoptosis.

Research has found that PUMA is more important than NOXA in DNA damage that induces activation of the p53-mediated mitochondrial apoptotic pathway. PUMA is located downstream of the p53 gene and has a powerful effect in promoting apoptosis and inhibition of cell growth. Low expression of PUMA exists in some tumor tissues and it is associated with the occurrence and development of tumor. Thus, increasing of PUMA can inhibit tumor growth.^[11] Long non-coding RNA p21 interacts with hnRNP-K to activate p21 to enforce cell cycle arrest at the G1/S phase. LincRNA-p21 is an important member of the cell cycle and its expression is directly induced by p53. The result of flow cytometry and apoptosis-related enzyme activity assay confirmed LincRNA-p21 induced apoptosis in the p53 dependent pathway, and related to the formation of tumor. PANDA is a noncoding RNA that inhibits expression of apoptotic genes by sequestering NF- κ B.

When DNA damage occurs, it activates expression of p53 and LincRNA-p21. In one aspect, the combination of p53 and LincRNA-p21 activates expression of PUMA. PUMA interact with members of the antiapoptotic family, and release Bax and Bak. Bax embedded in the outer membrane of mitochondria, leading to increased permeability of the outer mitochondrial membrane. Release of cytochrome C apoptotic factors activates the caspase cascade, eventually leading to cell death.

This study found that expression of PANDA, LincRNA-p21 and PUMA showed no significant difference in lung tissue of male and female ($P > 0.05$). Expression in squamous cell carcinoma and adenocarcinoma also showed no significant difference ($P > 0.05$), indicating that there is no gender difference or tissue specificity. Expression of LincRNA-p21 and PUMA in cancer tissues is not related but in the normal tissue it is positively correlated, perhaps due to existence of regulatory changes and disorder in cancer tissues.

In this study, we found that expression of LincRNA-p21 was higher in patients from non-Xuanwei regions with smoking index > 400 than in patients with smoking index < 400 and non-smokers, while in patients from Xuanwei, there was no significant difference among the three groups ($P > 0.05$). The probable reason might be that smoking is not the main reason for lung cancer in Xuanwei. The incidence of lung cancer is higher in women from Xuanwei, even though the majority of women were non-smokers. In an experiment, we exposed cells to the byproduct obtained from combustion of bituminous coal, the result of which was secretion of mediators of inflammation, damage to the

cell membrane, damage to mitochondria, and mutation in nuclear DNA and mt-DNA.

Expression of LincRNA-p21 in lung cancer patients from Xuanwei was higher than non-Xuanwei patients ($P < 0.05$). We observed that alveolar epithelial cells and bronchial epithelial cells were severely damaged in patients from Xuanwei due to serious air pollution caused by burning of coal. There was also damage to DNA from pollutants released as a result of combustion of coal. Our initial research in Xuanwei found that ash and smoke of bituminous coal contained large numbers of polycyclic aromatic hydrocarbons and nano-sized quartz particles,^[12-14] which were also found in lung tissues of lung cancer patients from Xuanwei. Polycyclic aromatic hydrocarbons and nano-sized quartz particles can lead to damage of nuclear DNA and mitochondrial DNA.^[15,16]

Expression of PUMA in lung tissues of Xuanwei patients was lower than non-Xuanwei patients ($P < 0.05$). The correlation analysis revealed that the expression of LincRNA-p21 had no correlation with expression of PUMA in normal lung tissues of Xuanwei patients, while normal lung tissues of non-Xuanwei patients were positively correlated. We considered that PUMA is a highly conserved gene in eukaryotes, possibly due to air pollution in Xuanwei causing damage to PUMA or effect the gene transcription process, but also polymorphism of LincRNA-p21 and PUMA in local population could not be ruled out, for which further research with larger sample is required.

We have successfully cultured a type of Xuanwei lung adenocarcinoma cell line (XW-05). We are now carrying out gene transfection and silencing experiments on XW-05 cells, human adenocarcinoma cells (A549), and human bronchial epithelial cells (BS2B) to observe whether XW-05 cells have specificity.

Part of this project was under the U.S. National Cancer Institute to study environmental exposure, dose-effect relationship, and epidemiology of lung cancer in Xuanwei. We are responsible for the questionnaire, collection of clinical data, specimen collection, treatment, follow-up, and prognosis. Genomic sequencing is carried out by Beijing Gene Square for National Cancer Institute. Currently, Beijing Gene Square is sequencing specimens of 421 cases, the findings of which should be reported soon.

Authors' contributions

The study's conception and design: K.Y. Yang, Z.Q. Shen

Extraction of RNA and PCR: Y.F. He, K.Y. Yang, K. Rizal
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Conflicts of interest

There are no conflicts of interest.

Patient consent

Patient consent was obtained.

Ethics approval

Ethics approval was obtained.

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A new twist to neurotransmitter receptors and cancer

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BACKGROUND

Nicotinic acetylcholine receptors (nAChRs) and beta-adrenergic receptors (β -ARs) are cell membrane receptors expressed in most mammalian cells where they function as the recipients of signals from the autonomic nervous system that maintains physiological homeostasis in the mammalian organism and regulates cell and organ responses to endogenous and exogenous signals. The neurotransmitter of the parasympathetic branch of the autonomic nervous system, acetylcholine, binds as an agonist to all members of the nAChR family, thus opening the ligand-gated ion channel of the receptors. The resulting depolarization of the cell membrane opens voltage-gated Ca^{2+} -channels (VOCs), allowing influx of additional Ca^{2+} that triggers the release of cell type-specific intracellular products via exocytosis.^[1] Influx of Ca^{2+} is particularly high in response to agonist binding to the homomeric (comprised of alpha subunits only) $\alpha 7$ nAChR due to the selectivity of its ion channel for Ca^{2+} whereas heteromeric (comprised of alpha and non-alpha subunits) nAChRs have non-selective ion channels. The mechanisms of nAChR-

mediated neurotransmitter release by the central and peripheral nervous system, their role in memory, cognition and stress responses and the nAChR-mediated mechanisms of nicotine addiction have been extensively studied.^[1,2]

Beta-adrenergic receptors are coupled to the stimulatory G-protein G_s that activates the enzyme adenylyl cyclase (AC) upon binding of an agonist to the receptor, leading to the formation of intracellular cyclic adenosine monophosphate (cAMP) that activates protein kinase A (PKA) and numerous PKA-dependent and independent intracellular signaling cascades in a cell type-specific manner.^[3] In addition, $\beta 1$ and $\beta 2$ -ARs can increase intracellular Ca^{2+} levels by a variety of mechanisms [Figure 1], including the PKA-induced upregulation of L-type Ca^{2+} -channels^[4] and release of Ca^{2+} from intracellular stores that can also be induced by the cAMP binding protein exchange factor directly activated by cAMP (Epac).^[5] Of particular importance for the regulation of cancer cells is the fact that activated PKA and/or cAMP stimulate the release of epidermal growth factor (EGF),^[6] arachidonic acid (AA),^[7,8] interleukins and vascular endothelial growth factor (VEGF),^[9] which jointly stimulate the development,



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progression and metastasis of numerous cancers. The neurotransmitters of the sympathetic branch of the autonomic nervous system, epinephrine (Epi) and norepinephrine (Nor) are the physiological agonists for β -ARs. Epi and Nor are additionally synthesized and released by the adrenal medulla and are often referred to as “stress neurotransmitters” because psychological stress triggers their simultaneous release from the sympathetic nervous system and adrenal gland.^[10,11] The release of stress neurotransmitters from the sympathetic nervous system and adrenal gland is regulated by nAChRs via Ca^{2+} influx that triggers their exocytosis.^[12,13] The biology of β -ARs as it relates to cardio-vascular disease has been extensively studied and beta-adrenergic receptor antagonists (beta-blockers) and VOC blockers are widely used as therapeutics for this disease complex.^[14-17]

Discoveries that nicotine induced the proliferation of human small cell lung cancer cells *in vitro*^[18] while inhibiting apoptosis,^[19] effects triggered by the nAChR-mediated release and re-uptake of the neurotransmitter 5-hydroxytryptamine (5-HT, serotonin),^[20] first pointed to nAChRs as important regulators of a subset of cancers. Reports that β -AR agonists stimulated the proliferation of lung adenocarcinoma cells *in vitro* and that this response was inhibited by β -blockers first implicated β -ARs in the regulation of another subset of cancers.^[21,22] The identification of the tobacco carcinogen nicotine-derived nitrosamine ketone (NNK) as a high affinity agonist for nAChRs^[23,24] as well as β -ARs^[7] subsequently provided a direct mechanistic link between the high carcinogenic potential of this agent and its interaction with neurotransmitter receptors. These studies also showed that NNK-induced β -AR signaling in lung adenocarcinoma cells and pancreatic ductal adenocarcinoma cells triggered the release of AA, resulting in the formation of cancer-stimulating AA metabolites while additionally trans-activating the epidermal growth factor receptor pathway.^[7,8,25] Collectively, these early findings represented the starting point for a new domain in cancer research: the role of neurotransmitters and their receptors in the initiation, progression and drug resistance of cancer and the development of novel therapeutic and preventive strategies that target this regulatory network.^[26-30]

It was initially thought that nAChRs and β -ARs expressed in non-neuronal cells and cancers derived from them were exclusively stimulated by the autonomic nervous system or by exposure to tobacco products. However, more recent studies have shown that numerous non-neuronal cells and the cancers derived from them synthesize and release their own

acetylcholine^[31] and are also able to synthesize and release Nor and Epi in response to acetylcholine self-stimulation or exposure to exogenous nAChR agonists.^[32-36] In addition, it has been shown that polymorphisms in genes CHRNA3 (encodes the $\alpha 3$ nAChR subunit) and CHRNA5 (encodes the $\alpha 5$ nAChR subunit) as well as a copy number variation that duplicates the $\alpha 7$ nAChR gene CHRNA7 are associated with an increased risk for lung cancer^[37-39] and that single nucleotide polymorphisms in the $\beta 2$ -AR gene are associated with adverse clinical outcomes of pancreatic cancer.^[40]

An important aspect of cancer regulation by neurotransmitters and their receptors is the significant influence of the mood on this regulatory network [Figure 1]. Preclinical investigations have thus shown that experimentally induced psychological stress or treatment with stress neurotransmitters have strong promoting effects on the majority of the most common human cancers via direct activation of cAMP-dependent intracellular signaling pathways by stress neurotransmitters downstream of $\beta 1$ and $\beta 2$ -ARs^[29,32,41-45] and the simultaneous suppression of the tumor suppressor gene *p53* by beta-arrestin-1 signaling downstream of $\beta 2$ -ARs.^[46] Moreover, chronic experimental stress suppressed the synthesis and release of the inhibitory neurotransmitter γ -aminobutyric acid (GABA).^[41,42] These findings are in accord with the reported suppression of the GABA system by chronic psychological stress^[47] and in anxiety disorders such as posttraumatic stress syndrome.^[48,49] GABA is the main inhibitory neurotransmitter in the mammalian body and inhibits the AC-dependent formation of cAMP as well as the activation of voltage-gated Ca^{2+} -channels^[50] under physiological conditions by activating inhibitory G-protein (G_i) signaling downstream of G_i -coupled GABA-B-receptors. In light of findings that the GABA-B receptor has tumor suppressor function in pancreatic^[42,51-53] and non small-cell lung cancer (NSCLC)^[35,41,54,55] while GABA also inhibits the *in vitro* growth of breast cancer and colon cancer,^[47,56] suppression of GABA by psychological stress has significant tumor promoting effects on these cancers.

Similar to chronic stress, smoking also increases the levels of cancer stimulating stress neurotransmitters^[57] while suppressing cancer inhibiting GABA,^[2] effects caused by the neuroadaptation of nAChRs to chronic nicotine, NNK and N'-nitrosonornicotine (NNN). In conjunction with the mutational activities of NNK and NNN at the *K-ras* and *p53* genes,^[58] the resulting prevalence of cancer stimulating beta-adrenergic receptor signaling contributes significantly to the increased cancer risk of smokers.

NOVEL FINDINGS

Three publications^[59-61] from the Research Institute of Pharmacological Sciences, College of Pharmacy, Seoul National University (Seoul, Republic of Korea) have recently revealed additional mechanisms of nAChR and β -AR-mediated lung cancer promotion that can potentially be exploited for the targeted prevention and therapy of lung cancer and numerous other cancers. These studies showed that NSCLC tissues from smokers expressed significantly higher levels of the phosphorylated insulin-like growth factor-1 receptor (IGF-1R) than NSCLCs from nonsmokers and that the nicotine-derived carcinogen NNK promoted NSCLC tumorigenesis *in vitro* and in a mouse model by inducing exocytosis of insulin-like growth factor 2 (IGF-2) that phosphorylated the IGF-1 receptor, effects inhibited by the neuronal nAChR antagonist mecamylamine, dihydropyridine blockers of L-type VOCs as well as by antagonists for β 1- and β 2-ARs.^[59] The investigators reported that the observed IGF-1R phosphorylation was caused by β -AR-mediated stimulation of IGF2

transcription.^[61] However, the molecular mechanisms of this effect have yet to be defined. Based on the inhibitory effects of mecamylamine and VOC blockers on EGF-1R phosphorylation, nAChRs were the upstream regulators of this β -adrenergic cascade by stimulating the release of Nor and Epi. In accord with established mechanisms of stress responses (nAChR-mediated opening of VOCs causing release of stress neurotransmitters by exocytosis from the sympathetic nervous system and adrenal glands), experimental chronic stress had significant tumor promoting effects on urethane-induced mouse NSCLC and on the development of this cancer type in transgenic Kras^{G12D/+} mice via IGF-2-mediated activation of the IGF-1R signaling cascade.^[60,61] In both animal models these effects were inhibited by the general beta-blocker propranolol or the dihydropyridine VOC blockers amlodipine or nifedipine. Propranolol also significantly prevented the development of NNK-induced lung tumors in A/J mice, an effect accompanied by suppression of phosphorylated IGF-1R.^[61] The authors conclude that beta-blockers and VOC blockers should be further explored for the prevention of lung cancer, a concept that could rapidly move into clinical trials because these drugs are already widely used for the long-term management of cardiovascular disease.

CONCLUSIONS AND FUTURE DIRECTIONS

The reported activation of the IGF-1R signaling cascade in NSCLC and their normal epithelial precursor cells by the joint actions of nAChRs, VOCs and β -ARs adds a novel aspect to the mechanisms of cancer regulation by neurotransmitter receptors. While cancer research on the regulatory function of these receptors has mostly interpreted their modulation of intracellular signaling pathways as direct events downstream of the receptors,^[20,24,30,45,62,63] the cited three publications^[59-61] instead take into consideration the physiological role of nAChRs and β -ARs in the release of cell type-specific products by exocytosis [Figure 1] in response to increased intracellular Ca^{2+} . In addition to IGF-2, β -AR-I agonists also induced the release of AA, EGF, VEGF, interleukin-6 as well as several cancer stem cell markers.^[36,64-66] In turn, these effects can be caused by elevated systemic levels of stress neurotransmitters in response to stress or tobacco exposure, by direct binding of NNK in tobacco products to β -ARs, or by medications that are beta-adrenergic agonists. In addition, epithelial cancer cells and their respective cancer stem cells synthesize and release their own Epi and Nor upon activation of nAChRs by nicotine or nicotine-derived nitrosamines.^[33,36] The proposed repurposing of beta-blockers and Ca^{2+} -channel blockers for lung cancer prevention would therefore inhibit

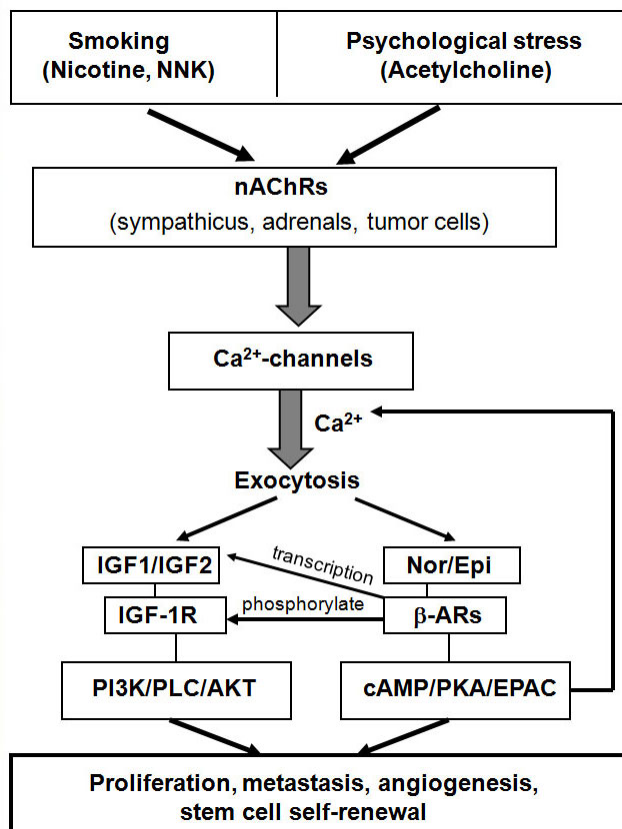


Figure 1: Working model illustrating the mechanistic interactions of nicotinic acetylcholine receptors, Ca^{2+} -channels, beta-adrenergic receptors and the IGF pathway in cancers associated with smoking and psychological stress. NNK: nicotine-derived nitrosamine ketone; nAChRs: nicotinic acetylcholine receptors; IGF-1R: insulin-like growth factor-1 receptor; PLC: phospholipase C; AKT: protein kinase B; cAMP: cyclic adenosine monophosphate; PKA: protein kinase A

differentiated cancer cells as well as cancer stem cells.

There is an ongoing international discussion on the potential usefulness of beta-blockers for cancer intervention, with numerous preclinical studies reporting significant cancer inhibition whereas clinical investigations have generated controversial data with some even reporting cancer promoting effects.^[28,29,64,67-71] The potential usefulness of beta-blockers for adjuvant cancer treatment has additionally been discussed in depth based by comprehensive reviews of published preclinical and clinical literature.^[67,72,73] By contrast, the current review analyzes mechanistic aspects of G_s -coupled receptors and their physiological inhibitors and their modulating effects on cancer. The discrepancies between preclinical and clinical findings are thus not only triggered by the potential sensitization of β -ARs in response to long-term beta-blocker therapy (decades of treatment in people as opposed to a few weeks in experimental animals), but also by the potential impact of factors unrelated to β -ARs. Preclinical studies that have employed agonists of receptors coupled to the inhibitory G-protein G_i (GABA-B receptors, opioid peptide receptors) for the inhibition of β -AR-mediated progression of adenocarcinoma of the lungs and pancreas *in vitro* and *in vivo* have repeatedly shown that increases in intracellular cAMP and the associated activation of its downstream effectors are key molecular events that activate β -AR-driven development and progression of both cancers and can be successfully inhibited by agonists of G_i -coupled receptors that inhibit the formation of cAMP by blocking the activation of adenylyl cyclase.^[41,42,51,54,55,66,74-76] A host of non- β -AR receptors coupled to the stimulatory G-protein G_s increase intracellular cAMP,^[3,77] a reaction not inhibited by beta-blockers but effectively counteracted by agonist-induced signaling of G_i -coupled receptors. There is also a host of non-beta-adrenergic agents that increase intracellular cAMP directly. Among such agents are caffeine, theophylline and theobromine contained in numerous beverages, weight loss medications, sweets and candies. These naturally occurring phosphodiesterase inhibitors block the enzymatic breakdown of cAMP which then accumulates inside the cells. In addition, pharmacological phosphodiesterase inhibitors are widely used for the therapy of chronic obstructive pulmonary disease because of their anti-inflammatory and broncho-dilating properties. None of the clinical investigations on beta-blockers and cancer conducted to date have adjusted their data to exclude the cancer promoting effects of such non-beta-adrenergic agents.

Beta-blockers should not be used for the general prevention/therapy of cancer because they are selectively effective only in cancers that are stimulated by beta-adrenergic agonists. In fact, without prior testing of patients for increased stress neurotransmitter and cAMP levels, beta-blocker treatment is contraindicated because it can promote certain cancers due to the fact that cAMP functions as a tumor promoter in some cancers while acting as a tumor suppressor in others. It has thus been shown that cAMP inhibits the growth/progression of squamous cell carcinoma,^[78] small cell lung carcinoma,^[79,80] medulloblastoma and basal cell carcinoma.^[81] The arbitrary use of Ca^{2+} -channel blockers for cancer prevention and therapy is equally ill advised. While preclinical investigations have identified cancer preventive effects of Ca^{2+} -channel blockers in a large spectrum of cancers,^[82-84] these agents not only suppress molecular targets studied in these cancers but additionally inhibit the release of Nor and Epi from sympathetic nerves,^[85] thereby suppressing the beta-adrenergic receptor-mediated formation of cAMP. In turn, this effect can selectively promote the development and progression of cancers in which cAMP has tumor suppressor function.

In summary, successful cancer prevention and improved therapeutic outcomes can be achieved by strategies that aim to maintain/restore cAMP homeostasis. Too much cAMP will promote the development and progression of cAMP-driven cancers (e.g. adenocarcinoma of the lungs, pancreas, colon, stomach and prostate) while too low cAMP levels will increase the risk for development and progression of cancers in which cAMP has tumor suppressor function (e.g. small cell lung cancer, squamous cell carcinoma, medulloblastoma, basal cell carcinoma). In analogy to the long-term management of diabetes by insulin injections that are based on blood glucose testing, this approach requires routine testing of cAMP levels. Beta-blockers will only be beneficial if elevated levels of Nor/Epi indicate hyperactive β -AR signaling.

Authors' contributions

H.M. Schuller contributed solely to the paper.

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There are no conflicts of interest.

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Chest wall metastasis in squamous cell carcinoma of buccal mucosa

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ABSTRACT

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Metastases from head and neck cancers is rare occurrence. The common form of failure/recurrence in these cancers are local site recurrence or nodal metastases. Distant metastases are very rare and are most commonly seen in the lung, brain, liver and bones, and the latent period between the development of the primary and the distant metastases is usually long. There are very few cases reported of chest wall metastases from squamous cell carcinoma of head and neck. This article reports such a case of squamous cell carcinoma of buccal mucosa metastasizing to the chest wall four months after primary therapy. The metastasis was treated with local palliative radiotherapy to the chest wall. This case is special as the present knowledge on this type of presentation is limited in the medical literature.

INTRODUCTION

Head and neck malignancy is a major burden of cancer worldwide being the sixth most common malignancy.^[1] It is the most common cancer in developing countries and its incidence shows an increasing trend.^[2] In southeast Asia region, the oral cavity is the most prevalent site of head and neck squamous cell carcinoma.^[3] Widespread use of smokeless tobacco products such as pan and supari in this population is the major cause of this high incidence.^[4] Advancement in the treatment of primary head and neck cancers has resulted in improvement of loco-regional control as well increased disease free survival and overall

survival of this subgroup of patients.^[5] Though distant metastasis are considered uncommon sites of failure for primary head and neck cancer, increased survival has resulted in an apparent increase in distant metastasis. The incidence of distant metastasis in patient of squamous cell carcinoma of oral cavity is low, i.e. approximately 15% to 20% of patients.^[6,7] Most common sites for distant metastasis in these cases are lung, liver, bone, thyroid.^[8] But distant metastasis to sites other than these, such as kidney, diaphragm, heart, brain, adrenal, thyroid pancreas and peritoneum are uncommon.^[9] The risk of distant metastasis increases with the increase in number of histologically positive lymph nodes and with the presence of extranodal spread.^[10] We report such



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an unusual case of squamous cell carcinoma of buccal mucosa that presented with distant metastasis to the chest wall.

CASE REPORT

A 20-year-old gentleman presented with an ulcer on the right cheek. The lesion was of 2-3 months duration. There was associated swelling on the right side of the neck region. He gave a history of tobacco chewing in the form of gutkha (a mixture of tobacco, betel nut, and lime) since last 7-8 years. A punch biopsy from the ulcer and subsequent histopathological examination of the biopsy sample confirmed the diagnosis of squamous cell carcinoma of buccal mucosa. The patient underwent wide local excision surgery of the primary lesion with right sided modified radical neck dissection in May 2016. Surgical pathology report was suggestive of a pathological staging $pT_1N_2bM_0$ with lymphovascular invasion and perinodal extension. The patient was advised adjuvant post-operative radiotherapy with concurrent chemotherapy (cisplatin weekly at 40 mg/m^2). He received 60 Gy by external beam radiotherapy in 30 fractions by conventional two-dimensional planning on a 6 MV linear accelerator with five cycles of concurrent cisplatin. Adjuvant therapy was concluded in August 2016. The patient was kept on follow-up, during which he was free of any symptoms and signs of the disease. After a disease-free survival of 4 months, he presented with swelling and redness over the right side of the chest wall in December 2016. On examination, the swelling was an indurated, erythematous, tender, hard, fixed mass of $7 \text{ cm} \times 7 \text{ cm}$ in the right upper chest wall away from the pectoral flap site [Figure 1]. A computed tomography scan of the neck, paranasal sinuses and thorax was suggestive of soft tissue opacity in the right upper chest wall and right axillary region [Figure 2]. There was also a recurrent lesion present involving the superior aspect of the flap in right retro-antral fat space in the oral cavity [Figure 3]. Fine needle aspiration cytology examination from the chest wall mass was suggestive of metastatic squamous cell carcinoma. Biopsy from the oral lesion confirmed recurrent squamous cell carcinoma [Figure 4]. No other lesion was present anywhere else in the body either by clinical examination or radiological investigations. The patient was advised palliative radiotherapy (30 Gy in 10 fractions) in view of unresectable disease and was treated with a conventional anteroposterior field on a telecobalt machine along with aspiration and drainage of the axillary collection. The patient was referred to palliative medicine for supportive care. He was lost to follow-up after January 2017.

DISCUSSION

Distant metastases from head and neck cancer are unusual. The commonest site is lungs, bones, and liver and usually, occur after a long latent period.^[9] The risk of incidence of distant metastasis depends on the age of patient, site of the primary cancer, loco-regional extension, tumor grade, and loco-regional control by primary treatment.^[11] The risk of developing distant metastasis in head and neck cancer increases with the development of regional metastasis and is associated with poor survival.^[12] Metastases to chest wall from head and neck cancer are extremely rare, with only a few cases reported in the literature till date. Metastasis to such an unusual sites may be due to the disruption of lymphatic system during surgery which resulted in the lymphatic dissemination of malignant cells to the region below the clavicle.^[13] Here recurrence at the pectoralis flap site due to



Figure 1: Clinical photograph of the chest wall lesion

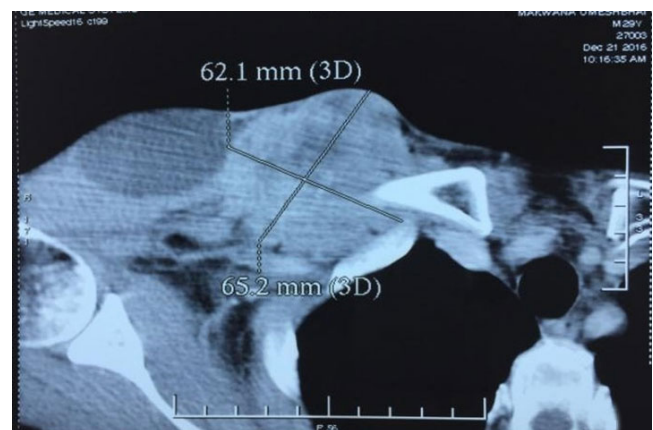


Figure 2: Computed tomography of thorax showing the chest wall lesion

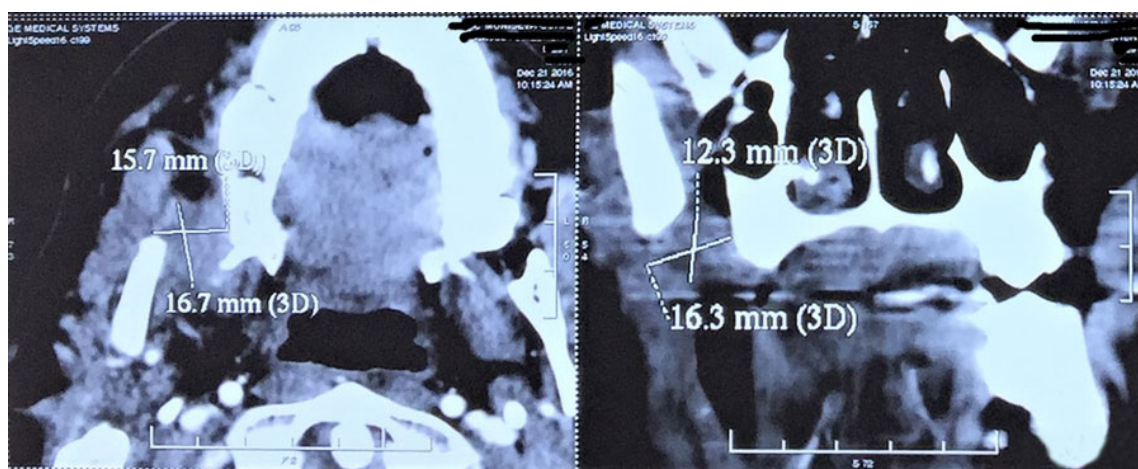


Figure 3: Computed tomography showing the local recurrent lesion at the flap site

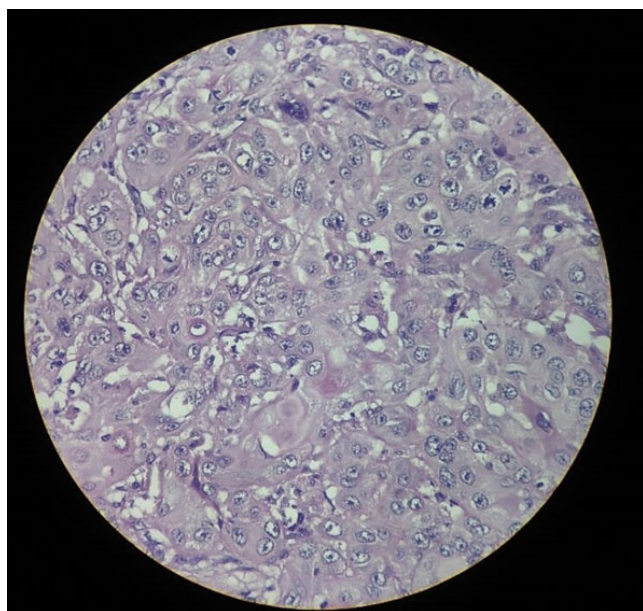


Figure 4: Histopathology slide showing malignant squamous cell (HE, x40)

surgical dissemination of primary tumor cell can be safely ruled out as the site of distant metastasis is not continuous with the surgical bed. Hematogenous spread can be another explanation for these rare recurrences.^[14] In literature, kidney, diaphragm, heart, brain, adrenal, thyroid, pancreas and peritoneum have been mentioned as rather rare sites of metastasis in head and neck squamous cell carcinoma.^[9] The prognosis of these cases remain poor and the intention of therapy is usually palliative with either excision, palliative local radiotherapy or palliative chemotherapy for widespread systemic metastasis.^[15] After thorough search of literature, we couldn't find a case report on buccal mucosa presenting with chest wall metastasis which makes this case extremely rare.

In conclusion, chest wall metastasis from squamous

cell carcinoma of buccal mucosa is a rare occurrence with no reported case in published literature. The present knowledge on this topic is very limited and this case report is intended to add to the existing information. This will help in better management of the patients who present with this rare incidence.

Authors' contributions

Concept, manuscript development and literature search: J.S. Singh, A.D. Sharma

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Patient consent was acquired.

Ethics approval

The study has been approved.

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Combination of insulin-like growth factor-1, IGF binding protein-3, chromogranin A and prostate specific antigen can improve the detection of prostate cancer

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Aim: Prostate cancer (PCa) is the second most prevalent male cancer worldwide and designated the sixth most frequent male cancer in Arab countries. Although prostate specific antigen (PSA) has become the best and most valuable biomarker for screening of PCa, elevated levels of PSA can reflect the presence of malignant cells but can overlap with benign prostatic diseases. There is a necessity to develop and improve current tools for early detection and diagnosis of PCa. This study was done to evaluate the validation of serum insulin-like growth factor-1 (IGF-1), IGF binding protein-3 (IGFBP-3), chromogranin A (CgA) and combination with PSA in treatment of benign prostatic hyperplasia (BPH) and PCa patients. **Methods:** The study included 72 patients with PCa, 70 BPH patients and 56 healthy male subjects of matched age. Full history and clinical data were recorded for all subjects. **Results:** Serum PSA attained sensitivity of 84% at 82% specificity with an accuracy of 83%, although IGF-1, IGFBP-3 and CgA did not recognize PCa patients. **Conclusion:** Combinations of IGF-1 and IGFBP-3 biomarkers with PSA were effectively differentiated between PCa and control groups as well as improving the overall value of sensitivity, specificity and diagnostic accuracy of PCa to 85% and 86% for IGF-1/PSA and IGFBP-3/PSA respectively.

INTRODUCTION

Prostate cancer (PCa) is ranked the second most prevalent male cancer worldwide^[1] and is currently the sixth frequent male cancer in Arab countries.^[2]

The annual worldwide estimate is 1.1 million new diagnosed PCa male patients, representing 15% of all male cancers and about 70% of the cases occurring in developed countries. PCa represents the fifth most common cause of male cancer death, accounting for



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6.6% of overall male deaths.^[3] In the United States, about 161,000 new PCa cases are anticipated in 2017 along with approximately 26,700 PCa deaths.^[4] Radical prostatectomy and radiotherapy are still curative therapeutic options for PCa, but are restricted to organ-confined tumors. Therefore, the successful treatment of PCa depends on detection of the disease at its earliest stages. There is a necessity to improve current methods for early detection and/or diagnosis of PCa and to distinguish men at risk for carcinogenesis.^[5] Screening can detect disease in its early or asymptomatic stage; in addition, screening tests of malignant tumors must have high sensitivity to detect the disease with sufficient specificity to protect patients with false-positive results from uncalled-for diagnostic interventions.^[6]

A single polypeptide, prostate specific antigen (PSA), exists in diverse molecular forms. It occurs in either normal or malignant prostatic tissue. About 70-90% of serum PSA combines with serum protein inhibitor, alpha 1 antichymotrypsin, and the rest remains unbound in free form (fPSA). Measurements of total and free/total PSA ratio are common analyses in diagnosing PCa. The lower the f/tPSA ratio, the higher the likelihood of malignancy. The protocol for PCa screening may involve PSA analysis and digital rectal examination. Trans-rectal ultrasonography has been associated with an increased false positive rate, making it not ideal screening tool. It has been recommended that, starting at age 50 years, a routine check-up with PSA analysis and digital rectal examination be carried out annually for men at high risk. Single polypeptide PSA is expressed in normal, benign and malignant prostatic tissues but not in any other human tissue.^[7] Although the introduction of PSA related PCa screening and PSA has become the best and most valuable biomarker for screening, detection, staging and monitoring of PCa; there are challenges. For example, elevated levels of PSA can reflect the presence of malignant cells but can also be related to non-malignant prostate disorders like benign prostatic hyperplasia (BPH), infection or chronic inflammations.^[8] Increased serum PSA levels have been seen in patients with PCa, as well as in BPH and prostatitis, producing a high rate of false-positive cases. There also remains a wide overlap between PCa and BPH, particularly in patients with marginally increased PSA concentrations, in the range of 4-10 ng/mL, a range which is said to be a "grey zone" where there is a dilemma in differentiation between benign and malignant prostatic diseases; this dilemma has strengthened the necessity to improve PCa specificity by developing, combining and validating other diagnostic biomarkers with consideration of the sensitivity.^[9] Consequently, the

clinical value of early detection of PCa has induced the search of many novel PSA-based diagnostic markers that might, singly or in combination, improve discrimination between PCa and BPH leading to help minimize the frequency of unnecessary and invasive biopsies.^[10]

The cell cycles of common human cells are tightly controlled and coordinated by intra-and extracellular signals, working in harmony and congruence to appropriately regulate cell proliferation, deterioration and apoptosis. As the combined signals of growth and inhibition boost proliferation, the cell attains mitosis.^[11] Insulin-like growth factors (IGFs) are important moderators of growth, development, and survival. They are synthesized by virtually any bodily tissue, and their action is accomplished by a network of complex molecules, including binding proteins, proteases and receptors, which all comprise the IGF system.^[12] IGF-1 and IGF binding protein-3 (IGFBP-3) play a pivotal function in the regulation of growth; controlling cellular proliferation and apoptosis. Circulating IGF-1 binds to the IGF-1 receptor and acts as a stimulus of signal transduction reactions, promoting proliferation and increased survival of cells. Such signalling components and reactions are fundamental to the tumorigenesis processes.^[13] The IGFBPs family exists in six different types; all with high affinity for IGF-1. IGFBP-3 is the superabundant type that influences serum levels of IGFs and has the highest affinity to IGF-1.^[11] Although IGFBPs are mainly synthesized in the liver, they can be expressed in many other normal and cancerous tissues such as lung, breast, and ovarian cancers. IGFBPs may impact carcinogenesis by various mechanisms. They regulate bioavailability of circulating IGF-1/2 as well as their activity and transportation mechanisms to target tissues.^[14] In many types of cancers, IGF-1 and IGFBP-3 have been related to tumor grade and stage as well as disease progression.^[15] Most circulating IGF-1 (99%) is bound to IGFBPs; less than 1% is carried in the unbound state in the circulation. IGFBP-3 is the most abundant IGFBP in the circulation and is produced by many types of cells, and is believed to regulate the availability of IGF-1^[16] by impairing IGF action and inhibiting cell growth by blocking free IGFs or through IGF-independent mechanisms.^[17] Moreover, IGFBP-3 has been found to elevate levels of the cell-cycle inhibitor p21/WAF1, leading to growth arrest in PCa cells.^[18] Many studies have shown the correlation between IGF-1 and PCa risk, and they have demonstrated the inclusion of the IGF network in the early stages of prostate carcinogenesis^[19] and other studies have found increased circulating IGF-1 and decreased IGFBP-3 levels correlated with an excess

risk of PCa development. However, in these studies, PSA remains the best PCa predictor.^[20] Another meta-analysis research found a positive relation between IGFBP-3 and PCa risk. However, the controversy over the contradicted results of IGF-1 and IGFBP-3 in these studies has been attributed to many factors such as race,^[21] study design and assay features.^[22]

Chromogranin A (CgA) is a member of the granin family, of 439 kDa glycoprotein, and exists in the secretory dense-core granules that contribute to the storage of peptide hormones and catecholamine in all endocrine and neuroendocrine (NE) cells. Thus, it can be released from NE cells in a heterogeneous circulating molecular form and is considered one of the most abundant components of secretory granules.^[23] While its function is unknown, through previous decades a growing body of evidence has suggested that CgA is released in abnormal amounts by many malignant NE cells, which may influence different components of the tumor stroma and engage with the regulation of tumor growth and progression. However, increased blood CgA levels have been established as a useful indicator in the diagnosis of many NE tumors, but the use of this marker for clinical management is still controversial.^[23,24] Therefore, research has been dedicated to its prognostic and diagnostic importance, but with little supporting evidence for its use beyond common screening methods.^[8] Several studies have reported that elevated CgA concentrations are associated with high-grade and advanced stage PCa. Some studies have indicated that increased serum CgA exceeded PSA increase as a marker of progression to hormone-refractory disease. Therefore, it is possible to use CgA to monitor metastatic PCa patients under androgen blockade.^[25] Some indicate CgA utility in early diagnosis, particularly when used in combination with free/total PSA ratio,^[26] however, some studies found that CgA does not precisely differentiate malignant disease.^[27] Similar debate occurs regarding CgA and tumor features^[28] and CgA does not show any advantage in the prognosis of PCa recurrence after radical prostatectomy or radiotherapy.^[29] Inconsistencies may be attributed to the transient and reversible process of neuroendocrine differentiation (NED) in most malignant prostate tumors; thus, these NE molecules are not constantly detectable.^[5,30]

This study is dedicated to evaluate the validity of IGF-1, IGFBP-3, CgA and combination with PSA in diagnosis of patients with localized and metastatic PCa. The secondary objective was to compare the advantage of these markers in differentiation of PCa patients.

METHODS

This study included 72 patients with PCa (mean age 70.8 ± 5.3 years), 70 BPH patients (mean age 69.5 ± 7.3 years) and 56 healthy males (mean age 67.3 ± 7.2 years) were randomly recruited among the volunteers of matched socioeconomic conditions and who did not have any known significant disease. Full history and clinical data were recorded for all subjects, and PCa patients were classified into localized PCa ($n = 54$) and metastatic PCa ($n = 18$). BPH and healthy male individuals ($n = 126$) were grouped as control. Patients with PCa underwent digital rectal examination, transrectal ultrasonography, guided biopsy of the prostate, computed tomography scanning of the pelvis, bone scanning, and histopathological examination to assess metastatic disease and determine disease stage.

Blood samples

A 10 mL blood sample was drawn in the morning after overnight fasting from healthy subjects and one week following digital rectal examination for patients with prostatic diseases at the time of diagnosis.

Serums of blood samples were separated and stored at -20°C in a deep freeze until the date of analysis. Serum levels of IGF-1, IGFBP-3, CgA, total (tPSA) and free PSA (fPSA) were measured by chemiluminescence ELISA technique IMMULITE, DPC (Diagnostic Products Corporation, Los Angeles, CA, USA).

Statistical analysis

Serum concentrations of CgA, IGF-1, IGFBP-3, tPSA and fPSA in addition to combination among parameters were expressed as arithmetic mean and standard deviation. Statistical analysis was accomplished by using the statistical package IBM SPSS V20. Data between the groups were compared and the statistical significance of mean values was determined by applying independent sample *t*-test and Mann-Whitney test. The significance level was established at the *P* value of < 0.05 . The validity (sensitivity and specificity), accuracy of each parameter and ratios thereof were calculated by area under curve (AUC) in receiver operating characteristics (ROC) curve analysis.

RESULTS

A total of 54 cases of localized PCa patients, mean age 70.3 ± 6.3 years, and 18 metastatic patients, mean age 71.1 ± 5.5 years, were grouped as PCa patients with a mean age of 70.8 ± 5.3 years ($P > 0.05$). Seventy BPH patients, mean age 69.5 ± 7.3 years, and 56 healthy male individuals, mean age 67.3 ± 7.2 years, were also included as a control group with a mean age of $68.3 \pm$

6.5 years ($P > 0.05$) as represented in Table 1.

Serum tPSA levels were significantly higher in the PCa group, 34.3 ± 21.1 ng/mL, as compared to the control group, 2.8 ± 1.9 ng/mL ($P < 0.005$) [Table 1], and the study showed significant differentiation between localized and metastatic PCa. Similarly, the f/tPSA ratio exhibited a significant difference among control, localized and metastatic PCa groups ($P < 0.005$, Table 1; Figure 1A and B). Surprisingly, while serum IGF-1 level showed no statistical difference between studied groups, as it represented 155.0 ± 44.2 ng/mL in PCa group compared to 148.4 ± 36.1 ng/mL in control group ($P > 0.05$) [Table 1 and Figure 1C], its combination with tPSA as IGF-1/tPSA ratio could differentiate significantly between PCa, 148.4 ± 36.1 , and control groups, 64.8 ± 22.3 ($P < 0.005$) [Table 1 and Figure 1C]. Similarly, mean serum IGFBP-3 and CgA did not statistically differentiate between the PCa and control groups, as IGFBP-3 represented $3,052 \pm 319$ ng/mL and $3,154 \pm 371$ ng/mL in PCa and control groups respectively ($P < 0.05$, Table 1 and Figure 1D) and CgA represented 65.4 ± 30.3 ng/mL and 62.1 ± 29.8 ng/mL in PCa and control groups respectively ($P < 0.05$, Table 1 and Figure 1D). On the other hand, statistical significance was noted for their ratios with tPSA, (IGFBP-3/tPSA and CgA/tPSA ratios), in distinguishing PCa and control groups ($P < 0.005$, Table 1 and Figure 1E).

The validity (sensitivity and specificity), and accuracy of each parameter for prediction of PCa occurrence were calculated by AUC in ROC curve analysis of

prediagnostic serum concentrations of tPSA, fPSA, IGF-I, IGFBP-3, and CgA and ratios thereof, for 72 patients with PCa and 126 control individuals. The AUC for tPSA, IGF-I, IGFBP-3, and CgA was 0.83, 0.58, 0.55 and 0.56 respectively. The AUC for f/tPSA, IGF-1/tPSA, IGFBP-3/tPSA and CgA/tPSA was 0.76, 0.85, 0.86 and 0.74, respectively. Thus, the combination of PSA with, IGF-1, IGFBP-3, and CgA improved the sensitivity, specificity and diagnostic accuracy for PCa patients [Table 2 and Figure 2].

DISCUSSION

The successful treatment of PCa depends on detection of the disease at its earliest stages. Since PCa is a heterogeneous disease, there is a need for supplementary biomarkers that add useful information and correctly prognosticate the existence and progression of PCa to eliminate unnecessary invasive biopsies and aggressive diagnostic tools, decrease morbidity rates, and reduce unnecessary expenses. Thus, many prospective PCa biomarkers will continue to develop and expand to improve and provide more diagnostic information and supplement PSA testing. A diversity of diagnostic and prognostic markers had been explored in different body fluids and tissue samples, although their clinical use still need further validation. The ideal PCa biomarkers should be prostate specific, readily detectable in the body fluids, reproducibly measured and analysed, and can effectively differentiate among normal, benign and cancerous prostatic diseases as well as have cogent

Table 1: Serum levels of selected parameters in study population (mean \pm SD)

Parameter	Control groups			PCa groups			P value
	BPH	Healthy	Total	Localized	Metastatic	Total	
Number (n)	70	56	126	54	18	72	-
Age (year)	69.5 ± 7.3	67.3 ± 7.2	68.3 ± 6.5	70.3 ± 6.3	71.1 ± 5.5	70.8 ± 5.3	> 0.05
IGF-1 (ng/mL)	149.8 ± 35.6	146.5 ± 37.4	148.4 ± 36.1	154.3 ± 44.2	157.6 ± 47.7^a	155.0 ± 44.2^b	$> 0.05^a$ $> 0.05^b$
IGFBP-3 (ng/mL)	$3,125 \pm 372$	$3,174 \pm 363$	$3,154 \pm 371$	$3,082 \pm 311$	$2,925 \pm 346^a$	$3,052 \pm 319^b$	$> 0.05^a$ $> 0.05^b$
CgA (ng/mL)	62.3 ± 27.6	61.9 ± 30.3	62.1 ± 29.8	64.0 ± 29.6	67.6 ± 33.9^a	65.4 ± 30.3^b	$> 0.05^a$ $> 0.05^b$
tPSA (ng/mL)	3.7 ± 2.0	1.6 ± 0.8	2.8 ± 1.9	27.8 ± 15.4	52.3 ± 22.6^a	34.3 ± 21.1^b	$< 0.005^a$ $< 0.005^b$
f/tPSA	0.24 ± 0.02	0.28 ± 0.03	0.26 ± 0.02	0.15 ± 0.02	0.11 ± 0.01^a	0.13 ± 0.02^b	$< 0.005^a$ $< 0.005^b$
IGF-1/tPSA	49.7 ± 17.6	102.8 ± 37.1	64.8 ± 22.3	6.7 ± 2.9	4.1 ± 2.4^a	4.9 ± 2.7^b	$< 0.005^a$ $< 0.005^b$
IGFBP-3/tPSA	893 ± 352	$1,710 \pm 524$	$1,310 \pm 422$	253 ± 197	186 ± 164^a	212 ± 178^b	$> 0.05^a$ $> 0.05^b$
CgA/tPSA	14.6 ± 5.4	24.7 ± 9.6	20.3 ± 8.5	3.1 ± 2.1	2.2 ± 1.6^a	2.8 ± 2.0^b	$< 0.005^a$ $> 0.05^a$
IGF-I/fPSA	348 ± 196	372 ± 228	364 ± 221	326 ± 216	296 ± 185^a	311 ± 201^b	$> 0.05^a$ $> 0.05^b$
IGFBP-3/fPSA	$3,727 \pm 1,739$	$3,918 \pm 1,865$	$3,811 \pm 1,788$	$3,395 \pm 2,216$	$3,210 \pm 1,984^a$	$3,304 \pm 2,107^b$	$> 0.05^a$ $> 0.05^b$

SD: standard deviation; PCa: prostate cancer; BPH: benign prostatic hyperplasia; IGF-1: insulin-like growth factor-1; IGFBP-3: IGF binding protein-3; CgA: chromogranin A; tPSA: total prostate specific antigen; f/tPSA: free/total prostate specific antigen; ^a: comparison between the localized and metastatic in PCa groups; ^b: comparison between total PCa groups and control groups

correlation to clinical data.^[5,11]

In the present study, serum tPSA and f/tPSA were significantly higher in the PCa than in the control groups and they can significantly be influenced by the tumor metastasizing. IGF-1 serum level was slightly increased and IGFBP-3 level was slightly decreased in patients with PCa, but they did not differentiate between PCa patients and control individuals; thus, no association of PCa risk were observed with prediagnostic serum concentrations of IGF-1, IGFBP-3 which may be attributed to the function of IGFBP-3 as a substrate for PSA. This is a member of the kallikrein family of serine protease^[31] and it is assumed that rising PSA levels during the natural history of PCa enhances the disease progression by proteolytically cleaving IGFBP-3, thereby increasing the amount of bioavailable IGF-1.^[32] Previous study on Arab males

indicated that IGF-1 and IGFBP-3 reached their peak levels during adolescence and gradually lowered with age.^[33] IGFBP-3, the most prevalent form of the IGFBPs, has been linked with prostatic growth. About 75% of IGF-1 is bound to IGFBP-3, while 20-25% is bound to the other binding proteins (IGFBP-1, 2, 4 and 5), and less than 1% is carried in the unbound state in the circulation.^[16] Therefore, relative IGFBP-3 concentrations may affect serum and likely prostatic tissue levels of IGF-1. Importantly, although the IGFBP-3-IGF-1 complex is a high molecular weight protein that cannot diffuse into tissues, complexes of IGF-1 and other IGFBPs have a lower molecular weight and can traverse the capillary membrane into tissues.^[16,34] Consequently, a decline of IGFBP-3 levels may give rise to an intension bind of IGF-1 to other IGFBPs, high diffusion into tissues, and an elevated tissue IGF-1 levels, leading to increased prostatic growth. Some

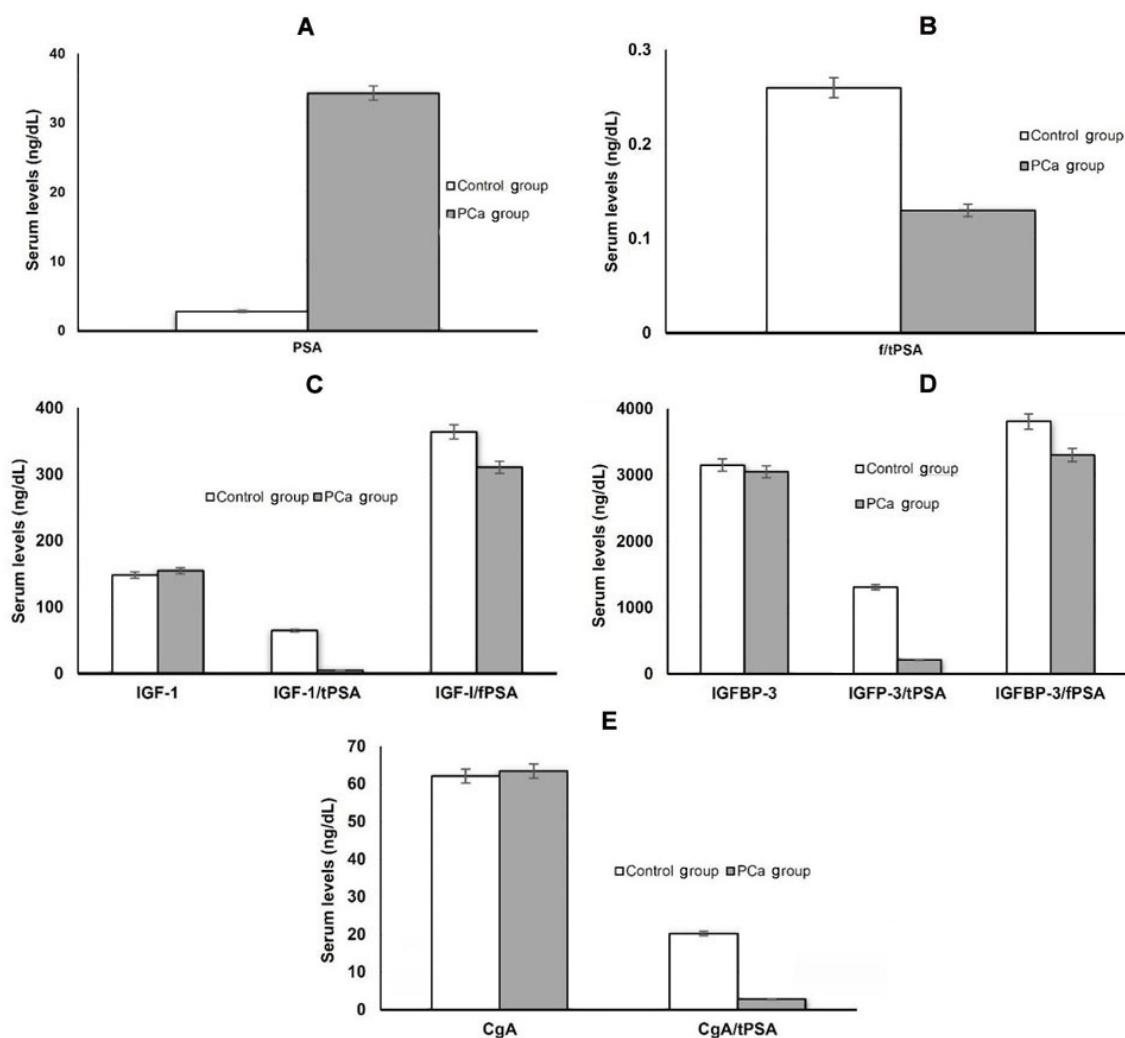


Figure 1: (A) Mean levels of tPSA in PCa and control groups; (B) mean levels of f/tPSA ratio in PCa and control groups; (C) mean levels of IGF-1, IGF-1/tPSA and IGF-1/fPSA ratios in PCa and control groups; (D) mean levels of IGFBP-3, IGFBP-3/tPSA and IGFBP-3/fPSA ratios in PCa and control groups; (E) mean levels of CgA and CgA/tPSA ratios in PCa and control groups. tPSA: total prostate specific antigen; PCa: prostate cancer; f/tPSA: free/total prostate specific antigen; IGF-1: insulin-like growth factor-1; IGFBP-3: IGF binding protein-3; CgA: chromogranin A

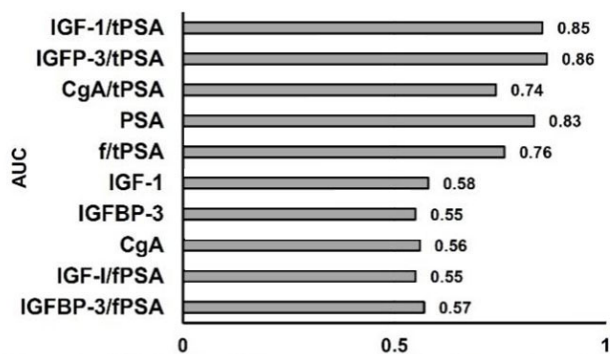


Figure 2: Validity (sensitivity and specificity) of parameters for prediction of future PCa occurrence, estimated by AUC. PCa: prostate cancer; AUC: area under curve; IGF-1: insulin-like growth factor-1; IGFBP-3: IGF binding protein-3; CgA: chromogranin A; tPSA: total prostate specific antigen; f/tPSA: free/total prostate specific antigen

Table 2: Validity* of selected markers in PCa detection

Parameter	AUC	95% CI	P value
IGF-1	0.58	0.51-0.66	> 0.05
IGFBP-3	0.55	0.48-0.63	> 0.05
CgA	0.56	0.49-0.64	> 0.05
PSA	0.83	0.76-0.90	< 0.005
f/tPSA	0.76	0.69-0.83	< 0.005
IGF-1/tPSA	0.85	0.78-0.92	< 0.005
IGFBP-3/tPSA	0.86	0.79-0.93	< 0.005
CgA/tPSA	0.74	0.67-0.82	< 0.05
IGF-1/fPSA	0.55	0.48-0.63	> 0.05
IGFBP-3/fPSA	0.57	0.49-0.64	> 0.05

*Validity (sensitivity and specificity) for prediction of PCa occurrence, estimated by AUC in ROC curve analysis of prediagnostic serum concentrations of tPSA, fPSA, IGF-1, IGFBP-3, and CgA and ratios thereof, for 72 patients with PCa and 126 control subjects. PCa: prostate cancer; ROC: receiver operating characteristics; AUC: area under curve; BPH: benign prostatic hyperplasia; IGF-1: insulin-like growth factor-1; IGFBP-3: IGF binding protein-3; CgA: chromogranin A; tPSA: total prostate specific antigen; f/tPSA: free/total prostate specific antigen

previous studies revealed that elevated concentrations of IGF-1 may increase the risk of some cancers,^[35,36] but results with respect to PCa have been discordant with other reports which revealed null associations similar to present study.^[37,38] As with the results of IGF-1 and IGFBP-3, although there was no statistical difference between CgA levels of PCa patients and control groups, CgA was slightly increased in patients with PCa than control groups. CgA is an excellent indicator of NE cells and of NED in PCa either in tissue or serum. The detection of CgA in the blood of patients with PCa indicates a NED, either of a primary tumour or an association with metastases.^[39] Tumors with NE features have displayed more aggression and are more resistant to hormone therapy.^[25]

Some studies have claimed that CgA is an independent prognostic marker for PCa,^[40] while others have conflicted with these findings.^[29,41] No PCa predictive values were seen for IGF-1, IGFBP-3 or CgA; AUC

were 0.58, 0.55 and 0.56, respectively. Similarly, some earlier reports found disappointing results for these biomarkers.^[42] Although the negative findings of the present study regarding IGF-1, IGFBP-3 or CgA serum levels in differentiating between the PCa and control groups; are notable, analysing the combination of these markers with PSA either in the PCa and control groups revealed that their combinations with serum tPSA level, (IGF-1/tPSA, IGFBP-3/tPSA and CgA/tPSA) were differentiated significantly among PCa, BPH patients and healthy individuals. These combinations could, potentially, effectively distinguish PCa patients from non-malignant individuals. Also, IGF-1/tPSA ratio can significantly differentiate between localized and metastatic PCa. Moreover, in our study, the ratios of IGF-1/tPSA and IGFBP-3/tPSA (AUC of 0.85 and 0.86, respectively) improved cancer detection, in comparison with PSA or f/tPSA ratio, (AUC of 0.83 and 0.76 respectively). Thus it would seem that circulating IGF-1 and IGFBP-3 concentrations are unlikely to be useful in differentiating patients with BPH from those with PCa, but their combinations with serum PSA level (IGF-1/PSA and IGFBP-3/PSA ratios) have improved the validity and correlation with the progression and clinical course of the disease. The strong correlation between the defective regulation of the IGF network and prostate carcinogenesis has been investigated previously by measurement of another member of the IGF family, IGF-2, in patients with PCa and BPH. Also no significant association was found between PSA and IGF-2 levels. However, the combination of PSA and IGF-2 improved the prognosis and discrimination of PCa and BPH.^[43]

The successful treatment of PCa depends on detection of the disease at its earliest stages. There is significant evidence for some novel PCa biomarkers to overcome the limitations of PSA; identifying these markers will allow more appropriate screening for early disease. However, few biomarkers have been appropriately validated and/or involved in clinical approach. To date, conflicting and insufficient data have indicated that there is still no biomarker likely to attain the desirable level of sensitivity and specificity. Potentially, combining use of biomarkers may improve the diagnostic accuracy of PCa which would impact treatment outcome.

In conclusion, although circulating IGF-1, IGFBP-3 and CgA are unlikely to be useful in differentiating healthy individuals or patients with BPH from those with PCa, or in identifying PCa metastasis, the combination of IGF-1 and IGFBP-3 with PSA has improved the overall sensitivity, specificity and diagnostic accuracy of PSA for prediction of the disease. Further prospective studies are needed concerning the correlation of

these serum markers and the aggressiveness of the PCa, either in terms of the pathological stage or of the Gleason score.

Authors' contributions

All authors equally contributed to this study.

Financial support and sponsorship

None.

Conflicts of interest

There are no conflicts of interest.

Patient consent

Patient consent was obtained from all participants. All participants were informed of the aim and procedures and were assured that they could discontinue their participation in the study without any effect on the medical care provided to them.

Ethics approval

The Ethical Research Committee at the Faculty of Medicine, Umm Al Qura University, and affiliated hospitals approved this study. Informed consent was obtained from all patients and healthy subjects; the aim of the study and the procedures that would be required were described to them beforehand. All subjects were assured that they could choose to discontinue their participation in the study without jeopardizing the medical care being given to them, including treatment and follow-up.

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Real-time quantitative PCR array to study drug-induced changes of gene expression in tumor cell lines

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ABSTRACT

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Key words:

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Aim: Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is still the "gold standard" for quantitative analysis of mRNA and the study of differentially expressed genes. **Methods:** The authors describe a RT-qPCR array that exploits SYBR Green dye-based detection to perform reliable gene expression analysis on 41 genes involved in several pathways linked to DNA damage response, cell cycle progression, cellular senescence, and programmed cell death. To validate the RT-qPCR array, the authors investigated changes of the gene expression profile of HeLa cells treated with two well-characterized antiproliferative molecules such as cisplatin (CDDP) and sodium butyrate (NaBu). **Results:** The results showed a gene expression profile compatible with both biological and gene expression data already reported in literature. **Conclusion:** Importantly, the assay allowed the monitoring of additional and not reported gene regulations, indicating that this custom-made RT-qPCR array is a cheap, robust, and rapid tool for the study of drug-induced effects in human biological models.

INTRODUCTION

The study of gene expression profile of cancer cells has become an essential tool to understand the biological alterations involved in disease development, to individuate new potential markers, to predict clinical outcome, to create personalized pharmacological therapies for patients, and to investigate the molecular effects of drug exposure with the aim of improving

treatment efficacy.^[1]

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)-based methods has emerged as the "gold standard" method for a rapid and robust analysis of gene expression.^[2] Currently, many PCR arrays are commercially available for the study of gene expression modifications involved in hundreds of molecular pathways. However, based on our



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experience, many of these RT-qPCR arrays are characterized by technical limits that could compromise the success of a gene expression study. The first limit is represented by the absence of technical replicates that could monitor the inter-well reproducibility for each gene transcript. The amplification of each target in a single well does not allow one to identify possible incidental mistakes that can occur, for example, during preparation of the PCR reaction mix. Another critical aspect is amplification of genes expressed at very low levels (e.g. CDK inhibitors) that is often difficult and requires a highly efficient DNA polymerase. In fact, the different DNA polymerase master mixes commercially available are not always able to amplify very low represented mRNA species in a detectable way.

The rationale of the study was to design and test a RT-qPCR array able to analyze the behaviour of 41 human genes involved in cell cycle regulation, DNA damage response, apoptosis, and senescence induction. Our goal was to develop a qPCR array that could overcome the technical limits described above (e.g. low efficiency of amplification and technical replicates controls) in order to obtain an inexpensive and easy-to-use tool for the reliable monitoring of transcriptional modulations induced by exposure of human cells to drug treatments.

We applied this qPCR array to investigate the response of HeLa cells to two different anti-proliferative drugs such as cisplatin [cis-diamminedichloroplatinum(II)-CDDP] and sodium butyrate (NaBu). CDDP remains one of the principal chemotherapeutic agents used for cancer treatment. CDDP cytotoxicity is mediated by its ability to form DNA adducts, primarily intra-strand adducts, which activate a DNA-damage cellular response and subsequent programmed cell death.^[3,4] Cellular exposure to CDDP, for example, is known to cause up-regulation of cyclin dependent kinase inhibitors (e.g. p27 and p21) and down-regulation of anti-apoptotic protein Bcl2 in cancer models.^[5,6] NaBu is a widely known histone deacetylase inhibitor and is considered the prototype of an epigenetic modulator. NaBu exerts anticancer effects by inhibiting histone deacetylase enzymes, thus inducing increased histone acetylation levels, elaboration of chromatin structure, and consequent reactivation of aberrantly silenced genes.^[7] Previous studies showed that several molecular pathways are affected by NaBu treatment. In particular, cyclin dependent protein kinase (CDK) down-regulation,^[8] cell-cycle inhibitors up-regulation, together with modulation of several apoptosis-related genes were observed.^[9,10]

Finally, the described modulation of gene expression

induced in HeLa cells, when subjected to CDDP or NaBu exposure, was compared with the results obtained by our RT-qPCR array using the same biological model of study.

METHODS

Cell culture and treatments

HeLa cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). All cellular populations were cultured in Dulbecco's Modified Eagle Medium (DMEM, Cambrex, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% glutamine.

HeLa cells in exponential phase of growth were subjected to treatment with 10 μ mol/L CDDP (Sigma-Aldrich, St Louis, MO, USA) or 5 mmol/L NaBu for 24 h and 16 h, respectively. At the end of the treatment period, viability of cellular populations was analyzed by the trypan blue dye exclusion method.^[11]

Primers design

Primer pairs, purchased from Primm S.r.l. (Milan, Italy) and listed in Table 1, were designed with Primer Express 2.0 Abi Prism software (PE Applied Biosystem, Foster City, CA, USA) as previously described,^[12] employing common design parameters [Table 2]. All amplicons primers, except for those amplifying an intronic region of GAPDH (control for possible genomic DNA contamination), were designed encompassing exon-exon boundaries to avoid genomic DNA amplification. The specificity of amplicons and primer pairs was checked *in silico* using BLAT (UCSC Genome Browser) and BLAST (National Center for Biotechnology Information) alignment tools.

RNA extraction

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and following supplier's instructions. Elution was performed in a solution of 1 volume of PBS and 5 volumes of RNeasy. For total RNA extraction RNase-free water and RNase-free supplies were used. Total RNA concentration was measured with Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) and RNA integrity evaluated through 1.3% agarose gel electrophoresis, as described.^[13]

Reverse transcription

One microgram of RNA from each sample was retrotranscribed (RT) using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and T Personal thermocycler (Biometra), according to

Table 1: List of genes amplified, relative primers, and main pathways

Gene	Forward primer	Reverse primer	Length	Pathways ^a
<i>ABL1</i>	5'-TACCCGGAATTGACCTGTC	5'-GGATTTTCAGCAAAGGAGGGC	151	Cell cycle
<i>ATM</i>	5'-GGTATAGAAAAGCACCAGTCCAGTATTG	5'-CGTGAACACCGGACAAGAGTTT	152	DNA damage
<i>ATP5B</i>	5'-GTCTTCACAGGTCATATGGGGA	5'-ATGGGTCCCACCATATAGAAGG	122	Housekeeping
<i>ATR</i>	5'-AGTAGCTTCCTTCGCTCCAAA	5'-ACTGACTCCGGCCACTCCAT	154	DNA damage
<i>BAX</i>	5'-CAAACCTGGTGCTCAAGGCC	5'-GGGCGTCCCAAAGTAGGAGA	151	Apoptosis
<i>BCL2</i>	5'-CTGGTGGAACAACATCGCCCT	5'-TCTTCAGAGACAGCCAGGAGAAAT	151	Apoptosis
<i>BIRC5</i>	5'-CCGGTTGCGCTTTCCTTTC	5'-CGCACTTCTCCGCGATTTTC	151	Apoptosis
<i>BRCA1</i>	5'-GCATGCTGAACTTCTCAACCA	5'-GTGTCAAGCTGAAAAGCACAAATGA	151	DNA damage
<i>BRCA2</i>	5'-AGACTGTACTTCAGGGCCGTACA	5'-GGCTGAGACAGGTGTGGAACA	151	DNA damage
<i>CCNA2</i>	5'-AGTAAACAGCCTGCGTTTACCC	5'-GAGGGACCAATGGTTTCTGG	151	Cell cycle
<i>CCNB1</i>	5'-ATGACATGGTGCCTTCTCTCC	5'-GCCAGGTGCTGCATAACTGG	151	Cell cycle
<i>CCNB2</i>	5'-GATAACGAAGATTGGGAGAACCC	5'-CCACTAGGATGGCAGCATG	151	Cell cycle
<i>CCND1</i>	5'-TGAAGGAGACCATCCCCCTG	5'-TGTTCAATGAAATCGTGCGG	151	Cell cycle
<i>CCNE1</i>	5'-AAATGGCCAAAATCGACAGG	5'-CGAGGCTTGACGTTGAGTT	151	Cell cycle
<i>CDC2</i>	5'-ACAGGTCAAGTGGTAGCCATGA	5'-ACCTGGAATCCTGCATAAGCA	151	Cell cycle
<i>CDC16</i>	5'-ATGCTGAGGCCTTGATTACC	5'-TCTCGCCTAAGACCAAGGGC	151	Cell cycle
<i>CDC20</i>	5'-AGATGGACGACATTTGGCCA	5'-ATTGGACTGCCAGGGACACC	151	Cell cycle
<i>CDC34</i>	5'-GGATTCCGCGTGACACTGGT	5'-ACCGAAAGGCTGGTGAGAG	151	Cell cycle
<i>CDK2</i>	5'-TTCTCATCGGGTCTCCACC	5'-TCGGTACCACAGGGTCACCA	151	Cell cycle
<i>CDK4</i>	5'-CTGTGCCACATCCGAACTG	5'-GCCTCTTAGAACTGGCGCA	151	Cell cycle
<i>CDK6</i>	5'-CCGAAGTCTTGCTCCAGTCC	5'-GGGAGTCCAATCACGTCCAA	151	Cell cycle
<i>CDK7</i>	5'-TCACATCTTCAGTGCAGCAGG	5'-TGGCAGCTGACATCCAGGT	151	Cell cycle
<i>CDK8</i>	5'-AGCGGGTCGAGGACCTGTTT	5'-CATGCCGACATAGAGATCCAG	151	Cell cycle
<i>CDKN1A</i>	5'-TACCCTGTGCCTCGCTCAG	5'-GGCGGATTAGGGCTTCCTCT	151	Cell cycle - senescence
<i>CDKN1B</i>	5'-AGACTGATCCGTCGGACAGC	5'-CACAGAACCGGCATTTGGG	152	Cell cycle - senescence
<i>CDKN2A</i>	5'-CAACGCACCGAATAGTTACGG	5'-CTGCCCATCATCATGACCTG	54	Cell cycle - senescence
<i>CDKN2B</i>	5'-ATCCCAACGGAGTCAACCG	5'-CTGCCCATCATCATGACCTG	58	Cell cycle - senescence
<i>CDKN3</i>	5'-TGAAGCCGCCAGTTCAATA	5'-CAACCTGGAAGAGCACATAAACC	151	Cell cycle
<i>CHEK1</i>	5'-GAGCGTTTGTGAACAAGATGTG	5'-GTTGGTCCCATGGCAATTCT	151	Cell cycle
<i>CHEK2</i>	5'-TCAGCAAGAGAGGCAGACCC	5'-ACAGCTCTCCCCCTTCCATC	151	Cell cycle
<i>CUL3</i>	5'-GGTAAACCAACACAGCGGGT	5'-CTGGGTGCGATTACCTTGT	151	Cell cycle
<i>DNMT1</i>	5'-AGAACGCCTTTAAGCGCCG	5'-CCGTCCACTGCCACCAAAT	110	Cell proliferation
<i>E2F4</i>	5'-GCATCCAGTGGAAGGGTGTG	5'-ACGTTCCGGATGCTCTGCT	151	Cell cycle
<i>GADD45A</i>	5'-GATGCCCTGGAGGAAGTGCT	5'-GAGCCACATCTCTGTCGTCGT	151	Cell cycle - senescence
<i>GAPDH</i>	5'-GCAAATTCATGGCACCGT	5'-TCGCCCCACTTGATTTTGG	106	Housekeeping
<i>KNTC1</i>	5'-ATAGTCAACCCAGAGTGGGCTGT	5'-TTTACGTTTTTCGTCCTGCG	151	Cell cycle
<i>MCM2</i>	5'-TGCCACTGTATCCTAGCCA	5'-GATGGAAGGAGCAATGCTGG	151	Cell cycle
<i>MKI67</i>	5'-TGTGCTGTCTCGACCCCTACA	5'-TGAAATAGCAGATGTGACATGTGCT	151	Cell proliferation
<i>PCNA</i>	5'-TTTGGTGCAGCTCACCTG	5'-CGCGTTATCTTCGGCCCTTA	151	Cell proliferation
<i>RB1</i>	5'-GACCCAGAAGCCATTGAAATCT	5'-GGTGTGCTGGAAGGGTCC	151	Cell cycle
<i>RPA3</i>	5'-TTCGTAGGGAGGCTGGAAAA	5'-CCTTGGCGGTTACTCTTCCAA	151	DNA damage
<i>RPLP0</i>	5'-TTCATTGTGGGAGCAGAC	5'-CAGCAGTTTCTCCAGAGC	156	Housekeeping
<i>TP53</i>	5'-GCGTGTTTGTGCCTGTCTCTG	5'-TGGTTTCTCTTTGGCTGGG	151	Cell cycle - senescence
<i>UBA1</i>	5'-CCATAAACGCCTTCATTGGG	5'-TGGAGGCACTTGCTCTGTG	151	Cell cycle

^aMain molecular pathways in which genes are known to be involved

manufacturer's protocol. Briefly, the RT assay was conducted with 1 µL Oligo (dT) 500 mg/mL, 1 µL dNTPs mix 10 mmol/L, each, 1 µg of total RNA and sterile RNase-free water up to 13 µL final volume. The RT mix was heated to 65 °C for 5 min, enriched with 4 µL of first-strand buffer 5X and 2 µL of DTT 0.1 mol/L, and then incubated at 42 °C for 2 min with addition of 1 µL SuperScript II RT. Lastly, 20 µL of final solution

was incubated at 42 °C for 50 min and successively warmed up to 72 °C for 15 min.

Quantitative real-time PCR

Quantitative real-time PCRs (qPCR) were performed with Rotor-Gene 6000 (Corbett Life Sciences, Sydney, Australia) in 100-wells Gene Discs, using a final volume reaction of 15 µL containing 0.3 µmol/L of each

Table 2: Parameters employed for primers design

Primer Tm requirements	Min Tm (°C)	58
	Max Tm (°C)	62
	Optimal Tm (°C)	60
	Max Tm Difference (°C)	2
Primer GC content requirements	Min GC (%)	40
	Max GC (%)	60
	Min length (bp)	12
Primer length requirements	Max length (bp)	40
	Optimal length (bp)	20
Amplicon requirements	Min length (bp)	150
	Max length (bp)	250

Tm: melting temperature

forward and reverse primer, 1x SYBR Green mastermix (as indicated in Results section) and 0.2 µL of cDNA solution, as described.^[14] The following thermal profile was applied: 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 10 s, 64 °C for 30 s, and 72 °C for 15 s. Melting curve analysis was performed ramping from 60 °C to 90 °C and rising by 0.5 °C every 2 s.

Gene expression variations were evaluated in term of fold induction respect to the untreated cellular population (control) by both the $2^{-\Delta\Delta CT}$ method and 'Comparative Quantitation' tool of the Rotor Gene 6000 Series software 1.7. Expression stability values of the different housekeeping genes were calculated by Norm Finder software^[15] to choose the best reference gene for normalization. Filtering of results was carried out as follows: genes were considered differently expressed when their change was greater than ± 2.5 fold respect to the transcript levels of untreated sample, as already described.^[16] All experiments were conducted in triplicate.

RESULTS

RT-qPCR array design

The RT-qPCR array was developed to study modulation of transcript abundance of 41 human genes involved in regulation of key cellular pathways, such as cell cycle, DNA damage, cellular proliferation, apoptosis, and senescence [Table 1]. The RT-qPCR array was designed exploiting 100-well discs compatible with the Rotor-Gene 6000 instrument [Figure 1], but it could be easily adapted to standard 96-well plates. Most importantly, our array was designed to harbor several technical controls to statistically evaluate final results and to exclude possible experimental bias. Six wells were reserved for no template controls (NTC) to monitor possible contamination (amplifying 3 housekeeping genes *GAPDH*, *RPLP0*, and *ATP5B*-yellow disc section, Figure 1). In addition, one primer pair was designed to amplify part of an intronic region of the *GAPDH* gene in order to detect possible genomic DNA contaminations resulting from the RNA extraction

procedure (purple disc section, Figure 1). The same *GAPDH*-primer pair was used to amplify commercial genomic DNA as both positive PCR reaction control and internal standard control to compare the efficiency of amplifications performed at different times in different discs (orange disc section, Figure 1).

In order to better control the results, and also to extend the applicability of the assay to different experimental conditions, we designed the RT-qPCR array, including primer pairs able to amplify the 3 stable and housekeeping gene transcripts *GAPDH*, *RPLP0* and *ATP5B*, to be used as reference for gene expression normalization. All reactions were placed in the 100-well disc in duplicate (red disc section, Figure 1).

Primers were designed following parameters reported in Table 2 in order to optimize and make uniform all PCR reactions of the array. To achieve the best results, we chose primer pairs with the lowest penalty value given by the PrimerExpress 2.0 software.

PCR conditions optimization

In order to optimize the PCR experimental condition, we evaluated: (1) primer efficiency by analyzing the slope of the real-time amplification curves; (2) absence of primer-dimer amplification; (3) specificity of the product; (4) absence of unspecific products by both agarose gel electrophoresis (AGE), and analysis of the melting curve profiles generated after PCR amplification. First, we compared 4 different commercially available master mixes and selected the 1 that, in our conditions, gave the best results in terms

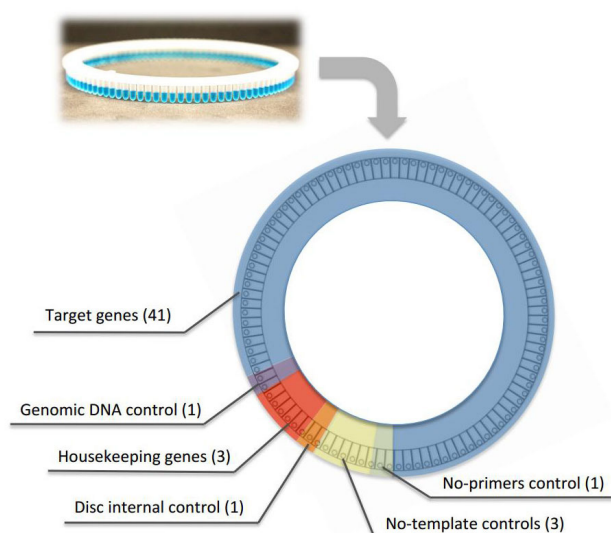


Figure 1: Schematic representation of the RT-qPCR array design. Distribution of the different primer pairs and relative experimental controls using the Rotor Gene 100-wells disc. RT-qPCR: reverse transcription-quantitative polymerase chain reaction

of product specificity and amplification efficiency (FastStart SYBR Green Master produced by Roche, Figure 2A-D). Then, we tested different thermal profiles by changing the annealing step temperature (from 56 °C to 66 °C, data not shown), identifying 64 °C as the best annealing temperature. We demonstrated that, at the selected qPCR condition, the slope of the amplification curves was comparable in all array samples [Figure 3A]. In addition, all the different primer pairs generated a unique PCR product as observed through both AGE separation [Figure 3B] and melting curves analysis (data not shown), highlighting the specificity of all the amplifications and the absence of primer-dimer products.

Modulation of the gene expression profile induced by cisplatin (CDDP)

Once optimized, the RT-qPCR gene array was used to study effects induced by CDDP on HeLa cells. To this end, cells were treated with sublethal doses of CDDP (10 μ mol/L for 24 h), obtaining a reduction of cell survival equal to 72.1%.

Total RNA was extracted from treated and untreated

HeLa cells and its integrity was evaluated by monitoring the 28S to 18S rRNA ratio through AGE (data not shown). After fluorometric quantitation, 1 μ g of RNA was retro-transcribed and the resulting cDNA used for qPCR analysis.

Gene expression profile modulations were evaluated comparing Ct values between treated and non-treated cells, using the $2^{-\Delta\Delta C_t}$ method. HeLa cells treated with CDDP showed a clear increase of the abundance of the 2 cell cycle inhibitors CDKN1A (+4.93 fold) and CDKN2B (+7.24 fold), as well as of GADD45A (+23.1 fold, Figure 4A). In addition, other genes playing key functions in both DNA damage response and cell cycle regulation were found to be up-regulated, such as BRCA1 (+2.73 fold) and cyclin dependent kinases 1 and 2 (CDK1, +3.12 fold; CDK2, +2.74 fold), while a significant down-regulation of anti-apoptotic gene *BCL2* was observed (-3.63 fold, Figure 4A). Notably, all these regulations were confirmed applying the comparative quantitation method available on Rotor Gene 6000 Series software 1.7 (data not shown). Amplification efficiency was checked by monitoring the slope of

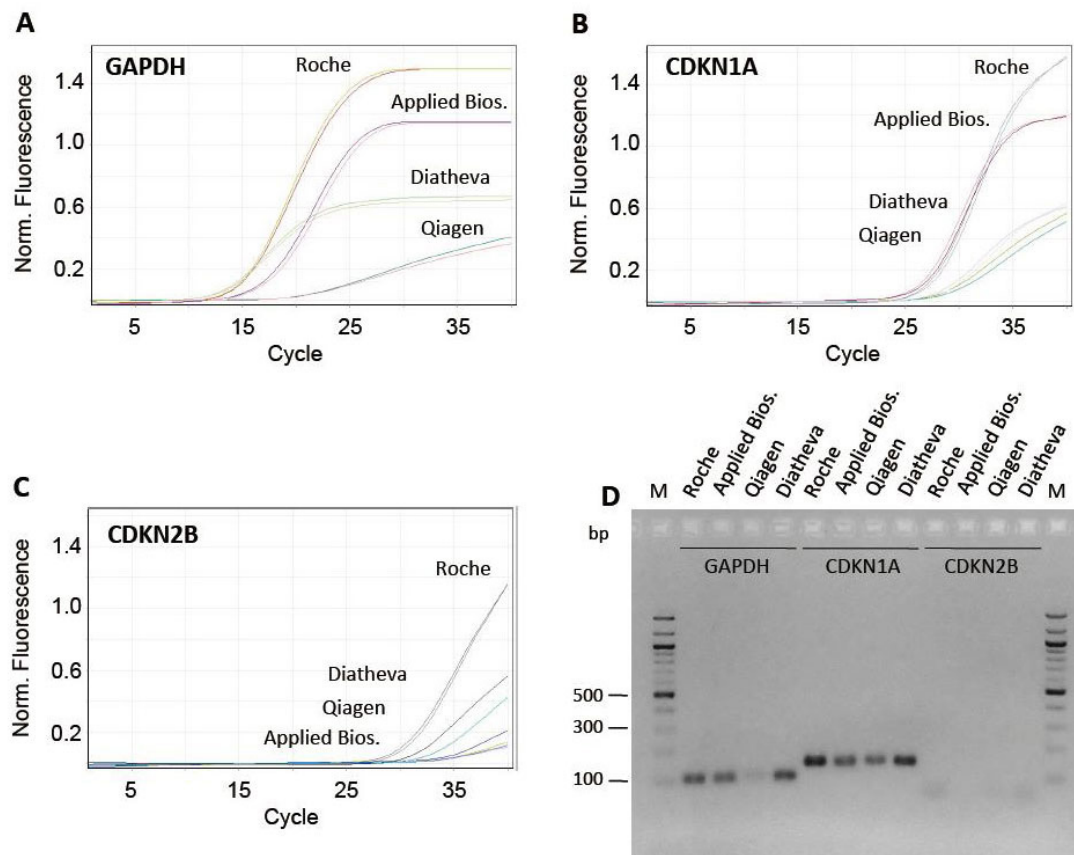


Figure 2: Test of different commercially available master mixes. (A-C) profiles relative to amplification of cDNA using primer pairs for GAPDH, CDKN1A and CDKN2B genes and the following commercially available SYBR-green master mixes: (1) FastStart SYBR Green Master (Roche); (2) SYBR Select Master Mix (Applied Biosystems); (3) RT2 SYBR Green FAST MasterMix (Qiagen); (4) 2X PCR Master Mix (Diatheva). (D) 2% agarose gel electrophoresis of GAPDH, CDKN1A and CDKN2B PCR products. PCR: polymerase chain reaction

amplification curves generated during real-time amplification [Figure 4B and C], while specificity was confirmed by analyzing the uniqueness of the PCR product by melting curve peaks analysis [Figure 4D and E] and AGE (data not shown).

Modulation of gene expression profile induced by sodium butyrate (NaBu)

Subsequently, the RT-qPCR array was used to investigate the effects induced by histone deacetylase inhibitor sodium butyrate (NaBu) on HeLa cells. Even in this case, cells were subjected to sublethal doses of NaBu (5 mmol/L for 16 h) to achieve a reduction of cell survival equal to 68.5%.

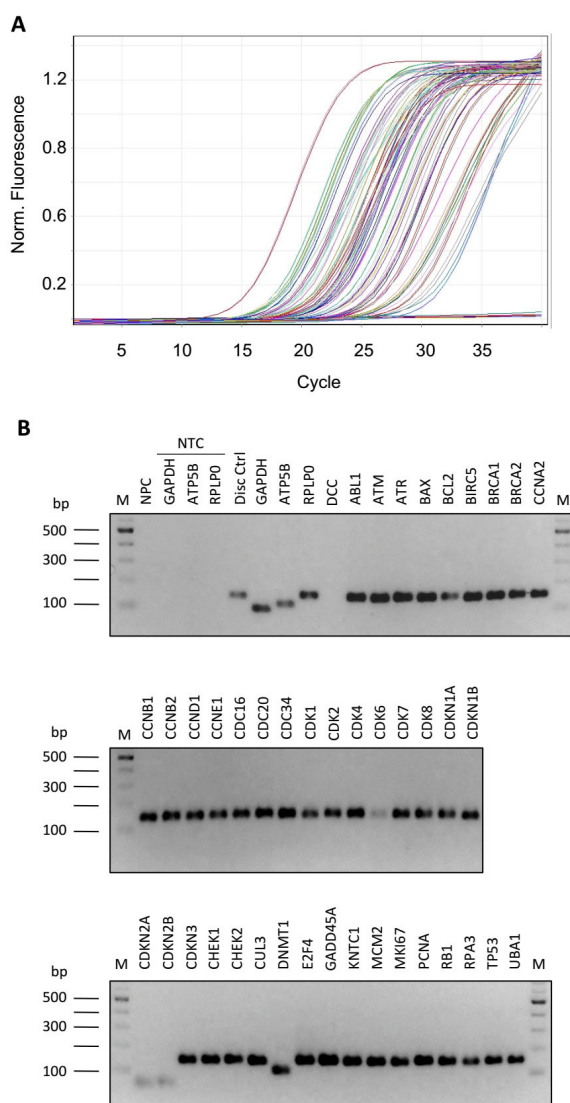


Figure 3: Optimization of PCR conditions. Amplification efficiency and specificity of all gene transcripts of RT-qPCR array. (A) Amplification plot showing profiles slope of different PCR reactions; (B) 2% agarose gel electrophoresis of all array amplicons. PCR: polymerase chain reaction; RT-qPCR: reverse transcription-quantitative polymerase chain reaction; M: 100 bp DNA ladder; NPC: no-primers control; NTC: no-template controls

Total RNA was extracted from treated and untreated HeLa cells and its integrity evaluated by monitoring 28S to 18S rRNA ratio through AGE (data not shown). After fluorometric quantitation, 1 µg of RNA was retro-transcribed and the resulting cDNA used for qPCR analysis.

As for CCDP treatments, gene expression profile modulations were evaluated comparing Ct values between treated and not treated cells, using the $2^{-\Delta\Delta C_t}$ method. NaBu-treated HeLa cells showed down-regulation of cyclins A2 (CCNA2, -2.86 fold), B1 (CCNB1, -2.78 fold), and D1 (CCND1, -9.09 fold), as well as of cyclin-dependent kinase CDK6 (-5.00 fold, Figure 5A). A significant reduction of transcript abundance was monitored also for marker of proliferation MKI67 (-4.76 fold). Moreover, the array showed up-regulation of genes encoding for cyclin-dependent kinase inhibitors CDKN1A (+2.82 fold), CDKN1B (+3.65 fold), and CDKN2B (+3.88 fold), and of GADD45A (+2.72 fold, Figure 5A). Again, all regulations emerged from these analyses were confirmed applying the comparative quantitation method mentioned above (data not shown).

Analysis of the amplification curves showed that the efficiency of the PCR reaction was similar between the different primer pairs and samples [Figure 5B and C], while the peaks of the melting curves demonstrated the specificity of the PCR product [Figure 5D and E].

DISCUSSION

Here we report the design and validation of a RT-qPCR array that allows the reliable study of gene expression modulations occurring in biological models exposed to drug treatments or to any other different culture condition. In order to better control the results, and also to extend the applicability of the assay to different experimental requirement, we designed a RT-qPCR array to include primers able to amplify 3 different housekeeping genes *GAPDH*, *RPLP0*, and *ATP5B*. The presence of these 3 control genes is extremely important mainly for two reasons: (1) it is unlikely that there exists a “universal” housekeeping gene whose expression can be considered as referee for RNA normalization in any biological model (or treatment) under investigation; (2) despite the control of both the amount and integrity of RNA extracted from different samples, the RT-qPCR could be impaired also by additional experimental variables (e.g. the limit of spectrophotometric analysis, not homogeneous RNA purity).

Furthermore, to monitor possible DNA contamination

from the RNA extraction procedure, we also designed a primer pair that amplified an intronic region of the housekeeping gene *GAPDH*. Importantly, since each sample of cDNA would be amplified in a separated 100-well disc, we also added an additional control to verify the “inter-disc” variability by using, as template, a known amount of human genomic DNA and a primer pair that amplified genomic *GAPDH*.

To further increase the reliability of the assay, amplification of each target gene was performed in duplicate. Most commercially available PCR arrays contain more target genes but lacks replicates (e.g. 84 genes using the RT² ProfilerTM PCR arrays by Qiagen, 92 genes using TaqMan Array Plates by

Thermo Fisher Scientific, 88 genes using real Time PCR Assay Panels by BioRad), increasing the risk of not identifying possible technical errors.

Prior to using the array in an experimental model, we tested 4 commercially available SYBR Green master mixes, focusing attention on 3 genes that can recapitulate 3 different levels of gene expression: high levels (*GAPDH*), medium/low levels (*CDKN1A*), and very low levels (*CDKN2B*). In fact, it is known that expression level of some genes involved in the DNA-damage response (e.g. genes that are involved in cell cycle regulation, such as CDK-inhibitors) are at the limit of the detection and thus require a highly efficient DNA-polymerase. Therefore, we optimized our RT-

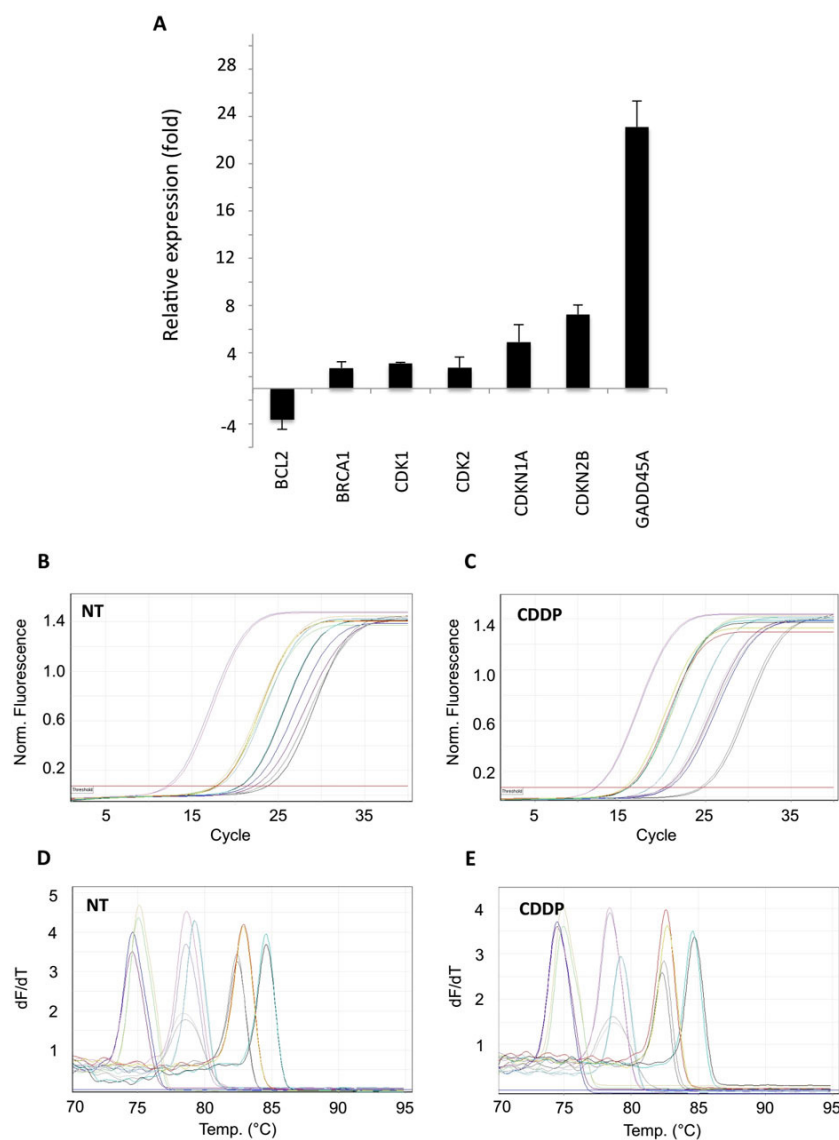


Figure 4: Application of PCR array to cisplatin (CDDP)-treated HeLa cells. Difference in transcriptional activity of cisplatin-treated HeLa cells, compared to non-treated cells, evaluated by $2^{-\Delta\Delta CT}$ method. Data reported as mean \pm standard deviation of 3 independent experiments. (A) Relative expression of differentially expressed genes; (B-E) examples of qPCR amplification plots and melting curves of genes found differentially expressed. PCR: polymerase chain reaction

qPCR array using SYBR-green master mix that showed the best efficiency in our conditions. This allowed us to monitor transcript levels of low-expressed genes (e.g. *CDKN2B*) [Figure 2B-D].

Then, the RT-qPCR array was validated using HeLa cells subjected to treatment with two different drugs, CDDP and NaBu, known to induce important modulations of the gene expression profile of this cellular model.

In particular, CDDP treatment was able to up-regulate growth arrest and DNA damage response genes *GADD45A* and *BRCA1*, as well as cell cycle inhibitors genes *CDKN1A* and *CDKN2B*. These transcriptional

elaborations have been widely documented in HeLa cells treated by CDDP.^[5,6,17,18] In addition, a significant down-regulation of anti-apoptotic gene *BCL2* also confirmed previously reported data.^[5,19] All these modulations were largely expected since CDDP is known to exert its activity by targeting DNA of cells and forming covalent adducts that lead to activation of the cellular DNA damage response.^[4] In fact, once damaged, cells respond by inhibiting its progression through the cell cycle and, in case of extensive damage, by activating the apoptotic cell death program.

Interestingly, we found that CDDP also induces up-regulation of cyclin dependent kinases 1 and 2 (CDK1 and CDK2). This result, which is to our knowledge the

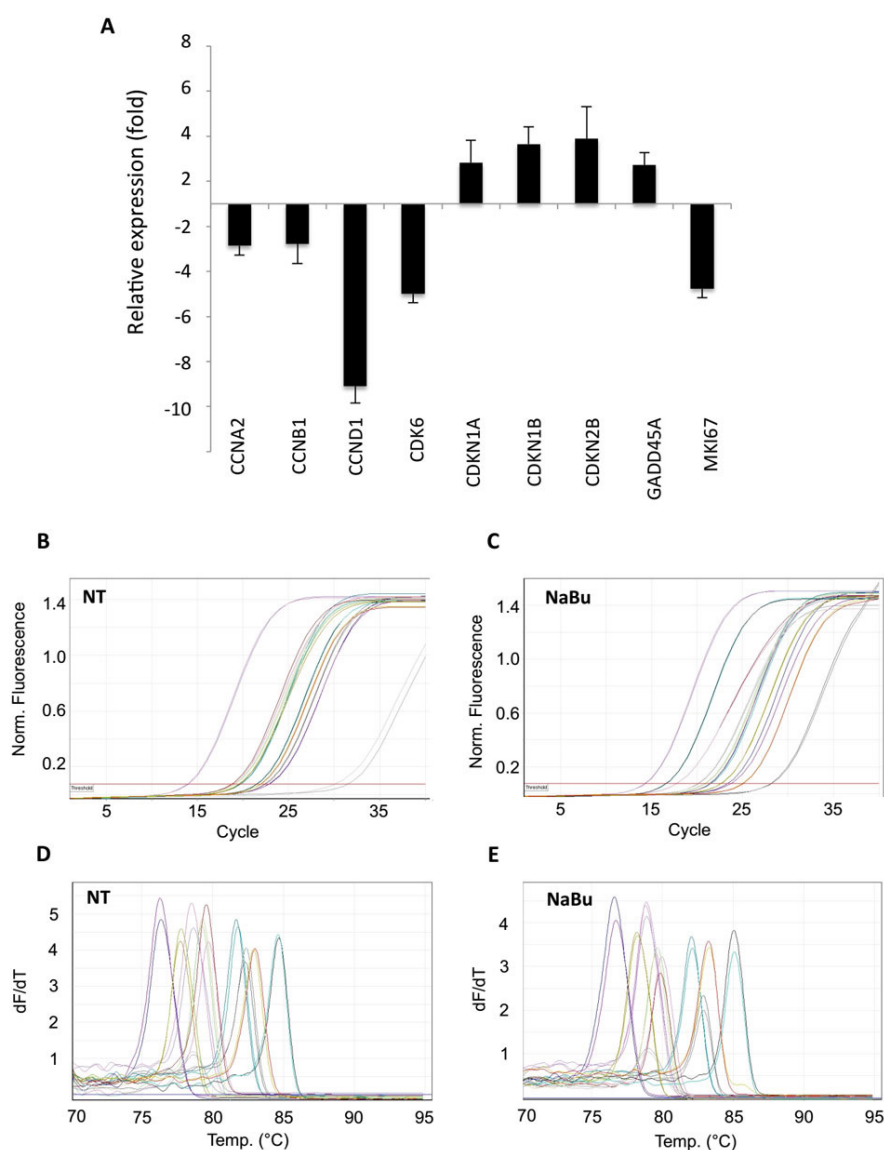


Figure 5: Application of the PCR array to sodium butyrate (NaBu)-treated HeLa cells. Difference in transcriptional activity of sodium butyrate-treated HeLa cells, compared to non-treated cells, evaluated by $2^{-\Delta\Delta CT}$ method. Data reported as mean \pm standard deviation of 3 independent experiments. (A) Relative expression of differentially expressed genes; (B-E) examples of qPCR amplification plots and melting curves of differentially expressed genes. PCR: polymerase chain reaction

first observation made in HeLa cells, could be put in relation with the known role of these CDKs in apoptosis, in addition to their known functions in regulation of cell cycle progression.^[20] This hypothesis is supported by evidence that CDK2 silencing by shRNA decreases cisplatin-induced apoptosis and PARP cleavage in embryonal carcinoma models^[21] and opens the door to possible future insights.

Regarding the effects of NaBu, our data confirm the down-regulation of cyclins and CDK6 and the up-regulation of three different CDK inhibitors,^[9,10] which reflect the long-known ability of this histone deacetylase inhibitor to regulate the progression of cells through the cell cycle.

Even in this case, the RT-qPCR array allowed us to provide new information about transcriptional activity of the genes under investigation. In fact, NaBu also induced in HeLa down-regulation of the gene encoding for the anti-proliferative antigen MKI67, and up-regulation of GADD45A, a typical sensor of stress involved in growth arrest and in the DNA damage response. These new observations, although already described in other cancer models (MKI67 in prostate cancer^[22,23] and GADD45A in colon carcinoma^[24]), have never been reported in HeLa cells subjected to NaBu treatment.

In summary, we have designed, optimized, and biologically validated an RT-qPCR array that can be exploited to robustly analyze expression of genes involved in cell cycle regulation, DNA damage response, apoptosis, and senescence induction.

This array represents a reliable, inexpensive, and rapid tool that could be exploited to provide an overview of molecular pathways activated by drug treatment in human cancer cells. Moreover, we believe that detailed description of the procedure will allow application of the RT-qPCR array in different experimental conditions and could contribute to understand the molecular mechanisms of action of new drugs in the context of pharmacologic studies.

Authors' contributions

Study conception and design: M. Fanelli, S. Amatori
Primers design and PCR conditions optimization: S. Amatori

Validation of the RT-qPCR array: G. Persico

Manuscript writing and editing: M. Fanelli, S. Amatori

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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Impact of health care insurance on overall survival of patients with multiple myeloma and monoclonal gammopathy of undetermined significance

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ABSTRACT

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Key words:

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outcomes,
survival

Aim: Health care insurance improves access to care and thus outcome in patients with solid tumors. Little information on the impact of health care insurance on hematological malignancies including multiple myeloma exists. The authors aimed to analyze the effect of health care insurance on the survival of patients with multiple myeloma (MM) and monoclonal gammopathy of undetermined significance (MGUS) at Louisiana State University Health Sciences Center in Shreveport, LA. **Methods:** Two hundred fifty seven patients were reviewed, of which 208 had MM and 49 had MGUS. **Results:** One hundred and seventy seven patients (69%) were funded and 80 (31%) were non-funded. Funded patients with MM had an overall survival (OS) of 6.2 years compared to 3.8 years for non-funded patients ($P < 0.001$). Survivals were not significantly affected by race or gender. The analysis demonstrates that funded patients with MM and MGUS patients have statistically significant increased OS compared to patients with no insurance. **Conclusion:** This study showed that patients with multiple myeloma and MGUS with health care insurance have longer overall survival when compared to non-funded patients.

INTRODUCTION

Multiple myeloma (MM) is the second most common hematological malignancy in the United States and the most common hematological malignancy in African-

Americans.^[1,2] MM is preceded by an asymptomatic premalignant proliferation of plasma cells termed "monoclonal gammopathy of uncertain significance (MGUS)".^[3-6] MM and MGUS are twice as common in African-Americans as in Caucasians and more



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common in males than females.^[7-10] The prevalence of MGUS is 3% in people older than 50 years and patients with MGUS have an annual 1% risk of progression to MM or related malignant disorder.^[11-14] Advances in therapeutic options such as proteasome inhibitors, immune modulatory drugs (IMDs) and stem cell transplantation (SCT) have improved the prognosis and survival of patients with MM.^[15,16] Several drugs belonging to the class of proteasome inhibitors such as Bortezomib, Carfilzomib, Ixazomib, immunomodulators such as Lenalidomide, Pomalidomide and monoclonal antibodies such as Daratumumab and Elotuzumab have changed the landscape of treatment of multiple myeloma in the last decade. Patients with MGUS undergo active surveillance for progression to MM and do not receive active treatment.

The impact of socioeconomic status has been studied in several solid organ malignancies including breast, colorectal, prostate and pancreatic cancer. Health care insurance has been shown to result in positive outcomes in patients diagnosed with these malignancies.^[17,18] Health insurance improves access to health care, early diagnosis and access to several advanced therapeutic options. Several studies have explored the influence of socioeconomic status on the survival of patients with leukemia and MM.^[19,20] However, these studies have not examined the effect of insurance on the outcome of these hematologic malignancies. We attempted to study the effect of health care insurance; in particular, on the outcomes of patients diagnosed with MM and MGUS at Louisiana State University Health Sciences Center (LSUHSC), Shreveport, LA, where approximately half the patient population is uninsured.

METHODS

We conducted a retrospective study of all the patients at our institution diagnosed with and treated for MM and MGUS between the years 1997 and 2012. MM and MGUS were defined using the International Classification of Disease for Oncology, 3rd edition (ICD-O-3) codes. All the data were obtained from the electronic medical records of the cancer center at our institution and Social Security death index. Approval for accessing the patient information was obtained from the Institutional Review Board (IRB) at our institution. Date of diagnosis, demographic factors such as age, gender, race, health care insurance status and staging at the time of diagnosis were available for each case. Durie-Salmon staging was used for staging the patients with MM. Patients with MM were classified as having high risk and standard risk disease according to the Mayo clinic risk stratification for myeloma.^[21]

Medical insurance status at the time of diagnosis was defined as funded if a patient had Medicaid, Medicare or commercial insurance and non-funded if the patient was coded as free-care or self-pay.

Descriptive statistics were used to describe the data. Product limit methods were used to estimate survival and Log rank test was used to compare survival difference for each factor. Statistical software SAS® 9.3 (SAS institute, Gary, NC) were used in the statistical data analysis. A *P*-value of < 0.05 was considered the threshold for statistical significance.

RESULTS

The medical records were reviewed of 257 patients with MM or MGUS diagnosed and treated at LSUHSC from 1997-2012. Of these patients 208 (80.9%) had MM and 49 (19.1%) had MGUS. The median age at diagnosis was 60 years for MM and 59 years for MGUS patients. Of all the patients diagnosed with MM and MGUS, 92 (37%) were Caucasians and 165 (63%) were African-Americans. There was a slight female predominance with 114 male (44%) and 143 female (56%) patients [Table 1]. Stratification of the patients according to the stage of MM revealed 49 (23.5%) to be stage 1, 23 (11%) to be stage 2, 95 (45.6%) to be stage 3A and 41 (19.7%) to be stage 3B disease. When patients were classified according to their health care insurance status, 177 (69%) were funded and 80 (31%) were non-funded. At presentation, 23.6% of non-funded patients had stage 3B disease compared to 18.5% in the funded group (*P* = 0.06). High-risk features were seen in 16.7% of the MM patients with insurance as compared to 27.2% of the non-funded patients (*P* = 0.29). However, the median survival was 6.2 years in the funded group compared to 3.8 years in the non-funded group (*P* < 0.001) [Figure 1] with a 5-year survival for MM patients of 60% in the funded group compared to 42% in the non-funded group. The 5-year survival for MGUS was 95% for patients with insurance and 62% for patients without insurance (*P* = 0.03). When the effect of race was examined the overall survival in the Caucasian group with MM was

Table 1: Patient demographics

	MM	MGUS
Age, years	59.7 (21.2-93.3)	61.0 (41.6-83.3)
Race		
AA	143 (66.5%)	20 (50%)
Cauc	72 (33.5%)	20 (50%)
Gender		
Male	95 (44.2%)	19 (47.5%)
Female	120 (55.8%)	21 (52.5%)
Insurance		
Funded	121 (56.28%)	25 (62.5%)
Non-funded	94 (43.72%)	15 (37.5%)

MM: multiple myeloma; MGUS: monoclonal gammopathy of undetermined significance; AA: African American; Cauc: Caucasian

noted to be 6.2 years compared to 5.1 years in the African American group ($P = 0.517$). Likewise, gender had little impact on survival as there was only a slight but not statistically significant increase in survival in female patients with MM with a mean survival of 5.4 years for females vs. 5.1 years for males ($P = 0.748$). Follow up of patients with MGUS at 8.3 years revealed that 73% of funded patients were alive as compared to 50% in the non-funded group ($P = 0.03$). The overall survival of patients when classified according to stage was 12.8 years for stage 1, 4.3 years for stage 2, 5.1 years for stage 3A and 3.3 years for stage 3B ($P < 0.0001$). Although the stage distribution was similar in the funded and non-funded groups at the time of diagnosis, within each stage the funded patients had improved survival. P -values to compare the survival difference between funded and non-funded groups for stage 1, 2, 3a, and 3b were 0.098, 0.267, 0.039, and 0.123, respectively.

DISCUSSION

We have conducted the first study to examine the

impact of health care insurance on the outcomes of patients with multiple myeloma. Previous studies examined the role of poverty and socioeconomic status on the outcome of patients with MM but until now there was no study looking into the role of health care insurance on the survival of patients with this disease. In our study, we found that the funded patients diagnosed with MM and MGUS, the precursor of MM, have significantly better median, 5-year and overall survivals than patients without insurance. The insurance status was analyzed at the time of diagnosis and did not take into account change of insurance status during the course of the illness. It could be presumed that funded patients had better access to medical care, thereby allowing for earlier diagnosis; less advanced disease, and hence improved survival. However, there was no statistically significant difference in the distribution of disease stage between the funded and non-funded groups. Another feature of MM that could potentially influence the outcomes is the biology of the disease as reflected by the presence of disease risk features. However, the Mayo Clinic risk stratification criteria were similarly distributed between

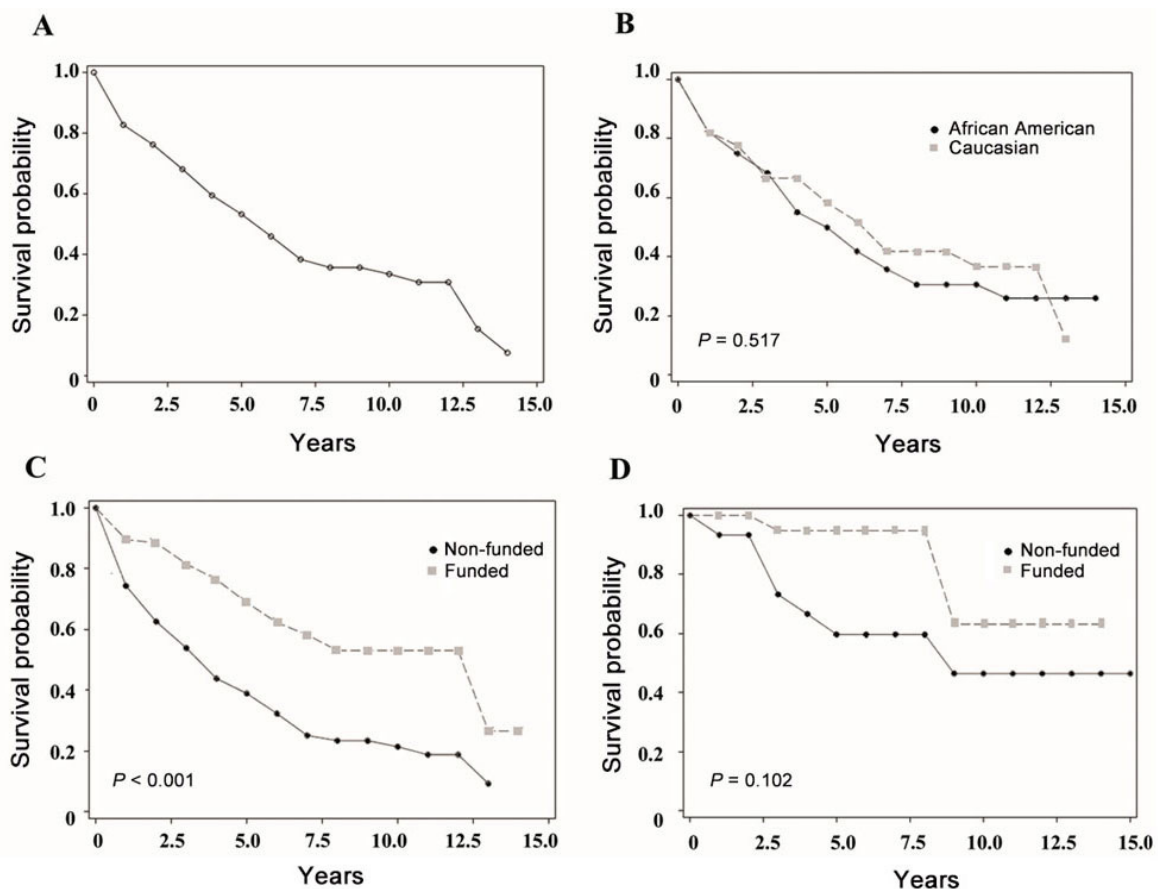


Figure 1: Two hundred and fifty-seven patients with multiple myeloma or MGUS diagnosed and treated. (A) Overall survival of patients with multiple myeloma; (B) comparison of overall survival of multiple myeloma based on race; (C) comparison of overall survival of multiple myeloma based on funding; (D) comparison of overall survival of MGUS based on funding. MGUS: monoclonal gammopathy of undetermined significance

the funded and non-funded patients.

While insurance did not appear to affect various parameters at the time of diagnosis, it is possible that subsequent treatment might be adversely affected. However, at our institution the same physicians treat patients in the same clinic setting, with the same treatment protocols regardless of payer status. As the possibility of treatment differences between the two groups leading to improved survival of one group over the other seems less likely, our results suggest that having health care insurance, with easier access to health care, is probably an independent predictor of survival in patients with MM and MGUS. We are currently investigating whether comorbid conditions at the time of diagnosis are affecting response to therapy and subsequent outcomes. It could be argued that the death of patients with MGUS is usually more likely to occur from comorbidities than from progression to MM, as the conversion rate of MGUS to MM is 1% per year, and hence since the impact of health care insurance on survival is greater in MM than MGUS, the effects are more likely to be direct (diagnosing earlier disease progression), particularly in view of the recent knowledge that MM is inevitably preceded by MGUS.^[3,4]

The survival differences relative to race and gender were not statistically significant. Prior studies have demonstrated either no difference or improved survival for one group or the other. Most recently, analysis of the SEER data demonstrated superior survivals in African Americans but with improving survival in Caucasians, but not African Americans, with time over the study period of 1973 to 2005.^[6,7] The last observation suggested differential access to newer therapies. However, we noted that survival was similar for all stages of disease and in a preliminary review of treatments that there were no differences in use of newer modalities. Our results suggest that the effects of socioeconomic status on outcomes can be overcome by the expertise of an academic center where patients are treated by faculty dedicated to the treatment of MM.

The strengths of our study are that it showed consistent results in regards to the impact of health care insurance in patients with both MM and MGUS. Our finding is not the consequence of differences in stage distribution or high-risk features, adding impetus to the independent effect of insurance status on the outcomes of MM. As the patients were treated in a health care facility with equal access regardless of payer status, we eliminated the confounding effects of the influence of advanced therapeutic options such

as SCT and IMDs. As the patients came from about 60 of Louisiana's 64 parishes (counties) it is difficult to ascertain the exact causes of death of all the patients, thereby making the calculation of disease specific survival rates difficult. With the advent of health care reform and insurance coverage for nearly all Americans, it is possible that outcome differences will be minimized in the future. However, the effects of insurance status on outcome presumably reflects the effect of various exogenous factors such as the lifestyle factors including diet, exercise, alcohol, tobacco, etc. as well as compliance issues and hence as these factors will not be eliminated by health care insurance it is important to understand how they influence the disease outcome.

In conclusion, our study showed that patients with multiple myeloma and MGUS with health care insurance have longer overall survival when compared to non-funded patients. There was no difference in survival in our patient population based on race or gender. Further studies are needed to explore the various factors through which health care insurance impacts the disease outcomes.

Authors' contributions

Designed the entire project, reviewed the data and drafted the manuscript: S.S. Devarakonda, F. Turturro, J. Glass

Contributed to finalizing the manuscript: R. Munker
Analyzed the data as cancer epidemiologist: R. Shi
Assisted in the preparation of the manuscript: E. Friday

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None.

Conflicts of interest

There are no conflicts of interest.

Patient consent

Waiver of consent and authorization was obtained from LSU Health Internal Review Board.

Ethics approval

The study has been approved by LSU Health Internal Review Board, IRB approval # E10-005.

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Management of choroidal metastasis using external beam radiotherapy: a retrospective study and review of the literature

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ABSTRACT

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Key words:

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acuity

Aim: Choroidal metastases are rare in the evolution of solid cancers and constitute exceptional metastatic sites involving functional visual prognosis. The authors conducted a retrospective study to determine the interest of external radiotherapy for the treatment of choroidal metastases. **Methods:** The authors reviewed the records of 28 patients with choroidal metastases who had breast ($n = 15$), lung ($n = 9$), ovarian ($n = 1$), kidney ($n = 1$), prostate ($n = 1$) cancer or carcinoma with unknown primitive at the moment of the diagnosis ($n = 1$). The median age was 58 years (extremes: 34-71 years). Tumor stage before the discovery of metastatic choroidal metastasis was 50% of patients. Ocular involvement was unilateral ($n = 22$) or bilateral ($n = 6$). The delivered doses ranged from 20 to 50 Gy fractionated with 3-5 Gy in 2D technique ($n = 5$), conformational ($n = 21$), intensity modulation ($n = 2$). The most widely used prescription scheme delivered 30 Gy in 10 fractions (64%) using two 6 MV photons beams. **Results:** At the end of irradiation, 13 patients (46%) showed an improvement of eye symptoms. For the others, a stabilization in symptoms was noted ($n = 15$). No patient had visual degradation. No acute or late grade 2-3 toxicities were objectified. The histological type did not influence the response ($P = 0.5$). There was no dose relationship-response in our series. **Conclusion:** External radiation therapy is a useful technique in the palliative treatment of choroidal metastases. Acute and late toxicities are acceptable.

INTRODUCTION

Choroidal metastases are rare in the development of solid cancers that can affect the visual prognosis

of the patient in the short to medium term. In several autopsy series, they have an incidence of 4% to 12% in patients with solid tumors.^[1,2] The uveal tract is the most common site of intraocular



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metastases, probably due to anatomical reasons and blood vessel supply.^[3] The treatment of these tumor localizations is not yet standardized but is based mostly on external radiotherapy, which with its sophisticated new techniques allows better results while protecting healthy organs around.^[4,5] We conducted a retrospective study and synthesis of the literature to determine the interest of radiotherapy in the management of choroidal metastases.

METHODS

Patient characteristics

We reviewed the cases of 28 patients treated in our center in the period between August 1996 to June 2015 and presenting choroidal metastases in a context of solid cancer, excluding melanomas. All information regarding the date and site of the primary tumor, status of the primary at the time when choroidal metastasis was diagnosed, date of choroidal metastasis diagnosis, symptoms, localization and number of lesions in the eye, radiation treatment and technical parameters was collected. All the ophthalmological examinations were done in the ophthalmology department of our center, and then sent to our department for oncological management by radiotherapy [Table 1].

Radiotherapy

For each patient, excluding those treated in two-dimensional radiotherapy (2D), a scanner with a slice thickness of 2.5 mm was performed in treatment position using a thermoformed contention mask to maintain a reproducible position throughout the treatment. Treatment plan was performed using the ECLIPSE TM treatment planning system (VARIAN®, Palo Alto, CA, USA). The treatment was delivered with a VARIAN CLINAC® Linear Accelerator. The doses varied from 20 Gy to 50 Gy with a fractionation of 3 Gy to 5 Gy in two-dimensional 2D ($n = 5$), conformational 3D ($n = 21$), intensity modulation ($n = 2$). The most used prescription regimen consisted of delivering 30 Gy into 10 fractions of 3 Gy ($n = 18$). The clinical target

volume CTV was the posterior uvea-choroid of the eye. The planning target volume PTV represented CTV with a margin varying between 0.5 cm and 1 cm. The use of magnetic resonance imaging (MRI) and merge it with the dosimetric scanner could help delineating the target volume with precision for a better planning of the treatment plan which will make it possible to give a large dose to the target volume to be treated (choroid) and sparing the risky organs beside it [Table 2].

Statistical analysis

SAS® JMP 11 software (SAS Institute Inc. Cary, NC, USA) was used for statistical analysis. An overall description of the population was carried out. For qualitative data, numbers and percentages were calculated. Quantitative data, median or mean by normality, as well as extreme values with minimum and maximum were estimated. Fisher's exact test was used to determine the association between two qualitative variables with a significance level $P < 0.05$.

RESULTS

Patients had cancer of breast ($n = 15$), lung ($n = 9$), ovarian ($n = 1$), kidney ($n = 1$), prostate ($n = 1$), with an unknown primitive at the moment of the diagnosis ($n = 1$). The median age was 58 years [extreme (E): 34 to 71 years]. Our cohort contained 19 women and 9 men (sex ratio = 0.47). The tumor stage before the discovery of choroidal metastases was metastatic for 48% of the patients. Eye involvement was unilateral

Table 2: Results of external beam radiotherapy in our cohort

Characteristics	Effective
Total dose (splitting)	
20 (4) Gy	4
20 (5) Gy	1
24 (4) Gy	2
30 (3) Gy	18
30.5 (2.5) Gy	1
37.5 (3.5) Gy	1
50 (4) Gy	1
Irradiation techniques	
IMRT	2
2D	5
3D	21
Acute toxicity	
No toxicity	22
Headache grade 1	4
Eye pain grade 1	1
Radiodermatitis grade 1	1
Latetoxicity	
No toxicity	28
Improvement of initial ophthalmologic symptoms	
Yes	13
No	15
Visual improvement based on dose	
20 (4) Gy	2 (50%)
20 (5) Gy	1 (100%)
24 (4) Gy	1 (100%)
30 (3) Gy	7 (35%)
30.5 (2.5) Gy	0 (0%)
37.5 (3.5) Gy	1 (100%)
50 (4) Gy	1 (100%)

Table 1: Clinical characteristics

Characteristics	Effective
Gender	
Man	9
Women	19
Median age	58 years (34-75 years)
Primary tumor localization	
Lung	9
Breast	15
Ovarian	1
Kidney	1
Prostate	1
Unknown primitive	1
Tumor stage before discovery	
Localized	13
Metastatic	15

($n = 22$) or bilateral ($n = 6$). The inaugural symptoms were variable: decreased visual acuity, visual field amputation with scotoma, photophobia, myodesopsia [Table 2].

At the end of the irradiation, 13 patients (46%) showed an improvement in ophthalmologic symptoms. For the others, a stabilization of the symptoms was noted ($n = 15$). No patients showed visual degradation. No acute or late grade 2-3 toxicity was objectified. The histological type was not significantly correlated with the response ($P = 0.5$) according to Fisher's exact test. Furthermore, there was no dose-response relationship in our serie. The response rates following delivered dose are shown in Table 2.

DISCUSSION

Choroidal metastases are frequently pauci symptomatic with unspecific visual signs (scotoma, myodesopsis, photophobia, ocular pain) or even strictly asymptomatic.^[5-8] The exact prevalence of this tumor localization is not known with certainty and can be very variable depending on the size of metastatic patients cohorts. The median age at diagnosis in the main published series^[6,7] is 55 years with a median time between diagnosis of primary cancer and choroidal metastasis of 49-month.^[5] Primary tumors are predominantly of mammary and pulmonary origin.^[6,7] In several series, women are predominantly involved. These data are consistent with the results of our study. However, others tumor localizations are providers of choroidal metastases such as thyroid, kidney, prostate, esophagus or melanoma cancers.^[7,8] In some cases, ocular involvement may be symptomatic in one eye and remain completely asymptomatic on the other,

raison why patients should systematically benefit from a complete specialized ophthalmologic examination, prior to the initiation of a treatment. According to some authors, bilateral involvement is associated with a shorter likelihood of survival.^[9] On ophtalmoscopy, choroidal metastases appear as flat orange lesions located most often at the posterior pole of the eye, which can induce focal retinal detachment. Anterior or posterior uveitis may sometimes be associated. Mode A (Amplitude) and B (Brightness) ultrasound as well as fluorescein angiography can assist in diagnosis. They can demonstrate hyperfluorescence at the late time (venous) and hypofluorescence at the early (arterial).^[10,11] The diagnosis of certainty by biopsy puncture is rarely obtained given the potential complications. It is based on clinico-radiological arguments and the clinical context (patient with metastatic solid cancer). On scan (CT), choroidal tumors appear as hyperdense heterogeneous lesions enhanced by the contrast medium. MRI is not essential for diagnosis but may be of interest in target volumes delineation for radiotherapy.^[12] The choroidal tumors appear as heterogeneous masses with hyper signal T1 and hypo signal T2 which can be enhanced with the injection of Gadolinium.

The main therapeutic option is external radiotherapy. A thermoformed mask is generally used in order to ensure reproducibility of the treatment. As discussed in the prospective study on the radiotherapy of choroidal metastases, the anatomo-clinical target volume, which is the choroid, can be treated via one or two direct beams of 6 Megavolt energy photons.^[13] A beam angulation of 5° to 10° can be performed in order to spare the contralateral choroid.^[14] Another irradiation ballistics is possible by the use of 3 beams (anterior, posterior,

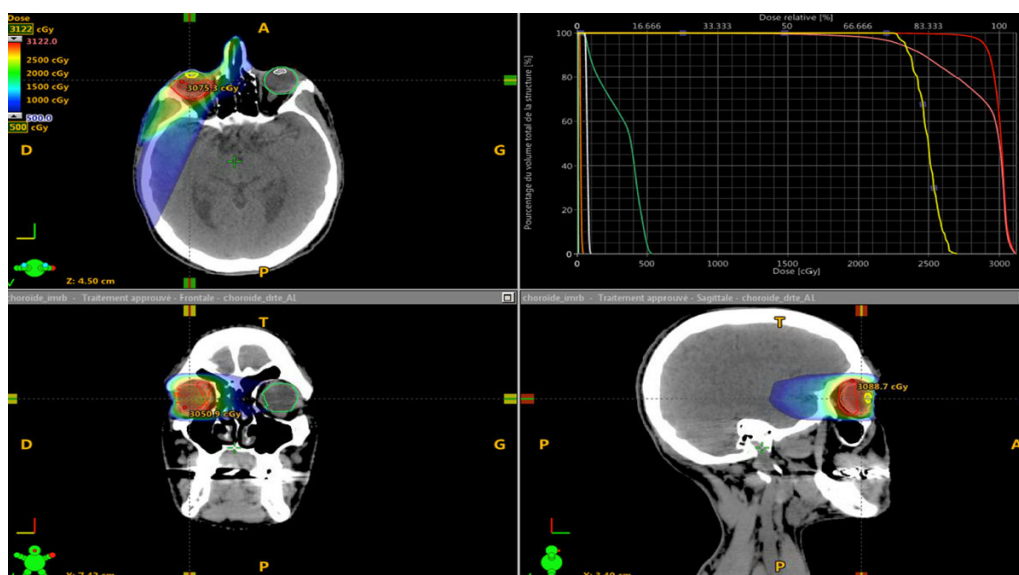


Figure 1: Intensity modulated radiotherapy using 3 beams of 6 MV photons

lateral). In our series, some patients benefited from an intensity modulation radiation therapy in archtherapy or static [Figures 1-3]. The contralateral choroidal sparing is controversial. It should be noted that the rate of recurrence in the untreated contralateral choroid is estimated to be low (less than 10%).^[15] However, some authors have reported a rate between 15% and 20%^[16,17] whereas it was zero in the German prospective trial ARO 95-08.

Potential acute side effects include radiodermatitis, focal alopecia, conjunctivitis, xerophthalmia, the severity of which is dose and field-dependent.^[18] Potential late side effects include cataract, glaucoma,

keratitis, dry eye syndrome, radiation-induced retinopathy depending on the technique, dose and especially the clinical context including the patient's life expectancy.^[19] In the German prospective trial of Wiegel *et al.*,^[20] The authors delivered a dose of 40 Gy in 20 fractions of 2 Gy and noted improvement or stabilization of symptoms in 86% of cases (an improvement in 50% and stabilization in 36% of cases). The best functional results were obtained for patients with breast cancer probably due to the use of chemotherapy after irradiation. Rosset *et al.*^[21] suggest a normofractionated dose of at least 35 Gy. However, in practice, hypofractionated regimens are preferred in routine clinical practice. In fact, they allow

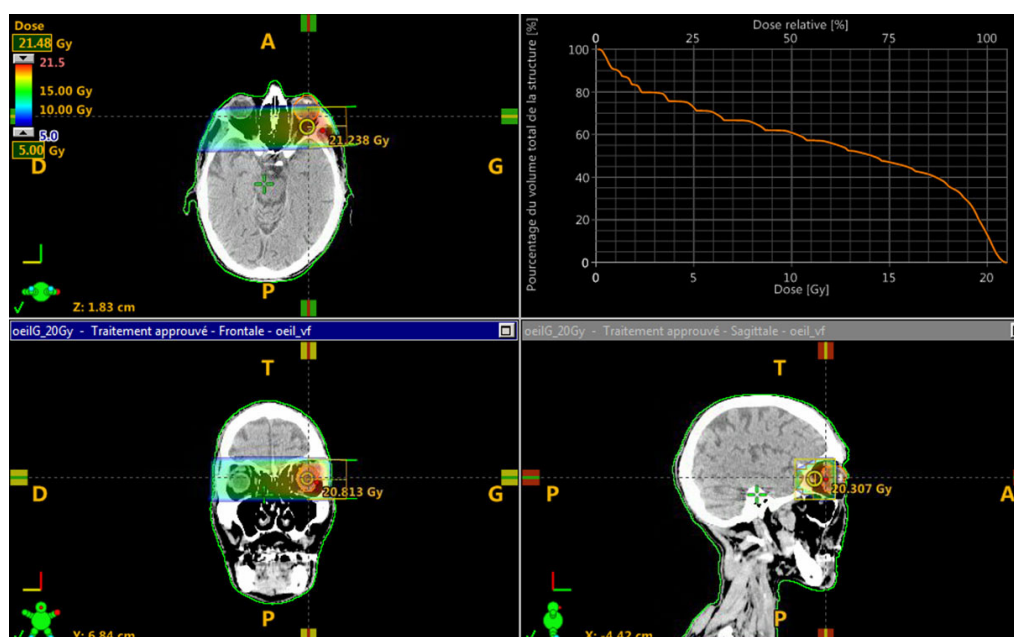


Figure 2: Conformal radiotherapy using two beams of 6 MV photons

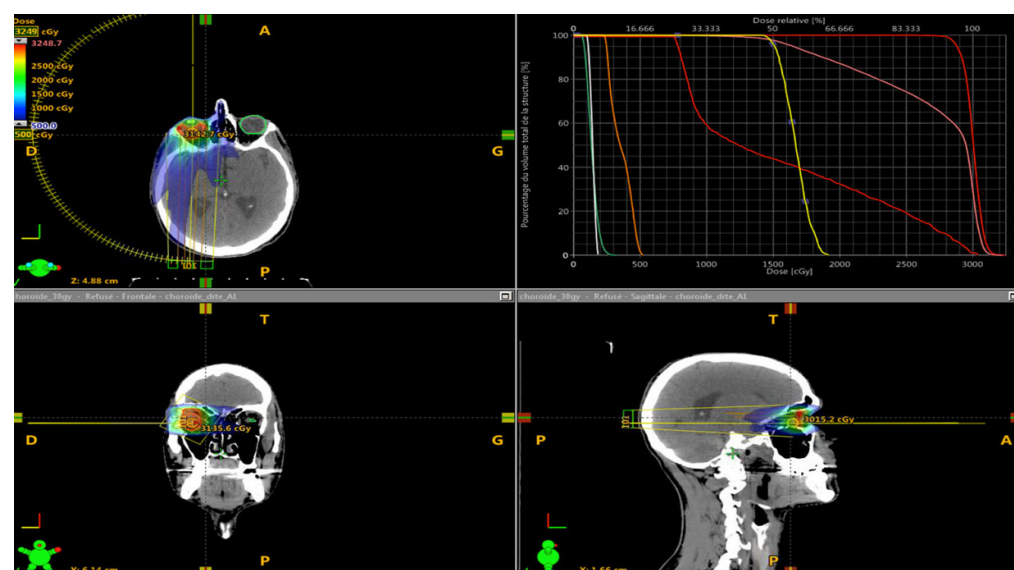


Figure 3: Volumetric modulated archtherapy using 6 MV photons

the patient to be treated quickly and, if necessary, reduce the time before resumption of any systemic treatment. The results of exclusive photon radiation therapy of the main cohorts published in the literature are reported in Table 3. According to the series,^[21] the rates of stabilization or improvement of symptoms vary from 57% to 100%. The results of our study are comparable.

Other therapeutic alternatives to conventional photon radiation therapy have been reported. Some teams have used stereotactic radiotherapy or proton therapy on series of patients with very limited numbers.^[22,23] The results functional results obtained do not appear to be superior in terms of local control of the disease compared to photonic radiotherapy for classical tumor sites (breast, lung). These techniques should be reserved for the most radioresistant tumors including melanomas.^[24-26]

Other local treatments such as plate brachytherapy,

dynamic phototherapy, laser or anti-angiogenic instillations are possible but external radiotherapy remains the oldest and proven technique in clinical routine.^[27,28]

In addition, systemic treatment alone with conventional chemotherapy or targeted therapy may have a local anti-tumor action on choroidal metastases.^[29] The optimal therapeutic attitude could be the sequential association of choroidal radiotherapy and systemic treatment.^[30,31]

In conclusion, radiotherapy is an effective treatment and low toxicity for the management of choroidal metastases. The hypofractionated regimens should be preferred in order to reduce the delay before the resumption of a possible systemic treatment which could also have a locoregional action on the choroidal metastases. This treatment fits perfectly into the palliative or curative management of oligo- or poly-metastatic disease.

Table 3: Results of external beam radiotherapy with photons

Authors	Effective	Primitif tumor (effective)	Doses (splitting)	Visual stabilisation or improvement
Burmeister <i>et al.</i> ^[23]	6	Breast (6)	21 to 27 Gy (3 to 3.4 Gy)	100%
Ratanatharathorn <i>et al.</i> ^[12]	19	Breast (19)	26 to 46 Gy (1.61 to 3 Gy)	100%
Nylén <i>et al.</i> ^[24]	17	Breast (14)		
		Lung (1)	20 to 45 Gy (2 to 4 Gy)	81%
		Others (2)		
		Lung (100)		
Röttinger <i>et al.</i> ^[11]	188	Breast (44)	30 to 40 Gy (2 to 3 Gy)	57%
		Others (44)		
		Breast (38)		
Rosset <i>et al.</i> ^[21]	58	Lung (10)	20 to 53 Gy (1.8 to 2 Gy)	81%
		Others (10)		
		Breast (31)		
Wiegel <i>et al.</i> ^[20]	50	Lung (13)	40 Gy (2 Gy)	86%
		Others (6)		
		Breast (88)		
d'Abbadie <i>et al.</i> ^[16]	123	Lung (11)	18 to 30 Gy (3 to 6 Gy)	68%
		Others (24)		
Demirci <i>et al.</i> ^[15]	129	Breast (129)	20 to 64 Gy (1.5 to 3 Gy)	82%
		Breast (11)		
Bajcsay <i>et al.</i> ^[25]	17	Lung (4)	42 to 51 Gy (np)	100%
		Others (2)		
		Lung (3)	30 Gy (3 Gy) or 20 Gy	
Bellmann <i>et al.</i> ^[13]	10	Breast (3)	SBRT (20 Gy)	100%
		Others (4)		
Amichetti <i>et al.</i> ^[14]	49	Breast (49)	16 to 60 Gy (1.8 to 3 Gy)	88%
Kreusel <i>et al.</i> ^[10]	18	Lung (18)	Unspecified	83%
		Breast (41)		
Konstantinidis <i>et al.</i> ^[22]	96	Lung (27)	Unspecified	94.30%
		Others (28)		

Authors' contributions

All authors have contributed to the realization of this work.

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Patient consent

The patient consent was obtained from the patients.

Ethics approval

Ethics approval was obtained prior to the commencement of the study.

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Report of primary leiomyosarcoma of renal pelvis and literature review

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ABSTRACT

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Primary sarcomas of kidney are exceptionally rare tumors, accounting for only 1-2% of all malignant tumors of kidney. Leiomyosarcoma (LMS) is the most common histological subtype among all renal sarcomas. The authors describe here a case of primary leiomyosarcoma of renal pelvis in a 50-year-old lady, presenting with flank pain. Based on triple phase cardio-electroencephalographic covariance tracing abdomen, presumptive diagnosis of renal cell carcinoma/renal sarcoma/neurogenic tumor was made and patient underwent radical nephrectomy. Microscopy reflected spindle cell tumor which showed strong positivity for desmin and smooth muscle actin with negative epithelial markers, thereby confirming the diagnosis of renal LMS. Owing to aggressive nature and low survival rates of LMS patient received adjuvant treatment in form of chemotherapy and radiotherapy. Patient is doing well 1 year post treatment.

INTRODUCTION

Primary sarcomas of kidney constitute 1-2% of all malignant tumors of kidney.^[1] LMS is most common histological subtype, accounting 60-70% of all sarcomas of kidney, commonly seen in females in 4th-6th decades.^[2] Radical nephrectomy remains the treatment of choice.^[3] The role of adjuvant treatment remains debatable due to paucity of data on treatment of this rare neoplasm. Herein, we report

a case of a 50-year-old lady with leiomyosarcoma originating in renal pelvis along with relevant review of literature.

CASE REPORT

A 50-year-old postmenopausal lady, known case of hypothyroidism on T Thyroxin for 3 years, presented with diffuse dull aching paroxysmal pain in left flank of 2 months duration. On physical examination PORT



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scar, due to laproscopic cholecystectomy done 2 years back was visualized. No mass palpable. Triple phase contrast-enhanced computed tomography (CECT) scan of whole abdomen revealed [Figure 1], well defined retroperitoneal mass in left para-aortic location with anterior displacement of left renal vein. Neurogenic origin/mesenchymal tumor. Patient planned for wide local excision of the mass, intra-operatively the mass was adherent to left renal vein and feeder vein, therefore decision taken to perform left nephrectomy along with adrenalectomy. Postoperatively, gross examination of the specimen reveals greyish white tumor measuring 8 cm × 4 cm × 2 cm, infiltration of the renal pelvis but not infiltrating into renal parenchyma. Renal sinuses, resected end of ureter and adrenal gland all were free of tumor. Microscopically tumor comprises of oval to spindle cells with moderate amount of cytoplasm and eosinophilic pleomorphic nuclei, tumor cells are arranged in interlacing fascicles and at places show cytoplasmic clearing, mitotic activity of 7-10/hpf, less than 50% necrosis, FNCLC grade 2. Immunopositive for desmin and smooth muscle actin (SMA), whereas immunonegative for Pan CK, CD-34, EMA, Bcl2, S-100, MIC-2. Overall features were suggestive of leiomyosarcoma [Figure 2]. Post operative CECT of chest, abdomen and pelvis were within normal limits. Patient received 6 cycles of adjuvant chemotherapy with single agent injection adriamycin 25 mg/m² day 1-3 followed by post operative radiotherapy to tumor

bed to a dose of 50 Gy/25#/5 weeks. Patient is doing well 1 year post treatment.

DISCUSSION

LMS are malignant neoplasm of smooth muscle origin. They are most commonly found in uterus, stomach, small intestine and retroperitoneum.^[4] LMS of renal origin are very rare and constitute only 0.12% of all malignant renal neoplasms.^[5] LMS of kidney was first described by Berry in 1919 but till date they have been reported only as case reports or as component

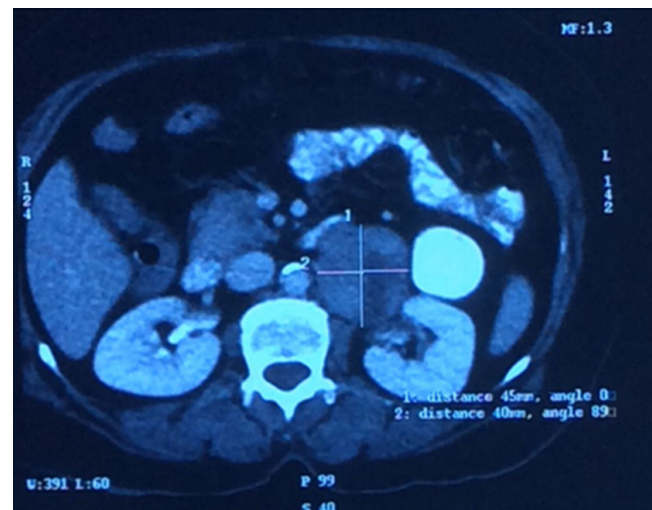


Figure 1: Contrast-enhanced computed tomography abdomen showing well defined retroperitoneal mass in left para-aortic location

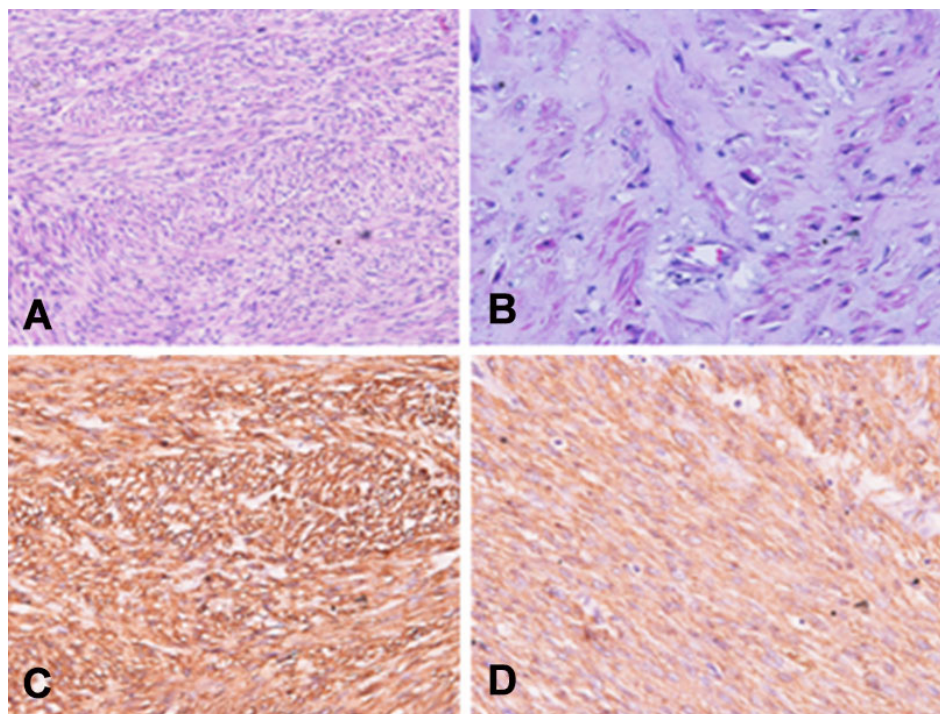


Figure 2: (A and B) Images comprising of oval to spindle tumor cells arranged in interlacing fascicles (A: 4x, B: 20x, HE); (C and D) images of desmin and smooth muscle actin immunopositivity, respectively (20x, HE)

Table 1: Summarizes clinic-radiological features, treatment and follow-up of previously reported cases of renal leiomyosarcoma in English literature in past 12 years

No.	Ref.	Year	Age (year)	Gender	Clinical symptoms	Side	CT diag	Size (cm ²)	Sx	Adj Rx	FU	LF	DF	Site of DF
1	[17]	2004	54	Male	Abd discomfort	Left	RCC	8 × 7.5	N	No	6 months	No	No	
2	[18]	2005	52	Female	Incidental	Left	-	2	PN	No	2 years	No	No	
3	[19]	2006	48	Female	Flank mass	Right	RCC	12.5 × 10.5	RN	No	Unknown	-	-	
4	[20]	2006	44	Male	Hematuria	Left	RCC	5 × 4	RN	No	3 years	No	No	
5	[21]	2007	42	Female	Incidental	Left	RCC	5 × 3	PN	No	Unknown	-	-	
6	[1]	2007	55	Female	Abd pain	Right	RCC	4 × 2	NSS	No	15 months	No	No	
7	[8]	2007	60	Male	Urinary frequency	Right	RCC	10 × 8	N	No	6 months	No	No	
8	[15]	2007	55	Male	Flank pain	Left	RCC	3 × 2.5	RN	Yes	6.5 years	No	No	
9	[6]	2009	42	Female	Flank pain	Right	RCC	15	RN	No	7 months	No	No	
10	[22]	2009	71	Male	Flank mass	Left	RCC	13 × 6.5	RN	Yes	7 months	No	No	
11	[23]	2009	65	Female	Flank mass	Right	RCC	15 × 11	RN	No	1 year	No	No	
12	[24]	2010	55	Female	Flank pain	Left	RCC	20 × 16	RN	No	Unknown	-	--	
13	[25]	2011	57	Female	Flank mass	Left	-	25 × 23	RN	No	3 years	No	No	
14	[26]	2011	65	Female	Flank pain	Right	-	13 × 11	RN	No	15 months	No	No	
15	[27]	2012	74	Female	Abd pain	Left	RCC	8 × 6	RN	No	1 month	No	No	
16	[28]	2013	70	Male	Flank pain	Right	RCC	4	LN	No	2 months	No	Yes	Bone
17	[29]	2013	69	Female	Flank mass	Right	RCC	18 × 15	RN	No	5 years	No	No	
18	[30]	2013	20	Male	Hematuria	Left	RCC	7 × 6	LN	No	2 months	No	Yes	Lung
19	[31]	2014	65	Female	Flank mass	Right	RCC	18 × 8	N	No	2 months	No	No	
20	[14]	2015	50	Female	Flank pain	Left	-	10 × 6	RN	No	1 year	No	No	
21	[32]	2015	39	Male	Flank mass	Left	RCC	16 × 14	RN	No	1 year	No	No	

Diag: diagnosis; Adj: adjuvant; Sx: surgery; Rx: treatment; FU: follow up; LF: local failure; DF: distant failure; Abd: abdomen; CT: computed tomography; RCC: renal cell carcinoma; N: nephrectomy; PN: partial nephrectomy; RN: radical nephrectomy; LN: laparoscopic nephrectomy; NSS: nephron sparing surgery

of larger series of renal sarcoma literature [Table 1].^[6] Histogenesis of renal LMS is believed to be from renal capsule or smooth muscle fibers in renal pelvis or from the renal vessels.^[7]

The renal LMS has preponderance in women, with women being twice more commonly affected than men and majority of patients presenting in 4th-6th decades of life.^[8] The cause of female preponderance is not fully known, but studies suggest that some malignancies are associated with genes located on X chromosome that escape X inactivation.^[9] Renal LMS is found to occur equally on both sides and also bilaterally, however the etiology remains obscure.^[10]

The patients of LMS usually presents with flank pain, hematuria and abdominal mass, thus mimicking renal cell carcinoma (RCC). It's difficult to differentiate between LMS and RCC. Ultrasound, tomography or magnetic resonance neither is able to differentiate between the two and diagnosis is usually made postoperatively.^[11] Because renal sarcoma is a rare condition; no effective treatment has yet been established. It is believed that surgery offers the best chance of cure therefore radical nephrectomy is treatment of choice, but recently partial nephrectomy has been shown to give good results in these patients.^[12]

Histologically, leiomyosarcoma has to be differentiated from sarcomatoid RCC, leiomyoma and angiomyolipoma. Leiomyosarcoma can be differentiated from leiomyoma by presence of mitosis and necrosis in malignant tumor although cellular pleomorphism can be seen in both. Renal angiomyolipoma shows fascicles of smooth muscle cells admixed with mature fat and thick walled blood vessels. Sarcomatoid RCC forms the closest differential diagnosis of renal LMS.^[13]

Diagnosis of sarcomatoid RCC can be made if typical RCC is seen somewhere in the tumor, so a thorough sampling of tumor is required to rule out any epithelial component of sarcomatoid RCC. Immunohistochemically tumor cells of leiomyosarcoma are positive for desmin, SMA calponin, H caldesmon and negative for CK, S-100 and HMB-45. The angiomyolipoma will show HMB-45 positivity while sarcomatoid variant of RCC will be CK positive.^[14]

To make diagnosis of primary renal sarcoma the following criteria should be met: (1) the patient must not have or have had sarcoma elsewhere to rule out metastasis; (2) gross must be compatible with origin in kidney rather than involvement due to retroperitoneal sarcoma; (3) sarcomatoid variant of RCC must be excluded.^[8]

Small size < 5 cm, low histological grade and renal limited disease are associated with more favorable outcomes. Histological grade of tumor is assigned based on mitotic count, necrosis and nuclear pleomorphism.^[8] The most important prognostic factor is tumor free resected margin. Large size and metastasis to adjacent organs at the time of diagnosis makes the prognosis poor.

Adjuvant treatment in form of chemotherapy and radiotherapy has been tried differently by different people. Sharma et al.^[15] prescribed chemotherapy with mesna, adriamycin, ifosfamide and dacarbazine regimen and sandwich radiotherapy with a dose of 44 Gy/22#/4.5 weeks to the renal bed and adjoining lymphatics. Beccia et al.^[16] prescribed vincristine (1.4 mg/m²) or cyclophosphamide (700 mg/m²) plus actinomycin D (0.04 mg/m²) to patients following surgery. As no randomized control trials have demonstrated their long term effects, treatment can be tailored individually. In our patient, we administered single agent doxorubicin (25 mg/m² day 1-3). Before administering treatment, we thoroughly explained our patient about potential benefits of adjuvant treatment, despite negative margin status and absence of metastasis. Our recommendations were based on high risk features like abdominal/retroperitoneal location, size greater than 5 cm, high grade histology and presence of necrosis.

To conclude, renal leiomyosarcoma is a rare tumor should be differentiated from sarcomatoid variant of renal cell carcinoma and angiomyolipoma. Radical nephrectomy is treatment of choice but in spite of successful resection it usually shows an unfavorable prognosis. Aggressive treatment with adjuvant chemotherapy and radiotherapy can offer better results. Paucity of cases and absence of long-term follow up with controlled randomized studies is hampering definitive treatment protocols.

Authors' contributions

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There are no conflicts of interest.

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Patient consent was obtained as per institutional policy.

Ethics approval

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Systemic humoral responses of non-muscle-invasive bladder cancer during BCG treatment: less is more

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multivariate analysis

Aim: Intravesical Bacille Calmette-Guérin (BCG) is the mainstay adjuvant treatment of non-muscle-invasive bladder cancer. However, one third of the patients on BCG regimen relapse within the first year of treatment. This study aimed at identifying biomarkers to predict response to BCG treatment. **Methods:** Gene expression was analyzed in blood cells of 58 patients treated with BCG through six consecutive weekly instillations and then at month 3, 6, 9, and 12. Cytokines tumor necrosis factor (TNF)- α , interleukin (IL)-10, interferon (IFN)- γ , IL-1 β , IL-2, IL-4, and IL-6; chemokines CCL2, CCL3, CCL8, CXCL9, and IP-10; and mediators of cytotoxicity CTLA4, Fas-L, Perf, GNLY, NOS2A, and HMOX-1 were analyzed before the 1st and the 6th week instillation and 24 h after to assess fast (within 24 h) and prolonged changes resulting from treatment. **Results:** BCG instillation led to fast-increased expression of *IL-1 β* , *TNF- α* , and *IL-10* genes. When compared to relapsing patients, patients with no relapses within one year showed significantly lower expression of IL-1 β at 1st week and less IFN- γ , HMOX-1, and GNLY at week 6. HMOX-1 and GNLY were independent predictive biomarkers, and values above the cut-off ≥ 110 and ≥ 13.0 % mRNA, respectively, were considered prejudicial factors. Patients with two HMOX-1 and GNLY factors had highest (66.7%) relapsing risk. **Conclusion:** Assessing immunomodulators' expression in blood allows the establishment of predictive cut-off values and identification of probabilities for patients' relapses after BCG treatment.



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INTRODUCTION

Non-muscle-invasive bladder cancer (NMIBC) has an increasing incidence and a high relapse rate, even after transurethral resection of the bladder tumor (TURBT), the initial standard treatment. Due to the frequent need for follow-up procedures, NMIBC significantly reduces quality of life and is one of the most expensive malignancies to treat. Post-TURBT intravesical instillation of Bacille Calmette-Guérin (BCG), the attenuate strain of *Mycobacterium bovis*, is the recommended treatment for intermediate and high-risk NMIBC,^[1] by preventing relapse and progression of NMIBC -- stages Ta, T1 and carcinoma *in situ*. While partially successful, 30% to 50% of the patients do not respond and experience relapse within the first year of BCG treatment. Of these patients, 15% develop tumor progression and form distant metastases.^[2]

Despite many efforts,^[3-5] there are currently no biomarkers that can predict patient's prognosis and that discriminate those who will respond to BCG treatment from those who would be best served by more aggressive therapy such as cystectomy or, alternatively, radio- and chemotherapy. BCG instillations can cause side effects ranging from inconvenient cystitis to sepsis and even death of patients in rare cases. This fact reinforces the need for selecting and accompanying patients who enroll in BCG immunotherapy.

Treatment with BCG was first used empirically by Morales in 1976.^[6] Today, it is well assumed that the efficacy of BCG is based on a massive, complex, and ongoing local immune activation. Hence, a healthy host immune system is a prerequisite to successful BCG therapy.^[1,7,8] Within bladder tissue, the leukocyte distribution before and after BCG therapy is remarkably different, and the induction of complex inflammatory cascade events, reflecting activation of multiple types of immune cells, is well known.^[9,10] The cascade of events results in secretion of an array of cytokines, chemokines, and cytotoxicity mediators that can be detected in tissue and in urine.^[7,11] These are summarized in Table 1. Examples are (1) pro-inflammatory, T helper type 1 (Th1) cytokines such as interleukin (IL)-1 β , IL-2, IL-6, tumor necrosis factor (TNF)- α , interferon (IFN)- γ ; (2) Th2 cytokines IL-4 and IL-10; (3) chemokines, as CCL2, CCL8, CCL3, IFN- γ -inducible protein (IP-10), CXCL9; (4) cytotoxicity mediators, such as nitric oxide (NO) released by macrophages, and mediators of cytotoxic response such as perforin (Perf), granzysin (GNLY), and Fas ligand (Fas-L) pathway released by cytolytic lymphocytes [natural killer (NK) and T

cytotoxic cells], and cytotoxic T lymphocyte antigen-4 (CTLA4) expressed by activated/exhausted T cell; and (5) molecules involved in stress homeostatic events such as heme oxygenase 1 (HMOX-1). The tight balance of such mediators is critical for the efficacy of BCG immunotherapy. Despite this knowledge, the precise immune mechanism involved in BCG therapy is still not completely clarified. Assessing immune response is thus fundamental to an understanding of BCG-induced antitumor mechanism and its prognostic value.

Interestingly, in many bacterial infections, analysis of immunomodulators detected in peripheral blood cells is extremely relevant to following a patient's immune response.^[12] Blood is the predominant sample substrate for systemic analysis, and it allows depiction of the overall inflammation burden that accompanies infection.

Significant changes in the number and function of peripheral blood cells have been reported over the course of BCG therapy, such as the increase in BCG-activated killer (BAK) cells.^[8] Few attempts have been made to assess the relevance of the systemic cytokine profile in peripheral blood cells.^[13,14] However, the expression of cytokines and other BCG-induced mediators that are systemically detected over the course of BCG instillation has never been properly addressed. In this work, we sought to profile the expression of key molecules expressed by blood cells of NMIBC patients during BCG treatment. We analyzed the expression of cytokines TNF- α ; IL-10; IFN- γ , IL-1 β , IL-2, IL-4 and IL-6; chemokines CCL2, CCL3, CCL8, CXCL9 and IP-10; mediators of cytotoxic response CTLA4, Fas-L, Perf, GNLY and NOS2A; and HMOX-1, which is involved in homeostatic events. We established predictive cut-off values and developed a predictive grouping system that allows us to identify the most likely patients to relapse after BCG treatment.

METHODS

Patients

From July 2005 until July 2007, 58 patients [mean age of 67.8 years (range 45 to 82)] were assisted at the Hospital São José, Lisbon, Portugal and diagnosed with NMIBC. Patients were BCG naïve and with high-risk tumors (T1 tumor, G3** [HG] tumor), CIS, multiple and recurrent and large (> 3 cm) Ta, G1G2 tumors, and they were treated with the same dose of BCG instillations (TICE® BCG) after TURBT. They entered this study after informed consent. BCG instillations were repeated once a week for 6 weeks

Table 1: List of molecules analyzed in this study

Name	Function	Expression in bladder cancer (methods)
IL-1 β	Important mediator of inflammatory response, involved in a variety of cellular activities, such as cell proliferation, differentiation, and apoptosis. Contributes to inflammatory pain hypersensitivity	Tissue (immunohistochemistry) ^[31]
IL-2	Necessary for T cell growth and function. Stimulates the production of IL-2 receptors on the surface of other immune effector cells, such as macrophages and B cells as part of the immune response	Present in urine during BCG immunotherapy ^[32] tissue (immunohistochemistry) ^[31]
IL-4	Important role in regulating antibody production, hematopoiesis and inflammation, and the development of effector T cell responses. Moreover, promotes tumor development by increasing tumor cell resistance to apoptosis	Tissue (immunohistochemistry) ^[31] bladder cancer cell lines ^[33] reduced function-polymorphism is associated with recurrence ^[7]
IL-6	Involved in the acute phase response, T cell proliferation, B cell maturation, macrophage maturation, and cytotoxic T-cell differentiation. Furthermore, contributes to proliferation of cytotoxic NK cells and promotes the differentiation of CD4 T cells into Th2 effector cells and it inhibits Th1 differentiation	Present in urine during BCG immunotherapy ^[32,34] bladder cancer cell lines ^[35]
IL-10	Pleiotropic effects in immunoregulation and inflammation, down-regulates the expression of Th1 cytokines, MHC class II presented antigens, and costimulatory molecules on APCs. It enhances B cell survival, proliferation, and antibody production	inhibitory role in BCG-induced macrophage cytotoxicity ^[36]
IFN- γ	Critical in innate and adaptive immune responses, with immunostimulatory and immunomodulatory effects, especially against viral and intracellular bacteria and participates in tumor control. Promotes Th1 responses, namely activating macrophages	Bladder cancer cell line ^[37]
TNF- α	Involved in systemic inflammation and stimulates the acute phase reaction. Regulation of immune cells. Endogenous pyrogen, able to induce fever, apoptotic cell death, sepsis, cachexia, inflammation, and inhibit tumorigenesis and viral replication	Present in urine ^[32,38] (immunohistochemistry) ^[31] bladder cancer cell line ^[37] reduced function-polymorphism is associated with recurrence ^[4]
CCL2	Known as monocyte chemoattractant protein 1. Displays chemotactic activity for monocytes and basophils. Implicated in the pathogenesis of diseases characterized by monocytic infiltrates, like psoriasis, rheumatoid arthritis, and atherosclerosis	Tissue (RT-PCR) ^[7]
CCL3	Known as macrophage inflammatory protein 1 alpha plays a role in acute inflammatory responses	Tissue (RT-PCR) ^[7]
CCL8	Known as monocyte chemoattractant protein 2. Chemoattractant which contributes to the local activation of many different immune cells, including mast cells, eosinophils, basophils, monocytes, T cells, and NK cells involved in the inflammatory response. Contribute to tumor-associated leukocyte infiltration	Tissue (RT-PCR) ^[7]
IP-10	Chemokine CXCL10. Secreted by several cell types in response to IFN- γ . Several roles, such as chemoattraction for monocytes/macrophages, T cells, NK cells, and dendritic cells, promotion of T cell adhesion to endothelial cells, antitumor activity, and inhibition of bone marrow colony formation and angiogenesis	Tissue (RT-PCR) ^[7] bladder cancer cell line ^[37]
CXCL9	Known as monokine induced by IFN- γ (MIG). T cell chemoattractant, which is induced by IFN- γ	Tissue (RT-PCR) ^[7]
Fas-L	Key effector molecule in cell-mediated cytotoxicity	BAK, LAK cells and PBCs stimulated with BCG or IL-2 (FACS analysis) ^[28]
GNLY	Present in cytotoxic granules of cytotoxic T cells and natural killer cells, with cytolytic and pro-inflammatory functions	
Perf	Key effector molecule in cell-mediated cytotoxicity	BAK, LAK cells, PBCs stimulated with BCG or IL2 (FACS analysis) ^[28]
NOS2A	It is the nitric oxide synthase inducible by certain cytokines. The formed product, nitric oxide, is a reactive free radical which acts as a biologic mediator in several processes, including neurotransmission and antimicrobial and antitumoral activities	Polymorphisms ^[39]
HMOX-1	Essential in heme catabolism. Highly expressed in various solid tumors, with an important role in rapid tumor growth	NMIBC tissue specimens (RT-PCR) ^[27]
CTLA4	Expressed on the surface of helper and cytotoxic T cells and transmits an inhibitory signal. Functions as an immune checkpoint, downregulating the immune system	

BCG: Bacille Calmette-Guérin; RT-PCR: real time polymerase chain reaction; BAK: BCG-activated killer; LAK: lymphokine-activated killer; PBC: peripheral blood cells; FACS: fluorescence-activated cell sorting; NMIBC: non-muscle-invasive bladder cancer; IL: interleukin; IFN: interferon; TNF: tumor necrosis factor; IP-10: IFN- γ -inducible protein; Fas-L: Fas ligand; GNLY: granulysin; Perf: perforin; HMOX-1: heme oxygenase 1; CTLA4: cytotoxic T lymphocyte antigen-4

followed by three weekly instillations at month 3, 6, 9, and 12. All the patients were followed for one year and the outcome variables were: tumor progression, relapse, and disease-specific survival. Patients who showed no relapses within a year after the beginning of treatment were named “BCG responders” [40 patients (3 women/37 men, mean age of 68, 3 years)]. Those patients who manifested relapses within that year were named “BCG-relapsing” [18 patients (1 woman/17 men, mean age of 67.5 years)]. In each case, four samples were collected before and after the first instillation and the week 6 instillation. The study has been approved by the ethical committee of the Hospital.

Isolation of RNA and real-time PCR

Blood specimens (2.5 mL) were collected in PAXgene™ tubes (Qiagen, Manchester, UK), incubated at room temperature for 4 h for RNA stabilization and then stored at -80 °C. RNA was extracted from whole blood using PAXgene™ Blood RNA System Kit (Qiagen), following manufacturer's instructions, and further purification of RNA was done with on-column DNase digestion. RNA concentrations and A_{260} -to- A_{280} ratios were measured in a spectrophotometer, and only samples with A_{260}/A_{280} ratios between 1.9 and 2.1 were further considered. 1 µg of total RNA was reverse transcribed with random primers, using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA).

Real time PCR gene quantification analysis was performed as described,^[7,15] using Taqman assays from Applied Biosystems: Hs00234140_m1 (*CCL2*), Hs00234142_m1 (*CCL3*), Hs00271615_m1 (*CCL8*), Hs00171065_m1 (*CXCL9*), Hs00171042_m1 (*IP-10*), Hs00175480_m1 (*CTLA4*), Hs00181225_m1 (*Fas-L*), Hs01110250_m1 (*HMOX-1*), Hs00167257_m1 (*NOS2A*), Hs00169473_m1 (*Perf*), Hs00246266_m1 (*GNLY*), Hs00174128_m1 (*TNF-α*); Hs00174086_m1 (*IL-10*); Hs00174143_m1 (*IFN-γ*), Hs00174097_m1 (*IL-1β*), Hs00174114_m1 (*IL-2*), Hs00174122_m1 (*IL-4*), Hs00174131_m1 (*IL-6*), and 4352935E (*β-actin*). Each reaction was performed in duplicate. All genes, including endogenous controls, were always analyzed in the same run to exclude between-run variations. The number of PCR cycles needed to reach fluorescence threshold in each sample was defined as the cycle threshold (Ct). The mRNA expression was normalized using *β-actin* gene expression as a reference according to our previous observations.^[15] Relative mRNA levels were calculated using formula $2^{-\Delta Ct} \times 1,000$ which infers the number of mRNA molecules of the gene of interest per 1000 molecules of endogenous control. ΔCt stands for the difference between cycle threshold

(Ct) of the amplification curve of target gene and that of endogenous controls. Reactions with a Ct value higher than 35 cycles were considered negligible and were not considered further. Efficiency of the amplification reaction for each primer-probe was above 95% (as determined by manufacturer).

Statistical analysis

The Kruskal Wallis test was used to compare ratios in patients with and without recurrence. Fisher's exact test was used to identify the factors that were significant in the univariate analysis. Multivariate logistic regression was used to identify the two factors that were retained in the multivariate analysis. A significance level of $P = 0.05$ was used to identify possible factors for predicting response to BCG therapy in the univariate analyses. No correction was made for multiple endpoints or testing.

RESULTS

BCG treatment induces significant systemic fast changes

To assess whether BCG treatment induced significant changes in the expression of specific cytokines and molecular effectors in peripheral blood cells, we analyzed its expression in blood samples from patients during BCG therapy. The selected cytokines and molecular effectors depicted in Table 1 represent the reported complex events known to locally accompany BCG treatment, i.e. to occur in the bladder. Blood samples were collected before and 24 h after BCG treatment to assess fast changes. To assess prolonged changes, samples were collected at the first instillation (week 1) and at week 6 of BCG treatment. Significant levels of mRNA were obtained from all blood samples. Before BCG treatment (pre-BCG stage), the lowest expression levels were observed for *IL-4* (0.024‰ mRNA) and maximum for *GNLY* (252.38‰ mRNA) [Figure 1]. During BCG treatment, we observed significant fast changes (24 h after the first treatment or after the instillation performed at 6th week) in *IL-1β*, *TNF-α*, *IL-10*, *GNLY*, and *Perf* [Figure 2]. No significant prolonged changes, i.e. during treatment, were observed [Figure 2]. In fact, 24 h after the first BCG instillation, significant expression changes were observed. *IL-1β* mRNA increased from 12.94‰ before treatment to 16.48‰ after treatment ($P = 0.033$), *TNF-α* mRNA increased from 3.70‰ to 4.23‰ ($P = 0.002$), and *IL-10* mRNA increased from 0.17‰ to 0.24‰ ($P = 0.026$) [Figure 2]. At week 6, changes were still observed and were sometimes more pronounced [Figure 2]. In fact, *IL-1β* mRNA increased from 11.46‰ to 20.76‰ ($P = 0.0001$), *TNF-α* mRNA increased from 3.12‰ to 4.57‰ ($P = 0.0045$), and *IL-10* increased

Table 2: Correlation values between fold changes in the transcripts observed within 24 h after Bacille Calmette-Guérin instillation

	1W IL-1 β	6W IL-1 β	1W IL-1 β	6W IL-1 β	1W IL-2	6W IL-2	1W IL-2	6W IL-2	1W IL-6	6W IL-6	1W IFN- γ	6W IFN- γ	1W TNF- α	6W TNF- α	1W Fas-L	6W Fas-L	1W GNLY	6W GNLY
1W IL-1 β	1																	
6W IL-1 β	-0.08	1																
1W IL-2	0.10	-0.03	1															
6W IL-2	0.21	-0.05	0.31	1														
1W IL-6	0.25	-0.02	0.14	0.70	1													
6W IL-6	0.01	0.75	0.88	0.24	1													
1W IFN- γ	0.15	-0.09	0.31	0.77	0.66	1												
6W IFN- γ	0.85	0.13	0.44	0.50	0.43	0.46	1											
1W TNF- α	0.74	0.05	0.29	0.55	0.82	0.38	0.59	1										
6W TNF- α	0.07	-0.16	0.58	0.38	0.32	0.74	0.39	0.70	0.38	1								
1W Fas-L	0.32	-0.12	0.34	0.56	0.75	0.47	0.72	0.51	0.76	0.48	1							
6W Fas-L	0.12	-0.19	0.52	0.24	0.21	0.68	0.31	0.64	0.27	0.92	0.40	1						
1W GNLY	0.22	0.70	0.26	0.42	0.85	0.44	0.53	0.53	0.70	0.53	0.84	0.44	1					
6W GNLY	0.12	-0.19	0.52	0.24	0.21	0.68	0.31	0.64	0.27	0.92	0.40	0.44	1					

Fold changes in the transcripts observed within 24 h represent the ratio between relative mRNA levels obtained after treatment in relation to those obtained before treatment. 1W: first instillation; 6W: instillation at week 6. Other molecules included in this study that did not show significant correlation are not shown. Correlation whose P values was less or equal than 0.05 is represented in bold. IL: interleukin; IFN: interferon; TNF: tumor necrosis factor; Fas-L: Fas ligand; GNLY: granulysin

from 0.13‰ to 0.23‰ ($P = 0.0004$). In addition, other significant changes were observed at week 6 of the treatment, such as decreased *GNLY* (159.2‰ to 111.9‰, $P = 0.008$) and *Perf* (49.74‰ to 43.26‰, $P = 0.01$) [Figure 2].

The observed fast changes in transcripts showed striking correlations [Table 2]. For example, there was positive correlation between pro-inflammatory cytokines: e.g. *IL-1 β* correlated with *TNF- α* ($P = 0.85$ or 0.74 at 1st or 6th week treatment). There was also correlation between cytotoxic mediators: e.g. *Fas-L* correlated with *GNLY* ($P = 0.92$ or 0.84 at 1st or 6th week treatment). Finally, correlation between pro-inflammatory cytokines and cytotoxic mediators was also observed: e.g. *IL-1 β* and *IL-6* correlated with *GNLY* cytotoxic mediator ($P = 0.70$ and $P = 0.85$, respectively). Overall, data suggested the involvement of pro-inflammatory and cytotoxic mechanisms in patient's response to BCG therapy.

Systemic molecular changes differ between BCG responders and relapsing patients

We then stratified our study population according to their response to BCG treatment. We considered two groups: patients who showed no relapses within a year after beginning treatment, i.e. "BCG responders" and patients who manifested relapses within that year, i.e. "BCG-relapsing." Stratification between BCG-responders and relapsing patients revealed interesting differences at week 6. After treatment, BCG responders showed significantly less expression of *IL-1 β* (18.54‰) than relapsing patients (25.61‰, $P = 0.018$) [Figure 3]. At week 6, before BCG treatment, there was significantly less expression in BCG responders of the following transcripts: *IFN- γ* (0.22‰/0.66‰ in responders/relapsing, $P = 0.04$); *HMOX-1* (10.80‰/15.81‰, $P = 0.006$), and *GNLY* (165.78‰/254.34‰, $P = 0.01$) [Figure 3]. Data suggest that the best response to BCG treatment involves the moderate contribution of specific immunomodulatory molecules.

Multivariate analysis revealed cut-off values for BCG response

To better discriminate BCG responders from relapsing patients, we used logistic regression analysis to establish cut-off values for each of the molecules identified above. We performed a univariate and a multivariate Cox analysis, whose results are represented in Table 3. In the univariate analysis, the variables that better distinguished responder vs. relapsing patients were *IL-1 β* , *IFN- γ* , *HMOX-1*, and *GNLY*. Indeed, we could define a cut-off value for mRNA levels, below which it predicted a good BCG

Table 3: Cut-off values of the relevant molecules with a predictive meaning for patients’ response to BCG. Multivariate logistic regression was used to identify the two factors retained in the multivariate analysis

		Good factor	Prejudicial factor
Univariate analysis	IL-1β at 6WAT	LE 12.5	GT 12.5
	IFN-γ at 6WBT	LE 0.17	GT 0.17
	GNLY at 6WBT	LE 110.0	GT 110.0
	HMOX-1 at 6WBT	LE 13.0	GT 13.0
Multivariate analysis	GNLY at 6WBT	LE 110.0	GT 110.0
	HMOX-1 at 6WBT	LE 13.0	GT 13.0

Values are relative mRNA molecules calculated by formula $2^{-\Delta Ct} \times 1,000$, which infers the number of mRNA molecules of each gene per 1,000 molecules of the endogenous control (β -actin). The designation of “good factor” and “prejudicial factor” is correlated with patients’ response to BCG, namely no relapse or relapse in less than one year after treatment. BCG: Bacille Calmette-Guérin; 6WAT: sample collected 24 h after BCG instillation at week 6; 6WBT: sample collected before BCG instillation at week 6; LE: less than or equal to; GT: greater than

response ($\leq 12.5\%$ to IL-1β; $\leq 0.17\%$ to IFN-γ, $\leq 110\%$ to GNLY, and $\leq 13.0\%$ to HMOX-1).

In the multivariate analysis, only HMOX-1 and GNLY were shown to be independent predictive biomarkers. As shown in Table 3, we established a cut-off, above which these biomarkers are considered prejudicial factors. We then subdivided patients according to whether they show no prejudicial factors, one prejudicial factor, or two prejudicial factors (see supplementary data). One in twelve patients with no prejudicial factors relapsed (8.3%). Four in twenty-three patients showing one prejudicial factor relapsed (17.4%) and finally, ten of fifteen patients showing two prejudicial factors relapsed (66.7%). This allowed us to establish predictive cut-off values and a predictive grouping system, thus identifying the probability of relapse after BCG treatment.

DISCUSSION

Immunotherapies boost patient’s immune response to improve its capacity to eliminate tumor. BCG is an immunotherapy, used as a standard of care to treat NMIBC patients to reduce cancer relapses. BCG instillations into the bladder attract antitumor effector immune cells to the tumor site,^[8,9] and three months after the BCG treatment course, the cellular infiltrate of T and B cells is concentrated in the persisting granulomas, focused on elimination of cancer cells.^[16] However, BCG treatment remains suboptimal because 30% to 50% of the patients show no response and/or relapse within the first year of treatment.^[17]

Although the underlying mechanisms of BCG therapy are not fully elucidated, it is known that a Th1 response is required to stimulate cell-mediated tumoricidal activity.^[18] The level of the Th1 cytokine, IL-2, expressed

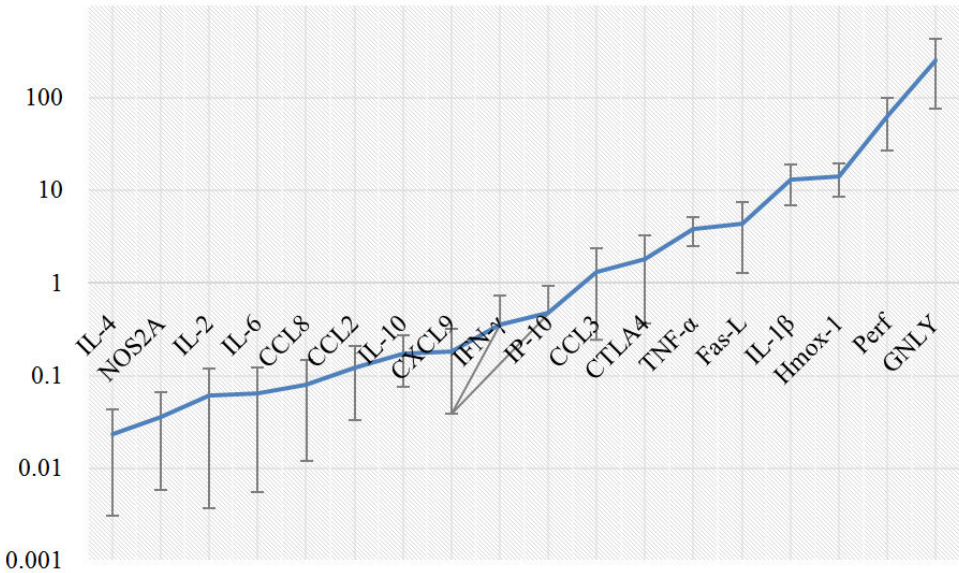


Figure 1: All the analyzed genes were significantly expressed at pre-BCG state, with the lowest mRNA levels observed for IL-4 and maximum for GNLY. Relative mRNA levels of IL-4, NOS2A, IL-2, IL-6, CCL8, CCL2, IL-10, CXCL9, IFN-γ, IP-10, CCL3, CTLA4, TNF-α, Fas-L, IL-1β, HMOX-1, Perf and GNLY were evaluated by real time PCR, as described in the Methods section. mRNA was obtained from blood samples of 58 patients, collected before any BCG treatment. Values were calculated, as referred to in the Methods section, by formula $2^{-\Delta Ct} \times 1,000$ and infers the number of mRNA molecules of a certain gene per 1,000 molecules of the endogenous control (β -actin). BCG: Bacille Calmette-Guérin; PCR: polymerase chain reaction

in situ can suggest patients at risk for bladder cancer relapses after a single course of BCG.^[19] Yet, despite efforts to analyze the immune response in bladder tissue before and after BCG therapy,^[4,7-9] there are currently no gold standard biomarkers to predict how effective or beneficial is the BCG-induced antitumor immune response. This knowledge is important to identify patients who may require more aggressive treatments such as cystectomy.^[20]

A mode of predicting response to BCG therapy has

been suggested through the analysis of IL-2 and IFN- γ mRNA in peripheral blood mononuclear cells during BCG treatment. IL-2 mRNA is increased in patients who responded with remission.^[14]

In this study, we have extended this methodology and analyzed the profile of several key molecules expressed by blood cells in NMIBC patients treated with BCG. Expression was analyzed before and after (24 h interval) the 1st and the 6th week instillation, to assess fast (within 24 h) and prolonged changes

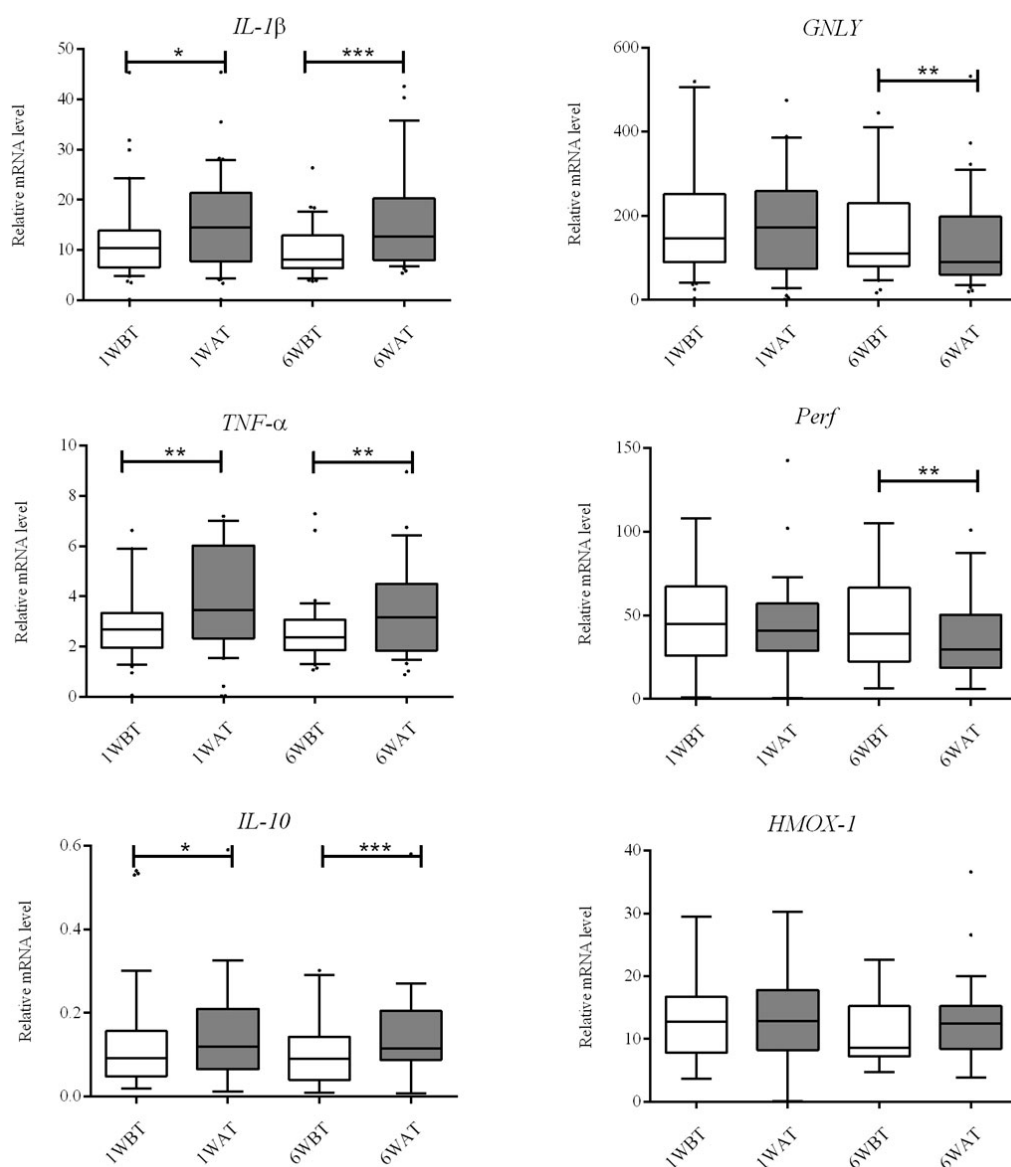


Figure 2: Expression of *IL-1 β* , *TNF- α* , *IL-10*, *GNLY* and *Perf* genes was significantly altered in the study population after BCG instillation. The relative mRNA levels of *IL-1 β* , *TNF- α* , *IL-10*, *GNLY*, and *Perf* were evaluated by real time PCR, as described in the Methods section. mRNA was obtained from blood samples of 58 patients, collected before (1WBT) and 24 h after (1WAT) the BCG treatment performed 1 week after TURBT and/or before (6WBT) and 24 h after (6WAT) the BCG treatment performed 6 weeks after TURBT. Values were calculated by formula $2^{-\Delta Ct} \times 1,000$ and infer the number of mRNA molecules of a certain gene per 1,000 molecules of the endogenous control (β -actin). Statistical significances (*P < 0.05, **P < 0.001 and ***P < 0.0001) refer to the differences between samples collected before (1WBT or 6WBT) and 24 h after (1WAT or 6WAT) BCG instillation. When not labelled, no significant differences were identified. BCG: Bacille Calmette-Guérin; PCR: polymerase chain reaction; TURBT: transurethral resection of the bladder tumor

during treatment.

During BCG treatment, significant fast changes were the expression of IL-1 β , TNF- α and IL-10, which increased at the 1st and 6th week, while the expression of GNLY and Perf decreased fast at 6th week. Correlations found between the fast changes of transcripts coding several pro-inflammatory cytokines and cytotoxic mediators further demonstrated that BCG promptly affects the systemic profile of mediators involved in both inflammatory and cytotoxic mechanisms. This coincides with previous studies demonstrating that BCG instillation influenced local immunological activity and a systemic immune response through both sorts of factors.^[21] For instance, a higher cytotoxic activity in the PBMCs after BCG instillation has been correlated to the appearance of IL-2 and IFN- γ in the serum.^[21]

Interestingly, our data showed that although the expression of IL-1 β was higher after BCG instillation, there was a significantly lower expression of this potent pro-inflammatory cytokine in BCG-responders than in

relapsing patients [Figure 3]. The increase in IL-1 β is an expected, normal physiological inflammatory response to BCG treatment that boosts immune response in bladder tissue. However, it is well known that prolonged exposure to inflammatory cytokines has also the potential to stimulate tumor growth through the promotion of proliferation, angiogenesis, DNA damage (due to their capacity to generate reactive oxygen and nitrogen species), and other events favorable to metastasis.^[22] In fact, high levels of cytokines induce reactive oxygen and nitrogen species.^[23] This may explain why patients with an excessive elevation of systemic expression of IL-1 β are more likely to relapse than patients who moderately express this cytokine.

In addition, it was observed that the basal expression profile of the immunomodulators also influences the response to BCG treatment. Indeed, patients who were considered BCG-responders, when compared with relapsing patients, exhibited significantly less expression of IFN- γ , HMOX-1 and GNLY immediately before the treatment at week 6 [Figure 3]. These data

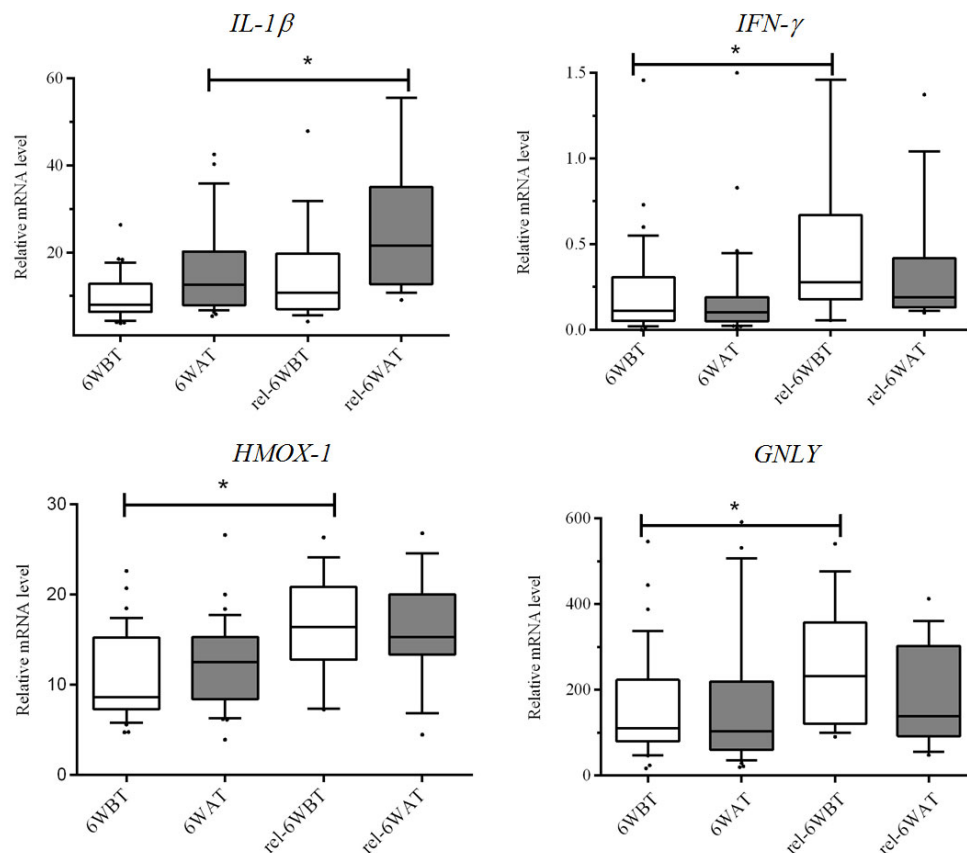


Figure 3: Expression of IL-1 β , IFN- γ , HMOX-1 and GNLY genes was significantly different between BCG responders and relapsing patients at week 6. Relative mRNA levels of IL-1 β , IFN- γ , HMOX-1, and GNLY were evaluated by real time PCR, as described in the Methods section. mRNA was obtained from blood samples of BCG responder and relapsing (rel-) patients, collected before (6WBT) and 24 h after (6WAT) BCG treatment performed 6 weeks after TURBT. Values were calculated by formula $2^{-\Delta C_t} \times 1,000$ and infer the number of mRNA molecules of a certain gene per 1,000 molecules of the average of the endogenous control (β -actin). Statistical significances (* $P < 0.05$) refer to differences between samples from BCG responders and relapsing patients collected before (6WBT) or 24 h after (6WAT) BCG 6 weeks treatment. BCG: Bacille Calmette-Guérin; PCR: polymerase chain reaction; TURBT: transurethral resection of the bladder tumor

agree with the idea that specific immunomodulatory molecules are involved in the success of BCG-treatment, but in moderate levels. Patients with relapsing tumors may be genetically prone to develop exacerbated chronic inflammation or cytotoxic responses during BCG treatment or during the disease itself.^[24] This exacerbated inflammation may add to the burden of the disease. For instance, IFN- γ is an essential component of cell-mediated immunity, and in BCG therapy it has an inhibitory effect on bladder cancer cells.^[25] However, depending on signalling intensity and microenvironmental factors, this cytokine can drive novel cellular and molecular inflammatory mechanisms that may underlie tumor initiation, immuno-evasion, and progression.^[26] Likewise, HMOX-1 catalyzes the degradation of heme and is considered a key enzyme to protect cells from stress and to regulate cell growth and proliferation. However, increased levels of HMOX-1 expression and activity were observed in various tumor tissues. The knockdown of HMOX-1 suppresses the growth of bladder cancer cells, and HMOX-1 expression was even proposed as an independent predictor of NMIBC recurrence and progression.^[27] This agrees with our data, showing that patients with lower levels of HMOX-1 before treatment are likely to respond better to BCG. On the other hand, granulysin is a cytolytic and pro-inflammatory molecule expressed by activated cytotoxic T cells and NK cells,^[28] necessary to kill target cancer cells. However, it has been described that granulysin kills bacteria^[29] and therefore it may lessen the efficacy of BCG therapy.

Our results point to the fact that the systemic expression of molecules that promote inflammation is involved in BCG host response. However, since inflammatory response shares various molecular targets and signaling pathways with the carcinogenic process, a good BCG response occurs only within limited levels of these molecules. Taking this into consideration, we define cut-off values for mRNA levels of IL-1 β , IFN- γ , HMOX-1, and GNLY, beneath which a good response to BCG is predicted. This allowed us to discriminate BCG responders from relapsing patients.

Univariate and multivariate analysis showed that IL-1 β , IFN- γ , HMOX-1, and GNLY expression levels could reliably predict responder vs. relapsing patients. Based on our data, we established cut-off values above which these biomarkers are considered a prejudicial factor. We subdivided patients into whether they showed no prejudicial factors, one prejudicial factor, or two prejudicial factors (see supplementary data). We also established a predictive grouping system to identify the probability that patients would

experience relapse after BCG treatment and to help design more personalized immune-based strategies. For instance, a promising avenue of clinical research in bladder cancer is the use of immune checkpoint inhibitors that target molecules involved in the balance and regulation of immune response, hence inducing proper T-cell anti-cancer response.^[30]

In conclusion, the analysis of mRNA expression of PBMCs by real-time PCR was a relatively simple, accurate technique for assessing the expression profile of several key immunomodulators in patients with NMIBC, as compared with classical histopathological evaluations and even with urine detection because not all patients have urine detectable levels.

Our results revealed that molecules with immunomodulatory roles, such as IL-1 β , IFN- γ , HMOX-1, and GNLY, have a role in BCG therapy but only at certain amounts, above which they appear to contribute to poor response to treatment. Thus, we established a cut-off value for mRNA levels of each molecule and propose the use of this information to predict which patients with NMIBC will be good BCG-responders, which patients will relapse when undergoing BCG treatment, and who therefore would benefit from alternative treatment strategies.

Authors' contributions

Conceived and designed the study: H. Trindade, D. Ligeiro, F.M. Calais da Silva, F.E. Calais da Silva
Performed literature search and prepared manuscript: P.A. Videira, M.G. Cabral
Acquired clinical data: F.M. Calais da Silva, F.E. Calais da Silva
Performed the experimental study and data acquisition: D. Ligeiro
Analyzed data and prepared presentation of manuscript: P.A. Videira
Performed statistical analysis: R. Sylvester
Revised the manuscript: F.M. Calais da Silva, P.A. Videira, D. Ligeiro, M.G. Cabral, R. Sylvester, F.E. Calais da Silva, H. Trindade

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Patients entered this study after written informed consent and were analyzed anonymously.

Ethics approval

The study has been approved by the ethical committee of the Hospital. All procedures followed the universal bioethical principles (Universal Declaration on Bioethics and Human Rights of UNESCO, 19 October 2005; The Charter of Fundamental rights of the EU, 2000; Ethical principles for medical research involving human subjects - Declaration of Helsinki (2008) with 2013 amendments.

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Role of adenosine in tumor progression: focus on A_{2B} receptor as potential therapeutic target

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ABSTRACT

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Adenosine receptors are a family of G-coupled receptors which mediate the anti-inflammatory and immune-suppressive effects of adenosine in a damaged tissue. A large number of evidence indicate that the accumulation of adenosine under hypoxic conditions favors tumor progression, helping cancer cells to evade immune responses. Tumor cells and/or lymphoid and myeloid cells can express the adenosine-generating enzyme CD73 and/or A_{2A} receptor, which in turn strongly suppresses an effective T-cell-mediated response, while promotes the activity of suppressive cells such as Treg and myeloid-derived suppressor cells. CD73 inhibitors and A_{2A} antagonists, either as single agents, or in combination with immune-checkpoints inhibitors such as anti PD-1 monoclonal antibodies, are currently in Phase I clinical trial in cancer patients. Recent studies show that A_{2B} receptor plays an important role in mediating the pro-tumor effects of adenosine, since its selective blockade can inhibit tumor growth in some murine tumor models. Targeting A_{2B} receptor reduces immunosuppression induced by myeloid cells and inhibits the stromal cells activity within the tumor microenvironment, limiting tumor angiogenesis and metastatic processes. Here, the authors review the current data on involvement of A_{2B} receptor in regulating tumor progression and discuss the development of A_{2B} receptor inhibitors as potential therapeutic agents in cancer treatment.

INTRODUCTION

Tumor microenvironment is populated not only by malignant cells but also by other stromal cells and immune cells that cooperate to the development of cancer.^[1,2]

In the eternal battle against cancer, several strategies have been developed. One of the first approach to treat cancer has been the antineoplastic chemotherapy which is made up of chemical substances that provide to halt directly the highly-replicating tumor cells by damaging their RNA or DNA.^[3] Radiotherapy is another important



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treatment currently used for several tumors, through which cancer cells are either directly killed upon DNA damage by depositing high physical energy of radiation, or indirectly due to the release of free radicals.^[4] Nowadays the most novel anti-cancer strategies are the targeted-therapy and immunotherapy. The cancer targeted-therapy uses small molecules that can block fundamental pathways or mutant proteins essential for tumor growth.^[5] Conversely, cancer immunotherapy is a therapeutic strategy that improves the host immune response against cancer cells, instead of acting directly on tumor cells.^[6]

Several chromosomal alterations, genetic mutations and genomic instability that occur in cancer cells provide a different set of antigens that the immune system can use to distinguish transformed cells from their own cells.^[7] However, tumor cells escape from host immune surveillance through different mechanisms, that include loss of immunogenicity and ineffective T-cell mediated responses. Moreover, several inflammatory mediators including chemokines [CC-chemokine ligand 2 (CCL2), CCL5, CXC-chemokine ligand 1 (CXCL1), CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, CXCL10 and CXCL12], cytokines [tumor necrosis factor (TNF), interleukin 1 (IL-1), IL-4, IL-5, IL-6, IL-10 and IL-13] and growth factors [granulocyte macrophage-colony stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), transforming growth factor- β (TGF- β)] are released by tumor cells and/or stroma and immune cells surrounding tumor tissue, generating a chronic inflammatory microenvironment. Chronic inflammation in cancer can facilitate tumor proliferation and invasion and drive the recruitment and activation of immunosuppressive cells, including T regulatory (Treg) cells, myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAM). In this context, many inhibitory receptors, known as “immune checkpoint molecules” such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) and programmed cell death-1 (PD-1), are upregulated on activated lymphocytes during an active immune response providing a negative feedback mechanism.^[7] CTLA4 binds to members of the B7 family on antigen-presenting cells (APCs) inhibiting T-cell activation, while PD-1 interacts with ligands PD-L1, expressed on different cell types including tumor cells, or PD-L2 on macrophages and dendritic cells, inhibiting T-cell functions.^[7,8] The development of agonist antibodies (for costimulatory pathways) or antagonist antibodies (for inhibitory pathways) which target lymphocyte receptors or their ligands is one of the most promising approach with the potential to modulate the tumor microenvironment and improve the efficacy of immune response/s against cancer

cells.^[8] The first class of immunotherapeutics approved by US Food and Drug Administration (FDA) for patients with metastatic melanoma includes antibodies against CTLA4 (Ipilimumab and Tremelimumab).^[9] Later on other immune checkpoint molecules have been discovered, such as antibodies against PD-1 (Nivolumab, Pembrolizumab and Atezolizumab), PD-L1, lymphocyte-activation gene 3 (LAG3, also known as CD223), B7-H3 (also known as CD276), B7-H4 (also known as B7-S1, B7x and VCTN1) and T-cell immunoglobulin domain and mucin domain 3 (TIM3).^[8] The therapeutic outcomes in cancer patients is improved by combining immunotherapeutics with chemotherapy.^[8] The concomitant blockade of different immune checkpoints may increase the success of immunotherapy in cancer patients.^[8] Hence, in the last few years many efforts have been made aiming to investigate novel therapeutic strategies to inhibit cancer-induced immune-suppression. It has become clear that in the tumor microenvironment there are several pathways that may play an important role in the tumor immune evasion process. Among them, extracellular adenosine, an ATP-derived molecule generated by the extracellular CD39/CD73 enzymes, has been identified as an immune checkpoint that critically impairs the anti-tumor immune response mainly via A_{2A} adenosine receptor subtype.^[10-12] Accordingly, selective inhibitors of adenosine signaling pathways have been tested in pre-clinical studies^[13,14] and some of them, including the antibody anti-CD73 and the A_{2A} receptor antagonists are currently in Phase I clinical trials in cancer patients, either as single agents, or in combination with immune checkpoints inhibitors such as anti PD-1 monoclonal antibodies [NCT02503774 and NCT02655822].

While the role in tumor immunity of CD73-A_{2A} receptor axis has been extensively examined, less is known about the role of A_{2B} receptor subtype in tumor development and progression. Compelling evidence suggest that this receptor contributes to the pro-tumor effects of adenosine within tumor microenvironment. In this article, we review the current data on the effects of adenosine in tumor progression, focusing on the emerging role of A_{2B} receptor in regulating tumor growth and discuss the therapeutic potential of targeting A_{2B} receptors in cancer treatment.

CRITICAL ROLES OF ADENOSINE IN TUMOR PROGRESSION

Adenosine is a key endogenous molecule produced at the extracellular level by two ectoenzymes, ecto-5'-nucleotidase (CD73) and ectonucleoside triphosphate diphosphohydrolase-1 (CD39) physiologically

expressed on both hematopoietic and non-hematopoietic cell types.^[15,16] Once released in the extracellular space, adenosine elicits its physiological responses by coupling and activating four membrane adenosine receptors (A₁, A_{2A}, A_{2B}, A₃) which contain seven transmembrane domains coupled to G proteins.^[17] Once bounded with its receptors, adenosine can inhibit (via A₁ and A₃) or stimulate (via A_{2A} and A_{2B}) the adenylyl cyclase resulting in a decrease or increase in cyclic AMP (cAMP) accumulation, respectively.^[18] cAMP activates the protein kinase A (PKA) and in turn the nuclear substrate cAMP responsive element-binding protein (CREB) that regulates the expression of several genes by binding to cAMP responsive elements and other cAMP effectors such as Epac, altering pro-inflammatory genes expression.^[19-22] A_{2B} receptor can also activate the phospholipase C (PLC) by coupling Gq protein.^[23,24] All adenosine receptors are involved in the modulation of mitogen-activated protein kinase (MAPK) activity.^[25]

The tumor milieu is characterized by high levels of adenosine triphosphate (ATP) due to the high proliferating rate of cancer cells. The ATP is rapidly converted at the extracellular level in ADP and AMP through two reversible steps via CD39, while the last irreversible step in adenosine is mediated by CD73.^[16] Under homeostatic conditions adenosine level is low but during pathophysiological events (including stress, infection, inflammation and cancer) extracellular adenosine levels can be increased from 10-200 nmol/L up to 10-100 μmol/L.^[26] In inflammatory-associated conditions, adenosine typically attenuates the inflammatory response.^[26,27] Importantly, studies by Ohta and Sitkovsky^[28] showed for the first time that A_{2A} receptor-deficient mice are unable to control inflammation, resulting in exaggerated immune responses which can trigger extensive tissue disruption with subsequent cell death. These effects of adenosine are dependent on the activation of the adenosine A_{2A} receptors on immune cells, which induce a wide range of singular immunosuppressive responses which regulate the uncontrolled inflammation to harmful insults.^[27,29,30] However, in the context of tumor while extracellular ATP increases the T-cell mediated effector function, high levels of adenosine mediates opposite effects favoring immune suppression that is associated with tumor growth and metastasis.^[31] Hypoxia, which is a common feature of the tumor microenvironment that promotes immunosuppression, is one of the main factors responsible of the increased production of adenosine within many solid tumors.^[11] Indeed, the expression and the enzymatic activities of CD39 and CD73, responsible of the adenosine generation, increased under hypoxic conditions, while the expression of the

adenosine kinase, which inhibits the metabolism of adenosine, is down-regulated.^[11] At the same time, the expression of adenosine receptors A_{2A} and A_{2B} is also up-regulated.^[32] Consequently, adenosine along with other HIF-induced immunosuppressive factors and cells, contributes to modulate the functions of tumor cells, tumor-infiltrating immune cells and/or other stroma cells.

Within the hematopoietic compartment, CD39 is expressed on B cells and monocytes, subsets of CD8⁺ T cells, CD4⁺ T cells and NK cells.^[15,33] CD73 is expressed on B cells and subsets of CD8⁺ T cells, CD4⁺ T cells and NK cells and small subsets of monocytes.^[15,33] CD39 and CD73 are co-expressed on B cells, Treg cells, Th17 cells, NK cells, neutrophils, tissue macrophages and myeloid-derived suppressor cells (MDSCs).^[15,33] CD39 and CD73 are also expressed on endothelial cells and on the surface of several types of cancer cells.^[14,15] Thus, CD73-expressing cells, including immune cells and/or stroma cells, produce adenosine that accumulate in the tumor microenvironment and profoundly impairs anti-tumor immune responses. Accordingly, a large number of evidence have proved that targeting adenosine-generating enzymes significantly reduces tumor growth by improving anti-tumor immune responses.

A_{2A} receptor is the most thoroughly characterized receptor involved in the adenosine-induced anti-inflammatory/immune-suppressive effects within the tumor microenvironment. A_{2A} receptor is highly expressed on lymphocytes, macrophages, dendritic cells, NK cells, and neutrophils. Activation of A_{2A} receptor significantly reduces T-cell receptor (TCR)-triggered effector functions, including proliferation and production of cytokines and chemokines, preventing T cells activation and function via cAMP/protein kinase cAMP-dependent (PKA) pathways.^[34-36] These effects occurs upon A_{2A} adenosine receptor stimulation in naïve CD4⁺ T cells as well as in CD8⁺ T cells. Furthermore, A_{2A} receptor stimulation reduces the expression of CD25 and CD40 ligand (CD40L) and increases the expression of PD-1 and CTLA-4 on T cells,^[37] inducing T cell anergy that promotes peripheral tolerance.^[35] Stimulation of A_{2A} receptor on myeloid cells can also affect the release of IL-12 and induce the production of IL-10,^[38] affecting significantly the T- and NK-cell responses in the solid tumor microenvironment.^[39] Additional evidence also show that A_{2A} adenosine receptor stimulation promotes the development of immune suppressive myeloid cells^[40] or Treg cells.^[41] The first *in vivo* genetic evidence of the role of A_{2A} receptor in tumor progression has been reported by Ohta *et al.*^[10] who showed that 60%

of A_{2A} receptor deficient mice completely rejected established immunogenic tumors in a CD8⁺ T-cell-dependent manner. However, 40% of tumor-bearing A_{2A} receptor deficient mice did not reject the tumor, possibly because of the expression of A_{2B} receptor on A_{2A} receptor deficient CD8⁺ T cells.^[10]

At the same time a large number of evidence show that inhibition of CD73 activity or CD73 knockdown on tumor cells inhibit tumor growth and metastasis by enhancing the anti-tumor T cell response.^[42-46] CD73-deficient mice are resistant to tumor and show an increased influx of CD8⁺ T cells^[44] and low number of Tregs within the tumor.^[47]

The expression of CD73 on various tumor cells from cancer patients, including breast,^[46] glioblastoma,^[48] prostate,^[49] ovarian,^[50] leukemia^[51] has been associated with poor prognosis. Notably, some chemotherapeutics are able to increase the expression of CD73 on cancer cells, which may in turn represent a putative mechanism of resistance to chemotherapeutics.^[46,49,51] On the other hand, targeting CD73 can improve the therapeutic potential of some conventional cancer treatments, including chemotherapy, radiotherapy and immunotherapy. For example, inhibition of CD73 in combination with doxorubicin prolonged the survival of mice with metastatic breast cancer.^[46] Adenosine can also impair the anti-tumor response induced by high dose of radiation therapy.^[52] Administration of CD73 inhibitor into mice with tumors exposed to radiation therapy can significantly reduce tumor growth.^[52] Notably, inhibition of CD73 may also improve the synergy of radiation therapy in combination with anti-CTLA4 monoclonal antibody.^[52]

Recent studies indicate that inhibition of adenosine/A₂ adenosine receptors axis synergizes with other immune checkpoints inhibitors reducing potently tumor growth in murine models of cancer. In particular, treatment of mice with monoclonal antibody anti-CD73 enhances the anti-tumor effects of antibodies anti-PD1 and anti-CTLA4.^[53] In support, other studies have demonstrated that selective blockade of A_{2A} adenosine receptor in combination with anti PD-1 antibody and anti-CTLA4 antibody potently reduced tumor growth.^[54-56] The therapeutic synergy of these combinations depends on the CD73 expression on tumor cells, proving that CD73-generating adenosine by tumor cells within the tumor microenvironment may affect the activity of immunotherapy. Furthermore, blockade of PD-1 enhances the expression of A_{2A} receptors on tumor-infiltrating CD8⁺ T cells, suggesting that adenosine via A_{2A} receptor limits the immune

response against cancer induced by inhibitors of immune checkpoints.^[54] More recently, it has been demonstrated that blockade of A_{2B} adenosine receptor subtype with a selective antagonist improves survival and the anti-metastatic effects of anti-PD1 and anti-CTLA4 monoclonal antibodies in both melanoma and mammary cancer models of metastasis with cells expressing CD73.^[57] The anti-metastatic effects of these combinations relies on the capacity of immune checkpoints inhibitors to boost immune responses and on direct effects of A_{2B} adenosine receptor inhibitor on cancer cell metastasis.^[57] Here the authors show that blockade of A_{2B} receptor in A_{2B} receptor deficient mice is able to reduce the metastasis of human triple negative breast cancer (TNBC) xenografts, confirming the critical role of A_{2B} receptor on cancer cells rather than host cells.^[57] Altogether these preclinical studies strongly support the therapeutic potential of targeting adenosine in cancer.

Experimental evidence suggests that also CD39 can represent a potential therapeutic target for cancer treatment. CD39 is highly expressed by Treg cells and together with CD73 generate adenosine in the tumor microenvironment.^[58] Elevated levels of CD39-expressing Treg cells have been found in some mouse tumor tissues, including melanoma and colorectal cancer.^[58] Inhibition of CD39 reduces the tumor growth, enhances the recruitment of T cells in the tumor lesions and improves the effector functions of CD8⁺ T cells and NK cells, by impairing the activity of CD39-expressing Treg cells.^[58] Although additional studies are needed to better clarify the therapeutic potential of targeting CD39 in cancer, the use of CD39 inhibitors might be useful to limit the immune suppression induced by Treg cells.

Selective agonists of A₃ adenosine receptor subtype have proved to directly inhibit proliferation of A₃-expressing tumor cells by arresting cell cycle progression and exert immunostimulatory effects in some murine tumor models in a NK- and T-cell-dependent manner, enhancing the production of Th1-like cytokines in the tumor microenvironment.^[59-63] A₃ adenosine receptor agonists have been tested indeed in some clinical trials for rheumatoid arthritis (NCT00280917, NCT00556894, NCT01034306, NCT02647762),^[64] hepatocellular carcinoma (NCTNCT00790218, NCT02128958) and hepatitis (NCT00790673),^[65] dry eye syndrome (NCT01235234, NCT00349466)^[66] and psoriasis (NCT01265667).^[67]

Nonetheless, emerging evidence suggest that A_{2B} receptor can mediate the pro-tumor effects of adenosine. It is known that A_{2B} receptor is important

in some patho-physiological conditions, including vascular injury,^[68] chronic lung disease,^[69] vascular leak,^[70] and ischemic disease.^[71] First studies performed by Ryzhov *et al.*^[72] in 2008 show that tumor growth in A_{2B} receptor deficient mice was reduced compared to that observed in wild type mice, providing the first genetic evidence for a pivotal role of A_{2B} receptor in tumor progression.

Up to now a number of selective A_{2B} receptor antagonists (such as MRS1754, ATL801, GS-6201, PSB603 and PSB1115) and selective A_{2B} agonists (Bay60-6583) have been synthesized, helping the study and the characterization of the role of this adenosine receptor in many patho-physiological conditions, including cancer, as discussed below.

EXPRESSION OF A_{2B} RECEPTOR

A_{2B} adenosine receptor is widely expressed in the entire organism, although its role is not completely understood. The A_{2B} receptor expression has been detected in type II alveolar epithelial cells,^[73] endothelial cells,^[74] chromaffin cells,^[75] astrocytes,^[76] neurons,^[77] and taste cells.^[78] Moreover, A_{2B} receptor is expressed also on many immune cell populations including mast cells,^[79] neutrophils,^[70] dendritic cells,^[80] macrophages,^[74] and lymphocytes.^[81]

Despite A_{2B} receptor binds adenosine with lower affinity (EC₅₀ = 24 μmol/L) than A_{2A} receptor,^[72,82] its relevance in regulating tumor growth is becoming clear both because its expression is highly influenced by the tumor milieu and because A_{2B} receptor can play different physiological roles compared to A_{2A} receptor.

The tumor microenvironment is characterized by high proliferating rate of cancer cells which contribute to hypoxia condition. Hypoxia is a very strong stimulus for up-regulating A_{2B} receptor expression through hypoxia inducible factor (HIF-1α) and hypoxia-dependent signaling pathways in endothelial cells, dendritic cells (DCs), muscles, fibroblasts and T cells.^[32,83-87] Indeed, a functional hypoxia-responsive region within the A_{2B} receptor promoter has been identified, confirming the selective transcriptional induction A_{2B} receptor by hypoxia.^[87] Transcription of A_{2B} receptor can be induced by bacterial lipopolysaccharide (LPS) or interferon (IFN)-γ in macrophages,^[88,89] by TNF-α in vascular smooth muscle cells,^[90] and by IL-1β in endothelial cells.^[91] Furthermore, a post-transcriptional regulation of A_{2B} receptor by inflammatory mediators has been demonstrated in endothelial and pulmonary epithelial cells^[92] and in colonic epithelial cells.^[93] Therefore, although A_{2B} is a low-affinity adenosine receptor,

under inflammatory-hypoxic conditions, its expression is up-regulated while the concentration of adenosine reaches highest levels. In this context, the A_{2B} receptor may play an important role in mediating adenosine-induced pathological effects.

A_{2B} RECEPTOR AND TUMOR IMMUNITY

Although the role of A_{2B} receptor in controlling T-cell-mediated response is not completely clear, compelling evidence indicate that this receptor may influence the features of some immune cell populations.

It has been demonstrated that A_{2B} receptor is involved in the differentiation of T cells under Treg skewing-conditions, since its inhibition is able to suppress the expression of FoxP3 and IL-10 production in a way completely independent from T cell activation.^[94]

To be activated and provide anti-tumor responses CD4⁺ T-cells need the expression of the major histocompatibility complex (MHC) class II. In several types of tumors, the loss of MHC class II is related to impaired levels of CD4⁺ T-cells.^[95] Moreover, the levels of either MHC class II or class II transactivator (CIITA) are altered in highly metastatic cancer cells.^[96] A_{2B} receptor stimulation by repressing CIITA can impair MHC class II transcription in IFN-γ-stimulated cells.^[97,98] Moreover, bone marrow-derived dendritic cells (BMDCs) express A_{2B} receptor and adenosine inhibits BMDCs IL-12p70 production via A_{2B} receptor. Depending on the levels of this cytokine, CD4⁺ T-cells can differentiate into Th1 or Th2 cells.^[99] The impaired production of pro-inflammatory cytokines (TNF-α and IL-12) and the increased IL-10 production induced by A_{2B} receptor activation leads to a lower expression of CD86 and MHC class II lowering CD4⁺ T cell stimulation.^[100]

A_{2B} receptor can also affect macrophages proliferation induced by macrophage colony-stimulating factor (M-CSF)^[101] and the differentiation of human monocytes, mouse peritoneal macrophages and hematopoietic progenitor cells (HPCs) into myeloid DCs with tolerogenic and angiogenic features.^[80] A_{2B} receptor activation promotes the expansion *in vitro* of MDSCs, that contribute to induce immunosuppression by producing adenosine.^[102] MDSCs potently suppress anti-tumor T-cell response and/or promote angiogenesis.^[103] Altogether, these studies strongly support a role of A_{2B} receptor in inducing the differentiation of hematopoietic progenitor cells into mature cells with tolerogenic and suppressive features. Subsequent studies performed *in vivo* show that A_{2B} deficient mice have reduced amounts of tumor-

infiltrating myeloid cells CD11b^{high}/Gr-1^{high}, suggesting that A_{2B} receptor suppresses immune surveillance.^[72] Later, Cekic *et al.*^[104] showed that the selective blockade of A_{2B} receptor inhibits bladder and breast tumor growth in mice, by inducing a T-cell mediated response in a CXCR3-dependent manner. In a mouse model of melanoma, selective blockade of A_{2B} receptor inhibits tumor growth.^[105] This effect was associated with lower levels of IL-10 and MCP-1 in the tumor tissue and reduced accumulation of tumor-infiltrating MDSCs.^[105] Notably, the levels of MDSCs in secondary lymphoid organs remained unchanged in mice treated with the selective A_{2B} receptor antagonist, consistent with a selective activity of the antagonist on the recruitment of MDSCs to tumor lesions rather than with a putative systemic effects.^[105] Blockade of A_{2B} receptor within the tumor microenvironment modulates the intra-tumoral levels of various inflammatory mediators and growth factors that could in turn influence the features of tumor-infiltrating immune cells, promoting the recruitment/accumulation of MDSC.^[106] Accordingly, the percentage of tumor-infiltrating CD8⁺ T cells upon A_{2B} receptor blockade enhanced in the tumor lesions.^[105] Furthermore, treatment of mice with the A_{2B} receptor antagonist PSB1115 in combination with dacarbazine, a chemotherapeutic agent commonly employed in melanoma patients, reduces tumor growth and significantly increases the number of CD8⁺ T cells in the melanoma lesions demonstrating the high potential of combining A_{2B} receptor blockade and chemotherapy for cancer treatment.^[105,106]

In conclusion, the experimental evidence in some tumor mouse models suggest that the selective blockade of A_{2B} receptor may ameliorate T cell-mediated immune surveillance by impairing the accumulation of suppressive cells and the levels of inflammatory factors in the tumor microenvironment.^[72,104-106] However, despite the relevance of these observations, more studies are needed to provide a detailed understanding of the role of A_{2B} receptor in modulating the immune responses in tumor environments.

A_{2B} RECEPTOR AND TUMOR STROMA

A number of studies indicate that A_{2B} receptor can directly affect the proliferation/migration of tumor cells and the function of other stroma cells that populate the tumor niche, including endothelial cells and fibroblasts.

A critical role for A_{2B} adenosine receptor in mediating proliferation and/or apoptosis in different cancer cell lines has been delineated. A_{2B} adenosine receptor is highly expressed in prostate cancer cell lines

and selective antagonist of A_{2B} adenosine receptors or silencing A_{2B} receptors blocked the proliferative effects induced by a non-selective adenosine analog NECA.^[107,108] Other studies indicate that A_{2B} adenosine receptor is highly expressed also in oral squamous carcinoma cell lines, as well as in human oral carcinoma tissues, where its expression is correlated with those of HIF-1.^[109] Studies by Gessi *et al.*^[110] demonstrate that in colon cancer cells, although at the mRNA levels A_{2B} receptor is more expressed than A₁, A_{2A} and A₃, the density of A₃ receptors is the highest among the adenosine receptor subtypes. Later, other studies have demonstrated that the adenosine A_{2B} receptor is up-regulated in colorectal carcinoma tissues and colon cancer cell lines compared with normal colorectal mucosa under hypoxic conditions.^[111] Antagonists of A_{2B} receptors inhibit cancer cell proliferation, suggesting that this receptor may be a potential therapeutic target for colorectal cancer.^[111]

In contrast, in gastric cancer cells A_{2B} adenosine receptor has been identified as target of miR-128b, a proto-oncogene miRNA down-regulated in gastric cancer tissues.^[112] In this work, the authors demonstrate that the down-regulation of miR-128b in gastric cancer cell is associated with an over-expression of A_{2B} adenosine receptor and decreased cell apoptosis rate.^[112] In osteosarcoma cells it has been demonstrated that p73 upregulates A_{2B} adenosine receptor and A_{2B} receptor agonists can enhance p73-dependent cell death in response to chemotherapy.^[113] Moreover, stimulation of A_{2B} receptor with a non-selective adenosine analog NECA induces apoptosis in ovarian cancer cells.^[114] Nonetheless, while a number of studies demonstrate that stimulation of A_{2B} adenosine receptor in some cancer cell types promotes proliferation, whereby knockdown or pharmacological inhibition of this receptor reduces tumor cell growth and promotes apoptosis,^[107-111] opposite results have been also described.^[112,113] The discrepancy might likely depend on the cancer cell types, the expression levels of this receptor on tumor cells and the selectivity and/or concentrations of pharmacological tools used in the experimental settings.

It has been demonstrated that agonists of A_{2B} receptor induce anti-proliferative and pro-apoptotic effects on glioblastoma cancer stem cells (CSCs).^[115] Furthermore, stimulation of A_{2B} receptors as well as A₁ receptors sensitize glioblastoma CSCs to chemotherapy.^[115]

A role of A_{2B} receptor in promoting the migration of tumor cells *in vitro* and *in vivo* has been clearly

demonstrated. Indeed, a number of studies show that adenosine may directly influence the migration/invasion of tumor cells via A_{2B} adenosine receptor. Stagg *et al.*^[42] have demonstrated that targeting the adenosine-generating enzyme CD73 inhibits tumor growth in mice and significantly delays the development of spontaneous lung metastasis. While the effect of anti-CD73 monoclonal antibody therapy on primary tumor growth relies on its capacity to improve immune surveillance, the anti-metastatic effects to the lungs is rather dependent on a direct effect of CD73-generating adenosine on breast tumor-cell migration via A_{2B} adenosine receptors stimulation.^[42] Consistent with the role of A_{2B} receptor in promoting metastasis of breast cancer cells to the lung, administration of selective or non-selective A_{2B} receptor antagonists into mice significantly reduced metastasis burden.^[42,104] Furthermore, antagonists of A_{2B} receptor preferentially inhibits the invasive capacity of breast cancer cells expressing Fos-related antigen-1 (Fra-1), a transcription factor overexpressed in human metastatic breast cancers.^[116] Therefore, the authors suggest that Fra-1 activity is a prognostic indicator of both breast cancer metastasis and responsiveness to pharmacological inhibitors, such as A_{2B} receptor antagonists.^[116]

In a recent paper it has been demonstrated that high expression of A_{2B} receptor is associated with poor survival in triple negative breast cancer (TNBC) patients.^[57] As mentioned above, these authors demonstrate that A_{2B} receptor antagonist prevents metastasis of A_{2B} receptor-expressing tumor cells and improves survival when administered in combination with chemotherapeutic agents and immune checkpoints inhibitors monoclonal antibodies in both experimental and spontaneous murine models of metastasis.^[57] The anti-metastatic effects of A_{2B} receptor antagonists is independent on lymphocytes and myeloid cells, whilst tumor A_{2B} receptor is critical.^[57] These evidence highlight that A_{2B} receptor may be an attractive target for treatment of breast metastasis.

A_{2B} adenosine receptor can also contribute to the pro-angiogenic effects of adenosine in the tumor milieu. Vascular endothelial growth factor (VEGF) is a well-known mediator critically involved in tumor progression and angiogenesis.^[117] A number of studies linked VEGF production to adenosine A_{2B} receptor in human endothelial cells,^[118,119] in some tumor cell lines^[120,121] and in host immune cells, including dendritic cells and myeloid-derived suppressor cells.^[72,80,122]

A_{2B} receptor is expressed on human endothelial cells and its stimulation promotes the expression of several

pro-angiogenic factors, including VEGF, IL-8 and basic fibroblast growth factor (bFGF).^[118] Importantly, under hypoxic conditions the expression of A_{2B} receptor in endothelial and smooth muscle cells increased and the stimulation of these receptors further enhance VEGF release.^[119] Hypoxia is a common feature of tumor and can induce angiogenesis. At the same time, adenosine, whose levels became elevated during hypoxia, further enhances angiogenesis by stimulating A_{2B} receptors, creating a positive feedback between hypoxia, adenosine and VEGF.

Other studies also indicate that adenosine promotes the release of angiogenic factors, namely VEGF and IL-8, in some cancer cells lines, via A_{2B} receptor including human melanoma cells^[120] and glioblastoma cells, which express high levels of A_{2B} receptor under hypoxic conditions.^[121]

Using A_{2B} receptor deficient mice, Ryzhov *et al.*^[72] firstly demonstrated the critical role of A_{2B} receptor in modulating the VEGF levels in tumor tissues. Importantly, vascularization and tumor tissue VEGF levels were significantly reduced in A_{2B} receptor deficient mice compared with WT mice.^[72] This effect was associated with reduced tumor infiltration of VEGF-producing myeloid cells, suggesting that A_{2B} receptor can modulate the release of VEGF either from tumor cells and from host tumor-infiltrating immune cells,^[72] that can contribute to promote tumor angiogenesis.^[123] As mentioned above, adenosine promotes the differentiation of dendritic cells precursors into a subset of DC that produce angiogenic factors, including VEGF, and other immunosuppressive factors via A_{2B} adenosine receptor.^[80] Notably, A_{2B}-stimulated dendritic cells are able to promote tumor growth when injected into mice.^[80] These observations strongly suggest that adenosine sustains tumor angiogenesis during tumor growth by stimulating the release of VEGF from endothelial cells, tumor cells and immune cells. Accordingly, targeting CD73 in mice impairs tumor angiogenesis and decreases VEGF levels, at least in part by lowering adenosine generation in tumor environment that activates A_{2B} receptors.^[124] Therefore, targeting CD73 and/or A_{2B} receptor may represent a potential therapeutic strategy to block angiogenesis. In support of this, the pharmacological blockade of A_{2B} receptor with a selective antagonist in mice significantly reduces the tumor levels of VEGF and CD31 positive cells within tumor lesions.^[122] Moreover, the anti-angiogenic effect of A_{2B} receptor antagonists is, at least in part, dependent on the lower frequency of tumor-infiltrating suppressive myeloid cells (MDSCs),^[72,122] breaking the positive feedback loop that promotes angiogenesis and MDSC-mediated

immune suppression in the tumor environment. Recent evidence indicate that A_{2B} receptor stimulation promotes the release of FGF-2 and C-X-C motif chemokine ligand 12 (CXCL12) from tumor-associated fibroblasts,^[125] that contribute to promote tumor growth and angiogenesis.^[126] These effects are associated with reduced expression of fibroblast activation protein (FAP), a common marker of tumor-activated fibroblasts termed cancer-associated fibroblasts (CAF), that promote tumor growth enhancing tumor immune evasion and tumor vascularization.^[127] A_{2B} receptor-induced CXCL12 by tumor-associated fibroblasts contributes to the pro-angiogenic effects of A_{2B} receptor via CXCR4, suggesting a link between tumor fibroblasts and endothelial cells.^[127] Moreover, fibroblasts express CD73, which is up-regulated under hypoxic conditions.^[127] Altogether, these evidence suggest that in the context of tumor A_{2B} receptor contributes to mediate multiple effects of adenosine on different types of cells that populate the tumor niche. Furthermore, blockade of A_{2B} receptor modulates the intra-tumoral levels of paracrine factors, which are critical in regulating intercellular crosstalk in the tumor microenvironment.

Although the predominant role of A_{2A} receptor in

mediating the immunosuppressive effects of adenosine in the tumor tissue and the high therapeutic potential of blocking adenosine generation and the A_{2A}-mediated effects, by using anti-CD73 monoclonal antibodies and A_{2A} selective antagonists, respectively, it is becoming clear that A_{2B} receptor may significantly affect tumor progression and metastasis. Its contribute to tumor development and growth is most likely dependent on its high expression levels on tumor cells, and/or endothelial cells and/or other tumor-infiltrating cells, in a rich adenosine environment.

CONCLUSION

Adenosine plays a critical role in tumor immunity, angiogenesis and metastasis process. Strategies aimed to inhibit tumor adenosine production and functions, by using CD73 inhibitors and selective blockade of A_{2A} adenosine receptor, are effective for cancer treatments, especially in combination with chemotherapeutic agents and immune-checkpoints inhibitors.

Nonetheless, compelling evidence support the role of A_{2B} receptor subtype in contributing to the pro-tumor effects of adenosine within the tumor

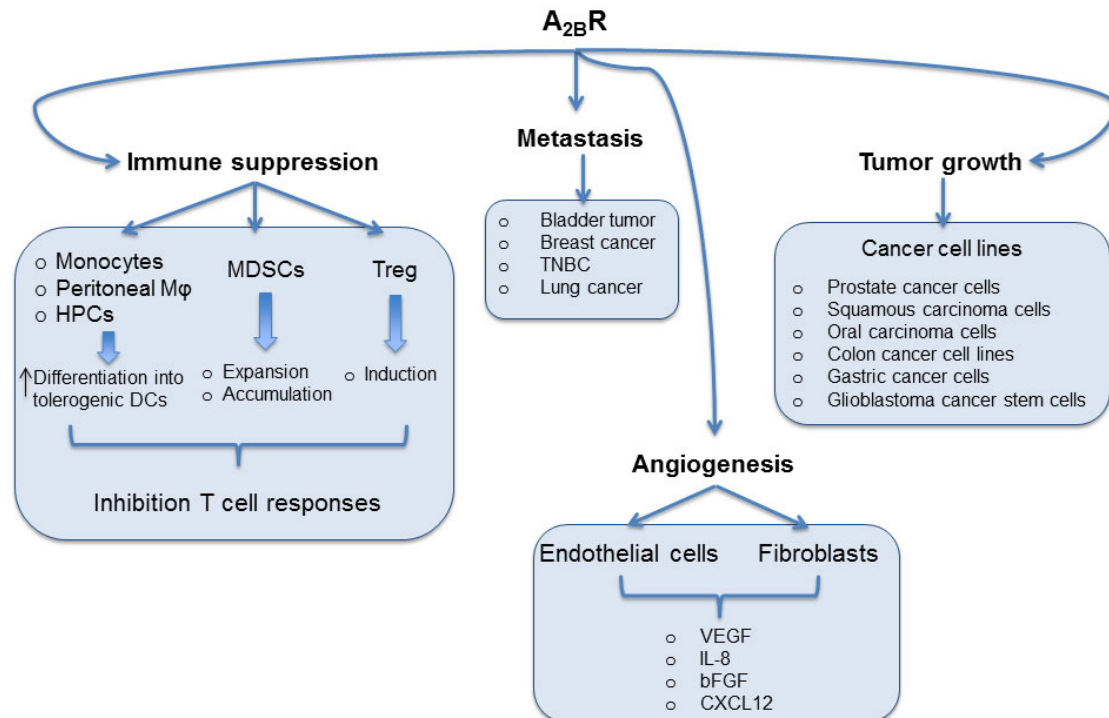


Figure 1: Multiple roles of A_{2B} adenosine receptors in cancer. A_{2B} receptor stimulation induces (1) the differentiation of human monocytes, mouse peritoneal macrophages (φ) and hematopoietic progenitor cells (HPCs) into tolerogenic dendritic cells (DCs); (2) the expansion and accumulation of MDSCs; (3) Treg differentiation, enhancing immune suppression that inhibits T-cell responses. Activation of A_{2B} receptors on stroma cells, including tumor cells, endothelial cells and fibroblasts promotes tumor proliferation or invasion and angiogenesis. TNBC: triple negative breast cancer; VEGF: vascular endothelial growth factor; IL-8: interleukin-8; bFGF: basic fibroblast growth factor; CXCL12: C-X-C motif chemokine ligand 12; MDSCs: myeloid-derived suppressor cells

microenvironment, including immune suppression, angiogenesis and metastasis [Figure 1]. Despite these evidence, further studies are needed to better investigate thoroughly the mechanisms by which blockers of this receptor limit tumor growth. Understanding the relative role of A_{2B} receptor in tumor, depending on the cell types, on its distribution and expression, will help to potentially apply A_{2B} receptor-targeting agents for cancer treatment.

Authors' contributions

Conceived and designed the study: C. Sorrentino, S. Morello

Performed literature search and prepared manuscript: C. Sorrentino, S. Morello

Revised the manuscript: S. Morello

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Conflicts of interest

There are no conflicts of interest.

Patient consent

There is no patient involved.

Ethics approval

This article does not contain any studies with human participants or animals.

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Large chest wall fibromatosis with challenging treatment plan

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ABSTRACT

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The primary mode of treatment for desmoid tumors is surgical excision. However, high recurrence rates (39-79%) have been reported when surgery is used alone. The role of adjuvant radiotherapy after surgical resection of primary disease is controversial and should be based on a balanced discussion of potential morbidity from radiotherapy and local recurrence. In this patient, the maximum dimension of tumor was 21 cm. This is a larger chest wall fibromatosis than has been reported thus far, to the best of our knowledge. In this case, post-operative margins were free, but in view of the large initial tumor size and potential morbidity in case of any future locoregional recurrence, post-operative adjuvant external beam radiation was delivered. An image guided intensity modulated radiotherapy technique was chosen to spare adjacent breast and lung parenchyma, and tolerance of these structures was well respected. This case provides insight into this treatment approach.

INTRODUCTION

Desmoid tumors, also termed as aggressive fibromatosis, are heterogeneous, benign tumors that originate from deep musculoaponeurotic structures. These rare tumors account for approximately 0.03-0.1% of all solid tumors and 3.6% of all fibrous tissue neoplasms.^[1] Fibromatoses of the chest wall are rare and only represent 10% to 20% of all fibromatoses.^[2,3]

These neoplasms can display local infiltrative growth but due to their benign nature they do not metastasize.^[4] Desmoid tumors are non-encapsulated and tend to extend along fascial planes. They also

have the potential to erode bone and surrounding blood vessels or nerves.

In this report, we present the case of a patient with a large chest wall aggressive fibromatosis, 21 cm in maximum dimension, who was treated with surgery followed by adjuvant modern image guided radiation therapy. Standard protocols and recent trends for the treatment of desmoid tumors are also discussed.

CASE REPORT

A 24-year-old female presented with complaints of pain in the right lower chest for 2.5 months and



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breathlessness for 2 months. Chest X-ray (PA view) reported dense homogeneity over the right middle and lower zones. A computed tomography (CT) scan of thorax and abdomen with contrast showed a large pleural based mass of approximately 12 cm × 13 cm × 19 cm in the right thoracic cavity, probably arising from right chest wall, extending into the mediastinum, with smooth indentation on pericardium and superior vena cava with no evidence of rib destruction [Figures 1 and 2]. Core needle biopsy of the mass showed a benign spindle cell tumor.

She underwent excision of the tumor along with a portion of ribs and intercostal muscle under general anesthesia on June 11, 2013. Intraoperative findings confirmed a large, firm mass in the right chest wall arising from anterior parts of the lower ribs. Lung, diaphragm and mediastinal structures were not infiltrated. Repair of the chest wall defect was done using double layer polypropylene mesh. Postoperative histopathology reported a benign spindle cell tumor of 21 cm × 15 cm × 5.5 cm with negative margins. On gross examination there was a single soft tissue piece with attached bone and skeletal muscle. Immunohistochemistry reports revealed tumor cells focally positive for SMA and negative for S-100 and CD 34, suggestive of extra abdominal fibromatosis.

The patient was prepared for postoperative adjuvant external beam radiation to the chest wall (postoperative bed) in view of the unusually large primary neoplasm and increased risk of recurrence. For immobilization, both thermoplastic mould and VACLOC of chest were made. The patient was kept in supine position with both arms abducted alongside of the head. For CT simulation, a radiation technologist accompanied the patient; the same. Positioning as during immobilization was followed. During CT simulation radio opaque markers were placed over the scar mark. A CT scan of the area of interest was taken using 2 mm slice thickness without intravenous contrast. The radiotherapy equipment used was dual-energy linear accelerator (Clinac iX, Varian Oncology System) incorporating asymmetric X and Y collimators, 120-leaf millenium-multileaf collimator, amorphous silicon-based electronic portal imaging, kilovoltage cone beam CT scanner, 3D beam planning computer workstation (Eclipse TPS ver 8.6.17) and networking (ARIA network).

After thorough discussions with the surgeon, radiologist, and based on preoperative images, contouring of the postoperative bed (clinical target volume) was done. All the organs at risk were contoured according to RTOG guidelines. Radiotherapy doses of 50.4 Gy in

28 fractions was delivered at the rate of 1.8 Gy per fraction, 5 fractions per week for 5 weeks to clinical target volume (CTV) by image guided radiotherapy technique. The doses delivered to CTV in the axial, coronal and sagittal sections are represented in Figures 3-5. Adjacent normal structures (right lung, heart, right breast, liver) were given dose constraints. We achieved a volume of 20 Gy (V20) as follows,

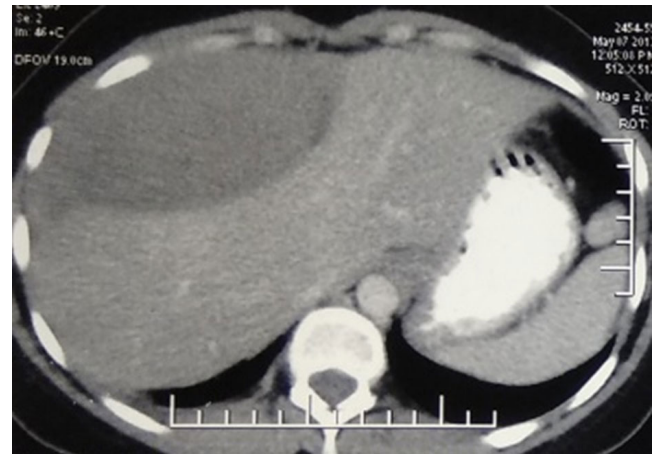


Figure 1: Axial image of computed tomography chest at level of liver



Figure 2: Axial image of computed tomography chest at level of heart

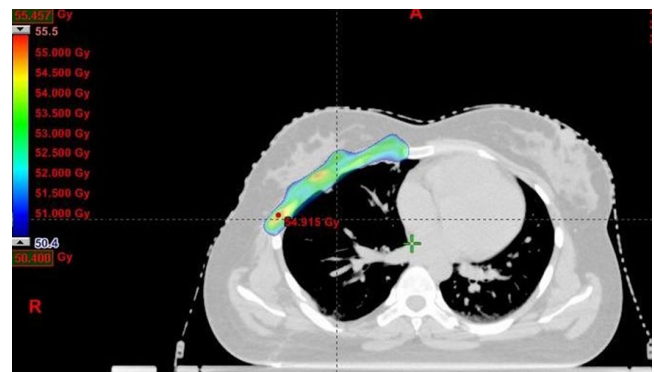


Figure 3: Axial image of radiation dose distribution

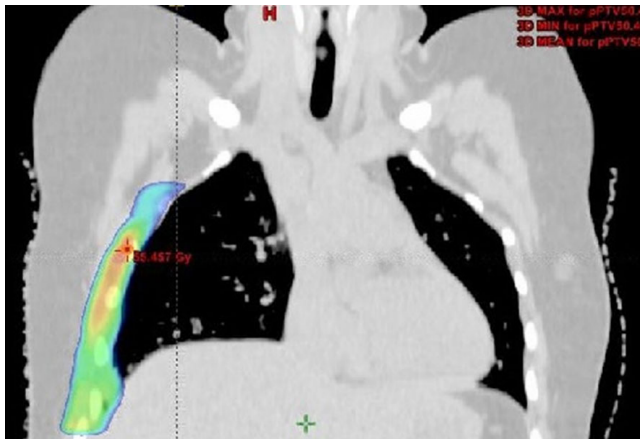


Figure 4: Coronal image of radiation dose distribution

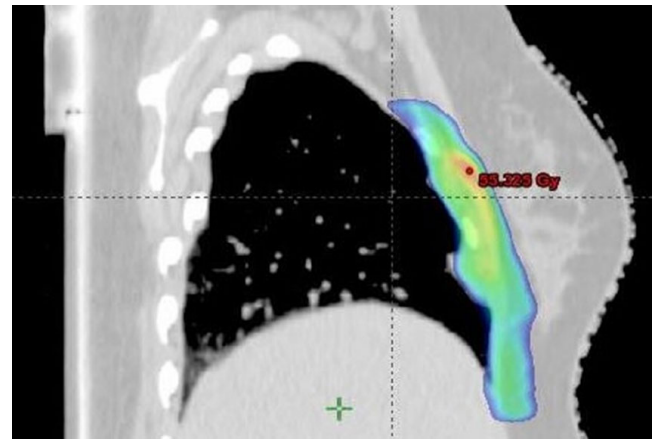


Figure 5: Sagittal image of radiation dose distribution



Figure 6: Axial image of magnetic resonance imaging chest at 3.5 years

21% for right lung, 16.6% for liver and 1.8% for heart. Maximum dose to left lung was 10.6 Gy. The patient tolerated the treatment well and did not report any toxicity. She is in regular follow-up since then. After a follow-up of 3.5 years, the patient is symptomatically free and clinical and radiological examination reveal results within normal limits as did a recent magnetic resonance imaging of the chest [Figure 6]. Presently the patient is not experiencing any skin fibrosis, pulmonary and cardiac toxicity. Bilateral breasts are also normal.

DISCUSSION

Desmoid tumors are an aggressive fibroblastic proliferation of well circumscribed, locally invasive, differentiated fibrous tissue. On gross examination, they appear as dense, rubbery gray-white masses.^[5] The most common locations for desmoid tumors include the shoulder, chest wall, and thigh. Males and females of all ages can be affected by desmoid tumors, but a propensity for fertile women has been noted by many authors.^[6] Possible risk factors include

estrogen status, pregnancy, physical and surgical trauma, radiation, skeletal abnormalities, and genetic determinants.^[7] Our patient was of a fertile age group but not pregnant.

Fibromatosis of the chest wall usually presents in the form of a tumor of various sizes which is often large. In the literature, the size of tumors has been reported as being from 5 cm to 10 cm and is rarely larger than 20 cm. In the series by Kabiri *et al.*,^[3] tumor size varied from 2 cm to 13 cm with a mean of 6 cm. In another series, the average tumor was 8.75 cm.^[8] In our patient maximum dimension was 21 cm. This is a larger chest wall fibromatosis than has been reported thus far. This fibromatosis has only become symptomatic due to the mechanical compression of neighboring organs.^[9] In our patient, the tumor was compressing a lung, hence, the patient was having breathlessness.

The primary mode of treatment for desmoid tumors is surgical excision. However, surgery alone has resulted in high recurrence rates.^[10] The goal of surgical excision is gross total resection with negative margins. Recurrence of desmoid tumors may be related to the age of the patient, the site of tumor, and the initial form of treatment. In a retrospective study of 142 patients by Fiore *et al.*,^[11] it was found that larger tumors and those tumors located on the trunk were associated with a higher risk of recurrence. Local control rates have been reported to be a function of tumor location, ability to obtain negative margins, and adjuvant therapy. In cases of R0 resection, a patient can be kept on observation but post-operative radiation is to be considered for larger tumors as it reduces the risk of loco-regional recurrence. For R1 resection (microscopic positive margins), either re-surgery or high dose radiation (66-70 Gy) is recommended. Post-operative radiotherapy reduces the risk of recurrence in patients with positive margins, improves local

Table 1: Chest wall fibromatoses treatment and results

Study	Number of patients	Treatment	Result
Zehani-Kassar <i>et al.</i> ^[8]	6	Surgery	Recurrence in 1 patient
Abbas <i>et al.</i> ^[18]	53	Surgery + radiotherapy	37.5% recurrence probability
Varghese <i>et al.</i> ^[14]	1	Surgery	No recurrence
Sakamoto <i>et al.</i> ^[19]	1	Surgery + radiotherapy	At 15 months: no recurrence

control and improves progression-free survival. For R2 resection (macroscopic positive margins), high dose radiation followed by boost (70-76 Gy) has been recommended.

Systemic therapy using NSAIDs, hormonal/biological agents, or cytotoxic drugs also plays an important role in patients with desmoid tumors.^[12] In a prospective study, tamoxifen along with sulindac has been used for disease stabilization in recurrent or progressive disease after surgery. Interferon-alpha, toremifene and doxorubicin, vinblastin and methotrexate-based chemotherapy and tyrosine kinase inhibitors (imatinib, sorafenib) have also been used in recurrent progressive tumors after surgery.

The overall rate of recurrence ranges between 25% and 75%. There is a huge variation in rate in the literature.^[9,13] Although survival at 5 years is nearly 93%, the probability of recurrence is an estimated 29%.^[14] Even though these tumors do not metastasize, they can result in significant morbidity and death from locoregional invasion.^[15] The role of adjuvant radiotherapy after surgical resection of primary disease is controversial and should be based on a balanced discussion of potential morbidity from radiotherapy and recurrence. The local control of desmoid tumor in the adjuvant setting is excellent, with total doses ranging from 50-60 Gy, with acceptable morbidity. Margin status is one of the most important predictor of recurrence after surgery in desmoid tumors. If a future local recurrence would incur even greater morbidity or would be potentially unresectable, then adding adjuvant radiotherapy would be reasonable. The benefit of radiotherapy has been claimed in several reports. In particular, a review by Nuytens *et al.*^[16] including more than 20 retrospective studies focusing on the role of the combination (surgery and radiotherapy), showed that surgery plus radiotherapy or radiotherapy alone could obtain a better local control rate (75% and 78%, respectively) compared with surgery alone (61%). However, this is an extremely debated topic. According to Gronchi *et al.*,^[17] these tumors represent a relatively benign condition and most of the patients are young; hence the authors suggest radiotherapy only for documented progressive disease and in absence of other alternatives. Table 1 shows some literature on chest wall fibromatoses with their

treatment and results.

In our patient, the clinical history was short, covering only two and half months. This patient was symptomatic (breathlessness along with pain). Tumor size was extremely large (maximum dimension 21 cm). The operating surgeon was also in favor of postoperative radiation due to potential morbidity in case of future locoregional recurrence.

Hence in spite of the post-operative margins being free, adjuvant external beam radiation was planned. Radiation by conventional techniques leads to increased doses to adjacent normal structures (lungs and breast in this case), which may lead to late complications in the form of fibrosis or secondary malignancies. However, with the use of newer techniques, it is possible to give homogenous dose distribution to the target volume, while keeping the dose to critical and normal structures within normal range. In our case, we chose image-guided intensity modulated radiotherapy technique to spare maximum normal tissues (adjacent breast and lung parenchyma). Tolerance of lungs and heart was well respected. This case provides valuable insights into potential treatment approaches in such a rare presentation.

Authors' contributions

Writing and conceptualization of this work: R. Agrawal, P. Choudhary

Supervising the work: V. Zamre, A.K. Goel, S. Agarwal, D. Singh

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Patient's consent was obtained as per institutional policy.

Ethics approval

The ethics approval was obtained from the institutional ethical committee for preparation and publication of this paper.

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Case Report

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Uterine large cell neuroendocrine carcinoma with unusual colonic metastasis

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ABSTRACT

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A 78-year-old female patient arrived at our practice complaining of progressive abdominal increase and presenting a clinical picture of intestinal obstruction. At physical examination, the abdomen appeared distended, moderately painful with the presence of a mass of hard consistency. Abdominal computed tomography scan showed a large hypodense pelvic mass that indicated a compression and lateral deviation of the uterus and bladder. Microscopically, the mass showed a uniform solid pattern, composed of medium and large-sized cells with hyperchromatic and pleomorphic nuclei demonstrating high mitotic activity and diffuse immunoreactivity for estrogen receptors and synaptophysin. A diagnosis of uterine poorly differentiated large cell neuroendocrine carcinoma, arising in the endometrium with an unusual colonic metastatic localization, was made.

INTRODUCTION

Uterine neuroendocrine carcinomas are rare and highly malignant tumors, morphologically subdivided into small and large cell according to their nuclear size, presenting as pure or combined forms, either associated with endometrioid adenocarcinoma or as a component of a malignant mixed müllerian tumor.^[1,2] It has been reported that the primary site of neuroendocrine tumors (NETs) is unknown in about 13% of patients,^[3] although the exact incidence of

unknown primary NETs has not been fully determined.^[4]

To identify the nature as well as the primary site of NETs, the immunohistochemical approach appears to be the most useful approach. It allows a correct characterization identifying site-specific transcription factors, such as thyroid transcription factor 1 (TTF-1) and CDX2.^[5] In particular, nuclear TTF-1 staining is effective in more than 50% of pulmonary carcinoids but only rarely in gastrointestinal NETs, while nuclear CDX2-staining is revealed in gastrointestinal NETs but seldom in pulmonary carcinoids.^[5] Moreover, in



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poorly differentiated neuroendocrine carcinomas, transcription factors are expressed irrespectively of primary neoplastic site, causing diagnostic problems.^[5]

We report a case of a large cell neuroendocrine carcinoma (LCNEC), initially developed in uterus and clinically silent, with an unusual colonic metastatic localization during the progression of the disease.

CASE REPORT

A 78-year-old female patient arrived in our practice complaining of progressive abdominal increase and presenting a clinical picture of intestinal obstruction. Her past medical history was characterized by an episode of intestinal perforation six months before in another hospital including right hemicolectomy. The histopathological examination of the surgical specimen suggested a poorly differentiated adenocarcinoma with serosal invasion (T4) and lymph node metastases (N1). The patient received 5 successive cycles of chemotherapy and underwent a follow-up examination. Six months later she was admitted to our hospital; at physical examination, the abdomen appeared distended, moderately painful with the presence of a mass of hard consistency. Tumor markers were normal. The patient underwent an abdominal computed tomography scan showing a large hypodense pelvic mass of 21 cm × 15 cm × 19 cm, with peritoneal implants, which caused an important distortion and lateral deviation of the uterus and bladder. Regional lymph nodes were enlarged. The patient underwent debulking surgery. A large neoplastic mass occupied

the abdomen from the transverse colon to the pelvis, infiltrating the small intestine, the anterior parietal and prevesical peritoneum, the sigmoid colon and proximal rectum. The small intestine was disease free for about 160 cm, being dislocated in the left upper quadrant. Surgeons isolated the lesion from the retroperitoneal structures and then removed en bloc the tumor, the small intestine, a small portion of the residual transverse colon, the sigmoid colon, the proximal rectum, the uterus, the uterine annexes, pelvic and anterior parietal peritoneum [Figure 1]. Finally, reconstruction of the digestive tract was performed by creating a mechanical termino-lateral ileo-transverse anastomosis and terminal colostomy. Postoperatively the patient was admitted to the intensive care unit for 7 days. Gastrografen enema, performed on the 8th post-operative day to control the ileo-colic anastomosis, demonstrated the absence of any leakage. Three months later the patient was alive, but was subsequently lost to follow-up.

Representative surgical specimens taken from uterus, colon, peritoneum and small intestine were fixed in 10% buffered formaldehyde for 24 h, completely sampled, routinely processed and paraffin-embedded at 56 °C. Four micron thick sections were cut and routinely stained with haematoxylin and eosin. Immunohistochemical stainings were performed with DAKO Link 48 automated system (DakoCytomation, Copenhagen, Denmark) using commercially obtained mono-or polyclonal antibodies [Table 1].

Microscopically the mass appeared to have developed

Table 1: Source, working dilution and immunostainings regarding the panel of utilized antisera

Antibody	Clone and dilution	Company	Staining
SMA	1A4, w.d. 1:100	DakoCytomation, Copenhagen, Denmark	-
Calretinin	DAK Calret 1, w.d. 1:50	DakoCytomation, Copenhagen, Denmark	-
CD10	56C6, w.d. 1:80	DakoCytomation, Copenhagen, Denmark	-
CD56	123C3, w.d. 1:50	DakoCytomation, Copenhagen, Denmark	-/+
CD117	C-kit, 1:400	DakoCytomation, Copenhagen, Denmark	-
CK	AE1/AE3, w.d. 1:50	DakoCytomation, Copenhagen, Denmark	-
CK 7	OV-TL 12/30, w.d. 1:50	DakoCytomation, Copenhagen, Denmark	-
CK 20	Ks 20.8, w.d. 1:50	DakoCytomation, Copenhagen, Denmark	-
CDX2	DAK-CDX2, w.d. 1:50	DakoCytomation, Copenhagen, Denmark	-
Chromogranin A	DAK-A3, w.d. 1:200	DakoCytomation, Copenhagen, Denmark	++
Desmin	D33, w.d. 1:100	DakoCytomation, Copenhagen, Denmark	++
EMA	E29, w.d. 1:100	DakoCytomation, Copenhagen, Denmark	-/+
ER	1D5, w.d. 1:50	DakoCytomation, Copenhagen, Denmark	+++
Ki67	MIB-1, w.d. 1:75	DakoCytomation, Copenhagen, Denmark	80%
MLH1	G168-728, w.d. 1:100	Cell Marque, Rocklin, California, USA	++
MSH2	G219-1129, w.d. 1:100	Cell Marque, Rocklin, California, USA	++
MSH6	SP93, w.d. 1:50	Cell Marque, Rocklin, California, USA	++
PAX-8	EP298, w.d. 1:500	Cell Marque, Rocklin, California, USA	-
PgR	PgR 636, w.d. 1:50	DakoCytomation, Copenhagen, Denmark	-/+
S100	Polyclonal, w.d. 1:50	DakoCytomation, Copenhagen, Denmark	-
Synaptophysin	DAK-SYNAP, w.d. 1:50	DakoCytomation, Copenhagen, Denmark	++
Vimentin	V9, w.d. 1:50	DakoCytomation, Copenhagen, Denmark	-

CK: cytokeratin; EMA: epithelial membrane antigen; ER: estrogen receptor; PgR: progesteron receptor; CD56: neural cell adhesion molecules; CD117: tyrosine kinase receptor; CDX2: caudal-related homeobox transcription factor; MLH1: MutL homolog 1 colon cancer, non polyposis type 2; MSH2: human homolog of the Escherichia Coli mismatch repair gene mutS; MSH6: protein similar to the MutS protein; PAX-8: protein member of the paired box family of transcription factors; SMA: smooth muscle actin; S100: protein S-100; w.d.: work dilution

in the uterine corpus involving the myometrium and serosal layer, with an infiltration of the colonic wall indicating lymph node metastases as well. The proliferation showed a uniform solid pattern, with complete absence of glandular differentiation and areas of geographic necrosis [Figure 2A]; it was characterized by medium and large-sized cells with hyperchromatic and pleomorphic nuclei, prominent nucleoli and high mitotic activity [Figure 2B]. Immunohistochemistry revealed a diffuse positivity for estrogen receptor (ER) [Figure 3A], chromogranin-A [Figure 3B], synaptophysin [Figure 3C], MLH1, MSH2, MSH6 and a partial staining for EMA, CD56 and progesterone receptor (PgR). No immunostainings

were present for CK, CK 7, CK20, CDX2, TTF1, Pax-8, CD10, vimentin, desmin and CD99. The growth fraction, assessed with Ki67, revealed a positivity of more than 80% of neoplastic elements. A diagnosis of infiltrating poorly differentiated LCNEC was made, based on synaptophysin, chromogranin-A, ER and PgR immunoreactivity. The diagnosis was classified as a primary tumor of the uterus, with extensive colonic and peritoneal spread. In light of these findings, we took the opportunity to re-examine the original neoplastic paraffin-block taken at the colonic level during the first surgical procedure. Histologically the colonic wall was extensively ab-extrinsico infiltrated by a highly cellular solid proliferation [Figure 4A], suggestive of a poorly differentiated adenocarcinoma, but absolutely unreactive for CK20 [Figure 4B], a marker usually positive in colonic cancer. Finally, a heterogeneous, well evident, cytoplasmic staining for chromogranin-A (Figure 4B, inset) was appreciable in neoplastic elements. These morphological data were consistent with a diagnosis of colonic parietal infiltration by aggressive neuroendocrine carcinoma.

DISCUSSION

NETs are more generally identified in the gastrointestinal tract, pancreas, lung and thymus, while in the female reproductive tract they account for about 2% of all gynecologic cancer.^[6,7] According to World Health Organization classification, NETs are classified in two principal groups: poorly differentiated neuroendocrine carcinomas (NECs) and well-differentiated NETs.^[8] NECs include small and large cell neuroendocrine carcinoma, while NETs include typical and atypical carcinoids.^[8]

Poorly differentiated LCNEC of the endometrium is a very uncommon tumor representing only 0.8% of endometrial cancers and they are considered particularly aggressive neoplasms with a tendency for early metastases and poor outcomes.^[9] Usually, endometrial NECs are combined with other epithelial neoplasms; in detail, 50-80% of cases are admixed with FIGO grade 1 or 2 endometrioid adenocarcinoma.^[6,7] To explain this intriguing association it has been hypothesized that some endometrial NECs may arise from the neuroendocrine component of endometrioid carcinomas.^[10] Although the possibility that an abdominal NEC may secondarily develop due to chemotherapy for an original endometrial carcinoma should be mentioned, nevertheless in the present case this option should be excluded since the first diagnosis and consequently the therapeutical approach were based on colonic poorly differentiated carcinoma. It has also been suggested that these tumors can be

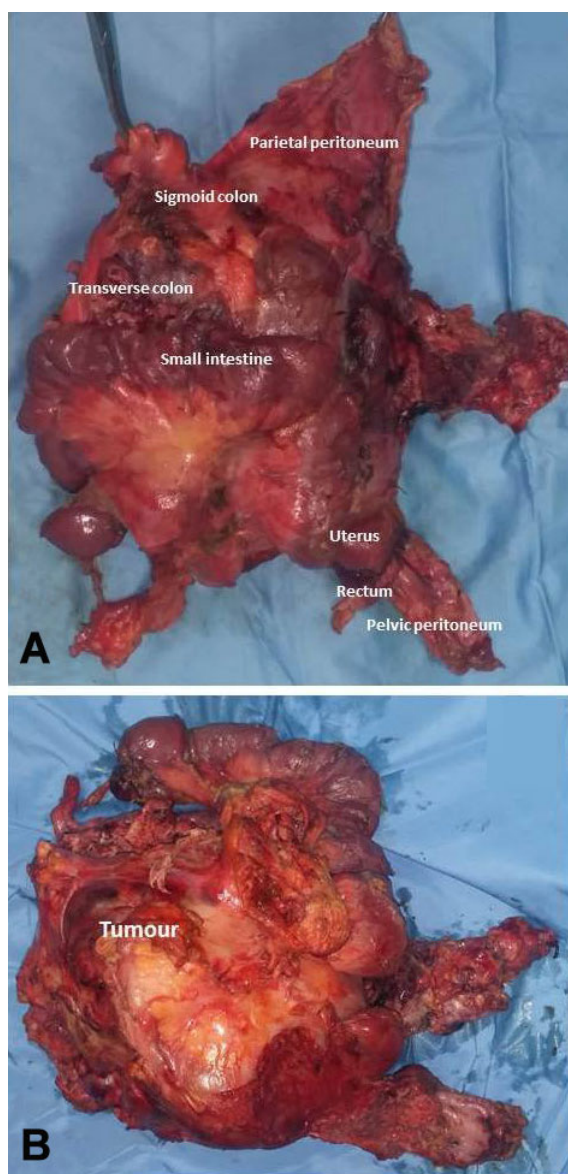


Figure 1: Grossly anterior and posterior appearance of the surgical specimen. (A) Macroscopically, in the anterior view, the relationships between neoplasia and adjacent anatomical structures are seen; (B) grossly, the tumor mass is easily appreciable at the posterior view of the surgical sample

derived from pluripotent stem cells with the possibility for both neuroendocrine and glandular endometrioid differentiation.^[11]

In the current literature, 15 cases of endometrial LCNEC have been described in patients with a mean age of 64 years, 8 of which cases are pure and 7 are associated with another component.^[8] In particular, the pure form LCNEC is characterized by solid sheets with organoid, trabecular or cord-like patterns including peripheral palisading and necrosis areas.^[8] The neoplastic cells have large and abundant eosinophilic cytoplasm with vesicular high-grade nuclei, prominent nucleoli and frequent mitotic figures.^[9] The confirmation of neuroendocrine differentiation is based on neuroendocrine markers,

such as chromogranin, synaptophysin and CD56.^[5] In this case, the diagnosis of endometrial LCNEC was based on neuroendocrine appearance, particularly the neuroendocrine marker expression (synaptophysin and partial CD56 reactivity). In differential diagnoses, endometrial NECs should be distinguished from other tumors characterized by nuclear high-grade features with a predominantly solid growth pattern, such as carcinosarcoma, undifferentiated endometrial sarcoma, solid pattern of serous carcinoma and undifferentiated endometrial carcinoma (UEC). However, the most problematic differential diagnosis is represented by UEC, in which a focal neuroendocrine differentiation (< 10%), with 1 or more neuroendocrine markers, has been demonstrated in 41% in UEC series;^[12] therefore, the expression of neuroendocrine markers in more

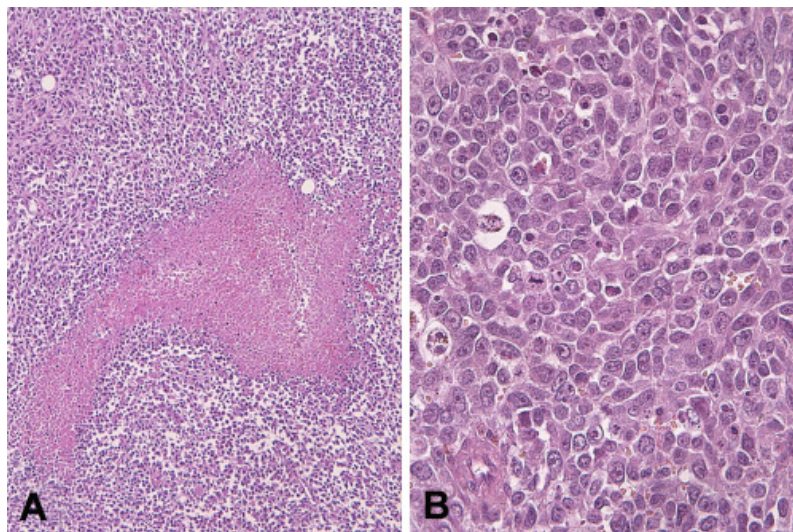


Figure 2: Microscopically the malignant proliferation presented a solid pattern with areas of necrosis (A, hematoxylin-eosin stain, $\times 200$), composed by medium or large-sized cells with hyperchromatic and pleomorphic nuclei, prominent nucleoli and high mitotic activity (B, hematoxylin-eosin stain, $\times 400$)

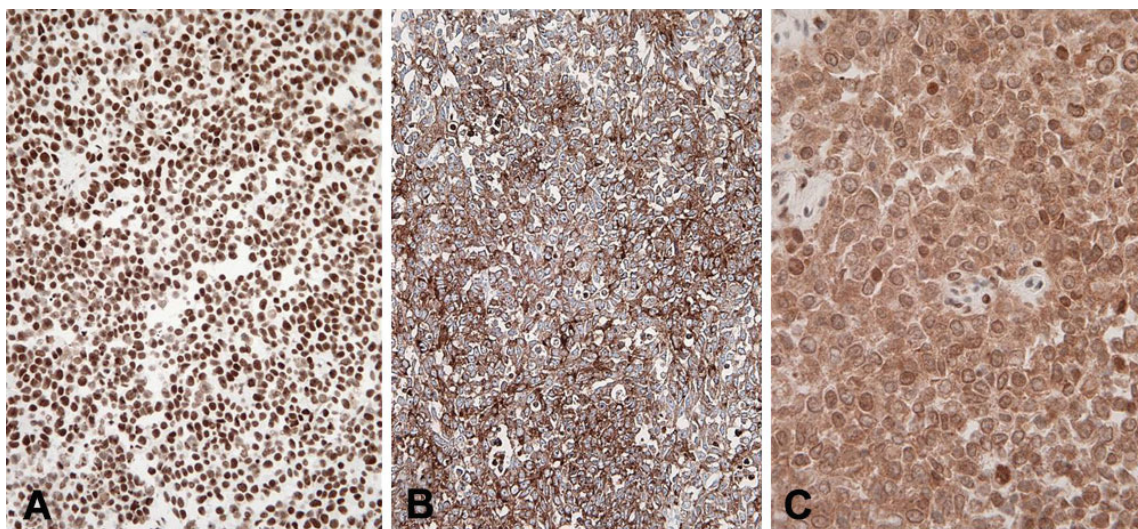


Figure 3: Immunohistochemistry revealed diffuse nuclear reactivity for estrogen receptor (A, $\times 400$); strong and uniform cytoplasmic staining for chromogranin-A (B, $\times 400$, haematoxylin nuclear counterstain) as well as synaptophysin (C, $\times 400$, haematoxylin nuclear counterstain)

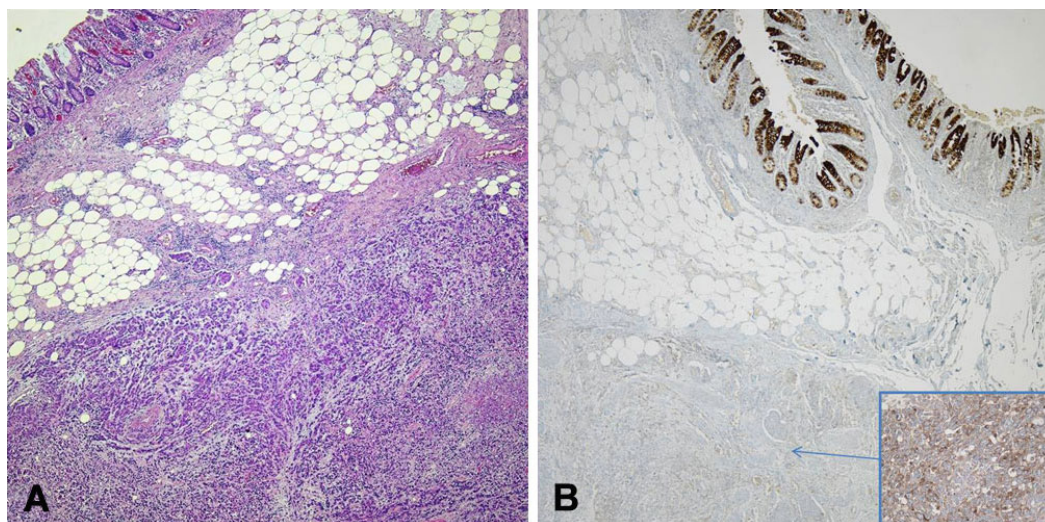


Figure 4: At lower magnification the neoplastic proliferation presented an infiltrative extrinsic pattern of the colonic wall in the first surgical sample (A, $\times 120$, hematoxylin-eosin stain); the neoplasm was unreactive for CK20, (note the positive control of the normal mucosa). The inset showed a patchy not uniform immunoreactivity for chromogranin-A in neoplastic elements ($\times 240$, haematoxylin nuclear counterstain)

than 20% of tumor cells is required to support the diagnosis of endometrial NECs.^[13]

In our case, the most intriguing difficulty was to discriminate between a primary uterine tumor and a uterine metastasis from the previous diagnosed colonic NEC. Consequently, we initially performed an immunohistochemical analysis with typical positive markers of the female genital tract, such as ER and PgR, since these receptors are usually considered useful to define the origin of unknown metastatic carcinoma.^[14,15] However, the neoplastic proliferation in the present case was strongly positive for ER and negative for CDX2; therefore, it was in contrast to gastrointestinal NETs, characterized by a variable nuclear CDX2 and a negativity for ER and PgR.^[5] According to these immunohistochemical findings, we have proposed to apply the same immunohistochemical panel to the colon specimens formerly removed in another hospital, that have confirmed our results supporting the diagnosis of primitive uterine LCNEC.

No prognostic data have been available until now for uterine LCNEC, while only survival data have been reported for cervical small cell neuroendocrine carcinoma (SCNEC). These have showed progression free survival and overall survival (OS) rates of 22% and 30%, respectively, and a median progression time of 9.1 months.^[16] Recently, uterine LCNEC cases have been associated with microsatellite instability (MSI);^[17] in detail, by immunohistochemistry it has been showed a mismatch-repair protein immunoexpression in about 44% of uterine NECs cases, with a prevalence of MLH1/PMS2.^[17] However, an intense nuclear positivity with MLH1, MSH2 and MSH6 was observed in our case. Even though it has been demonstrated

that a subset of gastrointestinal NECs exhibiting MSI showed a better prognosis than NECs without these features,^[18] in uterine NECs the presence of MSI does not appear to be associated with a good prognosis.^[17]

Currently, there is no consensus about the standard treatment of these tumors with either adjuvant chemotherapy or with radiotherapy.^[19] In cervical SCNEC cases, it has been suggested that patients who received platinum-based chemotherapy had both a 3-year recurrence-free survival (RFS) and a 3-year OS of 83%, while those not treated with chemotherapy exhibited RFS and OS of 0% and 20%, respectively.^[16] In our case, the surgical procedure was undertaken to debulk the colonic metastatic localization with additional chemotherapy; nevertheless, taking into consideration the aggressive course and poor prognosis of LCNEC, characterized by the low therapeutic response with a progression of disease, the opportunity of neoadjuvant chemotherapy approach prior to surgery should be considered in the future.

DECLARATIONS

Authors' contributions

Participated in the study design and analysis: A. Ieni, G. Angelico, F. Fleres, A. Macrì, G. Tuccari
Contributed to acquisition of literature data: G. Angelico, R. De Sarro, F. Fleres
Performed the surgical procedures: A. Macrì, F. Fleres
Realized the morphological study: A. Ieni, G. Angelico, R. De Sarro, G. Tuccari
Drafted the manuscript: A. Ieni, A. Macrì, G. Tuccari
Read and approved the final manuscript: A. Ieni, G. Angelico, R. De Sarro, F. Fleres, A. Macrì, G. Tuccari

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There are no conflicts of interest.

Patient consent

A patient's written consent was achieved for scientific institutional purpose.

Ethics approval

All procedures were performed in accordance with the Helsinki declaration. No further ethical approval was necessary to perform the diagnostic routine analysis as well as the immunohistochemical algorithm.

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TGF- β stimulation of EMT programs elicits non-genomic ER- α activity and anti-estrogen resistance in breast cancer cells

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tamoxifen resistance,
transforming growth factor- β

Aim: Estrogen receptor- α (ER- α) activation drives the progression of luminal breast cancers. Signaling by transforming growth factor- β (TGF- β) typically opposes the actions of ER- α ; it also induces epithelial-mesenchymal transition (EMT) programs that promote breast cancer dissemination, stemness and chemoresistance. The impact of EMT programs on nongenomic ER- α signaling remains unknown and was studied herein. **Methods:** MCF-7 and BT474 cells were stimulated with TGF- β to induce EMT programs, at which point ER- α expression, localization, and nongenomic interactions with receptor tyrosine kinases and MAP kinases (MAPKs) were determined. Cell sensitivity to anti-estrogens both before and after traversing the EMT program was also investigated. **Results:** TGF- β -stimulated MCF-7 and BT474 cells to acquire EMT phenotypes, which enhanced cytoplasmic accumulation of ER- α without altering its expression. Post-EMT cells exhibited: (1) elevated expression of EGFR and IGF1R, which together with Src formed cytoplasmic complexes with ER- α ; (2) enhanced coupling of EGF, IGF-1 and estrogen to the activation of MAPKs; and (3) reduced sensitivity to tamoxifen, an event reversed by administration of small molecule inhibitors against the receptors for TGF- β , EGF, and IGF-1, as well as those against MAPKs. **Conclusion:** EMT stimulated by TGF- β promotes anti-estrogen resistance by activating EGFR-, IGF1R-, and MAPK-dependent nongenomic ER- α signaling.

INTRODUCTION

Transforming growth factor- β (TGF- β) normally acts as a suppressor of mammary tumorigenesis by inducing cellular arrest, apoptosis, or differentiation,

and by creating a cell microenvironment that inhibits cell motility, invasion and metastasis. However, during breast cancer development, the tumor suppressing functions of TGF- β is frequently subverted, thus converting TGF- β from a tumor suppressor to a



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promoter of breast cancer growth and metastasis.^[1-3] Although the molecular mechanisms responsible for eliciting the dichotomous activities of TGF- β remain to be fully elucidated, accumulating evidence implicates canonical Smad2/3-dependent signaling in mediating tumor suppression by TGF- β and noncanonical Smad2/3-independent signaling in mediating its tumor promoting activities.^[1-3] Amongst the best characterized noncanonical signaling pathways operant in coupling TGF- β to tumorigenesis are: (1) integrins and components of focal adhesion complexes; (2) MAP kinase and small GTP-binding protein family members; and (3) PI3K/AKT and NF- κ B pathways;^[4] they also function to drive epithelial-mesenchymal transitions (EMT) stimulated by TGF- β , thereby promoting breast cancer dissemination, stemness, and chemoresistance.^[5]

Within the mammary gland, signaling by estrogen receptor (ER- α) plays an essential role not only during glandular development and differentiation, but also during the initiation and progression of luminal breast cancers.^[6-8] Indeed, the oncogenic activities of dysregulated ER- α signaling underlie the clinical success of anti-estrogen drugs (e.g. tamoxifen) as first-line therapies to treat ER-positive breast cancers. However, despite their initial efficacy, anti-estrogen drugs often become ineffective as patient tumors develop resistance and undergo disease recurrence.^[9,10] At present, the mechanisms resulting in acquired anti-estrogen resistance are not fully understood. However, compelling evidence implicates nongenomic ER- α signaling as a major culprit of resistance to anti-estrogen-based therapies.^[9,11-13] Likewise, aberrant expression of a truncated metastasis tumor antigen 1 (MTA1) mutant was found to bind and sequester ER- α in the cytoplasm, thus enhancing the nongenomic actions of ER- α and disease progression in breast cancers.^[14]

Given the pathophysiologic parallels that exist between nongenomic ER- α and noncanonical TGF- β signaling in driving breast cancer progression, we speculated that EMT programs induced by TGF- β may elicit nongenomic ER- α signaling and endocrine resistance in luminal breast cancers. The aim of this study was to test this hypothesis and further our understanding of how EMT programs drive disease progression and acquired resistance to anti-estrogen-based therapies in human breast cancers.

METHODS

Cell lines and chemical inhibitors

Human luminal A MCF-7 cells were obtained from ATCC

(USA) and cultured as previously described,^[15] while human luminal B BT474 cells were kindly provided by Dr. Mark W. Jackson (Case Western Reserve University, USA) and propagated in DMEM supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, USA) and 1% Pen/Strep (Invitrogen, USA). Pharmacological agonists and inhibitors used herein are described in the [Supplementary Table 1](#).

Cell biological assays

Analyzing the effects of TGF- β and EMT programs on ER- α signaling in MCF-7 and BT474 cells was determined as follows: (1) cell proliferation assays: cells were treated in the absence or presence of TGF- β 1 (5 ng/mL; R&D Systems, USA) for 72 h to induce EMT, at which point they were subcultured in 96-well plates (10,000 cells/well) for 5 days with either diluent or inhibitors to the TGF- β type I receptor (T β R-I; 100 ng/mL), the epidermal growth factor receptor (EGFR; 1 mol/L), the insulin-like growth factor 1 receptor (IGF1R; 1 mol/L), mitogen-activated protein kinase kinase (MEK; 10 mol/L), or ER- α (0.1 mol/L; [Supplementary Table 1](#)). Differences in cell growth and survival were determined by incubating the cells with MTS Plus reagent (20 μ L; Promega, USA) for 1 h at 37 °C, followed by measuring absorbance at 490 nm on a Promega Modulus II Microplate Multimode instrument (Promega, USA); (2) 3-dimensional (3D) growth assays: 3D-cultures were prepared by diluting pre- or post-EMT MCF-7 and BT474 cells in complete media supplemented with 5% Cultrex (Trevigen, Gaithersburg, USA), which subsequently were seeded onto solidified Cultrex cushions (500 μ L/well) contained in 6-well plates (150,000 cells/well). Afterward, the cells were cultured in the absence or presence of TGF- β 1 (5 ng/mL), estradiol (1 nmol/L), tamoxifen (0.1 nmol/L), or fulvestrant (0.1 mol/L; [Supplementary Table 1](#)) for 8 days, during which time they were fed every 3 days with full growth media supplemented with 5% Cultrex and pharmacological agents. Differences in organoid growth were calculated using NIH Image J; (3) luciferase reporter gene assays: pre- and post-EMT MCF-7 and BT474 cells were allowed to adhere overnight to 24-well plates (40,000 cells/well). The cells were transiently transfected as described previously^[16,17] with the following reporter plasmids: (a) pSBE-luciferase, which contains 4 copies of the Smad3/4-binding element (4X-CAGA) and serves as a direct measure of canonical TGF- β signaling; (b) p3TP-lux,^[18] which contains 3 copies of TPA-responsive elements and 96 bp of the PAI-1 promoter and responds to both canonical (i.e. Smad3/4) and noncanonical (i.e. AP-1) TGF- β signaling; (c) pERE-TATA-luciferase,^[19] which contains 3 copies of the estrogen response element (3X-GGTACAGTGACC)

and responds to estrogen; (d) pTopFlash-luciferase,^[20] which contains 7 copies of the TCF/LEF-binding sites (7X-AGATCAAAGGgggta) and responds to β -catenin activation; and (e) pCMV- β -gal, which was used to control for differences in transfection efficiency. Afterward, the cells were incubated in the absence or presence of TGF- β 1 (5 ng/mL), estradiol (0.1 nmol/L), 4-OHT (0.1 μ M), or T β RI inhibitor II (100 ng/mL; [Supplementary Table 1](#)) as described previously;^[16,17] (4) direct and indirect immunofluorescence: pre- and post-EMT MCF-7 and BT474 cells were cultured overnight in 8-well chamber slides (80,000 cells/well) prior to being treated for 96 h in serum-free media supplemented with TGF- β 1 (5 ng/mL), estradiol (0.1 nmol/L), 4-OHT (0.1 μ M), or fulvestrant (0.1 μ M; [Supplementary Table 1](#)). Afterwards, the cells were fixed in 4% paraformaldehyde and processed for direct immunofluorescence with phalloidin (0.25 μ M; Molecular Probes, USA), or for indirect immunofluorescence with either anti-E-cadherin (E-cad) or anti- β -catenin antibodies (1:250 dilution; BD Biosciences, San Jose, USA), followed by sequential incubation with biotinylated secondary antibodies (5 μ g/mL; Jackson ImmunoResearch, USA) and Alexa-streptavidin (1.2 μ g/mL; Molecular Probes, USA). Following extensive washing with PBS, the slides were mounted on glass slides with Prolong mounting medium (Molecular Probes) as described previously.^[16,17]

Semi-quantitative real-time PCR analysis

Total RNA from MCF-7 or BT474 cells were purified using the RNeasy Plus Mini kit (Qiagen, USA) according to the manufacturer's instruction. Afterwards, cDNAs were synthesized by iScript reverse transcription kit (Bio-Rad, USA), diluted 5-fold in H₂O, and employed in semi-quantitative real-time PCR reactions (20 μ L) using SYBR Green system (Bio-Rad, USA) that contained 5 μ L of diluted cDNA and 0.1 μ M of oligonucleotide pairs listed in [Supplementary Table 2](#). Differences in RNA concentration were controlled by normalizing individual gene signals to their corresponding β -actin or GAPDH as indicated.

Western blotting analyses

Pre- and post-EMT MCF-7 and BT474 cells were lysed in Buffer H/1% Triton X-100^[21] and solubilized for 60 min on ice. After microcentrifugation, the clarified cell extracts were resolved through 10% SDS-PAGE electrophoresis gels, transferred electrophoretically to nitrocellulose membranes, and blocked in 5% milk prior to incubation with primary antibodies listed in [Supplementary Table 3](#). The resulting immunocomplexes were visualized by enhanced chemiluminescence reactions, and differences in

protein loading were monitored by reprobing stripped membranes with antibodies against either β -actin, ERK1/2, or p38 MAPK as indicated.

Co-immunoprecipitation assays

Clarified cell extracts (1 mg/tube; see above) prepared from pre- and post-EMT MCF-7 cells were incubated under continuous rotation with 1 μ g of antibodies against either β -catenin, E-cad, ER- α , or Src overnight at 4 °C. The resulting immunocomplexes were collected by microcentrifugation, washed, and fractionated through 10% SDS-PAGE gels prior to their immobilization to nitrocellulose membranes, which subsequently were probed with antibodies against either phospho-tyrosine, β -catenin, EGFR, IGF1R, or ER- α [[Supplementary Table 3](#)]. Differences in protein loading and/or input were monitored by immunoblotting with corresponding antibodies as indicated.

Statistical analysis

Statistical values were defined using an unpaired Student's *T*-test, where a *P* value < 0.05 was considered significant. *P* values for all experiments analyzed are indicated.

RESULTS

TGF- β induces EMT in MCF-7 cells and potentiates noncanonical TGF- β signaling

EMT programs stimulated by TGF- β have been associated with the acquisition of motile and metastatic phenotypes, and with the generation of cancer stem cells and chemoresistance.^[4,22,23] With respect to cancers of the breast, these features of EMT are more commonly associated with basal-like/TNBC subtypes, with little evidence related to how TGF- β and its stimulation of EMT programs impact ER- α signaling in luminal breast cancers. To address this question, we administered TGF- β 1 to human MCF-7 cells (i.e. luminal A subtype) to induce an EMT program under 2D- and 3D-culture conditions. [Figure 1A](#) shows that MCF-7 cells readily transitioned from epithelial to mesenchymal morphologies when stimulated with TGF- β 1. Moreover, these morphological alterations exhibited by MCF-7 cells coincided with their (1) downregulated expression of the epithelial marker E-cadherin in 3D-cultures [[Supplementary Figure 1A](#)], and (2) upregulated expression of mesenchymal markers vimentin, N-cadherin, Cox-2, and MMP-9 [[Supplementary Figure 1B-D](#)], as well as that of the master EMT transcription factors, Snail, Twist, Zeb1, and Zeb2 [[Figure 1B and C](#)]. Likewise, post-EMT MCF-7 cells showed a striking loss of β -catenin from the plasma membrane [[Supplementary Figure 1E](#)] that reflected its enhanced phosphorylation on tyrosine

residue(s) and reduced capacity to bind E-cadherin [Figure 1D].

We also investigated the impact of EMT programs on MCF-7 cell behavior and intracellular signaling. To do so, we incubated MCF-7 cells in the absence (i.e. pre-EMT) or presence (i.e. post-EMT) of TGF- β 1 for 72–96 h to induce an EMT program, at which point pre- and post-EMT cells were subcultured and transiently transfected with the following reporter genes: (1) pSBE-luciferase, which monitors canonical Smad3/4 signaling stimulated by TGF- β ; (2) pTopFlash-luciferase, which monitors noncanonical β -catenin signaling stimulated by TGF- β ; and (3) p3TP-luciferase, which monitors

canonical Smad2/3/4 and noncanonical AP-1 signaling stimulated by TGF- β . Figure 1E shows that luciferase expression driven by the synthetic SBE promoter was significantly inhibited in post-EMT cells, suggesting that EMT programs suppress canonical signaling Smad-based signaling in response to TGF- β . Accordingly, the coupling of TGF- β to noncanonical β -catenin [Figure 1F] and AP-1 (Figure 1G; p3TP) activation was dramatically augmented in a manner reminiscent of EMT-induced signaling alterations observed previously in basal-like/TNBCs.^[4,24] Interestingly, EMT-associated events transpired in both culture systems (i.e. 2D- and 3D-cultures), although the magnitude of EMT response was typically greater in 2D-cultures, suggesting that

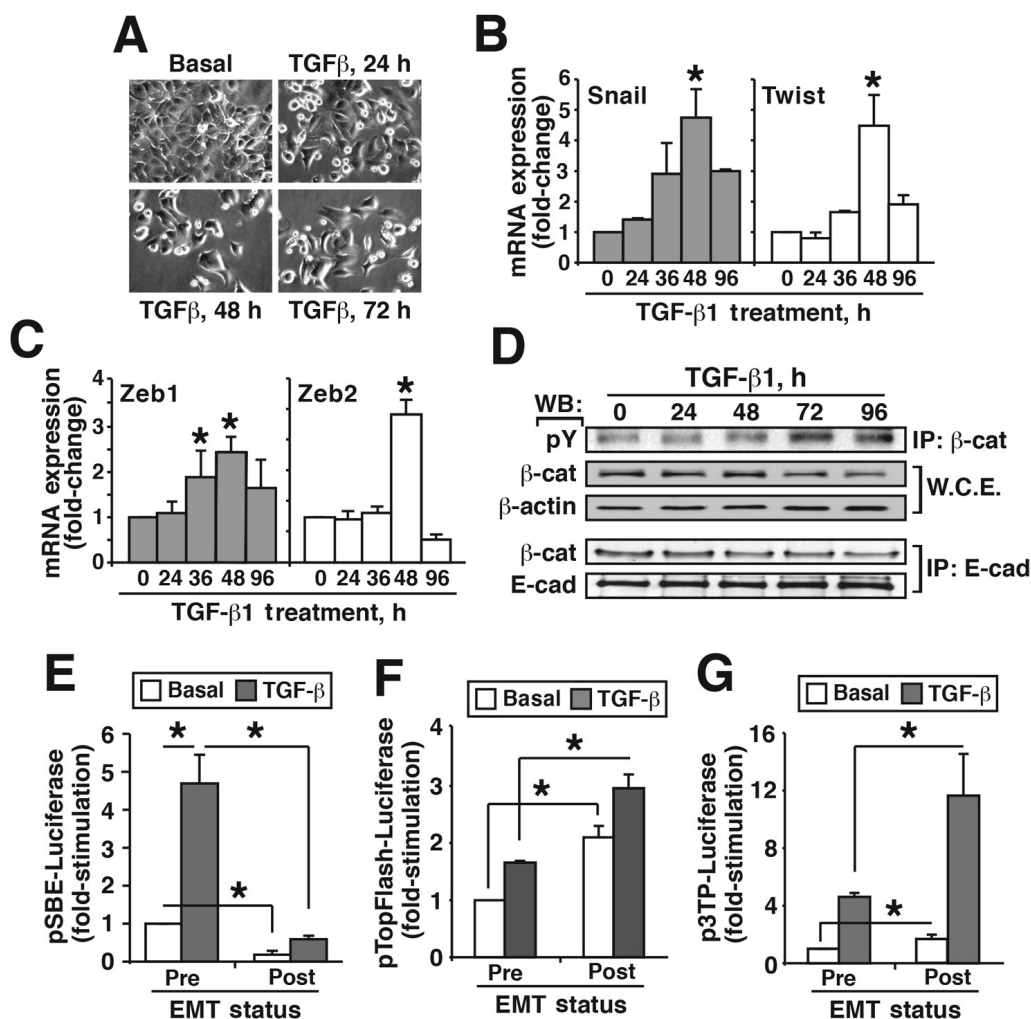


Figure 1: TGF- β induces EMT in MCF-7 cells and potentiates noncanonical TGF- β signaling. (A) MCF-7 cells were treated with TGF- β 1 (5 ng/mL) for 0–72 h to induce an EMT program. Photomicrographs depict accompanying alterations in cell morphology ($\times 400$); (B and C) MCF-7 cells were stimulated with TGF- β 1 (5 ng/mL) for 0–96 h, at which point total RNA was harvested and subjected to real-time PCR to monitor differences in the expression of Snail and Twist (B), and of Zeb1 and Zeb2 (C). Data are the mean fold-changes (\pm SE; $n = 3$; * $P < 0.05$; Student's t -test); (D) MCF-7 cells were treated with TGF- β 1 (5 ng/mL) for 0–96 h, at which point detergent-solubilized extracts were immunoblotted for phospho-Tyr (pY), β -catenin (β -cat), or β -actin as indicated (top). Additionally, E-cad immunocomplexes were captured and immunoblotted for β -catenin (β -cat) and E-cad as indicated (bottom). Images are representative of 3-independent experiments. (E–G) Pre- and post-EMT MCF-7 cells were transiently transfected overnight with the pSBE-(E), TopFlash-(F), or p3TP-(G) luciferase reporter genes, as well as the pCMV- β -galactosidase reporter gene to control for differences in transfection efficiency. Afterward, the transfectants were stimulated overnight with TGF- β 1 (5 ng/mL) prior to measuring luciferase and β -gal activities. Data are the mean fold-changes (\pm SE; $n = 3$; * $P < 0.05$; Student's t -test). TGF: transforming growth factor; EMT: epithelial-mesenchymal transition; SE: standard error

rigid microenvironments potentiate EMT programs stimulated by TGF- β .^[25-27]

Similar induction of EMT programs was observed in human BT474 cells (i.e. luminal B subtype) stimulated with TGF- β . For instance, BT474 cells displayed a more mesenchymal morphology in response to TGF- β [Supplementary Figure 2A]; they also exhibited robust cadherin switching as the cells transitioned from pre- (e.g. E-cadherin dominant) to post-EMT (e.g. N-cadherin dominant) states [Supplementary Figure 2B]. Likewise, BT474 cells undergoing EMT programs exhibited a time-dependent loss of epithelial markers (e.g. β -catenin, CK19, and ZO-1; Supplementary Figure 2C) and gain of mesenchymal markers (e.g. MMP9, Twist, and vimentin; Supplementary Figure 2D). Collectively, these events culminated in the redistribution of E-cadherin [Supplementary Figure 2E] and β -catenin [Supplementary Figure 2F] from the plasma membrane to the cytoplasm, consistent with the ability of TGF- β to induce EMT programs in luminal breast cancers.

Induction of EMT programs by TGF- β promotes ER- α accumulation in the cytoplasm of MCF-7 cells

We next investigated the interplay between TGF- β and ER- α in regulating EMT programs in MCF-7 cells, especially in light of the ability of TGF- β to inhibit ER- α signaling and *vice versa*.^[28] In doing so, we first determined whether ER- α signaling impacted the coupling of TGF- β to EMT programs in MCF-7 cells. Although estradiol treatment had no obvious effect on E-cad localization in MCF-7 cells, hormone administration did suppress the ability of TGF- β to induce the redistribution of E-cadherin from the plasma membrane [Figure 2A]. ER- α was the predominant estrogen receptor expressed in MCF-7 [Figure 2B] and BT474 [Supplementary Figure 3A], indicating that the actions of estrogen are mediated entirely by ER- α in these breast cancer cells. As such, we also monitored the impact of TGF- β on ER- α expression in MCF-7 and BT474 cells. Figure 2C shows that TGF- β transiently increased the synthesis of ER- α transcripts when MCF-7 cells were propagated in 2D-cultures, an event that also trended to occur in BT474 cells [Supplementary Figure 3B]. Conversely, ER- α transcript levels were dramatically repressed when MCF-7 organoids were stimulated with TGF- β in 3D-cultures [Figure 2D]. Although TGF- β clearly regulated ER- α transcription, activation of the TGF- β pathway had little-to-no effect on ER- α protein expression in both MCF-7 and BT474 cells [Figure 2E] despite remaining competent to inhibit luciferase expression driven by an estrogen-response element (ERE; Figure 2F and Supplementary Figure

3C). The antagonist activities of TGF- β on ER- α signaling are magnified in post-EMT MCF-7 cells as compared to their pre-EMT counterparts [Figure 2G]. Mechanistically, we observed TGF- β stimulation of EMT programs to rapidly promote ER- α translocation from the nucleus to the cytoplasm, resulting in ER- α being entirely excluded from the nucleus by 72 h [Figure 2H]. Collectively, these findings suggest that TGF- β and its stimulation of EMT programs inhibit the genomic functions of ER- α by inducing its cytoplasmic sequestration.

TGF- β stimulation of EMT promotes the interaction of ER- α with EGFR, IGF1R and Src in MCF-7 and BT474 cells

Previous studies have shown the ability of cytoplasmic ER- α to interact physically with EGFR and IGF1R, leading to the activation of MAP kinases and resistance to tamoxifen.^[9,11-13] Because EMT programs stimulated by TGF- β resulted in ER- α exclusion from the nucleus, we speculated that EMT programs may underlie the associations of ER- α with receptor tyrosine kinases (RTKs). To test this hypothesis, we determined the impact of TGF- β on the expression of EGFR and IGF1R in luminal breast cancer cells. As shown in Figure 3A-C, TGF- β significantly stimulated the synthesis of EGFR and IGF1R mRNA in MCF-7 cells propagated in either 2D- or 3D-culture. Similar increases in EGFR transcript levels were also observed in BT474 cells stimulated with TGF- β [Supplementary Figure 4A], while the abnormally high levels of IGF1R mRNA in BT474 cells [Supplementary Figure 4B] masked any apparent effects of TGF- β on IGF1R transcript levels. We also observed TGF- β stimulation of MCF-7 and BT474 cells to induce the production of EGFR proteins, which were readily captured in ER- α immunocomplexes [Figure 3D-F]. Likewise, IGF1R was also readily captured in ER- α immunocomplexes isolated from MCF-7 and BT474 cells stimulated with TGF- β [Figure 3F and G]. Finally, Src has been reported to facilitate the extranuclear activities and localization of ER- α in breast cancers.^[29] Accordingly, we also found Src to associate with ER- α in a TGF- β -dependent manner in MCF-7 cells [Figure 3H], suggesting a potential role for Src in facilitating the extranuclear localization and function of ER- α . Collectively, these findings suggest that TGF- β and EMT programs result in the upregulation of EGFR and IGF1R, which form cytoplasmic complexes with ER- α and Src.

TGF- β stimulation of EMT programs enhances EGFR, IGF1R, and MAP kinase signaling in MCF-7 and BT474 cells

The activation of MAP kinases by TGF- β is essential for its stimulation of EMT programs.^[4,30] Interestingly,

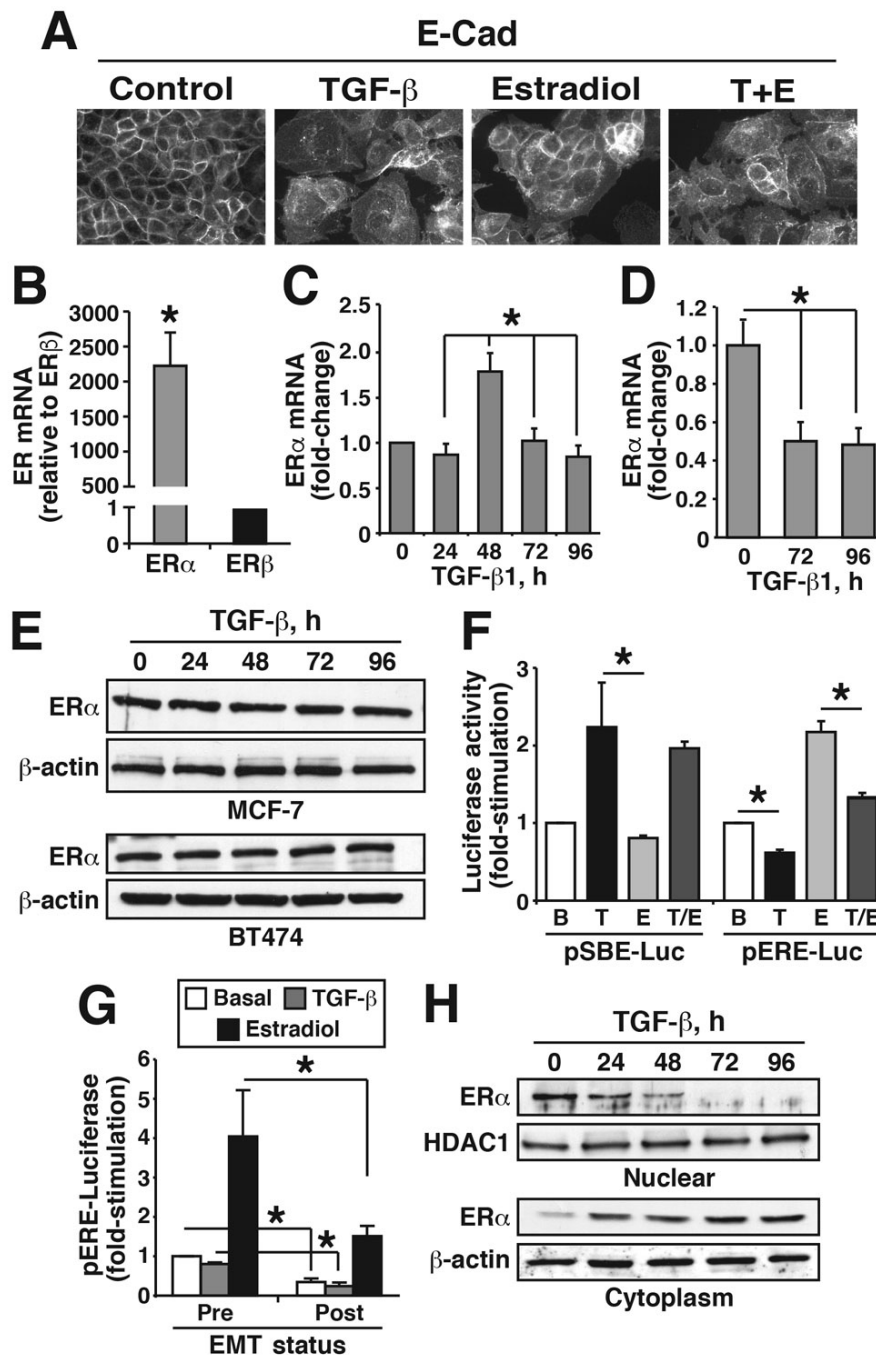


Figure 2: Induction of EMT programs by TGF- β promotes ER- α accumulation in the cytoplasm of MCF-7 cells. (A) MCF-7 cells were treated with either TGF- β 1 (5 ng/mL), estradiol (1 nmol/L), or both agonists for 96 h as indicated. Afterward, the cells were fixed in paraformaldehyde and processed for indirect E-cad immunofluorescence. Images are representative of 3-independent experiments ($\times 400$). (B and C) MCF-7 cells were treated with TGF- β 1 (5 ng/mL) in either 2D- (B) or 3D- (C) cultures for 0-96 h, at which point differences in ER- α expression were measured by real-time PCR. Data are the mean fold-changes (\pm SE; $n = 3$; $*P < 0.05$; Student's *t*-test). (D) Differential mRNA expression of ER- α and ER- β in quiescent MCF-7 cells. Data are the mean (\pm SE; $n = 3$; $*P < 0.05$; Student's *t*-test). (E) MCF-7 (top) and BT474 (bottom) cells were stimulated with TGF- β 1 (5 ng/mL) for 0-96 h. Afterward, ER- α expression levels were monitored by immunoblotting. Images are representative of 4-independent experiments. (F) MCF-7 cells were transiently transfected overnight with either the pSBE- or pERE-luciferase reporter genes, as well as with the pCMV- β -galactosidase reporter gene to control for differences in transfection efficiency. Afterward, the transfectants were stimulated overnight either singly or in combination with TGF- β 1 (T; 5 ng/mL) or estradiol (E; 0.1 nmol/L) prior to measuring luciferase and β -gal activities. Data are the mean fold-changes (\pm SE; $n = 3$; $*P < 0.05$; Student's *t*-test). (G) Pre- and post-EMT MCF-7 cells were transiently transfected overnight with pERE-luciferase and pCMV- β -gal-luciferase cDNAs, followed by 24 h treatment with either TGF- β 1 (5 ng/mL) or estradiol (0.1 nmol/L). Data are the mean fold-changes (\pm SE; $n = 3$; $*P < 0.05$; Student's *t*-test). (H) MCF-7 cells were treated with TGF- β 1 (5 ng/mL) for 0-96 h, at which point the expression of ER- α in cytoplasmic and nuclear cell fractions was determined by immunoblotting. Stripped blots were reprobbed with antibodies to either HDAC1 or β -actin to monitor integrity of nuclear and cytoplasmic fractions, respectively. Images are representative of at least 3-independent experiments. TGF: transforming growth factor; EMT: epithelial-mesenchymal transition; ER: estrogen receptor; SE: standard error

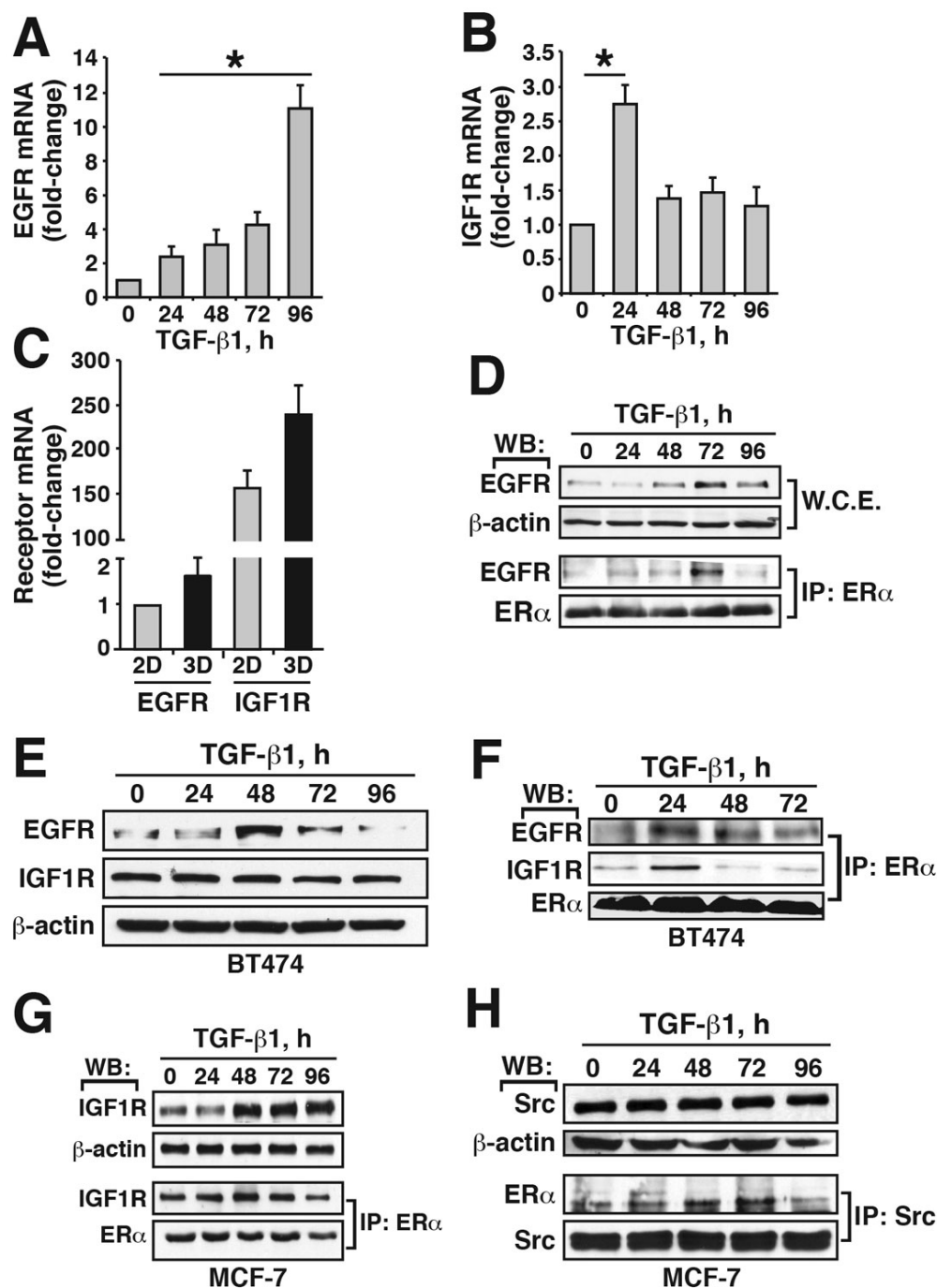


Figure 3: TGF- β stimulation of EMT promotes the interaction of ER- α with EGFR, IGF1R, and Src in MCF-7 and BT474 cells. (A and B) MCF-7 cells were treated with TGF- β 1 (5 ng/mL) for 0-96 h, at which point EGFR (A) and IGF1R (B) transcript levels were measured by real-time PCR. Data are the mean fold-changes (\pm SE; $n = 3$; * $P < 0.05$; Student's t -test). (C) MCF-7 cells were propagated in 2D and 3D cultures. Afterward, total RNA was isolated and subjected to real-time PCR to monitor the expression of EGFR and IGF1R. Data are the mean fold-changes (\pm SE; $n = 3$; * $P < 0.05$; Student's t -test); (D) MCF-7 cells were treated with TGF- β 1 (5 ng/mL) for 0-96 h, at which point detergent-solubilized extracts were immunoblotted with antibodies against either EGFR or β -actin as indicated (top). Additionally, ER- α immunocomplexes were captured and immunoblotted for EGFR and ER- α as indicated (bottom). Images are representative of 4-independent experiments; (E) BT474 cells were stimulated with TGF- β 1 (5 ng/mL) as indicated, and subsequently were subjected to immunoblot analyses to monitor the expression of EGFR, IGF1R, and β -actin. Images are representative of 3-individual experiments; (F and G) BT474 cells were stimulated with TGF- β 1 (5 ng/mL) for 0-72 h, while MCF-7 cells were stimulated with TGF- β 1 for 0-96 h. Afterward, detergent-solubilized extracts were immunoprecipitated with antibodies against ER- α and subsequently were immunoblotted with antibodies against IGF1R and ER- α . Images are representative of at least 3-individual experiments; (H) MCF-7 cells were treated with TGF- β 1 (5 ng/mL) for 0-96 h, at which point detergent-solubilized extracts were immunoblotted for Src or β -actin as indicated (top). Additionally, Src immunocomplexes were captured and immunoblotted for ER- α and Src as indicated (bottom). Images are representative of 4-independent experiments. TGF: transforming growth factor; EMT: epithelial-mesenchymal transition; IGF: insulin-like growth factor; ER: estrogen receptor; EGFR: epidermal growth factor receptor; SE: standard error

nongenomic ER- α signaling is mediated in part through its ability to activate MAP kinases, thereby contributing to the acquisition of tamoxifen resistance in ER-positive breast cancer cells.^[31] Given these parallels and reliance upon MAP kinases, we speculated that TGF- β and EMT programs would elicit the hyperactivation of MAP kinases downstream of upregulated expression of EGFR and IGF1R in post-EMT cells, leading to the initiation of nongenomic ER- α signaling. In testing this hypothesis, we first monitored the activation status of MAP kinases in MCF-7 and BT474 cells when stimulated by TGF- β . Although TGF- β did indeed elicit a modest and transient activation of ERK1/2 in MCF-7 cells [Figure 4A], its ability to stimulate both ERK1/2 and p38 MAPK was greatly potentiated as MCF-7 and BT474 cells transitioned through the EMT program [Figure 4B and C]. These events were specific for MAP kinases as no alterations in AKT phosphorylation were detected under both transient and prolonged TGF- β stimulations (data not shown). Interestingly, Figure 4D shows that administration of the T β R-I inhibitor II to inactivate T β R-I prevented both the upregulated expression of EGFR and the activation of MAP kinases (i.e. ERK1/2 and p38 MAPK) in MCF-7 cells stimulated with TGF- β . Moreover, administration of AG1478 to inactivate EGFR abrogated ERK1/2 activity in post-

EMT MCF-7 cells [Figure 4E]. Similar potentiation of ERK1/2 activity was also observed in post-EMT MCF-7 cells stimulated with either IGF-1, estradiol, or EGF [Figure 4F], a reaction partially dependent upon the greatly magnified activation of IGF1R in these post-EMT cells [Figure 4G]. Collectively, these findings demonstrate that EMT programs induced by TGF- β not only result in the robust stimulation of MAP kinases, but also elicit increased sensitivity and activation of post-EMT breast cancer cells to IGF1, estrogen, and EGF.

TGF- β stimulation of EMT programs promotes tamoxifen resistance in MCF-7 cells

The aforementioned findings showed that TGF- β and its stimulation of EMT programs engendered the nuclear exclusion of ER- α , leading to its (1) physical interaction with EGFR, IGF1R, and Src, and (2) enhanced activation of MAP kinases [Figures 2-4]. We next examined the functional consequences of these events on luminal breast cancer growth and their sensitivity to tamoxifen. In doing so, we first propagated pre- and post-EMT MCF-7 organoids in the absence of presence of ER- α modulators. Figure 5A shows that post-EMT MCF-7 organoids grew more robustly as compared to their pre-EMT counterparts; they were

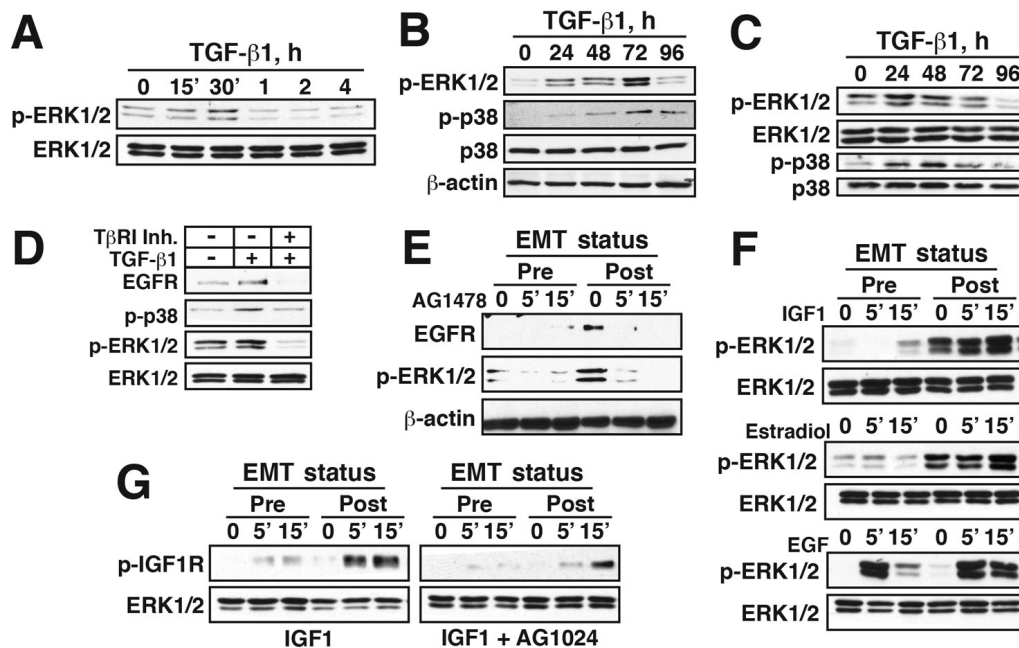


Figure 4: TGF- β stimulation of EMT programs enhances EGFR, IGF1R, and MAP kinase signaling in MCF-7 and BT474 cells. (A and B) MCF-7 cells were stimulated with TGF- β 1 (5 ng/mL) as indicated. Afterward, the activation status of ERK1/2 and p38 MAPK was determined by immunoblotting; (C) BT474 cells were treated with TGF- β 1 (5 ng/mL) for 0-96 h prior to monitoring the activation status of ERK1/2 and p38 MAPK by immunoblotting. (D) MCF-7 cells were stimulated with TGF- β 1 (5 ng/mL) in the absence or presence of the T β R-I inhibitor II (100 ng/mL) for 72 h. Afterward, the expression levels of EGFR and activation status of p38 MAPK and ERK1/2 were determined by immunoblotting; (E-G) pre- and post-EMT MCF-7 cells were treated with AG1478 (1 μ mol/L; E), with IGF-1 (100 ng/mL; top), estradiol (0.1 nmol/L; middle), and EGF (100 ng/mL; bottom; F), or with IGF-1 (100 ng/mL) in the absence or presence of AG1024 (1 μ mol/L; G) as indicated. Afterward, the expression levels of EGFR and activation status of ERK1/2 and IGF1R were determined by immunoblotting as indicated. Data are representative images from at least 3-independent experiments. TGF: transforming growth factor; EMT: epithelial-mesenchymal transition; IGF: insulin-like growth factor; EGFR: epidermal growth factor receptor

also significantly more sensitive to growth inhibition by administration of small molecule antagonists to either IGF1R (i.e. AG1024; [Supplementary Figure 5A](#)) or EGFR (i.e. AG1478; [Supplementary Figure 5B](#)), findings consistent with the ability of post-EMT cells to upregulate their expression of IGF1R and EGFR and activation of ERK1/2 [Figures 3 and 4]. Additionally, post-EMT MCF-7 cells also exhibited significantly increased cell growth and decreased sensitivity to tamoxifen-induced cell death [Figure 5B]. Importantly, co-administration of tamoxifen with small molecule inhibitors against either T β R-I (i.e. T β R-I inhibitor II), IGF1R (i.e. AG1024), EGFR (i.e. AG1478), or MEK1/2 (i.e. U0126) restored MCF-7 cell sensitivity to tamoxifen as determined by MTS [Figure 5B] or clonogenic

[Figure 5C and D] assays. Taken together, these results demonstrated that post-EMT cells acquire resistance to tamoxifen by upregulating EGFR and IGF1R expression and MAP kinase activation, culminating in extranuclear localization and nongenomic signaling of ER- α in MCF-7 cells.

DISCUSSION

The induction of EMT programs by TGF- β plays important roles in driving the progression, dissemination, and recurrence of human breast cancers; these events also underlie the development, expansion, and self-renewal of cancer stem cells, as well as the acquisition of chemoresistant phenotypes.^[4,22,23] Although EMT

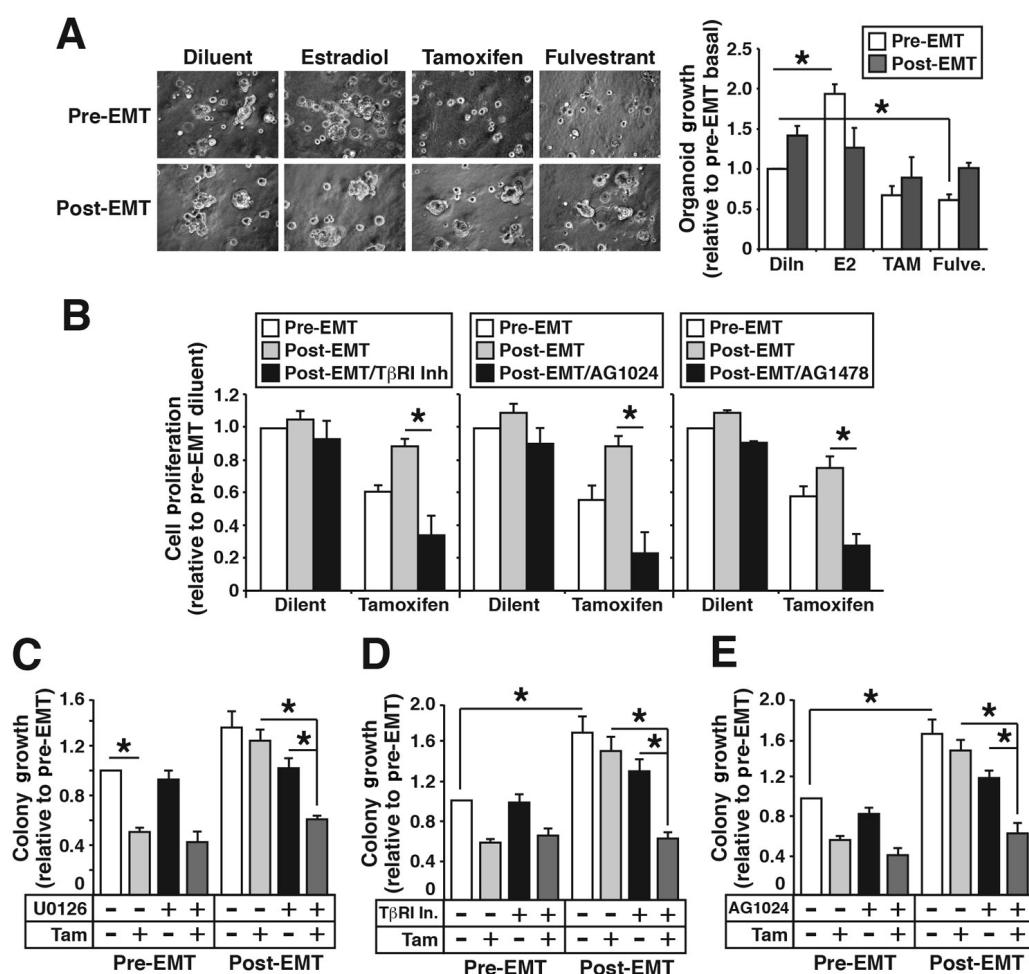


Figure 5: TGF- β stimulation of EMT programs promotes tamoxifen resistance in MCF-7 cells. (A) Pre- and post-EMT MCF-7 organoids were treated with estradiol (0.1 nmol/L), tamoxifen (0.1 μ mol/L), or fulvestrant (0.1 μ mol/L) for 8 days, at which point photomicrographs were captured and analyzed on Image J to assess differences in organoid growth. Images are representative of 3-independent experiments, while data are the mean fold-changes (\pm SE; $n = 3$; $*P < 0.05$; Student's t -test; $\times 400$); (B) pre- and post-EMT MCF-7 cells were treated with tamoxifen (0.1 μ mol/L) in the absence or presence of T β R-I Inhibitor II (100 ng/mL; left), of AG1024 (1 μ mol/L; middle), or of AG1478 (1 μ mol/L; right) as indicated. Afterward, differences in cell growth and survival were analyzed by MTS assays. Data are the mean (\pm SE; $n = 3$; $*P < 0.05$; Student's t -test) growth relative to untreated pre-EMT cells ($*P < 0.05$; Student's t -test); (C-E) pre- and post-EMT MCF-7 cells were treated with tamoxifen (0.1 μ mol/L) in the absence or presence of U0126 (10 μ mol/L; left), of T β R-I Inhibitor II (100 ng/mL; middle), or AG1024 (1 μ mol/L; right) for 10 days, at which point the number of surviving colonies in 11 random fields/plate was enumerated. Data are the mean (\pm SE; $n = 3$; $*P < 0.05$; Student's t -test). TGF: transforming growth factor; EMT: epithelial-mesenchymal transition; SE: standard error

programs are more commonly associated with basal-like/TNBCs, we show herein that luminal A (e.g. MCF-7) and luminal B (e.g. BT474) breast cancer cells not only undergo EMT in response to TGF- β , but that they also exhibit diminished sensitivity to tamoxifen that results from the: (1) upregulated expression of EGFR and IGF1R, which interact physically with ER- α ; (2) hyperactivation states of MAP kinases (e.g. ERK1/2 and p38 MAPK); and (3) extranuclear exclusion and nongenomic functions of ER- α . Importantly, the enhanced tumorigenic activities displayed by post-EMT cells were readily reversed by co-administration of tamoxifen with small molecule inhibitors against either T β R-I (i.e. T β R-I inhibitor II), IGF1R (i.e. AG1024), EGFR (i.e. AG1478), or MEK1/2 (i.e. U0126), thereby laying the foundation for future investigations related to how inactivating EMT programs can be harnessed to improve the clinical outcomes of breast cancer patients.

Several studies previously implicated the nongenomic actions of ER- α in promoting tamoxifen resistance and disease progression.^[9,11-13,32] For instance, the aberrant expression of truncated MTA1 mutants that remain competent to bind ER- α and sequester it in the cytoplasm clearly contribute to the generation of nongenomic ER- α activity.^[14] Indeed, MTA1 expression inactivates hormone-induced nuclear translocation of ER- α , an event that enhances tumor progression and correlates with a loss of ER- α in the nucleus.^[14] Along these lines, ER- α expression and activity also require interactions with growth factor signaling systems to enable luminal breast cancer cells to become insensitive to ER- α modulating agents. For instance, tamoxifen-resistant MCF-7 cells house cytoplasmic complexes comprised of ER- α , EGFR, and Src that elicit hyperactivation of MAP kinases by either EGF or estradiol. Consequently, targeted inactivation of either Src, EGFR, or MAPK not only restores ER- α to the nucleus, but also reestablishes the antitumor activities of tamoxifen in ER-positive tumors.^[12,32] Besides its ability to bind EGFR, ER- α also complexes with the IGF1R at the cell membrane, an interaction involving the adaptor protein Shc.^[31,32] Moreover, tamoxifen-resistant breast cancer cells can also undergo RTK switching as a means to evade targeted inactivation of either EGFR or IGF1R, thereby preserving nongenomic ER- α signaling.^[12] It should be noted that RTK switching is a mechanism associated TGF- β and its stimulation of EMT programs.^[33] Given the parallels between the aforementioned findings and those presented herein, it is tempting to speculate that EMT programs function as essential drivers of nongenomic ER- α signaling and disease progression in luminal breast cancer cells. Future studies need to determine the exact molecular

mechanisms whereby TGF- β and EMT programs elicit extranuclear exclusion of ER- α ; they also need to explore the linkages between EMT programs and ER- α mutations in regulating metastasis and disease recurrence.

As mentioned previously, EMT programs are closely associated with the acquisition of drug-resistant phenotypes.^[4,22,23] Indeed, tamoxifen-resistant MCF-7 cells exhibit post-EMT morphologies (i.e. mesenchymal/fibroblastoid-like) that reflect a loss of E-cadherin expression and a gain of β -catenin signaling, events that were readily reversed following administration of EGFR inhibitors.^[34] A similar dependence upon EMT programs was observed in pancreatic cancers as they acquired resistance to gemcitabine, 5-fluorouracil, and cisplatin, a reaction driven by the upregulated expression of Zeb1. Indeed, genetic inactivation of Zeb1 in post-EMT, chemoresistant pancreatic cancer cells was sufficient to induce a mesenchymal-to-epithelial transition (MET) that reinstated cellular sensitivity to conventional chemotherapeutic agents.^[35] Likewise, resistance to EGFR and FGFR3 inhibitors in cancers of the bladder coincides with their completion of EMT programs engendered by the loss of miR-200 family member expression. Importantly, restoring miR-200 expression in post-EMT bladder cancer cells not only induced their undertaking of MET programs, but also reactivated their sensitivity to EGFR and FGFR3 inhibitors.^[36] Our studies herein reinforce the central importance of EMT programs to elicit chemoresistance in developing and recurring cancers, particularly those arising in the breast. Moreover, we established TGF- β and EMT programs as drivers of tamoxifen resistance and nongenomic ER- α signaling in luminal breast cancers; we also provide a new and potentially impactful approach to eliminate tamoxifen-resistant, post-EMT breast cancer cells through the combined administration of tamoxifen with inhibitors against either T β R-I, EGFR, IGF1R, or MEK1/2, thereby restoring the effectiveness of anti-hormone therapies in ER-positive tumors.

DECLARATIONS

Authors' contributions

Conceived and designed the study: M. Tian, W.P. Schiemann

Performed molecular and cellular analyses: M. Tian

Performed literature search and prepared the manuscript: M. Tian

Revised the manuscript: W.P. Schiemann

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

The study was approved by Institutional Scientific and Ethical Committees.

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Papaya black seeds have beneficial anticancer effects on PC-3 prostate cancer cells

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ABSTRACT

Aim: The study investigated the effect of papaya seeds on prostate cancer (PC) using PC-3 cell line because papaya seeds have effects on the male reproductive system notably decreasing sperm concentration, motility, and viability, leading to azoospermia after short-to-long-term treatment. **Methods:** The black seeds from yellow (ripe) papaya and white seeds from green (unripe) papaya were harvested and then extracted in water, 80% methanol, and hexane. The cytotoxic effects of seeds extracts were determined using a WST-1 proliferation assay. The amount of total polyphenols was determined using Folin Ciocalteu reagent. **Results:** The methanol extracts from black seeds significantly ($P < 0.05$) decreased cell proliferation of PC-3 cells whereas hexane- and water-extracts have no effect. However, the water-extract from white seeds stimulated PC cell proliferation. The black seeds contained significantly more polyphenols than that of white seeds. The data suggest that black seeds from papaya have anticancer effects on PCs whereas white seeds stimulated prostate cancer proliferation. The anticancer effect of black seeds may be because of their high concentration of polyphenols. **Conclusion:** The black seeds from papaya may have a potential to reduce growth of prostate cells; however, consumption of white seeds should be avoided as they may stimulate pre-existing prostate cancer.

INTRODUCTION

The prostate cancer is the malignant tumor of the prostate gland of male reproductive organ, which may be life threatening, when spread to other body parts, predominantly towards lymph nodes, and bones.^[1] According to a report (2014) published by *Cancer Research*, UK, an estimated 14 million cases of cancer

reported worldwide and nearly half-8.2 million people (about 13% of the total worldwide deaths) died from cancer.^[2] The cases of cancer is expected to increase to 24 million by 2035, therefore, the death toll from cancer is expected to increase in the future.

The major factor contributing in prostate cancer is the age, because men who have the prostate cancer are



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between 65 and 80 years, and it is rare in men who are under than 40 years.^[3] The other contributing factor is genetics. For example, men of African-American descent are at a significantly higher risk of developing prostate cancer than white men. In fact, prostate cancer is the fourth most common reason overall for death in African-American men. About 19% of black men, (1 in 5) will be diagnosed with prostate cancer, and 5% of those will die from this disease.^[4] Also, a man who has a member of his family with prostate cancer is more likely to get the prostate cancer compared to another man.^[5] In addition, a man who inherited the faulty BRCA2 gene is more likely to get the more severe type of prostate cancer.^[5]

Obesity also contributes to prostate cancer.^[6] Sex hormones are also involved in prostate cancer development. Reduced testosterone levels have been related to obesity, metabolic syndrome (MS), benign prostatic hypertrophy and even prostate cancer.^[7] Diet rich in high-fat milk and red meat, saturated fats and omega-6 fatty acids was found to increase the risk of prostate cancer whereas diet rich in fruits and vegetable decreases the risk of prostate cancer.^[8-10] An inverse association between high intake of vegetables and/or fruits and incidences of cancer was reported by a number of epidemiological studies.^[11-17] Further, the preventive effect on prostate cancer risk was found for a diet, which was rich in tomato products and lycopene.^[18] Unfortunately, there are conflicting findings on the lycopene-prostate cancer risk relationship and the preventive role of tomato products.^[19,20]

Papaya fruits also contained a significant amount of lycopene and anticancer activities of papaya have been demonstrated in a number of *in vitro* studies.^[21] Papaya juice and pure lycopene caused cell death in the liver cancer cell line, Hep G2.^[22] Papaya seed extract exhibited anticancer activity in acute promyelotic leukemia HL-60 cells whereas papaya pulp extract did not have any effect.^[23]

Papaya has also been used as a traditional medicine in some cultures for male fertility, suggesting its direct role in male reproductive system.^[24] Papaya seeds are natural contraceptive for both man and women. It is traditionally used to affect the fertility in men in a reversible manner. Interestingly papaya seeds have not known for side effects, as these are common with pharmaceutical contraceptive.^[24,25] It appears that Papaya seeds have activity for male reproductive system; it is, therefore, possible that papaya seeds may have anticancer effect against cancer of prostate gland, a vital organ of male reproductive system. We hypothesized that papaya seeds can be effective

in inhibiting prostate cancer cells proliferation and, therefore, may be a good nutraceutical for preventing and/or treating prostate cancer in men. To determine the effect of papaya seeds extract on prostate cancer cell proliferation, we used both white and black seeds extracts for their anticancer activity using PC-3 prostate cancer cells.

METHODS

Materials

PC-3 cells (CRL-1435) and 3T3L1 cells (CL-173) were purchased from ATCC (Manassas, VA 20110), F-12K and DMEM media was purchased from Gibco (Grand island, NY14072), Fetal Bovine Serum (FBS-BBT) was purchased from RAMBIO (Missoula, Montana), antibiotics penicillin and streptomycin (BP2959) and Phosphate Buffered Saline (PBS; BP399-550) was purchased from Fisher (Fair lawn, New Jersey 07410). Green papaya was obtained from Randolph Farm at Virginia State University, whereas golden papaya was purchased by Tex State Distributing LLC (Alaneo, TX). WST-1 (MK400) was purchased from Talkara (Shiga, Japan).

Preparation of papaya seeds extracts

The papaya was washed with distilled water, blotted dry with paper towel, and was cut into half to access the seeds. The seeds were scrapped and washed 3 times with distilled water. The washed seeds were spread on a plastic trays and left for drying in a chemical hood until a constant weight was obtained. The dried seeds were grounded to a fine powder using a mortar and pestle with liquid nitrogen added to keep the powder frozen. The dried powder was flashed with nitrogen and stored at -80 °C until used. A known quantity (5 g) of dried papaya powder was mixed with 100 mL of distilled water, 80% methanol, or hexane and placed on a shaker at room temperature overnight.

The next day, the mixture was centrifuged at 1,500 g for 20 min using a Thermo Scientific centrifuge (Waltham, MA). The supernatant was collected and the residues washed 2 times by suspending them again in the respective solutions, mixing, and placing on shaker overnight. The collected supernatant was pooled together and the residues were discarded. The hexane and methanol extracts were dried in a nitrogen evaporator (Organomation Associates, Inc, Berlin, MA) to dryness and then subjected to freeze drying over night to ensure removal of traces of solvents. The water extract was freeze dried. The dried extract was stored in a -20 °C freezer.

Cell culture

The PC-3 prostate cancer cells were cultured in F-12K media whereas 3T3L1 cells were cultured in DMEM media. Both media were supplemented with 10% FBS and 1% penicillin and Streptomycin. The cells were incubated in a humidified incubator at 37 °C with 5% CO₂. Media was changed every 3 days and cells were subcultured when they became confluent.

Cell proliferation assay

Effect of papaya seeds extract on cell proliferation was determined using a WST-1 assay as per manufacturer instructions. The assay is based on the reduction of WST-1 dye (brown color) by mitochondrial dehydrogenases in viable cells. The reduced dye changes to an orange color and the intensity of color is proportional to number of living cells, which can be estimated by reading at 420 nm in a spectrophotometer. Cells (10,000/well) were initially incubated for 24 h in a 96 well plate as described above. For treatment with extracts, media was replaced by serum-free media containing varying amounts of papaya seeds extract. The dried water extract was dissolved in serum-free F-12 or DMEM media whereas dried methanol and hexane extracts were initially suspended in 50% DMSO. The concentration of stock solution was 250 mg/mL. The extracts were diluted with serum-free F-12 or DMEM media to make 0-250 µg/mL concentrations for treatment. The concentration of DMSO did not exceed to 0.05% and has no effect on cell viability.

Determination of total polyphenols

The extracts of papaya seed was used to determine the total polyphenols as described previously.^[26] Briefly, the extracts was incubated with Folin Ciocalteu reagent (Sigma Chem. Co., St. Louis, MO) and the formation of a blue chromophore from the reduction of phosphotungstic phosphomolybdenum was determined at 765 nm. The total phenolic content was calculated from a calibration curve using Gallic acid as a standard, and the result are expressed as mg Gallic acid equivalent per g dry weight of sample.

Data analysis

The data is expressed as mean ± SD for at least 3 replicates. All comparisons were made by one-way ANOVA with Tukey's-HSD-*post-hoc* test using SPSS Statistics 20 software. All significant differences are reported at $P < 0.05$ and indicated by “*”.

RESULTS

When cells were treated with water extract of papaya black seeds, the cells viability initially decreased

slightly by 20% (non-significant) at 25 g/mL. The cells viability did not change further on increasing the papaya seeds extract [Figure 1A]. When cells were treated with methanol extract of papaya black seeds, the cells viability initially decreased significantly to 60% ($P < 0.05$) in a concentration depends manner up to 25 µg/mL; however, the cells viability was not

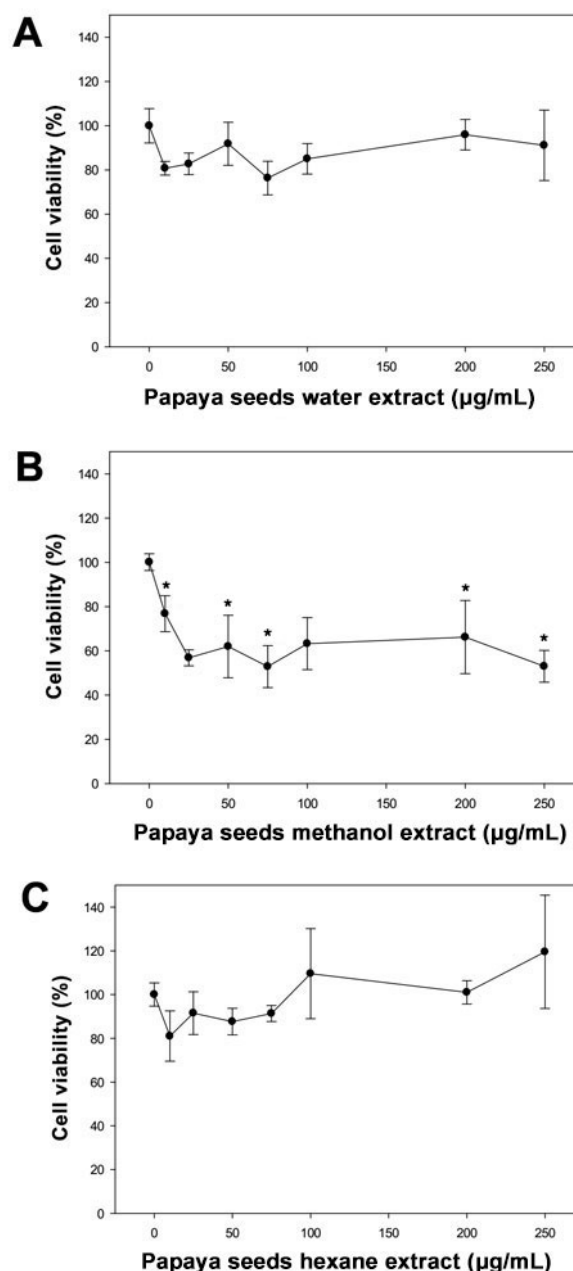


Figure 1: The effect of black papaya seeds on PC-3 prostate cancer cells. Cells (10,000/well) were incubated with different concentration of water (A), methanol (B), and hexane (C) extracts of papaya black seeds in a CO₂ incubator at 37 °C for 24 h. After treatment, cell viability was determined using a WST-1 assay. Results are expressed as mean ± SD for at least 3 replicates. All comparisons were made by one-way ANOVA with Tukey's-HSD-*post-hoc* test using SPSS Statistics 20 software. *Significant differences between treated and untreated groups were reported as $*P < 0.05$

decreased further by increasing the concentration of methanol extract over 25 $\mu\text{g/mL}$ [Figure 1B]. However, when cells were treated with hexane extract of papaya black seeds, the cells viability didn't show any significant change [Figure 1C].

When cells were treated with water extract of papaya white seeds, the cells viability initially did not change up to 50 $\mu\text{g/mL}$; however, by further increasing the concentration of papaya extract, the cells proliferation increased significantly ($P < 0.05$) up to 140% [Figure 2A] at 250 $\mu\text{g/mL}$. When cells were treated with methanol extract or hexane extracts of papaya white seeds, the cells viability initially decreased slightly by 20% or 10% (non-significant), respectively at 50 $\mu\text{g/mL}$. The cells viability did not change further on increasing the concentration of papaya seeds extract [Figure 2B and C].

We have further tested methanol, hexane, and water extracts of black and white seeds on non-cancerous undifferentiated 3T3L1 fibroblasts [Figure 3]. The hexane extracts of either black or white seeds almost have no cytotoxic effect on the growth of 3T3L1 fibroblasts with the exception of black seeds extract that exhibited a marginal increase in cell proliferation ($P < 0.05$) at 250 $\mu\text{g/mL}$. The methanol extract of white seeds was also slightly cytotoxic (10-15%, $P < 0.05$) at 150 $\mu\text{g/mL}$ or higher doses. In contrast, water extracts from both white and black seeds caused cytotoxic effects in these cells in a dose dependent manner. The white seed inhibited cell proliferation by 50% at 250 $\mu\text{g/mL}$ whereas black seeds have a lesser effect and inhibited cell proliferation only by 25% at this concentration.

Data presented in Figure 4 indicates that black seeds contained significantly more polyphenols than that of white seeds. The total polyphenols were almost twice in black seeds than that in the white seeds (black seeds, 5.2 mg/g dry powder vs. white seeds 2.5 mg/g dry powder; $P < 0.05$).

DISCUSSION

The present study was conducted to examine the effects of papaya seeds extracts on prostate cancer. The seeds account for only 7% of papaya weight but typically discarded. Different parts of papaya have been used to prevent or treat a number of diseases. Oil extract of papaya seed have shown antifungal activity.^[27] The fatty acids identified in the seed extracts of papaya (from ripe fruit) were able to reduce the number of *Trypanosoma cruzi* parasites from both parasite stages, blood trypomastigote and amastigote

(intracellular stage) in mice.^[28] The chloroform extract of the papaya seeds showed contraceptive efficacy without adverse toxicity, mediated through inhibition of sperm motility.^[29] The methanolic extract of the seeds also showed antifungal activity against *Aspergillus*

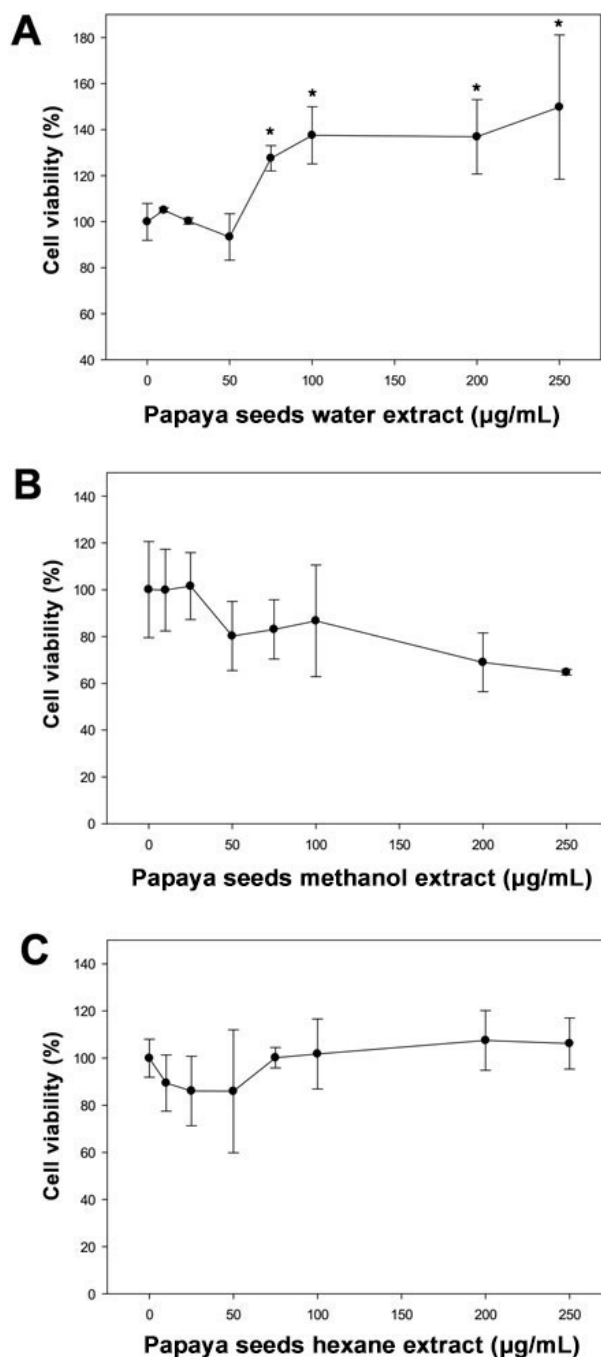


Figure 2: The effect of white papaya seeds on PC-3 prostate cancer cells. Cells (10,000/well) were incubated with different concentration of water (A), methanol (B), and hexane (C) extracts of papaya white seeds in a CO_2 incubator at 37 $^{\circ}\text{C}$ for 24 h. After treatment, cell viability was determined using a WST-1 assay. Results are expressed as mean \pm SD for at least 3 replicates. *Significant differences between treated and untreated groups were reported as $*P < 0.05$

flavus, *Candida albicans* and *Penicillium citrinum*.^[30] Furthermore, treatment of methanolic extract of the papaya seeds in rats induced gastroprotection without signs of toxicity. This effect seems to involve sulfhydryl compounds, increased mucus, and reduced gastric acidity.^[31]

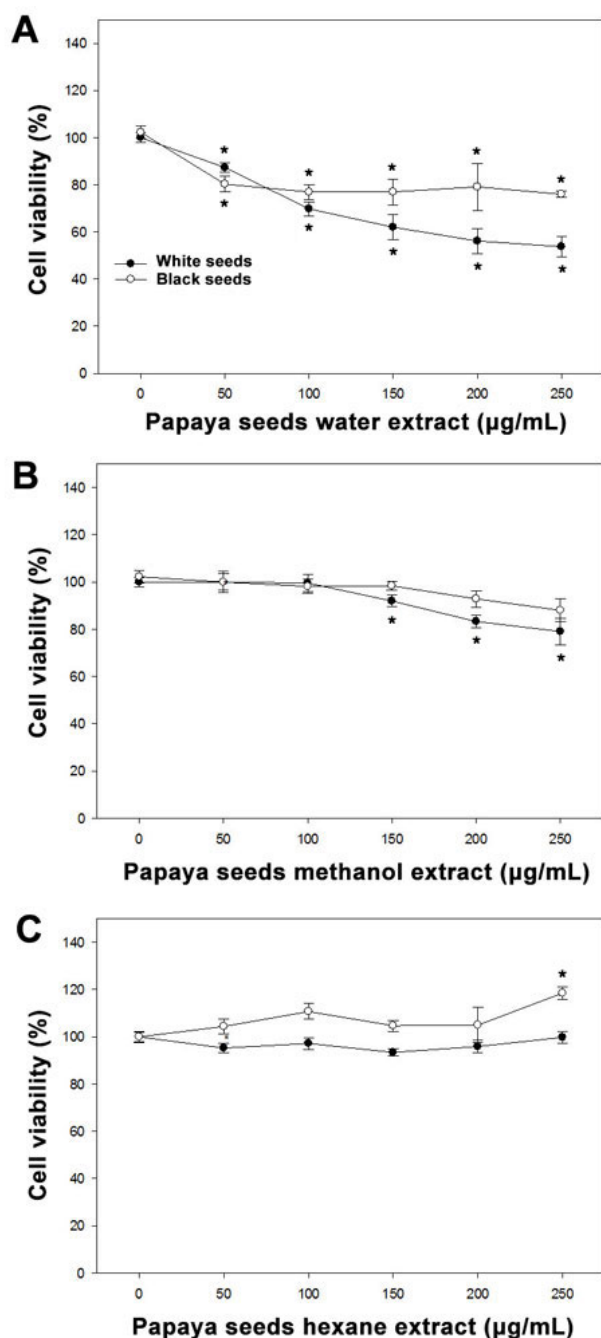


Figure 3: The effect of Papaya seeds extract on non-cancerous undifferentiated 3T3L1 fibroblasts. Cells (10,000/well) were incubated with different concentration of water (A), methanol (B), and hexane (C) extracts of papaya white or black seeds in a CO₂ incubator at 37 °C for 24 h. After treatment, cell viability was determined using a WST-1 assay. Results are expressed as mean ± SD for at least 3 replicates. *Significant differences between treated and untreated groups were reported as **P* < 0.05

In addition to various biological activities described above, anticancer activities have been demonstrated by papaya extracts in a number of *in vitro* studies.^[21] Papaya juice and pure lycopene, a component present in papaya, caused cell death in the liver cancer cell line, Hep G2, with the half maximal inhibitory concentration (IC₅₀) of 20 µg/mL and 22.8 µg/mL, respectively.^[22] Papaya seed extract also exhibited anticancer activity in acute promyelotic leukemia HL-60 cells at IC₅₀ of 20 µg/mL whereas papaya pulp extract did not have any effect even at a concentration of 100 µg/mL.^[23] In contrast, Garcia-Solis *et al.*^[32] observed that papaya pulp inhibited proliferation of MCF-7 cells after 72-h treatment. In our studies, we have observed almost 50% growth inhibition at 25 µg/mL of methanol seeds extract on PC-3 cells. These results are in the similar range of concentration as shown by other studies.^[22,23] This effect does not appear to be due to residual methanol contamination because the methanolic seeds extract after dryness under nitrogen gas was subjected to freeze drying under vacuum over night to ensure complete removal of methanol. Furthermore, from both seeds extracts only methanolic white seeds extract has a small (15% inhibition) effect (*P* < 0.05) on 3T3L1 fibroblasts [Figure 3], again suggesting unlikely effect of methanol in the extracts.

The effect of papaya on cancer growth inhibition are further evident from studies performed by Morimoto *et al.*^[33] who patented the extracts of different parts of papaya for the prevention, treatment, or improvement of many types of cancer, including stomach, lung, pancreatic, colon, liver, ovarian, neuroblastoma, lymphoma, leukemia, and other blood cancers. Effect of papaya leaves have

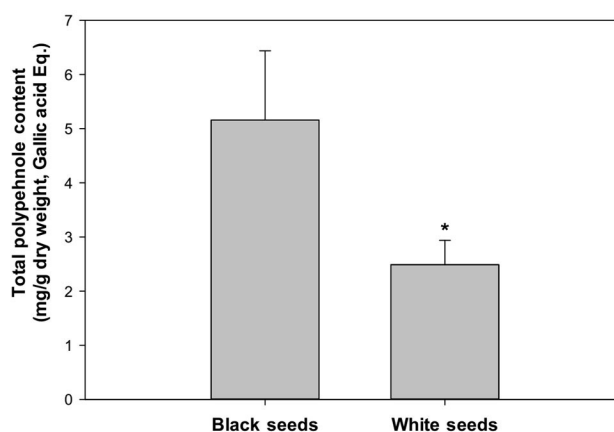


Figure 4: Total phenolic contents in black and white papaya seeds. The phenolic contents in black and white extracts were determined using a Folin Ciocalteu reagent (Sigma Chem. Co., St. Louis, MO) and the total phenolic content was calculated from a calibration curve using Gallic acid as a standard, and the result are expressed as mg Gallic acid equivalent per g dry weight of sample. *Significant differences between black seeds and white seeds were reported as **P* < 0.05

also been characterized on T-cell lines, Burkitt's lymphoma cell lines, chronic myelogenous leukemia cell line, cervical carcinoma cell line, hepatocellular carcinoma cell lines, lung adenocarcinoma cell line, pancreatic epithelioid carcinoma cell line 1, pancreatic adenocarcinoma cell line, mesothelioma cell lines, plasma cell leukemia cell line, anaplastic large cell lymphoma cell line, breast adenocarcinoma cell line (MCF-7), and mesothelioma cell line.^[34] Other studies have shown effect of the aqueous extract of papaya seeds on human kidney epithelial cells, human colon epithelial cells, human lung fibroblasts, and human pancreatic cells.^[35] Interestingly no human clinical trials were performed as yet; however, survival was observed in patients with lung cancer, stomach cancer, breast cancer, pancreatic cancer, liver cancer, and blood cancer after drinking papaya leaf extract.^[21] One report suggested that papaya can be effective against prostate cancer because of its lycopene content,^[36] however, to our knowledge no study has been performed to test the effect of papaya on prostate cancer. Furthermore, water extracts from both white and black seeds exhibited cytotoxic effects on non-cancerous undifferentiated 3T3 fibroblasts whereas only methanolic white seeds extracts have a marginal effect and hexane seeds extracts has no cytotoxic effects. The cytotoxic effect (15%) in the methanolic extracts from white seeds occurred at 250 µg/mL concentration which is about 10 times higher than a 50% inhibition which was observed only 25 µg/mL of methanolic black seeds extract. Typically all cancer drugs exhibit some degree of cytotoxic effects on normal cells. Although deviated from normal path, many pathways for cellular replication and repair are similar between cancer and normal cells. However, as cancer cells divide more rapidly than normal cells, the cytotoxic drugs more selectively kill cancer cells whereas the normal cells are able to adapt and recover from toxicity.^[37]

We hypothesized that papaya seeds may possess anticancer activity on prostate cancer because of its effects on male reproductive system. The aqueous extracts from papaya seed have been reported to reversibly decrease the testicular weight and to suppress spermatogenesis, and fertility in rats.^[38] This study suggested that water extract of papaya seeds suppresses the activities of steroidogenic enzymes in the testis of rats, and that this may contribute to reversible suppression of spermatogenesis, a property that gives a possible male contraceptive potential.^[38] Other studies have shown that the seed extract resulted in a significant dose dependent suppression of cauda epididymal sperm motility coinciding with a decrease in sperm count and viability with no developmental

toxicity and teratogenicity which could affect pregnancy, implantation, and gestation.^[39] However, another study reported that low dose aqueous crude extract of papaya seeds did not adversely affect prenatal development, whereas high doses of papaya extracts resulted in abortifacient property indicating that papaya toxicity can adversely affect the fetus.^[40] These studies suggest that papaya has wide range of activities on cellular targets in male reproductive system. We, therefore, tested the effect of papaya seeds on prostate cancer cells.

Unripe papaya contains white seeds whereas ripe papaya contains black seeds. The different color of seeds indicates that as papaya matures its compositions changes. The presence and absence of different compounds in papaya seeds, therefore, may have different biological properties. We used hexane, methanol, and water to extract compounds of different chemical nature. The hexane was used to extract non-polar compounds. During this study we did not see any effect of hexane extract from either black or white seeds on prostate cancer cells. However, it has been shown that hexane extract of the papaya seed was highly effective in inducing apoptosis or cell death in leukemic HL-60 cells.^[23] Papaya seeds contain considerable amount of oil (27.0%),^[41] comprising 45.9% of oleic acid, 24.1% of palmitic and 8.52% of stearic acid.^[28] Among phytochemicals, the major constituent is benzyl isothiocyanate (99.36%).^[27] The apoptotic effect of papaya seeds on HL-60 cells were comparable to those of authentic benzyl isothiocyanate.^[27] However, lack of hexane effect on prostate cancer cells suggest that benzyl isothiocyanate may not has effect in these cells.

Our data indicate that methanol extract from black seeds was effective in inhibiting cell proliferation of prostate cancer cells whereas the methanol extract from white seeds extract was not effective. Alcoholic extracts are commonly used to extract phytochemicals as it solubilize most of the polyphenols including flavonoids and alkaloids.^[42,43] The Phenolic compounds are a main class of secondary metabolites in plants and possess a number of potent biological activities including anti-oxidation, anti-cancer, anti-bacterial, and immune modulating activities.^[44] We found that black seed extract have almost 2.5 times more polyphenolic contents then white seeds. It is, however, not known what compound is predominantly present in black versus white seeds. It is, therefore, difficult to predict a tentative active compound in our methanolic extract that resulted in prostate cancer cell growth inhibition. Further experiments are required to perform a comparative analysis of phytochemicals

present in the black and white seeds to identify and test the potential anticancer agent in black seeds of papaya.

In contrast to methanolic seeds extract from papaya back seeds, we found that the aqueous extract of papaya white seeds, surprisingly, stimulated prostate cancer cell proliferation whereas aqueous extract of black seeds has no effect. Papaya seeds are rich in various types of phytochemicals including saponins, tannins, polyphenols, flavonols, glucosides, alkaloids, triterpenes, amino acids, sugars, proteins, and vitamins.^[45] Some of the known phytochemicals identified by LCMS-MS technique in the aqueous extract of papaya seeds include 5-hydroxy feruloyl quinic acid, acetyl p-coumaroyl quinic acid, quercetin-3-O-rhamnoside, syringic acid hexoside, 5-hydroxy caffeic quinic acid, peonidin-3-O-glucoside, sinapic acid-O-hexoside, cyaniding-3-O-glucose and methyl feruloyl glycoside.^[46] Again, it is not clear if these phytochemicals are present in black seeds or white seeds. It is apparent from our study that some tumor promoting substance is present in white seeds which is no longer present as the white seeds mature into black seeds when papaya becomes ripe. Clearly, further studies are required to resolve this issue.

To our knowledge we performed first study to demonstrate the effect of papaya seeds on prostate cancer cells. Additional studies are required using other prostate cancer cell lines and cancer cells lines of different origin to validate the specificity of papaya black seeds extracts. The future studies will also be required to characterize phytochemical profile of papaya seeds, to identify targets of intracellular signaling pathways, to determine pharmacokinetics of the active compounds, and toxicological safety of the bioactive fraction in an *in vivo* animal model. These studies will be essential to pave the way for a successful clinical translation.

DECLARATIONS

Authors' contributions

Performed the experiments: K.S. Alotaibi

Analyzed the data: H. Li

Cultivated papaya and contributed in the experimental design: R. Rafi

Conceived and designed the study, and prepared the manuscript: R.A. Siddiqui

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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The synergy of *Helicobacter pylori* and lipid metabolic disorders in induction of Th17-related cytokines in human gastric cancer

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ABSTRACT

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interleukin-17A,
ROR γ t

Aim: To study the impact of *Helicobacter pylori* (*H. pylori*) and lipid metabolic disorder on the expression of Th17-related cytokines in gastric cancer (GC). **Methods:** GC specimens were randomly collected from 42 patients, of whom 15 had *H. pylori* infection and 27 were without. Tumor RNA was extracted for reverse transcription quantitative polymerase chain reaction quantification of gene expression. **Results:** The mRNA levels of interleukin (IL)-6 and leptin, which are known to regulate Th17 differentiation, were upregulated by 20 and 6 folds, respectively, in *H. pylori*-infected compared to uninfected patients. IL-17A and granulocyte-macrophage colony-stimulating factor, two cytokines produced by Th17 cells, were 5- and 6-fold higher in tumors with *H. pylori* infection, respectively. Consistently, ROR γ t, a transcription factor regulating Th17 differentiation, was increased 6-fold in *H. pylori*-positive vs. negative tumors. Further elevation of ROR γ t was seen in advanced *H. pylori*-associated tumors. In addition, *H. pylori* infection was also associated with enhanced expression of CXCL1 (5 folds), chemotactic factor capable of driving bone marrow-derived immature myeloid cells. Interestingly, we observed that *H. pylori*-associated increase of IL-17A was enhanced in the group with higher plasma triglycerides. **Conclusion:** The findings demonstrate a cross-talk and synergistic role of *H. pylori* infection and abnormal lipid metabolism in GC development, at least partly via cooperative induction of Th17 differentiation and activation.



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INTRODUCTION

Gastric cancer (GC) is currently the third leading cause of cancer-related death worldwide due to the highly metastatic property and poor prognosis.^[1,2] The overall 5-year survival rate of GC patients is only between 15 to 35%.^[3] Epidemiological studies show that persistent *Helicobacter pylori* (*H. pylori*) infection accounts for approximate 75% of confound risk factors for GC.^[4-6] Understanding the underlying mechanism of GC development associated with *H. pylori* infection will be important for developing novel therapeutic methods.

H. pylori, a gram-negative spiral-shaped pathogenic bacterium, specifically colonizes and induces damage to the gastric epithelium leading to chronic gastritis, ulcers and even cancer.^[2,7,8] Considerable studies have demonstrated that a mixed response of Th1 and Th17 cells plays a critical role in *H. pylori*-induced inflammatory gastric diseases and cancer.^[9,10] The phenotypes of T helper subsets are determined by the local cytokine milieu and their lineage-specific transcription factors.^[11-13] *H. pylori* elicits Th1 response to produce interferon- γ and tumor necrosis factor- α causing chronic gastritis and ulcers.^[9,13] Th17 cells are also frequently recruited by *H. pylori* to the gastric mucosa, and are characterized by expression of interleukin (IL)-17A/F, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-21, IL-22 and IL-23, and the transcription factor of ROR γ t.^[11,14] While activation of Th17 cells contributes to bacterial eradication,^[15] Th17-mediated immune-response can be detrimental to gastric epithelium during gastritis.^[9,14] Th17 cells can be further activated in tumor microenvironment due to involvement of IL-6 and transforming growth factor- β .^[16,17] Although activation of Th17 cells might have antitumor activity by facilitating the recruitment of other effector immune cells,^[18] Th17-derived IL-17A favors angiogenesis and tumor growth through inducing IL-6 that in turn activates STAT3 signaling to promote tumor survival and angiogenesis.^[19,20]

It has been reported that obesity and diabetes can worsen the process of *Helicobacter*-associated GC.^[19,21] However, the cross-talk between *Helicobacter* infection and metabolic disorders in the gastric carcinogenesis remains not completely understood. We recently demonstrated that high fat diet (HFD) and obesity could strongly enhance *H. felis*-induced GC in mice.^[19] We observed that *H. felis* infection potently stimulates stomach Th17 recruitment and development, and enhanced mobilization of bone-marrow derived IMCs via CXCL1

expression. Interestingly, the local Th17-associated gastric inflammation results in increased IL-17A in blood and causes adipose inflammation in HFD-fed obese mice. In turn, fat-derived IL-6 and leptin can promote gastric Th17 expansion, thus forming a positive loop in Th17 activation. These findings suggest that Th17 and IL-17A play a critical role in the synergy of *Helicobacter* infection and metabolic abnormalities in accelerating GC progression. In present study, we used clinical GC specimens to document Th17-related cytokines and explore the roles of *H. pylori* infection and lipid metabolic disorders in GC development. Our results suggest that dysregulated lipid metabolism may synergize with *H. pylori* to promote GC development.

METHODS

Clinical specimens

Forty-two GC specimens were randomly collected from Fujian Provincial Cancer Hospital in China. *H. pylori* infection was clinically diagnosed and confirmed with the expression of CagA, VacA or both.^[22,23] The patients were also divided into high and normal lipid groups with a diagnostic cut-off of 1.7 mmol/L of plasma triglyceride (TG).^[24] All tumors were histological diagnosed according to the World Health Organization classification. The pathological TNM stage and clinical stages were also recorded.^[25]

Extraction of RNA and quantitative real-time PCR

Total RNA was extracted using Triazol kit (Invitrogen Company, USA) with slight modifications of protocol. The RNA was reverse transcribed using Hifair™ III 1st Strand cDNA Synthesis Kit (Yesen Company, China). The cDNAs were then used in quantitative polymerase chain reaction (qPCR) quantitative analysis of IL-6, leptin, IL-17A, GM-CSF, CXCL1 and ROR γ t mRNA expression levels in ABI 7500 system (Applied Biosystems, Foster, CA) by using Hieff™ qPCR SYBR® Green Master Mix (Yesen Company, China). Their relative levels were normalized to β -actin expression. Specific primers used in this study were listed in Table 1.

Statistical analysis

Data analysis was conducted by using Graph pad 6.0 Software. After log transformation, normal distribution was analyzed. Comparison between the two groups was done using *t*-test and Spearman analysis of correlation was performed between the groups. The contingency were analyzed by using Chi-square testing.

RESULTS

The effects of metabolic milieu on *H. pylori*-induced IL-17A expression

Since obesity was suggested to play an important role in *H. felis*-induced GC by stimulating Th17 response in mice,^[19] we determined the effects of *H. pylori* infection and lipid metabolic disorders on Th17-related cytokines in human GC. Our results showed that IL-17A was 5-fold higher in *H. pylori*-infected than uninfected patients [Figure 1A], while approximately 1.8-fold increase in IL-17A levels was seen in patients with high TG (> 1.7 mmol/L) comparing to those with normal TG [Figure 1B]. However, abnormal TG could not alone induce IL-17A expression as evidenced in *H. pylori*-negative patients [Figure 1C]. *H. pylori* could increase IL-17A expression by 3.2 folds in patients with normal plasma TG, but could further increase IL-17A expression (5.5 folds) in the milieu of high TG content [Figure 1C]. However, the contingency analysis showed that there was no significant

correlation between *H. pylori* infection and metabolic factors including plasma glucose, cholesterol, low density lipoprotein, high density lipoprotein and TG [Table 2]. Taken together, the data demonstrated that *H. pylori* infection and lipid metabolic disorders could synergistically increase IL-17A expression.

H. pylori infection contributes to Th17 differentiation and response

IL-6 and leptin have been reported to promote Th17 differentiation and play roles in tumor progression.^[19,26] Consistently, our results showed that the levels of IL-6 and leptin expression were increased 20 and 6 folds, respectively, in *H. pylori*-infected vs. uninfected tumors [Figure 2A and B]. This data suggested that *H. pylori* infection promoted the expression of factors that regulate Th17 differentiation.

GM-CSF is an additional cytokine released by Th17 cells, and importantly, has been strongly linked to pathogenicity of Th17 cells in other disease states.^[27] Thus, we next examined whether GM-CSF was

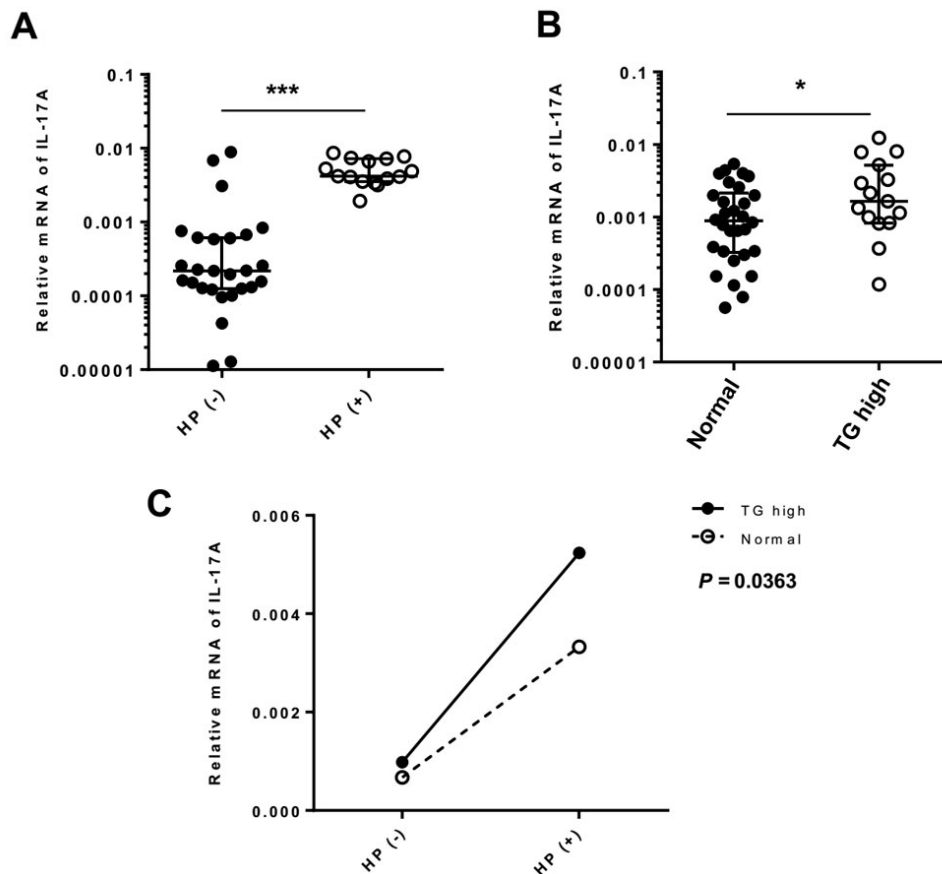


Figure 1: IL-17A expression in *H. pylori*-associated GC was enhanced in abnormal lipid milieu. (A) *H. pylori* induced IL-17A expression. RNA was extracted from GC specimens. The levels of IL-17A were quantitated with RT-qPCR and compared between HP (+) and HP (-) groups; (B) the effect of high TG content on *H. pylori*-induced IL-17A expression. The GC patients were divided into groups with high TG (> 1.7 mmol/L) and normal TG (\leq 1.7 mmol/L). IL-17A levels were compared between these two groups; (C) the synergistic effects of *H. pylori* and aberrant lipid metabolism on IL-17A induction. Regression analysis was employed. *** $P < 0.001$, HP (+) vs. HP (-); * $P < 0.05$, high TG vs. normal TG. *H. pylori*: *Helicobacter pylori*; GC: gastric cancer; IL: interleukin; RT-qPCR: reverse transcription quantitative polymerase chain reaction; TG: triglyceride

associated with *H. pylori* infection in human GC. Indeed, higher levels of gastric GM-CSF were seen in *H. pylori*-positive vs. negative tumors [Figure 3A]. Further, we observed that *H. pylori*-induced GM-CSF expression was closely associated with enhanced IL-17A expression [Figure 3B]. Our results suggest that *H. pylori* infection may activate Th17 responses as evidenced by the induced expression of IL-17A and GM-CSF.

CXCL1 has been demonstrated to be secreted by inflamed stomach and adipose associated with *H. pylori* infection,^[19] acting as a potent mobilizer of bone marrow-derived IMCs. Consistently, our present results showed that the expression of CXCL1 was significantly increased in *H. pylori*-positive vs. negative patients [Figure 3C], and a close correlation of CXCL1

with IL-17A expression was established [Figure 3D].

H. pylori induces tumor progression and ROR γ t expression

In agreement with previous reports,^[28,29] we observed that *H. pylori* infection was related to tumor progression, as *H. pylori*-associated tumors were usually more aggressive than the tumors from uninfected individuals. Tumors larger than 4 cm were seen in 67% of *H. pylori*-infected patients but only in 44% of uninfected patients [Figure 4A]. Consistently, approximately 80% *H. pylori*-associated tumors advanced to T3/T4 stages, whereas 69% of tumors of

Table 1: The primers used in RT-qPCR

Target gene	Primer sequence (5' to 3')
<i>IL-6</i>	AGACAGCCACTCACCTCTTC TTTCACCAGGCAAGTCTCCT
<i>IL-17A</i>	AATCTCCACCGCAATGAGGA ACCAGTATCTTCTCCAGCCG
<i>CXCL1</i>	TCACAGTGTGTGGTCAACAT AGCCCTTTGTTCTAAGCCA
<i>GM-CSF</i>	ATTCTACAAGCCCAGCCAG CCCTCCTTGGCTGAACAGAG
<i>CagA</i>	GAAGCAATCAATCAAGAACC GACTCCCCATTAACACAGAA
<i>VacA</i>	CGGTATCAATCTGTCCAATC AATTCACAAATCTTCCAAA
β -actin	GCGTGACATTAACCACAAGC CCACGTCACACTTCATGATGG

RT-qPCR: reverse transcription quantitative polymerase chain reaction; IL: interleukin; CXCL1: chemokine (C-X-C motif) ligand 1; GM-CSF: granulocyte-macrophage colony-stimulating factor

Table 2: Altered glycolipid metabolic factors and *H. pylori* infection

	HP (+)	HP (-)	P value
GLU			
High	6	14	0.53
Normal	9	13	
CHO			
High	7	10	0.74
Normal	8	17	
LDL			
High	10	18	0.74
Normal	5	7	
HDL			
Low	1	3	1
Normal	14	22	
TG			
High	7	8	0.32
Normal	8	19	

The contingency was analyzed by using Chi-square testing. The cut-offs for diagnosis of metabolic abnormality were: GLU > 6.1 mmol/L, CHO > 5.7 mmol/L, LDL > 3.07 mmol/L, HDL < 0.9 mmol/L, TG > 1.7 mmol/L, according to clinical criteria. *H. pylori*: *Helicobacter pylori*; GLU: glucose; CHO: cholesterol; LDL: low density lipoprotein; HDL: high density lipoprotein; TG: triglyceride

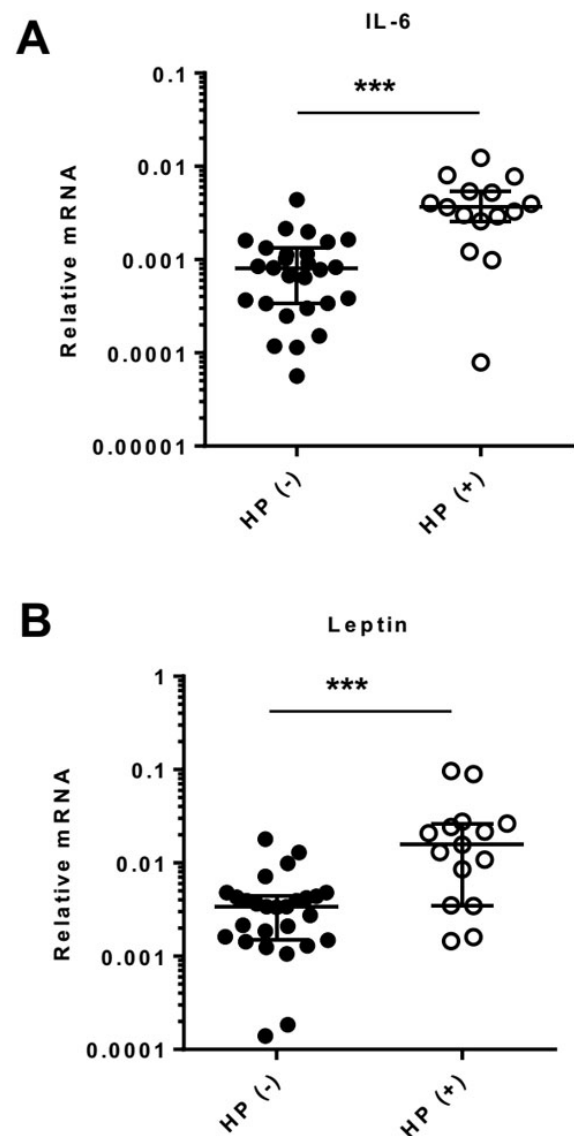


Figure 2: *H. pylori* induced IL-6 and leptin expression. (A) The levels of IL-6 expression was quantitated by RT-qPCR and compared between HP (+) and HP (-) GCs; (B) leptin was similarly assayed to determine the effects of *H. pylori* infection. *** $P < 0.001$, HP (+) vs. HP (-). *H. pylori*: *Helicobacter pylori*; GC: gastric cancer; IL: interleukin; RT-qPCR: reverse transcription quantitative polymerase chain reaction

uninfected individuals did so [Figure 4B].

ROR γ t is the most important transcription factor for the differentiation and activation of Th17 cells.^[30] We thus analyzed ROR γ t expression in *H. pylori*-associated GC and explored its potential role in tumor progression. We found that an overall 6-fold increase of ROR γ t in *H. pylori*-infected vs. uninfected GC [Figure 4C], and that *H. pylori*-associated expression of ROR γ t and IL-17A were positively correlated [Figure 4D]. In the absence of *H. pylori* infection, the levels of ROR γ t were not different between early and advanced tumors [Figure 4E and F]. In contrast, ROR γ t expression was further enhanced in *H. pylori*-associated tumor progression, with higher expression in larger tumors (> 4 cm) [Figure 4E] and those with more metastatic capability (T3/T4 stages) [Figure 4F].

DISCUSSION

It has been long recognized that unresolved inflammation induced by *H. pylori* will favor gastric carcinogenesis. Eradication of *H. pylori*

has been shown to be beneficial in preventing GC development.^[31,32] Obesity and diabetes have become a great problem in modern societies, which profoundly increase the frequencies of malignant neoplasms, including GC.^[21,33,34] Although *H. pylori* infection and metabolic disorders can independently promote tumor progression, there are considerable evidences showing that they can also exert a synergistic effect on tumorigenesis.^[19] However, the molecular mechanisms behind this synergy remain elusive. We previously reported that *H. felis*-induced GC in obese mice can be influenced by the gastric homing and activation of Th17 cells, which trigger a series of inflammatory responses in both stomach and adipose tissues through releasing IL-17A.^[19] Our current results further the concept that chronic *H. pylori* infection and aberrant lipid metabolism can interact to activate Th17 responses and facilitate GC.

We demonstrate that *H. pylori* infection is associated with striking elevation of IL-17A content in GC [Figure 1A]. The expression of IL-17A is also increased in the patients with abnormal high plasma

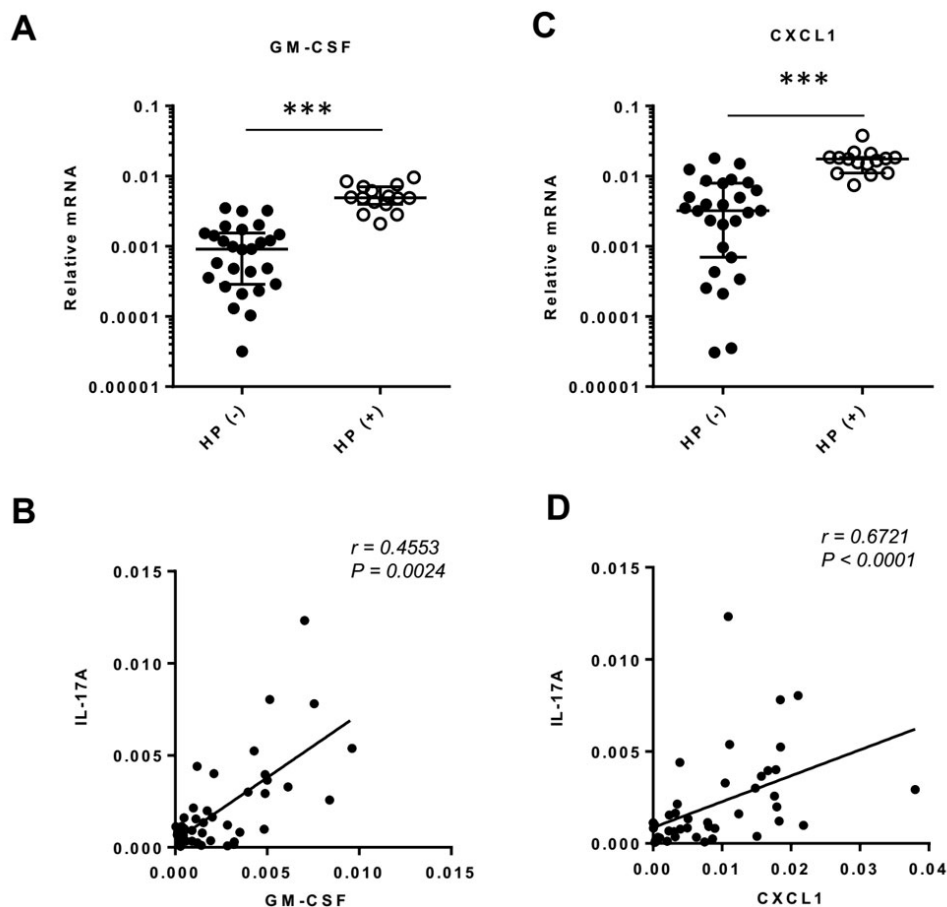


Figure 3: *H. pylori* induced GM-CSF and CXCL1 expression. (A and C) The quantitative assays of GM-CSF (A) and CXCL1 (C) were performed by RT-qPCR. *** $P < 0.001$, HP (+) vs. HP (-); (B and D) the correlation of GM-CSF (B) and CXCL1 (D) with IL-17A expression was analyzed. *H. pylori*: *Helicobacter pylori*; RT-qPCR: reverse transcription quantitative polymerase chain reaction; IL: interleukin; CXCL1: chemokine (C-X-C motif) ligand 1; GM-CSF: granulocyte-macrophage colony-stimulating factor

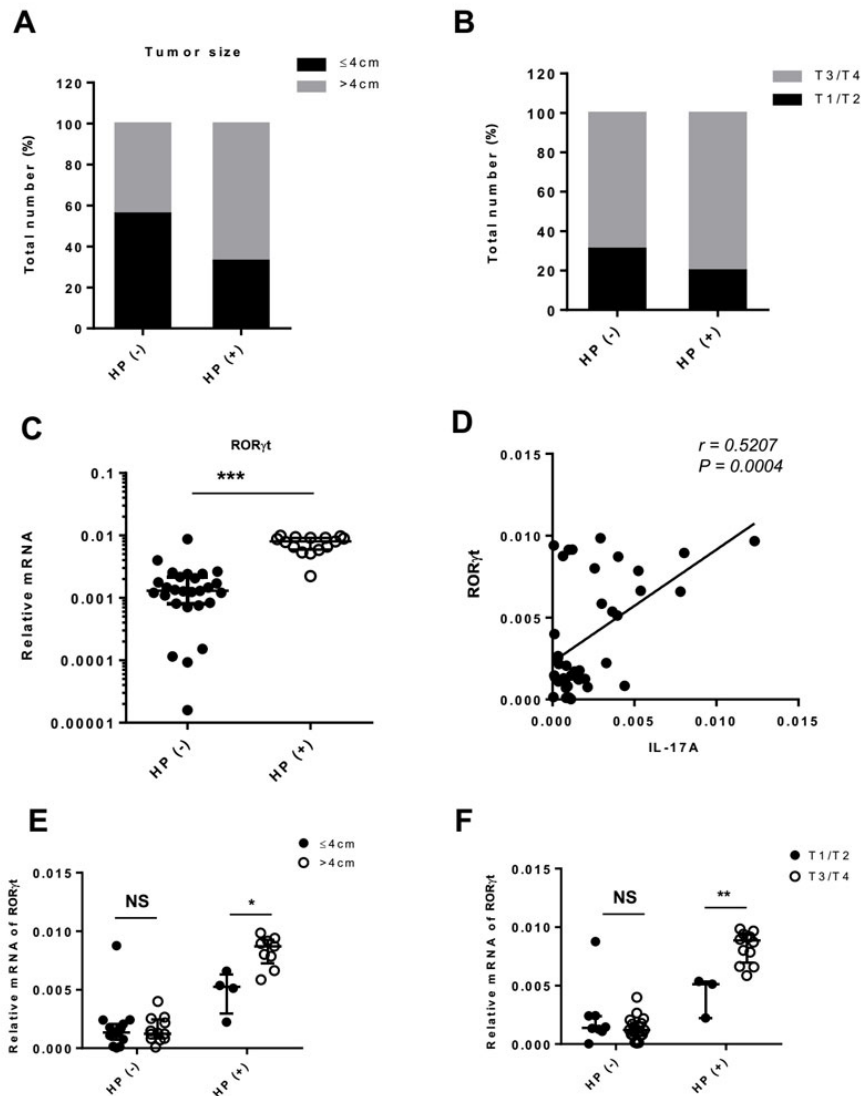


Figure 4: Roles of *H. pylori* and ROR γ t in tumor progression. (A and B) The association of *H. pylori* infection with tumor sizes (A) and stages (B); (C) ROR γ t was enhanced by *H. pylori*. ROR γ t was assayed via RT-qPCR; (D) the correlation of ROR γ t with IL-17A; (E and F) the expression levels of ROR γ t were compared in *H. pylori*-related and unrelated tumors. The levels of ROR γ t were also compared in tumors with different sizes and different stages. HP (+) vs. HP (-): *** $P < 0.001$; ≤ 4 cm vs. > 4 cm: * $P < 0.05$; T1/T2 vs. T3/T4: ** $P < 0.01$. *H. pylori*: *Helicobacter pylori*; RT-qPCR: reverse transcription quantitative polymerase chain reaction; IL: interleukin

levels of TG, though to a lesser extent compared to that of *H. pylori* infection [Figure 1B]. We consistently observe a significant synergy between *H. pylori* infection and abnormal lipid metabolism in producing IL-17A [Figure 1C]. CD4 $^{+}$ T-cells are widely observed in *Helicobacter*-associated GC, and of these, IL-17A is predominantly produced by the Th17 subset.^[35] However, future studies are needed to understand whether IL-17A is also produced from other sources, such as CD8 $^{+}$ T-cells and/or innate lymphoid cells (ILCs). Nevertheless, given the observation that other Th17-related cytokines, such as IL-6 and GM-CSF, are also increased, our results suggest that *H. pylori* infection and altered TG metabolism cooperate in enhancing the Th17 response.

As a pro-inflammatory subset of T cells, it is possible that Th17 cells can also activate antitumor immunity.^[19,37] Myeloid-derived suppressor cells (MDSCs) are known to home to the site of tumors and facilitate their avoidance of cytotoxic T cells. Thus, the production of CXCL1 and GM-CSF may be critical members of the cytokine milieu, as these have been reported to promote the recruitment and function of MDSCs, respectively.^[38] In agreement with previous observations, we found that both GM-CSF and CXCL1 were greatly increased in *H. pylori* positive tumors [Figure 3A and C] and their upregulation was coincident with elevated IL-17A [Figure 3B and D]. In addition, it has been shown that inflamed adipose and stomach tissues induced by *H. felis*/HFD can enhance IL-6 and leptin production to stimulate Th17

differentiation and stabilization.^[19,39] In present study, we note that significant amount of IL-6 and leptin are seen in *H. pylori*-associated GC tissues, suggesting that tumor microenvironment may be sufficient foster Th17 development.

We finally demonstrate that ROR γ t, a transcriptional activator of IL-17A,^[40,41] is extensively induced in *H. pylori*-infected patients [Figure 4C], and the expression is further enhanced in advanced tumors [Figure 4E and F]. ROR γ t has been widely investigated in Th17 cells but seldom in malignant diseases. Thus, future studies should include analysis of the important association of ROR γ t and *H. pylori* infection.

In summary, we demonstrate that *H. pylori* infection and abnormal lipid metabolism can exert a synergistic role in Th17 activation and response to promote GC development. These observations are important to understand GC pathogenesis and can be of therapeutic significance.

DECLARATIONS

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Authors' contributions

Conceived and designed the study: J.Z. Zeng, W. Han, A.S.T. Wong, R.E. Ericksen

Performed the study: H. Wang, G. Chen, J. Liu, M. Yang, Z.X. Wu

Prepared the manuscript: J.Z. Zeng

Revised the manuscript: W. Han, R.E. Ericksen

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Informed consents were signed by patients.

Ethics approval

Our experimental protocols were approved by The Hospital Ethics Committee and The Ethics Committee of Xiamen University, China.

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Malignant field expression signatures in biopsy samples at diagnosis predict the likelihood of lethal disease in patients with localized prostate cancer

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ABSTRACT

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clinical management of early-
stage prostate cancer,
malignant field effect

Aim: Overtreatment of early-stage low-risk prostate cancer patients represents a significant problem in disease management and has significant socio-economic implications. Changes in prostate cancer screening and treatment practices in the United States have been associated with the recent decline in overall incidence and concomitant significant increase of the annual incidence of metastatic prostate cancer has been documented. Therefore, development of genetic and molecular markers of clinically significant disease in patients diagnosed with low grade localized prostate cancer would have a major impact in disease management. **Methods:** Identification of gene expression signatures (GES) associated with lethal prostate cancer has been performed using microarray analyses of biopsy specimens obtained at the time of diagnosis from 281 patients with Gleason 6 (G6) and G7 tumors in a Swedish watchful waiting cohort with up to 30 years follow-up. The performance of GES has been validated in independent cohort of 568 prostate cancer patients of the Cancer Genome Anatomy Project Prostate Cancer database. **Results:** GES comprising 98 genes identified 89% and 100% of all death events 4 years after diagnosis in G7 and G6 patients, respectively. At 6 years follow-up, 83% and 100% of all deaths events were captured in G7 and G6 patients, respectively. Remarkably, the 98-gene signature appears to perform successfully in patients stratification with as little as 2% of cancer cells in a specimen, strongly indicating that it captures a malignant field effect in human prostates harboring cancer cells of different degrees of aggressiveness. In G6 and G7 tumors from prostate cancer patients of age 65 or younger, GES identified 86% of all death events during the entire follow-up period. In G6 and G7 tumors from prostate cancer patients of age 70 or younger, GES identified 90% of all death events 6 years after diagnosis. **Conclusion:** Classification performance of the reported in this study 98-genes GES of lethal prostate



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cancer appeared suitable to meet design and feasibility requirements of a prospective 4 to 6 years clinical trial, which is essential for regulatory approval of diagnostic and prognostic tests in clinical setting. Prospectively validated GES of lethal PC in biopsy specimens of G6 and G7 tumors will help physicians to identify, at the time of diagnosis, patients who should be considered for exclusion from active surveillance programs and who would most likely benefit from immediate curative interventions.

INTRODUCTION

In the United States, widespread implementation of the prostate-specific antigen (PSA) screening programs enabled diagnosis of more than 200,000 cases of prostate cancer each year.^[1] Clinically localized prostate cancer represents the vast majority of new cases.^[2] Therefore, one of the most significant benefits of the widespread use of PSA screening is that the prevalence of the late stage, advanced and high grade prostate cancer at diagnosis has declined dramatically and the vast majority of newly diagnosed prostate cancers are early stage and low grade tumors.

The natural history of early stage clinically localized prostate cancer is considered favorable^[3] and other types of cancer such as lung cancer are considered hundreds times as deadly. Despite this seemingly “indolent” nature, prostate cancer is the second leading cause of cancer-related deaths and accounts for 3.5% of all male deaths.^[4] Development of clear, consensus guidelines for physicians’ decision-making process in clinical management of early stage localized prostate cancer is one of the most significant public healthcare problems. Inevitable and fast approaching demographic changes in the Western world underscore the critical economic and logistical needs for a rational, evidence-based approach to the clinical management of the early stage localized prostate cancer. A path to solutions to this problem is complicated by a multitude of competing positions attempting to emphasize the perceived shortcomings and benefits of different approaches and need to balance multiple variables such as public health care costs, individual patients’ benefits, interests, socio-economic status, ethical and professional responsibilities of the medical personnel, and humanitarian considerations.

Conclusive statistical evidence of the life-saving therapeutic benefits of radical prostatectomy versus watchful waiting in early prostate cancer have been documented in a randomized multicenter clinical trial: radical prostatectomy reduces disease-specific mortality, overall mortality, and the risks of metastasis and local progression.^[5-7] Immediate curative interventions are the predominant therapy choice and 168,000 prostatectomies are performed each year to treat prostate cancer.^[8] It seems reasonable to conclude, that early detection of prostate cancer facilitated by PSA screening and aggressive use of

radical prostatectomy for treatment of early prostate cancer have contributed to a significant extent to the reported 98-100% 5-year survival rates since 1998 in the United States (SEER 13 areas statistics).

However, there is a lack of consensus regarding the benefits of a population-scale PSA screening and a controversy about the potential for overdiagnosis and overtreatment of clinically insignificant disease that would not likely to become life-threatening in a man’s lifetime.^[9] Further socio-economic arguments in support of significant overdiagnosis and overtreatment have been presented in studies indicating that prevention of one prostate cancer death would require active treatment of 48 men for 9 years or 12 men for 14 years.^[10,11] Outcome studies from contemporary population-based cohorts reported cumulative 10-year prostate cancer-specific mortality in patients with low-risk disease 2.4% and 0.7% in the surveillance group and curative intent groups, respectively,^[12] which indicates that the surveillance may be a suitable treatment option for majority of patients with low-risk prostate cancer. Clinical evidence that active surveillance may be a safe, perhaps preferred option for older men diagnosed with a very low-grade or small-volume form of prostate cancer were published recently by Tosoian *et al.*^[13] Therefore, active surveillance with curative intent for low-risk prostate cancer is under active consideration as a potentially safe alternative to immediate curative intervention with the expectations that it may reduce overtreatment and therapy-associated adverse events. It certainly would reduce the escalating economic burden of cost of prostate cancer treatment. The major limitation of these studies is a short follow-up time [for example, in the John Hopkins study,^[13] the total cohort has a median follow-up of 2.7 years (range 0.01 to 15)] which requires the use of biochemical recurrence or other “proxy” end-points for disease-specific mortality. This limitation is particularly relevant for early prostate cancer because the overall survival benefits of radical prostatectomy versus watchful waiting are not statistically apparent until 10 years follow-up^[5-7] due to the fact that a majority of death events in the watchful waiting cohorts of early prostate cancer occurs at or after 10 years follow-up (this study).^[5-7] Furthermore, significantly longer follow-up data are required because most patients currently diagnosed with localized prostate cancer are aged 60-70 years and have a life expectancy of more than 15 years.^[12]

Most importantly, there are no genetic or molecular methods prospectively defining low-risk or indolent prostate cancer at diagnosis with sufficient specificity and selectivity to ensure the safety of patients and allow physicians to make informed, ethical, evidence-based disease management decision of not treating prostate cancer. Given the natural history of early prostate cancer and long-term survival data from watchful waiting cohorts, conclusive prospective validation of laboratory methods defining low-risk indolent disease in Gleason 6 and 7 patients would require at least 10 years. Based on the analysis of the long-term survival data of prostate cancer patients from watchful waiting cohorts with up to 30 years follow-up, we reasoned that more feasible and clinically-relevant approach would be an attempt to identify genetic markers of lethal prostate cancer in patients with Gleason 6 and 7 tumors which would capture a vast majority of all cancer-related death events 4-6 years after diagnosis. Here we report identification of gene expression signatures (GES) of lethal prostate cancer in biopsy specimens obtained at the time of diagnosis from patients with Gleason 6 and 7 tumors in a Swedish watchful waiting cohort with up to 30 years follow-up. In retrospective analysis, best-performing GES of lethal prostate cancer identify 89% and 100% of all death events 4 years after diagnosis in Gleason 7 and Gleason 6 patients, respectively. GES appear to perform successfully in patients' stratification with as little as 2% of cancer cells in a specimen. In Gleason 6 and 7 prostate cancer patients of age 65 or younger, GES identifies 86% of all death events during the follow-up. In Gleason 6 and 7 prostate cancer patients of age 70 or younger, GES identifies 90% of all death events 6 years after diagnosis. Reported in this study GES of lethal prostate cancer in biopsy specimens of Gleason 6 and 7 tumors should help practicing physicians to identify at the time of diagnosis prostate cancer patients who should be considered for exclusion from the active surveillance programs and who would most likely benefit from immediate curative interventions.

METHODS

Patients

This study is based on prostate cancer patients from the population-based Swedish Watchful Waiting cohort of men with localized prostate cancer.^[5-7,14] Distinguishing feature of this cohort is that it represents patients diagnosed with symptomatic early prostate cancer at the time when no PSA screening programs were in place: these men had symptoms of benign prostatic hyperplasia (lower urinary tract symptoms) and were subsequently diagnosed with prostate

cancer. All men in this study were determined at the time of diagnosis to have clinical stage T1 and T2, Mx, and N0, according to the 2002 American Joint Commission Committee TNM staging system.^[5-7,14] The prospective follow-up time in this cohort is now up to 30 years and the study cohort was followed for cancer-specific and all-cause mortality until March 1, 2006.^[11] Deaths were classified as cancer-specific when prostate cancer was the primary cause of death as determined through a complete review of medical records by a study end-point committee.^[5-7,14] Importantly, that in addition to the histopathological examination at the time of diagnosis, slides and corresponding paraffin-embedded formalin-fixed blocks were subsequently retrieved and re-reviewed to confirm cancer status and to assess Gleason scores using review, examination, and grading procedures blinded with regard to disease outcome.^[14]

Gene expression analysis, evaluation, and selection of GES

GES were developed based on a publicly available microarray analysis of a Swedish Watchful Waiting cohort with up to 30 years of clinical follow up using a novel method for gene expression profiling (cDNA-mediated annealing, selection, ligation, and extension method) which enabled the use of formalin-fixed paraffin-embedded transurethral resection of prostate (TURP) samples taken at the time of the initial diagnosis. Details of the experimental procedure can be found in a recent publication^[14] and in Gene Expression Omnibus (GEO: <http://www.ncbi.nlm.nih.gov/geo/>) with platform accession number: GPL5474. Full data set and associated clinical information is available at GEO with accession number: GSE16560.

Feature selection was performed without assessment of differential gene expression between deceased and surviving patients. All 6,144 genes were evaluated for association with clinical and pathological variables (except survival status) using correlation analysis. Different thresholds on the *P*-values (0.05; 0.01; 0.001) were used for selection of gene sets with common patterns of association and concordance analysis was performed using expression profiling data of snpRNA-driven cell line-based models of prostate cancer predisposition^[15,16] to identify concordant and discordant GES in cell lines and clinical samples.^[17-20] GES were built based on selection of co-regulated transcripts in various experimental conditions and clinically-relevant models, including prostate cancer predisposition and longevity models.^[16-20] Underlying concept at this stage of the analysis was to identify GES with concordant expression profiles across multiple data sets.^[17-20] Cox regression analysis was

carried out to identify statistically significant candidate GES associated with patients' survival status. Cut-off threshold of *P*-values was set based on the *P*-value of the best-performing clinico-pathological parameter (Gleason score) in univariate Cox regression analysis (*P* = 0.0113). Genes from statistically significant GES were split, combined, and permuted using random iteration process to find novel statistically significant combinations based on univariate Cox regression analysis. GES scores were derived directly from measurements of expression values of each gene by calculating a single numerical value for each patient. GES scores represent the difference between sums of expression values of genes with common co-regulation profiles which is defined by up-regulation and/or positive correlation values versus down-regulation and/or negative correlation values. GES with *P* values < 0.01 were selected for further evaluation using multivariate Cox regression analysis of classification models which include GES and clinico-pathological co-variants (age and Gleason score). Cut-off threshold of *P*-values for candidate GES selection was set based on the *P*-value of the best-performing clinico-pathological model (age and Gleason score) in multivariate Cox regression analysis (*P* = 0.0052). Candidate GES that outperformed clinico-pathological models in multivariate Cox regression analysis were selected for further consideration using a split-sample validation procedure for classification threshold selection and GES classification performance evaluation as previously described.^[17-20]

Gene expression-based classification models were designed and evaluated through a split-sample validation procedure which enables the unbiased estimation of the performance of a classifier since the evaluation is performed on an independent data set.^[21] Specifically, the entire data set of 281 patients was split into training and test sets (141 and 140 patients, respectively), with approximately equal proportion

of men with lethal and indolent prostate cancer and statistically undistinguishable clinical and pathological variables, e.g. age and time of diagnosis, follow up time, Gleason scores, percent of cancer cells in specimens [Table 1]. The training set of 141 samples was utilized to identify and select the best classifier, whose performance was evaluated on the test set of 140 samples without any further adjustments to the threshold selection and classification protocols using Kaplan-Meier survival analysis essentially as previously described.^[17-20] Best-performing GES classifiers were further evaluated in various clinically-relevant patients' sub-groups, including only Gleason 6 patients (*n* = 83), only Gleason 7 patients (*n* = 117), Gleason 6 and 7 patients (*n* = 200), with further subdivision of patients in additional validation screens based on age at diagnosis (age 65 and younger; age 70 and younger) and percent of cancer cells in the samples (2%; 5% or less; 10% or less; 20% or less; 40% or less; and 50% or more). In all these secondary validation screens no further adjustments to the threshold selection and classification protocols were made. Ninety-eight genes classifier that remains statistically significant in all these validation screens is reported in this paper.

Statistical significance of the Pearson correlation coefficients for individual test samples, clinical variables, and the appropriate reference standard were determined using GraphPad Prism version 4.00 software. We calculated the significance of the differences in the numbers of death events and surviving patients between the groups using two-sided Fisher's exact test and the significance of the overlap between the lists of differentially-regulated genes using the hypergeometric distribution test.^[22]

Validation analyses of GES were performed using the most recent release of web-based tools, the UCSC Xena (<http://xena.ucsc.edu/>) to explore and visualize

Table 1: Clinical characteristics of prostate cancer patients in the training and test sets

Characteristic	Training set (<i>n</i> = 141)	Test set (<i>n</i> = 140)
Years of diagnosis, range (years)	1977-1998	1977-1998
Years of diagnosis, mean ± SD (years)	1991 ± 4.1	1991 ± 4.0
Age at diagnosis, range (years)	51-91	55-91
Age at diagnosis, mean ± SD (years)	74.5 ± 7.5	73.5 ± 7.0
Follow-up time, range (months)	6-274	7-259
Follow-up time, mean ± SD (months)	102.3 ± 57.2	101.9 ± 55.7
Percent of cancer in samples, range (%)	2-90%	2-90%
Percent of cancer in samples, mean ± SD (%)	22.9 ± 22.7	24.0 ± 25.5
Gleason scores, <i>n</i> (%)		
Gleason 6	42 (29.8)	41 (29.3)
Gleason 7	62 (44)	55 (39.3)
Gleason 8-10	37 (26.2)	44 (31.4)
Clinical outcomes, <i>n</i> (%)		
Deceased	105 (74.5)	101 (72.1)
Alive	36 (25.5)	39 (27.9)

SD: standard deviation

Table 2: Receiver operating characteristic area under the curve analysis of training and test data sets

Data sets and survival time	10 years	7 years	6 years	5 years	4 years
Training set (<i>n</i> = 141)	0.85	0.854	0.814	0.788	0.794
Test set (<i>n</i> = 140)	0.826	0.801	0.786	0.758	0.759

Table 3: Percent of all death events at different follow-up time in lethal prostate cancer groups of training and test data sets

Data sets and survival time	10 years	7 years	6 years	5 years	4 years
Training set (<i>n</i> = 141)	75%	83%	82%	84%	84%
Test set (<i>n</i> = 140)	83%	88%	87%	84%	84%

the comprehensive functional cancer genomics datasets of thousands annotated clinical samples of the Cancer Genome Anatomy Project (TCGA) (<https://xenabrowser.net/datapages/>). The classification performance of the 98-genes GES was further validated using TCGA Prostate Cancer cohort of 568 clinical samples with known therapy outcomes after the initial treatment. Importantly, in contrast to biopsy samples analyzed in the population-based Swedish Watchful Waiting cohort, tumors tissues of the cotemporary TCGA Prostate Cancer cohort comprise the prostatectomy samples which were analyzed using the state of the art Illumina Next Generation Sequencing technology.

RESULTS

Clinical characteristics of the training and test sets are provided in Table 1, and further details for the entire Swedish Watchful Waiting cohort are available in a recent publication^[14] and in Gene Expression Omnibus (GEO: <http://www.ncbi.nlm.nih.gov/geo/>) with accession number GSE16560. All of the 281 patients in the Swedish cohort had clinical symptoms and were diagnosed from TURP or adenoma enucleation samples and thus were staged depending on the proportion of the tissue that was cancerous either T1a or T1b.^[14] Analysis of survival data in the entire cohort of 281 patients indicates that prostate cancer patients with different Gleason scores have markedly distinct timelines of death events during the extended up to 30 years follow-up [Figure 1]. Most striking indicator is that only 6% of untreated Gleason 6 prostate cancer

patients died at 5 years; 14% died between 5 to 10 years; and a majority of deaths (~ 35%) occurs 10-23 years after diagnosis. This analysis suggests that a majority of all death events (> 60%) in untreated Gleason 6 prostate cancer patients is occurring more than 10 years after diagnosis and during the sufficiently long follow-up period more than 50% of these patients will die [Figure 1]. Long-term survival timelines for untreated Gleason 7 prostate cancer patients with symptomatic prostate cancer appear even more alarming: 27% died at 5 years follow-up; 22% of deaths occurred between 5 to 10 years; and > 70% died during the entire follow-up period [Figure 1]. When compared with active surveillance patients from the PSA screening era these mortality figures seem very high, particularly because the survival references were made to prostate cancer-specific mortality. At least in part, it might be attributed that in this cohort all Gleason 7 prostate cancer patients were clinically symptomatic in contrast to predominantly asymptomatic Gleason 7 prostate cancer patients diagnosed during the PSA screening era. These apparent differences indicate what would likely to happen to prostate cancer specific mortality if the population scale PSA screening practices will be changed or abandoned.

Collectively, the analysis of timelines of death events in a watchful waiting cohort indicates that a majority of patients with symptomatic Gleason 6 and 7 prostate cancers will eventually develop clinically significant disease during sufficiently long follow-up period which further underscore the critical need to reliably define

Table 4: Classification performance of the 98-genes GES in the TCGA cohort of 550 prostate cancer patients with known therapy outcomes after the initial treatment

Categories	Therapy outcomes after the initial treatment (number of patients with adverse events)		
	Relapse	Biochemical recurrence	New tumors
Patients' sub-group/adverse events			
Poor prognosis (<i>n</i> = 275)	33	44	60
Good prognosis (<i>n</i> = 275)	10	18	20
Patients' sub-group/adverse events	Therapy outcomes after the initial treatment (percent of patients with adverse events)		
Poor prognosis (top 50% scores)	12.00	16.00	21.82
Good prognosis (bottom 50% scores)	3.64	6.55	7.27
<i>P</i> value*	0.0004	0.0006	< 0.0001

**P* values were estimated using 2-tailed Fisher's exact test. At the date of the analyses, the median follow-up time in the prostate cancer TCGA cohort was 2.1 years. GES: gene expression signatures; TCGA: the Cancer Genome Anatomy Project

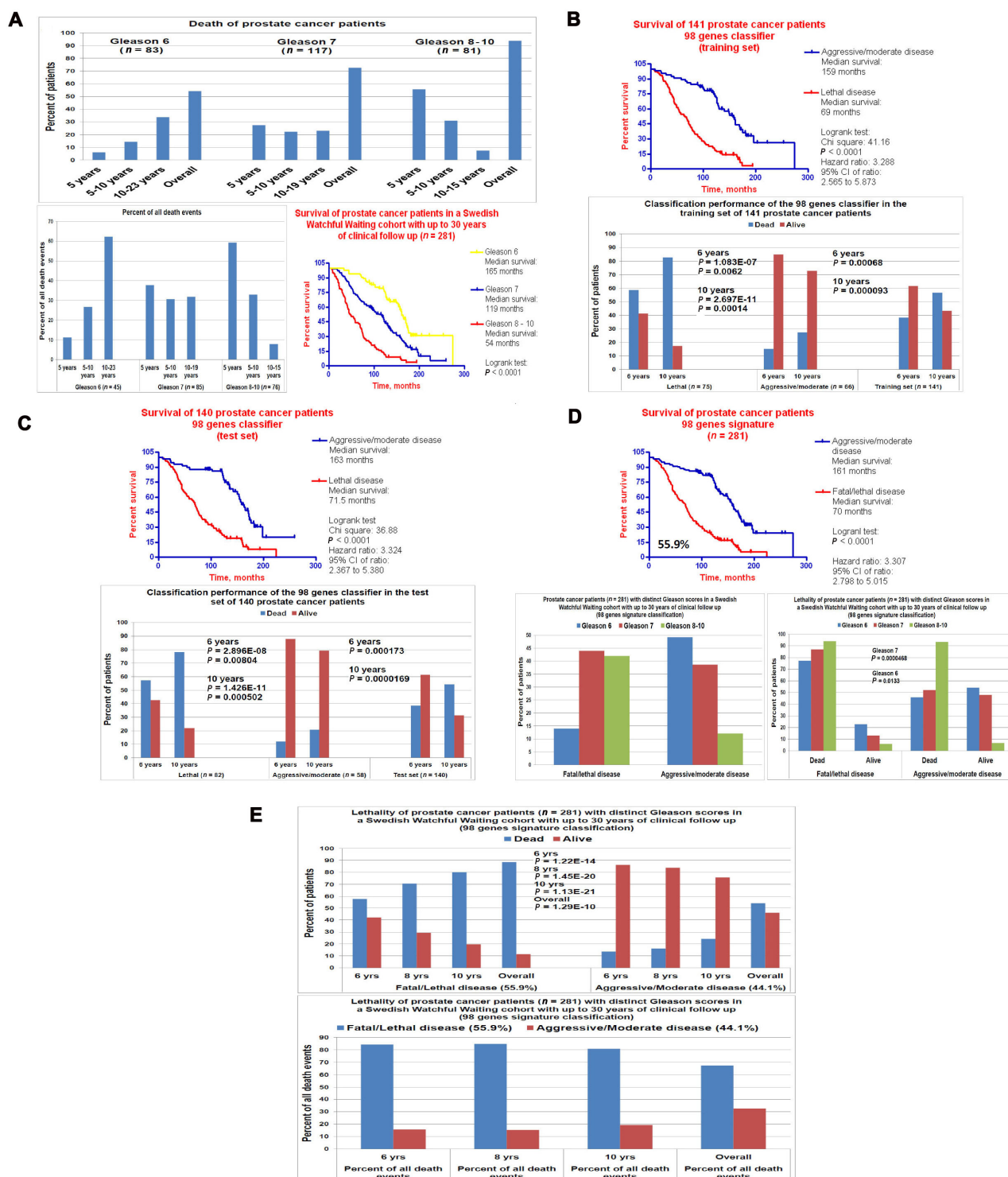
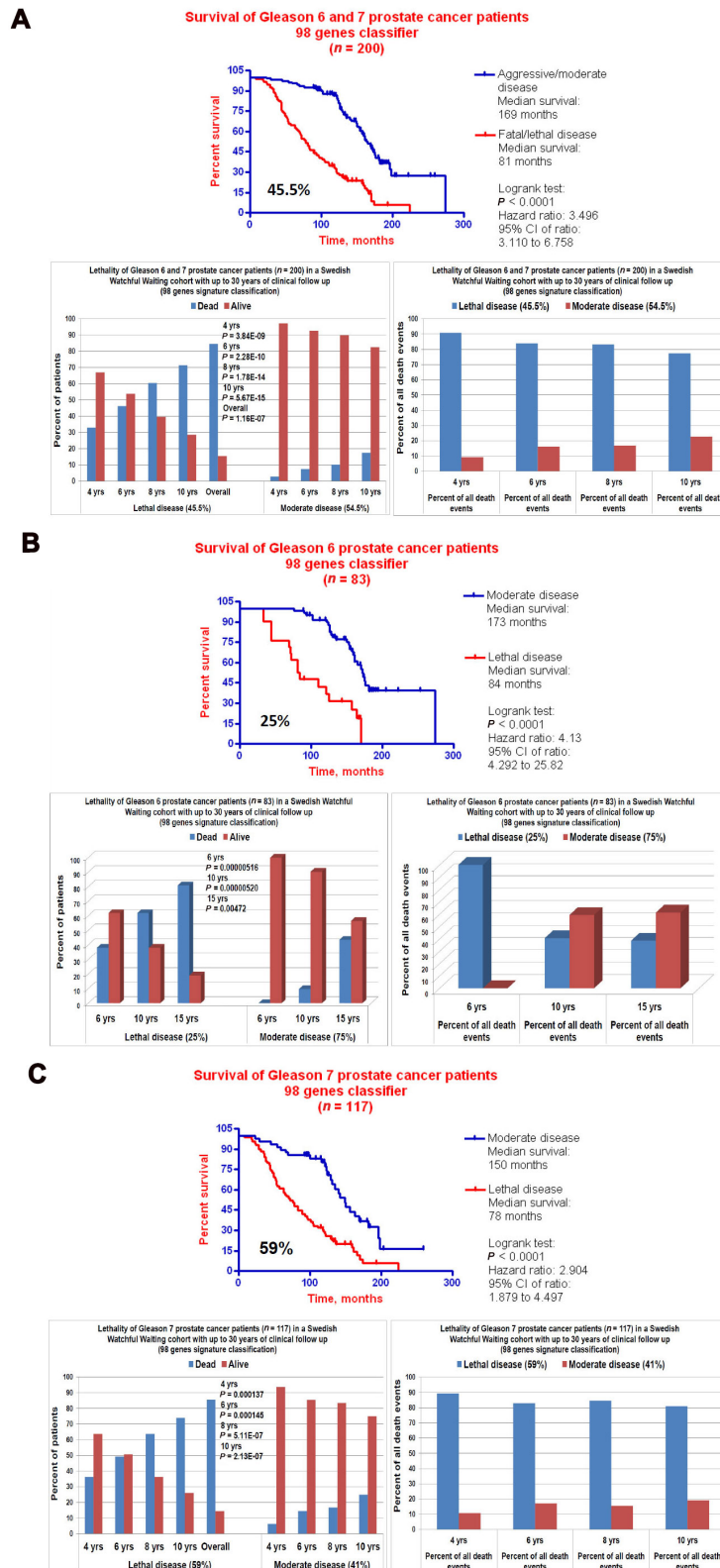


Figure 1: Natural history of prostate cancer progression in patients' population from a Swedish watchful waiting cohort with up to 30 years follow-up (A) and classification performance of the 98 genes signature of lethal disease in prostate cancer patients (B-E). (A) Cancer-specific survival data in the entire watchful waiting cohort are presented to illustrate markedly distinct survival timelines of non-treated prostate cancer patients diagnosed with different Gleason scores prostate cancer. Kaplan-Meier survival analysis of the classification performance of the 98 genes GES in the training set (B), test set (C), and pooled cohort of 281 patients (D, E). Classification threshold 98 genes GES score of 270.43 units was chosen using the training set of 141 prostate cancer patients and consistently applied in all subsequent validation screens using the Kaplan-Meier survival analysis to stratify the patients into lethal disease sub-groups (score ≥ 270.43) and moderate/aggressive disease sub-group (score < 270.43). Percent value indicates the proportion of patients in the lethal disease sub-group. P values indicate the significance of the differences in the numbers of death events and surviving patients between the groups which was determined using two-sided Fisher's exact test. GES: gene expression signatures



lethal prostate cancer at diagnosis. We applied the univariate Cox regression analysis to the entire cohort of 281 patients to identify several GES with the P value < 0.01 which appear to perform better than the best clinico-pathological co-variate, Gleason score ($P = 0.0113$; [Supplemental Table 1](#)). Most of these GES outperformed the clinico-pathological classification model in multivariate Cox regression analysis as well [[Supplemental Table 2](#)].

Separating the cohort of 281 patients into training and test cohorts and using the Kaplan-Meier survival analysis, we identified 98 genes GES that manifest the highly significant classification performance in the training set, retained highly consistent classification performance in the test set, and remained a highly significant classifier in the pooled cohort [[Figure 1](#)]. It is important to note that in all secondary validation screens following the training set analysis no further adjustments to the threshold selection and classification protocols were made.

Notably, prostate cancer patients with identical Gleason scores (e.g. Gleason 6 patients and Gleason 7 patients) which were segregated into lethal and moderate disease sub-groups based on 98 genes GES classification had highly significant differences in the survival rates [[Figure 1](#)]. These data suggest that 98 genes GES may be useful in identifying lethal disease in patients diagnosed with low grade localized prostate cancer [[Supplemental Table 3](#)]. To test this hypothesis, we performed Kaplan-Meier survival analysis based on 98 genes GES classification in the cohort of 200 patients with Gleason 6 and 7 prostate cancer [[Figure 2](#)]. We found that 98 genes GES is a highly significant classifier of Gleason 6 and 7 prostate cancer patients into sub-groups with lethal and moderate disease [[Figure 2](#)]. Ninety-eight genes GES of lethal prostate cancer performs as a highly significant after segregation of patients into separate Gleason 6 and Gleason 7 sub-groups: 89% and 100% of all death events were identified 4 years after diagnosis in Gleason 7 and Gleason 6 patients, respectively; at 6 years follow-up, 83% and 100% of all deaths events were captured in Gleason 7 and 6 patients, respectively [[Figure 2](#)].

Age at diagnosis is considered among very important clinical determinants guiding the decision making process in clinical management of prostate cancer. This is particularly important for relatively younger patients because patients diagnosed with prostate cancer at age < 65 years are more likely to benefit from the immediate curative therapies.^[7] We therefore attempted to determine whether 98 genes GES will

identify lethal disease in prostate cancer patients of differing ages. Remarkably, Kaplan-Meier survival analysis has determined that 98 genes GES performed very efficiently in stratification of prostate

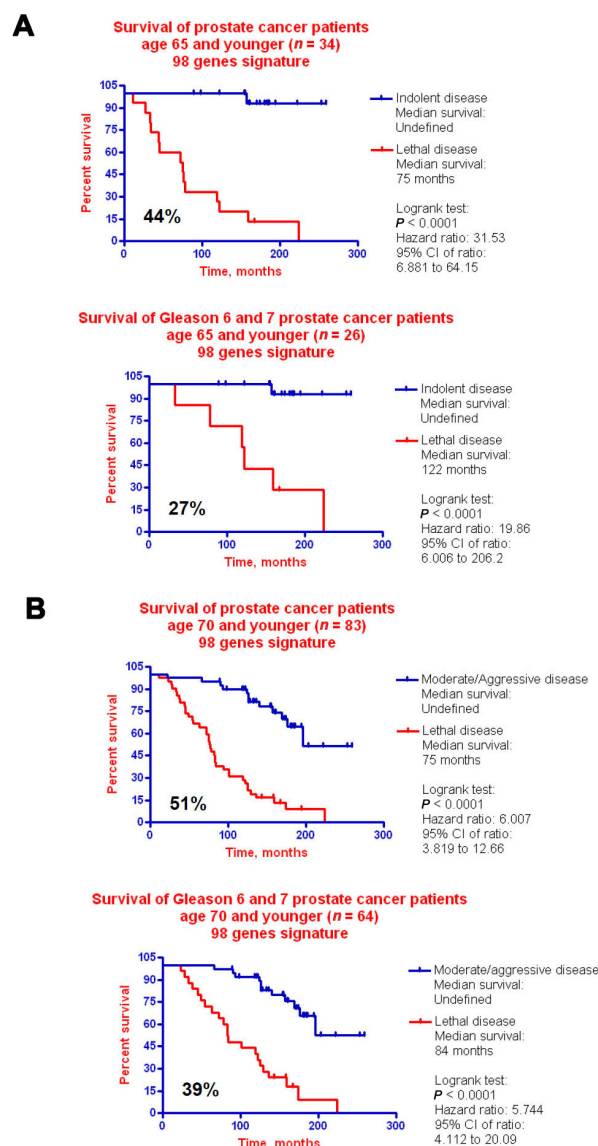


Figure 3: GES-based identification of lethal disease in prostate cancer patients with different age at diagnosis. Kaplan-Meier survival analysis of the classification performance of the 98 genes GES in 34 prostate cancer patients of age 65 or younger (A), 64 prostate cancer patients of age 70 or younger (B). Bottom figures in both A and B panels show the results of Kaplan-Meier survival analysis for Gleason 6 and 7 patients only of corresponding age groups. Classification threshold 98 genes GES score of 270.43 units was chosen using the training set of 141 prostate cancer patients and consistently applied in all subsequent validation screens using the Kaplan-Meier survival analysis to stratify the patients into lethal disease sub-groups (score ≥ 270.43) and moderate/aggressive disease sub-group (score < 270.43). Percent values indicate the proportion of patients in the lethal disease sub-group. P values indicate the significance of the differences in the numbers of death events and surviving patients between the groups which was determined using two-sided Fisher's exact test. GES: gene expression signatures

cancer patients of 65 years or younger [Figure 3]: in Gleason 6 and 7 prostate cancer patients of age 65 or younger, GES identifies 86% of all death events during the follow-up. In Gleason 6 and 7 prostate cancer patients of age 70 or younger, GES identifies 90% of all death events 6 years after diagnosis [Figure 3].

Proportion of cancer cells in biopsy samples is highly variable and these variations may have significant impact on performance of gene expression-based classifiers. In biopsy samples from the population-based Swedish Watchful Waiting cohort the reported

percent of cancer cells in a sample varied dramatically from 2% to 90%. We therefore set out to determine whether the number of cancer cells in biopsy samples would have an impact on classification performance of the 98 genes GES of lethal prostate cancer. We applied the 98 genes GES classifier to prostate cancer patients which were segregated into distinct sub-groups based on the percent of cancer cells in a biopsy sample. Kaplan-Meier survival analysis demonstrates that 98 genes GES performs successfully in patients' stratification regardless of the number of cancer cells in biopsy samples [Figures 4 and 5]. Remarkably

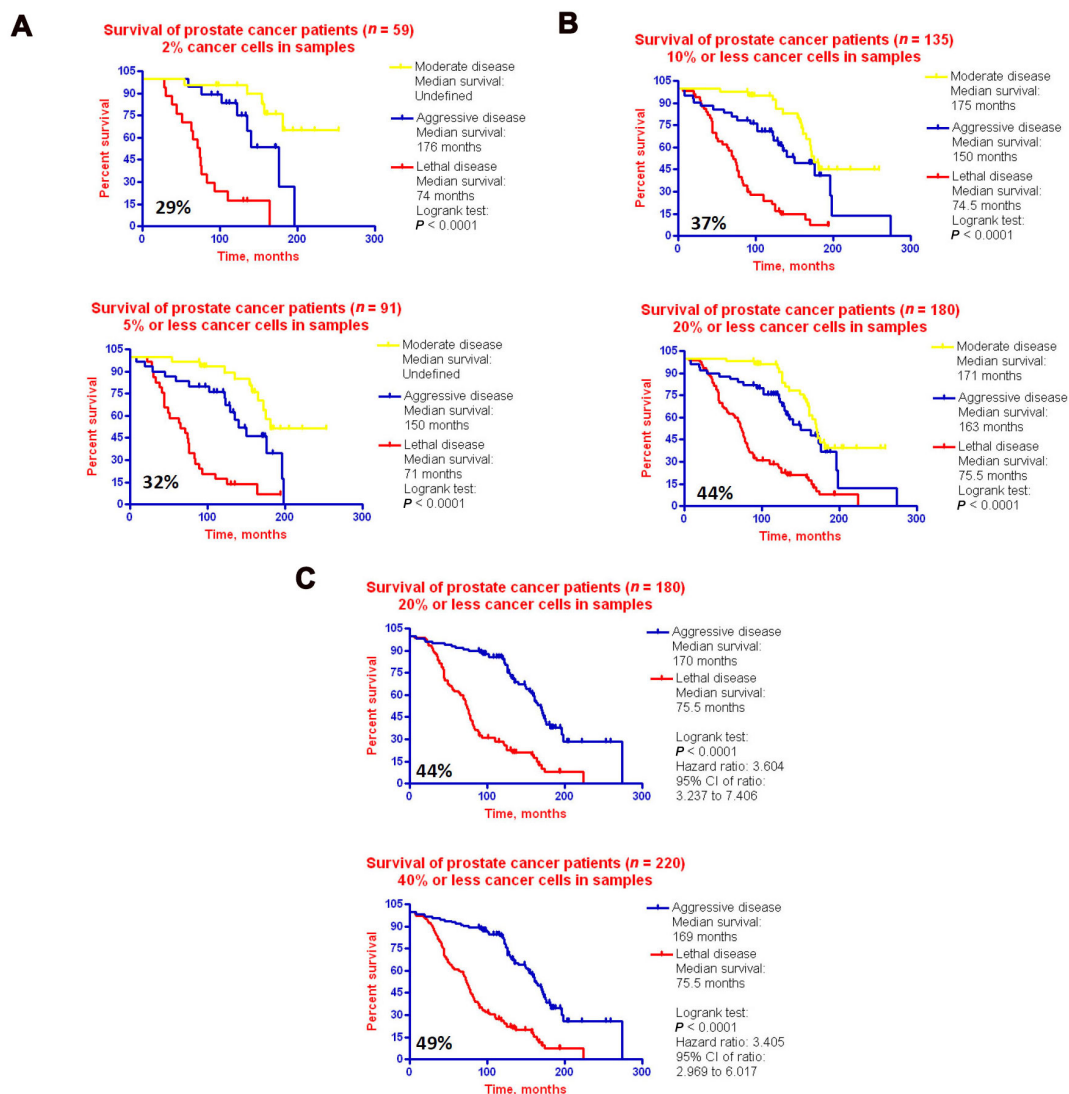


Figure 4: GES-based identification of lethal disease in prostate cancer patients with distinct numbers of cancer cells in biopsy samples. Kaplan-Meier survival analysis of the classification performance of the 98 genes GES in 59 prostate cancer patients having 2% cancer cells in biopsy samples (A, top), 91 patients having 5% or less cancer cells in biopsy samples (A, bottom), 135 patients having 10% or less cancer cells in biopsy samples (B, top), 180 patients having 20% or less cancer cells in biopsy samples (B, bottom; and C, top), 220 patients having 40% or less cancer cells in biopsy samples (C, bottom). Classification threshold 98 genes GES score of 270.43 units was chosen using the training set of 141 prostate cancer patients and consistently applied in all subsequent validation screens using the Kaplan-Meier survival analysis to stratify the patients into lethal disease sub-groups (score ≥ 270.43) and moderate/aggressive disease sub-group (score < 270.43). Percent values indicate the proportion of patients in the lethal disease sub-group. P values indicate the significance of the differences in the numbers of death events and surviving patients between the groups which was determined using two-sided Fisher's exact test. GES: gene expression signatures

98 genes GES appear to identify lethal disease in Gleason 6 and 7 prostate cancer patients with as little as 2% of cancer cells in a biopsy specimen [Figure 5]. The conclusions reached based on the Kaplan-Meier survival analyses were confirmed using the receiver operating characteristic area under the curve analysis of the patients' classification based on the 98-genes signature score in training ($n = 141$) and test ($n = 140$) groups (A) and different clinically-relevant sub-groups (B-D) of patients [Figure 6; Tables 2 and 3]. Collectively, the results of the present analyses strongly indicate that the 98-genes GES captures a malignant field effect in the human prostates harboring cancer cells

with markedly different clinical aggressiveness.

The most recent release of web-based tools, the UCSC Xena (<http://xena.ucsc.edu/>), provides powerful resources to explore, analyze, and visualize the comprehensive functional cancer genomics datasets of thousands annotated clinical samples of TCGA (<https://xenabrowser.net/datapages/>). The classification performance of the 98-genes GES was further validated using TCGA Prostate Cancer cohort of 550 clinical samples with known therapy outcomes after the initial treatment [Table 4]. Notably, the 98-gene GES successfully stratified prostate cancer

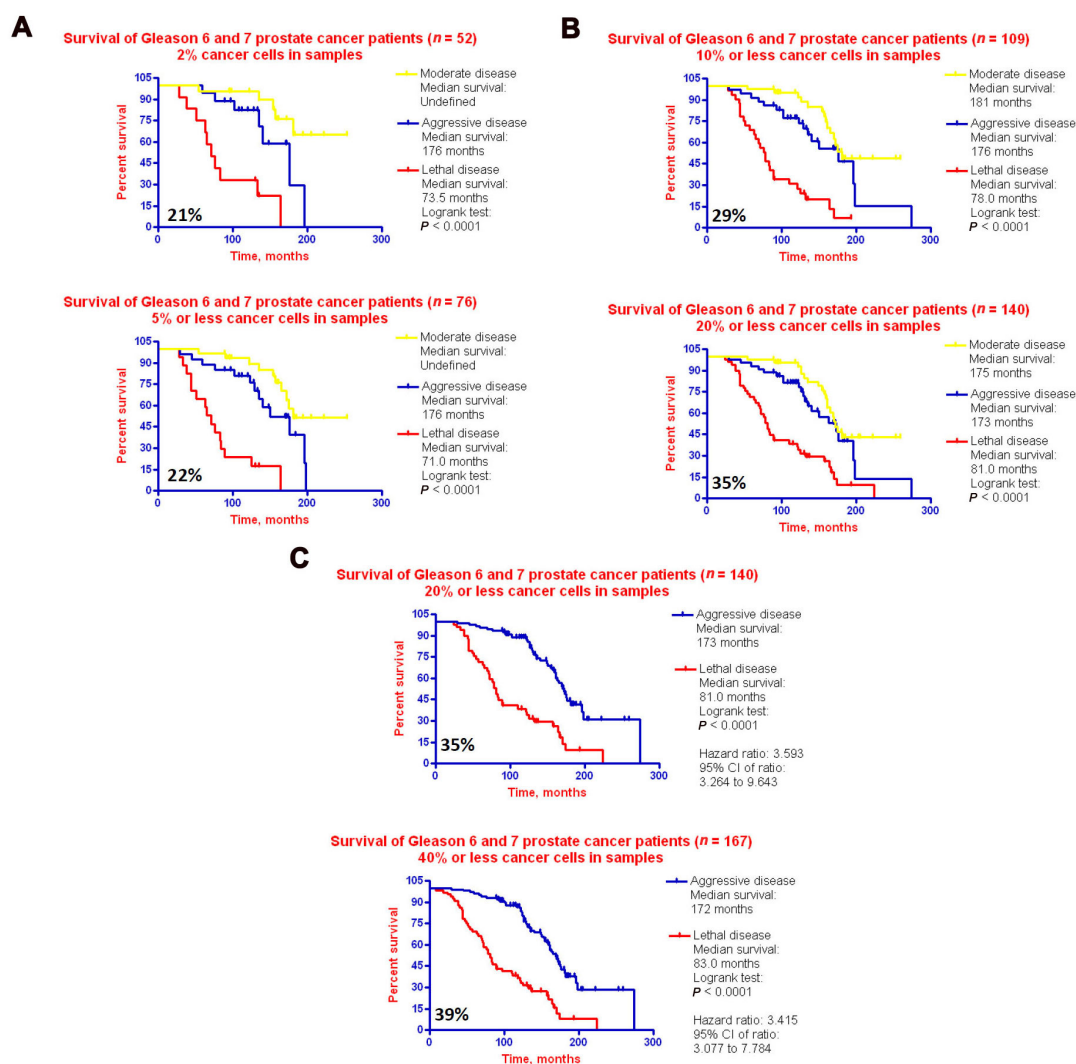


Figure 5: GES-based identification of lethal disease in Gleason 6 and 7 prostate cancer patients with distinct numbers of cancer cells in biopsy samples. Kaplan-Meier survival analysis of the classification performance of the 98 genes GES in 52 prostate cancer patients having 2% cancer cells in biopsy samples (A, top), 76 patients having 5% or less cancer cells in biopsy samples (A, bottom), 109 patients having 10% or less cancer cells in biopsy samples (B, top), 140 patients having 20% or less cancer cells in biopsy samples (B, bottom; and C, top), 167 patients having 40% or less cancer cells in biopsy samples (C, bottom). Classification threshold 98 genes GES score of 270.43 units was chosen using the training set of 141 prostate cancer patients and consistently applied in all subsequent validation screens using the Kaplan-Meier survival analysis to stratify the patients into lethal disease sub-groups (score ≥ 270.43) and moderate/aggressive disease sub-group (score < 270.43). Percent values indicate the proportion of patients in the lethal disease sub-group. P values indicate the significance of the differences in the numbers of death events and surviving patients between the groups which was determined using two-sided Fisher's exact test. GES: gene expression signatures

patients into subgroups with markedly distinct therapy outcomes after the initial treatment defined by different indicators of clinical progression such as biochemical recurrence, disease relapse, and appearance of recurrent tumors [Table 4].

DISCUSSION

Decision making process in clinical management of low-risk localized prostate cancer is likely to affect life and death of thousands of patients. The problem is confounded by the fact that statistically significant survival benefits of curative therapy are evident only 10 years after diagnosis of the early-stage prostate cancer. Therefore, any genetic or molecular tests designed to aid physicians and patients in this process would require the regulatory approval following the successful prospective clinical trial. Classification performance of the reported in this study 98 genes GES of lethal prostate cancer appears highly suitable to meet design and feasibility requirements of the prospective 4 to 6 years clinical trial. One of the most remarkable features of the 98-gene signature is that it appears to perform successfully in patients' stratification with as little as 2% of cancer cells in a specimen, indicating that this GES captures a

malignant field effect in human prostates harboring tumors of different degrees of aggressiveness. It will be of interest to investigate the molecular and genetic mechanisms of this phenomenon. Prospectively validated GES of lethal prostate cancer in biopsy specimens of Gleason 6 and 7 tumors will help practicing physicians to identify at the time of diagnosis individual patients who should be considered for exclusion from the active surveillance programs and who would most likely benefit from the immediate curative interventions.

One of the distinguishing features of this unique 281 patients' cohort that will never be replicated for ethical and humanitarian reasons, is that prostate cancer patients were never treated and just subjected to the long-term follow-up observations. In this context, the outcome data on the prostate cancer-specific death of these patients reveal what would happen to prostate cancer patients who will not be treated (i.e. subjected to "watchful waiting"). Importantly, it demonstrates that a majority of prostate cancer patients diagnosed with Gleason 6 and 7 tumors will die from prostate cancer when left untreated. A distinguishing feature of the patients' cohort analyzed in this study is that it represents patients diagnosed with symptomatic early

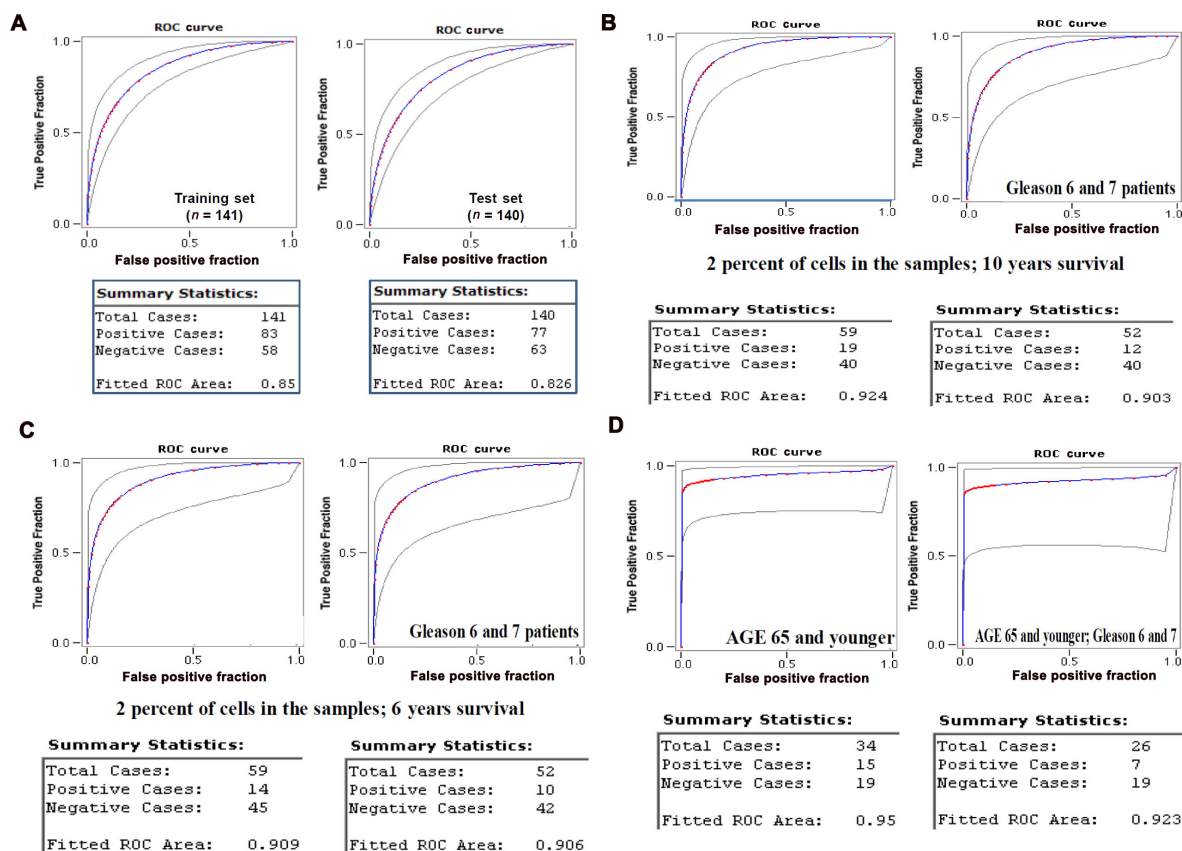


Figure 6: ROC area under the curve analysis of the patients' classification based on the 98-genes signature score in training ($n = 141$) and test ($n = 140$) groups (A) and different clinically-relevant sub-groups (B-D) of patients. ROC: receiver operating characteristic

prostate cancer at the time when no PSA screening programs were in place: these men had symptoms of benign prostatic hyperplasia (lower urinary tract symptoms) and were subsequently diagnosed with prostate cancer. No PSA analyses were performed and these patients did not receive treatment for prostate cancer. Therefore, the prostate cancer-specific death reported in this study represent the realistic natural history of prostate cancer with respect to the assessment of what happen to the prostate cancer patients if they will not be treated and just subjected to observations. In this contribution we evaluated the prostate cancer-specific death as the main outcome.

It has been reported that changes in prostate cancer screening practices in the United States have been associated with the recent decline in the overall incidence of prostate cancer and concomitant significant increase of the annual incidence of metastatic prostate cancer from 2007 to 2013.^[23] Statistically significant increase in the annual incidence of metastatic prostate cancer in the United States from 2007 to 2013 has been consistently documented in all age groups with the overall increase of 72% in 2013 compared to 2004. Particularly alarming is the evidence of the greatest increase of the incidence of metastatic prostate cancer in men of the age group of 55 to 69 years who experienced 92% increase in the incidence of metastatic disease from 2004 to 2013.^[23] These patients are likely to benefit most from definitive curative treatment of prostate cancer, suggesting that relaxed screening protocols and transition to active surveillance with curative intent strategy as a predominant approach for treatment of early-stage prostate cancer should be considered with extreme caution for this group of men, particularly in the absence of validated genetic tests reliably discriminating indolent prostate cancers from the clinically significant disease.

It is outside of the scope of this contribution to compare the diagnostic, prognostic, therapy-outcome assessments or targeted therapy-selection performances of GES. These questions were extensively explored and debated in the literature. There is no need to attempt a “horse race” comparing the signatures against each other while the state of the art comprehensive microarray and/or RNA-seq platforms enable the analyses of all of the signatures in one run and score all of the signatures simultaneously can be made for the specifically-defined benefits of the patients. It is reasonable to expect that no single signature will fit all clinically-defined disease diagnosis and management criteria and different signatures will address more adequately and resolve more efficiently

the specific needs, which will be ultimately tailored to the need of the particular individual patient.

DECLARATIONS

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Authors' contributions

G.V. Glinsky contributed solely to the paper.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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Using circulating tumor cells to advance precision medicine in prostate cancer

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ABSTRACT

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The field of circulating tumor cell (CTC) enrichment has seen many emerging technologies in recent years, which have resulted in the identification and monitoring of clinically relevant, CTC-based biomarkers that can be analyzed routinely without invasive procedures. Several molecular platforms have been used to investigate the molecular profile of the disease, from high throughput gene expression analyses down to single cell biological dissection. The established presence of CTC heterogeneity nevertheless constitutes a challenge for cell isolation as the several subpopulations can potentially display different molecular characteristics; in this scenario, careful consideration must be given to the isolation approach, whereas methods that discriminate against certain subpopulations may result in the exclusion of CTCs that carry biological relevance. In the context of prostate cancer, CTC molecular interrogation can enable longitudinal monitoring of key biological features during treatment with substantial clinical impact, as several biomarkers could predict tumor response to AR signaling inhibitors (abiraterone, enzalutamide) or standard chemotherapy (taxanes). Thus, CTCs represent a valuable opportunity to personalize medicine in current clinical practice.

INTRODUCTION

Recent advances in the treatment of metastatic prostate cancer (PC) led to the FDA-approval of many effective therapies (abiraterone acetate, enzalutamide, radium-223), which demonstrated a significant survival benefit for patients with castrate resistant PC^[1,2]. Nevertheless, patients' clinical response is only transient, owing to the development

of drug resistance, which remains a major clinical challenge. Moreover, we are yet unable to predict response to a specific therapy in an individual patient and the optimal sequence of these therapies needs to be clarified. The results of two large phase III clinical trials (STAMPEDE trial and CHAARTED trial) support the use of taxane chemotherapy in combination with standard androgen deprivation therapy (ADT) in patients with hormone-sensitive metastatic PC^[3,4].



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In the same clinical setting, two other recent studies (STAMPEDE trial and LATITUDE trial) indicate a significant advantage in survival when abiraterone acetate is added to standard ADT^[5,6]. These new results expand the selection of treatment strategies in PC, thus prompting the need to identify predictive factors to select ideal candidates for anticipated therapies.

A greater understanding of the molecular abnormalities underlying PC progression and the associated mechanisms responsible for treatment resistance may reveal potential biomarkers related to treatment failure, adding a valuable tool to the clinician's arsenal. However, the clinical validation of these molecular features as predictive biomarkers is still an unmet need, as personalized medicine continues to be elusive, due in part to the lack of tumor tissue to profile these alterations.

The use of circulating tumor cells (CTCs) as a “liquid biopsy” to interrogate the molecular profile of single patients is extremely encouraging, as these cells originate from primary tumor and metastatic sites, providing a comprehensive fingerprint of a given tumor at any time during the course of the disease with a simple blood draw^[7]. This is particularly important in PC, as the primary biopsy tissue available is usually collected at diagnosis many years before the development of metastases and may not mirror the biological state of the current disease.

Thus, the longitudinal isolation and characterization of CTCs could supply the necessary information to tailor treatment to the individual. The present review discusses current CTC-enrichment techniques with the specific downstream analyses that can be performed given each method with a particular focus on PC, and the clinical applications that can be guided by the molecular and functional analyses of CTCs. The most updated results for the use of CTCs to investigate the role of potential predictive biomarkers in PC will also be included.

FROM RARE CELL CAPTURE TO INSIGHT INTO PATIENTS' TUMORS

The isolation and analysis of patient-derived CTCs has received enormous attention from the biomedical community based on their applications towards personalized therapy^[8,9]. It is now established that CTCs offer a suitable source of tumor material as there are numerous reports showing that CTC molecular profiles concur with the profiles from metastatic sites and primary tumors from the same patient^[10-12].

CTC isolation and downstream molecular characterization are a powerful tool that has the possibility to grant physicians insight into each individual patient's tumor. Furthermore, repeat sample collection could provide the chance of monitoring tumor evolution and could offer the potential to guide therapy.

The rarity of CTCs in peripheral whole blood requires multi-step isolation techniques to maximize the capture of cells of malignant origin while minimizing contamination from circulating blood cells that could mask or limit the clinical utility of CTCs^[13]. However, due to the often-intense sample processing during CTC isolation, not all enrichment techniques lend themselves to all downstream analyses. For example, sample fixation during blood collection or the presence of antibody-conjugated magnetic beads may prevent the implementation of certain assays, such as real time polymerase chain reaction (RT-PCR) or CTC culture, thereby limiting the type of information that can be extracted from the isolated CTCs. Therefore, the sample processing involved in CTC enrichment is of important consideration given the desired biomarker analyses that will follow [Figure 1; Table 1]. Even with these limitations, the wide variety of isolation methods currently available allows researchers to perform a multitude of downstream assays and reveal important clinically relevant information from CTCs.

CTC enrichment based on physical properties

A basic approach to enrich CTCs from circulation uses the physical properties of cancer cells, such as size and density, to differentiate them from the circulating hematopoietic cells. Density-gradient centrifugation effectively separates CTCs from whole blood by taking advantage of their larger size and distinct shape in relation to other components of whole blood. By combining blood with a density-gradient solution (e.g. Ficoll-Paque®, GE Healthcare Life Sciences) and subsequent centrifugation, the blood separates into distinct layers of plasma, mononuclear cells, and anucleated cells. CTCs are retained in the mononuclear cell layer with other peripheral blood mononuclear cells (PBMCs).

Since physical isolation strategies do not rely upon the expression of cell surface cancer-specific antigens, these techniques could effectively capture all CTC subtypes including those that may have potentially lost their epithelial features due to epithelial-mesenchymal transition (EMT), a biological process by which epithelial cells undergo molecular changes and lose the cohesive interaction among cells down-regulating the expression of common plasma membrane epithelial markers, such as epithelial cell

Table 1: Overview of main methodologies for CTC isolation and potential research implementation in prostate cancer

Isolation principle	Technique	Commercially available?	Surface antigen requirement	Cell output	Downstream analyses
Physical	Density gradient centrifugation	Yes	-	Live cells	IF, RT-PCR
	EPIC Sciences™ HD-CTC assay	Yes	-	Fixed cells	IF, FISH, CTC enumeration
Biological	ScreenCell® filter	Yes	-	Live cells	IF, FISH, CTC enumeration
	ISET® filter	Yes	-	Live cells	IF, FISH, CTC enumeration
	CellSearch®	Yes	EpCAM	Fixed cells	IF, FISH, CTC enumeration
	MagSweeper	No	EpCAM	Live cells with bound beads	IF, FISH, CTC enumeration, RT-PCR, RNA-seq
	AdnaTest	Yes	EpCAM/HER2	RNA	RT-PCR
	RosetteSep™ CD45 depletion	Yes	CD45	Live cells	IF, FISH, CTC enumeration, RT-PCR, RNA-seq, CTC-organoid culture and PDX models, <i>ex-vivo</i> drug treatment
Microfluidics	CTC-Chip	No	EpCAM	Live cells on device	IF, FISH, CTC enumeration, RT-PCR, RNA-seq, <i>ex-vivo</i> drug treatment
	HB-Chip	No	EpCAM	Live cells on device	IF, FISH, CTC enumeration, RT-PCR, RNA-seq, <i>ex-vivo</i> drug treatment
	iChip	No	-	Live cells	IF, FISH, CTC enumeration, RT-PCR, RNA-seq, CTC-organoid culture and PDX models, <i>ex-vivo</i> drug treatment
	GEDI Chip	No	PSMA	Live cells on device	IF, CTC enumeration, RT-PCR, RNA-seq, <i>ex-vivo</i> drug treatment
	VERSA	No	EpCAM	Live cells	IF, CTC enumeration, RT-PCR, RNA-seq, <i>ex-vivo</i> drug treatment
<i>In vivo</i>	GILUPI®	Yes	EpCAM	Live cells on device	IF, CTC enumeration, RT-PCR, RNA-seq, <i>ex-vivo</i> drug treatment

CTC: circulating tumor cell; IF: immunofluorescence; RT-PCR: real time polymerase chain reaction; PDX: patient-derived xenograft; HD: high definition; HB: herringbone; GEDI: geometrically enhanced differential immunocapture; EpCAM: epithelial cell adhesion molecule; PSMA: prostate specific membrane antigen

adhesion molecule (EpCAM) and E-cadherin^[14,15]. Importantly, these approaches yield live, unaltered cells, which can be then used in a wide variety of downstream analyses. However, the low purity of the CTC population obtained, due to the presence of many contaminating hematopoietic cells that outnumber the CTCs by several logs, compromises the sensitivity of this technique^[16].

To better differentiate CTCs from peripheral blood cells, immunofluorescence is commonly used in conjunction with density-based separation. CTCs are typically identified as nucleated cells (positive for DAPI staining) that express an epithelial marker [e.g. cytokeratin (CK), or prostate specific membrane antigen (PSMA) in the case of PC CTCs], and are negative for expression of the hematopoietic marker, CD45.

In practice, following a “no cell left behind” philosophy, the Epic Sciences™ high definition (HD)-CTC assay screens for CTCs amid all blood nucleated cells plated onto custom glass slides and identifies epithelial

cancer cells using an immunofluorescence-based algorithm, which measures CK and CD45 intensities, as well as cell physical properties including nuclear and cytoplasmic size and shape^[17]. This assay has been extensively used clinically and identifies CTCs in several cancer types, including NSCLC, breast and prostate cancers^[17,18]. Interestingly, the HD-CTC test recognizes distinct categories of CTCs based on morphologic characteristics of the cells (traditional CTCs, small CTCs, CTC clusters and apoptotic CTCs), whose clinical relevance has yet to be determined^[19]. The versatility of the method is exemplified by the possibility to assess a wide range of protein biomarkers via immunofluorescence (e.g. androgen receptor in prostate cancer or PD-L1 in bladder cancer), specific driver genomic alterations (by FISH) and genome-wide copy number alterations^[20]. Unfortunately, the lack of a robust multiplexing of the technique does not allow for concomitant investigation of more than one or two biomarkers within the same slide and, together with a low resolution used in image acquisition, limits the clinical power of the assay.

Taking advantage of the larger size of CTCs compared to hematopoietic cells (15-25 μm vs. less than 12 μm), many different microfiltration devices have been developed and tested clinically for the isolation of CTCs. These devices employ small pore membranous filters that select CTCs apart from the contaminating PBMCs by size^[21]. ScreenCell® has developed a range of devices based on microporous membrane filters, which are engineered to either capture CTCs for cytological studies, molecular and genetic analysis, or for CTC culture *in vitro*^[22]. Another largely clinically used filter-based approach, ISET® (Isolation by Size of Epithelial Tumor cells, Rare cells Diagnostics), uses membranes with 8 μm pores to retain CTCs allowing smaller blood cells to pass through and be discarded^[23].

Overall, all filtration-based CTC isolation techniques have the advantage of being “antigen agnostic”; as these methods do not discriminate based on expression of plasma membrane antigens, molecularly diverse CTC subpopulations can be retained, including

those undergone EMT potentially missed by epithelial antigen-based approaches. In a direct comparison of performance in CTC enumeration in breast, lung and prostate cancers, the ISET assay isolated CTCs in higher numbers than CellSearch®, suggesting that size-based methods could isolate more than the merely EpCAM positive CTCs^[24]. The ability of ISET to retain CTCs with EMT molecular features is more directly supported by other evidence in the literature that show how ISET-isolated CTCs can express antigens of mesenchymal origins with concomitant lack of epithelial markers^[25,26]. In addition, all these size-based approaches provide the advantage of isolating CTC-clusters, which proved to be critical in metastasis initiation^[27].

Similarly to simple density gradient centrifugation, microfiltration devices produce live, unaltered CTCs with the added benefit of higher purity. These CTCs lend themselves to a wide variety of downstream assays, which can reveal clinically meaningful

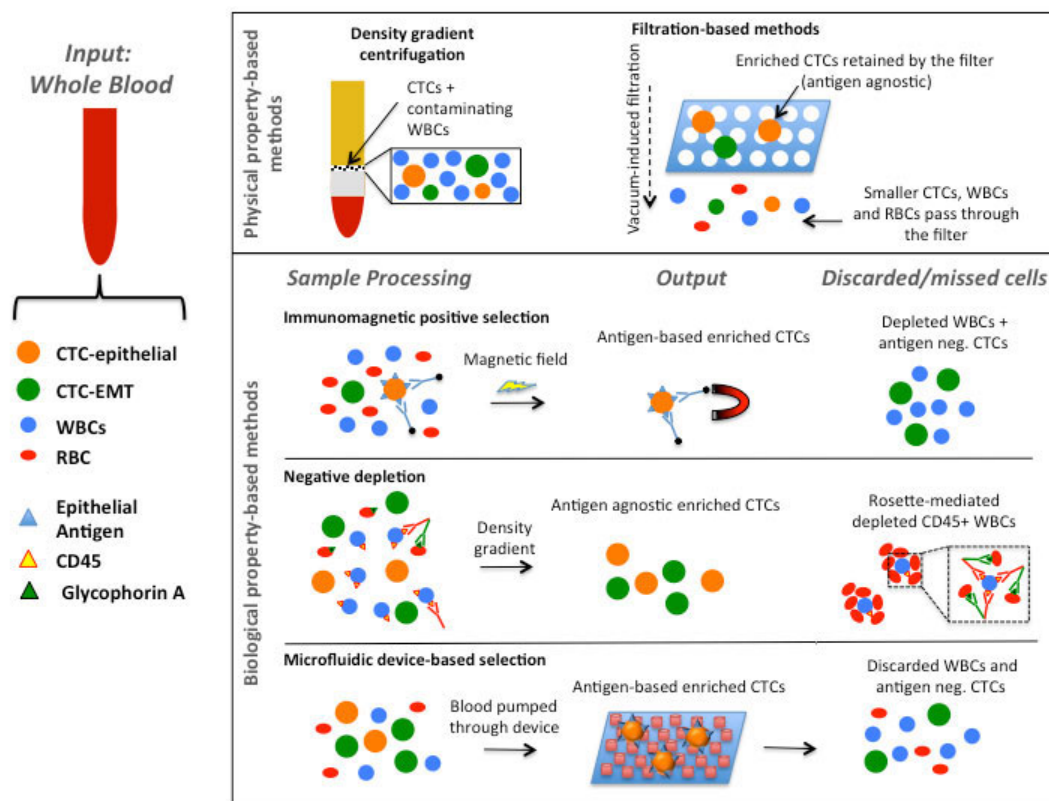


Figure 1: Descriptive overview of the main methodologies to isolate CTCs from the peripheral blood of cancer patients. CTCs can be isolated and enriched from the contaminating WBCs based on either their physical properties (e.g. size, density) or their biological properties (i.e. expression of tumor-selective markers on the plasma membrane). Physical property-based techniques have the potential advantage of isolating molecularly heterogeneous CTC subpopulations, thus including CTCs undergone EMT with low/absent expression of epithelial surface markers. Presence of contaminating leukocytes represents the major limit. Biological property-based methods rely on positive selection of CTCs based on the expression of cancer-specific markers on the surface of circulating tumor cells; alternatively, negative depletion leaves out the unwanted contaminating leukocytes, based on immunomediated depletion of cells expressing the leukocyte-specific CD45 marker. Biological property-based technologies are characterized by high purity of the obtained CTC population, with the caveat of missing CTC subpopulations lacking the expression of the surface marker when positive selection is adopted. CTC: circulating tumor cell; EMT: epithelial-mesenchymal transition; WBC: white blood cells; RBC: red blood cell

biological information about a patient's disease state. On the other hand, a potential downside of the size-based technologies is represented by the high heterogeneity in diameter CTCs can display, with subsets of CTCs with diameters as low as 11 μm , which is virtually indistinguishable from the diameter of leukocytes^[28,29]. These smaller CTCs may be of clinical importance but they are likely missed by the size based filtration techniques.

CTC enrichment based on cell-surface antigen expression or other biological properties

Many CTC isolation techniques exploit the expression of tumor-selective cell surface antigens by using an antibody-antigen interaction for CTC enrichment. The most established and the only FDA-cleared method for CTC isolation is the CellSearch[®] CTC Test system. Starting with 7.5 mL of peripheral blood, CTCs are positively selected using anti-EpCAM-coated magnetic microbeads and subsequently embedded and fixed onto a magnetic cartridge for subsequent immunofluorescence staining with antibodies against CK, CD45, and DAPI^[30]. CTC enumeration by CellSearch[®] has been established as a test predictive of patient outcomes in metastatic breast^[31], colorectal^[32] and prostate cancer patients^[33]. Following the initial excitement for a prognostic test based on a liquid-biopsy, it soon became apparent that enumeration alone could not guide therapy, as there was no information regarding the molecular CTC profiles. Nevertheless, these early clinical studies using CellSearch[®] have provided proof-of-principle regarding the use of EpCAM for the detection of epithelial-type cells in the bloodstream. To date, the majority of antigen-specific CTC enrichment techniques rely on the use of EpCAM. The MagSweeper CTC enrichment represents another example of platforms, which also utilize EpCAM-based immunomagnetic cell isolation^[34].

These two methodologies have been extensively used in several studies for the purposes of CTC enumeration and identification of tumor-specific biomarkers using immunofluorescence or FISH analyses [Table 1]. MagSweeper-based single CTC mRNA seq has also been performed and provided a detectable transcriptional signature of PC tissue^[35]. Despite their extensive use, there are significant concerns associated with the EpCAM-based CTC enrichment techniques. Importantly, the dependence on EpCAM expression for CTC capture could specifically select for only a subset of CTCs resulting in the potential loss of EpCAM-low or -negative CTCs and thus underestimate CTC numbers; it has been indeed shown that the loss of epithelial markers

such as EpCAM could define a more mesenchymal subpopulation of CTCs, whose increase has been associated with tumor relapse during treatment in solid tumors^[36]. In addition, the presence of anti-EpCAM coated beads, which remain bound to the captured CTCs, can interfere with functional applications of the isolated cells. Finally, the added steps of fixation and immunofluorescence staining performed for cell enumeration purposes limits the molecular analysis that can be performed.

Another antigen-based CTC isolation method is the AdnaTest (QIAGEN[®]), which uses anti-EpCAM and -HER2 antibodies conjugated to Dynabeads[™] magnetic particles^[37,38]. CTCs with bound beads are magnetically separated and subsequently processed for gene expression profiling (Prostate Cancer Select) or for PC-specific gene quantification (Prostate Cancer Detect). A major advantage of the AdnaTest is the combination of two isolation markers, EpCAM and HER2, to capture CTCs; the addition of HER2, which can be found expressed in up to 78% of metastatic castrate prostate cancers, could potentially include the EpCAM-/HER2+ CTCs in the enrichment process, a subpopulation of cells, which an EpCAM only based approach may easily miss^[39,40]. Interestingly, pretreatment CTC identification by using the AdnaTest correlated with radiologic progression to chemotherapy in a small cohort of Castration-Resistant PC (CRPC) patients, supporting the clinical relevance of the test^[38]. The main property of the AdnaTest is having nucleic acids as a direct output of the assay for specific downstream analyses (e.g. RT-PCR, DNA sequencing, gene expression profile)^[41]. Even if this characteristic precludes the direct visualization of the captured cells, it provides flexibility to incorporate and explore additional markers, like androgen receptor (AR) splice variants or stem cell markers^[38,42]. Although potentially more comprehensive in CTC isolation than EpCAM only strategies, this method is still based on the expression of tumor-selective antigens, an approach that may still omit certain CTC subpopulations.

To take advantage of both the physical and biological properties of CTCs, several microfluidic devices have been developed to capture CTCs from whole blood. The design of many of these devices takes advantage of the blood flow through the device that is engineered to facilitate the collisions of the larger CTCs with microposts, eliminating the smaller leukocytes; the posts are coated with an anti-epithelial marker antibody to specifically retain tumor epithelial cells. The CTC-Chip utilizes an array of microposts coated with an antibody against an epithelial marker (EpCAM, HER2, EGFR) to

capture cells of epithelial origin and proved efficient in isolating CTCs from different tumor types^[43]. Improvements to the fluid dynamics within the CTC-chip led to the development of the herringbone-chip, which generates microvortices to multiply the number of interactions between CTCs and the antibody-coated chip surface^[44]. The geometrically enhanced differential immunocapture chip employs a 3D geometry with microposts functionalized with a monoclonal antibody to the cell-surface antigen PSMA to enhance the capture of CTCs from PC patients^[45]. All these microfluidic devices have been shown to capture live cells that can be used for many subsequent molecular assays (IF, FISH, DNA or RNA sequencing), including CTC *in vitro* culturing and *ex-vivo* drug treatment^[46].

The novel microscale platform VERSA (Versatile Exclusion-Based Rare Sample Analysis) optimizes the potentially harmful washing, cell transfer, and centrifugation steps of many microfluidic devices by coupling EpCAM-coated paramagnetic particles (PMPs) with immiscible barriers that aid in the removal of contaminating PBMCs and unbound PMPs through surface tension over gravity at the microscale on the single device^[47]. This innovative approach demonstrates very versatile and is able to perform different assays, as it can either isolate live cells for subsequent nucleic acid extraction and analysis, or perform cell fixation and immunofluorescence directly within the wells of the device, which is engineered for CTC microscopy.

In order to avoid the pitfalls of selecting for CTCs based on positive expression of epithelial markers, negative depletion techniques have emerged that target and remove the contaminating leukocytes, thus allowing the enrichment of a pool of heterogeneous subpopulations of CTCs.

RosetteSep™ Circulating Epithelial Tumor Cells enrichment kit (STEMCELL™ Technologies) employs anti-CD45/anti-glycophorin A antibody complexes that create crosslinks between red blood cells and white blood cells (immunorosettes), that are then eliminated by gradient-based separation, leaving behind a CTC-enriched cell population^[48]. A major benefit of this approach is the possibility to enrich live CTCs, without sample fixation, allowing live cell studies such as development of CTC-derived xenografts (PDX) and gene expression profiling^[49].

The CTC iChip captures CTCs using the two-stage process of deterministic lateral displacement, inertial focusing and magnetophoresis to achieve leukocyte depletion and CTC enrichment, in an antibody

independent manner. The device can then release the enriched live CTCs, which can be used for standard cytopathologic evaluations, RNA or DNA-based analysis and to generate CTC-derived cell culture^[36,50].

All of the approaches described above isolate CTCs from a tube of peripheral blood collected from the patient. The GILUPI CellCollector® is a medical guide wire functionalized with anti-EpCAM antibodies that is designed to capture CTCs directly from the circulating blood of patients after it is inserted in the cubital vein of patients for 30 min. This device can interact with an estimated 1.5-3 liters of blood^[51], and has the advantage of being able to isolate live CTCs from volumes of blood larger than what usually reported in the literature and obtained from patients with either localized or advanced PC^[52,53].

CTC isolation techniques are constantly seeing new and improved methods to separate these rare tumor cell populations from other nucleated blood cells. Particular emphasis has been given to methods that move away from antigen-dependent capture and can isolate live CTCs, regardless of the expression of specific surface markers, like EpCAM, as cells with low or complete loss of expression of EpCAM likely hold clinically meaningful information^[36]. In addition to enabling a diverse array of downstream biomarker analyses, live CTCs can also be expanded through culturing methods or organoid development, opening new exciting scenarios in tumor biology^[54,55]. Organoids originating from CRPC CTCs have been successfully generated and reproduce some of the major molecular features commonly found in the primary tumor, such as expression of SPYKN1, STEAP1 and TMPRSS2, confirming CTCs are a precious readily available source of tumor material that can be used to generate *in vitro* PC models to explore and understand disease pathogenesis and progression^[55].

CTC AS SOURCE OF TUMOR TISSUE FOR BIOMARKER INVESTIGATION

The biomarker-based approach has considerably transformed the treatment strategies of cancer, improving response rates and overall survival; however, the availability of tumor tissue is required to study and understand the biology of these biomarkers and to follow the evolution of these molecular alterations over time.

Using CTCs as source of tumor material to characterize the molecular profile of the disease represents the most significant and exciting impact for the clinical practice, especially in the current era

of personalized medicine. Importantly, CTCs provide the ideal tool to analyze and longitudinally monitor the expression and/or the dysregulation of several tumor related biomarkers, associated with response or resistance to investigational antitumor agents and to the already FDA-approved drugs currently used in clinical practice.

In the next paragraphs, we will describe the most recent results regarding the CTC-based analysis and validation of the most clinically relevant biomarkers in PC [Table 2].

CTCs as tool to unveil tumor biology in the single patient

By filling the gap of chronic lack of tumor tissue, CTCs provide the opportunity to perform broad-spectrum genomic analyses to unveil the molecular characteristics of the disease in the individual patient, a key point for a precision medicine-based approach.

To investigate the use of CTCs to overcome the inaccessibility of metastatic tissue for high throughput genomic testing, Lohr *et al.*^[12] investigated the role of CTCs in comprehensive analyses of cancer genomes by developing a census-based whole exome sequencing method to analyze single EpCAM-positive CTCs isolated from CRPC patients. The CTC-data were then compared to the genetic profiles of the primary tumor and one metastatic site. The authors found that CTCs harbored up to 73% of the somatic single nucleotide variants identified in the primary tumor and in the metastatic site. Gupta *et al.*^[56] also conducted a whole genomic copy number analysis of CTCs and matched leukocytes from 16 men with CRPC using array-based comparative genomic hybridization (aCGH) and observed CTC-specific genomic gains (i.e. AR, FOXA1, ERG) and genomic losses (i.e. PTEN, RAF1, GATA1) in key regulators of PC progression.

Interestingly, Miyamoto *et al.*^[57] performed RNA sequencing profiles of single CTCs isolated from 13 metastatic PC patients; they recognized CTC-specific up-regulation of molecular pathways linked to cell growth and adhesion, found AR point mutations associated with AR inhibitors resistance and identified high inpatient CTC heterogeneity for AR alterations, showing that more than half of the patients had multiple AR splice variants within different CTCs.

These studies provide proof of the feasibility of CTC-based high throughput sequencing in individual cancer patients using a high-throughput analysis, thus, offering a minimally invasive look into the mutational landscape of metastatic PC.

Evaluation of androgen receptor expression and signaling in CTCs

AR is a transcription factor, which plays a key role in prostate homeostasis and drives growth and progression of PC cells. AR-targeting drugs (ADT, abiraterone acetate, enzalutamide) are current standard of care for PC treatment and exert their anti-tumor activity mostly by inhibiting AR activation and its consequent nuclear translocation, ultimately impeding AR mediated tumor growth^[1,2]. Although most patients initially respond to these therapies, treatment-related resistance often occurs through a variety of mechanisms^[58]. AR dysregulation (i.e. amplification, mutations, alternative splicing) represents the key molecular alteration in PC, leading to aberrant activation of the receptor and its downstream pathways with consequent tumor progression. Clinical assessment of these molecular alterations has been challenging and currently there is no way to clinically predict which patients are more likely to respond to AR-targeting therapies.

AR expression and nuclear localization, considered readouts of AR activation, have been subject of translational studies as biomarkers of response to AR-targeting drugs in PC. Many research groups have used CTCs as a liquid biopsy to longitudinally assess these markers and correlate them with treatment outcome.

Reyes *et al.*^[59] evaluated AR expression levels and subcellular localization in CTCs isolated from a cohort of 20 CRPC patients to develop a platform for CTC protein interrogation. In this study, cells were isolated by gradient medium-based separation and were fluorescently stained for EpCAM, CD45 and AR. AR expression and nuclear accumulation were then analyzed in confirmed CTCs by ImageStream[®]X. Results revealed a significant increase in AR expression levels in CTCs of patients who progressed on abiraterone compared to patients who were abiraterone-naïve. The authors also looked at AR nuclear localization in the isolated CTCs and observed high inter- and intra-patient heterogeneity without a clear correlation with treatment response. Similarly, Crespo *et al.*^[60] quantified AR nuclear expression in CTCs isolated from 48 subjects grouped by absence of prior exposure or resistance to abiraterone or enzalutamide. After CellSearch[®]-based enrichment, CTCs were analyzed for the detection of AR by arranging a user-defined assay based on the CellTracks Autoprep system. No difference was observed in nuclear AR expression between treatment naïve and resistant patients; however, when analyzing eight patient-matched CTC samples collected before and after treatment, the authors found an increase in

Table 2: CTC-based molecular markers investigated in prostate cancer

Enrichment strategy	Biomarker	Assay	Patient cohort	Main findings	References
Ficoll	ERG	RT-PCR	72 pts treated with taxane chemotherapy (50 with docetaxel and 22 with cabazitaxel)	Detection of TMPRSS2-ERG was significantly correlated with shorter PSA-progression free survival	[81]
FACS sorting	AR	IF	20 pts (10 abi-treated, 10 abi-naïve)	Increase in median AR staining intensity in patients with prior abi exposure	[59]
	Ki-67	IF	16 pts for AR expression and 10 pts for AR nuclear localization	Increased AR expression and nuclear localization associated with elevated Ki-67	[59]
	Steroidogenic enzymes	RT-PCR	5 pts	Detection of AKR1C3, SRD5A1, CYP17A1 in CTCs from pts with CRPC	[82]
ScreenCell®	EMT-related genes	RT-PCR	308 CTCs from 8 pts	Subset of EMT-related genes found in CTCs of CRPC; less frequently in castration-sensitive PC	[86]
HD-CTC assay (EPIC Sciences™)	AR	IF	27 pts (12 with NEPC, 5 with atypical clinical features suggestive of NEPC, 10 with CRPC)	Low or absent AR expression, lower cytokeratin expression, and smaller morphology in CTCs from NEPC pts	[87]
	ARv7	IF	191 samples from 161 pts	Nuclear expression of ARv7 in CTCs associated with superior survival on taxane therapy over AR inhibitors	[69]
CellSearch®	AR	IF	Taxane treated pts: 17 samples from responding/stable pts; 18 samples at progression	13/18 (72%) of progression samples showed CTCs with nuclear AR; 12/17 (71%) of responding/stable samples showed CTCs with cytoplasmic AR	[62]
		IF	8 pts (baseline and at progression after abi or enza)	5/8 (63%) patients showed increase median AR expression at progression	[60]
		FISH	9 pts with > 5 CTCs	Marked AR amplification in 5/9 (56%) pts (all with CTC > 50)	[88]
		FISH	33 pts with > 4 CTCs	28/33 (85%) pts had CTCs with < 3 copies of AR, 10/33 (30%) with < 4-5 copies, 15/33 (45%) with > 5 copies	[76]
	ERG	FISH	49 pts with > 4 CTCs	ERG rearrangement in 23/49 (47%) pts (matched ERG gene status of tumor tissue in all cases)	[76]
	PTEN	FISH	49 pts with > 4 CTCs	PTEN loss in 13/49 (27%) pts; (6/13 (46%) pts had homozygous loss of PTEN in all CTCs)	[76]
	EGFR	IF	20 pts with > 5 CTCs	18/20 (90%) pts with EGFR positive CTCs	[88]
AdnaTest	ARv7	RT-PCR	62 pts (31 treated with abi, 31 treated with enza)	0/18 (0%) pts with detectable levels of ARv7 had PSA response to either abi or enza	[42]
		RT-PCR	37 pts receiving taxane chemotherapy	No difference in PSA response between pts with or without detectable ARv7	[68]
MagSweeper	Prostate-specific markers	RT-PCR	20 CTCs from 4 pts	19/20 (95%) of CTCs positive for at least one prostate marker (AR, PSA, KLK3, TMPRSS2)	[35]
EpCAM-conjugated magnetic beads	ERG	RT-PCR	41 pts with CTCs	TMPRSS2-ERG fusion detected in 15/41 (37%) of pts	[76]
VERSA	AR	IF	17 pts (4 treatment-naïve, 10 responding to therapy, 3 progressing on therapy)	Pts responding to AR-targeted or chemotherapy treatments showed lower nuclear AR percentage compared to pts progressing	[47]
	ARv7	RT-PCR	26 pts (19 treated with AR targeted therapies)	ARv7 was detected in 5/7 (71%) pts who had evidence of radiographic progression compared to 1/19 (5%) pts without evidence of radiographic progression	[47]

Continued...

Enrichment strategy	Biomarker	Assay	Patient cohort	Main findings	References
RosetteSep™ CD45 depletion	PTEN	aCGH	1 pt derived organoid from CTCs (MSK-PCa5)	Homozygous loss of PTEN	[55]
	RB1	aCGH	1 pt derived organoid from CTCs (MSK-PCa5)	Heterozygous loss of RB1	[55]
	TP53	aCGH	1 pt derived organoid from CTCs (MSK-PCa5)	Heterozygous loss of TP53	[55]
CD45 depletion	AR variants	RT-PCR	73 samples from 46 pts	ARv7 in 50/73 (68%) and ARv567s in 23/73 (32%) of samples; strong association of ARv positivity with a history of second line hormonal therapy	[70]
Collagen-adhesion matrix fractionation	Genome aberrances	aCGH	3 pts with matched CTC and WBC DNA samples (2 pts with matched primary tumor sample)	Greater percentage of genome aberrance in CTCs compared to WBC; similar genome aberrance in CTCs and primary tumors	[89]
GEDI Chip	AR	IF	26 taxane treated pts with available CTCs at C1D1 and C1D8	Pts who experienced PSA response had lower CTC-AR nuclear after only one week of treatment localization than pts who did not respond	[63]
	ERG	IF	2 pts (1 ERG+ and 1 ERG-)	CTCs of ERG-pt showed increase in taxane induced microtubule bundling when treated ex-vivo with docetaxel or cabazitaxel whereas CTCs of ERG+ pt showed no microtubule changes	[80]
HB-Chip	AR	IF	4 ADT-naïve pts with metastatic PC; 17 pts treated with abi	ADT-naïve pts with metastatic PC had a majority of CTCs with “AR-on” phenotype and initiation of ADT led to conversion to a “AR-off” within 3 months; 4/17 (24%) pts treated with abi showed a decrease in “AR-on” CTCs; CRPC pts had mixed phenotypes	[61]
	ERG	RT-PCR	2 pts with matched primary tumors	TMPRSS2-ERG fusion in CTCs of pt with translocation in primary tumor; no TMPRSS2-ERG in CTCs from patient without fusion in primary tumor	[44]
CTC iChip	AR expression	RNA-seq	77 CTCs from 13 pts	AR transcript in 60/77 (78%) of CTCs from 12/13 (92%) patients	[57]
	AR T877A mutation	RNA-seq	77 CTCs from 13 pts	T877A mutation in 5/9 (56%) CTCs from 1/13 (8%) pts	[57]
	ARv	RNA-seq	77 CTCs from 13 pts	33/73 (43%) CTCs with at least one ARv: 26/73 (36%) CTCs with ARv7; 18/73 (25%) CTCs with ARv567es; 7/73 (10%) CTCs expressed ARv1, ARv3, or ARv4	[57]
	Wnt	RNA-seq	77 CTCs from 13 pts	Enrichment for non-canonical Wnt signaling in patients who progressed on enza	[57]

CTC: circulating tumor cell; RT-PCR: real time polymerase chain reaction; IF: immunofluorescence; AR: androgen receptor; EMT: epithelial-mesenchymal transition; pts: patients; abi: abiraterone acetate; enza: enzalutamide; WBC: white blood cell; HD: high definition; HB: herringbone; GEDI: geometrically enhanced differential immunocapture; aCGH: array-based comparative genomic hybridization

nuclear AR expression at progression compared with the pre-treatment assessment.

Sperger *et al.*^[47] evaluated AR expression and subcellular localization in CTCs isolated from 17 patients with CRPC using an EpCAM-based enrichment embedded in the VERSA device. They found that patients responding to AR targeted therapies or docetaxel-based chemotherapy showed lower percentages of AR in the nucleus compared to patients who progressed on treatment. When combining single cell data points from multiple patients, they also showed that CTCs from patients responding

to treatment generally had lower AR expression and lower percentage of AR in the nucleus.

Miyamoto *et al.*^[61] explored AR signaling activity in CTCs isolated from PC patients, by using an EpCAM-based herringbone microfluidic device, and observed that AR-dependent pathway was active in untreated patients, became inactive after ADT, and could be reactivated at the time of progression, supporting CTCs as a dynamic biomarker that could reflect the drug-induced changes of the therapeutic target in real time.

Taken together, these observations demonstrate that the interrogation of AR expression and subcellular localization in CTCs is not only feasible but could also be informative as predictive therapeutic biomarker for AR-targeting treatments. More extensive prospective validation is required but initial findings are very encouraging.

Taxanes (docetaxel, cabazitaxel) are the only class of chemotherapy drugs that improve survival in CRPC patients. Even though traditionally considered as anti-mitotic drugs, taxanes exert their PC-specific mechanism of action by stabilizing microtubules and consequently impairing the microtubule-dependent AR nuclear translocation and the consequent signaling activation^[62]. The combined inhibition of the microtubule-AR axis mechanistically supports the unprecedented clinical benefit of survival observed in the CHARTEED and the STAMPEDE trials, in which taxane treatment and ADT are combined to treat hormone-sensitive metastatic PC^[3]. Hence, AR cytoplasmic sequestration has been proposed as marker of response to taxane activity. Darshan *et al.*^[62] retrospectively correlated AR subcellular localization in CTCs with clinical response in CRPC patients receiving taxanes and showed that 72% of subjects who progressed after treatment had AR nuclear localization and, on the contrary, 70% of responding patients had cytoplasmic AR localization. These data have been prospectively validated with the analysis of the TAXYENERGY trial, a phase II randomized trial in which AR subcellular localization was prospectively assessed in CTCs of taxane-treated CRPC patients. In this trial, subjects were randomized 2:1 to first line docetaxel or cabazitaxel and CTCs were used as a source of tumor tissue to monitor longitudinally potential predictive biomarkers including AR nuclear localization, AR variants, presence of intra-tumoral drug-target engagement. Of the 63 patients enrolled in the study, 26 had CTC evaluable before the first cycle of treatment (C1D1) and after one week (C1D8); in these subjects, taxane-induced decrease in AR nuclear localization (C1D8 vs. C1D1) was associated with a higher rate of biochemical response ($\geq 50\%$ PSA decrease at cycle 4, $P = 0.009$), suggesting that AR nuclear localization assessment can serve as early biomarker of clinical benefit in patients treated with taxanes^[63].

These results strongly support the use of CTCs as source of tissue to interrogate AR subcellular localization in tumor cells as marker to predict response to taxane chemotherapy.

Detection of AR alternative splicing variants in CTCs

Alternative splicing of AR has emerged as one of the main mechanisms of disease progression in PC. The clinically relevant ARv7 and ARv567 are truncated versions of the receptor, which partially or entirely lack the C-terminal ligand-binding domain and are constitutively active, independent of the ligand^[64,65]. Recently, AR splice variants have been repeatedly associated with resistance to standard ADT and second-generation AR signaling inhibitors, abiraterone and enzalutamide^[66].

Several studies have been reported, in which CTCs are used as liquid biopsy to detect AR variants in PC patients and monitor their expression longitudinally during treatment. In a single institution prospective study, Antonarakis *et al.*^[42] used a modified version of the EpCAM-based AdnaTest Prostate Cancer Detect assay to evaluate ARv7 mRNA levels by RT-PCR in CTCs isolated from CRPC patients receiving enzalutamide ($n = 31$) or abiraterone ($n = 31$). They detected ARv7 transcript in 39% of the enzalutamide cohort and in 19% of the abiraterone cohort, and showed that ARv7-positive enzalutamide-treated patients had significantly lower biochemical response (0% vs. 53%) and shorter progression-free (PFS) and overall survivals (OS) than ARv7 negative subjects (2.1 months vs. 6 months and 5.5 months vs. unreached, respectively). The authors observed similar results among the ARv7-positive subjects receiving abiraterone, who experienced lack of PSA response (0% vs. 68%) and significantly shorted PFS and OS (2.3 months vs. not reached and 10.6 months vs. not reached, respectively) compared to ARv7-negative men.

Following previously reported *in vitro* data that associated ARv7 positivity with lack of response to taxane treatment^[67], Antonarakis *et al.*^[68] used the above-mentioned CTC-based RT-PCR assay to investigate a clinical correlation between pre-treatment ARv7 status and response to taxanes in CRPC patients. Although no significant difference was detected between ARv7-positive and ARv7-negative patients in terms of PSA response to treatment, PFS and OS, a clear trend towards inferior response rate to taxane was observed in ARv7-positive men. The sample size of this study was too small and the median follow-up was relatively short to obtain any meaningful clinical conclusion and validation in a larger cohort study is expected.

Sperger *et al.*^[47] also investigated the expression of ARv7 in CTCs. Using the VERSA platform on 26

patients with mCRPC (19 of whom had previously received or were being treated with AR signaling inhibitors), they found that detectable expression of ARv7 was significantly higher in patients with radiographic progression compared to patients with only PSA progression or PSA response to AR signaling inhibitors (71% vs. 5%, $P = 0.007$).

To address the role of ARv7 as marker of response to AR targeting drugs and taxanes in CRPC, Scher *et al.*^[69] adopted a protein-based CTC assay in which the isolated cells were fluorescently stained for the presence of the variant by using the Epic Sciences™ HD-CTC platform. The authors observed that the frequency of ARv7-positive CTC detection increased with subsequent therapies ranging from 3% prior to first-line treatment to 31% prior to third or subsequent lines. This protein-based approach confirmed that patients with ARv7-positive CTCs were more resistant to AR signaling inhibitors, with no significant difference for biochemical response and PFS after taxane treatment, thus supporting the results of the other CTC-based correlative studies. Even though probably less sensitive and specific than RT-PCR-based methods, this protein-based methodology provides information of the AR variants' status at a single CTC resolution and could potentially offer the possibility to perform CTC subpopulation analysis based on other informative molecular and phenotype characteristics, such as EMT markers.

Taken together, these studies strongly encourage the use of CTC-based approaches to profile AR splice variants and support the development of more sensitive methodologies to detect and quantify these tumor-related biomarkers.

Recently, other assays have been proposed to identify and quantify AR splicing variants from CTCs. Liu *et al.*^[70] developed a CTC-based RT-PCR platform to assess both clinically relevant alternative transcripts, ARv7 and ARv567 and tested it in 73 whole blood samples from 46 CRPC patients. The authors found ARv7 and ARv567 in 68% and 32% of the samples, respectively, and detected a strong association of higher AR variants' expression levels with patients who had received hormonal therapies compared to hormone-naïve men. Qu *et al.*^[71] developed a highly sensitive and specific digital droplet PCR assay and quantified ARv7 expression levels in PBMCs of 132 PC patients; ARv7 transcripts could be detected in more than 95% of the subjects and a significant correlation was found between high ARv7 levels and worse outcome after treatment with AR signaling inhibitors.

Detection of TMPRSS2-ERG fusion and ERG expression in CTCs

ERG is a transcription factor, which is over-expressed in approximately half of primary PCs, following the gene rearrangement that fuses the AR-regulated TMPRSS2 promoter with the coding region of ERG gene^[72]. ERG rearrangements and over-expression define the largest of the molecular classes of PC based on molecular oncogenic drivers and have been repeatedly associated with accelerated disease progression and worse prognosis^[73,74]. More recently, many reports have correlated response to treatment to ERG expression, which became a compelling biomarker to investigate clinically.

The use of CTCs for ERG assessment has been extensively explored in both gene-targeted approaches (e.g. FISH, RT-PCR) and broad-spectrum techniques (aCGH)^[56,75]. To date, several studies have focused on the role of CTC-ERG as predictive marker to treatment response in PC, with conflicting data correlating ERG expression and outcome after AR signaling inhibitors. Attard *et al.*^[76] investigated ERG fusion by FISH in CellSearch®-isolated CTCs from therapy-naïve PC patients and found a significant correlation between ERG rearrangement and PSA decline after abiraterone therapy. Subsequently, ERG rearrangement status, in particular the duplication of the fusion of TMPRSS2 to ERG sequences (2+ Edel), was associated with a better clinical response to abiraterone treatment, supporting ERG assessment in CTCs as predictive biomarker^[77]. In contrast, Danila *et al.*^[78] could not find any correlation between TMPRSS2-ERG status and clinical outcome after abiraterone therapy, by using a RT-PCR-based approach to detect ERG fusions in CTCs from docetaxel-resistant CRPC patients. The discrepancies in the observed results concerning ERG in CTCs and treatment outcome could be attributed to significant differences in the methodologies adopted to evaluate the biomarker and in the cohorts of patients analyzed. Further prospective evaluation of the role of ERG expression in predicting response to AR-targeted therapies is currently ongoing^[79].

ERG fusion status also showed to correlate with lack of response to taxane treatment^[80]. Reig *et al.*^[81] measured TMPRSS2-ERG mRNA expression by RT-PCR in PBMCs isolated from 50 docetaxel-treated CRPC patients by gradient medium separation and showed that TMPRSS2-ERG significantly correlated with lower biochemical response, radiological PFS and overall survival. Even though performed without any form of enrichment for epithelial CTCs, these findings support the clinical role of ERG assessment to tailor treatment strategies.

Investigation of other molecular makers in CTCs

Intratumoral androgen synthesis has been identified as one of the mechanisms PC cells adopt to activate AR signaling in presence of castrate levels of circulating androgens. Several steroidogenic enzymes are involved in this process and the expression profiling of these molecules could be found altered in CRPC, with overexpression of enzymes involved in androgen synthesis (SRD5A1, ARK1C3) and down regulation of enzymes implicated in androgen inactivation (SRD5A2, CYP3A4, CYP3A5). Mitsiades *et al.*^[82] evaluated the levels of expression of AKR1C3, SRD5A1 and CYP17A1 by RT-PCR in CTCs isolated from CRPC patients following EpCAM-based PBMCs sorting. Even though no clinical correlations were made between the expression levels of these enzyme and tumor response to ADT, the authors demonstrated the feasibility of profiling key molecules in androgen metabolism allowing the assessment and monitoring of potentially druggable targets.

EMT is a biological process that allows molecular changes and enhancement of the migratory and invasive capabilities of the epithelial cancer cells.^[15] Many molecules and pathways have been implicated in the regulation of the EMT-dependent cellular plasticity and of its role in tumorigenesis^[83]. Recently, Ware *et al.*^[84] identified a novel role of the epithelial plasticity marker Snail in the induction of enzalutamide resistance in PC. Even though a direct clinical evaluation of Snail in CTCs of PC patients has not been reported yet, Li *et al.*^[85] proved the feasibility of Snail expression levels assessment by immunofluorescence in CTCs isolated from hepatocellular carcinoma patients.

CONCLUSION

CTCs first showed their clinical relevance more than a decade ago, when it was reported that elevated CTC counts were associated with tumor prognosis and that changes in enumeration correlated with treatment response in solid tumors. Nevertheless, CTC count is far from being used in the everyday practice for lack of clinical utility.

CTCs rapidly demonstrated their full potential to function as a true liquid biopsy and provide relevant biological information to describe the molecular portrait of disease.

It's true that not all enrichment techniques are equal and it is necessary to distinguish between platforms that are purely scientific tools and methods that have to be regarded as clinically validated and

commercially available assays. Most of these platforms are already commercially accessible to the public as research tools to enrich CTCs for downstream molecular analysis (i.e. RosetteSep™ epithelial tumor cells enrichment kit, ISET® filters). Conversely, only CellSearch® received FDA clearance for CTC enumeration in solid tumors and few other methods are performed in CLIA-certified laboratories to interrogate specific key molecular tumor markers (i.e. AdnaTest Prostate Detect and Epic Sciences™ for AR-V7) but the vast number of technologies currently available for the enrichment of CTCs enables researchers to examine nearly any biological property of this cell population and potential implement that information into clinical practice.

The evaluation of tumor-specific alterations in CTCs could potentially reveal clinically relevant information to guide treatment selection based on the molecular features of the disease in individual patients. CTCs could be used to inform medical oncologists about the major biological alterations of PC that have been shown to significantly affect treatment outcome. EpCAM-based approaches have been mostly adopted to perform these molecular analyses, however other emerging technologies could also play a significant role in dissecting the biologic properties of PC CTCs, especially in diseases with more aggressive features.

Clinical studies are currently ongoing to prospectively clarify the role of CTC-based assessment of these molecular markers to predict patient response and contribute to the era of precision medicine. However, the clinical utility of a liquid biopsy-based evaluation of a predictive marker still needs to be addressed with clinical trials in which any therapeutic strategy is planned according to the liquid biopsy analysis.

The role of CTC enumeration has also been tested in localized PC to identify those patients more likely to relapse after radical treatment, and even in this setting a clear correlation between CTC detection and prognosis has been identified. However, whether or not adding CTC status in the panel of the standard prognostic marker still remains an unanswered question.

In conclusion, CTC analyses could provide crucial information on tumor biology, with the potential to enhance the quality and the efficacy of treatments with the ultimate goal of improving patient survival.

DECLARATIONS

Authors' contributions

Concept and design: G. Galletti, D. Worroll, D.M. Nanus, P. Giannakakou

Literature search: G. Galletti, D. Worroll

Manuscript preparation: G. Galletti, D. Worroll

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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Adenosine A2B receptor: novel anti-cancer therapeutic implications

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Extracellular adenosine is a product of the metabolism of nucleotides such as ATP and ADP and mediates a wide range of events under normal and pathological conditions^[1,2]. Adenosine receptors belong to the G-coupled signalling receptors and are broadly expressed in normal tissues in 4 subtypes (A1, A2A, A2B, A3).

While A2B has traditionally been considered of less relevance in comparison to A2A due to lower affinity of the ligand for this adenosine receptor subtype, recent evidence strongly suggests a specific role of this receptor in cancer and other pathological conditions^[3,4].

The authors of this review are experts in the field of adenosine signalling. In this work they analyse the oncogenic role of the A2B receptor, also proposing and discussing its targeted blockade as a new anti-cancer therapeutic option.

As they explain, the mechanisms thought to be involved in A2B-mediated tumour progression are multiple. Adenosine is responsible for modulating the tumour microenvironment and the phenomenon of angiogenesis via production of growth factors,

cytokines and chemokines. Also, it is involved in the regulation of dendritic cells and macrophages differentiation and function, aspects, these, crucial for tumour immune-surveillance. Lastly, via its A2B receptor, adenosine modulates the inflammatory response to the tumour and promotes tumour cells migration and therefore metastasis.

The effects of protracted inflammation can be devastating on normal tissues. Adenosine modulates inflammation by enhancing differentiation of T-regulatory and myeloid derived suppressor cells which are able to induce T-cells anergy^[5]. Also, through its A2B receptor, adenosine induces anti-inflammatory cytokines such as IL-10, further limiting the amplification of the inflammatory biochemical cascade. On the other hand, activation of the A2B receptor can be associated with pro-inflammatory effects through activation of mast cells, fibroblasts and other epithelial cells, such as intestinal cells^[6]. The pro- or anti-inflammatory action of adenosine has been subject to extensive study and debate through the recent years and it seems to be dependent on specific cell type and extracellular microenvironment^[7,8].



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Phylogenetically, both the anti-inflammatory and the pro-inflammatory actions of adenosine have a protective function, the first keeping in check the cascade of events typical of inflammation and thus avoiding tissue damage, the second facilitating the reaction to and elimination of foreign pathogens.

Exactly as it is the case for other immune-checkpoints, tumour cells can exploit these defence mechanisms to induce immune suppression and cancer-tolerance. In this context, the expression of A2B in immune cells has attracted a lot of attention, as the receptor seems to drive the expansion of immunosuppressive, pro-angiogenic and cancer tolerant cells^[9]. For these reasons, adenosine may be considered itself an immune-modulatory checkpoint molecule^[10]. This hypothesis is further strengthened by evidence of a synergic anti-tumour effect elicited combining anti-PD-1 (or CTLA-4) and adenosine signalling inhibitors^[11-14]. This synergism requires CD73 (one of the nucleotidases involved in adenosine generation) expression on tumour cells, suggesting that the adenosine produced in the context of the tumour could interfere with the effect of targeted immunotherapies^[13]. The addition of A2A- or A2B-receptor inhibitors to current targeted immunotherapies could therefore represent a means to overcome acquired resistance to such treatments.

It is worth noting that, besides the effects of adenosine and A2B on the immune system, the expression of this receptor on the surface of cancer cells seems to mediate important oncogenic effects in a variety of cancers. Pharmacological inhibition or knockdown of A2B decreases proliferation of tumour cells^[15-17] and a role for A2B in tumour progression and metastasis is supported by multiple studies in bladder, breast, colon, prostate and other cancers^[14,15,18-20].

In particular in triple negative breast cancer, expression of CD73 is associated with poor prognosis and pharmacological resistance to doxorubicin^[21]. Similarly, high expression of A2B in cancer cells increases invasiveness and metastasis and is a predictor of poor prognosis and shorter survival in triple negative breast cancer (TNBC)^[19]. On the other hand, presence of A2B in the host immune cells does not impact the metastatic potential of TNBC tumour cells in the same metastatic mouse models of breast carcinoma, as demonstrated by the fact that blockade of tumour-expressed A2B receptor, in A2B receptor-deficient mice, reduces the metastatic burden from TNBC cell lines xenografts.

Moreover, constitutive activation of the adenosine receptor A2B in response to a hypoxic

microenvironment has been associated with increased proliferation of prostate cancer cells *in vitro*^[20].

Lastly, the A2B receptor has been shown to activate downstream oncogenic pathways frequently mutated in cancer such as mitogen-activated protein kinase^[18,19], as well as phospholipase C, cathelicidin antimicrobial peptide, *NFκB1* and arachidonic acid signalling. Moreover, A2B is also a downstream target of the transcription factor *Fos-related Antigen-1 (Fra-1)*, a gene involved in the development of metastasis^[16]. A2B pharmacological blockade in *Fra-1* positive breast cancer cells inhibited metastasis to the lungs in a mouse model of metastatic breast cancer. In the near future, it is possible that identification of *Fra-1* positive tumours will guide the stratification of patients that are most likely to respond to A2B inhibitors.

Notwithstanding the mounting evidence in favour of an oncogenic role for A2B, most of the data supporting this hypothesis come from pre-clinical studies *in vitro*. Whilst A2A small molecule inhibitors are already in clinical development (NCT02403193 and NCT02655822), the same cannot be said for A2B receptor inhibitors. All the A2B receptor inhibitors have been tested *in vitro* and *in vivo*, but their pharmacokinetic characteristics are still mostly unknown.

A2B has the potential to become a therapeutic target, at least in tumours overexpressing the protein. However, more studies are needed to explore all the functions of the A2B receptor and its ligand, particularly with the aim of gaining a better understanding of the multiple A2B receptor-independent metabolic effects of adenosine^[22].

Moreover, due to the extensive cross-talk and the number of molecular targets involved with the adenosine signalling pathway, and considering the ubiquitousness of the receptors, it is conceivable that side effects of the inhibition of CD73, A2A and A2B could represent an issue in the clinical setting.

Finding strategies to specifically target receptors expressed on tumour cells could help mitigate the toxicity of these agents. Also, using these drugs in combination with other targeted agents, as it is already the case with A2A-inhibitors, will hopefully further decrease toxicities by exploiting the synergism shown in combination therapy.

Finally, pharmacologically modulating metabolic conditions such as hypoxia should likely increase the effectiveness of these molecules.

Accumulating knowledge of the adenosine signalling targets drives the identification of biomarkers and predictors of response/resistance, opening therefore the possibility of a personalised therapeutic approach.

DECLARATIONS

Authors' contributions

Concept and design of the article: S.P. Corona, N. Sobhani, D. Generali

Definition of intellectual content, literature search and manuscript preparation: S.P. Corona

Manuscript editing: S.P. Corona, D. Generali

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Health-related quality of life and its correlates among rectal cancer survivors, Northwest of Iran

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ABSTRACT

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Aim: The objective of this study was to examine the health-related quality of life (HRQOL), and its correlates among rectal cancer survivors. **Methods:** This cross-sectional study was conducted in the northwest of Iran. Rectal cancer survivors were selected from teaching hospitals. HRQOL was estimated using the European Organization for Research and Treatment of Cancer Quality-of-Life Questionnaire C30. Information about socio-demographic, lifestyle and clinical features of disease was obtained by trained interviewers. **Results:** A total of 96 patients were included in this study with mean age of 57.31 ± 14.15 years, 54% were male and 59% over 55 years of age. Women performed poorer than men in many dimensions of HRQOL ($P < 0.05$). Total score of symptoms was higher in those who had a higher stage of the disease. Participants with insufficient physical activity had a lower score in physical and role dimensions and a higher score of pain and fatigue ($P < 0.05$). In multiple regression models, treatment, stage of disease, and physical activity were important predictive factors of HRQOL. **Conclusion:** Some clinico-epidemiological factors were associated with a reduced score of HRQOL and its dimensions in this study. Overall, better performance in the presence of a modifiable factor; physical activity, is an opportunity for interventional strategies to improve the HRQOL.

INTRODUCTION

Colorectal cancer (CRC) is the second common cancer in female and the third in men, with an estimation of about 1.5 million incident cases and almost 700,000

deaths in 2012^[1]. There is a geographical variation in the incidence of CRC around the globe with over 10-fold difference between the highest and lowest estimated incidence in 2012. The highest incidence has been reported from Australia, New Zealand, Europe and



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North America and the lowest incidence rates belonged to Africa and south-central Asia^[1]. Variation might be explained by differences in genetic susceptibility, dietary habits and environmental exposures^[2]. Recent advances in treatment and management of CRC have improved control and disease-free survival^[3].

Nowadays, the standard strategy in the management of CRC is a multidisciplinary approach to treatment especially for management of rectal cancer which is one of the great challenges and responsibility of the colorectal surgeons. The main goal is to improve survival, to minimise morbidity and to maximise the quality of life of the rectal cancer patients^[4,5].

Health-related quality of life (HRQOL) is a multidimensional concept that covers a range of the subjective perceptions from physical, emotional, social, and cognitive functions to disease symptoms and treatment side effects among cancer patients^[6]. In CRC, the assessment of HRQOL is critical, and a range of various factors including socio-demographic characteristics, lifestyle, surgical procedures, and health-related factors are associated with HRQOL of patients with CRC^[7]. Although, HRQOL assessment is important in evaluating the treatment options for rectal cancer (RC) patients, data on assessment of the quality of life among RC patients in the presence of a range of socio-demographic, lifestyle behaviours and clinical factors are scarce.

There are few cross-sectional studies which have assessed the quality of life among this group of patients in which some included few cases, or focused on treatment options only or did not include lifestyle factors^[7-9]. There is a relatively large prospective study that showed the anterior resection and non-stoma patients, despite suffering micturition and defecation problems, had a better quality of life scores than abdominoperineal extirpation and stoma patients^[10]. A longitudinal study with 19 months follow-up, evaluated HRQOL in patients with RC using the functional assessment of cancer therapy-colorectal instrument and reported no differences in HRQOL by either tumour location or type of surgery, at either 9 or 19 months after diagnosis. These prospective studies were also focused on clinical aspects of QOL.

In Iran, CRC is one of the common cancers and is ranked the third and fifth most common cancer in women and men respectively. The age-standardized incidence rates in men and women were 11.31 and 10.89 respectively^[11]. Previous studies showed an increase in trend of this cancer in north of Iran and poor quality of life among CRC survivors^[12,13]. However, the data was not specific to RC survivors because of the

small sample size.

The studies on assessing HRQOL among CRC patients are limited, and we could not find any publication related to the quality of life of RC patients. The aim of this study was to assess the HRQOL and its determinants in patients diagnosed with RC referring to the specialty teaching hospitals in East Azerbaijan, northwest of Iran.

METHODS

This cross-sectional study was conducted in the city of Tabriz, East Azarbaijan province located in the northwest of Iran between 2014 and 2015. All newly diagnosed patients aged 18 and over who had been diagnosed less than one year presenting at the teaching hospitals regardless of stage at diagnosis and plans for treatment, were included in this study.

Demographic information including age, the level of education, employment status and place of residency was collected via self-report. Age was categorised into two categories; < 55 and ≥ 55 years, the level of education was classified as illiterate, literate, occupation classed into (paid work or out of work) and finally place of residency specified as either urban or rural. Patients were also asked whether they experienced various comorbid conditions (including heart disease, hypertension, chronic back pain, arthritis, stroke, osteoporosis, asthma, chronic obstructive pulmonary disease, stomach and/or intestinal condition). Three different treatment regimens were used in this cohort of stage I-III rectal cancer patients: (1) surgery only, surgery plus chemotherapy (surgery + CT), surgery plus radiotherapy (surgery + RT); (2) surgery plus adjuvant chemo-radiotherapy (surgery + CRT); and (3) CT only/RT only/CRT only.

European organization for research and treatment of cancer quality-of-life questionnaire (QLQ) C30 was completed from all participants by two trained interviewers. This scale is the core questionnaire for evaluating the QOL of cancer patients. It is a 30-itemed instrument with a four-point scale, from "not at all" to "very much", for items 1 to 28; and a seven-point scale for items 29 and 30. The QLQ-C30 dimensions include the physical functioning (PF), role functioning (RF), cognitive functioning (CF), emotional functioning (EF), social functioning (SF), the general level of QOL and the symptoms scale (i.e. fatigue, pain). Each patient's scores were transformed into a 0-to-100 scale, where 0 denotes the worst and 100 the best on functioning scales. In contrast, the reverse scoring system was applied for symptoms where zero point denotes the

best and 100 the worst on symptom scales.

Statistical analysis

Descriptive analysis was used to present data, mean and standard deviation (SD) was used for quantitative variables, and numbers and percentages were provided for categorical data. Data checked for normality and linearity where it was required. Total score of HRQOL and its dimensions score were as dependent variables in this study. Univariate and multivariate analysis performed to assess the association between a range of factors and HRQOL. Variables with a *P* value less than 0.1 were included in the multiple linear regression models to identify predictors of HRQOL. A *P*-value of less than 0.05 was predetermined to be mean statistical significance, and SPSS version 21 was used for all data analyses.

Table 1: Clinico-epidemiological characteristics of patients with rectal cancer referring to specialty teaching hospitals in Tabriz, Northwest of Iran 2014-2015

	Number	%
Age group		
< 55 years	39	40.6
≥ 55 years	57	59.4
Gender		
Male	52	54.2
Female	44	45.8
Marital status		
Single/widowed	19	19.8
Married	77	80.2
Place of residence		
Urban	81	54.4
Rural	15	15.6
Education		
Illiterate	42	44.7
Literate	52	55.3
Occupation		
No working	38	39.6
Working	58	60.4
Comorbidities		
Yes	55	57.9
No	40	42.7
Smoking		
Yes	28	29.5
No	67	70.5
Physical activity		
Adequate	15	15.6
Inadequate	81	84.4
Income		
< 7,000,000 R	27	36.0
> 7,000,000 R	48	64.0
Stage of disease		
I & II	66	68.8
III & IV	17	17.7
Treatment		
CT only/RT only/CRT	15	15.6
Surgery only/surgery + CT/ surgery + RT	36	37.5
Surgery + CRT	44	45.8
Stoma		
Yes	36	62.1
No	22	37.9

Total number might be different due to missing values. CT: chemotherapy; RT: radiotherapy; CRT: chemo-radiotherapy

This study received ethics approval from Tabriz University of Medical Sciences; Ethics Committee and all patients completed an informed consent form before the interview session.

RESULTS

A total of 96 newly diagnosed patients with rectal cancer were included in this study with a mean age 57.31 ± 14.15 years (min: 27, max: 83). The majority of them were male (54%), 59% were over 55 years of age and more than two-third resided in urban areas (84%). About 45% of them had no education, and about 40% were out of work. Many of them (84%) reported inadequate physical activity, 30% smoked either a cigarette or waste pipe, 58% had at least one comorbidity, and the stage of the disease was I/II in most of the cases (69%), 46% had received surgery plus CRT as the treatment of choice [Table 1].

Table 2 shows the mean (SD) score for different dimensions of QOL according to socio-demographic factors. As it can be seen, the score of total QOL and its dimensions were not different between younger and older age groups. Women performed significantly poorer than men in EF, PF, and RF dimensions ($P < 0.05$). Those who were not engaged in any work had a lower score in EF and RF dimensions ($P < 0.05$). Participants with insufficient physical activity had a lower score in PF and RF dimensions ($P < 0.05$). Women, those who were not working, and those with insufficient physical activity had higher scores in symptom total, pain and fatigue. Scores of total QOL and its dimensions were not different according to the place of residency and smoking. Although the study participants with no education had poorer scores in total QOL and all dimensions, and showed the higher scores of symptoms total, pain and fatigue, the difference was not statistically significant compared to literate participants. Patients with lower income had significantly higher scores of total QOL, and EF dimension ($P < 0.05$). They performed better in all other dimensions and had a lower score in symptom-total, pain and fatigue, but it was not statistically significant.

Table 3 shows the mean (SD) score for different dimensions of QOL according to clinical factors. Only treatment option had a significant association with total QOL score. Total score of symptoms was higher in those who had a higher stage of disease, and those who had undergone CT/RT/or CRT only. Those who had undergone surgery plus CRT performed better in all subscales except CF, and they had lower scores in symptom total, pain and fatigue ($P < 0.05$). Comorbidities were associated with higher scores in

Table 2: Total score of HRQOL and its dimensions according to various factors, rectal patients referring to the specialty teaching hospitals, Tabriz, Northwest of Iran, 2014-2015 (mean \pm SD)

	QOL total	EF	PF	CF	SF	RF	Symptom total	Pain	Fatigue
Age group									
< 55 years	50 \pm 26.35	46.15 \pm 27.42	56.23 \pm 32.5	71.36 \pm 29.6	46.15 \pm 32.54	49.57 \pm 33.65	38.53 \pm 22.99	48.71 \pm 35.12	50.42 \pm 34.18
\geq 55 years	50 \pm 24.39	57.16 \pm 29.52	49 \pm 30.35	70.76 \pm 25.64	47.36 \pm 29.67	42.98 \pm 36.45	37.51 \pm 21.18	48.53 \pm 33.81	51.46 \pm 33.3
P value	0.99	0.068	0.26	0.91	0.85	0.37	0.82	0.98	0.88
Sex									
Female	44.88 \pm 26.7	44.7 \pm 28.87	42.87 \pm 32.41	67.45 \pm 31.22	43.93 \pm 30.72	35.60 \pm 35	43.77 \pm 23.27	61.36 \pm 35.54	58.08 \pm 34.54
Male	52.32 \pm 22.9	59.45 \pm 27.71	59.61 \pm 28.39	74.35 \pm 22.97	49.35 \pm 30.72	54.16 \pm 33	33 \pm 19.4	37.82 \pm 29.17	45.08 \pm 31.68
P value	0.066	0.01	0.008	0.2	0.39	0.01	0.01	0.001	0.058
Residency									
Urban	49.4 \pm 24.9	51.44 \pm 27.6	50.86 \pm 30.33	71.39 \pm 26.77	46.29 \pm 30.61	44.23 \pm 35.27	38.3 \pm 21.02	49.3 \pm 33.89	51.3 \pm 32.5
Rural	52.77 \pm 26.6	59.44 \pm 36.3	57.77 \pm 36.57	68.88 \pm 3.12	50.00 \pm 32.12	53.33 \pm 35.71	35.9 \pm 26.50	44.45 \pm 36.55	49.62 \pm 39.36
P value	0.64	0.33	0.43	0.74	0.67	0.36	0.70	0.61	0.86
Education									
Illiterate	50.39 \pm 22.58	55.95 \pm 28.76	52.38 \pm 31.64	76.98 \pm 18.61	45.23 \pm 32.12	38.89 \pm 34.69	41.77 \pm 24.71	54.76 \pm 37.32	50.79 \pm 36.27
Literate	46.66 \pm 22.44	42.77 \pm 26.51	47.11 \pm 23.43	74.44 \pm 25.09	48.89 \pm 31.15	50.00 \pm 37.26	44.10 \pm 23.04	48.88 \pm 31.16	60.00 \pm 35.58
P value	0.63	0.16	0.59	0.73	0.73	0.36	0.77	0.62	0.45
Occupation									
No-working	47.15 \pm 27.49	44.52 \pm 30.34	42.46 \pm 33.26	66.67 \pm 31.72	44.30 \pm 30.58	33.33 \pm 36.92	45.22 \pm 24.27	61.84 \pm 36.12	59.36 \pm 36.56
Working	51.87 \pm 23.42	58.05 \pm 27.13	58.16 \pm 28.53	73.85 \pm 23.59	48.56 \pm 30.95	53.74 \pm 32.0	33.16 \pm 18.79	39.94 \pm 30.1	45.59 \pm 30.42
P value	0.37	0.025	0.15	0.23	0.5	0.005	0.007	0.002	0.048
Income									
Low (< 800,000 T)	55.92 \pm 24.03	59.86 \pm 28.33	56.66 \pm 30.75	77.63 \pm 25.78	49.56 \pm 31.35	46.49 \pm 31.27	34.68 \pm 19.56	46.92 \pm 35.69	45.61 \pm 31.19
Acceptable (> 800,000 T)	45.06 \pm 25.31	47.68 \pm 28.19	48.02 \pm 32.17	66.97 \pm 27.18	44.13 \pm 30.22	43.51 \pm 38.36	41.36 \pm 22.91	51.54 \pm 33.53	56.58 \pm 34.48
P value	0.041	0.045	0.2	0.06	0.40	0.68	0.14	0.52	0.12
Smoking									
Yes	47.91 \pm 25.82	50.29 \pm 29.26	54.52 \pm 29.87	72.02 \pm 24.45	42.85 \pm 32.21	52.38 \pm 31.0	36.26 \pm 20.24	43.45 \pm 30.87	52.77 \pm 30.63
No	50.37 \pm 24.76	52.98 \pm 28.78	50.15 \pm 28.78	70.15 \pm 28.35	47.76 \pm 29.71	42.53 \pm 36.95	39.11 \pm 22.34	51.24 \pm 35.45	50.91 \pm 34.71
P value	0.66	0.68	0.53	0.76	0.47	0.21	0.56	0.31	0.80
Physical activity									
Inadequate	48.76 \pm 25.14	51.85 \pm 29.58	47.32 \pm 30.67	69.75 \pm 26.88	45.6 \pm 30.09	39.91 \pm 34.22	40.23 \pm 22.35	53.29 \pm 34.3	53.9 \pm 33.66
Adequate	56.66 \pm 24.43	57.22 \pm 26.51	76.88 \pm 21.8	77.77 \pm 28.63	56.66 \pm 33.21	76.66 \pm 23.40	25.47 \pm 13.37	23.33 \pm 19.72	35.55 \pm 28.85
P value	0.26	0.51	0.001	0.29	0.18	0.00	0.002	0.00	0.051

HRQOL: health-related quality of life; PF: physical functioning; RF: role functioning; CF: cognitive functioning; EF: emotional functioning; SF: social functioning

Table 3: Total score of HRQOL and its dimensions according to clinico-pathological factors, rectal patients referring to the specialty teaching hospitals, Tabriz, Northwest of Iran, 2014-2015 (mean \pm SD)

	QOL total	EF	PF	CF	SF	RF	Symptom total	Pain	Fatigue
Comorbidities									
Yes	46.96 \pm 25.82	50.15 \pm 32.01	48.6 \pm 31.88	68.18 \pm 29.79	47.87 \pm 31.92	43.93 \pm 34.59	39.58 \pm 21.49	50.9 \pm 33.39	57.37 \pm 32.9
No	53.33 \pm 23.55	55 \pm 23.78	55.33 \pm 29.82	74.16 \pm 22.94	44.16 \pm 28.38	47.5 \pm 36.89	36.48 \pm 22.07	46.25 \pm 35.5	43.33 \pm 32.78
P value	0.22	0.4	0.3	0.29	0.55	0.63	0.49	0.51	0.043
Stage of disease									
I & II	52.02 \pm 25.1	57.95 \pm 26.89	55.95 \pm 31.07	72.47 \pm 26.71	52.77 \pm 30.74	47.97 \pm 35.5	34.11 \pm 21.22	43.18 \pm 33.33	45.62 \pm 32.5
III & IV	48.04 \pm 25.09	42.64 \pm 30.74	44.31 \pm 30.36	70.58 \pm 26.04	31.37 \pm 24.21	50.00 \pm 38.18	48.29 \pm 21.4	61.76 \pm 36.68	64.05 \pm 34.14
P value	0.56	0.045	0.17	0.79	0.009	0.83	0.016	0.048	0.042
Treatment									
CT only/RT only/CRT	39.44 \pm 19.78	45.00 \pm 26.31	48.44 \pm 25.63	66.67 \pm 33.92	47.78 \pm 26.63	42.22 \pm 35.56	42.22 \pm 14.92	54.44 \pm 32.41	56.03 \pm 26.38
Surgery only/surgery + CT/surgery + RT	45.83 \pm 28.97	43.29 \pm 26.71	40.37 \pm 31.99	67.06 \pm 26.24	33.33 \pm 26.73	34.72 \pm 32.7	48.37 \pm 20.03	60.19 \pm 34.43	66.36 \pm 30.8
Surgery + CRT	56.81 \pm 21.81	62.69 \pm 29.28	62.88 \pm 29.55	75.76 \pm 25.02	57.95 \pm 31.44	55.3 \pm 35.54	27.33 \pm 20.62	36.74 \pm 31.66	35.86 \pm 31.69
P value	0.009	0.006	0.016	0.17	0.03	0.054	0.001	0.012	0.002
Stoma									
Yes	51.28 \pm 27.2	54.27 \pm 30.64	53.67 \pm 32.55	64.95 \pm 30.77	41.02 \pm 31.02	41.88 \pm 38.01	36.88 \pm 21.84	47 \pm 34.38	49 \pm 32.5
No	47.02 \pm 23.84	47.61 \pm 27.0	47.46 \pm 29.78	74.6 \pm 24.48	45.63 \pm 27.8	46.42 \pm 35.41	43.47 \pm 20.82	54.76 \pm 34.58	60.05 \pm 33.74
P value	0.45	0.3	0.37	0.12	0.48	0.58	0.16	0.31	0.13

HRQOL: health-related quality of life; PF: physical functioning; RF: role functioning; CF: cognitive functioning; EF: emotional functioning; SF: social functioning; CT: chemotherapy; RT: radiotherapy; CRT: chemo-radiotherapy

fatigue. Those with stage III and IV had significantly lower scores in EF and SF dimensions and had significantly higher scores of symptoms total, pain and fatigue compared to patients with lower scores. Patients with stoma had better scores of QOL and reported less pain, and fatigue and had a lower score of symptom total.

Table 4 shows the results of a multivariate linear regression model that was performed to identify predictors of HRQOL. As it can be seen in this table income and treatment option were predictors of QOL ($R^2 = 9\%$). Income was negatively predictive of QOL score. Stage of disease and treatment option were associated with EF scores ($R^2 = 18\%$). Stage of disease was negatively predictive of poorer EF score, however surgery plus CRT was a predictor of better EF functioning. Gender, treatment option and physical activity remained significant predictors of better PF functioning ($R^2 = 23\%$). Stage of disease and treatment option were predictors of SF dimension score ($R^2 = 11\%$). Physical activity was the only predictors of RF functioning score ($R^2 = 17\%$). Treatment option and physical activity were negatively predictive of total symptom score ($R^2 = 22\%$).

Predictors of pain were a treatment option, physical activity and stage of disease ($R^2 = 25\%$). Treatment other than surgery + CRT, insufficient physical activity and higher stage of disease were associated with higher scores of pain. Treatment option, comorbidities, disease stage, and physical activity were predictors of fatigue score ($R^2 = 27\%$).

DISCUSSION

The aim of the current study was to assess the predictors of HRQOL among patients with rectal cancer. To our knowledge, limited studies examined the HRQOL among CRC patients including a range of different clinico-epidemiological factors, and especially among patients with rectal cancer separately.

We found that the overall score of QOL was low in our patients (48.2). Which was lower the total score reported from studies regarding CRC patients in other countries^[14,15] and in Iran which reported the total scores of higher than 70^[16,17]. The global QOL in a study by Engel *et al.*^[10] among rectal cancer patients was reported 65.3 in the first year of diagnosis. It was reported 54.5 in a study by Zajac *et al.*^[18] among patients with stoma due to rectal cancer. Studies showed that the score of QOL is getting better over time^[19]. It has been shown that the QOL among disease-free survivors of rectal cancer after two years

Table 4: Association between HRQOL and its dimensions and sociodemographic and clinical factors

Dependent variable	Covariates	Regression coefficient, <i>R</i>			95% CI	
		B (SE)	Beta	<i>P</i>	Lower bound	Upper bound
QOL total	Income	-11.44 (5.14)	-0.22	0.029	-21.66	-1.23
<i>R</i> ² = 9%	Treatment	8.73 (3.47)	0.25	0.014	1.83	15.63
EF	Stage of disease	-14.99 (7.1)	-0.21	0.03	-29.14	-0.83
<i>R</i> ² = 18%	Treatment	8.81 (4.09)	0.23	0.035	0.65	16.96
PF	Sex (male/female)	14.55 (5.84)	0.23	0.015	2.94	26.16
<i>R</i> ² = 23%	Treatment	9.07 (3.91)	0.21	0.023	1.29	16.85
	Physicalactivity	26.07 (7.67)	0.31	0.001	10.82	41.32
SF	Stage of disease	-22.42 (7.90)	-0.29	0.006	-38.15	-6.68
<i>R</i> ² = 11%	Treatment	9.42 (4.32)	0.22	0.032	0.80	18.03
RF	Physicalactivity	32.66 (9.32)	0.33	0.001	14.15	51.18
<i>R</i> ² = 17%						
Symptom total	Treatment	-9.14 (2.94)	-0.32	0.003	-15.00	-3.27
<i>R</i> ² = 22%	Physicalactivity	-15.51 (5.98)	-0.26	0.011	-27.44	-3.59
Pain	Treatment	-8.83 (4.63)	-0.18	0.06	-18.05	0.39
<i>R</i> ² = 25%	Physicalactivity	-31.18 (9.41)	-0.31	0.001	-49.93	-12.43
	Stage of disease	21.146 (8.33)	0.24	0.013	4.55	37.74
Fatigue	Treatment	-14.94 (4.56)	-0.33	0.002	-24.03	-5.85
<i>R</i> ² = 27%	Physicalactivity	-22.68 (9.29)	-0.25	0.017	-41.19	-4.17
	Stage of disease	23.66 (8.23)	0.29	0.005	7.26	40.06
	Comorbidities	-14.49 (6.73)	-0.21	0.035	-27.90	-1.07

HRQOL: health-related quality of life; CI: confidence interval; PF: physical functioning; RF: role functioning; EF: emotional functioning; SF: social functioning

was higher than that in the general population^[20]. The difference between our results and other studies might be explained by the time of recruitment of the study population. In our study, all patients were diagnosed less than one year and some were receiving active treatment, and some patients with advanced stages were also included. Our results showed that younger and older patients had almost the same score of the overall QOL and its dimensions except emotional dimension which was lower in younger patients. It in line with the results of other studies which showed the poorer emotional performance of younger patients^[9,21]. We found that females generally had poorer QOL than men, the same reported by Li *et al.*^[9] but some studies reported the lower social wellbeing score among men, that might be because they used different instrument for assessment of the QOL^[21].

In the current study income and treatment options were predictors of the total score of QOL. Income was negatively predictive of QOL score, surgery plus CRT was positively related to the higher score of the QOL total score. The QOL of the long-term survival group was associated with lifestyle factors, symptoms and usual activity, and the presence of a stoma was not the matter. However, QOL one year after surgery was associated with adjuvant therapy^[22].

In this study, stage of disease was negatively predictive of EF and SF, but positively predictive of pain and fatigue. Treatment option was predictive of all QOL dimensions (except CF) and pain and fatigue. Those who received surgery plus CRT had better

performance and lower pain and fatigue. There is evidence that type of surgery affects the QOL after surgery among patients with cancer of the rectum. Evidence showed that cancer-free patients with rectal cancer who had no terminal abdominal stoma showed a better score in all categories of the QOL 30 after five years^[23]. In addition, it has been shown that sphincter sparing operations are higher among patients who undergone neoadjuvant chemo-radiotherapy and they show better scores in QOL^[24]. However, in this study we combined neoadjuvant and adjuvant therapies, therefore the reason for such results cannot be clearly concluded. In this study, patients with comorbidities had poorer scores on total QOL and its dimensions and showed higher pain and fatigue, however, in the final model it was predictive of only fatigue. Studies also showed a poorer performance of QOL among those with comorbidities^[25].

Another finding of this study was the association between physical activity and the score of PF and RF dimensions, those with sufficient physical activity had better scores in these dimensions, and it was also negatively predictive of symptom total, pain and fatigue. Those with sufficient physical activity had lower scores in symptom total, had lower pain and fatigue. Studies demonstrated the positive effect of physical activity on quality of life among patients with CRC^[26].

This study has some limitations, we included patients from teaching hospitals, those who were admitted to private hospitals might be from higher socioeconomic

status, therefore association between income and score of QOL might be affected. At the time of this study we could not access to surgery profile of the patients, then the information about preserving sphincter was not available, however evidence showed that sphincter-ablating procedures do not necessarily reduce QOL in patients with rectal cancer^[21].

This study reported a relatively low score of QOL among patients with rectal cancer compared to studies from other countries. In general treatment option and stage of disease, and physical activity were important predictive factors of QOL. The presence of a modifiable factor is an opportunity for interventional strategies to improve the QOL via physical activity modification. Organised screening is recommended to improve the stage at presentation and concordance with treatment guidelines is also recommended.

DECLARATIONS

Authors' contributions

Involved in design of the protocol of the study and all drafts of the manuscript, and reviewed and agreed the final draft of the manuscript: N. Aminisani, M. Fatemi, P. Sarbakhsh, A. Nikanfar, A. Eftekharsadat, E. Jafari Did data collection and analysis: M. Fatemi, E. Jafari Supervised and supported data collection: N. Aminisani, A. Nikanfar, A. Eftekharsadat Supervised the data analysis: N. Aminisani, P. Sarbakhsh

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Conflicts of interest

There are no conflicts of interest.

Patient consent

At the beginning of the study, informed consent was obtained in written from all of the participants.

Ethics approval

The study was conducted in accordance with the declaration of Helsinki and had ethics approval from the Tabriz University of Medical Science Ethical Review Committee (Ethical ID numbers: TBZMED.REC.5/4/11460).

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Introduction to the Special Issue on Cancer Immunotherapy

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The biotechnology revolution started in early 1970s following advances in molecular biology, specifically: (1) sophisticated methodologies for manipulating DNA in mammalian cells; (2) hybridoma technology for the generation of preselected monoclonal antibodies; (3) genomic and recombinant DNA technology that allowed the production of large quantities of specific proteins; and (4) improved understanding of cancer immunology. In the 1980s, we further witnessed another wave of technological revolution which includes our ability to molecularly clone many growth factors, cytokines and immunogenic molecules, and to discover immune checkpoint molecules such as cytotoxic T-lymphocyte antigen 4, programmed death 1 and programmed death ligand-1 and their inhibitors, to develop various vaccines (dendritic cells, personalized human leukocyte antigen-binding peptides and RNA mutanomes), and to expand and/or genetically modify effector cells for adoptive cell based immunotherapy, such as chimeric antigen receptor T-cell therapy. There are major scientific, clinical and regulatory hurdles that still need to be overcome to bring the full potential clinical benefits of immunotherapy to cancer patients, particularly when an individualized approach is under consideration. The next 20 years should be very exciting period to the development of this field.

As Guest Editor of this special issue, I would like to express my sincere thanks to those who have contributed a series of articles to the issue, each representing either a commentary, original article, or review. I was so pleased that the birth of this issue which was finally turned into reality. Without the sustained enthusiasm and persistence of all the contributors and editorial staff, the completion of this issue would not have been possible.

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S.K. Liao contributed solely to the paper.

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Cancer immunity and therapy using hyperthermia with immunotherapy, radiotherapy, chemotherapy, and surgery

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ABSTRACT

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Hyperthermia is a type of medical modality for cancer treatment using the biological effect of artificially induced heat. Even though the intrinsic effects of elevated body temperature in cancer tissues are poorly understood, increasing the temperature of the body has been recognized as a popular therapeutic method for tumorous lesions as well as infectious diseases since ancient times. Recently accumulated evidence has shown that hyperthermia amplifies immune responses in the body against cancer while decreasing the immune suppression and immune escape of cancer. It also shows that hyperthermia inhibits the repair of damaged cancer cells after chemotherapy or radiotherapy. These perceptions indicate that hyperthermia has potential for cancer therapy in conjunction with immunotherapy, chemotherapy, radiotherapy, and surgery. Paradoxically, the anticancer effect of hyperthermia alone has not yet been adequately exploited because deep heating techniques and devices to aggregate heat effects only in cancer tissues are difficult in practical terms. This review article focuses on the current understanding concerning cancer immunity and involvement of hyperthermia and the innate and adoptive immune system. The potential for combination therapy with hyperthermia and chemotherapy, radiotherapy, and surgery is also discussed.

INTRODUCTION

Cancer is one of the most fatal diseases in the world, inducing various conditions such as organ disorders from primary lesions or metastatic lesions and cachexia. Three standard therapeutic methods include surgery, chemotherapy, and radiotherapy; however, these are not satisfactory on the whole. Difficulties in treating

cancer are due to its distinctive abilities for immune escape, metastasis, and tolerance to cancer therapies. These abilities result from the heterogeneity of cancer cells^[1] and the anticancer efficacy of conventional treatment methods has limitations. Recent progress has been made in cancer immunotherapy, a fourth cancer therapeutic method, including activated T-cell therapy and dendritic cell (DC) vaccines; however,



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their therapeutic efficacy is still limited^[2,3]. The more recent discovery of immune check-point inhibitors has demonstrated sensational long-term benefits in patients with advanced cancer and has highlighted the importance of immune responses to cancer, but its efficacy has been recognized only in a minority of patients^[4,5]. Hence, current therapeutic methods need to be improved or new therapeutic cancer therapy developed for single or combination use. Recently, many reports have shown that an appropriate heat effect has potential anticancer efficacy and can enhance the efficacy of other cancer therapies. Nonetheless, fever itself is a complex physiological response^[6], with the intrinsic effects of elevated body temperature regarded as an important defense system for the body by increasing the immune reaction not only against infectious disease but also against cancer.

Hyperthermia

Even though the efficacy of intrinsic effects of elevated body temperature in tissues to cells is still being studied, it has been disclosed that the survival rate of cells is reduced by heating at 39–42 °C, and it is amplified remarkably by heating at ≥ 42.5 °C for ≥ 1 h. There is no variation in tolerance between tissue types^[7,8]. Hyperthermia is a type of cancer treatment using this feature to target cancer cells and their surrounding environment^[9]. In the early days, hyperthermia alone at 42–44 °C was performed against recurrent tumors derived from head and neck cancer and breast cancer, which appeared on the surface of the body. The objective antitumor response in this set of superficial tumors was around 40%^[10–12]. However, most cancers, including primary sites as well as recurrent or metastatic sites, are located deep inside the body. This makes hyperthermia alone less effective because it is quite difficult to heat only cancer tissues to more than 42 °C using currently available heating devices. Recently, the usefulness of mild hyperthermia with 39–42 °C (fever-range hyperthermia) for 1–2 h has been reported for combination use with other cancer therapies^[13]. This method takes advantage of the difference in sensitivity to heat stress between normal tissues and cancerous tissues. The logic behind its use is that normal tissues have enough vascular distribution to drain the congestion of fever and avoid tissue damage in these shorter time periods. In contrast, in cancerous tissues, fever and heat stress tend to accumulate. Consequently, an anticancer effect can be obtained within the fever range while normal tissues endure^[14]. Nevertheless, irradiation for a long period with a higher temperature than body temperature until cancer is eliminated is still harmful to normal tissues and homeostasis of the body.

In widespread use, the term hyperthermia generally

includes regional hyperthermia, whole-body hyperthermia, and hyperthermic intraperitoneal chemotherapy (HIPEC). Ablation therapy, which uses microwave or a laser at 80–100 °C, leading to direct cancer cell death by heat denaturation of proteins or necrosis^[15], may also be categorized as hyperthermia in the broad sense. Regional hyperthermia is a less invasive method of thermal therapy. In this method, heat effects are limited to the range of irradiation and have an expected role as a chemosensitizer or radiosensitizer used to augment the efficacy of chemotherapy or radiotherapy *in situ*^[9]. The currently popular method for hyperthermia in clinical practice is mild hyperthermia applied to the regional cancer area by using an 8 MHz^[16,17] or 13.56 MHz^[18] radiofrequency capacitive heating device, applied to the surface of the body directly above the cancer. In contrast, whole body hyperthermia heats areas of body and increases the systemic body temperature of patients^[19]. This method is suitable for patients with metastases in multiple organs, including carcinomatosis. Increased effects of immune cells such as T cells and DCs located in the peripheral organs or circulation are also expected, in addition to the effect of regional hyperthermia^[20].

T-cell-based immune responses to cancer

T-cells are key immune cells with regard to specific immune responses against cancer. The pivotal events involved in the induction of successful T-cell mediated immune responses and those in the immune effector phase are shown in Figure 1. DCs are the major antigen-presenting cells (APCs), which are capable of initiating T-cell mediated immune response^[21]. These cells usually reside in the epidermis of the skin and mucosal tissues to prepare to combat foreign enemies. This includes cancer antigens, which are fragments of tumor cells generated as a consequence of natural death or the interaction of tumors and innate immune cells such like natural killer (NK) cells^[22,23]. DCs process and present these fragments on their cell surface along with major histocompatibility complex (MHC) antigens after capturing cancer antigens. The complex of cancer antigens and MHC class I antigens are presented to CD8⁺ cytotoxic T lymphocytes (CTLs), while the complex of cancer antigens and MHC class II antigens engage to stimulate CD4⁺ helper T-cells. The CD4⁺ helper T cells enhance the differentiation of CTLs into effector T-cells by secreting cytokines such as interferon- γ (IFN- γ) and interleukin-2 (IL-2).

Naïve T-cells receive the presentation of cancer antigens from DCs in the T-cell zones of lymphoid organs to acquire an appropriate immune response^[24]. To achieve antigen presentation, these two cells constantly migrate to the lymphoid organs (homing).

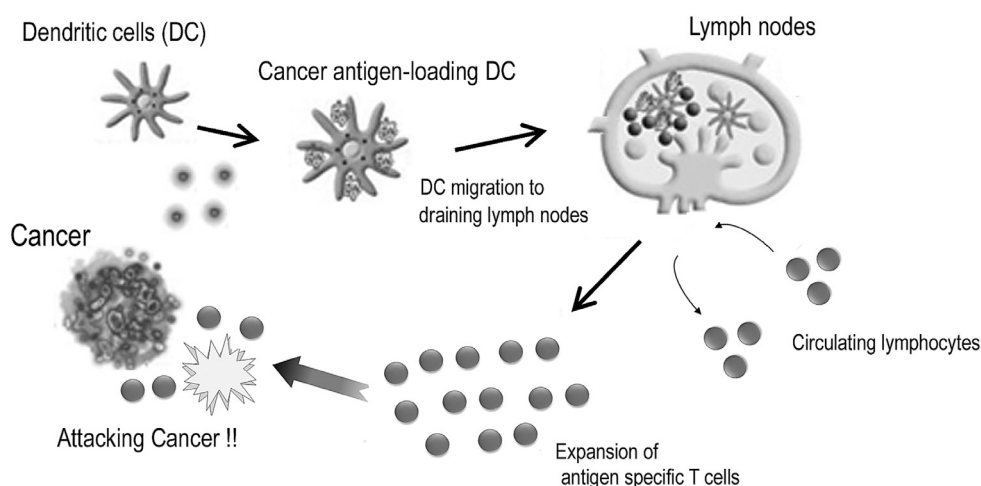


Figure 1: T-cell-mediated immune responses against cancer^[76]

The homing mechanism is regulated by chemokines and the chemokine receptor axis. CCL21 and CCL19 are homeostatic chemokines that are constantly secreted from secondary lymphoid organs, including lymph nodes and Peyer's patches, without any exogenous stimulation^[25-27]. Meanwhile, C-C chemokine receptor 7 (CCR7) is a concomitant chemokine receptor of CCL21 and CCL19^[25]. Cells expressing CCR7 can migrate to organs where CCL21 and CCL19 are secreted depending on the concentration gradient^[28]. Most naïve T-cells constantly express CCR7. DCs matured by exogenous stimulation with antigens derived from bacteria or cancers begin to express CCR7, whereas immature DCs do not express CCR7^[24,29]. Thus naïve T-cells and antigen-presenting DCs can migrate to secondary lymphoid organs from peripheral organs. As a final step in homing, these immune cells need to carry out transvasation through high endothelial veins to infiltrate into the lymphatic organs from the lymphatic system. To complete homing through the interval between endothelial cells, expression of adhesion molecules such as L-selectin and integrin is up-regulated for rolling and adhesion with intercellular adhesion molecule (ICAM), which is a co-receptor for integrin^[30,31]. After antigen presentation, T-cells are induced to proliferate and differentiate into effector T-cells. The expression of CCR7 on T-cells is then down-regulated to leave the lymphoid tissue for migration to cancer tissues.

Effector T-cells recognize the complex of cancer antigens and MHC class I antigens expressed on cancer cells through a T-cell receptor (TCR). Then, the lethal effects against cancer cells are triggered along two different pathways: granule exocytosis and death ligand/death receptor system. Granule exocytosis induces the secretion of perforins and granule enzymes

(granzymes)^[32,33]. Even though the nature and the role of these proteases in response to cancer is still unclear, perforins generally act as a carrier for the delivery of granzymes and build pores in the plasma membrane of target cells to allow granzymes to gain entry to the target cell cytosol. Granzymes are considered to execute the target cells through the cleavage of factors required for replication and defense^[33]. Death ligands are proteins including Fas ligand (FasL) that is expressed on effector T-cells. FasL can engage target cells through the Fas receptor, which belongs to the tumor necrosis factor (TNF) superfamily, to induce target cell death by apoptosis^[34]. The main role of FasL is to regulate the immune system, but cancer cells may over-express FasL spontaneously or as chemotherapy resistance to make a countercharge by inducing the apoptosis of tumor-infiltrating lymphocytes (TILs) to escape from the immune system^[34].

T-cell based immunotherapy for cancer

As has been mentioned, the human immune system always works in response to antigens expressed on cancer cells, thus distinguishing cancer cells from noncancerous cells. This causes the induction of TILs to be found in the tumor microenvironment^[35]. However, anticancer immunity is usually not enough to overcome the tumor's growing speed owing to the low immunogenicity of cancer cells because these cells are derived from an individual's own cells. Thus, it was inevitable that immunotherapy would be developed to overcome the low immunity against cancer. With regard to T-cell-based immunotherapy, adoptive transfer of CD3-activated T-cells has been induced traditionally as a compulsory activation stimulus to compensate for the reduction in stimulation frequency due to low antigenicity. Moreover cancer-specific-antigen loaded DC vaccines are also utilized to induce more types of cancer-specific T lymphocytes.

The more recent discovery of immune check-point inhibitors achieved outstanding progress in cancer immunotherapy by showing sensational long-term benefits in patients with advanced cancer^[4,5]. The purpose of this medicine is to inhibit immune suppressive signals between cancer cells and T-cells; thus the agent that eliminates a cancer during the final phase is T-cells^[36,37]. Immune check-point molecules such as programmed death-1 (PD-1) and T-lymphocyte-associated antigen 4 (CTLA4) are expressed on T cells and play a vital role in limiting the exaggerated immune response in both adaptive immune response and autoimmune response to maintain homeostasis by acting as an inhibitory signal against APCs. Recently, it has been disclosed that cancer cells take advantage of this mechanism to survive. For example, cancer cells express PD-L1, which is a concomitant ligand against PD-1, to attenuate T-cell-based immune reactions in association with cancer progression. With the discovery of this mechanism, immune check-point inhibitors have been shown to carry great promise. However, its efficacy has only been recognized still in a small number of patients, and PD-L1 expression on tumor cells has been regarded as a negative prognostic factor^[4,5].

Hyperthermia enhances immune systems in response to cancer

Body temperature elevation has been considered an important phenomenon associated with regulation in both innate and adaptive immune responses^[38]. Hyperthermia elicits various effects in several steps of the immune reaction for cancer. It up-regulates the homing of immune cells and the function of adhesion molecules on both immune cells and endothelial cells, activating immune cells including CTLs, DCs, and NK cells, and inhibiting immune suppression. In this

section, we discuss how thermal stress up-regulates the immune system.

Hyperthermia, especially whole body hyperthermia, has the potential to increase the homing of immune cells. Continuous secretion of homeostatic chemokines including CCL21 and the expression of adhesion factors including selectin, integrin, and ICAM regulate immune homeostasis by maintaining the homing of these immune cells. Thermal effect can enhance the expression of ICAM-1 and CCL21 in high endothelial venules (HEVs)^[39] and can up-regulate L-selectin- and integrin-dependent adhesive interaction to induce the adhesion and migration of DCs and T-cells toward HEVs^[40]. Additionally, increases in the migration capacity of DCs *ex vivo* has been reported^[41].

We reported previously that heat treatment stimulated cytokine production from peripheral T-cells *in vitro* and *in vivo* in fresh peripheral venous blood obtained from 5 healthy volunteers^[42]. We first incubated peripheral blood mononuclear cells (PBMCs) separated from obtained blood samples at 37 °C or 39 °C for 2 h in a water bath, then PBMCs were co-cultured with anti-CD3/CD28 monoclonal antibodies for 24 h at 37 °C. To evaluate the secretory properties of cytokines in T-cells, IFN- γ and IL-2 levels in the supernatant were measured. Results showed that both cytokine production levels were significantly increased (approximately twofold) when PBMCs were cultured at 39 °C [Figure 2]. Next, the volunteers underwent whole body hyperthermia until the rectal temperature reached 38.5 °C (generally it required 1 h of treatment). After terminating heating, volunteers were covered with a leather tent for 60 min as a heat-retention phase. Blood samples were obtained four times: before the treatment, at the end of the heat-retention phase, and then 24 and 48 h after

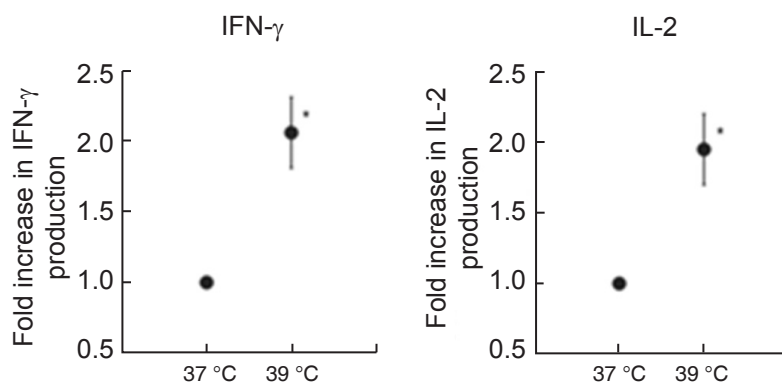


Figure 2: *In vitro* hyperthermia stimulates IFN- γ and IL-2 production from T-cells stimulated with monoclonal antibodies against CD3 and CD28. Blood samples were collected and incubated at 37 °C and 39 °C for 2 h, then PBMCs were extracted and co-cultured with monoclonal antibodies against CD3 and CD28 to measure IFN- γ and IL-2 production levels in each supernatant. Results are shown as twofold over the control (37 °C) for the average of five separate donors, and expressed as mean \pm SEM. Statistical differences from the control were evaluated using paired *t*-test. *P* < 0.05 was recognized as statistically significant^[42]. IFN: interferon; IL: interleukin; PBMCs: peripheral blood mononuclear cells

treatment. IFN- γ and IL-2 levels in each supernatant were measured in response to monoclonal antibody against CD3 and CD28. The results showed significant increases in the production of both IFN- γ and IL-2; these were observed not only immediately after but also 24 h after whole body hyperthermia. At 48 h after whole body hyperthermia, the production levels of both cytokines had returned to the pretreatment levels [Figure 3].

The potential mechanisms that stimulate cytokine production after hyperthermia may be explained by an increase in the membrane fluidity of T-cells. It was reported that physiological heat stress enhanced the membrane fluidity of T-cells. It also showed an increase in the cluster formation of the GM1⁺ CD-microdomain in CD8⁺ T-cells, clustering TCR β and the CD8 co-receptor, and enhanced conjugate formation between T-cells and APCs in mice^[43]. These results suggest that a heat-stress-induced increase in membrane fluidity is one of the primary events, and it subsequently triggers a cascade of molecular events that eventually make T cells crosstalk more rapidly and efficiently with APCs. These cellular events, including the formation of TCR microclusters, consist of several adhesion and signaling molecules^[44], which accumulate at the immune synapse^[45]. This is also known as the central supramolecular activation complex^[46].

Heat-shock proteins (HSPs) have been considered to play an important role in the effects of heat treatment on T-cell function. Indeed, the synthesis of HSPs was shown to increase with elevated body temperature in fever-range whole-body hyperthermia^[47]. The essential function of HSPs is known to involve their actions as molecular chaperones. As part of this function, HSPs are involved in antigen presentation and cross-

presentation in DCs by delivering chaperoned antigenic peptides to MHC class I molecules, thereby inducing antigen-specific T-cell activation^[48-50]. It was reported that the presence of recombinant Hsp60 allows antigen-dependent T-cell activation with antigen-specific IFN- γ secretion in conditions when even stimulation is not sufficient to activate T-cells^[51].

In contrast, Hsp70 is also expressed in cancers and acts as an effective inhibitor of apoptosis caused by heat stress, thereby participating in tumor progression^[52]. Hsp70 can prevent aggregation, remodel folding pathways, and regulate activity of cancer cells^[53]. However, the effects of HSPs on DCs and T-cells are still contradictory^[54]. Thus, the function of HSPs must continue to be investigated in order to clarify whether and how HSPs are involved in antigen presentation between T-cells and DCs during heat stress.

NK cells can behave as a spearhead of the innate immune response toward exogenous antigens and can make an initial attack against targets without prior exposure to the specific antigens. Basically, normal cells express MHC class I molecules, whereas aberrant cells such as cancer cells extinguish the expression of MHC class I molecules on themselves^[55]. This phenomenon was especially observed in pancreatic^[56], cervical^[57], breast^[58], prostatic^[59], and penile cancer^[60]. Down-regulation of MHC class I molecules on cancer cells is one of the steps for immune escape from cancer-specific immune response by T-cells, because abnormal antigens must be presented with MHC class I molecules when T-cells recognize them. In contrast to T-cells, NK cells target cells that have lost the expression of MHC class I molecules, because NK cells express inhibitory receptors that engage with MHC class I antigens. Hence, the anticancer ability of

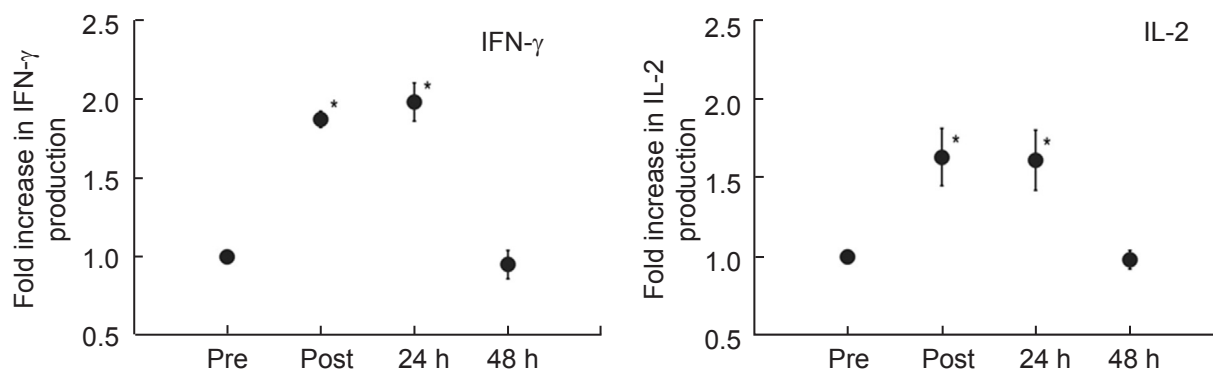


Figure 3: Whole-body hyperthermia stimulates IFN- γ and IL-2 production from T-cells. Blood was obtained from donors before (Pre), immediately after (Post), 24 h after (24 h), and 48 h after (48 h) whole-body hyperthermia. PBMCs from each time point were extracted and co-cultured with monoclonal antibodies against CD3 and CD28 to measure IFN- γ and IL-2 production levels in each supernatant. Results are shown as fold over the control (Pre) for the average of five separate donors, and expressed as mean \pm SEM. Statistical differences from control was evaluated using paired *t*-test. *P* < 0.05 was recognized statistically significant^[42]. IFN: interferon; IL: interleukin; PBMCs: peripheral blood mononuclear cells

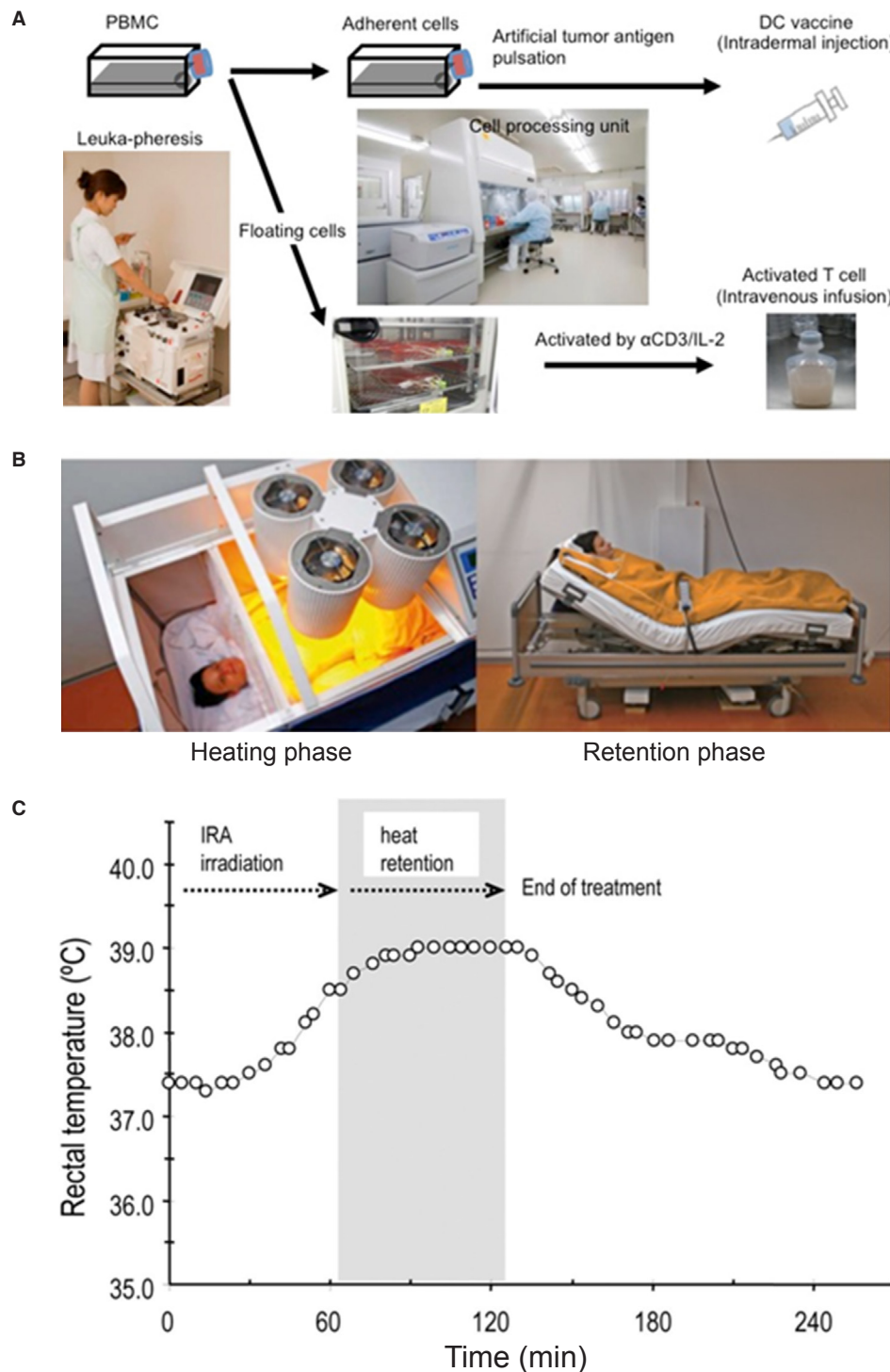


Figure 4: Approach for immunotherapy with whole-body hyperthermia. (A): cell preparation for DC vaccine and activated-T-cell therapy; (B): fever-range hyperthermia using heckle HT-3000 device; (C): representative data of body temperature during whole-body hyperthermia. DC: dendritic cell; PBMCs: peripheral blood mononuclear cells

NK cells is exercised at this time to complement T-cell-based immune reactions owing to attenuated inhibitory signals between NK cells and cancer cells^[61]. Activated NK cells have nonspecific anticancer potential by secreting cytotoxic molecules including perforin and granzyme^[33] and death receptors such as FasL, TRAIL,

and TNF- α ^[62,63]. Additionally, heat stress can enhance the distinct clustering of NK cell-activating receptors such as NKG2D on the surface of NK cells and the expression of NK cell-activating ligands, including major histocompatibility complex class I-related chain A (MICA)^[64,65]. Moreover, an increase in the expression of

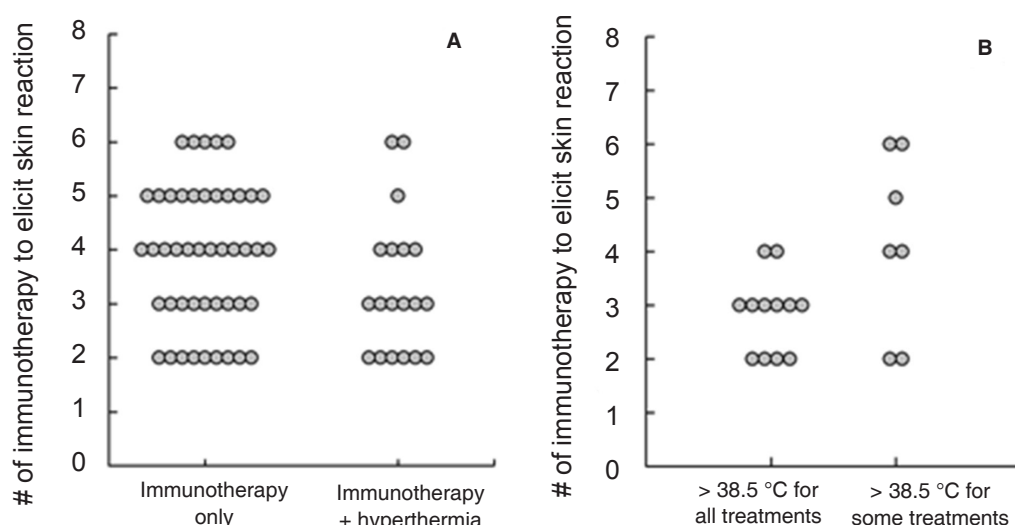


Figure 5: Required number of immunotherapy injections with DC vaccine and activated T-cells to elicit DTH-like skin reaction. (A): comparison between patients who received immunotherapy alone or in combination use with hyperthermia; (B): comparison of patient's rectal temperatures during hyperthermia^[76]. DC: dendritic cell; DTH: delayed type hypersensitivity

MICA and increased cytotoxicity of NK cells were also observed in several cancer cell lines^[66]. Concerning the contribution of NK cells to cancer-specific immune responses, after an initial attack, fragments including cancer antigens of tumor cells are generated as a result of the interaction of the tumor and NK cells. These cancer antigens are recognized by DCs for T-cell-based cancer-specific immune responses. Additionally, activated NK cells secrete cytokines including IFN- γ and IL-2 to enhance acquired immune responses with immunoglobulin production from B-cells and activation of T-cells^[55,61]. This perception leads to the conclusion that increasing the function of NK cells by hyperthermia could be expected to result in the augmentation not only of nonspecific anticancer immune reactions regulated by NK cells but also of specific anticancer immune reactions regulated by T-cells and DCs.

To avoid exaggerated immune responses that cause harmful effects on the body, the immune system is regulated to limit adaptive immune responses and prevent autoimmune responses and auto-inflammatory reactivity in the normal situations. To achieve this, our immune system combines immunological tolerance system. Regulatory T cells (Tregs) are a subpopulation of T-cells, expressing CD4, CD25, and FOXP3, which negatively modulate both innate and adopted immune response by down-regulating or suppressing induction and proliferation of immune cells including T cells, DCs, and NK cells^[67-69]. Even though Tregs usually account for about 4% of CD4⁺ T cells, they can make up as much as 20-30% of the total CD4⁺ population in the tumor microenvironment and are associated with poor prognosis in many cancers, such as ovarian, breast,

renal, and pancreatic cancer^[70]. Depletion of Tregs in animal models has been shown to increase the efficacy of immunotherapy. So, achieving the depletion of Tregs is one of the pivotal targets of recent research and therapy associated with cancer immunology^[71]. The potential effect of hyperthermia is considered to enhance the cytotoxicity of NK cells against Tregs, and to inhibit the induction of Tregs while the apoptosis of Tregs is induced. A significant decrease in the number of Tregs was observed while NK cell activity and the percentage of NK cells increased in peripheral blood samples of healthy volunteers after irradiation of fever-range hyperthermia to the upper abdominal region^[72]. Moreover, combination therapy of intratumoral injection of immature DCs and local hyperthermia for patients with advanced malignant melanoma demonstrated decreased infiltration of Tregs and increased infiltration of activated CTLs, even though there was no statistical difference in overall survival time^[73].

The efficacy of hyperthermia in down-regulating the expression of PD-L1 in some cancer cell lines was reported. In this study, decreased expression of PD-L1 in cancer cell lines was shown when samples were exposed to temperatures between 40 °C and 43 °C^[74]. Further accumulation of data associated with this new experimental model is eagerly awaited.

Combination therapy with immunotherapy

Hyperthermia has been reported to enhance the efficacy of DC vaccines by up-regulating IFN- γ secretion to stimulate naïve T-cells, enhancing DC migration toward lymphatic organs and protecting DC from apoptosis^[75]. We introduced a whole body hyperthermia device

(HECKEL HT3000, Heckel medizintechnik GmGH, Esslingen, Germany), and have performed combination therapy with adoptive transfer of CD3-activated T-cells and cancer-antigen loaded DC vaccines. Patients with various solid tumors were vaccinated once a week with DC vaccine prepared from autologous monocyte-derived DCs, which were pretreated with tumor antigens. The DC vaccine was injected intradermally near the inguinal lymph nodes and CD3-activated T-cells were administered intravenously [Figure 4A]. Some patients underwent whole-body hyperthermia at the same time, with the target rectal temperature set at 38.5 °C and heat-retention for another 1 h [Figure 4B and C]. To evaluate the induction of immune responses in patients who received antigen-loaded DC vaccination, we examined the onset of skin reaction at the vaccination site because this reaction indicates antigen-specific T-cell responses against tumor antigens presented by DC vaccine. Then, we examined how much DC vaccination was required in each patient up to the point when delayed hypersensitivity like skin reaction sizes 48 h after DC vaccination became consistently larger than 1.5 cm in diameter. The average number of vaccinations to induce skin reaction was 3.87 and 3.32 in patients without and with whole-body hyperthermia, respectively [Figure 5A]. Moreover, 12 of 19 patients who underwent whole-body hyperthermia successfully elevated their core body temperature above 38.5 °C in every treatment and displayed earlier expression of skin reaction [Figure 5B]. This result indicated that the combined use of hyperthermia with a DC vaccine and activated T-cells had a positive impact on the induction of T-cell based immune responses^[76].

Combination therapy with radiotherapy

The enhancement of anticancer efficacy of combination use of radiotherapy and hyperthermia was clinically recognized in cervical, breast, and head and neck cancer and so on^[77]. Even though radiological cytotoxicity induces DNA damage of cancer cells^[78], some cancer cells can come back into existence (termed sublethal damage repair or lethal damage repair)^[79,80]. In the analysis of the cell cycling, quiescent tumor cells were more resistant to irradiation because cells in this stage have the potential for lethal damage repair^[81]. In contrast, hyperthermia can inhibit the repair of radiation-induced damage in cancer cells, so that combination use of hyperthermia can enhance the anticancer efficacy of radiotherapy^[82,83]. Cells in the synthesis (S) phase are also relatively radio-resistant, while they are the most sensitive to hyperthermia. Additionally, hypoxic cells in tumors are also radio-resistant, while hyperthermia improves the anaerobic condition by oxygen delivery due to increased blood

flow. These perceptions indicate the synergetic effect of combination use of radiotherapy and hyperthermia^[7]. Moreover, additional use of DNA repair inhibitors was reported to further enhance its efficacy^[84].

Combination therapy with chemotherapy

Chemotherapy is the most popular therapeutic method for patients with inoperative cancer and recurrent or metastatic cancer; however, there are serious problems including its uncertain efficacy, drug resistance, and adverse effects. To improve therapeutic results, combination use of hyperthermia was tested, and increased anticancer effect was reported in paclitaxel, docetaxel, gemcitabine, oxaliplatin, and irinotecan^[85]. The mechanism of interaction of chemotherapy and hyperthermia was considered as follows: increased drug uptake into cancer cells by causing damage to the membrane of cancer cells and reducing oxygen radical detoxification. Eventually, DNA damage increased while DNA repair decreased. Additionally, hyperthermia was reported to have a potential ability to avoid drug resistance^[86,87]. In addition, it is also expected that elevated blood flow could result in a relative increase in anticancer drug concentration within the tumor. Moreover, adverse effects can be decreased because increased drainage of the drugs may accelerate in normal cells due to the up-regulation of metabolism. On the other hand, some anticancer drugs, including 5-fluorouracil, gemcitabine, and oxaliplatin, are considered to enhance cancer immunity by inducing the infiltration of CTLs while reducing Tregs in the tumor^[88]. Accordingly, enhancing the efficacy of chemotherapy will result secondarily in up-regulation of cancer immunity.

Combination therapy with surgery

Chemotherapy is usually performed for peritoneal metastases, but its prognosis is nonetheless bad, because blood flow to the peritoneum is poor owing to the presence of the peritoneal-plasma barrier^[89]. Hyperthermic effects are considered to impair the peritoneal-plasma barrier and result in increased resorption of anticancer drugs in peritoneal tumors. Hence, the combination of hyperthermia and chemotherapy by intraperitoneal administration resulted in more anticancer drug accumulation in peritoneal tumors than after chemotherapy alone^[90]. Using this concept, the effectiveness of cytoreductive surgery with subsequent HIPEC has been reported for peritoneal metastasis from gastric^[91], colorectal^[92,93], appendiceal^[94], and adrenal cancer^[95]. Generally HIPEC is performed after resection of the cancer lesion with or without systemic peritonectomy by intraperitoneal administration of an anticancer drug containing saline, which is heated in advance to maintain the peritoneal

surface at around 43 °C while irrigating the drug solution.

Adjuvant chemotherapy is often given after surgery for certain types of cancer, such as pancreatic, colorectal, and breast cancer, to improve prognosis by reducing the potential for recurrence and metastasis^[96-99]. However, in some cancers, including intrahepatic cholangiocarcinoma (ICC), the prognosis of patients is extremely poor because the recurrence rate after curative operation is very high and there is no standard adjuvant setting. We reported previously that postoperative adjuvant immunotherapy with intradermal administration of a DC vaccine and intravenous administration of activated T-cells would be a feasible and effective treatment for preventing recurrence and achieving long-term survival in patients with ICC. In this study, the median 5-year progression-free survival and overall survival were 18.3 and 31.9 months in the patients receiving adjuvant immunotherapy, and 7.7 and 17.4 months in the group with surgery alone ($P = 0.005$ and 0.022 , respectively). Additionally, patients whose skin reactions at the vaccine site were ≥ 3 cm showed dramatically better prognosis in patients receiving adjuvant immunotherapy^[100]. As has been explained above, hyperthermia can elicit early skin reactions when used in conjunction with immunotherapy including with DC vaccines and activated T-cell transfer^[76]. Hyperthermia can also augment the efficacy of adoptive immunotherapy by up-regulating IFN- γ secretion to stimulate naïve T-cells, and enhancing homing of DCs and T-cells^[75]. For the reasons stated above, hyperthermia is considered to be useful for adjuvant settings.

Neoadjuvant chemotherapy and chemoradiotherapy have become well established, especially for esophageal cancer patients^[101]. These neoadjuvant therapies improved the long-term survival rate, but the therapeutic benefit was sometimes countered by a significant increase in adverse effects^[102]. In addition, postoperative complications, including cardiac diseases and pulmonary diseases, are much more severe after chemoradiotherapy^[103,104]. Preoperative radiotherapy increases the risk of postoperative anastomosis leakage, an unfavorable complication. As has been mentioned above, hyperthermia has the potential to augment the effects of chemotherapy or radiotherapy. Combination use of hyperthermia with chemotherapy or radiotherapy may be useful even in the neoadjuvant setting to suppress the possibility and seriousness of adverse effects and complications by reducing the dose of chemotherapy or radiotherapy required while maintaining or increasing its anticancer effects. Indeed, in 1995, the result of a randomized phase III study for patients with resectable

squamous cell carcinoma of the thoracic esophagus was disclosed. Patients underwent neoadjuvant chemoradiotherapy with or without radiofrequency wave local hyperthermia, and the 3-year survival rate was 24.2% and 50.4%, respectively. There were no procedural complications^[105]. Additionally, in 2010, the results of a randomized phase III trial of patients with high-risk soft-tissue sarcoma were reported. Patients underwent neoadjuvant chemotherapy consisting of etoposide, ifosfamide, and doxorubicin with or without local hyperthermia. The treatment response rate in the group that received regional hyperthermia was 28.8%, compared with 12.7% in the group with chemotherapy alone ($P = 0.002$)^[106].

During the postoperative period, immunity is suppressed by operative invasion^[107] and the administration of anesthetic drugs such as opioids^[108], and it results in the encouragement of postoperative cancer metastasis^[109]. Concerning the biological effect, preoperative fever-range whole-body hyperthermia was reported to augment postoperative cancer immunity by increasing the blood level of TNF α and HSP60^[77]. Thus, hyperthermia increased the benefit of neoadjuvant therapy.

Adverse effects of hyperthermia

Adverse effects of acute or chronic periods of regional hyperthermia do not develop often and are usually minor, owing to a recent development of heating techniques and thermometry, or treatment schedule^[110]. Adverse effects of hyperthermia include skin burns and skin pain, but these events usually heal spontaneously^[111]. In terms of combination therapies with hyperthermia, radiation toxicity is not increased, but toxicity of chemotherapy might be enhanced depends on the increase in drug efficacy^[110]. In rare settings of combination therapy with chemotherapy, the formation of severe subcutaneous fat or muscle necrosis that required surgery to cure was reported. Adverse effects of regional hyperthermia vary with the type of targeted organ or heating device and techniques. Whole body hyperthermia is a slightly more invasive hyperthermal method accompanied by a feeling of heat, tiredness, and loss of sweat due to a rapid elevation of the core body temperature. Dehydration, heat illness, cardiac disease, or thrombosis might appear depending on underlying disease or physical condition. In addition, whole body hyperthermia has a risk for toxicity to the peripheral nervous system. Hence, this method is contraindicated for patients with neurodegenerative diseases, such as multiple sclerosis^[112].

On the other hand, no reports that describe hyperthermia having a positive impact on cancer progression in

regard to biological effects are found until now.

Overall, hyperthermia is considered a convenient therapeutic method so long as it is used appropriately. Paradoxically, the safety of hyperthermia is maintained by avoiding excess irradiation with the purpose of deep heating, because it is still difficult to aggregate the heat effect only in cancer tissue. By using hyperthermia in combination with chemotherapy or radiotherapy, the dose of these therapies may be reduced to ease their side-effects without reducing therapeutic effects, because hyperthermia has the potential to augment the effect of chemotherapy or radiotherapy in a less invasive manner.

CONCLUSION

This report shows that hyperthermia increases the advantage of the following biological features. Heat stress lowers the survival rate of all cells, but normal tissues are better able to tolerate this than cancerous tissues. Heat has a potential to augment immune responses while decreasing immune suppression. Heat inhibits the recovery of cancer cells from DNA damage. Heat enhances the resorption of anticancer drugs into cancer cells. The sensitivity of cancer cells against heat and radiation differs depending on the condition of cancer cells in the cell cycle. The anticancer efficacy of hyperthermia alone with currently available heating devices is not enough to suggest its use as a standalone therapy. However, some studies have shown that combination therapy with conventional methods including immunotherapy, radiotherapy, chemotherapy, and surgery improves its anticancer efficacy *in vitro* and *vivo*.

Perspective

Currently, clinical experience and data of oncological hyperthermia are still limited because both information and devices for hyperthermia have not become common. Therefore, multicenter clinical trials for cancer treatment including hyperthermia should be done to provide convincing data. The combination hyperthermia with immune check-point inhibitors should be involved in these studies to achieve fuller anticancer efficacy with fewer adverse effects. Development of drugs such as DNA repair inhibitors or regulators of HSPs is also expected to augment the efficacy of hyperthermia itself. Additionally, further efforts will be required to solve the mechanism of the involvement of cancer and hyperthermia to optimize cancer therapy. The development of heating devices and thermometry is also needed to achieve more appropriate heat delivery that is limited to the tumor lesion.

DECLARATIONS

Authors' contributions

Designed and wrote the manuscripts: Y. Yagawa
Conducted IFN- γ and IL-2 production assays: Y. Kobayashi
Provided suggestions on the revision of the manuscripts: M. Yamamoto
Built Figures 1, 4 and 5 and revised the manuscript: K. Tanigawa

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There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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Immunological aspect of the liver and metastatic uveal melanoma

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ABSTRACT

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Uveal (eye) melanoma is the most common primary eye malignancy in adults. Despite optimal treatments for primary uveal melanoma, up to 50% of patients subsequently develop systemic metastasis, often in the liver. Once hepatic metastasis develops, the survival of patients is generally short and currently available treatments fail to show meaningful improvement of survival. Recent development of immune checkpoint blockades revolutionized immunotherapy for metastatic cutaneous (skin) melanoma. Unfortunately, metastatic uveal melanoma is unresponsive to this approach, thus there is an unmet need to improve the treatment of metastatic uveal melanoma. One unique characteristic of uveal melanoma is that the majority of metastases first develop in the liver. The liver is highly specialized in development of immune tolerance to food-derived antigens and consequently serves a unique function in the immune system. Understanding the mechanisms by which the liver orchestrates immune-related responses is important to the development of an effective immunotherapy for hepatic metastases such as metastatic uveal melanoma. In this review article, the authors overview the immunological aspects of the liver and discuss approaches to improve immunotherapy for metastatic uveal melanoma.

INTRODUCTION

Uveal melanoma (UM) originates from the uveal tract of the eye (iris, ciliary body, and choroid). The estimated incidence of UM is 5 per million in the United States, and between 2 to 8 per million in Europe^[1]. Despite shared embryologic origin, UM differs from the cutaneous melanoma in biological behavior, epidemiology, prognostic features, and molecular profiles^[2,3]. Previous

investigators have identified categories of patients with a higher risk of systemic recurrence. Such risk factors include: large tumor size, epithelioid cell type, extra-scleral extension, loss of chromosome 3 (monosomy 3), and chromosome 8q amplification^[4]. Up to 50% of patients with UM develop metastases within 10 years of diagnosis^[1]. UM disseminates homogeneous, as there is no significant lymphatic drainage from the eye. The most common sites of metastasis are the liver (80-90%), then lung and bone^[1,5]. Hepatic metastasis is an



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important determinant of clinical course and survival. After development of hepatic metastasis, the median survival of patients is reported to be 6 to 12 months^[2].

The liver copes with the bacterial pathogens, toxins, and food antigens transported through the portal vein from the gastrointestinal tract. The immune cells in the liver serve diverse functions ranging from immunity against bacteria and tolerance to food antigens.

Circulating tumor cells (CTCs) that enter the liver encounter a unique immune system. Interaction between the liver immune system and cancer cells provide a complex tumor microenvironment. Newly developed immunological treatment strategies such as immune checkpoint blockade have appreciably improved the survival of non-hepatic metastatic cutaneous melanoma patients; however, response of hepatic metastases is less robust. Likewise, UM patients with hepatic metastases have not derived meaningful survival benefit from these immunotherapies.

In this review article, we first overview the immune microenvironment of primary UM and that of the liver. We then summarize ongoing immunotherapies against metastatic UM and discuss possible approaches to improve the efficacy of immunotherapy against metastatic UM.

IMMUNOLOGICAL MICROENVIRONMENT OF PRIMARY UVEAL MELANOMA

The eye is considered an immune-privileged organ. It has a unique ability to defend against uncontrolled inflammation that could damage sight. Anatomical constraints to the development of an immune response in the eye include the absence of lymphatics that limit the traffic of immune cells to the eye. Immune cells that enter the eye encounter immunosuppressive factors such as transforming growth factor beta (TGF- β), α -melanocyte stimulating hormone (MSH), retinoic acid (RA), and indoleamine 2, 3-dioxygenase (IDO)^[6]. These factors suppress T cell proliferation and effector function, and could induce immunosuppressive regulatory T (Treg) cells.

A lymphocyte-rich tumor microenvironment generally indicates a good prognosis in various types of cancer. Paradoxically, in UM high densities of immune cells are associated with poor prognostic factors. Primary UMs with monosomy 3, in comparison to those with disomy 3, are associated with a more vigorous inflammatory response, with infiltration by a variety of immune cells, including CD8⁺, CD4⁺, CD3⁺CD8⁺Foxp3⁺ T cells and CD68⁺CD163⁺ M2 macrophages^[7,8]. Infiltration of

immune cells occurs more frequently in epithelioid-cell-type UM. An increased number of macrophages were associated with epithelioid tumor cells ($P = 0.025$), heavy pigmentation ($P = 0.001$), and high microvascular density ($P = 0.001$). The 10-year melanoma-specific mortality rate increased with increasing numbers of macrophages (0.10 for low vs. 0.57 for high numbers, $P = 0.0012$)^[9]. It has been reported that Treg cells are recruited into tumors by chemokines, CCL17 and CCL22 that are produced by M2 macrophages. Furthermore, tumor-produced CCL2 and CCL22 have a role not only in attracting tumor-promoting macrophages, but also in promoting their survival and M2 polarization^[7].

While UM cells possess tumor-associated antigens and tumor-infiltrating CD4⁺ and CD8⁺ cells are present in the primary UM, Treg cells are also present in the tumor. One study has identified that the frequency of CD4⁺, forkhead box P3 (FoxP3)⁺ Treg cells within primary UM is correlated with the development of systemic metastasis^[10]. The presence of Treg cells and cyclooxygenase-2 expression in the tumors is especially correlated with poor prognosis^[10]. In terms of the role of NK cells in primary UM, down-regulation of HLA class I, which is a common mechanism for evading CD8⁺ cells, renders tumors more susceptible to NK cell-mediated lysis. However, while the NKG2D ligands (MIC-A and B) are expressed by 50% of primary UM, none of the metastases express these ligands, indicating that metastatic UM might not be controlled by NK cells^[11].

The mechanism of inflammatory cell infiltration to the primary UM and the reason for contradictory clinical outcomes remains speculative. Accumulative evidence indicates that tumor-microenvironment crosstalk facilitates cancer cells to modulate the inflammatory response. Cancer cells interact with both the innate and the adaptive immune systems and skew the acquired T cell response from the T helper 1 (TH1) type to the TH2 type. Cancer cells also skew the phenotype of macrophages and neutrophils to a type 2 differentiation and attract myeloid-derived suppressor cells (MDSCs) as well as Treg cells to tumor sites^[12]. UM cells may utilize these immune cells for their survival and protection from immunological attack. It is possible that UM cells already induced tolerance against them when they left from the eye. Immuno-modulatory microenvironment in the liver could further protect escaped UM cells from systemic immune surveillance.

MECHANISMS OF METASTASIS TO THE LIVER

The mechanisms for development of metastases in

the liver are still highly speculative. It is assumed that multiple factors contribute to development of metastasis and growth of UM cells in the liver. The proposed mechanisms are summarized as follows.

Slow hepatic blood circulation

The liver sinusoids are located at a confluence of arterial (hepatic artery) and venous (portal vein) blood, mixing the oxygen-rich blood from the hepatic artery and the nutrient-rich blood from the portal vein. They are a type of capillary-like blood vessels with fenestrated, discontinuous endothelium. Slow flow in the liver sinusoids maximizes the contact between hepatic cells and pathogenic molecules to filter them prior to circulation. The slow and tortuous sinusoidal blood flow can trap UM cells in the liver^[13,14].

Interaction between chemo-attractants and their receptors

The chemokines produced in the liver might attract UM cells to the liver and interact with chemokine receptors on their surface. A typical example is the interaction between CXCR4 and its ligand CXCL12 that is rich in the liver^[15]. Primary UM cell lines express CXCR4. Blockage of CXCR4 on UM significantly reduced migration to human liver extract^[16]. An alternative explanation for chemokine-related liver tropism is the loss of chemokine receptors in the liver. It has been reported that extracts from the liver down-regulated the expression of CXCR4 and CCR7 on primary UM cell lines^[16]. Retention of UM cells in the liver may not solely be related to a chemokine gradient toward the liver, but could also be related to the loss of chemokine receptors once melanoma cells reach the liver.

Another example is c-Met, a receptor for hepatocyte growth factor (HGF). c-Met-expressing UM cells interact with HGF produced in the liver^[14]. Primary UM cells that metastasized had higher levels of c-Met expression than tumors that did not metastasize. The expression of c-Met in the primary UM specimens significantly increased the risk of subsequent liver metastasis^[14].

Growth factors rich in the liver

The insulin-like growth factor-1 (IGF-1) plays a major role in tumor transformation, maintenance of malignant phenotype, promotion of cell growth, and prevention of apoptosis. It is mainly produced in the liver. High expression of IGF-1 receptor (IGF-1R) has been detected in UM hepatic metastasis specimens^[17]. The association between the expression of IGF-1R on tumors and the progression of UM also has been reported^[18]. Additionally, HGF could facilitate the growth of c-Met-expressing UM cells in the liver.

Chromosomal and genetic abnormalities

UM has unique genetic abnormality profiles compared to cutaneous melanoma (CM). Mutation of BRCA1-associated protein 1 (BAP1), located on chromosome 3p21, was frequently identified in metastatic UM^[19-21]. Particularly, it was reported that BAP1 mutation in UM cells may cause the liver tropism^[22]. However, this might be an over-simplified explanation for the liver tropism as a certain fraction of metastatic UM retained their BAP1 expression and monosomy 3 is not always seen in hepatic metastasis. Polysomy 8q is rather a common feature of metastatic UM, and the role of this chromosomal abnormality on hepatic metastasis should be further explored^[23,24].

The expression of adhesion molecules in the sinusoid

Vascular cell adhesion molecule-1 (VCAM-1) is expressed on sinusoidal endothelial cells and might trap tumor cells in slow blood flow^[13,25]. VCAM-1 is expressed on endothelial cells under inflammatory conditions, and mediate rolling and adhesion of various subsets of leukocytes as well as tumor cells for the recruitment and settlement of these cells from the blood stream. In animal models, partial-hepatectomy induced expression of inflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and IL-6 as well as the expression of VCAM-1 and facilitated liver metastasis^[26]. Endothelial cell expression of VCAM-1 showed adhesion of human malignant melanoma cells that expressed very late activation antigen-4 (VLA-4) on their surface^[27].

Angiogenesis factors rich in the liver

IL-8 and vascular endothelial growth factor (VEGF) are rich in the liver and could promote the angiogenesis of tumor in the liver microenvironment^[28]. Hepatic stellate cells (HSCs) in the tumor stroma predominantly produce IL-8, and neutralizing IL-8 with antibody dramatically reduces angiogenic effects^[28]. IL-8 also induces the expression of VEGFR2 and VEGF on endothelial cells through NF κ B activation, and mediates autocrine and paracrine stimulation of vascular endothelium.

Immuno-modulatory microenvironment

As stated in the following section, the liver is considered to be an immuno-modulatory organ^[29,30] and this immunologically complex microenvironment could promote tumor metastasis and growth in the liver.

IMMUNOLOGICAL ASPECTS OF THE LIVER IMMUNE MICROENVIRONMENT

The liver has a complex immune microenvironment. It is continually exposed to foreign pathogens such

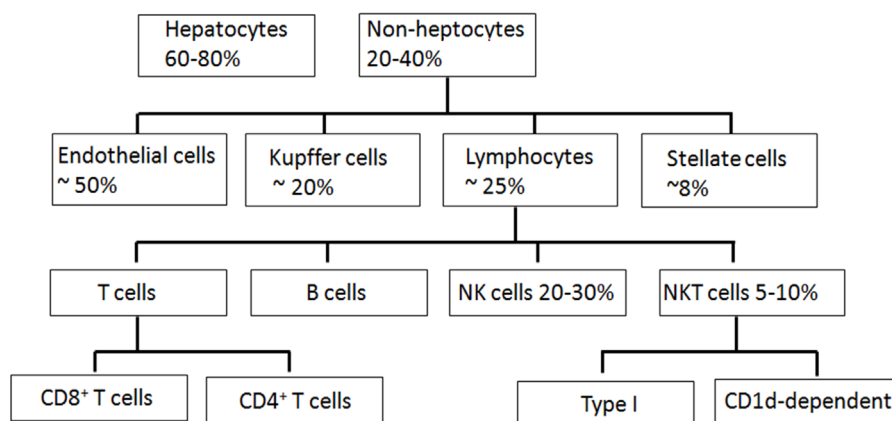


Figure 1: Cell population in the liver. The percentages indicate the estimated frequency of each population relative to the total number of parenchymal and non-parenchymal cells in the normal liver

as food antigens and low levels of endotoxin, many of which are derived from the gut. The local immune system must constantly provide secure mechanisms to eliminate those pathological antigens and toxins while it maintains tolerance to dietary antigens. In addition, the liver is subject to invasion by infectious pathogens from intestinal mucosa and the liver immune system must eliminate these infectious pathogens to protect the host from systemic infection. Thus, liver immunity exists in a delicate balance between the tolerance of essential elements and the defense against pathological agents.

RESIDENTIAL CELLS IN THE LIVER

Homeostatic immune microenvironment is tightly controlled by various residential non-immune cells and immune cells in the liver. There is a diverse population of residential cells in the liver, including the liver sinusoidal endothelial cells (LSECs), Kupffer cells (KCs), HSCs, and hepatocytes [Figure 1].

LSECs

LSECs separate the underlying hepatocytes from the blood in the sinusoidal lumen by the space of Disse [Figure 2]. LSECs do not have a basement membrane. This structure allows for the quick exchange of molecules between blood and hepatocytes. HSCs reside in the space of Disse. The lymph is collected from this space and flows into lymphatic vessels that run into the draining lymph nodes via portal tracts. LSECs have the capacity for endocytosis and phagocytosis through receptors, and present antigens as antigen-presenting cells (APCs)^[29]. LSECs are efficient in cross-presentation of antigens, allowing both CD4⁺ and CD8⁺ T cells to be activated by blood-derived antigens. Upon stimulation, LSECs secrete chemokines, CXCL9 and CXCL10, and recruit lymphocytes. On the other hand, LSECs are

able to express PD-L1 triggered by cognate interaction with activated T cells for elimination of these T cells. In contrast, the exposure to soluble molecules such as IL-10 and prostaglandin E2 (PGE2) derived from KCs can reduce the expression of major histocompatibility complex (MHC) and costimulatory molecules on LSECs that promotes immune tolerance in the liver^[30].

KCs

KCs comprise 80-90% of all tissue macrophages in the body and account for 20% of non-hepatocytes in the liver^[29]. Granulocyte macrophage colony stimulating factor (GM-CSF) appears to be most important for the development of mature KCs^[31]. Large KCs are predominantly located in the region of liver acini near the portal triads and have higher lysosomal enzyme activities and a greater phagocytic capacity. The large KCs also produce TNF- α , PGE2, IL-10 and IL-1, while small KCs near the central veins produce high level of nitric oxide (NO)^[32].

One of the primary function of KCs is to discriminate “self” from “non-self” particles, playing a prominent role as APC as well as a scavenger of microorganisms. One of the molecules that recognizes “self” and “non-self” is Dectin-2, a C-type lectin receptor of the innate immunity receptor family. It is known to recognize high-mannose carbohydrate structures present on bacteria and fungi. This receptor also recognizes tumor cells. Once tumor cells are recognized via Dectin-2, KCs increase phagocyte activity against tumor cells, which contributes to the suppression of metastasis in the liver^[33].

HSCs

Eighty percent of total body vitamin A is stored in HSCs as intra-cytoplasmic lipid droplets. Upon their activation, HSCs metabolize vitamin A and all-trans

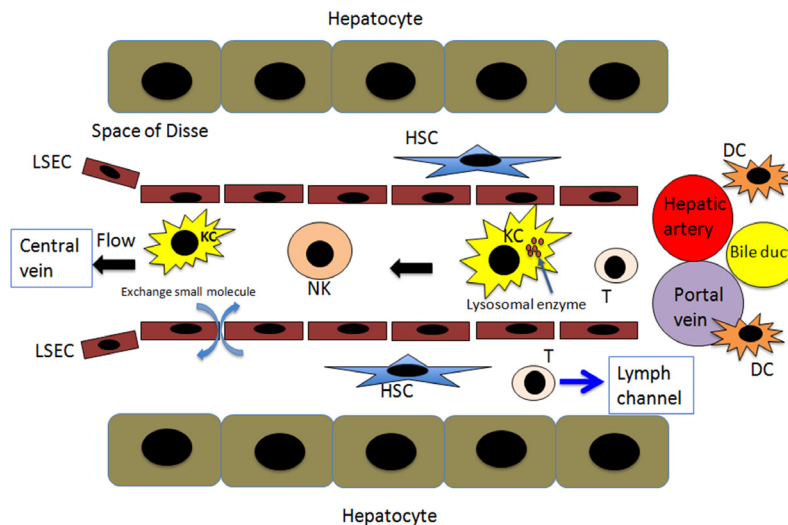


Figure 2: Immune microenvironment in the liver. Liver sinusoids are lined by a fenestrated monolayer of LSECs. HSCs reside in the space between hepatic cells and LSECs, called the Space of Disse. Blood flow in the Space of Disse goes toward the lymphatic vessels that run through the portal tracts to the draining lymph nodes. Blood from the hepatic artery and the portal vein goes through capillary-like vessels, called sinusoids, to the central vein. Dendritic cells are mainly located in the portal tracts. Large Kupffer cells, which have higher lysosomal enzyme activity, are located predominantly in the periportal region. Smaller Kupffer cells are located more close to the central vein. NK cells and T cells patrol the sinusoid. Hepatic NK cells are found in the sinusoidal space, while CD3 cells are mainly present in the periportal area. LSEC: liver sinusoidal endothelial cell; KC: Kupffer cell; HSC: hepatic stellate cell; DC: dendritic cell; NK: natural killer cell; T: T cell

retinoic acid, and are differentiated into myofibroblasts (MFs), which produce extracellular matrix and play a central role in hepatic fibrosis and cirrhosis. $\text{TNF-}\alpha$, IL-6 and $\text{TGF-}\beta$ promote activation and proliferation of HSCs to produce different extra-cellular matrix (ECM) components including α -smooth muscle actin (α -SMA) and type I collagen for tissue remodeling^[34]. The activation of HSCs from their resting/quiescent state to a profibrotic state triggers production of HGF, which results in recruitment of c-Met-expressing cells. It also contributes to proliferation of c-Met-expressing tumor cells and prevents apoptosis^[14,30,35]. Furthermore, secretion of IL-8 from HSCs contributes to tumor angiogenesis, which facilitates the growth of metastases in the liver^[28,36].

Hepatocytes

Hepatocytes comprise 80% of all liver cells. Hepatocytes express low levels of MHC class I and co-stimulatory molecules. However, under inflammatory conditions some hepatocytes express MHC class II^[30] and initiate an adoptive immune response. Hepatocytes produce IL-6 upon stimulation by HGF, lipopolysaccharide, or bacterial hepatotoxins^[37].

CIRCULATING IMMUNE CELLS IN THE LIVER

Immune homeostasis is dependent on the ability of the immune system to respond to pathogens. A variety of circulating immune cells in the liver interact with residential liver cells and modulate immune responses

in the liver as well as in the peripheral sites. These populations of immune cells reside in various locations in the liver^[38].

NK cells

NK cells represent only a small fraction of circulating lymphocytes, but account for up to 50% of lymphocytes in the liver. It has been reported that the human liver contains two NK cell subsets: conventional NK cells (cNK), which circulate freely, and liver-resident (lr) NK cells. There are two non-overlapping NK cell populations in human lrNK cells: CD49a^+ (integrin $\alpha 1$)⁺ NK cells and Eomes^{hi} (largely $\text{CD56}^{\text{bright}}$ and CXCR6^+) NK cells^[39]. Eomes^{hi}Tbet^{lo} NK cells account for 50% of human liver NK cells and they reside in sinusoidal space. This type of NK cells is completely absent in peripheral circulation. Eomes^{hi} lrNK express fewer receptors for human targets, suggesting that they would recognize non-human targets such as bacteria or bacterial products. In contrast, CD49a^+ NK cells are mainly found in the parenchyma and express cytotoxic effector molecules and receptors for MHC class I; thus, it seems likely that they recognize and kill virally infected or cancerous cells. It has been suggested that these lrNK cells have immune memory against specific antigens^[39].

NKT cells

NKT cells are activated by self- or microbial-lipid antigens, or by signaling through toll-like receptors (TLR), and act as a bridge between innate and adaptive

immunities. Following activation, NKT cells rapidly secrete either pro-inflammatory or anti-inflammatory cytokines and chemokines, and thereby determine the direction for subsequent immunity or tolerance.

Type I invariant NKT cells expressing specific T cell receptors (TCRs) comprise 95% of liver NKT cells, while type II NKT cells expressing diverse TCRs make up less than 5% of them. NKT cells recognize non-peptide antigen targets such as lipid and glycolipid components. They are activated by IL-12 or by interaction between NKG2D and its ligands on target cells. The role of NKT cells in cancer is rather controversial. In patients with hepatocellular carcinoma (HCC), CD4⁺ Vα24/Vβ11 type I NKT cells secreting Th2 cytokines, accumulated in tumor sites, and inhibited tumor-specific CD8⁺ T cell responses^[40].

T cells

The normal resident lymphocytes of the human liver consist of more CD8⁺ T cells relative to CD4⁺ T cells. Circulating T cells pass through the liver sinusoids and can interact with KCs and LSECs. Antigens that are expressed in the liver might be taken up by immature dendritic cells (DCs) and then presented to CD4⁺ and CD8⁺ T cells either in lymphoid-tissue aggregates in the portal tracts or in secondary lymphoid tissues. Alternatively, antigens might be presented *in situ* by LSECs, KCs and, possibly by hepatocytes. The outcome of antigen recognition by T cells in the liver could induce the proliferation of T cells or activation-induced T-cell apoptosis. Antigen recognition could also result in immune deviation to a suppressive or regulatory phenotype.

The determination of outcome depends on upregulation and expression of an extensive panel of T-cell interacting molecules including intercellular adhesion molecule 1 (ICAM1), MHC class II molecules, VCAM1, co-stimulatory molecules of the B7 family, and CD95 (FAS). These molecules might also modify cell trafficking, priming, and the induction of tolerance.

In general, with the production of immune modulatory cytokines such as IL-10 and TGF-β, the antigen-presentation by LSECs and many DCs in the liver is biased strongly towards the induction of CD4⁺ T cells with a regulatory phenotype^[41], whereas both CD8⁺ T cells that are activated systemically and naive CD8⁺ T cells that first encounter antigen in the liver are predisposed to undergo apoptosis. The liver sequesters activated T cells in an antigen-independent manner, and the high apoptotic rate of such cells has given rise to the idea that the liver might be a “graveyard” for systemic T cells. Activated antigen-specific T cells

expressing PD-1 interact with PD-L1 on LSECs to become tolerogenic or apoptotic^[42]. Activated T cells have been shown to be short-lived in the liver^[29].

It is of note that tryptophan 2,3-dioxygenase (TDO) is predominantly expressed in the liver. In contrast, indoleamine 2,3-dioxygenase (IDO) is found in many tissues and induced by interferon (IFN)-γ. TDO and IDO are responsible for metabolism of tryptophan (TRP). The metabolite of TRP, kynurenine (KYN), binds to the aryl hydrocarbon receptor (AHR) on T cells to suppress their activity. Effector T cells are particularly sensitive to low TRP levels. Local depletion of TRP suppresses T cell proliferation and induces cell death^[43]. AHR activation reportedly induces differentiation of Treg cells^[44].

INTERACTIONS BETWEEN CTCs AND LIVER MICROENVIRONMENT

CTCs can enter the liver through both the hepatic artery and portal vein. CTCs in the liver encounter various populations of residential cells that are specialized to carry out various immunological functions in the liver. Actual mechanism of establishment and progression of hepatic metastasis in UM are mostly speculative. Based on the published literatures^[13,29,30,41,45-49] and our limited institutional experience, we propose the following two-phase growth model for metastatic UM in the liver.

Microvascular phase

This phase starts with tumor cell arrest in the sinusoidal space. The final fate of surviving tumor cells can be determined by the interactions between tumor cells and immune cells. These interactions can lead to tumor cell death, or the growth of the tumor in the liver. Obstruction of the sinusoidal vessels by clusters of CTCs can result in transient blockade of blood flow and ischemia. This could result in tumor cell destruction caused by mechanical stress and deformation-associated trauma. Additionally, VCAM-1 expression on LSEC increases and traps melanoma cells that enter the liver^[50]. It was reported that VCAM-1 expression on LSEC increased significantly within 24 h of melanoma cell entry into the liver^[50], and blocking VCAM-1 by antibodies decreased microvascular retention of tumor cells and metastasis. Tissue ischemia induces the local release of NO and reactive oxygen species, and kills tumor cells.

LSEC and KCs are likely to be the first resident cells that CTCs encounter in the liver. The tumor cells can be eliminated by local, tumoricidal KCs. KCs can also activate other innate immune response cells such as NK cells, NKT cells, and neutrophils. NK cells can mediate antitumor cytotoxicity by secreting perforin/granzyme or through

CD95/CD95L pathway. Cytokines and chemokines such as TNF- α , IL-8, and CXCL10 can activate resident tumoricidal macrophages, as well as recruit host immune cells with anti-tumor activities^[51] [Figure 3A].

Growth phase

While an efficient first line defense can defeat some CTCs trapped in the liver, local inflammatory response can also promote tumor cell adhesion to LSECs and subsequent trans-endothelial migration of tumor cells, which results in escape from the cytotoxic resident KCs and NK cells. IL-10 production from KC or LSECs enhances the expression of the chemokine receptor CCR5, but down-regulates CCR7 expression by DCs thus preventing their homing to the secondary lymphoid tissue^[41]. E-selectin, VCAM-1, and ICAM-1 play essential roles in tumor cell arrest and extravasation into the hepatic parenchyma^[52]. In particular, E-selectin facilitates diapedesis of tumor cells and subsequent invasion into the hepatic parenchyma. Invasion of tumor cells into the extra-sinusoidal space triggers the recruitment of HSCs and macrophages into the tumors. These macrophages are polarized by IL-4 and IL-13 towards M2 type macrophages expressing arginase-1^[48] [Figure 3B]. Recruited HSCs release growth factors, cytokines, and matrix metalloproteinases (MMPs), and increase production of collagen. As a result, recruitment of vascular endothelial cells, assembly and turnover of extracellular matrix, and proliferation tumor cells are promoted. Tumor cells also produce VEGF to promote angiogenesis^[47] [Figure 3C]. Hepatocytes contribute to fibrosis and neovascularization through secretion of IGF-1 and IGF-2, factors that promote HSC recruitment and activation. IGF-1 can also directly enhance tumor cell growth.

In addition to interacting with various residential cells in the liver, MDSCs are recruited to tumor sites in response to mediators released by tumor and/or resident hepatic cells. There are two different types of MDSC: polymorphonuclear MDSC (PMN-MDSC) and monocytic MDSC (M-MDSC). In the tumor sites, M-MDSCs are more prominent than PMN-MDSC, and M-MDSC rapidly differentiate to tumor-associated macrophage (TAM) to enhance tumor growth. CD68⁺/CD163⁺ TAMs are observed in metastatic UM in the liver^[49]. MDSCs produce immunosuppressive cytokines, such as IL-10 and TGF- β , and induce Tregs^[53]. Subsequently, immunological equilibrium between tumor cells and host immune responses is shifted to the escape (growth) phase [Figure 3D]. More vascular endothelial cells are subsequently recruited to the tumor site and the tumors become further vascularized. Eventually, the vascularization of tumor results in rapid growth of metastasis.

Recently, microscopic investigation on inflammatory cells in advanced metastatic uveal melanoma tissue specimens was reported by Coupland's group^[49]. They reported that CD3⁺ T lymphocytes were noted both within tumor and surrounding tissues. Of note, CD8⁺ T lymphocytes were "few" in number within metastatic UM and were predominantly seen peritumorally at the tumor/normal liver interface. In contrast, CD4⁺ T lymphocytes showed a high perivascular density within melanoma. The characteristics of CD4⁺ T cells were not further investigated; however, it is possible that these CD4⁺ T cells might be Treg cells recruited from the peripheral circulation. Furthermore, CD68⁺ and CD163⁺ TAMs of "indeterminate" morphology were observed in metastatic UM, suggesting the presence of the pro-tumorigenic M2 phenotype. It has also been reported that tumor infiltrating T cells obtained from metastatic UM were difficult to expand *ex-vivo* despite the lack of PD-L1 expression in tumor tissues^[54]. Lack of PD-L1 expression by metastatic UM cells and marginalization of CD8⁺ T cells suggests an impaired anti-tumor immune response in metastatic UM.

Grossniklaus *et al.*^[38] proposed two growth patterns of hepatic metastasis: "infiltrative" and "nodular". They hypothesized that primary UM cells, expressing high levels of c-Met and/or CXCR4, aggregate in the liver which contains HGF and CXCL12. These metastatic UM cells have a CD133⁺ tumor stem cell-like phenotype, and give rise to the infiltrative or nodular growth patterns depending on whether the tumor is in the sinusoidal space (infiltrative) or periportal area (nodular). The infiltrative growth pattern showed cell growth within the sinusoidal space. The nodular growth pattern predominantly contained nodules of tumor that effaced, rather than infiltrated, the adjacent hepatocytes. Hepatic metastasis with infiltrative pattern showed the lack of VEGF protein in the tumor, but tumor cells induce MMP9 expression in monocytes and dissect through the tissue planes and creates "pseudo-sinusoidal spaces". On the other hand, UM cells that metastasize to the periportal areas in the hepatic triadcoopt the portal venules for nutrition and hypoxia resulting in MMP production and VEGF expression for angiogenesis. The role of the immune system in development of these two growth patterns needs to be further investigated.

CURRENT IMMUNOTHERAPIES FOR METASTATIC UVEAL MELANOMA

The major difference between CM and UM is the low mutational burden in UM compared to the high mutational burden in CM^[19]. Metastatic UM is highly resistant to traditional systemic chemotherapies, and

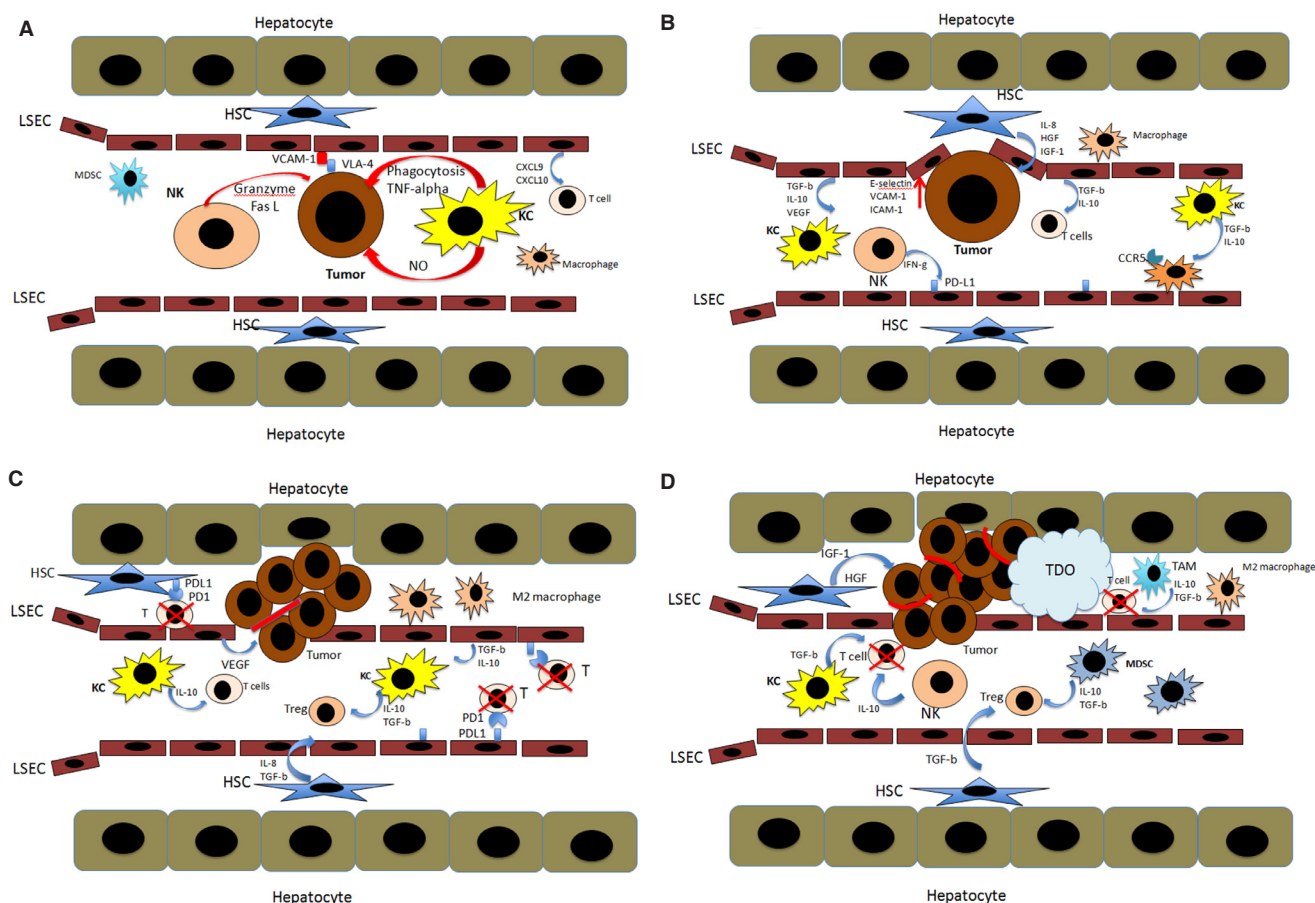


Figure 3: Liver immune microenvironment and tumor growth. **A:** immune attack on circulating tumor cells. Circulating tumor cells entering the sinusoidal area are attacked by immune cells in the sinusoid, especially Kupffer cells and NK cells. These cells eliminate tumor cells via phagocytosis, cytotoxic granules, death-receptor pathways, nitric oxide, or ROS; **B:** extravasation of tumor cells into the hepatic parenchyma. Following firm attachment to LSEC via adhesion molecules such as E-selectin, VCAM-1, and ICAM-1, tumor cells escape from the sinusoidal space and invade into the extrasinusoidal space, which is rich in various growth factors such as HGF and IGF-1; **C:** remodeling of hepatic parenchyma and angiogenic sprouting. Tumor cell invasion into the extrasinusoidal space triggers HSC and M2 macrophage recruitment into the tumors and increases production of collagen in and around hepatic metastases. HSC recruited into the metastases as myofibroblasts release growth factors, cytokines, and MMPs. IL-8 produced by HSC induces the expression of VEGFR2 and VEGF on endothelial cells and mediates autocrine and paracrine stimulation of vascular endothelium; **D:** rapid growth of hepatic metastasis. Vascular endothelial cells are further recruited to the tumor site and tumors become further vascularized. The vascularization of tumor results in rapid growth of metastasis. Local production of Th-2 type cytokines, deprivation of tryptophan, and elimination of activated T cells via PD-L/PD-L1 interaction result in non-T cell inflamed immune microenvironment in the hepatic metastasis. CXCR9: chemokine (C-X-C motif) ligand 9; CXCR10: chemokine (C-X-C motif) ligand 10; CCR5: C-C chemokine receptor 5; Fas L: fas ligand; HSC: hepatic stellate cell; HGF: hepatocyte growth factor; IL-6: interleukin 6; IL-8: interleukin 8; IL-10: interleukin 10; IFN- γ : interferon gamma; IGF-1: insulin growth factor-1; KC: kupffer cell; LSEC: liver sinusoidal endothelial cell; NO: nitric oxide; PD-1: programmed death 1; PD-L1: programmed death ligand 1; TDO: tryptophan 2,3 dioxygenase; TGF- β : transforming growth factor beta; T reg: regulatory t Cell; VCAM-1: vascular cell adhesion protein 1; VEGF: vascular endothelial growth factor; VLA-4: very late antigen 4; ROS: reactive oxygen species

currently approved signal inhibitors for CM do not work for metastatic UM. In addition, the liver is one of the most tolerogenic immune microenvironments, especially in regard to the T cell immune system. Taken together, metastatic UM is one of the most challenging tumors and convincing survival benefit of systemic and local treatments remains to be seen.

There have been several clinical trials using immunotherapy for metastatic UM [Table 1]. These clinical trials have provided important insights into the immune microenvironment of metastatic UM and have

identified a direction for future immunotherapy truly for metastatic UM.

LOCOREGIONAL TREATMENT

The liver is the first and dominant site of metastasis in UM, therefore it is reasonable to consider locoregional immunotherapy to directly destroy the tumor and provide tumor-related antigens to the systemic immune system. We have developed one such approach called, "immunoembolization (IE)". This consists of embolization of the tumor-feeding hepatic artery by

Table 1: Immunotherapy clinical trials in metastatic uveal melanoma

Study	Phase	Identifier	Targets and approaches	Status
Nivolumab and ipilimumab in treating patients with MUM	Phase II	NCT01585194	PD-1 and CTLA-4, MoAb	Recruiting
Pembrolizumab in treating patients with advanced uveal melanoma	Phase II	NCT02359851	PD-1, MoAb	Ongoing but not recruiting
Glembatumumab vedotin in treating patients with metastatic or locally recurrent uveal melanoma	Phase II	NCT02363283	gpNMB, ADC	Recruiting
A study of the intra-patient escalation dosing regimen with IMCgp100 in patients with advanced uveal melanoma	Phase I	NCT02570308	gp100, TCR-CD3 Ab fusion protein	Recruiting
Yttrium90, ipilimumab and nivolumab for uveal melanoma with liver metastases	Phase I, Phase II	NCT02913417	PD-1 and CTLA-4 + radiospheres	Recruiting
Dendritic cells plus autologous tumor RNA in uveal melanoma	Phase III	NCT01983748	DC plus mRNA	Recruiting
Immunotherapy using tumor infiltrating lymphocytes for patients with metastatic ocular melanoma	Phase II	NCT01814046	TIL	Ongoing but not recruiting
Trial of nivolumab in combination with ipilimumab in subjects with previously untreated metastatic uveal melanoma (GEM1402)	Phase II	NCT02626962	PD-1 and CTLA-4	Ongoing but not recruiting
Epacadostat and vaccine therapy in treating patients with Stage III-IV melanoma	Phase II	NCT01961115	IDO1 inhibitor + MELITAC 12.1 Vaccine	Ongoing but not recruiting

MUM: metastatic uveal melanoma; MoAb: monoclonal antibody; ADC: antibody-drug conjugate; TIL: tumor infiltrating lymphocytes; DC: dendritic cells; IDO: indoleamine 2, 3-dioxygenase. As of May 2017 [Clinical Trials.gov].

gelatin sponge particles following arterial infusion of GM-CSF emulsified in ethiodized oil. In theory, metastatic UM cells will be killed by the ischemic effect of embolization; GM-CSF stimulates APCs and promotes uptake of tumor antigens, leading to the induction of T cell activation in the liver and at a secondary lymph node. This approach could lead to the development of systemic immunity against melanoma and delay development and progression of extra-hepatic metastasis. This concept was subsequently investigated in a clinical trial setting. Compared with chemoembolization with 1,3-bis (2-chloroethyl)-1-nitrosourea (CE), IE induced significantly better overall survival (OS) (20.4 vs. 9.8 months, $P = 0.005$) and systemic progression free survival (PFS) (12.4 vs. 4.8 months, $P = 0.001$)^[59].

In subsequent randomized double-blinded clinical trials, IE was compared with embolization of hepatic tumor with normal saline solution with ethiodized oil, "bland embolization (BE)"^[56]. Overall survival was 21.5 months (95% CI: 18.5-24.8 months) with IE and 17.2 months (95% CI: 11.9-22.4 months) with BE. The degree of proinflammatory cytokine production was more robust after IE. TNF- α , IL-6, and IL-8 levels in serum were increased with IE 1 h and 18 h after the embolization procedures. On the other hand, IL-6 and IL-8 levels in serum in BE were mildly increased 18 h after the procedures. The higher degree of cytokine release after IE was correlated with longer time to "systemic" extrahepatic progression. In the IE group, higher IL-6 levels at 1 h ($P < 0.001$) and IL-8 levels at 18 h after the procedure ($P < 0.001$) were significant predictors of longer systemic PFS. We are currently in the process

of initiating a new phase 2 study, in which IE will be combined with the immune checkpoint inhibitors, ipilimumab and nivolumab.

Since hepatic metastasis is life-limiting in the majority of patients and the induction of anti-tumor response by traditional chemotherapies or immunotherapies is difficult, various liver-directed treatments have been investigated including percutaneous hepatic perfusion with melphalan, intrahepatic arterial infusion with fotemustine. The impact of these liver-directed treatments on tumor immune microenvironment in the liver remains to be investigated.

SYSTEMIC IMMUNOTHERAPY

Immune checkpoint blockade

Development and approval of immune-modulatory antibodies against cytotoxic T lymphocyte associated antigen 4 (CTLA-4) and PD-1/PD-L1 resulted in a paradigm shift in the treatment of metastatic CM^[57-59] and provided hope for patients with this disease. Unfortunately, this new approach did not improve the outcome of metastatic UM. Disappointing response rates have been reported with anti-CTLA-4 antibody as well as with anti-PD-1/PD-L1 antibody treatments in metastatic UM^[60,61]. In the retrospective collection of data on 82 assessable UM patients who received ipilimumab, the fully human monoclonal antibody against CTLA-4, at 3 mg/kg, every 3 weeks for a maximum of 4 doses, 4 (5%) had an immune-related objective response and 24 (29%) had immune-related stable disease lasting ≥ 3 months. With a median follow-up of 5.6 months,

median OS was 6.0 months and median PFS was 3.6 months. The 1-year rates of OS and PFS were 31% and 11%, respectively^[61].

Another retrospective study including 34 patients who received 3 mg/kg ipilimumab and 5 patients who received 10 mg/kg ipilimumab showed 1 complete response (CR) and 1 late partial response (PR) for an immune-related response rate of 5.1^[62]. The OS from the first dose of ipilimumab was 9.6 months (95% CI: 6.3–13.4 months). Survival ranged from 1.6 to 41.6 months in this study. Retrospective investigation of 56 patients treated with anti-PD-1 or PD-L1 antibodies also showed disappointing results^[60]. Among 56 UM patients, objective tumor responses were observed in only 2 patients for OS of 3.6% (95% CI: 1.8–22.2%). Stable disease (≥ 6 months) was observed in 5 patients. The median PFS was 2.6 months (95% CI: 2.4–2.8 months), and the OS was 7.6 months (95% CI: 0.7–14.6 months). The result may be correlated with low expression of PD-L1 in metastatic UM, compared to that of non-hepatic metastatic CM^[63]. It is of note that a poor response to anti-PD-1 antibody therapy has also been reported in CM patients with hepatic metastasis^[64]. This further supports the hypothesis that the liver immune microenvironment itself hampers T-cell immune response against cancer cells.

Adoptive immunotherapy using tumor-infiltrating T cells

Tumor-infiltrating T cells (TIL) treatment has been highly successful in metastatic UM with durable response and regression of bulky metastasis. In a study at National Cancer Institute (NCT01814046), 21 metastatic UM patients who were HLA-A2 positive were treated with TIL therapy in phase II clinical trial^[65,66]. Seven of 20 evaluable patients showed objective tumor regression including 6 patients with PR and one patient with CR, ongoing at 21 months post therapy. No significant difference was seen among responders and non-responders in terms of mutation burden in tumors. On the other hand, TIL products with either less than 3% tumor-reactive T cells, less than 2×10^9 tumor-reactive T cells, or less than 100 pg/mL of tumor-induced IFN- γ release yielded poor clinical responses^[65]. This study indicated that adoptive transfer of autologous TILs can mediate objective tumor regression in patients with metastatic UM.

DC vaccine

Melanocyte-associated proteins including gp100, MART-1, tyrosinase, and TRP-1 were also expressed in the majority of human UM^[67]. These tumor-

associated antigens constitute an appropriate target for immunotherapy for metastatic disease. DCs are antigen-presenting cells with the unique ability to activate naive T cells, and hence are suitable for inducing immunologic antitumor responses. A group from the Netherlands treated 14 metastatic UM patients with therapeutic DC vaccines loaded with gp100 and tyrosinase. Patients were required to have HLA-A*02:01 phenotype or HLA-DRB*01:04 and needed to have metastatic UM expressing gp100 and tyrosinase. All patients were vaccinated with autologous DCs loaded with gp100 and tyrosinase in various ways (mutated or wild type peptides, or mRNA), 3 times, biweekly. One to 2 weeks after the last vaccination, a skin test was performed. In the absence of disease recurrence, patients received a maximum of 2 maintenance cycles of vaccine at 6-month intervals. T cells specific for gp100 and tyrosinase were detected by tetramer assay after DC vaccination in 4 (29%) of 14 patients. DC-vaccinated patients with metastatic disease showed a median overall survival of 19.2 months. No serious treatment-related adverse events (common toxicity criteria grade 3 or 4) were observed. Clinically, no regression of metastasis was observed. Ten patients showed stable disease at the first evaluation point, 3 months after start of vaccination, but 7 patients subsequently progressed before the first maintenance cycle of vaccine was started at 6 months. Seven (50%) patients survived more than 2 years after start of DC vaccination for metastatic UM^[68]. The efficacy of this therapeutic DC vaccine remains to be investigated in a large prospective study with a more uniform antigen-loading method to DC.

TCR and anti-CD3 antibody fusion protein

IMCgp100 is a fusion protein containing gp100-specific T cell receptor (HLA-A*02:01) and anti-CD3 scFv. IMCgp100 binds to UM cells that express gp100 peptides on MHC Class I and then tags CD3⁺ T cells to the other end via anti-CD3 antibody. Soluble TCRs have been engineered and modified to possess extremely high affinity to gp100 peptides on HLA-A*02:01 molecules. The early phase clinical studies showed encouraging results^[69,70] and a pivotal large-scale randomized phase 2 study for metastatic UM is planned to start.

Antibody target glycoprotein NMB

Glembatumumabvedotin (also known as CDX-011 and CR011-vcMMAE) is an antibody-drug conjugate (ADC) that targets cancer cells expressing transmembrane glycoprotein NMB (gpNMB). It is a fusion molecule containing fully-human IgG2 monoclonal antibody against gpNMB and the cytotoxic drug monomethyl auristatin E (MMAE). The anti-gpNMB antibody binds to gpNMB expressing tumor cells and, upon internalization,

the antibody releases MMAE, a potent cytotoxic agent. gpNMB is overexpressed by multiple tumors including melanoma and breast cancer. Eighteen of 21 primary UM tissue specimens (85.7%) evaluated by immunohistochemical analysis (IHC) expressed gpNMB in 10-90% of tumor cells with variable intensity^[71]. A phase 2 clinical study for Glabatumumab vedotin in metastatic or locally recurrent UM (NCT02363283) has been recently concluded^[72]. The final results are awaited.

CONCLUSION

There is no standard care for the treatment of patients with metastatic UM. The effectiveness of treatments for metastatic UM seems to be very limited, and induction of immunity against UM cells may be the major challenge. We are just beginning to understand the immune suppressive pathways involved in metastatic UM and their tumor microenvironment. As we described in this review article, immune reactions can be generated against cancer cells under specific circumstances such as the presence of cognitive antigens, an increasing level of APCs, high affinity of interactive TCR, and depletion of inhibitory immune cells or molecules in the liver. Considering potential pre-existing immune tolerance against UM cells, low mutational burden, and an immune-modulating microenvironment in the liver, mechanical perturbation of hepatic metastasis with or followed by immunostimulatory molecules would be a reasonable approach. Alternatively, the usage of ex-vivo activated tumor-specific T cells or mimicking approaches such as bispecific fusion proteins would be a promising approach against metastatic UM as suggested by on-going clinical trials. Inhibitors of key signal pathways unique to metastatic UM might also be helpful in increasing immunogenicity of UM cells, which in turn might improve the efficacy of currently available immune checkpoint blockades. Further collaboration between basic immunology researchers and clinical scientists is required for the development of more effective immunotherapy strategies against metastatic UM.

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Authors' contributions

Drafting and writing the manuscript: M. Terai
Manuscript's conception, revision and supervised: M.J. Mastrangleo, T. Sato

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There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

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Cell-mediated immunotherapy for hepatocellular carcinoma

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ABSTRACT

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Hepatocellular carcinoma (HCC) is the most common primary liver cancer. Most of the time, these tumors are diagnosed at late stages. Because no effective treatments exist for patients with advanced stage HCC, there is an urgent need for novel, effective treatments. Cancer cells originate as a consequence of abnormal expression of oncogenes or loss of tumor suppressor genes. Often, neoplastic transformation results in a hyper-mutated cellular genome, which in turn produces neo-antigens from mutated genes. These tumor-specific or tumor-associated antigens can be recognized by antigen-presenting cells and trigger T-lymphocytes to elicit anticancer immunity. Immune responses to cancers are often rendered ineffective by tumor immune-editing and immune-suppressive mechanisms. Yet, therapeutic strategies to stimulate anti-cancer immunity have had remarkable success in several solid and hematological malignancies. Among the various strategies for cancer immunotherapy, cell-mediated immunotherapy holds considerable promise to overcome anergy and systemic immune suppression. This brief review will focus on cell-mediated immunotherapy for HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common malignancy in the world^[1]. The treatment modalities for HCC included liver transplantation^[2,3], liver resection^[4], local ablation^[5], transcatheter arterial chemoembolization (TACE)^[6,7], molecular target therapy^[8,9], radiotherapy^[10], chemotherapy^[11], and so on. According to Barcelona Clinic Liver Cancer (BCLC) classification, HCC can only be cured by liver

transplantation, liver resection, and radiofrequency ablation (RFA) in the very early and early stages of the disease^[12]. Even when tumors are completely removed by liver resection and liver transplantation, or by complete ablation by RFA, tumor recurrence is still not preventable. When tumors are in the intermediate stage, they can only be controlled by TACE. When tumors are in more advanced stages, patients can only be treated by molecular target therapy or supported by best care. There are as yet no effective treatments for



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patients with advanced stage HCC, so the search for effective treatments is a crucial one.

Cancer cells occur as a consequence of enhanced or aberrant expression of oncogenes or from loss of tumor suppressor genes. Cancer cells with genetic change will express new antigens^[13]. These tumor-specific or tumor-associated antigens might be recognized by antigen-presenting cells and trigger T-lymphocytes to conduct anti-cancer immunity^[14-16]. Immunotherapy has been studied as an attractive and novel therapeutic strategy to treat cancer since a few decades ago. This brief review will focus on cell-mediated immunotherapy for HCC.

IMMUNITY IN CANCER

The immune system is the most important protection for a host in defending itself from foreign invaders and cancer development. As noted, cancer cells occur as a consequence of enhanced or aberrant expression of oncogenes or loss of tumor suppressor genes. The cancer cells with genetic change express new antigens and the new antigens may be captured and processed by dendritic cells (DCs) to trigger T cell-mediated immunity^[17,18].

Dendritic cells, the most potent and professional antigen-presenting cells, constitutively express major histocompatibility complex (MHC) class I and II and high levels of costimulatory molecules CD40, CD80, and CD86. When DCs meet antigens, they capture antigen, process it, and present the antigen to activate antigen-specific cytotoxic T-cells. Clinically, DC-based immunotherapy has been applied to treat end-stage patients with B-cell lymphoma^[19], melanoma^[20], renal cell carcinoma^[21], prostate cancer^[22] and other tumors^[23]. The results are promising^[24]. DC-based immunotherapy offers a hope of successful eradication of cancer [Figure 1].

However, DC-based immunotherapy yields only a 20% response rate in most of the clinical trials for advanced cancer diseases^[25]. These results suggest that, even though DCs are the most powerful antigen-presenting cells, the immune system of most advanced cancer patients cannot be activated or may only be activated to a limited extent by DC. It is hoped that exploration of immunosuppressive mechanisms in tumor-bearing patients will improve the success of DC-based or cell-mediated immunotherapy.

IMMUNODEFICIENCY IN CANCER PATIENTS

T-cells are the direct effector cells that attack and eradicate cancer cells. The cytotoxic ability of

Dendritic cells -- Cancer vaccine

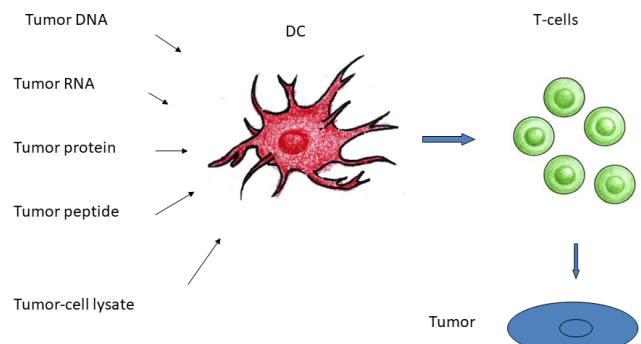


Figure 1: Dendritic cells can be pulsed by tumor DNA, RNA and proteins to activate antigen-specific T-cells. These activated T-cells can produce antigen-specific cytotoxicity to eradicate tumor cells

activated T-cells is directly related to efficacy of cancer treatment. In animal studies, tumor-infiltrating lymphocytes in tumor-bearing hosts have been proved anergic to cancer cells. Cancer cells may also induce T-cell apoptosis or regulatory T-cells, which conduct peripheral tolerance to cancer cells^[26,27]. Clinically, we have already observed that the percentage of lymphocytes decreases along with tumor growth in HCC patients^[25]. In patients with significant numerous tumor mass, the percentage of lymphocytes is always below normal range. Immediately before patients die of HCC, lymphocytes cannot even be detected. Obviously, lymphocytes are suppressed by HCC through currently unidentified mechanisms.

Regulatory T cells are immune suppressive cells^[28]. In animal models, depletion of regulatory T cells causes inflammatory colitis, and restoration of regulatory T cells can prevent inflammatory colitis^[29]. Therefore, natural regulatory T cells, CD4⁺CD25⁺CD45RB^{low}, are considered important cells in maintaining peripheral tolerance. Regulatory T cells are also recognized as playing an important role in cancer diseases^[30]. For gastric^[31,32], esophageal^[33], and other gastrointestinal malignancies, regulatory T cells were increased in peripheral blood. For breast cancer, regulatory T cells were increased in peripheral blood and in the tumor microenvironment^[34]. For lung cancer, regulatory T cells selectively inhibited host immune response and may have contributed to disease progression^[35]. For HCC, CD4⁺CD25⁺ regulatory cells are also found in the tumor by immunohistochemical staining, and the number of regulatory T cells is correlated to the prognosis. In our previous study, regulatory T-cells were identified in the tumor microenvironment. The number of regulatory T-cells was correlated to tumor size and contributed to prognosis. These regulatory T cells also appeared to suppress the DC-mediated

immune responses^[36].

Regulatory T-cells are not the only immunosuppressive cells in hosts with cancer. In a murine cancer model, a group of cells expressing CD11b and Gr-1 in the spleen was noted when the cell population of spleen was analyzed. These cells are currently called myeloid-derived suppressor cells (MDSC)^[37]. Actually, MDSC are a population of cells of myeloid origin, including myeloid progenitors, immature macrophages, immature granulocytes, and immature dendritic cells. MDSC are characterized by production of reactive oxygen, nitrogen species, and arginase I to suppress immunity^[38,39]. The CD11b⁺/Gr-1⁺ cells were expanded in mice bearing large tumors^[40]. Deletion of CD11b⁺/Gr-1⁺ cells *in vitro* or *in vivo* reverses the depression of CD8⁺ T-cell function.

Many researchers attempt to decrease or deplete MDSC to enhance cancer treatment^[41-44]. Kusmartsev *et al.*^[45] implanted slow-releasing all-trans-retinoic acid subcutaneously in order to decrease MDSC from 27% to 11%. De Santo *et al.*^[46] used nitro-aspirin, which released NO to interfere MDSC inhibitory enzymatic activity. Gemcitabine, amino-biphosphonase, and celecoxib all have been used to reduce the number of MDSC in order to enhance cancer treatment^[47]. Moreover, all-trans-retinoic acid has been employed to treat renal cancer patients with metastasis, and there was a clinical response in 1 patient (1/18)^[48]. These data in animal models and a limited number of clinical trials implied that depletion or decrease of MDSC might be helpful in cancer treatment.

CLINICAL CELL-MEDIATED IMMUNOTHERAPY FOR HCC

DC-based immunotherapy

As mentioned, DCs are the most potent antigen-presenting cells. Theoretically, DCs can capture HCC-associated antigens, process the antigens, and activate antigen-specific T-cells to get rid of the tumors. However, the function of DCs is defective in advanced HCC, and antigen-specific T-cells cannot be activated properly. Therefore, DCs are cultured and matured *ex vivo* for immunotherapy.

In a phase I trial, Iwashita *et al.*^[49] enrolled 10 patients to receive autologous DC to treat unresectable HCC. DC was administered by injection into inguinal lymph nodes. Seven of 10 patients experienced delayed-type hypersensitivity response and one patient had tumor response. It was concluded that DC administered was safe and no major toxic effects were found. In another study, Palmer *et al.*^[50] used autologous DC pulsed

with liver tumor cell line lysate (Hep G2) to treat 35 patients with advanced HCC. Twenty-five patients who received at least 3 courses of DC infusion were assessed for tumor response. Disease control rate was 28%. Qiu *et al.*^[51] conducted a phase II clinical trial using α -1,3-galactosyl epitopes pulsed DC to treat stage III HCC patients. They enrolled 9 patients to have DC vaccination and 9 patients as control. The results showed that all patients had delayed hypersensitivity. Three of the 9 patients with DC vaccination had partial response. Compared to control, mean survival was prolonged from 10.1 to 17.1 months^[51]. In our previous study, DC progenitors from peripheral blood monocytes (PBMC) were cultured in granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4, pulsed with autologous tumor lysates and matured by cytokine cocktail (tumor necrosis factor- α , IL-1, IL-6 and PGE2). These DCs were positive for CD83 and expressed high levels of CD40, CD80, CD86 and HLA-DR. In *in-vitro* study, these DCs could activate T-cells. These *ex-vivo* prepared DCs were applied intravenously to treat 31 patients with advanced HCC. Among 31 patients, 4 (12.9%) patients had partial response and 17 (54.8%) patients had stable diseases. Disease control rate was 67.7%. Compared to the same stage patients without DC treatment, the 1- and 2-year survival rates were significantly prolonged^[25]. DC can be injected into the tumors directly. Nakamoto *et al.*^[52] enrolled 10 patients to receive autologous DC infusion into tumors following TACE. DC could be detected in the tumors for up to 17 days when the DCs were labelled with ¹¹¹Indium. Tumor antigen-specific lymphocytes could be found around the tumors. However, no clinical benefit was found in this study.

Checkpoint inhibitor immunotherapy

Activation of naïve T-cells by DC is through ligation of MHC class I/II and T-cell receptor (signal 1) and costimulatory molecular pathways (signal 2). Costimulatory molecular pathways may deliver positive or negative signals to T-cells and result in T-cell activation or T-cell anergy to specific antigens. In immunotherapy of cancer, checkpoint inhibitors can block negative costimulatory molecular pathways and enhance T-cell-mediated immunity. Clinically, checkpoint inhibitor immunotherapy already has obtained promising results in treating advanced melanoma. Tremelimumab, a blockade of cytotoxic T-cell antigen 4 (CTLA-4), was used to treat hepatitis C patients with HCC in a clinical trial^[53]. Twenty patients were enrolled and 17 patients were available to assess therapeutic responses. Partial response rate was 17.6%, stable disease was 76.4%, and time to progression was 6.48 months. Nivolumab, anti-

programmed cell death protein-1, is another checkpoint inhibitor employed in a clinical trial of HCC treatment. In total, 262 patients were enrolled in dose-escalation and dose-expansion phases. The objective response rate was 20%. Complete response rate was 1%, partial response was 18%, and stable disease was 45%. The median progression-free survival was 4 months^[54]. Currently, several clinical trials of checkpoint inhibitor immunotherapy are ongoing. Results will be published in the near future.

T-cell immunotherapy

T-cells are direct effector cells to attract cancer cells. Activated T-cells can be applied to treat cancers^[55]. Takayama *et al.*^[56] conducted a randomized clinical trial by infusion of T-cells to prevent HCC recurrence after curative resection of HCC. One hundred and fifty patients were enrolled and randomized: 76 received adoptive immunotherapy with activated T-cells, and 74 patients received no adjuvant treatment. Autologous lymphocytes were activated by recombinant IL-2 and anti-CD3 antibody and could be expanded more than 1000-fold. Compared to control, the frequency of tumor recurrence was decreased by 18%, and time to the first tumor recurrence was longer. However, overall survival was not significantly affected.

Jiang *et al.*^[57] conducted a phase I trial using autologous tumor-infiltrating lymphocytes (TIL) to prevent tumor recurrence after curative resection for HCC. TIL was obtained from adjacent-tumor tissue and could be expanded by IL-2 and anti-CD3 in 15 of 17 patients. When the expanded TIL was infused back into the patients, only grade 1 flu-like symptoms and malaise were noted. After a median follow-up of 14 months, 12 patients were tumor-free and 3 patients had tumor recurrence. Basically, immunotherapy with expanded autologous TIL was safe and the toxicity was low. Future clinical trials may be conducted by the authors.

Cytokine-induced killer cell therapy

Autologous cytokine-induced killer (CIK) cells were also used to provide cell-mediated immunotherapy for HCC. Shi *et al.*^[58] conducted a phase I clinical trial using CIK cells to treat HCC. CIK cells were expanded from PBMC *ex vivo* by interferon- γ in the first day and followed by anti-CD3, IL-1 α , and IL-2. After CIK cells were infused, the populations of CD8⁺ cells and DCs were both increased. Tumor volume was decreased in 3 of 13 patients. The authors concluded that infusion of CIK cells was safe and immunological status could be improved. Since CIK cells might show anti-tumor activity for HCC, Hao *et al.*^[59] conducted a randomized trial to compare the treatment efficacy of combination of TACE and CIK cells infusion vs. TACE alone for

unresectable HCC. They enrolled 72 patients treated by combination of TACE and CIK cells infusion and 74 patient treated by TACE alone. The results showed progression-free survival and overall survival were both improved. The 1- and 2-year survival were 71.9% and 62.4% for combination therapy of TACE and CIK cells infusion, compared to 42.8% and 18.8% for therapy with TACE alone. Su *et al.*^[60] collected 7 randomized controlled trials and one controlled clinical trial to perform a meta-analysis study of comparison between DC + CIK cells + TACE/RFA treatment for HCC and control. A total of 349 patients had DC + CIK cells + TACE/RFA treatment, compared to 344 patients as control. The results showed that DC + CIK cells + TACE/RFA treatment improved 1- and 2-year overall survival.

Tumor neo-antigens

A successful DC-based immunotherapy for cancers needs specific tumor-associated antigens to promote anti-cancer immunity. HCC-associated antigens were well reviewed by Hong and Huang^[61] and Sun *et al.*^[62]. Among the reported antigens, α -fetoprotein, glypican-3 (GPC3), and multidrug resistance-associated protein-3 (MRP-3) were frequently expressed on HCC and were employed as tumor antigens to conduct clinical trials. However, the clinical responses were not satisfactory. Recently, Aref *et al.*^[63] found HCA519/TPX2 was an HCC-associated antigen. When DCs were pulsed with this peptide, cytotoxic T-cells could be activated. Zhu *et al.*^[64] found the levels of cytokeratin (CK) 10 in HCC cell lines were higher than in normal liver tissue. CK 10 is a potentially targetable tumor-associated antigen. Whether these antigens can be presented by DC to enhance anti-cancer immunity needs to be proved by clinical trials.

CONCLUSION

HCC is an aggressive cancer and can recur even when tumors are completely removed. Effective treatments for advanced stage HCC are still lacking. Cell-mediated immunotherapy is an attractive therapy for HCC with few toxicities. However, tumor response rates are only around 20% because immunosuppressive factors or cells interfere with the effects of immunotherapy. The combination of increasing immunity and depleting immunosuppressive factors shows promise for future success in conducting cell-mediated immunotherapy for HCC.

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Authors' contributions

W.C. Lee contributed solely to the paper.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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Cancer immunotherapy: a brief review of the history, possibilities, and challenges ahead

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ABSTRACT

The knowledge that the body possesses natural defenses to combat cancer existed long before the modern period, with multiple anecdotal reports of tumors miraculously disappearing, sometimes spontaneously or after a febrile or infectious episode. Spontaneous tumor regression of untreated malignant tumors is currently a well-accepted albeit rare phenomenon, and it is recognized that immunosuppression is associated with a higher cancer risk. The treatment of bladder carcinoma by intravesical administration of live attenuated *Bacillus Calmette-Guérin* bacteria was shown to be very effective in 1976 and is now standard treatment. Effective immunity against cancer involves complex interactions between the tumor, the host, and the environment. Cancer immunotherapy uses various strategies to augment tumor immunity and represents a paradigm shift in treating cancer, since attention has become more focused on the “biologic passport” of the individual tumor rather than the site of origin of the tumor. The different types of cancer immunotherapies discussed here include biologic modifiers, such as cytokines and vaccines, adoptive cell therapies, oncolytic viruses, and antibodies against immune checkpoint inhibitors, such as the co-inhibitory T-cell receptor PD-1 and one of its ligands, programmed death-ligand 1.

INTRODUCTION

Cancer immunotherapy (CI) is rapidly advancing and can now be considered to be the “fifth pillar” of cancer therapy, joining the ranks of surgery, cytotoxic chemotherapy, radiation, and targeted therapy. The CI which has sparked the most interest involves antibodies to inhibitory immune checkpoint molecules. Although they have produced dramatic results in only a subset of

some malignancies to date^[1], it is difficult to not be very excited about their potential. In a recent meta-analysis of ipilimumab, a monoclonal antibody that targets cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), a type of immune checkpoint receptor or negative regulator of T-cell immune function, more than 20% of patients with metastatic melanoma who received a single round of treatment were alive 10 years later with no evidence of disease^[2]. Before this treatment, the 10-year survival



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rate was less than 10%^[3]. Combination therapy with another immunotherapeutic agent shows even greater promise, as seen when the addition of another checkpoint inhibitor antibody, nivolumab, a monoclonal antibody (mAb) which targets the PD-1 receptor on T-cells, produced a 50% response rate in metastatic melanoma^[4,5]. CI has also shown effectiveness in other types of malignances, and combinations with different treatment modalities (“immuno-oncology”) are also showing remarkable benefits^[6,7].

HISTORY OF OUR KNOWLEDGE OF THE IMMUNE SYSTEM'S ROLE IN CANCER

It has been known for many years that the immune system plays a major role in neoplastic development and control, since patients who are immunosuppressed have a higher risk of cancer, and spontaneous regression of many types of malignant tumors is a rare but well-recognized phenomenon-occurring in approximately 1 in every 60,000 to 100,000 cancer cases^[8-10].

Throughout history there are multiple accounts about tumorous growths regressing or disappearing after an infectious and/or high febrile episode, having been reported from ancient Egypt up to the early 18th century in Europe, but the scientific basis for attempts at modulating the immune system to treat cancer can find its modern roots only in the second half of the 18th century, when histologic confirmation of a malignancy became possible. More than 135 years ago the German physicians Busch^[11] and Fehleisen^[12] independently noticed regression of tumors in cancer patients after accidental infections by erysipelas. In 1868, Busch was the first to intentionally infect a cancer patient with erysipelas and he noticed shrinkage of the malignancy. Fehleisen^[12] repeated this treatment in 1882 and he also eventually identified *Streptococcus pyogenes* as the causative agent of erysipelas^[12,13]. In 1891, an American surgeon, William Coley, of the Bone Tumor Service at Memorial Hospital in New York, followed up on his own independent observation of a long-term regression of a sarcoma after an erysipelas infection by starting a 43-year-old project involving the injection of heat-inactivated bacteria (“Coley’s toxins”) into patients with inoperable cancers^[14]. He reported a significant number of regressions and cures in more than 1,000 patients, many or most with sarcomas, and the method started gaining wide acceptance and notoriety^[15] [Figure 1]. His toxins gradually disappeared from use because of several factors, including his failure to follow good scientific protocols and inability to consistently obtain reproducible results. The development of radiation therapy and chemotherapy also contributed to the loss of interest in using this type of therapy to treat

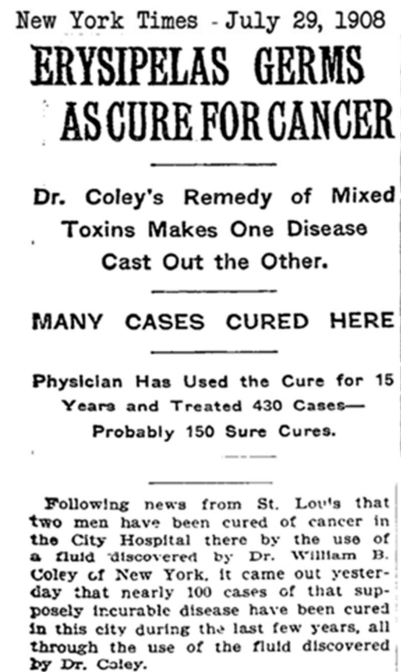


Figure 1: An article in a major U.S. newspaper printed in 1908 reflects the widespread attention given to Coley's toxins

cancer. Nonetheless, after no fewer than five marked shifts in attitude toward CI since the 1890s^[16], Coley's principles have been shown to be correct, and the use of bacteria finally found sound justification in 1976 when Morales *et al.*^[17] established the effectiveness of the bacterium *Bacillus Calmette-Guérin* (BCG) in the treatment of superficial bladder cancer. The underpinnings for this clinical trial include a 1959 study by Old *et al.*^[18] showing the anti-tumor effects of BCG in a mouse model. Besides his work on BCG, Old also performed extensive research on other CI-related topics, and was a discoverer of tumor necrosis factor in 1975^[19]. Due to their foundational discoveries and lifelong dedication to the field, Coley and Old have each been referred to as the “Father of Immunotherapy”, a title which is perhaps best shared.

Even viral infections were believed to have a cancer-suppressive effect as far back as 1904 when George Dock at the University of Michigan described a 42-year-old woman with acute leukemia who experienced a temporary remission after a presumed infection with influenza in 1896^[20]. At the same time, a better understanding of the immune system was being developed, including discoveries about cellular and humoral immunity [Table 1].

THE IMMUNE SYSTEM'S ROLE IN ELIMINATING OR CONTROLLING MALIGNANT CELLS

As a brief background review, the immune system is

Table 1: Timeline of selected key events in modern cancer immunology, 1868-2017

Year	Event
1868	First report of an intentional infection of a cancer patient with erysipelas by Wilhelm Busch, with notable shrinkage of the tumor ^[11]
1883	Elie Metchnikoff publishes a key paper describing phagocytic cells (macrophages) in frogs ^[102] ; awarded Nobel Prize in 1908
1890	Discovery of antibodies (diphtheria and tetanus) by Emil von Behring and Kitasato Shibasaburō ^[103] ; Nobel Prize awarded to von Behring in 1901
1891	William Coley injected his first of many cancer patients with bacteria, reporting tumor regressions in many of them ^[14]
1895	Discovery of complement, by Jules Bordet ^[104] ; awarded Nobel Prize 1919
1897-1901	Paul Ehrlich "Side-chain" theory of antibody specificity (adaptive immunity, autoimmunity) ^[105] ; awarded Nobel Prize in 1908
1901	Serological discovery of blood groups, by Karl Landsteiner ^[106] ; awarded Nobel Prize in 1930
1901-08	Rejection of transplanted tumors in mice, reported by Carl Jensen & Leo Loeb ^[107,108]
1914	Genetic basis for the rejection of transplantable tumors, reported by Clarence Little ^[109]
1909-20	Establishment of inbred strains of mice by Leonell Strong and Clarence Little ^[110]
1948	First report of histocompatibility antigens being the basis for transplant rejection, by P. Gorer, S. Lyman, & G. Snell ^[111] . 1908 Nobel Prize awarded jointly to G. Snell, B. Benacerraf, & J. Dausset in 1980
1955	Natural-Selection Theory of Antibody Formation, first formulated by N. Jerne ^[112] ; awarded Nobel Prize in 1984
1956	Discovery of acquired immunological tolerance by R. Billingham, L. Brent & P. Medawar. Nobel Prize awarded to Medawar & F. Burnet in 1960 ^[113,114]
1957	Immune rejection of transplanted syngeneic tumors (i.e., each tumor is antigenically unique) ^[115] . Reported by Richmond Prehn & Joan Main.
1957	Interferon discovered, described as a factor that conferred the property of viral interference ^[116] , reported by Alick Isaacs and Jean Lindenmann. Its anti-leukemic effect is reported in 1984.
1959	Immune surveillance of cancer theory by Lewis Thomas & F. Macfarlane Burnet ^[30-32]
1959	Chemical structure of antibodies, by Gerald Edelman & Rodney Porter ^[117-120] ; Nobel Prize awarded to both in 1972.
1959	BCG shown to have anti-tumor effects in a mouse model, reported by Lloyd Old, Donald Clark, & Baruj Benacerraf ^[18]
1973	First description of dendritic cells, by Ralph Steinman & Zanvil Cohn ^[121] ; Steinman awarded Nobel Prize in 2011 for discovery of the dendritic cell and its role in adaptive immunity
1974	First reports of the specificity of cell-mediated immunity by Peter Doherty and Rolf Zinkernagel ^[122,123] ; Nobel Prize awarded to both in 1996
1975	Monoclonal antibodies manufactured by George Kohler & Caesar Milstein ^[124,125] ; Nobel Prize awarded to Kohler, Milstein & N. Jerne (for his theoretical contributions) in 1984
1975	Discovery of tumor necrosis factor, reported by Lloyd Old, with Elizabeth Carswell, Robert Kassel, S. Green, N. Fiore, & B. Williamson ^[19]
1975	First description of NK cells on a functional basis according to their ability to lyse tumor cells in the absence of prior stimulation ^[126] . Reported by Ronald Herberman, Myrthel Nunn, Howard Holden, & David H. Lavrin.
1976	Discovery of the genetic principle for generation of antibody diversity, by Susumu Tonegawa ^[127,128] ; awarded Nobel Prize in 1987.
1982	Discovery of the T-cell receptor in 1982, reported by James Allison, B. McIntyre, & D. Bloch ^[101]
1984	First report of interferon response in patients with hairy cell leukemia ^[129]
1991	First report of a human tumor antigen recognized by T-cells, reported by Pierre van der Bruggen, C. Traversari, P. Chomez, <i>et al.</i> ^[36]
1996	Discovery that CTLA-4 blocking antibodies could treat tumors in animal models, reported by Dana Leach, Matthew Krummel & James Allison ^[72]
1998	Discoveries regarding the activation of innate immunity, by R. Medzhitov, P. Preston-Hurlburt, C. Janeway; & B. Beutler; Beutler awarded Nobel Prize in 2011 ^[130,131]
2001	Rag2 ^{-/-} immunodeficient mice, with no B or T cells, show increased susceptibility to spontaneous and carcinogen-induced tumors, reported by V. Shankaran, with L.J. Old, R. Schreiber, <i>et al.</i> ^[132]
2005	Memory T-cells in colorectal tumors shown to predict clinical outcome, reported by F. Pagès, A. Berger, M. Camus <i>et al.</i> ^[133]
2010	First autologous cell-based cancer vaccine (sipuleucel-T) is approved by the FDA for the treatment of metastatic, asymptomatic stage IV prostate cancer ^[43,134]
2010	First successful use of gene-edited T-cells for the treatment of CD19+ hematologic malignancies in humans, reported by W. Qasim, H. Zhan, S. Samarasinghe <i>et al.</i> ^[135]
2011	Anti-CTLA-4 (ipilimumab), is the first inhibitory checkpoint inhibitor (ICI) approved by the FDA for treatment of stage IV melanoma ^[136]
2012	Discovery of the CRISPR/Cas9 system, a simpler and more efficient method of genome editing, reported by J.A. Doudna & E. Charpentier, with M. Jinek, K. Chylinski, I. Fonfara, & M. Hauer ^[49]
2013	First use of CRISPR/Cas9 technique in eukaryotic cells, reported by F. Zhang, with L. Cong, F. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, P. Hsu, X. Wu, W. Jiang, & L. Marraffini ^[50]
2016	A second class of ICIs, anti-PD-1 (pembrolizumab), is approved for the treatment of melanoma ^[137]
2016	First characterization of the role of dendritic cell CTLA-4 in Th-1 immunity, reported by M. Halpert, V. Konduri, D. Liang <i>et al.</i> ^[138]
2016	A third class of ICIs, PD-L1 (atezolizumab), is approved for treatment of bladder cancer ^[139]
2016	First test in humans of CRISPR gene-editing technique for CAR T-cell therapy ^[51]
2017	Phase I/IIa study of an inhibitor of indoleamine 2,3-dioxygenase (IDO1), a non-membrane-attached enzyme with a checkpoint inhibitor function, shows promise ^[95]

Not all of the events listed are discussed in the text, but all are referenced to the pertinent literature

classically considered to be comprised of the innate and adaptive arms, although this is a simplification since these arms have overlapping functions and are intimately related. The innate immune system includes dendritic cells, natural killer cells (NK), macrophages, neutrophils, eosinophils, basophils, and mast cells. Innate immune cells do not require prior stimulation by antigens and act as a first line of defense against foreign antigens. The adaptive immune system includes B lymphocytes, CD4⁺ helper T lymphocytes, and CD8⁺ cytotoxic T lymphocytes (CTLs), and requires formal presentation by antigen-presenting cells (APCs) for its activation^[21]. The adaptive immune system generates antigen-specific T- and B-cell lymphocytes. The immune system is highly variable between individuals but relatively stable over time within a given person^[22].

Each cell is estimated to experience over 20,000 DNA damaging events each day^[23], which are normally repaired by specific DNA repair pathways with no lasting effects^[24]. Cells which are not repaired and which acquire malignant or potentially malignant changes are then usually recognized and killed by the tumor immunosurveillance system. This involves predominantly cell-mediated mechanisms that can differentiate between self and non-self antigens. Since a malignant cell can have more than 11,000 genomic mutations, many new tumor-associated antigens (TAAs) may be expressed^[25]. TAAs include products of mutated proto-oncogenes, tumor suppressor genes, overexpressed or aberrantly expressed proteins, tumor antigens produced by oncogenic viruses, oncofetal antigens, altered glycolipids and glycoproteins, and cell type-specific differentiation antigens. These new TAAs, or fragments thereof, are presented on the cell surfaces with their major histocompatibility complex (MHC) molecules. However, recognition of an antigen-MHC complex by a T-cell antigen receptor is insufficient for the initial activation of naive T-cells, requiring additional costimulatory signals that are provided by the engagement of the CD28 receptor on the T-cell surface with B7 ligand molecules (two of which are CD80 and CD86) on the APCs. This CD28 receptor/B7 ligand combination or “immunological synapse” stimulates the proliferation and function of the T-cells. Many other receptor/ligand combinations are possible between activated T-cells and other cells, including tumor cells, and some of these interactions are inhibitory, such as PD-1/PD-L1 and CTLA-4/B7^[26-29], and are discussed later in this monograph.

Some malignant cells are able to evade the tumor immunosurveillance system by manipulating their own characteristics as well as the cells in their microenvironment to become “successful” tumors;

these evasive mechanisms represent the major area of interest in current CI research. The concept that the immune system is capable of detecting and killing nascent “non-self” malignant cells was first developed by Burnet^[30,31] and Thomas^[32] in their cancer immunosurveillance hypothesis. The concept was not accepted initially but it is now considered a component of cancer immunoediting, whereby the surveillance system can determine or “shape” the immunogenicity of the tumor cells which are not eliminated initially^[33]. The immunoediting process has been formally divided into three main phases: elimination, equilibrium, and escape. The elimination phase refers to the initial damage and possible destruction of tumor cells by the innate immune system, followed by presentation of the tumor antigens in the cellular debris to dendritic cells which then present them to T-cells and thereby create tumor-specific CD4⁺ and CD8⁺ T-cells. These help destroy the remaining tumor cells if elimination is complete. The equilibrium phase occurs when any tumor cells survive the initial elimination attempt but are not able to progress, being maintained in a state of equilibrium with the immune cells. In the escape phase, cancer cells grow and metastasize due to loss of control by the immune system. The cancer cells which are not eliminated and which escape may do this by expressing fewer antigens on their surfaces or even by losing their MHC class I expression^[34]. They may also show the ability to protect themselves from T-cell attack by expressing immune checkpoint (IC) molecules on their surfaces like normal cells; these IC molecules are upregulated by cytokines produced by activated T-cells and are part of a normal negative feedback loop to control excessive tissue damage from inflammation by downregulating or suppressing T-cells^[35].

The dynamic that exists between the immune system and tumor antigens is a phenomenon recognized relatively recently, since it was only in 1991 that van der Bruggen and colleagues first reported the existence of a human tumor antigen recognized by T-cells^[36]. They were able to clone the melanoma antigen-encoding gene (MAGE), which encodes an antigen recognized by cytotoxic T-cells. This provided not only proof that the immune system was capable of seeking and destroying tumor cells but also provided the first identification of a molecular target.

The ability of cancer cells to evade immune destruction has been proposed as the eighth hallmark of cancer^[37]. As noted above, a tumor is able to do this not only by modulating its own cellular characteristics but also by creating its own “tumor microenvironment” by recruiting apparently normal immune cells to help shield it from attack by the immune system. Through the production of various cytokines and chemokines, successful

cancers and their metastatic derivatives are able to generate an immunosuppressive, protumorigenic, and prometastatic microenvironment by recruiting and “training” immune cells, including macrophages, regulatory T-cells, immature myeloid cells (which become “myeloid-derived suppressor cells”), T helper 17 cells, regulatory B cells, and leukocytes^[38]. Even before they metastasize, tumors can influence the systemic environment by altering hematopoiesis as well as the tissue parenchyma of organs at distant sites, thereby forming “pre-metastatic niches”^[39]. While some cancer immunotherapies have had marked successes in manipulating these tumor microenvironments, the loss of MHC class I expression by a tumor represents a major immunotherapy treatment challenge^[40].

The intrinsic immunological ability of an individual to combat cancer has been called the “cancer–immune set point”, and is influenced by a complex set of factors involving the tumor, the host, and environmental factors^[41]. Clinical studies are trying to better characterize these factors to help predict a person’s response to immunotherapy, as discussed in the following paragraphs.

BIOLOGIC MODIFIERS: CYTOKINES AND VACCINES

The term “immunotherapy” encompasses a wide variety of concepts and methods. Older and non-specific immunotherapies include immunostimulatory cytokines such as interleukin-2 (IL-2) and interferon (IFN). L-MTP is a synthetic analogue of a bacterial cell wall that is capable of activating monocytes and macrophages and has had limited success in cancer treatment as reported in other countries; it is not approved by the Food and Drug Administration (FDA) of the USA^[42]. The only vaccine for cancer which has received approval by the FDA is sipuleucel-T for metastatic castrate-resistant prostate carcinoma. Dendritic cells from the patient are exposed to prostatic acid phosphatase and granulocyte-macrophage colony-stimulating factor (GM-CSF) and reinfused into the patient. Treatment results in a 4-month increase in median survival^[43]. Sipuleucel-T is a dendritic cell vaccine, while other types of vaccines employ killed tumor cells or selected tumor antigens, and various vaccines may use microorganisms as vectors for delivery. Vaccine trials using multiple neoantigens specific to an individual patient’s tumor have shown promise in two small early trials^[44]. The goal of all of these tumor vaccines is try to expose patients to those tumor antigens which can provoke an antitumor immune response via the generation of tumor specific antibodies and/or T-cells. Vaccines are one type of biologic response modifier, and BCG was

the first one to be used in cancer therapy, for treatment of bladder carcinoma^[45], where it indirectly increases the expression of tumor antigens after the tumor cells internalize the bacteria. This induces an intense and complex coordinated release of multiple cytokines, including those from T helper 1 cells (IL-2, IL-12, IFN- γ , tumor necrosis factor), as well as those from T helper 2 cells (IL-4, IL-5, IL-6, IL-10). Macrophages, epithelial cells, and fibroblasts contribute IL-8, and T helper 17 cells release IL-17^[46]. This wide array of cytokines then induces antitumor activity mediated by cytotoxic T lymphocytes, natural killer cells, neutrophils, and macrophages.

ONCOLYTIC VIRUSES

Oncolytic viruses are an emerging class of cancer therapeutics which lie at the junction of biologic therapy and immunotherapy. These viruses are genetically modified to lack virulence against normal cells but are able to invade and lyse cancer cells which have sacrificed many of their normal anti-viral cellular defenses in order to amplify their growth potential. Lysis is only one of multiple mechanisms involved in the viral-induced destruction of cancer cells, which undergo further attack by an immune system stimulated by a plethora of tumor antigens released by lytic destruction^[47]. The oncolytic virus which was approved by the FDA in 2015 to treat advanced melanoma is a herpes simplex-1 virus (HSV-1) named “T-VEC”, modified to express GM-CSF which further stimulates proliferation of immune cells. T-VEC is injected directly into areas of melanoma that a surgeon cannot remove. Clinical trials are underway with other oncolytic viruses for treatment of different types of cancer, with some of these trials combined with other types of cancer therapies.

ADOPTIVE CELL THERAPY

Adoptive cell therapy (ACT) is another type of immunotherapy which mostly involves the isolation and *in-vitro* expansion of tumor-specific T-cells, followed by infusion back into the cancer patient. These efforts have also extended to using natural killer cells, since they display rapid and potent immunity to solid tumor metastasis and hematological cancers^[48].

There are many forms of ACT, including those using techniques such as culturing tumor-infiltrating lymphocytes obtained directly from the tumor; isolating and expanding one particular T-cell or clone; or using T-cells that have been engineered *in vitro* to potentially recognize and attack tumors, which technique is known as chimeric antigen receptor T-cell (CAR T-cell) therapy. The revolutionary CRISPR/Cas9 (or “CRISPR” for

short) technique is a much simpler and more efficient method of editing genes than previous methods, and was first reported in 2012^[49]. The acronym stands for “Clustered Regularly Interspaced Short Palindromic Repeats”, which refers to a method normally used by bacteria and archaea for protection against the invading nucleic acids of viruses and plasmids. In 2013, the method was adapted for use in eukaryotic cells^[50], and in late 2016 a group at Szechuan University became the first to use CRISPR-edited cells in humans^[51]. Other similar trials are scheduled to start in 2017 in the United States. Prior vaccination with a cancer vaccine can also be used, in an attempt to “prime” rare tumor-specific T-cells^[52]. Although ACT has produced remarkable results in clinical trials with melanoma and hematologic malignancies as well as with solid cancers, some deaths have occurred in the trial phases secondary to marked cytokine release (“cytokine storm”, or “cytokine release syndrome”) and cerebral edema^[53]. Researchers are still studying other ways of modifying T-cells to treat cancer. Relapsed and refractory B-cell acute lymphoblastic leukemia in pediatric and young adult patients is the first disease to receive approval from the FDA for CAR T-cell therapy, outside of clinical trials^[54].

IMMUNE CHECKPOINTS

In order to ensure that an immune inflammatory response is not constantly activated once foreign or tumor antigens have stimulated a response, multiple controls or “checkpoints” are in place or activated. These checkpoints are mostly represented by T-cell receptor binding to ligands on cells in the surrounding microenvironment, forming immunological synapses which then regulate the functions of the T-cell, which become specialized, or “polarized”, to perform different activities. As noted earlier, initial T-cell activation involves antigen presentation by the MHC molecules on the antigen-presenting cells (APCs) to the corresponding T-cell receptor (TCR) on naive T-cells. The interaction of the costimulatory T-cell receptor CD28 with the B7 ligand is required for full activation, which is tightly regulated or suppressed by inhibitory checkpoint receptor/ligand pairs to avoid collateral damage from autoimmunity^[35].

This type of suppression or induced dysfunctionality of T-cells is also referred to as “T-cell exhaustion” and is different from anergy or senescence. Although it is a mostly reversible physiologic protective mechanism against autoimmunity, the first observation of it was made in mice infected with a chronically persistent strain of lymphocytic choriomeningitis virus^[55]. This T-cell dysfunction was later discovered to exist in multiple other conditions involving persistent antigen exposure by other viruses such as HIV, hepatitis B, and hepatitis

C, or by cancer, thereby allowing these agents to avoid detection and destruction by immune cells^[35,56-59].

More than twenty checkpoint molecule pairs, both co-stimulatory and co-inhibitory, have been discovered, including TIGIT/CD155, LAG-3/MHCII, and TIM3/Gal-9, which are variably expressed not only by T-cells but also by other cells of both myeloid and lymphoid derivation^[56,60]. Some of these molecules are similar to more common or better-known membrane moieties but with important differences: for example, lymphocyte-activated gene-3 (LAG-3) is structurally homologous to CD4 but has a higher binding affinity to MHC class II antigens than CD4. Since these checkpoint molecules are upregulated in suppressed T-cells, they can also be used as markers of “T-cell exhaustion”. The two pairs of inhibitory receptor/ligands which have received the most attention in recent years are cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) receptor with B7 ligand, and programmed cell death protein 1 (PD1) receptor with PD1-L1 ligand. The expression of CTLA-4 receptors on activated effector T-cells and regulatory T-cells was reported in 1987^[61]. CTLA-4 has very high homology to CD28, with a higher competitive binding affinity to B7, causing inhibition of proliferation and IL-2 secretion by T-cells^[62]. PD-1 was cloned in 1992^[63], and its ligands PD-L1 and PD-L2, which are members of the B7 ligand family, were later characterized^[64-66]. Unlike CTLA-4, PD-1 does not interfere with costimulation, but generates signals that prevent phosphorylation of key signaling intermediates in the T-cell, which reduces their activation^[67]. While B7 ligands are expressed by professional APCs (includes dendritic cells, macrophages and B cells), PD-L1 can be expressed on many cell types, including T-cells, epithelial cells, endothelial cells, and tumor cells after exposure to interferon-gamma, produced later in the immune response by activated T cells. PD-L2 is primarily expressed on dendritic cells and monocytes, but can be induced in a wide variety of other immune cells and nonimmune cells^[68].

Since the CTLA-4/B7 synapse acts earlier than the PD1/PDL-1 synapse in the immune response, CTLA-4 is considered the “leader” of the immune checkpoint inhibitors, because it stops potentially autoreactive T-cells at the initial or priming stage of naive T-cell activation, occurring chiefly within lymph nodes^[62,69]. The PD-1/PD-L1 pathway functions during the later effector phase in the periphery and protects the cells found there from T-cell attack, including tumor cells which express PD-L1^[70]. The PD1/PD-L1 pathway represents an adaptive immune resistance mechanism that is exerted by tumor cells in response to endogenous anti-tumor activity^[71].

ANTIBODIES TO IMMUNE CHECKPOINT MOLECULES

In 1996, Leach, Krummel and Allison reported that CTLA-4-blocking monoclonal antibodies (Mabs) could treat tumors in animal models^[72]. These Mabs became known as “immune checkpoint inhibitors” (ICIs), although they should really be called “anti-immune checkpoint inhibitors” if traditional usage in nomenclature could be easily changed. They are receiving much attention recently because they are much less toxic than conventional cancer therapies, are easier to prepare and administer than other types of cancer immunotherapeutics, and have great potential for widespread application.

Mabs that have been approved for clinical use target either PD-1, PD-L1, or CTLA-4, which “block the negative blocking” of the T-cells, with a consequent boost of the immune response against cancer cells. Assays of PD-L1 protein expression by immunohistochemistry are used to determine which tumors would best be treated with an anti-PD-L1 antibody, but it is an imperfect measurement practice because there is lack of standardization of methods and it can sometimes be difficult to differentiate PD-L1-positive tumor cells from the other PD-L1-positive cells in the tumor microenvironment^[73]. Moreover, immunohistochemistry has a lower sensitivity compared to studies measuring PD-L1 mRNA expression^[74].

Anti-PD-1 and anti-PD-L1 antibody treatments are currently the most investigated ICIs because they have shown less severe toxicity, or high-grade “immune-related adverse effects” (irAEs), than anti-CTLA-4 antibody treatments (5-20% compared to 10-40% respectively)^[75-79]. The wide ranges in the percentages of adverse effects reported reflect the variabilities associated with single or multiple drug regimens, dosage levels, and types of malignancies treated. The more common side effects are fatigue (with or without associated endocrinopathies), dermatologic and mucosal toxicities, diarrhea/colitis, and hepatotoxicity. Corticosteroids or other immunomodulators can reverse nearly all of the toxic manifestations of these drugs^[75-78]. Pneumonitis is an uncommon but potentially severe complication, and rarely deaths have occurred^[80]. As the authors noted in one comprehensive review article about management of immunotherapy toxicities, “This new family of dysimmune toxicities remains largely unknown to the broad oncology community^[77]”.

These drugs have powerful effects, as seen when a Phase I trial using an antibody to the CD28 ligand nearly cost the lives of all six healthy volunteers in a British

study when a cytokine storm was provoked, associated with multiorgan failure and resuscitation in the intensive care unit^[81]. Since this reaction occurred after the very first infusion of a dose 500 times smaller than that found safe in animal studies, this study raised awareness of the need to develop better animal models which more closely mimic drug behavior in humans. In addition, there was increased appreciation of the wisdom of restricting the initial testing of a new pharmaceutical to only a few human subjects^[82].

The less toxic antibodies to checkpoint inhibitors have shown a great deal of promise and are now approved by the FDA for six malignancies which are in advanced stages - melanoma, lung cancer, renal cell carcinoma, head and neck cancer, urothelial cancer, and Hodgkin's lymphoma - with many other tumor types being investigated in clinical trials^[83,84]. Some of these trials are using specific antibodies to modulate the function of the more recently discovered inhibitory and co-stimulatory checkpoint molecules.

The immunotherapy/immuno-oncology field has shown such exponential gains in recent times, associated with an accumulation of a dizzying array of complex results arriving from numerous clinical trials, that mechanistic patient studies are necessary to best advance understanding. This is essentially “reverse translational research”, or the opposite of the usual “bench to bedside” philosophy, requiring genetic, phenotypic, functional, and immunohistochemical studies of pre-treatment, on-treatment, and post-treatment tissues. These are necessary in order to generate hypotheses that can then be tested in animal models and thereby provide more precise biologic pathways about tumor immunity and rejection^[85].

Some of the complexities of interacting with the immune system include timing of administration of ICIs to coincide with a high inflammatory microenvironment in the tumor to ensure the presence of many potential tumor-fighting CD8⁺ T-cells^[86,87]. This often correlates with tumor necrosis provoked by prior conventional chemotherapy, and is also related to the number of mutations present in the tumor (“mutational burden”), which is associated with higher antigenicity and correlates positively with response to ICIs^[88]. Lung cancers occurring in smokers have a higher mutational burden and have shown more responsiveness to ICIs^[89]. Microsatellite instability-high colorectal cancers, which tend to have a high-mutation/high-neoantigen load based on owing their genesis to a deficiency in DNA repair, have also been proven to respond well to ICIs^[90,91]. Even the patient's intestinal bacteria needs to be considered, as noted in two recent reports: in one study of patients with metastatic melanoma treated with anti-PD-1 antibody, the diversity

and composition of the gut microbiome differed in responders versus non-responders, with the non-responders showing less diversity and higher abundance of Bacteroidales, while the responders had higher diversity and a higher abundance of Clostridiales^[92]. Another study of patients with metastatic renal cell carcinoma showed faster tumor progression in the patients who had received broad-spectrum antibiotics up to one month before treatment with ICIs^[93].

Not all immune checkpoint or immunomodulatory molecules take the form of a receptor or ligand. Some may be expressed by the cell in a free soluble form, such as indoleamine 2,3-dioxygenase (IDO1), an enzyme produced by some activated macrophages and also overexpressed by many tumors^[94]. The enzyme depletes tryptophan in the microenvironment, with production of the catabolite kynurenine, which harms the cytotoxic T-cells. Phase I/IIa studies of one IDO1 inhibitor show promise^[95].

CONCLUSION

With the development of the field of cancer immunotherapy, the focus of treatment has shifted from treating the disease site to treating the specific tumor biologic characteristics and its interaction with the intrinsic immunological ability or “cancer immune set-point” of the patient to combat the disease. Since the immune system has the capacity to remember and the ability to detect and destroy tumor variants as they emerge, immunotherapy will always possess inherent advantages over other therapies that lack these two key attributes. The challenges ahead are to discover why immunotherapy treatments work so dramatically well in some cancers and in some patients while not at all in others, and how tumors which were once sensitive to treatment can acquire resistance. Specifically, to be effective, cancer immunotherapy needs to find ways to manipulate the immune system in the (probable majority of) patients who show little or no immune response to their tumors, even to the point where the tumor microenvironment is an “immune desert” with no tumor-infiltrating T-cells^[86,87]. Breakthrough discoveries will be necessary to be able to consistently elevate a patient’s cancer immune set point and to recover MHC class I antigens in those tumors that downregulate them. One recent study with large therapeutic and prognostic implications used the new CRISPR technique to reveal multiple mutations in the tumor genes of individual patients who failed immunotherapy^[96]. Some of these identified genes may be associated with loss of tumor antigen expression, while others may involve disturbances in tumor cytokine production or T-cell co-stimulation.

The pharmacoeconomics of these treatments also needs to be considered. The cost of the typical treatment using antibodies to ICIs is near \$150,000 a year. A combination of ipilimumab and nivolumab, approved by the FDA for advanced or inoperable melanoma, has a cost of \$256,000 a year for patients who respond to the treatment^[97]. Some relief may be obtained by the entry of at least some less costly biosimilars, which are biological products that must be “highly similar” to the original reference product, per FDA regulations. Biosimilars are usually made by a different/competing company^[98].

Immunotherapy drugs are now approved for treatment of multiple cancer types either as first-line treatment or when standard first-line treatment has failed. The FDA has recently approved the anti-PD-1 antibody pembrolizumab for the treatment of any unresectable or microsatellite instability-high or mismatch repair-deficient solid tumors that have progressed after prior treatment and who have no other satisfactory treatment options^[99]. This is the first time the agency has approved a cancer treatment based on a common biomarker rather than the location of the body where the tumor originated.

Immunotherapies do not yet represent a panacea in cancer therapy since only a minor subset of some cancers respond to some of these treatments, and it is difficult or impossible to determine precisely who will benefit.

Before finishing this brief review, it is proper to recognize the work of Dr. James Allison of Houston’s MD Anderson Cancer Center in Texas, the winner of the 2015 Lasker Award, since he is the one who discovered the T-cell receptor in 1982 and went on to develop the field of checkpoint blockage, leading to the breakthrough drug ipilimumab^[100,101].

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Topic: How does the prostate cancer microenvironment affect the metastatic process and/or treatment outcome?

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Getting closer to prostate cancer in patients - what scientists should want from clinicians

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ABSTRACT

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For scientists pursuing drug development for prostate cancer, it is critical that an appropriate *ex vivo* or *in vitro* model system is available for study. Cancer research has generally consisted of: (1) finding the means to arrest fast growing cancer cells; or (2) (as a compromise) to slow down the excessive rate of cell growth; or in the best case (3) to kill the cancer cells whilst sparing the surrounding normal tissues. As the knowledge of the biological nature of the cancer cell improves, it has become increasingly apparent that such a simplistic attitude to cancer therapy development or indeed diagnosis is rapidly outdated, and a closer liaison between the clinic and the laboratory studies is more important than ever as the author seeks to target specific gene expression pathways, specific signaling pathways, cancer specific mutations and indeed the interactions between cancer cells and their micro-environment, all of which provide a tremendous potential for novel therapeutic development.

INTRODUCTION

Not all cells within a cancer are fast growing. Indeed a proportion of cells within every tumor are probably quiescent and impervious to drugs targeting cell cycle activity^[1,2]. To accommodate this cellular heterogeneity we really require new tissue mimetic cancer models^[3]. For many scientists, the availability of “off the shelf” cancer cell lines is a facility which they use as a matter of expediency. The reasoning which makes a basic laboratory scientist choose a cancer model are: (1) robust cells which grow

quickly (minimizing the amount of time required to do the experiment); (2) cells which are easy to infect or transfect; and (3) cells which express the gene or the signaling pathway of interest. In relatively few cases has any consideration been made of the stage of disease that a particular cell line represents. The study of “prostate cancer” is actually the study of a number of different diseases^[4-8], for example: (1) low Gleason grade tumors which have a weak capacity to invade; (2) high Gleason grade cancers which remain sensitive to the effect of male sex hormones but which are known to invade at least locally and



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also have the capacity to spread at an early stage of the cancer; and (3) castration resistant disease (after hormone treatment) which has been shown in gene expression and mutational studies^[9-11] to represent a completely different type of cancer. Although castration-resistant prostate cancer shares some trunk or driver mutations^[12], it has developed a completely new mutation sub-set and ultimately the fatal lesion in prostate cancer, which often has a partly or wholly neuroendocrine phenotype and is impervious to most chemotherapy treatments^[13,14]. To simply pick a cell line to represent “prostate cancer” is not sufficient, as is becoming increasingly apparent with our depth of analysis. If we now look back through the online scientific literature, which contains more than half a million references to LNCaP cells together with “prostate cancer treatment” in key words, one must begin to doubt the validity of many of these previous experiments.

There is also the question of timeliness. The castration resistant cancer of the 1980s was very different from that developed after the current generation of androgen therapies. For example, LNCaP was derived from a patient who had failed on the combination drug treatment of Estramustine (estrogen and nitrogen mustard, both hormone suppressive and strongly mutagenic)^[15].

Origins of the LNCaP cell line

The LNCaP cell line was derived (1977) from a 50-year-old Caucasian male patient with stage D prostate cancer, and strong evidence of extraprostatic disease as determined by high serum levels of prostatic acid phosphatase (> 20 times normal). The patient had been treated with oral estrogens, orchiectomy and an aggressive course of estramustine chemotherapy. He also presented with metastases to the bone. At this point a biopsy from the supraclavicular lymph node was taken and cultured in RPMI medium containing 15% fetal calf serum and antibiotics. The cells grew rather slowly, although a single clone emerged with a population doubling time of 24 h, known as the fast growing clone which is now supplied by the ATCC for research purposes.

The cells grow well (from a 107 cell inoculum) in immunocompromised mice to form poorly differentiated adenocarcinomas, and whilst tumor take is better in male compared to female mice, the rate of tumor growth is independent of the sex of the recipient mouse. No distal metastases are found with the parental line unless co-inoculated with fibroblast cells, although highly malignant sublines have subsequently been derived.

LNCaP cells have been in culture for more than 40 years with a hypotetraploid karyotype (84-87 chromosomes) and are genetically unstable. Both cell growth and secreted proteins (such as PSA) are sensitive in a biphasic manner to androgens, but also to estrogens, as the androgen receptor gene carries a mutation (T877A - selected/induced by the estrogenic/mutagenic treatment of the patient?) which broadens the hormone binding capacity of the AR protein.

To overcome the latter property and to render LNCaP more sensitive to antiandrogen drugs, derivatives have been generated by transfection of extra copies of the AR gene (LNCaP-AR). AR low/negative (C4-2) and neuroendocrine derivatives can be readily generated by growth in selective media, specific gene knockout, and application of epigenetic modifiers such as histone deacetylase inhibitors.

It is therefore no surprise that the LNCaP genome is packed with secondary mutations, but also that LNCaP itself has a propensity to grow in estrogen supplemented medium. This is partly due to the original culture/xenografting procedure which is described in the original paper from 1980^[15], often forgotten by current day researchers, who simply treat it as an androgen responsive cell line.

THE FUTURE: TEAM INTEGRATION AND STRATIFIED MEDICINE

The steep and downhill learning curves: beware of the next bandwagon

When developing new anti-androgen receptor drugs, LNCaP has been the preclinical and basic science tool of choice. Given its responsiveness to estrogens and the presence of a mutant androgen receptor, this is perhaps surprising. To develop the new generation of androgen receptor inhibitors such as Enzalutamide, a variant of LNCaP into which extra copies of the androgen receptor gene (in an un-mutated wild type form) had been inserted, was used^[16]. The consequences of extra androgen receptor expression within the cells appeared to make them especially sensitive to the anti-androgen drugs such as Enzalutamide. Does this represent a true reflection of the drug efficacy? It is somehow redesigning the test system to fit the drug. Whilst there is no doubt that Enzalutamide is a powerful and virtually irreversible inhibitor of androgen receptor in the clinic^[17-21], perhaps there are more complexities in real cancer tissues than seen in this now, increasingly artificial experimental cancer model. In our own experiments, LNCaP has proven to be hypersensitive to treatment by Docetaxel compared to every primary

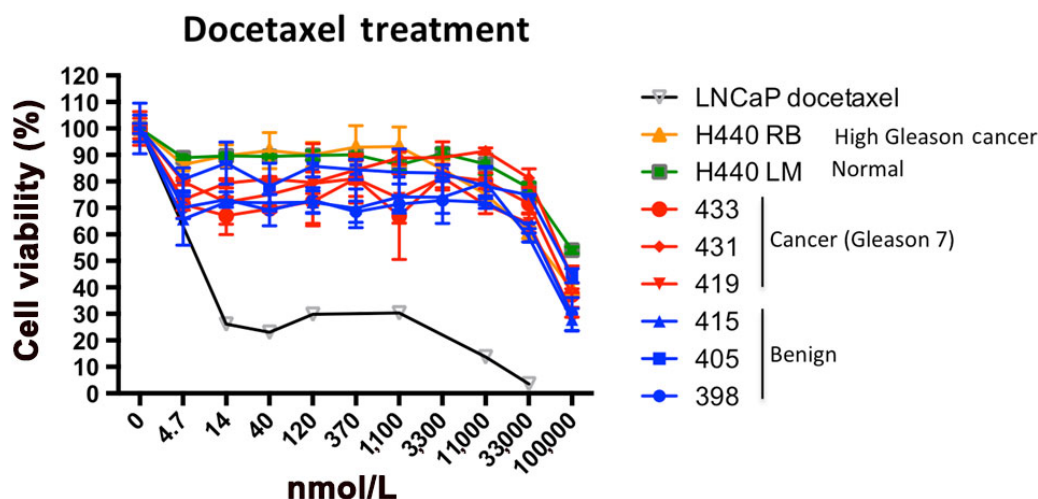


Figure 1: Response of primary patient cultures and the LNCaP cell line to docetaxel treatment. Cells were plated at 5,000 cells per well of 96-well plates, treated with several doses of docetaxel and measured using alamar blue assay at 24 h post-treatment. Primary prostate epithelial cells cultured from patient samples from different disease grades and LNCaP cells were used (Data from Dr. Fiona Frame, University of York)

prostate culture, which we have treated with the same concentration of drug [Figure 1]. Unsurprisingly this is also true for a number of signaling pathway inhibitors, such as inhibition of Akt and PI3 Kinase. LNCaP is null for the PTEN gene, which renders the signaling pathways considerably more active. In most prostate cancer patients the situation is less clear cut with only very advanced prostate cancers having lost both copies of the PTEN gene. Most earlier stage cancers contain a single copy or express the PTEN protein at lower but still biologically active levels, a phenomenon known as haplo-insufficiency. In this case our experiments indicated that LNCaP has inactivated a number of emergency or salvage pathways during the 40 years in which LNCaP has been cultured on a nutrient rich medium of initially 15% and more recently 10% fetal calf serum. These pathways remain active in primary cancers^[22].

The final argument against designer cell lines is that of clonal selection. Prostate cancers are incredibly heterogeneous, containing multiple sub-clones with a restricted number of mutations, but with distinct differentiated states and epigenetic levels of control. To treat all of these with a single drug is over ambitious. However, to derive models such as those previously described, the scientist is reliant on the ability of some or all of these cancer cell types to grow in the laboratory. As a population of cells begins to grow from a cancer, those which initiate growth first are inevitably going to dominate the final culture by means of the exponential growth of cells. Exponential growth means that even within 3-4 days there could be 8 to 16 fold more of a fast growing cell compared to a slower growing cell in the same population. Thus long

established cultures are sometimes representative only of the cells that will grow in the laboratory, which may not be the cells that grow quickly in the patient.

Overcoming clonal bias

Clearly the best method is to develop a closer liaison between clinicians working with patient samples and the scientists themselves. There are however a number of scientific arguments against this. Working with primary samples is time consuming and perceived to be prone to failure. To achieve statistical significance, ironing out the natural patient to patient variation in clinical samples, requires multiple samples far beyond the normal "journal requirements" (currently for at least 2 independent prostate cancer cell lines). How then should those representative cell lines be chosen? Are they selected because the results are consistent with the original hypothesis, or should they cover the same or different prostate cancer phenotypes? One sample each from castration resistant and hormone sensitive cells is by no means statistically significant, however the analysis of 10-12 tumors of a similar Gleason grade in patients with a similar hormone naïve background should provide statistically relevant results, which also can reflect, in their diversity, the patient specific variation we see in responses to many drugs^[23]. Although clonal selection can also be an argument against primary cultures, they at least do have a heterogeneous phenotype (several cell subpopulations are represented), they can be differentiated in 2D culture to give rise to other, more luminal, cell populations and when routinely used at low passages the amount of time-dependent selection pressure is reduced.

Establishing a multidisciplinary team

Thus truly translational research requires an ability to access fresh human cancer tissues, rather than employ the current laboratory “formula” for medical research, where detailed experiments are performed on cell lines, and then (often summarily) tested with a number of patient samples for the same response in formalin fixed tissue for antigen expression. Is this really valid as a critical assay of a longer term treatment for prostate cancers?

Certainly the availability of large numbers of tissue sections in array format should revolutionize the correlative part of such a study, but this resource is not available to all, and is of variable quality between different sources and centers depending on the time of surgery, fixation and processing required to produce a grid of perhaps 96 disease-focused tissue sections. It is also largely dependent on the skill of the histopathologist in obtaining the tumor localized blocks. When fresh unfixed tumor biopsies are required, this becomes just as much a multidisciplinary team effort as the treatment of prostate cancer. It involves the surgeon who removes the

biopsy, the pathologist who decides the area for sampling, the research nurse, technician or tissue procurement officer who takes the samples and finally the research team, who process and analyze the sample (see below). Finally the clinical history of the patient is required to ensure that a particular patient who has been studied intensively is not an anomaly within the general patient population, or indeed has been subject to an initial misdiagnosis.

In many cases, a basic science investigation does not fit within the National Health Service (NHS) ethical framework (and paperwork) in the UK, where the procedure is more often designed with a clinical trial of drug efficacy in patients, from whom tissue/blood samples are required. The elapsed time from application to approval is also rather long and incompatible with some grant deadlines. However, it remains essential to have such approval in place before funding is approved. Again the multidisciplinary team (MDT) approach and closer clinical science liaison is the best approach. Ethical compliance from the very start is not only desirable, but essential now for many grant-giving bodies.

The complexities of tissue processing from tissue biopsies

The next stage in the procedure involves the processing of the tissues from primary samples. Over many years my laboratory has developed the means to fractionate prostate tissues into many constituent cell types. This is illustrated in Figure 2. Although individual populations can for example be studied in isolation for a particular gene expression pattern or a cancer target expression, this may not be the ultimate analytical system in which to determine whether a patient's tumor is indeed susceptible to such treatment. The mere act of dissociating the tissue biopsy could be sufficient to modify gene expression in the short term. For example, the stress of homogenisation and the time taken may well affect individual gene expression patterns/cell types. The loss of infiltrating macrophages and lymphocytes from the tissues is also of concern, given our current knowledge of their influence on not only each other, but also on the whole tumor micro-environment.

Therapeutic development strategies which involve growing cells from prostate tissues in the laboratory are inevitably a compromise. Purification of different cell types and their culture removes micro-environmental influences. The mere induction of cell proliferation within a cell population also induces new patterns of gene expression, as we have recently shown in benign prostatic hyperplasia^[24]. Such a reductionist approach

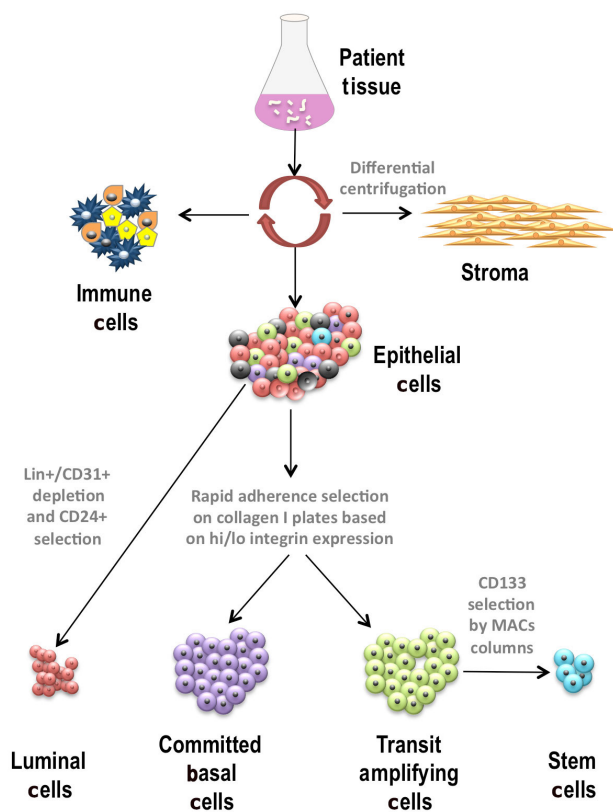


Figure 2: Selection of cells from prostate tissue. Protocols have been developed to extract, enrich and select different cell types from fresh human prostate tissue. The cell types include immune cells, stromal cells and epithelial cells. Luminal cells and basal cells, which include transit amplifying progenitor cells, committed basal cells and stem cells, can be selected

is nevertheless useful and in the long term critical to determine treatment targets, in an easily controlled biological system. At the next level of complexity, the ability to co-culture multiple different populations from primary tissues offers something closer to the original patient, but is confounded by resolution of the individual cell types both during treatment and after treatment. Here the critical step has to be the inclusion of (1) a vehicle or non-treated control and (2) if possible a control of normal tissue from the same region of the prostate from the same patient. The latter remains a very rare occurrence in published accounts of experimental prostate cancer treatments, which often consist of a cancer cell line (or two) and one of the rare normal or non-malignant prostate cell lines (such as PNT2^[25]) but more often involves a primary culture of “normal” prostate which can be purchased commercially^[26]. Any scientist setting out on a similar experimental programme must remember that the culture media on which such primary cultures are maintained is often serum free and of a different calcium content to that required to culture the common prostate cells such as PC3, DU145 and LNCaP. How convincing would such a comparison be in another biological system when the effects of foetal calf serum in growth medium are also so manifold? The entire point of a defined culture medium has been to reduce the reliance on the “black box” effect of serum constituents - which often vary between suppliers, and indeed in batches from the same supplier.

The next level of complexity for treatment studies is the culture of tissue slices on artificial extra cellular matrix, or indeed on collagen sponges^[27]. These have the added advantage of retaining haematological cell infiltrates, and include prostate stroma. However such studies can only be considered as “window” treatment studies over a short period of time. In our own studies of this system, the type of cell which proliferated within the tissue slice was critically dependent on the choice of growth medium. For example, in RPMI medium, growth of the stromal component was apparent whereas in higher calcium medium, the epithelial component would react. Supplementation of the culture medium with cholera toxin removed the stromal proliferation but ultimately resulted in tissue degeneration. A short period of time (3-7 days) is fine, but longer exposure/culture is likely to bias the final output. Tissue slicing strategies often also suffer from the stress imposed upon the tissues at the periphery of the sample. Thus only cells within the centre of the slice are credible candidates for treatment analyses as those on the outside are often too damaged and respond totally differently (as in a wound response) to be considered typical of the intact tissues.

Compensation and redundancy in cell signaling

The transmission of external stimuli to the nuclei of cancer cells has always been considered as a “pathway” by molecular biologists. A better description however is as a network or system. The naïve linear view of such signaling, for example when a growth factor binds to (one of) its receptors on the cell surface, has now been dispelled by current targeted inhibition studies. In a cell line, whose growth has become dependent on supplied growth factors, over perhaps a 20-year period of stress and adaptation in culture, such signaling networks have been degraded: what a cell does not require is frequently down-regulated, and even mutated - a situation which is more prevalent in advanced cancer cells and tissues with DNA replication and repair defects. Examples of the different drug sensitivities of multiple tissue derived cells, and the weak “model” provided by the industry standard LNCaP cells for this are shown in Figure 1. The same was true during the development of the now standard enzalutamide, as discussed earlier^[16].

The relevance of pathway intermediates can be approached by gene knockout (by CRISPR for example) or gene expression knockdown (Si and ShRNA). Such techniques are readily applied to fast growing, clonogenic and easily transfected established cell lines, but their application in primary cultures of more clinical significance is substantially harder. In addition, the “clean” statistics from a couple of cell lines is not applicable in primary tissue-derived material. Primary cultures are often heterogenous (containing normal and malignant cells, as well as cells at different stages of differentiation), but there is also substantial inter-patient variability in the response to genetic manipulation. Therefore, sufficient numbers of patient samples are required to achieve a “consensus” view - if such an endpoint is actually achievable.

The ideal clinical trial from a scientist's viewpoint

The last statement brings me appropriately to the title and aim of the article. If we assume that the ultimate aim of translational research in cancers is to understand what happens in the patient, the MDT for cancer treatment now has to extend beyond the clinic to the scientific researchers. Back in 2010, I asked a genome sequencer, who was tracking oncogenic mutations in prostate cancer, what type of cancer their group needed for their studies. Expecting an answer of “high gleason grade”, or “castration-resistant”, or perhaps “a homogeneous mass”, the reply was actually “a BIG one”. Apparently the quality of material was less important, as the cellular

composition could all be sorted out in the analysis - all that mattered was quantity. Of course we now know that many of these early next-generation RNA/DNA sequencing projects are confounded by cellular heterogeneity or by the unexpectedly high error rate in the sequencing itself^[28]. Sequences of “model systems” reflect the enormous genetic drift (and selection) imposed by decades in cell culture. The quantities of nucleic acids required for the exercise are diminishing monthly: but there is still in my opinion insufficient control of homogeneity and quality. At this level, minor populations would be excluded and mutations in subpopulations perhaps missed altogether. Even single cell sequencing has the inherent bias introduced by comparison to a canonical genome or cancer cell expression pattern^[29]. The individual cells that fail to match this can be excluded as abnormal, unrepresentative or even “normal”. Cell calling will improve, but at present we are working with an imperfect resource, which I believe can only be solved by closer clinical-scientific collaboration.

The perfect requirements for truly translational study of prostate cancer:

1. Provision of fresh human tissues. Most translational projects operate by “confirming” cell lines studies in arrays of archival and fixed human tissues. Perhaps a better model for study should be establishment of hypotheses in primary human tissues and subsequently confirming mechanisms in representative primary cells or cell lines.

Fresh human tissues impose a higher requirement for clinical-science cooperation. In our laboratory we have observed that, for some purposes, tissue which is more than 3 h from biopsy has significantly altered properties, and that storage overnight destroys most of the infiltrating lymphocytes, for example.

2. Transportation of tissues, apart from rapidity, requires a specialist medium. In some laboratories, an enriched cell culture medium containing high calcium and fetal calf serum is used. In addition, prevention of opportunistic infections can be almost eliminated by the presence of anti fungal and antibiotic agents. Transport medium should be relatively neutral and isotonic such as RPMI: to eliminate false growth and differentiation effects of calcium and calf serum. Rapid chilling to 4 °C is advantageous, but not always essential.

Personnel: key members of a clinical team for translational research

When establishing such a team I always stress that each member should do exactly what they are trained to do best [Figure 3]. However, it is important that by regular communication, the individual specialities should understand something of the procedures for obtaining and the subsequent analysis of tissue-derived material. This feeds back to the key principle of co-authorship in ultimate publications in addition to obtaining ethical permission and design of the studies.

The urological surgeon

For benign, normal and organ confined or lymph node biopsies of tissues, the cooperation of the surgeon has been essential. The first priority should be to ensure patient wellbeing, and the provision of fresh tissues should not in any way compromise this. The presence of a research nurse or a junior surgical team member, who has been well briefed, is a major bonus for this procedure (see below) and in fact this person can act as the all-important bridge between the lab and the clinic.

We have also found it essential to plan ahead and to receive an operation list 1-2 weeks before surgery.

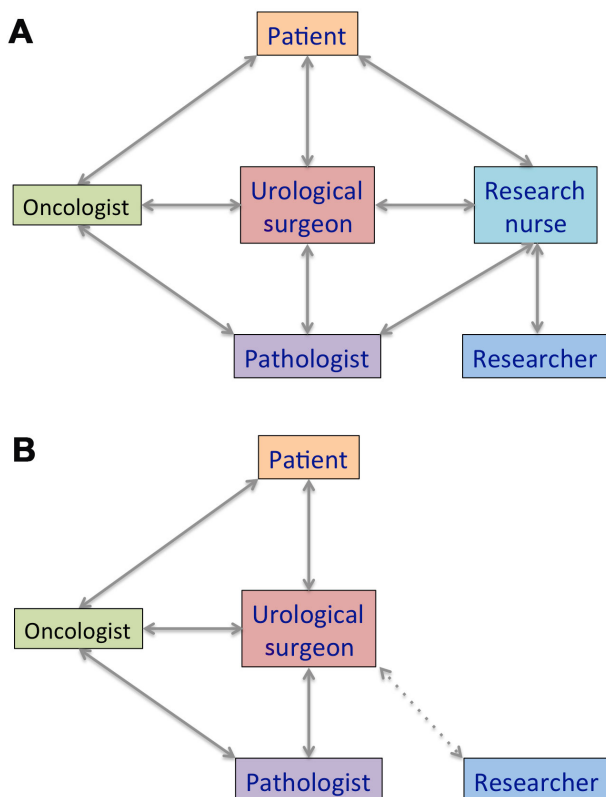


Figure 3: Components of the team required for a clinic to laboratory collaboration. (A) For a Lab-Clinic collaboration to work, collaboration and communication between several willing parties is required; (B) one key member of staff is a research nurse or technician who can liaise between several different members of the team. Without this member, connection between the team members can be more challenging and there is more pressure on the urological surgeon

Rather than take all tissues, we can then decide those which are required to be part of each specific scientific study. Prior warning is also important for provision of patient consent as part of our ethical requirements.

In this sense the surgeon is a vital part of the research team, although he may only feel that he is “supplying tissues”. I prefer to refer to a surgeon’s role as supplying first class tissue biopsies, and such a procedure is clearly worthy of an authorship on any scientific publications. The decision making, review of patient’s details are all essential components in a translational study. As with all co-authors, the collaborating surgical team is sent final drafts (before submission) of relevant academic publications. They may claim not to understand all of the science, but hopefully can pick up on any clinical inaccuracies.

Lastly and perhaps most often neglected is the retention of strictly limited but anonymised clinical information about the patients not only prior to the first operation, but also over time - longer term clinical outcomes are essential when working on biomarkers, for example. Our collaborations have now been in place for a sufficient time to see the recurrence of tumors resected during the first periods of tissue collection: enabling longitudinal studies of tumor progression.

Trainee surgeon or research nurse

Such is the pressure on surgical time, the involvement of another team member radically boosts the quality (and quantity) of materials supplied to the research laboratory. There are two strategies possible, both of which involve the provision of part or full time salary, eligible to be funded by a scientific research grant.

Translational research has often been the result of the needs of a clinical trainee, who exploits the materials in his/her MD or PhD studies. Alternatively, funding a dedicated research “nurse” with an NHS or University employment contract can provide a service by taking postoperative biopsies (guided by the consulting histopathologist, see below). This person can also handle the shipping of materials in insulated packages. We also supply a dedicated refrigerator adjacent to the Operating Facility, which contains aliquoted transport media. The nurse/trainee liaises with both the laboratory and couriers to ensure rapid transfer for tissue processing.

Again this team member’s importance goes beyond tissue provision. It begins by discussion of the research with the patient, when he is first scheduled for surgery. This not only involves supplying the required information sheets and forms for signature, but also the time to explain what will happen, including in our

case withdrawal of 5-10 mL of venous blood. In our experience taking the time, and making personal contact ensures a high participation rate amongst patients, and their close relatives. The information provision is key both before and after surgery, and I strive to thank all of the contributing patients in any press releases and in journal paper acknowledgements. We are frequently asked on the regular visits to my laboratories by patient support groups, “Could that culture be from ‘my’ prostate cancer”.

Urological histopathology

As discussed earlier in this review, there remains an enormous disconnection between the study of prostate cancer in a few cell lines, and the disease in real patients, as provided by histopathology analysis prior to surgery: decision making about treatment options. Where surgery is selected, then the analytical role of the pathologist does not end at this point. Firstly they must regularly review the post-operative biopsy procedure. Our intention is always to do nothing to harm the histological analysis, performed to confirm the initial biopsy based treatment decision. This secondary analysis can on occasion differ from the pre-operative one, and for scientific purposes it is really useful to both mark the research biopsy location (using inks) and/or to repair the sample location with a compatible glue filler. In both cases further analysis will confirm the precise section of tissue under scientific study - particularly important in a heterogeneous tumor such as prostate cancer.

Pathology also has a further role: to confirm that the patient matched “normal” tissue biopsies we take, when the tumor seems confined and relatively homogeneous, was indeed from a normal region of the prostate. For this approach to work, there is a certain amount of faith and extra effort from the science laboratory. On a number of occasions a normal or tumor biopsy has turned out to be incorrectly diagnosed, only after extensive processing [Figure 2] according to the histopathology analysis. The tissue is then relabeled, or even removed from the study as unreliable.

Clinical and medical oncologists

Once a prostate cancer patient has relapsed, or has chosen radiotherapy/brachytherapy, they are treated by oncologists. At this point a biopsy (from the patient rather than a post-operative specimen) is more difficult to justify ethically. However, this is the very population that we need to understand more - and to learn how to treat. We are now discovering that the application of chemotherapy/radiotherapy together with hormone treatment at an earlier stage in prostate cancer disease progression, provides startling

improvements in survival. We will still be unable to determine which patients will benefit, without detailed longitudinal study of clinical tissues. Artificial cell line models of “progression” help little in this regard. I firmly believe that the future of cancer treatment lies in a patient/tumor-specific strategy, and without the clinical material to back this up, from both an early stage in the treatment cycle, and after the almost inevitable relapse, we are limiting our understanding of the disease. Every man appears to be different in his responses, although there are some overarching changes in response to therapy. The “fatal lesion” provided at rapid autopsy has been a goal of some major centers in the USA. This is an expensive exercise, which often lacks both pre-treatment normal tissues (chemotherapy affects all tissues in the patient, so a blood draw of lymphocytes from a patient after extensive chemotherapy will reflect the populations which survived the treatment) and the treatment naïve cancers as comparators. The depth of analysis of which we are now capable, simply demands better tissues to be used to their fullest capacity. It is these very tumor biopsies, which scientists really require from the oncology community, not just from patients referred to large research centers, but the “every prostate cancer patient” samples, from truly standard of care environments.

CONCLUSION

For almost 40 years, a limited set of established prostate cancer cell lines have dominated basic research in prostate cancers. Whilst they retain a number of key properties of the cancer in patients, they represent prostate cancer in an era before androgen therapies and targeted radiotherapy. In addition, the cells from LNCaP, DU145 and PC3 have been passaged repeatedly in different growth media and in immune-compromised mouse hosts. To study prostate cancer in the current decade we require models that represent contemporary disease.

By establishing a collaboration with the clinic, basic scientists can begin to ask the correct questions, whilst retaining the capacity to test mechanisms of action or hypotheses in appropriate established cell lines. However, we should be framing our hypotheses in actual cancers, either in human tissue biopsies or primary cell cultures (or both). Using multiple primary cultures or biopsies is the equivalent of an *in vitro* clinical trial for new drugs. Only by embracing the heterogeneity of the prostate cancer patient population can we begin to approach personalized, effective cancer medicine with an ultimate goal of long-term treatments for men with prostate cancer.

DECLARATIONS

Authors' contributions

N.J. Maitland contributed solely to the paper.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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Topic: How does the prostate cancer microenvironment affect the metastatic process and/or treatment outcome?

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Getting better at treating prostate cancer: what clinicians should want from scientists

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ABSTRACT

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If the treatment landscape for prostate cancer is to be transformed, clinicians and scientists must work together ever more closely. Prostate cancer defeats physicians when patients are not accurately stratified according to patients' risk of dying of disease, when the effects of tumor heterogeneity are insufficiently understood, and when attempts at therapy by clinicians spur further disease evolution and the emergence of new resistance mechanisms. At the same time, clinicians' over-treat men who in reality do not need it, and some of those men needlessly suffer long term side effects as a result. This commentary is aimed at stimulating debate about how we as clinicians and scientists can assist one another and improve our knowledge to the benefit of patients dying from metastatic disease.

WHAT DO CLINICIANS WANT TO KNOW?

"Is cure possible? Is cure necessary? Is cure possible only when it is not necessary?" This - now almost legendary - quote by the late American Urologist Willett Whitmore neatly sums up the entire clinical dilemma that is prostate cancer^[1]. The concept of "overdiagnosis" and "over treatment" as it relates to early prostate cancer is now widely accepted. One commonly used and useful, though scientifically imprecise, analogy, when talking to patients is that prostate cancers can either be "tigers" or "pussy cats".

In a brief survey of clinicians in the UK National Cancer Research Institute's prostate cancer Clinical Studies Group (Mason, unpublished), the distinction between the two was the most frequent item on the "wish list" that these clinicians cited. Conversely, for patients with metastatic prostate cancer, the most common cause of death - by some margin - is due to prostate cancer^[2]. For patients with "significant" disease, and particularly those with metastatic disease, cure is currently virtually impossible, and there is an urgent imperative to improve treatment. The oft-repeated platitude that a man is "more likely to die with his prostate cancer than of it" is completely inappropriate for someone



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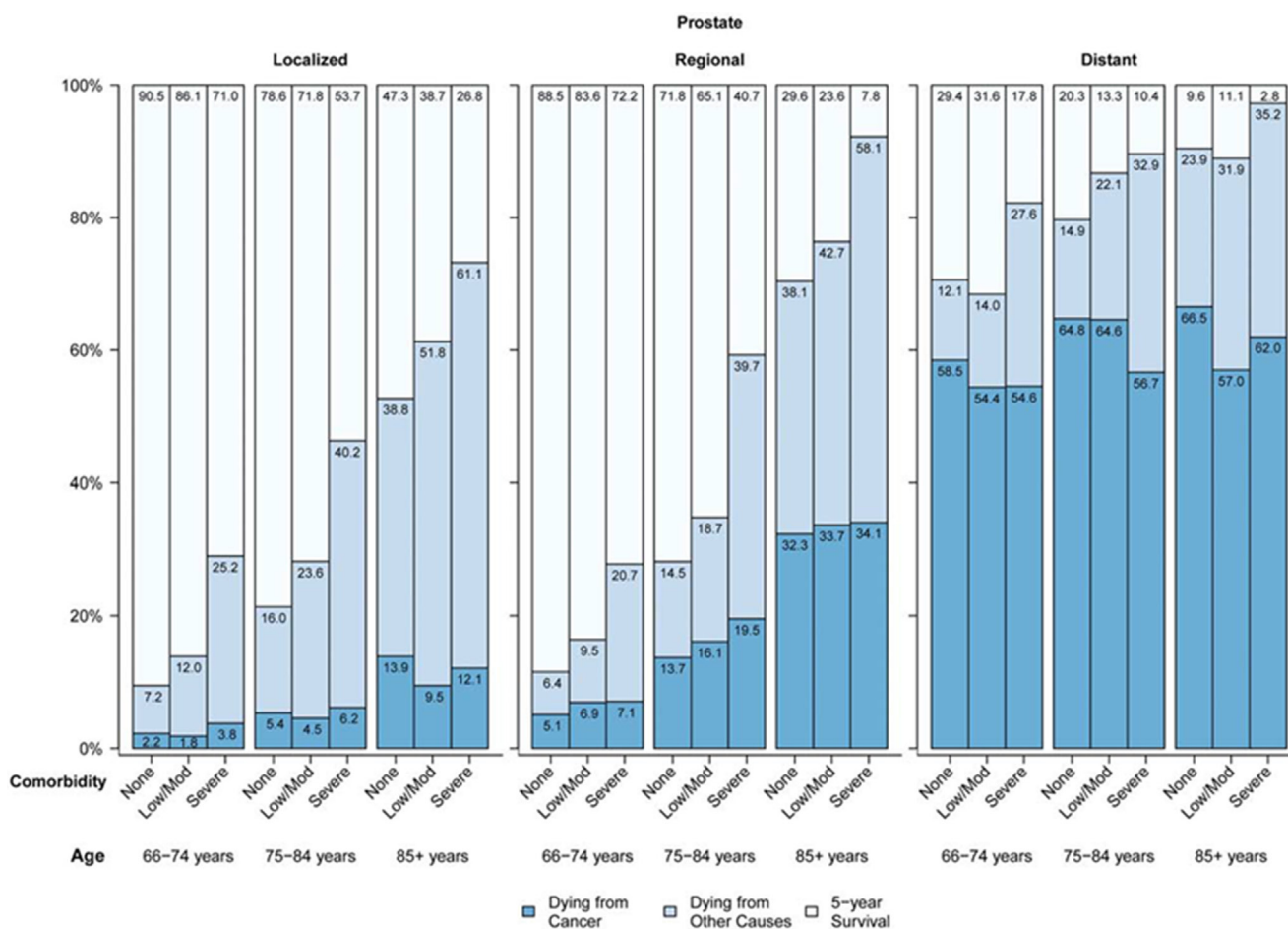


Figure 1: Probabilities of dying from cancer, dying from other causes, and survival are stratified by stage, comorbidity status, and age among men who were diagnosed with prostate cancer between 1999 and 2005. Mod indicates moderate. (Reprinted with permission from Edwards *et al.*^[3])

with metastatic disease as illustrated in Figure 1^[3]. For advanced (metastatic) disease, some form of hormone therapy, or more properly, androgen deprivation therapy (ADT), has remained the cornerstone of treatment. Usually this is given as luteinizing hormone releasing hormone (LHRH)-agonist injections, which decrease testosterone levels by virtue of their strong affinity for the LHRH receptors in the pituitary, preventing native LHRH from binding. More recently, other approaches have been developed, including orally administered drugs which bind antagonistically to the androgen receptor.

LOCALISED PROSTATE CANCER - WHO NEEDS TREATMENT, WHO CAN BE TREATED?

Treatment for “early” prostate cancer, that is, cancer confined to the prostate and entirely within the gland, tends to fall into three main categories: surgery, radiotherapy, or active surveillance. The last of these

deserves some explanation; its philosophy is based on the assumption that if a patient harbours an indolent cancer (a “pussy cat”), it would be safe to monitor him carefully, but to defer curative treatment until and unless there is evidence that his disease is progressing. In the absence of firm evidence, there has always been a tendency for specialists to recommend their own treatment modality to a patient^[4]. Efforts to establish which of the two major options - surgery or radiotherapy - is superior were unsuccessful for decades, and in the vacuum created by the lack of evidence, unsubstantiated opinion was present in abundance. However, this has changed recently with the publication of the first results of the UK ProtecT trial. In this trial, 1,643 patients with early prostate cancer were randomly allocated to treatment with surgery, radiotherapy, or active monitoring (a slightly different approach to active surveillance in that in the latter, often includes a re-biopsy of the prostate after a few years^[5-7]). After a median follow-up time of 10 years, the trial allows clinicians to make several important

observations:

1. Very few patients, of the sort selected for this trial, die of prostate cancer, a least over a 10 year period. It should be stressed that the patients in this trial had early, localised disease, apparently confined to the prostate (categorised as stage cT1-2, N0, M0). Prostate cancer-specific survival rates in all 3 arms of the trial were 99%.
2. The outcomes after surgery and radiotherapy were the same, and both treatments were roughly equivalent in the degree to which their side effects affected quality of life.
3. However, more patients managed by active monitoring suffered progression of their disease, including the subsequent development of metastatic disease, though this has not, yet, translated into a worsening of their 10-year overall survival rate. It should also be stressed that, although the numbers progressing after active monitoring were double the numbers after surgery or radiotherapy, the absolute excess was only of the order of 4%.

Looking at the patients who died of prostate cancer, one might reasonably expect to have been able to pick them out retrospectively, based on the conventional clinical parameters of tumor stage, prostate-specific antigen level, and Gleason grade. Unfortunately, such complacency would be misplaced. For example, of 17 patients who died of prostate cancer, 8 had Gleason scores of 6 at diagnosis, and 9 had scores > 7. The numbers are very small, and some patients with apparently Gleason 6 could have had more aggressive tumors missed due to sampling errors, some of which might have been identified on modern imaging such as multi parametric magnetic resonance imaging, which among other things is capable of detecting anterior tumors that might not have been biopsied in this cohort. Even so, it seems inconceivable that these clinical parameters, which we use to stratify patients into “low”, “intermediate”, and “high” risk groups, are sufficient to enable us to determine which patients with early prostate cancer need treatment, and which ones do not. This is a major, and urgent clinical priority - solving it would, among other things, revolutionise the approach to prostate cancer screening. As described by Maitland in an accompanying answer in this themed issue, the scientific answer to the question of how to distinguish tigers and pussy cats will not come from cell lines, but will require a combination of biobanking of tissues from patients with early prostate cancer, combined with meticulous collection of associated clinical outcome data. Without the latter, the former are

rendered relatively meaningless in this context.

Turning to a different category of prostate cancer, locally advanced disease (where the cancer has spread beyond the capsule of the gland, or into the adjacent seminal vesicles, but no metastatic spread), the prevailing clinical bias was different. Early studies had already shown that, in the context of “old fashioned” radiotherapy, outcomes were less good than for localized disease. In retrospect, many patients who were then labelled as “locally advanced” might today be recognised as having still more advanced disease. The pivotal study by the European Organisation for Research and Treatment of Cancer (EORTC) showed that the addition of ADT to radiotherapy substantially improved survival^[8], but it left an open question about the role of radiotherapy. Nihilists argued that patients with locally advanced disease actually had occult metastatic disease, and that the important modality was the ADT. This was refuted in two randomised trials, of similar design, in which patients with - predominantly - locally advanced disease (some had high risk localised disease) were randomly allocated to ADT alone, or to ADT plus radiotherapy^[9,10]. These trials showed unequivocally that radiotherapy - a locally directed, potentially curative treatment, improved survival. This probably means that some patients with locally advanced disease can be cured with local treatment. Moreover it means that as a group they do, indeed, “need” to be cured - but we should not forget that other explanations leading to improved survival without “cure” are not impossible.

After more than two decades, we can begin to answer Whitmore's questions: for patients with early prostate cancer, cure is apparently not necessary in many cases, at least over a 10-year period. Our dilemma is now that, though we know that this does not apply to all such men, we do not know how to identify the all-important minority of such men who do need treatment. Two other studies, previously published, have randomised patients to surgery, or to “watchful waiting”^[11,12]. The Swedish study reported improved survival with surgery, but the benefits appear to be restricted to patients under 65 years of age. The American study showed no evidence of a survival benefit overall, though suggested some benefit in some men in a higher risk category. In contrast, the studies of locally advanced disease not only show that this category of disease is both life-threatening and yet curable, but they also point to a category of disease which is deserving of more basic scientific attention than perhaps it has had. All these studies carry the same implication: the need for better biomarkers to enable us better to stratify patients. To test this

hypothesis a lab programme which compared tissue from early disease with those from locally advanced disease would surely bear fruit?

WHAT HAVE WE LEARNED FROM CLINICAL TRIALS ABOUT LIFE-THREATENING PROSTATE CANCER?

Generations of oncologists and urologists in training were taught that advanced prostate cancer was characterised by a phase during which the disease would respond to hormone therapy of some sort, an observation that dates back over 70 years^[13]. After a period, which in the case of metastatic disease might have been only of the order of 18 months, the disease progressed, and it was perfectly reasonable to ascribe the label “hormone resistant” to this latter phase. A “favourite” question that clinicians want to ask of their scientific colleagues is why this should be, and what might be the mechanism of disease progression after first line treatment with ADT. The multitude of explanations seem to fall into two categories: one in which some sort of acquired hormone insensitivity emerges, probably as a result of additional mutations, or other changes in key molecules such as the androgen receptor^[14]. An alternative possibility is that disease progression results from the clonal expansion of a subgroup of cells, present at the time of the initial ADT, but insensitive to treatment *ab initio*. Support for the latter possibility comes from a randomised trial conducted by the EORTC, in which patients, who were not fit enough to receive curative therapy, were planned to commence long-term ADT and were randomised between immediate therapy, and treatment delayed until further disease progression^[15]. There was no difference in prostate cancer mortality, though there was some improvement in mortality from any cause (the reasons for this are still debated). However, strikingly, the time course to the onset of disease progression after first line ADT, was identical, irrespective of whether the ADT was given immediately, or delayed^[16]. Why might the time at which so-called “hormone resistant” disease is detectable be independent of when ADT was given? Almost the only explanation, if the findings are generalisable, is that resistant disease has emerged from a resistant sub-population that was present at the time of the initial therapy. Is this true? We need our scientists to answer this question.

Of the novel anti-androgens described above, one in particular deserves mention. Abiraterone acetate is an inhibitor of androgen synthesis, via dual inhibition of the 17 α -hydroxylase/C17,20-lyase enzymes, and it reduces testosterone levels in untreated men^[17]. For

some years, this drug was, effectively, put “back on the shelf”, because it was not obvious what advantages it might offer compared to existing anti-androgens such as flutamide, although the mechanism of action is different; flutamide is a competitive blocker of the AR, while abiraterone inhibits androgen synthesis. Part of the reason for the clinical uncertainty was that in the late 1990s, when this decision was made, it was not appreciated that prostate cancer cells contained low levels of androgen, even in advanced cases, and that they were even capable of synthesising their own androgen^[18]. Once this was recognised, there was a new rationale for testing abiraterone in patients progressing after firstline ADT. This was done in two pivotal randomised trials, comparing abiraterone with placebo and showing unequivocally that abiraterone improved survival^[19,20]. As well as the obvious clinical benefits, these studies confirmed, and extended the initial laboratory observations; prostate cancer growth, even in advanced cases, remains driven by the androgen receptor. Mutations in the AR may allow cancer cells to respond to minutes levels of androgen, to different ligands, or even to be ligand-independent, but at its heart, advanced prostate cancer is anything but “hormone-resistant” - if anything, it is often “hormone super-sensitive”. This finding drove the recent change in nomenclature, from “hormone-resistant” to “castrate refractory”, a term which we must acknowledge is hated by our patients. We must also remember, though, that the survival benefits in these advanced patients are modest- of the order of a few months’ only, and that disease progression after abiraterone (and similarly after novel and more potent AR blockers such as enzalutamide) is inevitable^[21,22]. The scientific imperative for clinicians is to understand what other pathways co-operate with AR-mediated signalling to drive subsequent disease progression.

One way to overcome the complex effects of multiple, diverse, and - within a tumor - heterogeneous mutations is to treat patients earlier in the course of their disease, and this was the thinking behind the STAMPEDE trial, which tests a number of additional therapies, given alongside first-line ADT. This has already borne fruit, with chemotherapy using docetaxel being recognised as the new standard of care, in combination with ADT, for patients with metastatic disease who have not yet had long-term hormone therapy, following reports from the STAMPEDE and CHAARTED trials that docetaxel given at this time improved overall survival with a 25% reduction in the odds of death^[2,23]. Results from the addition of abiraterone to ADT in the STAMPEDE trial, and also in a second trial called LATITUDE, are expected imminently. Many questions arise from these studies: what is the mechanism of the benefit showed by docetaxel? Which patients benefit, as surely not all

patients do so? Are there other agents which might be further combined?

HOW CAN OUR SCIENTISTS HELP US TO BETTER TREAT OUR PATIENTS?

If we are to transform the treatment landscape for prostate cancer, clinicians and scientists must work together ever more closely. Prostate cancer defeats us when we do not accurately stratify patients according to their risk of dying of disease, when we fail to overcome the effects of tumor heterogeneity, and when our attempts at therapy spur further disease evolution and the emergence of new resistance mechanisms. At the same time, we over-treat men who in reality do not need it, and some of those men needlessly suffer long term side effects as a result. We have all recognised the need for the development of better biomarkers that will characterise disease states. I suggest that as clinicians we have a duty to help our scientific colleagues, especially focusing our efforts on several areas:

1. The various and peculiar clinical phenomena which we observe in our patients, through clinical trials, and clinical observations. Another critical observation has been the emergence of new patterns of disease, maybe following the selection pressures on tumor cells resulting from more diverse and novel therapies.
2. The provision of tissue and blood samples in a meaningful way. Samples from patients are usually characterised in the crude terms that we use in the clinic; but, for localised disease, what do the terms “low risk”, “intermediate risk” and “high risk” actually mean? The data from the ProtecT trial, though from very small numbers of dying patients, argue that these terms are no guarantee that scientists will really be studying cells that are indolent, or aggressive, if they use samples based on these labels. The best we can say is that tumors that are “high risk” are more likely to harbour cells with metastatic potential than say tumors that are “low risk”. We know that prostate cancers are often multifocal, and heterogeneous; how do we overcome this? Perhaps circulating tumor products, including but not restricted to circulating tumor DNA, will in time give some sort of precis of the profile of a tumor population. What about tumor evolution? This would argue for repeated sampling in order to give a longitudinal profile of tumor behaviour. This does, however, carry some significant implications

for patients; biopsies from metastatic sites may need special procedures such as computed tomography-guided biopsies; they may be unpleasant - biopsy in bone is notoriously painful and may even require hospitalisation or a general anaesthetic, especially, if multiple sites are to be biopsied at the same time. As well as the ethical implications, the resource implications for the NHS are far from trivial.

3. Prostate cancer therapy, as with other types of cancer, must evolve from an era of empiricism, to the era of precision medicine^[24]. The utopian vision, whereby a clinical sample somehow gets to the lab, and a subsequent analysis leads to a report that precisely determines the required treatment, will be difficult at best and maybe impossible to fully realise. It may not be helped if the laboratory analysis is based only on a random sample from a primary tumor, maybe taken years before the onset of metastatic disease, which is the objective of the study, though when such samples yield cells with the characteristics of stem cells, the insights can be striking^[25]. Nonetheless, we may need to grapple with the practical, ethical, and clinical challenges posed by metastatic biopsies - and maybe not just once, but repeated during the course of a patient's illness, in order to get a profile of their tumor that reflects the current status at a time when therapeutic decisions are being made. If we can, in the future, safely and reliably inhibit multiple signalling pathways in tumor cells, then a more aggressive clinical approach to tumor sampling might be justified.

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Authors' contributions

M. Mason conceived the idea and wrote the manuscript.

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There are no conflicts of interest.

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Not applicable.

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Topic: How does the prostate cancer microenvironment affect the metastatic process and/or treatment outcome?

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HOX transcription factors and the prostate tumor microenvironment

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ABSTRACT

It is now well established that the tumor microenvironment plays an essential role in the survival, growth, invasion, and spread of cancer through the regulation of angiogenesis and localized immune responses. This review examines the role of the *HOX* genes, which encode a family of homeodomain-containing transcription factors, in the interaction between prostate tumors and their microenvironment. Previous studies have established that *HOX* genes have an important function in prostate cancer cell survival *in vitro* and *in vivo*, but there is also evidence that *HOX* proteins regulate the expression of genes in the cancer cell that influence the tumor microenvironment, and that cells in the microenvironment likewise express *HOX* genes that confer a tumor-supportive function. Here we provide an overview of these studies that, taken together, indicate that the *HOX* genes help mediate cross talk between prostate tumors and their microenvironment.

INTRODUCTION

In addition to cancer cells, tumor tissue contains a variety of host cells, extracellular matrix components, and secreted proteins that together constitute the tumor microenvironment^[1]. Crosstalk between the tumor and its microenvironment has an important role in tumor development, including the recruitment of immune cells and vascular cells, both of which can have profound effects on the survival and spread of the tumor and are therefore targets for cancer therapy^[2-4]. In this review, we consider the role of the

HOX family of transcription factors in the interaction between prostate tumors and their microenvironment.

THE HOX GENES

Early embryonic development is characterized by a number of overlapping signaling events that give rise to stable transcriptional states and these in turn confer specific identities at both the cellular and tissue level. Many of the transcription factors that are responsible for regulating embryonic development were originally characterized by the distinct phenotypes caused by



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mutations in either their reading frame or regulatory regions, and one of the most notable examples of this are the *HOX* genes^[5]. The *HOX* genes encode transcription factors that are characterized at the protein level by a highly conserved DNA-binding domain, known as the homeodomain, and their expression defines the identity of cells primarily along the anterior to posterior axis of the embryo, both in the main body and within organs and appendages^[6]. Mammals have 39 *HOX* genes that are organized in 4 distinct chromosomal clusters named A, B, C, and D. The *HOX* genes are named on the basis of which cluster they are found in, and their position within the cluster. Thus for example *HOXB1* is the 3' most member of the *HOXB* cluster, and its immediate 5' neighbor is consequently named *HOXB2*^[7]. The clusters arose through multiple duplication events during the evolution of vertebrates, and consequently *HOX* genes at equivalent positions within each cluster (e.g. *HOXA1*, *HOXB1*, *HOXC1*, and *HOXD1*) share high levels of sequence identity beyond the conserved homeodomain region, and are referred to as paralogues^[5]. The sharing of enhancer regions within clusters confers unusual regulatory properties on *HOX* genes, whereby the 3' members are expressed earlier in development (temporal collinearity) and more anteriorly (spatial collinearity) than their 5' neighbors^[8].

HOX proteins can bind as monomers to DNA, although the affinity and specificity of binding are considerably increased through an interaction with other homeodomain-containing transcription factors including Pre-B-cell Leukemia Homeobox (PBX) and Myeloid Ecotropic Viral Integration Site 1 Homolog (MEIS) proteins^[9]. Of these, PBX can bind to *HOX* proteins from paralogue groups 1-11^[10-12], whilst MEIS binds to *HOX*9-13 proteins^[13]. Despite this increased binding specificity, *HOX* proteins exhibit high levels of functional redundancy in some contexts due to extensive sequence identity between paralogue group members and 3' and 5' neighbors^[14].

HOX gene expression generally reduces before birth and many adult cells show either low levels of expression, or no expression. Exceptions include cells that maintain proliferative capacity in the adult, for example stem cells, and most notably hematopoietic stem cells (HSCs), which are dependent on the continued expression of *HOXB4* for proliferation^[15]. The subsequent differentiation of HSCs along different lineages and ultimately to mature blood cells is also dependent on distinct patterns of *HOX* gene expression^[16]. Other adult processes that are known to be at least partly dependent on *HOX* genes

include the menstrual cycle^[17] and the differentiation of mesenchymal stem cells^[18]. Over the last 20 years it has become increasingly clear that *HOX* genes are also very highly dysregulated, and usually strongly over expressed in a wide range of haematological and solid malignancies compared to the cells from which these cancers originate^[19,20]. The *HOX* genes have multiple functions in cancer, and can act both as tumor suppressors and oncogenes. Examples of the former include *HOXA5*, which can promote expression of the p53 tumor suppressor protein^[21], and *HOXC12*, which promotes cellular differentiation in follicular lymphoma^[22]. However, the majority of reports indicate that *HOX* genes have a pro-oncogenic role, including functions that support tumor growth and invasion such as angiogenesis, metastasis, and immune evasion^[23]. At the cellular level, a generalized role for many *HOX* proteins in cancer appears to be to prevent apoptosis by inhibiting *cFos*^[24-27] and dual specificity protease 1 (*DUSP1*) expression^[26,28,29]. *DUSP1* is a tumour suppressor gene^[30], and whilst *cFos* is generally considered to be a proto-oncogene, *cFos* protein can also induce apoptosis through the activation of the cell death ligand, *FAS1*^[31-35]. Additional cellular functions of individual *HOX* proteins include DNA repair^[36] and the regulation of the cell cycle^[37]. It has also become apparent that the *HOX* genes function to modify the tumour microenvironment, and it is this aspect of their biology that we focus on here.

HOX GENES IN PROSTATE CANCER

The role of *HOX* genes in prostate cancer has in general been more extensively studied than for other solid malignancies. *HOXC4*, *HOXC5*, *HOXC6*, and *HOXC8* have all been found to be highly expressed in lymph node metastases^[38], and *HOXC6* and *HOXC8* overexpression has also been demonstrated in primary tumors^[25]. *HOXC8* expression was also shown to be higher in tumors with a higher Gleason score^[39]. Of these 4 *HOX* genes, *HOXC6* is reported to be the most highly upregulated in primary, metastasized, and castrate-resistant prostate cancer, and the presence of *HOXC6* RNA in urine might be a diagnostic marker for prostate cancer and a potential monitoring tool for disease progression^[40], and was shown to distinguish between high and low grade prostate tumors with a very high specificity when used in conjugation with a second urinary marker, *DLX1*^[41]. In addition, disrupting the interaction between *HOX* proteins and their PBX cofactor using the competitive antagonist HXR9^[23] causes apoptotic cell death in the prostate cancer-derived cell lines LnCaP, DU145, and PC3, and was shown to block the growth of PC3 tumors in a mouse xenograft model^[25].

The most extensively studied *HOX* gene in prostate cancer is *HOXB13* due to its apparent role in androgen sensitivity. It has been shown to be highly expressed in androgen receptor (AR) positive prostate cancer-derived cell lines, but only at a very low level in AR negative cell lines^[42,43], and to be strongly expressed in hormone-refractory tumors after initial treatment^[44]. Furthermore, mutations in *HOXB13* are associated with an increased risk of prostate cancer. The G84E variant was found to significantly increase the risk of hereditary prostate cancer^[45], and was present in around 5% of families with at least one affected member^[46]. A second variant, G135E was found to be associated with an increased risk of prostate cancer in Chinese men^[47]. At the cellular level the functional significance of these variants remains unclear; for example, *HOXB13* G84E was not found to result in an appreciably different phenotype to the wild type gene when expressed in PNT2 cells^[48]. However, a clear mechanistic basis for the pro-oncogenic role of *HOXB13* has arisen over the last few years [Figure 1]. *HOXB13* protein can function both as a repressor and activator of transcription. It represses the p21WAF1/CIP1 (*p21*) tumor suppressor gene, which can block

androgen-stimulated cell proliferation^[49], and has also been shown to bind directly to the enhancer region of the *RFX6* gene, the product of which inhibits the proliferation, migration, and invasion of prostate cancer cells^[50]. *HOXB13* additionally represses prostate derived Ets factor (*PDEF*) expression, which in turn blocks the expression of matrix metalloproteinase 9 (MMP-9) and the anti-apoptotic protein survivin, and thus reduces the invasive potential of cells^[51]. A further pro-oncogenic effect of *HOXB13* is exerted through the upregulation of zinc transporters that in turn results in lower intracellular zinc concentrations. This reduces the level of inhibitor of NF- κ B alpha ($\text{I}\kappa\text{B}\alpha$) and promotes NF- κ B signaling leading to increased invasion and metastasis^[52]. Thus, *HOXB13* exerts multiple tumor-promoting effects through the regulation of specific target genes.

In addition to their roles in regulating the proliferation and survival of prostate cancer cells, it has become apparent that the *HOX* genes are also instrumental in promoting changes to the tumor microenvironment that support metastasis and angiogenesis [Figure 2]. Each of these aspects will be considered in detail in

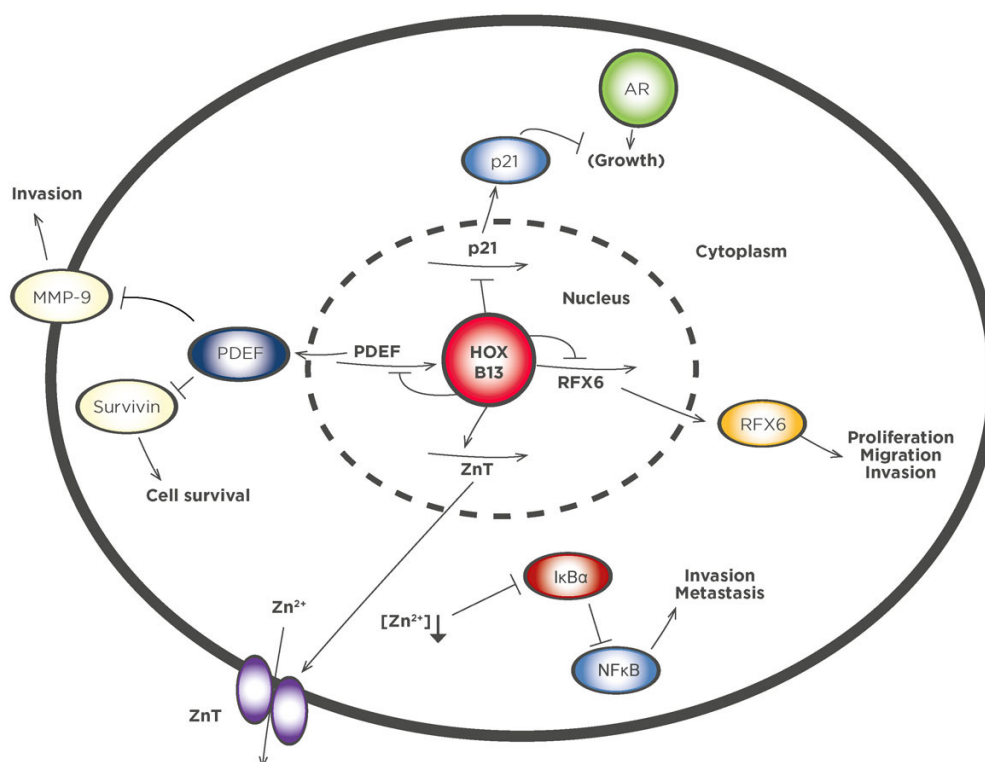


Figure 1: *HOXB13* exerts multiple tumor-promoting effects through the regulation of specific target genes. *HOXB13* protein can function both as a repressor and activator of transcription. It represses the p21WAF1/CIP1 (*p21*) tumor suppressor gene, which can block androgen-stimulated cell proliferation and has also been shown to bind directly to the enhancer region of the *RFX6* gene, the product of which inhibits the proliferation, migration, and invasion of prostate cancer cells. *HOXB13* additionally represses prostate derived Ets factor (*PDEF*) expression, which in turn blocks the expression of matrix metalloproteinase 9 (MMP-9) and the anti-apoptotic protein survivin, and thus reduces the invasive potential of cells. A further pro-oncogenic effect of *HOXB13* is exerted through the upregulation of zinc transporters resulting in lower intracellular zinc concentrations. This reduces the level of inhibitor of NF- κ B alpha ($\text{I}\kappa\text{B}\alpha$) and promotes NF- κ B signaling leading to increased invasion and metastasis. Right pointing arrows in the nucleus indicate transcription. AR: androgen receptor

the remainder of this review.

HOX TRANSCRIPTION FACTORS AND METASTASIS

Metastasis is a complex, multi stage process and the tumor microenvironment plays a key role both at the earliest stages, in facilitating the movement of cells away from the primary tumor, and in the final stages in allowing metastatic cells to generate a new tumor at a distant site. One of the most important mechanisms by which tumors can modify the microenvironment is through the release of matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases that can modify the extra cellular matrix (ECM)^[53]. Two of the most extensively studied of these enzymes with respect to prostate cancer are MMP-2 and MMP-9, both of which are members of the gelatinase subgroup of MMPs characterized by a fibronectin-like, gelatin-binding domain^[54]. MMP-2 expression is higher in prostate tumors compared to normal prostate tissue, and has also been shown to be secreted by the former^[55], and reducing its expression in mouse melanoma B16F10 cells resulted in significantly fewer lung metastases^[56]. Both MMP-9 and MMP-2 expression is directly activated by the binding of HOXC11 protein to

the enhancer region^[57], and HOXC11 is expressed in multiple prostate cancer cell types^[25] [Table 1]. MMP-9 expression has also been shown to be activated by HOXB7 in breast cancer cells^[58], and both MMP-9 and HOXB7 are over expressed in prostate cancer^[25,53]. The most frequently used prostate cancer-derived cell lines are LNCaP, DU145 and PC3, of which PC3 has by far the higher capacity for invasion *in vitro* and shows a significantly higher level of MMP-9 expression compared to the other cell lines^[59]. Correspondingly, the invasive capacity of LNCaP increased significantly when MMP-9 was experimentally over-expressed in these cells^[60], and invasion by DU145 and PC3 was reduced after MMP-9 expression was knocked-down using siRNA^[61].

In addition to the gelatinase class MMPs, the expression of two other MMPs, MMP-3 and MMP-14, is activated by HOX transcription factors^[62,63]. MMP-14 differs from other MMPs as it is membrane bound through a transmembrane domain with its catalytic center on the outside of the cell^[64]. Its expression in prostate cancer cells is associated with androgen independence^[65] and aggressiveness^[66]. Prostate tumors primarily metastasize to bone, and MMP-14 has a particularly important role in this process due to

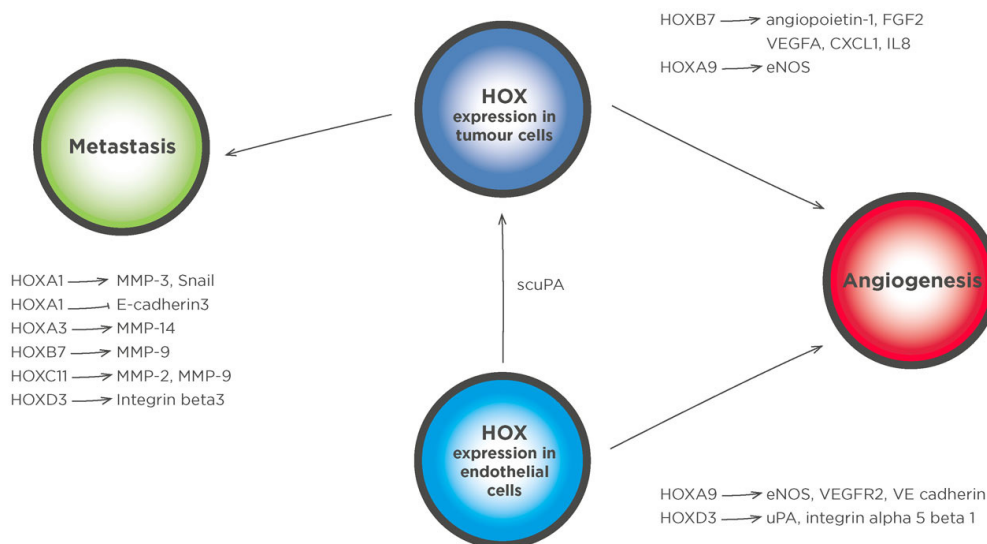


Figure 2: HOX transcription factors regulate genes in prostate cancer cells that modify the tumor microenvironment, as well genes in stromal cells that support tumor growth. HOX transcription factors have multiple roles in regulating genes that drive angiogenesis and metastasis. HOX targets with a key role in metastases include MMPs 2, 3, 9, and 14, as well as genes such as Snail and E-cadherin that are involved in the epithelial to mesenchymal transition. Genes involved in angiogenesis are also regulated by HOX transcription factors both in tumor cells and in endothelial cells. HOXD3 drives the expression of integrin alpha 5 beta 1 in endothelial cells which in turn leads to immature, leaky vessels. A number of HOX transcription factors can also drive the expression of proangiogenic secretory factors, including HOXB7, which regulates the transcription of FGF2, VEGFA, CXCL1, and IL8. An additional proangiogenic gene upregulated by HOXB7 is angiopoietin-1, the product of which plays a crucial role in stabilizing newly formed vasculature. Other proangiogenic genes that are regulated by HOX transcription factors include eNOs and uPA. HOXA9 expression in progenitor endothelial cells is necessary for their commitment to an endothelial lineage as it directly regulates endothelial specific genes such as eNOs, VE cadherin, and VEGFR2. HOXD3 has also been shown to have a role in vessel formation by endothelial cells through the activation of uPA transcription. In addition to an extracellular activity, a scuPA can be taken up by cancer cells in which it binds directly to HOXA5. MMP: matrix metalloproteinase; FGF2: fibroblast growth factor 2; VEGFA: vascular endothelial growth factor A; CXCL1: C-X-C motif ligand 1; IL8: interleukin 8; eNOs: endothelial nitric oxide synthase; uPA: urokinase plasminogen activator; scuPA: single chain form of uPA

Table 1: Direct and indirect regulation of target genes by HOX transcription factors in the context of the tumor microenvironment

HOX protein	Target gene	Direct or indirect regulation	Reference
HOXA1	<i>MMP-3</i>	Unknown	[63]
HOXA1	<i>Snail</i>	Unknown	[63]
HOXA1	<i>E-cadherin3</i>	Unknown	[63]
HOXA3	<i>MMP-14</i>	Unknown	[62]
HOXA9	<i>eNOS</i>	Direct	[99]
HOXA9	<i>VEGFR2</i>	Direct	[99]
HOXA9	<i>VE cadherin</i>	Direct	[99]
HOXB7	<i>MMP-9</i>	Unknown	[58]
HOXB7	<i>Angiopoietin-1</i>	Unknown	[58]
HOXB7	<i>FGF2</i>	Direct	[58,85]
HOXB7	<i>VEGFA</i>	Unknown	[58]
HOXB7	<i>CXCL1</i>	Unknown	[58]
HOXB7	<i>IL8</i>	Unknown	[58]
HOXC11	<i>MMP-2</i>	Direct	[57]
HOXC11	<i>MMP-8</i>	Direct	[57]
HOXD3	<i>Integrin beta 3</i>	Indirect	[75]
HOXD3	<i>uPA</i>	Unknown	[100]
HOXD3	<i>Integrin alpha 5 beta 1</i>	Direct	[82]

its ability to degrade collagen^[67]. Accordingly, LNCaP cells overexpressing MMP-14 were shown to form significantly larger bone lesions in mice^[67]. MMP-14 has been shown to be upregulated by *HOXA3* expression^[62], and *HOXA3* is overexpressed in a number of cancers, including prostate cancer^[25]. Another *HOX* gene linked to the progression of prostate cancer is *HOXA1*, the expression of which promotes the proliferation, invasion and metastasis of prostate cancer cells^[63]. A number of key downstream target genes of *HOXA1* have been identified, including *MMP-3*, which has itself been linked to prostate tumor progression in a number of studies^[68-71], and polymorphisms in the *MMP-3* gene have been identified as a risk factor for the development of prostate cancer^[72].

In addition to the *MMPs*, *HOX* transcription factors regulate a number of other target genes involved in the interaction of prostate cancers cells with the ECM. These include *HOXA1*, which inhibits the expression of *E-cadherin*^[63], a major component of the epithelial adherence junctions that mediate intercellular interactions^[73]. The downregulation of *E-cadherin* expression is one of the changes that occurs during the epithelial to mesenchymal transition, the activation of which in cancer cells is a key step in tumor invasion and metastasis^[74]. The loss of *E-cadherin* also results in the disruption of the cytoplasmic cell adhesion complex, releasing proteins that can further modify the tumor microenvironment^[73]. Another protein with a key function in cell adhesion is integrin $\beta 3$, elevated expression of which is positively associated with high levels of *HOXD3* expression^[75]. Integrin $\beta 3$ has a role in tumor progression, invasion, and metastasis^[76-78], and

is also associated with more aggressive behavior of prostate cancer bone metastases^[79]. Correspondingly, integrin antagonists have been shown to reduce bone degradation in clinical trials^[80].

HOX TRANSCRIPTION FACTORS AND ANGIOGENESIS

Angiogenesis is a fundamental event in the natural history of tumors, allowing for their growth beyond a size restricted by the diffusion limits of nutrients and oxygen, and ultimately their systemic spread to form metastases^[81]. *HOX* transcription factors have multiple roles in regulating the secretion of factors from tumor cells that drive this process in the microenvironment, and are also expressed in the cells of the tumor microvasculature in which they promote tumor-supportive functions. For the latter, *HOXD3* has been shown to be particularly significant as it drives the expression of integrin alpha 5 beta 1 in endothelial cells which in turn leads to immature, leaky vessels that are typical of many tumor types^[82]. Conversely, *HOXA5*, the expression of which results in more stable and less permeable vessels, is absent from tumor vessels^[83,84]. Within tumor cells it has been shown that a number of *HOX* transcription factors can drive the expression of proangiogenic secretory factors. One of the earliest identified examples of this is *HOXB7*, which drives fibroblast growth factor 2 (*FGF2*, also known as *bFGF*) expression in multiple cancer types^[58,85]. *FGF2* is a well characterized proangiogenic factor, and has been shown to induce tubule formation by endothelial cells when secreted from a prostate tumor in a rat model of this disease^[86]. In addition to *FGF2*, *HOXB7* drives the expression of vascular endothelial growth factor A (*VEGFA*), C-X-C motif ligand 1 (*CXCL1*), and interleukin 8 (*IL8*)^[58]. A role for *IL8* in angiogenesis and its potential as a therapeutic target in cancer was demonstrated using fully-humanized antibodies to this protein in a mouse model of melanoma^[87], and it was subsequently shown that *IL8* increases expression of the key proangiogenic ligand *VEGF* in endothelial cells resulting in a self-reinforcing, autocrine loop through the *VEGF* receptor 2 (*VEGFR2*) expressed on the surface of these cells^[88]. Correspondingly, polymorphisms in the *IL8* gene were shown to be associated with more aggressive prostate cancer^[89]. *CXCL1* is also a proangiogenic cytokine and has a potential role in the development of tumor resistance to anti-*VEGF* based therapy^[90], and in gastric cancer has been shown to promote tumor growth through the *VEGF* pathway^[91]. Correspondingly, the down regulation of *CXCL1* has been shown to mediate the enhancement of the antiangiogenic effects of docetaxel by dexamethasone in *in vitro* and *in vivo* models of prostate cancer^[92].

Its proangiogenic effects are also mediated through non-VEGF pathways, including the downregulation of fibulin-1 in castrate resistant prostate cancer^[93]. It is targeted by the tumor-suppressor microRNA (miR)-200 that blocks angiogenesis and inhibits metastasis in multiple tumor types^[94].

An additional proangiogenic gene upregulated by HOXB7 is angiopoietin-1 (*Ang-1*)^[58], the product of which plays a crucial role in stabilizing newly formed vasculature. The binding of Ang-1 protein to its receptor on endothelial cells promotes their adherence to mural cells such as pericytes and smooth muscle cells^[95-97]. Correspondingly, Ang1 secretion by prostate cancer cells in a xenograft model was shown to enhance tumor growth through an increased level of branching in the neovasculature^[98].

Additional proangiogenic genes that are regulated by HOX transcription factors include endothelial nitric oxide synthase (eNOs)^[99] and urokinase plasminogen activator (uPA)^[100]. *HOXA9* expression in progenitor endothelial cells within the tumor microenvironment was shown to be necessary for their commitment to an endothelial lineage, and it was also shown to directly regulate endothelial specific genes such as eNOs, *VE cadherin*, and *VEGFR2*^[99]. In this context *HOXA9* was identified as a key target of histone deacetylases (HDACs), as its expression was significantly reduced after HDAC inhibitor treatment and this in turn blocked angiogenesis both in mice^[99] and in a clinical trial of combined HDAC and VEGF inhibitors for multiple cancers including advanced prostate cancer^[101]. *HOXD3* has also been shown to have a role in vessel formation by endothelial cells through the activation of uPA transcription^[100]. uPA is involved at all stages of angiogenesis, including endothelial cell division, migration, the formation of stable vessels, and the regulation of vascular permeability through proteolytic degradation of the extracellular matrix^[102-104]. This is mediated through intracellular signaling initiated by its binding to receptors including uPA receptor (uPAR; CD87), low-density lipoprotein receptor-related protein receptor (LRP/ α 2MR), and specific integrins^[105-110]. In addition, uPA converts plasminogen into serine protease plasmin^[111,112], which in turn breaks down matrix proteins and activates a number of MMPs^[113-116]. uPAR-bound uPA has been shown in a number of studies to be localized to the leading edge of migrating cells^[117-119] to help ensure a focused degradation of the ECM and liberate matrix-bound proangiogenic factors, including VEGF^[120-122] and FGF2^[123,124]. In addition to an extracellular activity, a single chain form of uPA can be taken up by cancer cells and be translocated to the nucleus^[125] where

it binds directly to HOXA5 protein and prevents it from activating the transcription of the key tumor suppressor gene *p53*^[21]. Taken together, these studies imply the existence of a HOX-mediated feedback mechanism from the developing neovasculature to the tumor whereby HOXD3 promotes uPA expression in the endothelial cells, and this in turn blocks *p53* expression in the tumor, promoting cell proliferation and survival.

CONCLUSION

The evidence from previous studies indicates that the expression of *HOX* genes in the prostate tumor modifies the microenvironment in a manner that supports metastasis through degradation of the ECM, and angiogenesis through the secretion of proangiogenic cytokines. This is complemented by the expression of *HOX* genes in the microenvironment, particularly in endothelial cells, that promotes tumor-supportive functions including angiogenesis and the secretion of proteins that directly influence the malignant phenotype. Thus, targeting the function of HOX proteins may not only have a direct effect on tumor cells, but could also help reverse changes in the tumor microenvironment that would otherwise promote cancer progression.

DECLARATIONS

Authors' contributions

Performed the literature search and drafted the manuscript: R. Morgan

Helped write the manuscript and provided further interpretation of the referenced studies: H.S. Pandha

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None.

Conflicts of interest

The authors are shareholders in HOX Therapeutics Ltd., a company which is developing novel HOX/PBX binding antagonists, although these reagents are not discussed in this review.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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Topic: How does the prostate cancer microenvironment affect the metastatic process and/or treatment outcome?

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Prostate cancer exosomes as modulators of the tumor microenvironment

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ABSTRACT

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Researchers are currently trying to understand why some men with prostate cancer go on to develop aggressive disease whilst others maintain slow growing tumors. Although endogenous genetic anomalies within the tumor cell are important, the prevailing view is that the tissue microenvironment as a whole is the determinant factor. Many studies have focussed on the role of soluble factors in modulating the nature of the tumor microenvironment. There is however a growing interest in the role of extracellular vesicles, including exosomes, as regulators of disease progression. A variety of resident cells, as well as infiltrating cells, all contribute to a heterogeneous population of exosomes within the tumor microenvironment. Studies focussing on the role of exosomes in prostate cancer are however relatively rare. In this review, evidence from various cancers, including prostate, is used to present numerous potential roles of exosomes in prostate cancer. Whilst further validation of some functions may remain necessary it is clear that exosomes play a major role in intercellular communication between various cell types within the tumor microenvironment and are necessary for driving disease progression.

INTRODUCTION

Prostate cancer is the most common form of cancer to affect men in the UK. Current survival rates suggest that of those men who develop the disease approximately eighty four percent will survive for 10 or more years. For some men, however, the disease is far more aggressive. Ongoing studies are in place to try to understand the mechanisms responsible for

this difference between slow growing, indolent tumors, and the aggressive disease. Many of these studies have focussed on the role of soluble growth factors as modulators of the tumor microenvironment thereby supporting aggressive metastatic forms of the disease. There is, however, a growing precedent to explore the role of extracellular vesicles (EV) in this process.

All cells are capable of secreting vesicles into the



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extracellular space. Vesicle secretion becomes elevated when cells are subjected to cellular stress^[1,2], which can also result in altered molecular cargo within the vesicle^[3]. This is particularly relevant in cancer where stress can come from the hypoxic environment, nutrient deficiency, altered extracellular matrix, and other environmental factors. Such vesicles are often regarded as one of two broad subtypes, microvesicles and exosomes. Microvesicles are large, tending to be greater than 200 nm in diameter, dense, and are formed from outward budding of the plasma membrane. Exosomes are much smaller, typically 30 to 150 nm in diameter, float at a characteristic density of 1.1 to 1.2 g/mL^[4] and originate within multivesicular endosomes^[4]. The secretion of small, exosome-like, vesicles has also been reported from the plasma membrane^[5]. It remains a challenge to accurately define vesicle subtypes based on size alone. To aid researchers, the International Society for Extracellular Vesicles has released a position paper detailing the minimal experimental requirements for defining EV^[6]. Although, the challenge of defining EV subtypes remains, and is further compounded by overlap in EV composition^[7], hence the term EV is often used. The majority of EV present within both cell conditioned media or biological fluids tend to be small^[8], suggesting a predominant exosome-like population. The biological significance of any one EV subtype compared to another, however, remains unknown.

The role of EV in cancer has been studied intensively over recent years^[9]. Relatively few of these studies have focused on the potential role of EV, and more specifically exosomes, in prostate cancer. In this current article, we review past studies into the role of exosomes, in diverse malignancies, to identify their potential functions in disease processes of relevance to prostate cancer.

EXOSOME-MEDIATED ANGIOGENESIS

Angiogenesis, or the formation of new blood vessels from pre-existing vasculature, is a vital component in numerous physiological and pathological responses. A variety of angiogenic signals are required to drive endothelial maturation and subsequent re-organisation with vascular smooth muscle cells and pericytes to form a functional vessel network^[10], thereby allowing nutrient and waste product exchange^[11,12]. In cancer, multiple modulators of vascular remodelling contribute to tumor growth and progression^[13]. Once a tumor lesion forms it will become hypoxic and nutrient deprived. The secretion of growth factors activates normal surrounding quiescent cells, to initiate a cascade of events that become quickly dysregulated.

This involves an “angiogenic” switch, regulated by both anti- and pro-angiogenic cytokines, examples of which include endothelial growth factor, fibroblast growth factor (FGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF)^[14-16]. These responses may initially provide the tumor with more nutrients and oxygen, however, the structural organization of the vessel network is poor, and the continuously remodeled tumor vasculature is disorganized and leaky^[17]. This causes irregular blood flow and provides invasive tumors with access to the circulatory system.

PRO-ANGIOGENIC ACTIVITY OF EXOSOME-ASSOCIATED PROTEINS

Cancer cell-derived EV have been shown in several studies to promote angiogenesis. In the case of prostate cancer it is well established that c-Src tyrosine kinase, insulin-like growth factor 1 receptor (IGF-1R) and focal adhesion kinase (FAK) play important roles in tumor growth and disease progression^[18]. Src-family kinases are normally expressed in prostatic epithelium and reported to transform normal cells when constitutively active and up-regulated during disease initiation and progression^[19]. Cross-talk between Src and IGF-1R has previously been shown to promote angiogenesis^[20]. It has been reported that Src, IGF-1R and FAK are enriched in prostate cancer exosomes^[21]. Src and c-Src are also present in plasma exosomes derived from prostate tumor bearing mice; suggesting that Src-enriched exosomes can promote angiogenesis *in vivo*. Src is known to stimulate transcription of VEGF and modulate angiogenesis^[22] whilst IGF-1R has been demonstrated to induce VEGF-C expression and stimulate angiogenesis^[23]. These observations suggest that prostate cancer exosomes enriched with c-Src, IGF-1R and FAK may be able to stimulate angiogenic activity within the tumor microenvironment.

Prostate cancer EV are also likely to be capable of delivering growth factors with known pro-angiogenic function. For instance, EV from aggressive prostate cancer cells have been shown to contain urokinase-type plasminogen activator (uPA)^[24], known to be involved in activation of the protease plasminogen which is responsible for vascular remodeling^[25]. Addition of uPA positive vesicles to less aggressive prostate cancer cells stimulated cell migration and invasiveness^[24]. Although this study did not investigate the impact of uPA positive vesicles on the ability of treated cells to drive angiogenesis, it is conceivable that prostate cancer derived EV can support endothelial tubule formation via delivery of pro-angiogenic growth factors. Additional pro-angiogenic factors have been

identified on EV from a variety of different cancer cell types and are summarized in Table 1. Further studies are required to ascertain whether these factors are present on prostate cancer EV.

DELIVERY OF PRO-ANGIOGENIC RNAS BY EXOSOMES

Whilst direct evidence of RNA delivery by prostate cancer EV is currently lacking, EV from several cancer types are known to be enriched with mRNA transcripts related to pro-angiogenic function that can then be translated by recipient cells^[26,27]. Similar studies have shown an enhanced proliferative impact on endothelial cells^[28,29] and enhanced tubule formation within 3D cell cultures^[28]. The transfer of exosomal miRNA, such as miRNA-92a and miR-17-92, may also play a role in this process^[30] and miR-17-92 may play a role in this process^[30]. Furthermore, transmittance of the miR-17-92 cluster from EV to endothelial cells has been shown to attenuate endothelial expression of integrin α_v , resulting in enhanced endothelial cell migration and tube formation^[30]. Numerous studies highlight a role of cancer exosomes in delivery of RNAs to endothelial cells, thereby promoting angiogenesis, and it is therefore likely that prostate cancer exosomes share this functionality.

HYPOXIC TUMOR-DERIVED EXOSOMES ENHANCE ANGIOGENESIS

As a tumor grows diffusion distances from the existing vascular supply increase, resulting in hypoxia.

Sustained growth of the tumor mass often requires new blood vessels to provide rapidly proliferating tumor cells with an adequate supply of metabolites and oxygen. Under hypoxic conditions the cellular secretome becomes altered and a proportion of these changes may reside within the exosome fraction. Exosomes derived from solid tumors, which have been cultured in hypoxic conditions, become enriched with hypoxia-regulated mRNAs and proteins such as Caveolin 1, IL-8, matrix metalloproteinase (MMP) and PDGF, and are capable of promoting angiogenesis^[31]. Similarly, under hypoxic conditions, the secretion of exosomes from breast cancer^[32] or leukemic cells^[33] demonstrate elevated levels of exosomal miR-210, with the capacity to enhance HUVEC tube formation compared to exosomes from normoxic conditions. Although EV from hypoxic prostate cancer cells are yet to be investigated, based on this evidence, it is highly likely that the cargo of prostate cancer exosomes is also influenced by hypoxic conditions. The impact of hypoxia-derived vesicles on angiogenesis and subsequent development of prostatic tumors remains unknown.

INDIRECT EXOSOME-MEDIATED ANGIOGENESIS

In addition to direct modulation of angiogenesis within the tumor microenvironment, exosomes have the potential to regulate angiogenesis indirectly through interactions with various non-endothelial cell types. Prostate cancer exosomes, expressing transforming growth factor beta (TGF β), can activate fibroblasts

Table 1: EV-associated pro-angiogenic proteins

Protein	Pro-angiogenic function	Cancer cell of EV origin	Reference
Angiogenin	Translocates to the nucleus of recipient cells and enhances RNA transcription, stimulating expression of pro-angiogenic proteins	Multiple myeloma	[131]
EGFR	Induces VEGF expression in recipient cells through Akt signaling	Lung, glioma	[132,133]
FAK	Interactions between FAK, IGF-1R and Src result in various downstream signaling events and modulation of angiogenesis	Prostate	[21]
FGF2	Promotes proliferation and differentiation of endothelial cells	Multiple myeloma	[131]
IGF-1R	Interactions between FAK, IGF-1R and Src result in various downstream signaling events and modulation of angiogenesis	Prostate	[21]
MMP-2, MMP-9	Degradation of extracellular matrix components	Ovarian	[134]
Src	Activation of FAK, and subsequent formation of focal adhesions between endothelial cells	Prostate, myeloid leukemia	[21,135]
Tspan8	Induces uPA, VEGFR and vWF in recipient endothelial cells	Pancreatic	[134,135]
uPA	Activation of plasminogen leading to vascular remodeling	Prostate	[24]
VEGF	Rearranges the cytoskeleton through the FAK/paxillin pathway, induces capillary formation via RhoA/ROCK signaling and controls vascular permeability through PLC γ	Multiple myeloma, ovarian	[131,134]

A selected overview of pro-angiogenic factors previously identified on EV. Association of pro-angiogenic proteins with EV has been demonstrated in multiple cancers, but the precise involvement of some such proteins in prostate cancer remains unclear. EV: extracellular vesicles; EGFR: epidermal growth factor receptor; VEGF: vascular endothelial growth factor; Akt: protein kinase B (serine/threonine specific protein kinase); FAK: focal adhesion kinase; IGF-1R: insulin-like growth factor 1 receptor; Src: proto-oncogene tyrosine-protein kinase; FGF2: fibroblast growth factor 2; MMP: matrix metalloproteinase; Tspan8: tetraspanin-8; uPA: urokinase-type plasminogen activator; VEGFR: vascular endothelial growth factor receptor; vWF: von willebrand factor; RhoA: Ras homolog gene family, member A; ROCK: Rho-associated, coiled-coil containing protein kinase; PLC γ : phospholipase C gamma

resulting in elevated secretion of multiple pro-angiogenic factors including VEGF, HGF, FGF-2, and uPA^[34,35]. Furthermore, prostate cancer exosomes were shown to induce pro-angiogenic function within primary prostate stromal cells and were shown to facilitate *in vivo* tumor growth^[35]. Other studies have reported cancer-associated myofibroblasts can secrete pro-angiogenic growth factors and promote angiogenesis at the primary tumor site^[36-39]. Also, HGF and stromal cell-derived factor-1 derived from these myofibroblasts can indirectly enhance angiogenesis by inducing the secretion of angiogenic factors from tumor cells^[40,41]. Collectively, these studies demonstrate that exosomes derived from solid tumors, including prostate cancer, drive activation of fibroblasts to a pro-angiogenic phenotype.

The ability of prostate cancer exosomes to trigger secretion of pro-angiogenic factors extends to bone marrow-derived mesenchymal stem cells (BM-MSCs), which can also gain exosome-induced pro-angiogenic function^[42]. Exosome-activated MSCs were shown to secrete elevated levels of HGF, VEGF and MMPs, and support the formation of endothelial vessel-like structures. Exosomes from metastatic melanomas have also been shown to interact with bone marrow progenitor cells via the tyrosine kinase MET^[43], which induced vascular leakiness at pre-metastatic sites and reprogrammed bone marrow progenitors towards a pro-vasculogenic phenotype. This “reprogramming” of the bone marrow progenitors resulted in significantly increased tumor vascular density *in vivo*.

A wide range of studies have demonstrated various roles of cancer exosomes in promoting angiogenesis either through direct or indirect interaction with endothelial cells. Prostate cancer exosomes are likely to be dynamic in response to hypoxia and may act as a means to deliver a variety of factors capable of supporting the formation of tumor-associated vasculature *in vivo*.

EXOSOME-DRIVEN TUMOR-STROMA INTERACTIONS

Stromal cells surrounding a tumor can undergo a desmoplastic response, characterized by aberrant cell growth and morphological transformation of the stroma, resulting in a more aggressive tumor microenvironment^[44]. A key feature of this tumor reactive stroma is the presence of cells with a myofibroblast-like phenotype^[45]. Myofibroblasts are contractile cells, characterized by the formation of a smooth muscle actin (α SMA) stress fibers^[46], loss of the spindle phenotype and formation of a hyaluronic acid pericellular coat^[47].

During wound healing myofibroblasts are present to aid wound closure. In various cancers, however, a chronic wound response can occur resulting in sustained presence of myofibroblasts within the tumor microenvironment^[48].

Cancer associated myofibroblasts display an altered phenotype compared to wound associated myofibroblasts^[49] and have been termed activated fibroblasts, tumor associated fibroblasts and cancer associated fibroblasts. There is conflicting evidence as to whether myofibroblasts promote or suppress tumorigenesis. Rhim *et al.*^[50] observed that removal of myofibroblasts from the stroma of pancreatic ductal adenocarcinoma (PDAC) *in vivo* results in more aggressive tumors and reduced mouse survival rates. However, the prevailing view is that stroma rich in myofibroblasts has an increased ability to drive tumor growth, angiogenesis, metastasis and treatment resistance^[45,51,52].

ACTIVATION AND MODULATION OF STROMAL CELLS BY EXOSOMES

Fibroblast differentiation is known to be induced by TGF β 1 via SMAD dependent and independent signaling pathways^[53-55]. It has been established that exosomes secreted by prostate cancer cells express latent TGF β 1^[56], tethered to the exosome surface via proteoglycans and capable of activating SMAD3 dependent signaling^[34]. The authors demonstrate that prostate cancer derived exosomes, with greater than 6 pg TGF β 1/ μ g exosome, can induce fibroblast differentiation^[34]. Differentiation could be sustained for at least 2 weeks in the absence of further exosome treatment, indicating the resulting myofibroblast-like phenotype is self-maintaining. In contrast, EV originating from MDA-MB231 breast cancer cells and u87 glioblastoma cells could only induce transient fibroblast differentiation^[57], potentially suggesting differences between EV from distinct tissue types.

A subsequent study by Webber *et al.*^[35] identified that exosomal TGF β 1 induces a more aggressive, pro-angiogenic myofibroblast phenotype compared to the soluble form of the growth factor. These results were replicated in primary stromal cells from normal prostate tissue, resulting in a myofibroblast-like phenotype that matched that found within disease-associated stromal tissue. Furthermore, pre-treating normal stroma with prostate cancer derived exosomes prior to administration enhanced tumor growth in mice. In contrast, pre-treatment with soluble TGF β 1 led to tumor control. Consistent with this report, a separate study showed that metastatic rat prostate tumor EV are

capable of activating primary rat prostate fibroblasts, leading to upregulation of α SMA, HGF and VEGFA^[58]. Exosomes therefore appear to play a crucial role in communication between prostate cancer cells and the surrounding stroma, with exosome-associated TGF β 1 essential for inducing fibroblast differentiation towards a disease supporting phenotype.

The exact origin of disease-associated myofibroblasts remains unclear, and it has been shown that other cells are capable of myofibroblastic differentiation. MSCs, a multipotent cell type capable of generating many different types of connective tissue, can also differentiate into myofibroblasts in response to secreted factors from tumors^[40]. MSCs make up 1.1% of cells within the prostate cancer stroma^[59] and exhibit similar tumor promoting effects to cancer associated stroma^[40,60,61].

Exosomes secreted by breast^[62], ovarian^[63] and gastric^[64] cancer cells induce TGF β 1-dependent differentiation of adipose or cord blood derived MSCs to myofibroblasts. In addition, chronic lymphocytic leukemia exosomes have been shown to enhance tumor growth *in vivo* by inducing differentiation of BM-MSCs^[65]. Adipose derived MSCs, meanwhile, differentiate in response to EV from metastatic breast cancer in 2D and 3D culture. This process was shown to require TGF β dependent MAPK signaling involving phosphorylation of ERK1/2 and JNK1/2^[66].

Chowdhury *et al.*^[42] demonstrated that prostate cancer exosomes can also drive BM-MSC differentiation, resulting in myofibroblasts with increased VEGFA, HGF and MMP secretion, capable of enhancing cancer cell growth. Exosome differentiated BM-MSCs drove prostate cancer cell invasion in a 3D spheroid model and stimulated endothelial cell migration, proliferation and angiogenic potential. As previously observed, exosomal TGF β 1 treatment resulted in myofibroblasts with an enhanced pro-tumorigenic phenotype compared to soluble TGF β 1. Interestingly, exosomes also modulated BM-MSC derived myofibroblast expression of ITGB6 and ITGB8, encoding for components of integrins α v β 6 and α v β 8, which are involved in converting latent TGF β 1 to the active form^[67,68]. This may therefore explain how latent TGF β 1 delivered by exosomes becomes functionally active. The predominant population of myofibroblast precursors remains unclear. Regardless of the precursor cell, it is evident that prostate cancer exosomes can trigger differentiation to a stromal phenotype with disease promoting properties.

Delivery of TGF β 1 is not the only mechanism by which exosomes can stimulate pro-tumorigenic phenotypes

in stromal cells. EV transfer of mRNA, miRNA and membrane proteins have all been implicated. For instance, acute myeloid leukemia cell exosomes promote proliferation and migration of bone marrow stromal cells via transfer of IGF-IR mRNA^[69]. Similar results have been shown in solid cancers whereby exosomal miRNAs regulate stromal cell behavior. Metastatic breast cancer cells, for example, were shown to enhance vascular permeability, and promote tumor metastasis, via the suppression of the tight junction protein ZO-1 by exosome delivered miR-105^[70]. Gastric cancer exosomes stimulate primary mouse liver myofibroblasts and hepatic pericytes by exosome mediated delivery of the membrane protein epidermal growth factor receptor (EGFR)^[71]. After insertion into the stromal cell membrane, where it co-localizes with E-cadherin, EGFR activates HGF secretion by potentially suppressing upstream miRNAs such as miR-26a/b. The subsequent increase in HGF secretion promotes gastric cancer cell proliferation, migration and invasion.

Exosomes are not the only EV subgroup shown to alter the prostate stroma phenotype. Prostate cancer cells also secrete large oncosomes, EV between 100-400 nm in diameter^[72], which have sustained AKT1 activity^[73,74]. A recent study by Minciacchi *et al.*^[75] reported that internalization of large oncosomes by prostate fibroblasts resulted in the induction of a α SMA-positive myofibroblast phenotype. Interestingly, induction of other myofibroblast markers, such as MMP1, thrombospondin-1 (TSP-1) and TGF β 1 did not occur, potentially suggesting that oncosomes induce a distinct myofibroblast-like phenotype. Analysis of transcription factor DNA binding in treated prostate fibroblasts highlighted that MYC binding was essential for this induction of a myofibroblast-like phenotype. The mechanism by which large oncosomes stimulate MYC-DNA binding has not yet been elucidated, however, as MYC has not been found to be present inside the EV it appears MYC is activated rather than delivered. This study also explored the impact of large oncosomes *in vivo* and found that prostate fibroblasts pre-treated with oncosomes facilitated enhanced tumor growth. These findings are similar to the earlier results obtained with exosomes and lend support to the critical role of diverse vesicle subtypes in tumor-stroma communication in prostate cancer.

SECRETION OF STROMA-DERIVED EXOSOMES

Stromal cells activated by cancer cell secreted EV can initiate a positive feedback mechanism via release of stromal cell EV which promote

tumorigenesis after internalization by tumor cells. A study by Jossion *et al.*^[76] highlighted this cyclical system in prostate cancer. Activated prostate fibroblasts were shown to release miR-409 containing EV, which are taken up by prostate cancer cells. Upon EV internalization miR-409 downregulates the tumor suppressors Ras suppressor 1 and stromal antigen 2, promoting cancer cell tumorigenesis and stimulating EMT and stemness in epithelial cells. This effect can also be observed in other tissues. For example, activated PDAC fibroblasts secrete ANXA6 positive EV containing the ANXA6/LRP1/TSP1 complex. Uptake of these EV by PDAC cells was shown to enhance tumorigenesis by stimulating cancer cell migration and driving tumor growth *in vivo*^[77]. Activation of the Wnt-planar cell polarity (PCP) pathway, and subsequent stimulation of cell motility and metastasis, can also be induced by stromal cell EV. Luga *et al.*^[78] determined that CD81⁺ vesicles secreted from activated fibroblasts are capable of activating the Wnt-PCP pathway in breast cancer cells via transfer of Wnt11.

Stromal cells can also confer chemoresistance on surrounding tumor cells via EV communication. Activated fibroblasts resistant to the chemotherapy drug Gemcitabine (GEM) release exosomes containing miR-146a and mRNA for its upstream transcription factor Snail^[79]. Incubation of PDAC cells with exosomes from GEM treated fibroblasts results in increased levels of Snail mRNA and miR-146a in the cancer cells, leading to cell proliferation and chemoresistance. Similar findings have been observed in colorectal^[80] and breast cancers^[81], with the latter study identifying activation of antiviral signaling pathways through stimulation of the pattern recognition receptor RIG-I by exosomal RNA. RIG-I activates STAT1 dependent signaling which cooperates with NOTCH3 to mediate NOTCH target gene transcription, supporting maintenance of therapy resistant tumor initiating cells.

Activation of stromal cells by cancer cell-derived exosomes results in a pro-proliferative and pro-angiogenic stromal phenotype. In turn, EV and exosomes from activated stromal cells may then drive surrounding cancer cells towards a more aggressive, chemoresistant, phenotype. This suggests a network of reciprocal communication based on EV exists to exacerbate disease.

EXOSOME MODULATION OF MYELOID CELLS

There have been numerous studies demonstrating immunological control by EV, as reviewed previously^[9].

Despite such studies, there is a surprising paucity of information relating to prostate cancer exosomes and their influence on myeloid cells. This topic is highly relevant, however, as the presence of CD14⁺ macrophages and chronic inflammation within the microenvironment is a key risk factor in prostate cancer^[82].

EXOSOME-MEDIATED ANTIGEN PRESENTATION

Some of the early discoveries of exosome function have centered on their potential as immune-activating factors^[4], where professional antigen presenting cells derived from monocyte precursors were able to secrete exosomes carrying MHC-peptide complexes that were functional in T cell stimulation^[83]. Antigen presenting cells (APC), educated with cancer antigens in the form of protein or peptide fragments, therefore produce nanovesicles as APC-surrogates to disseminate the activation of T cells. Isolated APC-exosomes can also be manipulated directly, by pulsing with antigenic peptides of desired specificity, and this scheme has been proposed as a cancer vaccine strategy^[84]. APC can, however, also receive a complex set of antigenic information in the form of exosomes secreted by tumor cells^[85,86], providing not only tumor-associated antigens but importantly additional information such as cellular stress signals (e.g. heat shock proteins^[87]), or even encapsulated RNA^[88], to modulate APC-phenotype and control subsequent functions. Some researchers argue that cancer cell-derived exosomes may be an advantageous form of antigen delivery to APCs *in vivo*^[89]. There are, however, conflicting examples where the interaction of cancer-exosomes with myeloid cells may lead to disease exacerbating effects.

Amongst the earliest examples are reports detailing the skewing of dendritic cell differentiation away from a competent antigen presentation phenotype, and towards TGFβ producing myeloid cells capable of negatively regulating T cell responses^[90,91]. More recent reports also point to this phenomenon, where monocytes stimulated with cancer cell-derived EV become alternatively-activated/M2-type macrophages, expressing elevated levels of VEGF, IL6, Cox2, and arginase-1 amongst many other tumor-supportive factors^[92,93]. Similar modulation of myeloid cells are seen using pancreatic cancer exosomes, giving a suppressive CD14⁺HLA-DR^{low/neg} phenotype akin to those elevated within the circulation of patients^[94]. Similarly, myeloma-derived EV present within the bone marrow microenvironment can activate myeloid-derived suppressor cells (MDSC) and promote progression^[95]. In acute myeloid leukemia, vesicles may play a role

in modulating normal myelopoiesis and select for cells destined for suppressor-like differentiation^[96,97]. It currently remains unclear as to whether this latter phenomenon is also true of solid cancers.

MECHANISMS OF MYELOID ACTIVATION BY EV

Whilst there remains much to be learned about how EV exert such influences on myeloid cells, evidence points to delivery of EV-associated ligands to trigger signaling cascades mediated through toll-like^[98,99] or other receptors^[100,101]. Moreover, there is a likely additional effect of EV-encapsulated RNAs which may also be delivered to myeloid cells. In one elegant experimental system, cancer cells were engineered to express Cre-recombinase. Cre mRNA was detectable in various EV sub-fractions secreted by these cells, with the predominant EV type appearing to be exosome-like. Transplantation of these cells into mice with a Cre-reporter background led to recombination events at the tumor site, as indicated by β -galactosidase expression following receipt of vesicular Cre mRNA. These Cre-recombined cells were 90% CD45⁺ leukocytes, principally of a Gr1⁺CD11b⁺ MDSC phenotype^[102]. The MDSC which had taken up vesicular RNA exhibited more potent suppressive functions compared to their counterparts that had not. The study highlights the *in vivo* transfer of vesicle-encapsulated RNA to myeloid cells within the tumor microenvironment, resulting in enhanced immune-suppressive function of MDSC.

The influence of EV may, however, not be limited to the local environment. In a highly metastatic breast cancer model EV were again taken up principally by CD45⁺ bone marrow-derived cells present at distant sites of the lung and liver. These myeloid cells were implicated thereafter in aiding the colonization of these organs by metastasizing cells. Part of this effect may also be due to localized natural killer and T cell suppressive effects attenuating anti-cancer immunity in the premetastatic organs^[103]. Dissemination of EV may be more limited in some other cancer types, like glioma, where influences on myeloid phenotypes are not always found in the periphery^[104]. In one study, attenuating TLR2-dependent interaction between cancer exosome and MSDS was an effective strategy for limiting MDSC numbers and activation *in vivo*, and in fact potentiated the effect of chemotherapeutics that would otherwise lead to heightened release of MDSC-activating vesicles. Preventing vesicle effects on MDSC may be a worthwhile therapeutic approach to consider^[99].

If lessons are to be gained from these studies of other diverse cancer types, it is indeed likely that EV of prostate cancer origin may also exert local and possibly distant influences on the myeloid cell components of tissues, and profoundly impact the course of the disease. New studies are, however, required in order to examine this further.

EXOSOME DRIVEN METASTASIS AND MULTIDRUG RESISTANCE

A key step in the progression of various cancers is the invasion of cancer cells into surrounding tissues and subsequent metastasis from the primary tumor site. The 5-year survival rates of patients with prostate cancer drop dramatically following metastasis from the primary tumor. The primary site of metastasis of prostate cancer is the bone; such metastasis remains incurable. An increased concentration of circulating microvesicles has been reported in *in vivo* models of metastatic prostate cancer^[74] and studies by Peinado *et al.*^[43] have demonstrated a role of exosomes in the support of tumor metastasis to the bone.

EXOSOME-MEDIATED REGULATION OF MMPs

Cancer cell invasion, and disease progression, has been linked to an altered expression of MMPs, key regulators of the extracellular matrix. Fibronectin-mediated binding of exosomes to myeloma cells has been shown to activate p38 and ERK signaling, resulting in elevated expression of DKK1 and MMP-9 and subsequent myeloma progression^[105]. More recently, it has been shown that prostate cancer exosomes can regulate MMP-9 expression within osteoclast precursor cells and impair osteoclastic differentiation^[106]. Collectively these studies suggest a role of prostate cancer exosomes in the modulation of the bone environment, and subsequent preparation of the metastatic site.

Proteomic analyses have revealed that both cell surface-anchored and soluble matrix metalloproteinases are present in EV isolated from either cell conditioned media or from biofluids^[107]. Such vesicular-associated MMPs have been shown to be proteolytically active, and may play a variety of functional roles including direct interaction or cleavage of extracellular matrix proteins or removal of membrane-anchored receptors from target cells^[108]. This is supported by further evidence from Hakulinen *et al.*^[109] demonstrating that cancer exosomes can express functionally active MMP-14.

It has long been recognized that platelets can play a role in tumor progression by promoting angiogenesis, resulting in leaky capillaries, and therefore facilitating tumor metastasis. The mechanism of their action has however remained unclear until relatively recently. In a study by Janowska-Wieczorek *et al.*^[110], it was shown that platelet-derived EV, and exosomes released from α -granules, can contribute to metastatic spread via phosphorylation of mitogen activated protein kinase p42/44 and serine/threonine kinase as well as the expression of membrane type 1-MMP (MT1-MMP). The authors also showed that platelet-derived EV are capable of inducing MMP-9 mRNA expression. This study demonstrates that platelet-derived EV can simultaneously activate MT1-MMPs and induce de novo expression of MMPs within cancer cells.

Collectively, these studies suggest that exosomes may be capable of direct contribution to matrix remodeling both within the tumor microenvironment and potentially at distant sites away from the primary tumor.

EXOSOME-REGULATED METABOLISM AND DRUG RESISTANCE

Altered cell metabolism is a hallmark of cancer, with many cancer cells demonstrating an increase of aerobic glycolysis. This results in subsequent lowering of pH, leading to increased tumor invasion, proliferation, migration and drug resistance^[111,112]. There is growing interest in the role of exosomes, and other EV, as modulators of cancer cell metabolism. It has been reported that pH of the tumor microenvironment is a key factor in regulating both the release and uptake of exosomes by cancer cells^[113], suggesting a positive-feedback mechanism resulting in elevated secretion of EV from the tumor microenvironment.

Several studies have demonstrated a link between altered cell metabolism and the development of multidrug resistance in multiple cancer types^[114-116], including prostate^[117]. Prostate cancer progression is a complex process. In early stage disease the cancer remains androgen sensitive and can be treated with androgen-deprivation therapy. Over time, however, the cancer cells become androgen insensitive. Chemotherapeutic agents, such as docetaxel, can be used to treat androgen-independent disease^[118]. By this stage, however, disease relapse is extremely likely and the development of multidrug resistant cancers results in impaired treatment. Several factors have been linked to multidrug resistance^[119] including the overexpression of transporter proteins such as P-glycoprotein^[120], a well characterized

ATP-binding cassette transporter that is involved in the transportation of various substances across the plasma membrane.

Drug-resistant prostate cancer cell lines can transfer drug resistance to non-resistant cells via uptake of exosomes^[121], and other EV^[122], shed from drug-resistant cells. The initiation of drug-resistance is triggered by vesicular-mediated metabolic alteration of drug-sensitive cells towards a drug-resistant phenotype, with an increase in glycolysis and glycolytic capacity^[123]. Such changes in metabolic profile may also be reflected in cargo of EV secreted from the cancer, and may represent a source of biomarkers useful for both diagnosis and monitoring prognosis of disease^[124].

In addition, cancer-associated fibroblasts can also regulate metabolic processes within neighboring cancer cells^[125]. It was recently shown that cancer-associated fibroblast-derived exosomes can reprogram prostate cancer cell metabolism by downregulating mitochondrial function^[126]. Specifically, fibroblast-derived exosomes were shown to inhibit mitochondrial oxidative phosphorylation, resulting in an increase in glycolysis. This may be in part due to delivery of metabolite cargo consisting of lactate, acetate, amino acids, tricarboxylic acid cycle intermediates and lipids from fibroblast-exosomes^[126]. Activated stromal cells therefore appear capable of inducing the Warburg effect^[127,128], an increased rate of glycolysis followed by lactic acid fermentation, in surrounding cancer cells through EV mediated processes. Despite further studies being required to clarify the effects of metabolic change on cancer progression, stromal cell EV appear to contribute to cancer proliferation and survival in environments low in oxygen and nutrients.

CONCLUSION

As studies into the role of exosomes in prostate cancer continue, we are likely to learn of further ways in which exosomes regulate disease progression. Whilst studies specifically on prostate cancer/stroma-derived exosomes may appear limited in number there is a great wealth of knowledge on the role of exosomes within other solid cancers that remain useful in informing us of the potential role of exosomes in prostate cancer [Figure 1].

Prostate cancer exosomes have been shown to regulate angiogenesis, which may occur through exosome-mediated delivery of growth factors or RNAs. Prostate cancer exosomes have also been shown to further regulate the tumor microenvironment through

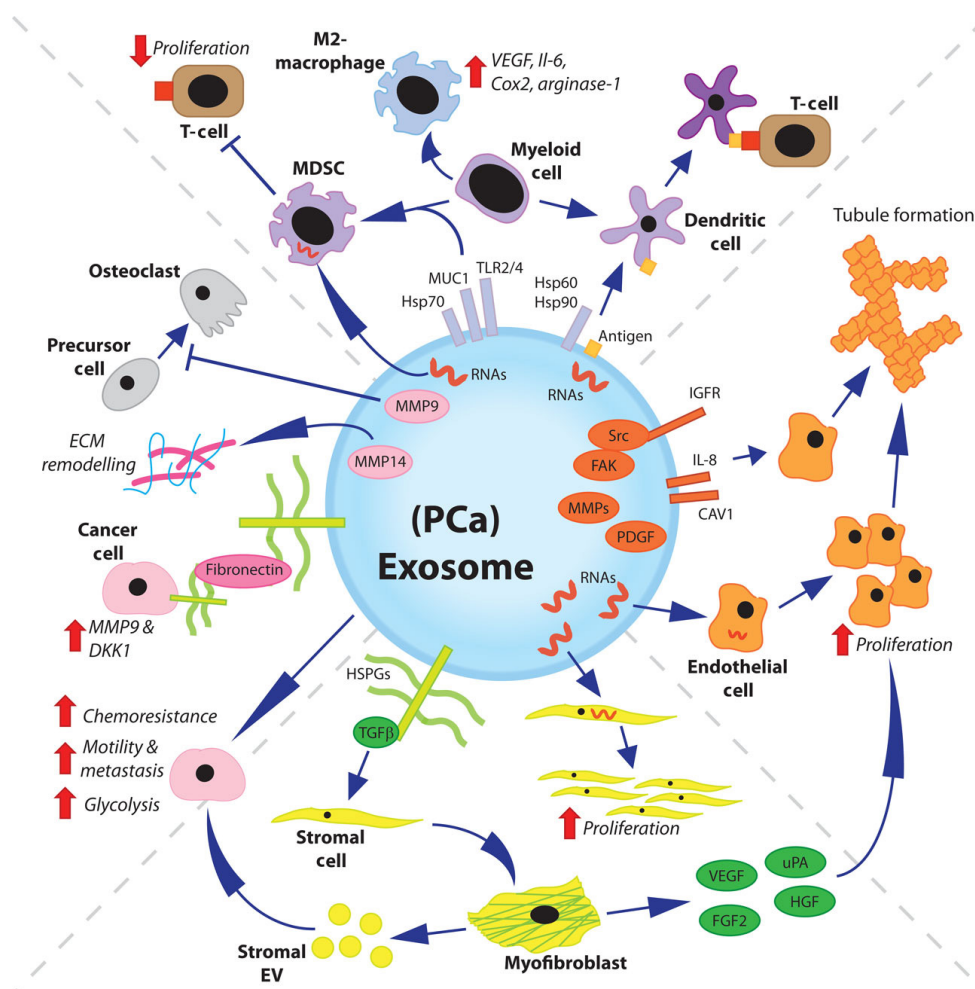


Figure 1: Overview of multiple roles of exosomes in prostate cancer. The previously described roles of prostate cancer exosomes are varied. Many other potential roles demonstrated for exosomes, and/or EV, from other cancer types may also be applicable to prostate cancer exosomes. Cancer exosomes can modulate the immune system. They can transmit tumor antigens to DC, or direct differentiation of myeloid cells towards MDSC/anti-inflammatory (M2) macrophage phenotypes. Exosome-mediated delivery of RNAs can induce endothelial cell proliferation, and exosome-associated proteins can induce endothelial tubule formation. Exosomal-TGF β can induce differentiation of stromal fibroblasts or MSC towards a pro-angiogenic and tumor supporting myofibroblast-like phenotype. Stromal cell-derived EV can transfer chemoresistance to cancer cells and modulate both cancer cell metastasis and metabolism. Disease progression is further enhanced by cancer exosomes, which have been shown to drive extracellular matrix remodeling and impair osteoclastic differentiation. EV: extracellular vesicles; DC: dendritic cells; MDSC: myeloid-derived suppressor cell; MSC: mesenchymal stem cell; VEGF: vascular endothelial growth factor; MMP: matrix metalloproteinase; FAK: focal adhesion kinase; IGFR: insulin-like growth factor receptor; Src: proto-oncogene tyrosine-protein kinase Src; FGF2: fibroblast growth factor 2; uPA: urokinase-type plasminogen activator; HGF: hepatocyte growth factor, PDGF: platelet-derived growth factor; TGF β : transforming growth factor beta

activation of stromal cells to a disease-supporting myofibroblast-like phenotype and may be capable of modulating myeloid cells, thereby regulating immune and inflammatory responses within the tumor microenvironment. There is sufficient evidence to suggest that exosomes are capable of regulating cancer cell metabolism and tumor metastasis, and are capable of transferring drug resistance from one cell to another. Such exosome-mediated effects, may impact tumor progression through direct or indirect mechanisms. Furthermore, it is not just cancer cell-derived exosomes, but also exosomes from other cell types within the tumor microenvironment, which may facilitate cancer progression. Whilst we may currently only be scratching the surface in terms of the possible

roles for exosomes in prostate cancer, it is clear that exosomes are present and actively contribute to the disease process.

It remains unclear why some men with prostate cancer have slow growing, indolent, tumors whilst others develop aggressive late stage disease that is resistant to treatment. There is therefore a growing demand for improved assays capable of predicting those men who are likely to develop aggressive disease. Due to the elevated secretion of exosomes from neoplastic cells, their altered cargo, and their presence within numerous biological fluids, there is substantial interest in the use of exosomes as biomarkers for both diagnostic and prognostic monitoring of disease. Methodologies for

isolation of exosomes from biofluids, such as urine and plasma^[129], already exist and early testing of exosomes as potential biomarkers of prostate cancer appear promising^[130]. Further studies are however required to validate the clinical utility of such assays and to fully understand the relationship between EV, biomarkers, and disease outcome.

DECLARATIONS

Authors' contributions

Conceived the study: A. Clayton, J.P. Webber

Performed literature searches and prepared the manuscript: A.P. Shephard, V. Yeung, A. Clayton, J.P. Webber

Revised the manuscript: J.P. Webber

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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Topic: How does the prostate cancer microenvironment affect the metastatic process and/or treatment outcome?

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Tumor heterogeneity and therapy resistance - implications for future treatments of prostate cancer

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ABSTRACT

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Aim: To develop new therapies for prostate cancer, disease heterogeneity must be addressed. This includes patient variation, multi-focal disease, cellular heterogeneity, genomic changes and epigenetic modification. This requires more representative models to be used in more innovative ways. **Methods:** This study used a panel of cell lines and primary prostate epithelial cell cultures derived from patient tissue. Several assays were used; alamar blue, colony forming assays, γ H2AX and Ki67 immunofluorescence and comet assays. Ptychographic quantitative phase imaging (QPI), a label-free imaging technique, combined with Cell Analysis Toolbox software, was implemented to carry out real-time analysis of cells and to retrieve morphological, kinetic and population data. **Results:** A combination of radiation and Vorinostat may be more effective than radiation alone. Primary prostate cancer stem-like cells are more resistant to etoposide than more differentiated cells. Analysis of QPI images showed that cell lines and primary cells differ in their size, motility and proliferation rate. A QPI signature was developed in order to identify two subpopulations of cells within a heterogeneous primary culture. **Conclusion:** Use of primary prostate epithelial cultures allows assessment of therapies whilst taking into account cellular heterogeneity. Analysis of rare cell populations and embracing novel techniques may ultimately lead to identifying and overcoming treatment resistance.

INTRODUCTION

Tumor heterogeneity and therapy resistance are

two sides of the same coin; because there is tumor heterogeneity, therapy resistance is inevitable. There are many different kinds of heterogeneity [Figure 1],



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and so developing new treatments is an increasingly complex task. Not only do we have to consider the differences between patients, giving rise to the need and hope of patient stratification, but we also have to consider that the tumor that has been biopsied may be the larger more detectable one but not necessarily the most aggressive one. Alongside that, we have our dependence on hormone treatments for metastatic prostate cancer, whilst knowing that there are tumor cell subpopulations that are not responsive or develop acquired resistance to those treatments^[1]. There is a poor choice of chemotherapy available for prostate cancer and it is typically a last resort, although some progress is being made in this area^[2]. Even with all these known variations, only in recent years has the true complexity of prostate cancers emerged^[3-6] with genomic and transcriptomic sequencing^[7] as well as clonal tracking^[8-10]. So, if we set out to test current treatments or develop novel therapies for prostate cancer, we must consider our current drug pipeline from bench to bedside; what models are used, do they take into account the different layers of heterogeneity and are they fit for purpose?

Here, we present a study that highlights the variation in results that can be acquired when using different cell line models, and also in comparison to primary prostate epithelial cells cultured from patient tissue. We consider how to tackle the cellular heterogeneity within tumors by assessing cell subpopulations rather than a heterogeneous mixture, as well as introducing a new technique that might be instrumental in assessing drug response whilst simultaneously taking into account cell heterogeneity.

METHODS

Culturing of cell lines

PNT1a, PNT2-C2 and LNCaP cells were cultured in Roswell Park Memorial Institute medium (RPMI) with 10% fetal calf serum. BPH-1 cells were cultured in RPMI with 5% fetal calf serum^[11]. PC3 cells were cultured in Hams-F12 media with 7% fetal calf serum. P4E6 cells were cultured in Keratinocyte Serum-Free medium (KSFM) with supplements (bovine pituitary extract 50 mg/mL and human recombinant epidermal growth factor 5 ng/mL) and with 2% fetal calf serum^[12]. To all media, Glutamine (2 mmol/L) was added. No antibiotics were used in the media. Cells were grown in an incubator at 37 °C in a humidified atmosphere containing 5% CO₂.

Culturing of primary prostate cells

Tumor tissue was obtained by targeted needle biopsy following radical prostatectomy. Following collection from the hospital, tissue was digested overnight in collagenase, followed by a trypsin digest. Primary prostate epithelial cells derived from patient tissue were cultured in stem cell media (SCM). SCM contains KSFM plus supplements (bovine pituitary extract 50 mg/mL and human recombinant epidermal growth factor 5 ng/mL) with the addition of 2 ng/mL stem cell factor, 100 ng/mL cholera toxin, 1 ng/mL granulocyte macrophage colony-stimulating factor and 2 ng/mL leukemia inhibitory factor. Cells were cultured on Biocoat collagen I 10 cm dishes with irradiated Sandoz inbred strain, thioguanine- and ouabain-resistance (STO) feeder cells. A detailed method of the whole procedure has been published^[13]. Patient samples used in this study are listed in Table 1.

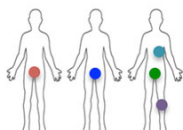
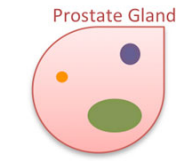
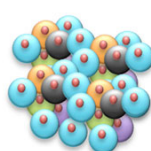
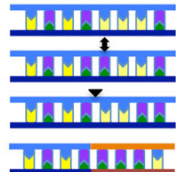
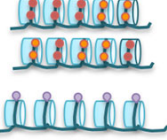
Types of heterogeneity				
Patient	Tumour	Cellular	Genomic	Epigenetic
	 Prostate Gland Tumours			 ● Methylated ● Acetylation ● Unmethylated
Different primary tumours in different patients. Different metastatic tumours in different locations.	Different tumours in the same prostate of a single patient (multi-focal disease). Recurrent tumours that have been altered or selected for by treatment.	Different cell types within each tumour mass. These range from stem cells to terminally differentiated cells.	Different mutations (small nucleotide polymorphisms, insertions, deletions or genome rearrangements).	Different methylation and acetylation patterns between the same genes in the same cells, between normal and cancer cells and between different cancer cells.

Figure 1: Heterogeneity in prostate cancer. When considering the task of improving current prostate cancer treatments or developing novel therapies, multiple types of heterogeneity have to be taken into account. These include patient tumor heterogeneity, multi-focal disease, intra-tumor cellular heterogeneity, genomic heterogeneity including mutations and gene fusions and finally epigenetic heterogeneity with inherent differences between cell populations but also the possibility of therapy-induced epigenetic changes

Selection of stem cells, transit amplifying cells and committed basal cells

Following trypsinisation of primary cultures, cells were first selected using collagen adherence. stem cells (SC) and transit amplifying (TA) cells are $\alpha 2\beta 1$ integrin^{hi} and committed basal (CB) cells are $\alpha 2\beta 1$ integrin^{lo}. A stringent selection of TA cells can be achieved with 5 min adherence to collagen I. Any non-adherent cells can be passed on to another plate, then any cells not adhered after 20 min are the committed basal cells. A slightly less stringent selection of TA cells can be achieved with a 20 min adherence where any non-adherent cells represent the committed basal population. This latter selection can be used when trying to achieve maximum stem cell ($\alpha 2\beta 1$ integrin^{hi}/CD133⁺) yield. To select stem cells, positive selection using a CD133 microbead kit (Miltenyi Biotec) was used^[14].

Ethics approval and patient consent

Patient samples were collected with ethical permission from Castle Hill Hospital (Cottingham, Hull) (ethics number: 07/H1304/121). Use of patient tissue was approved by the Local Research Ethics Committees. Patients gave informed consent and all patient samples were anonymized.

Alamar blue assay

The alamarBlue[®] reagent (ThermoFisher scientific) was used as an assessment of cell viability. Briefly, cells were plated at 5,000 cells per well in a 96-well plate and treated with drug. Radiation of cells was carried out prior to plating. The alamar blue assay was carried out 24-72 h post-treatment. Cells were in 200 μ L and a 1:10 dilution of alamar blue reagent was added. Fluorescence was measured on a plate reader 2 h after addition of reagent.

Table 1: Patient samples

Sample	Operation	Patient age (years)	Diagnosis
209/12 LA	RP	64	Normal
329/13 R	RP	53	Normal
434/14 LM	RP	68	Normal
048/11	RP	-	GI6 (3+3)
018/11	RP	-	GI7
035/11	RP	-	GI7 (3+4)
054/11	RP	58	GI7 (3+4)
665	RP	53	GI7 (3+4)
049/11	RP	-	GI7 (3+4)
087/11	RP	68	GI7 (3+4)
031/10	RP	-	GI7 (3+4)
034/11	RP	-	GI7 (3+4)
517/15 RM	RP	65	GI7 (3+4)
329/13 L	RP	53	GI7 (3+4)
209/12 RA	RP	64	GI7 (4+3)
545/15 LB	RP	69	GI7 (4+3)
307/13 LB	RP	65	GI7 (4+3)
545/15 RM	RP	69	GI7 (4+3)

RP: radical prostatectomy

Colony forming assay

Selected cells (SC and TA) were plated at 100-500 cells per well on a collagen I-coated 6-well plate and treated with 30 μ mol/L etoposide or an appropriate dilution of DMSO for 45 min at 37 °C, washed twice with phosphate buffered saline (PBS) and fresh SCM was added to each well. Cells were kept at 37 °C and SCM was changed every second day. An appropriate amount of irradiated feeder cells were added to keep the wells confluent. After 6-14 days SCM was removed and cells were washed once with PBS then stained with crystal violet (1% crystal violet, 10% ethanol in PBS) for 20 min, and after a final PBS wash, the number of colonies was determined. Colonies with < 32 cells and \geq 32 cells (5 population doublings) were counted. Colony forming assays were also carried out with radiation and Vorinostat treatment. For combination treatments, cells were treated with 0.625, 2.5 or 10 μ mol/L of Vorinostat for 30 min then treated with a range of radiation doses.

Treatments with radiation and drugs

An RS2000 X-Ray Biological Irradiator was used, which contains a Comet MXR-165 X-Ray Source (Rad-Source Technologies Inc. GA, USA). A range of radiation doses were administered with a dose rate of 0.02 or 0.08 Gy/s. Addition of Vorinostat (Cambridge Bioscience) was carried out at three concentrations: low, 0.625 μ mol/L; medium, 2.5 μ mol/L; high, 10 μ mol/L.

Comet assays

The comet assay was carried out as previously described^[15,16]. Briefly, drug-treated cells were resuspended in 25 μ L of PBS and mixed with 225 μ L of low melting point agarose. Following mixing, the cells and agarose were spread onto a glass slide that had been pre-coated with 1% agarose in PBS. A clean coverslip was placed on top until the cell-agarose mixture had set. Slides were placed in lysis buffer overnight and then incubated in alkaline solution for 40 min at 4 °C then electrophoresed at 23V/300 mA in the alkaline solution for 40 min on ice. This was followed by two washes in neutralising buffer. SYBR Gold was applied at a concentration of 1:10,000 in TE buffer to stain the DNA. Following collection of images on a fluorescent microscope (Nikon Eclipse TE300), comets were quantified using CometScore freeware (TriTek Corp, VA, USA).

Immunofluorescence

Immunocytochemistry was carried out to stain selected populations for DNA damage [γ H2AX - anti-phospho-Histone H2A.X (Ser139) clone JBW301, Millipore, UK], proliferation (Ki67 - ab15580, abcam) and a cell

marker (CD49b - anti-human CD49b:RPE, Serotec MCA743PET). Primary cells were plated at 10,000 cells per well in collagen I coated 8-well chamber slides or in the case of rare stem cells, all stem cells collected were plated on the slide. Staining of γ H2AX was carried out as described previously^[15]. Fixation for CD49b and Ki67 staining was with 4% paraformaldehyde, with no permeabilisation step when staining CD49b and with permeabilisation using 0.3% Triton X-100 for Ki67 staining. Alexa Fluor secondary antibodies (goat anti-mouse and goat anti-rabbit) with fluorescent tags were used at a concentration of 1:1000.

Flow cytometry

Flow cytometry was used to measure expression levels of CD49b on primary cells. All cell populations (WP, TA and CB) were harvested and resuspended in 300 μ L MACs buffer. Control (REA control (I)-APC) and target (CD49b-APC human clone REA188) antibodies were used (Miltenyi Biotec). Ten μ L of antibody was added and incubated with rotation for 10 min at 4 °C. Cells were washed, resuspended in MACs buffer and analyzed by flow cytometry including a cell only control to set gates.

Image capture using ptychography and image analysis using cell analysis toolbox

Quantitative phase imaging (QPI) was carried out using a VL21 Live Cell Imaging System (Phase Focus Limited, Sheffield, UK), which utilises a method known as ptychography in image formation. The high contrast images generated by the system are label-free and exempt from focal drift, allowing extended time-lapse imaging^[17-19]. The high-contrast nature of the images facilitates automated individual cell segmentation and tracking with the Cell Analysis Toolbox[®] software, which outputs extensive and specific feature measurements for each cell. As a result, data analysis can include information on cell populations in addition to individual cell information such as cell morphology and cell kinetics.

Statistical analysis

Alamar blue assays were performed in triplicate and data presented as % cell viability with percentage standard error. Significance calculations were carried out using the unpaired, nonparametric Mann-Whitney *U*-test. The *P* values indicating statistical significance are displayed (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.001).

RESULTS

A combination of Radiation and Vorinostat treatment on a panel of cell lines and on primary prostate cells

shows a varied response and reduces colony forming ability of primary prostate cells more effectively than either treatment alone.

A previous investigation had shown that prostate cancer stem-like cells were more radio-resistant than progenitor (TA) cells and more differentiated (CB) cells from primary prostate epithelial cells cultured from patient tissue^[15]. Pre-treatment with a low dose of a histone modifier, Trichostatin A, resulted in radio sensitisation of the stem-like cells, which was observed as an increase in DNA damage and a decrease in colony forming ability. To follow on from this, a clinically approved histone modifier, Vorinostat, was tested in combination with radiation treatment. First, a panel of cell lines including normal prostate (PNT1a), benign (BPH-1), localized cancer (P4E6) and metastatic cancer (PC3), were tested using alamar blue assays to measure viability following treatment with a combination of seven drug concentrations (0.156/0.3125/0.625 /1.25/2.5/5/10 μ mol/L) and six radiation doses (2, 5, 10, 25, 50, 75 Gy) with measurements taken at 24, 48 and 72 h. The percentage viability of each of the highest doses alone and in combination in the cell line panel is shown in [Figure 2A-C](#). There is a significant decrease in viability in all cell lines with the combination treatment compared to drug only, however the effect on PC3 cells is minimal, whilst the effect on the cell line derived from the localized cancer, P4E6, is most significant. Viability of primary prostate epithelial cell cultures (*n* = 6) was then measured following single and combination treatments [[Figure 2D and E](#)]. There was a significant reduction in viability with the combination treatment in normal and cancer cells, however the cancer cells showed less of a reduction in viability. We have previously shown that radiation can cause senescence of primary prostate epithelial cells rather than cell death, and so the small reduction in viability as measured by alamar blue could be because the cells are senescing rather than dying^[20]. Therefore, we tested the effect of three drug doses with and without 2 Gy of radiation on colony formation [[Figure 2F](#)]. As previously seen, 2 Gy of radiation results in a 50% surviving fraction. We used 0.625, 2.5 and 10 μ mol/L of Vorinostat, with and without 2 Gy radiation. Drug alone only reduced the surviving fraction by 10%-50% with patient variability observed. The combination treatments reduced surviving fraction by 65%-95%.

Cancer stem-like cells from patient tumor tissue are more resistant to etoposide than the progenitor cells due to a quiescent phenotype.

Previous studies had identified the cancer stem-like cells of primary prostate epithelial cell cultures

as being more radiation-resistant^[15]. One report showed that stem-like cells from the Du145 cell line were more resistant to etoposide^[21]. However, there is currently no experimental evidence determining the effect of chemotherapeutic agents specifically on primary prostate cancer stem-like cells. Therefore,

the colony forming ability of selected subpopulations of primary prostate epithelial cells, including stem-like cells and TA cells, were analyzed following treatment with etoposide [Figure 3A]. Cancer stem-like cells showed increased ability to form colonies compared to TA cells post-treatment. Since etoposide is known to

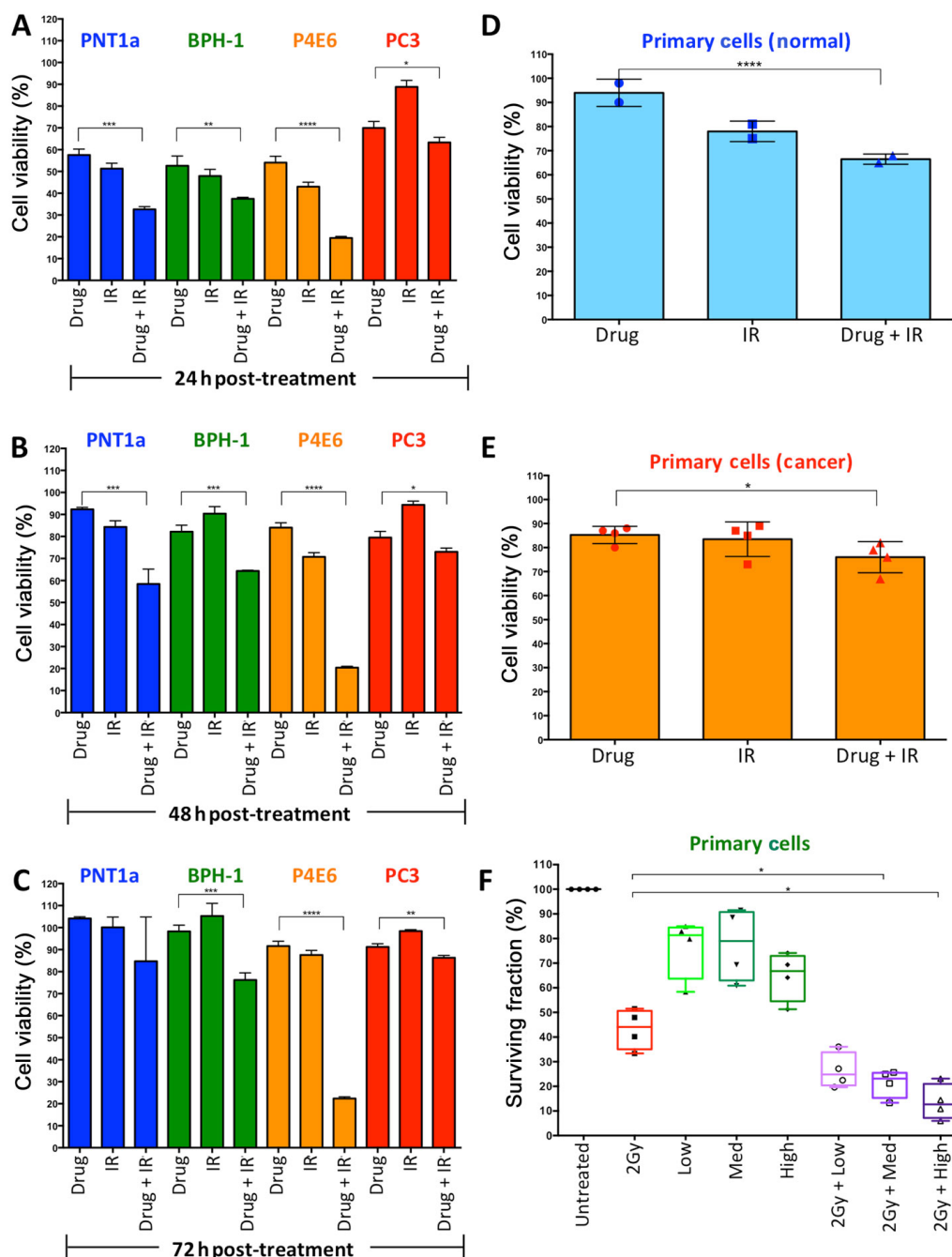


Figure 2: A combination of radiation treatment and Vorinostat has varied effects on viability and colony forming ability in a panel of cell lines and primary prostate epithelial cells. PNT1a, BPH-1, P4E6 and PC3 cells were treated with Vorinostat (10 $\mu\text{mol/L}$) or radiation (75 Gy) or both and measured using alamar blue assay at 24 h (A), 48 h (B) and 72 h (C) post-treatment. Primary epithelial cultures from six patients, normal (2 samples) (D) and prostate cancer (4 samples) (E) were treated with Vorinostat (10 $\mu\text{mol/L}$) or radiation (75 Gy) or both and measured using alamar blue assay at 24 h post-treatment. Each symbol represents a different patient sample; (F) primary epithelial cultures from four patients were treated with 2 Gy radiation or three concentrations of Vorinostat (low: 0.625 $\mu\text{mol/L}$, medium: 2.5 $\mu\text{mol/L}$, high: 10 $\mu\text{mol/L}$) or both and assessed for colony forming ability 10-14 days after growth. Colony forming ability is presented as % surviving fraction

cause DNA damage, this was measured in two ways, comet assays [Figure 3B] and γ H2AX foci [Figure 3C]. Both methods of measurement showed that stem-like cells sustained less DNA damage following etoposide treatment. Finally, Ki67 staining was carried out, and this indicated that TA cells were more proliferative (50%-90% Ki67-positive cells) than stem-like cells (10%-60% Ki67-positive cells), with patient variability being observed [Figure 3D].

Use of QPI to compare growth and proliferation of cell lines to primary prostate epithelial cells cultured from patient tissue.

What these results and previous studies have shown us is that the model that is used can strongly impact

the conclusions. This is why we advocate the use of a panel of cell lines, with an understanding of the origin of those cell lines, such that the relevance of the result can be best understood. Cell lines are excellent tools to establish methods and make an initial determination of mechanism of action and effectiveness of a compound. However, our hypothesis is that use of patient-derived primary prostate epithelial cell cultures is more clinically relevant and is more representative of intra- and inter-tumor heterogeneity^[13,15,22,23]. Cell lines are usually characterized by expression of certain markers, for example whether they are androgen receptor positive or negative^[24,25]. However, an alternative strategy to compare the different cell types might be to look at cell behavior. In order to do this we used a pychographic

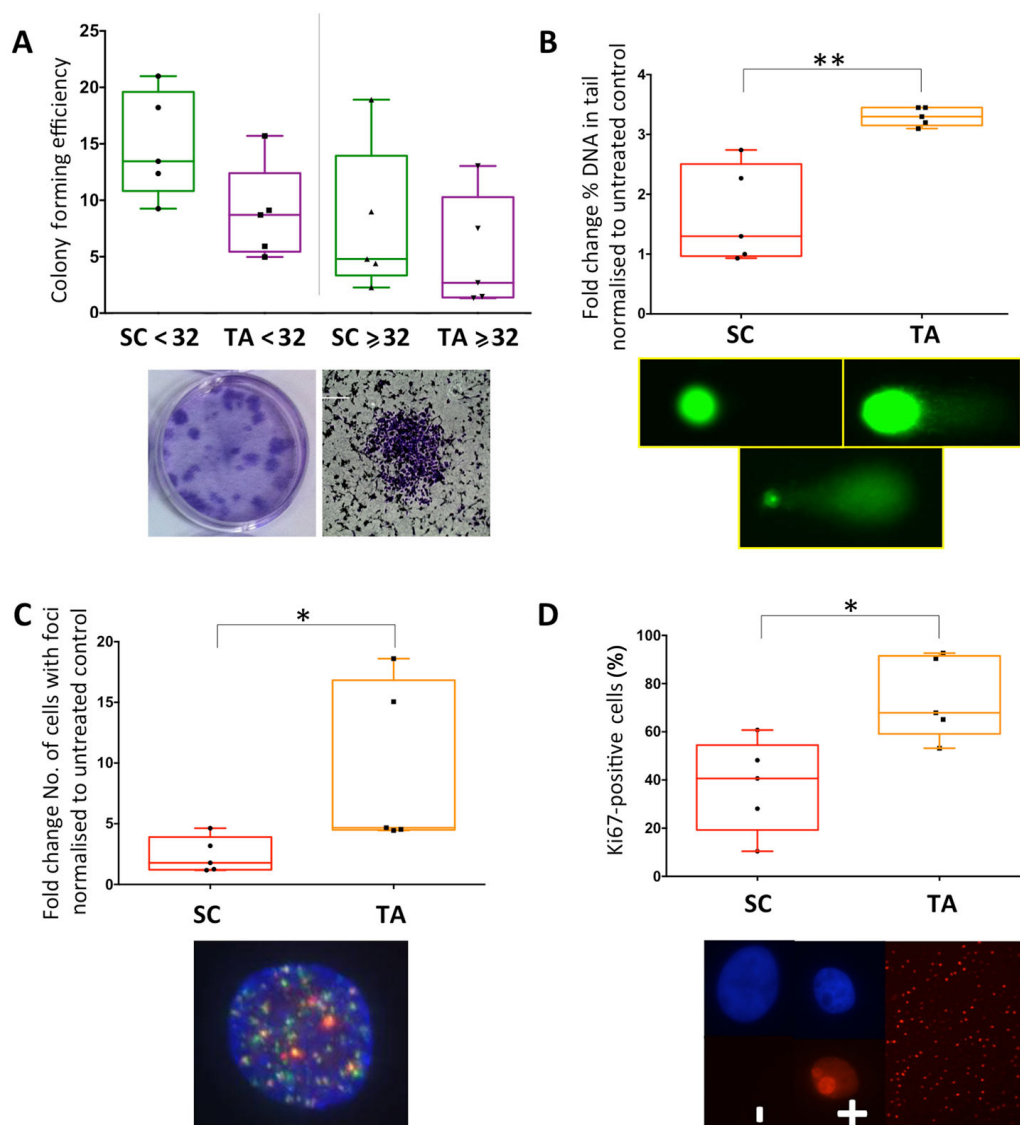


Figure 3: Prostate cancer stem-like cells (SC) sustain less DNA damage and form more colonies than progenitor cells following etoposide treatment, which correlates with less proliferation. Cancer SC and transit amplifying (TA) cells were selected from primary prostate epithelial cell cultures, treated with 30 μ mol/L of etoposide and assessed for (A) colony forming ability, (B) comet assay, (C) γ H2AX foci formation and (D) Ki67 expression. Each symbol represents a patient sample

QPI label-free imaging technique. We used a panel of cell lines from a variety of sources, PNT2-C2 (normal), BPH-1 (benign), P4E6 (localized cancer), PC3 (bone

metastasis), LNCaP (lymph node metastasis) and compared them to a primary culture [Figure 4A]. A 72-h time-lapse experiment was performed (images

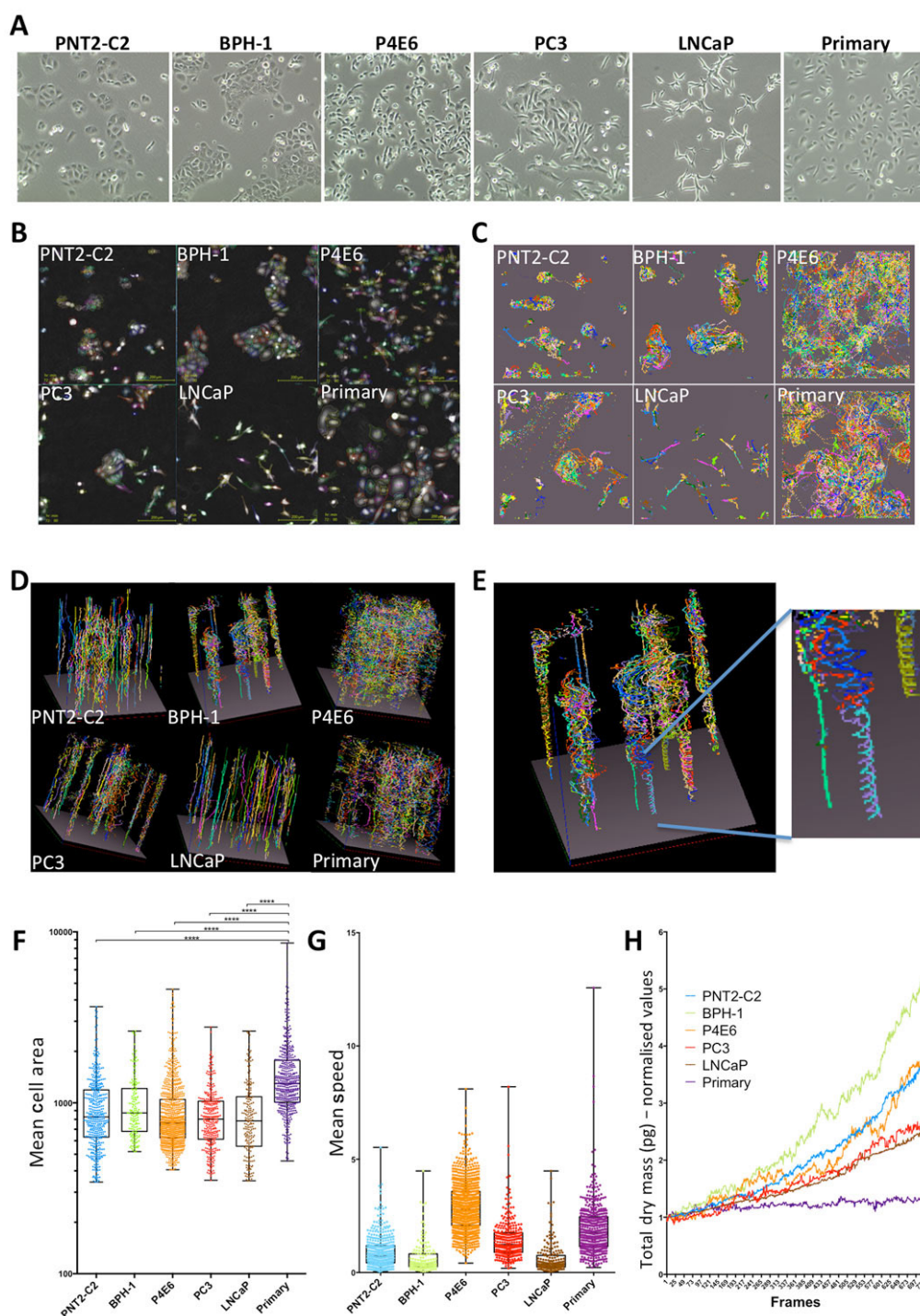


Figure 4: Label-free quantitative phase imaging (QPI) shows that primary prostate cultures divide less frequently than cell lines but undertake significantly more movement in 2D culture. A panel of prostate cell lines was grown alongside a primary prostate epithelial culture in a 6-well dish and time-lapse imaging was carried out. (A) Brightfield images of each cell type; (B) QPI images of each cell type with cell segmentation outlines (colored lines) and cell tracking ID (colored numbers) shown; (C) 2D representation of tracking of each cell type (X-axis, x position; Y-axis, y position); (D) 3D representation of tracking of each cell type (as for 2D but including a Z-axis, time); (E) close up view of the 3D rendition showing the trace of two cells spinning round each other; (F) mean cell area is plotted for each cell type. Each dot represents a single cell track; (G) mean speed is plotted for each cell type. Each dot represents a single cell track; (H) the total dry mass of each frame of the time-lapse video is plotted, which is indicative of cell growth and proliferation

collected every 6 min). Segmentation and tracking of every cell was carried out, during which each cell track is assigned an identification number [Figure 4B]. The movement of every cell was tracked and measured over time, and tracks were observed as 2D [Figure 4C] and 3D representations [Figure 4D]. The representations demonstrate that the automated tracking procedure used by the Cell Analysis Toolbox (CAT) software is capable of following individual cells. For example with BPH-1 cells a doublet of cells circled round and round each other, which is observed as a spiral over time [Figure 4E]. Morphological measurements (e.g. area, thickness, volume, radius, sphericity) and kinetic measurements (speed, displacement, meandering index) can be extracted from CAT. Of note, cells in the primary cultures are significantly larger on average than all the cell lines [Figure 4F]. In addition, most of the cell lines were much less motile than the primary culture apart from P4E6, which is the closest cell line to a primary culture [Supplementary Video 1]. Even though the primary cells are significantly more motile, which can be measured as mean speed of a track [Figure 4G], they actually show slower growth and proliferation than the cell lines. Indeed, there is a range of growth and proliferation rates in all cell lines measured. The growth and proliferation rate is one example of a unique QPI measurement that takes into account the whole population rather than individual cells and in this case is represented as total dry mass over time^[17] [Figure 4H].

Ptychographic label-free imaging can distinguish between cell populations in heterogeneous primary epithelial cell cultures.

Although ptychographic QPI can measure detailed morphological and kinetic measurements to distinguish between different cell populations, the power of the technique is to harness these individual cell measurements to take into account cell heterogeneity. We sought to determine whether QPI can distinguish between cell populations within a primary prostate epithelial cell culture. We already know that within these cultures, which have a predominantly basal epithelial cell phenotype, there are three subtypes; rare stem-like cells - $CD133^{+}/\alpha_2\beta_1\text{integrin}^{\text{hi}}$, TA cells - $CD133^{-}/\alpha_2\beta_1\text{integrin}^{\text{hi}}$ and CB cells - $\alpha_2\beta_1\text{integrin}^{\text{lo}}$. First, we enriched for TA and CB cells using rapid collagen adherence to select the TA cells (which also contains the rare stem cell population). Immunofluorescence staining highlights the high expression of $\alpha_2\beta_1\text{integrin}$ in TA cells and the low expression in CB cells [Figure 5A]. Staining of the whole population shows a mixture of cells with different fluorescent intensities. Staining the cells with CD49b and analyzing by flow

cytometry also shows the separation of the two populations [Figure 5B]. After selection, QPI was carried out [Figure 5C] and an analysis using the CAT was completed. A QPI signature was established for each cell type [Figure 5D and E], indicating that CB cells had a larger mass and size [Figure 5D and E] than the TA cells. The TA cells had a higher value relating to cell sphericity compared to the CB cells [Figure 5F]. Significantly, once these parameters were established, a heterogeneous (unselected) culture of primary prostate epithelial cells was analyzed. The area measurement from the ptychographic signatures of each cell type was applied to the images of the mixed culture and the software was able to identify TA and CB cells within the culture [Figure 5G].

DISCUSSION

These studies highlight that the use of a single cell line is insufficient to make a conclusion about efficacy and mechanism of action of a treatment. In addition, using a panel of cell lines may also not be a great improvement because results from experiments in cell lines have been seen here and in other studies to be quite different from primary cells^[26-28]. Cells in primary cultures have compensatory signaling pathways that have been lost in cell lines, and so an inhibitor that works well in cell lines may be less effective or ineffective in primary cultures^[28]. This is one explanation for such high attrition rate in the drug pipeline; weak, incomplete, unrepresentative or inappropriate models. Also, it has previously been shown that the DNA methylation profile is quite different in cell lines compared to primary cells and indeed between different primary cell subpopulations^[29,30], thus impacting how cells respond to various treatments. Indeed, epigenetic changes can also be induced in response to treatments such as radiation, which relates to radioresistance and radiosensitization^[31]. The use of primary cells from both normal and cancerous patient tissue as part of the drug pipeline may be at least part of the solution. Indeed, use of this model, *in vitro* primary cell culture, was critical in the development of an oncolytic adenovirus for prostate cancer^[32-34], which is currently in clinical trials.

Results presented here and previous studies looking at cancer stem-like cells^[15] suggest that a combination treatment of Vorinostat and radiation may be more effective in treating prostate cancer than radiation alone. Since Vorinostat is already clinically-approved^[35], a move to clinical trials for the combination treatment could be swift. However, before this could happen, a prognostic indicator and/or a measurement output, other than overall survival,

to show any differential response of the combination treatment would be required. This could be something similar to the PORTOS score; predictor of response

to postoperative radiotherapy in prostate cancer^[36]. Alternatively, a pre-treatment prognostic gene signature could be of use to decide which patients

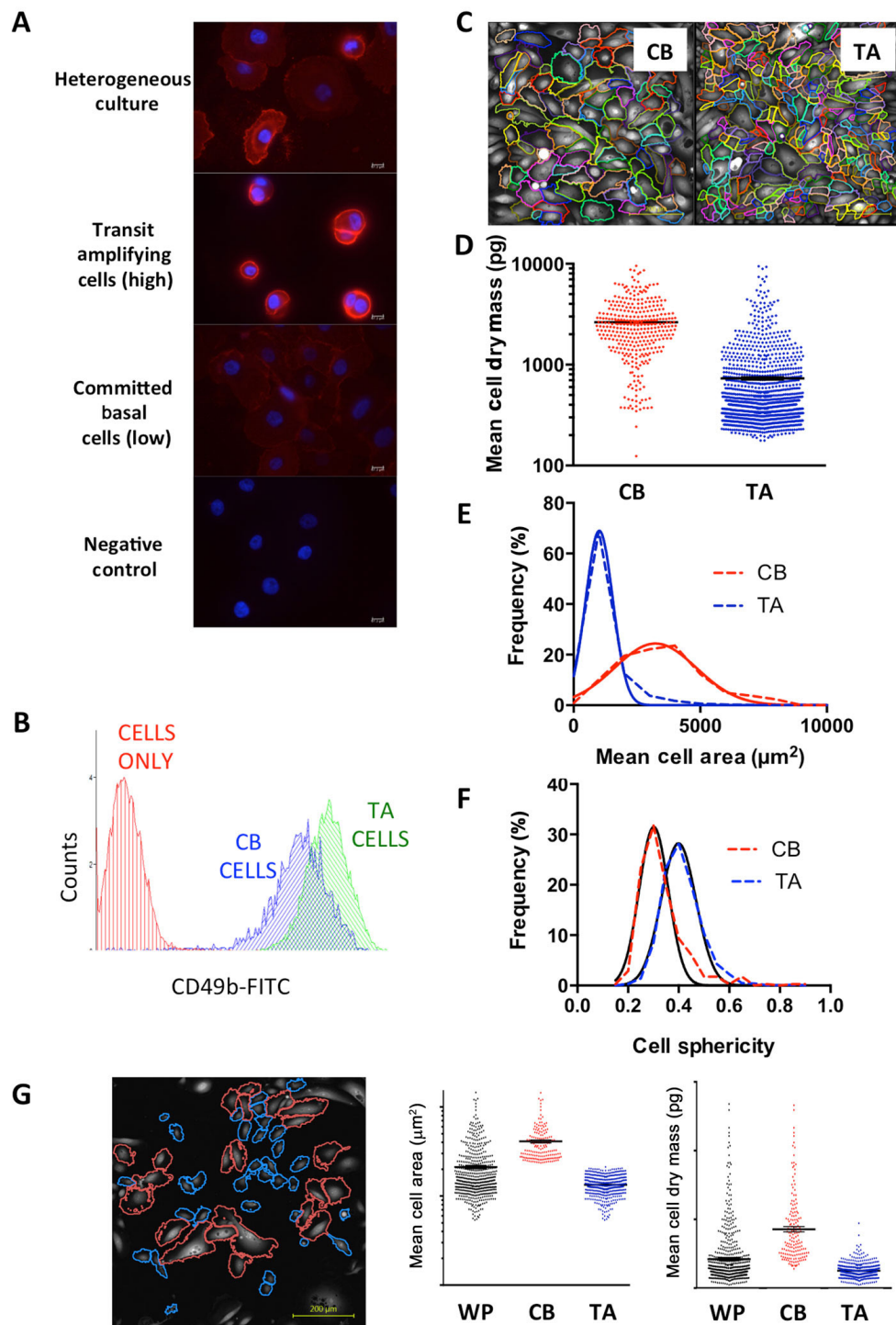


Figure 5: Signatures of two populations of cells within primary prostate cultures can be characterized from quantitative phase imaging (QPI) data and used to identify different cell populations within heterogeneous cultures. (A) Immunofluorescence of CD49b in a mixed culture of cells, transit amplifying (TA) cells and committed basal (CB) cells; (B) flow cytometry of TA and CB cells using the CD49b surface marker; (C) QPI images of TA and CB cells showing cell segmentation outlines (colored lines). Data from QPI analysis of each cell type was measured including (D) mean cell dry mass, (E) mean cell area and (F) cell sphericity; (G) analysis of a mixed culture of cells with gates applied to separate out the two cell populations on the basis of cell area. Data from the whole population (WP) and each cell type was measured and plotted as mean cell area and mean cell dry mass

would benefit from a new combination treatment^[37]. We carried out a preliminary analysis to measure gene expression, using “DNA damage signaling pathway” polymerase chain reaction arrays, in primary cultures. Cell subpopulations (SC/TA/CB) selected and enriched from primary cultures derived from different disease states (benign prostatic hyperplasia, Gleason 7 prostate cancers and high Gleason prostate cancers) were used, both untreated and treated with radiation (2 Gy). The results illustrated variation between patients, between disease state, and between each cell type (SC, TA, CB). Exploring the heterogeneity of gene expression between disease states and between cell types with and without treatment may ultimately lead to novel drug targets being exploited^[38,39].

This is the first report of chemotherapy resistance of cancer stem-like cells from primary prostate epithelial cultures. This study only shows the resistance to etoposide, however we anticipate that this would also be true for other chemotherapeutic drugs that

act as cell cycle inhibitors since it appears that the reduced proliferation rate of the stem cells is acting as a resistance mechanism. This result also highlights the need to enrich and/or sort for subpopulations of cells within the patient cell cultures^[22] to observe the response of rare populations of cells, since they can be masked when looking at the whole population.

The use of QPI illustrates behavioral differences between cell lines and primary cells. By making measurements encompassing morphological, kinetic and population data a cell signature for each cell type can be established. One significant observation is the larger size of primary cells. Also, the different growth and proliferation rates of the cell lines and primary cells will impact the length of time for drugs to take effect. In addition, it will be of interest to explore the meaning behind the increased cell motility of the primary cultures. Since, ptychography is able to identify heterogeneous populations within a culture, the hope for this technique is to use analysis post-treatment to

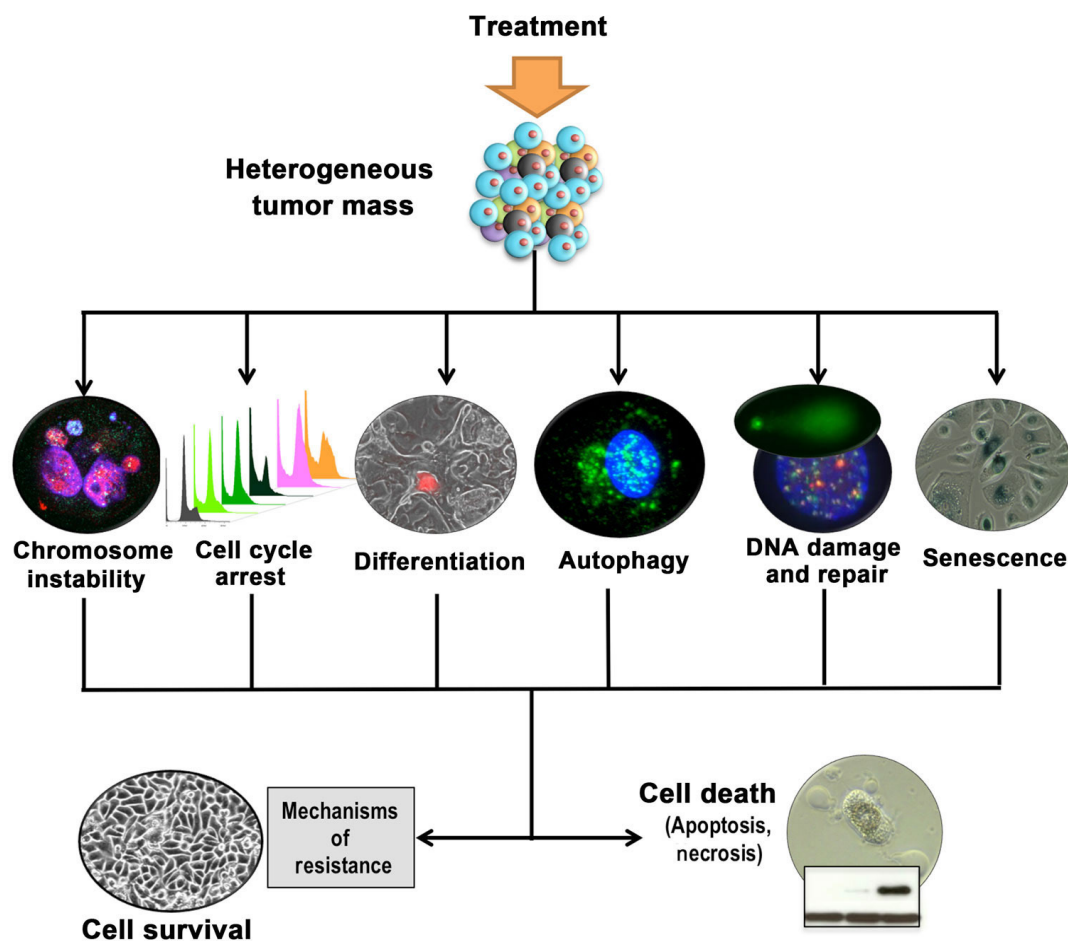


Figure 6: Consequences of treatment; paths to resistance or death. Inducing cell death in cancer cells is not a single pathway and initial treatments may push cells to many different outcomes. To overcome cell thresholds and safeguards and push cells towards cell death, other stimuli may be required. This could involve sensitizers to make the initial treatment more effective or it could include inhibitors to prevent activation of cell survival mechanisms. Heterogeneity of response dictates that combination treatments are likely to be more effective

observe cell behavior in real-time and to detect any inherently resistant cells within the heterogeneous primary cultures.

Going forward, using primary prostate epithelial cultures, as part of the lab to clinic pipeline, has many advantages including patient variation, current and follow-up pathology, correlation with patient outcome, representation of modern disease, close-to-patient, clinically relevant and less adapted to tissue culture conditions than cell lines. The model is also flexible since the cultures with typically a basal phenotype can be pushed to differentiate and express luminal markers^[40,41] and 3D spheroid culture is also possible^[42]. Even in the 2D culture, microenvironment studies can be carried out using the STO feeder cells as a stromal mimic. This technique has been used to elegant effect, where STO feeder cells were engineered to express human IL-4, and this resulted in an increase in clonogenic potential of primary prostate epithelial cells through the STAT6 signaling pathway^[43].

Several recent studies using primary prostate epithelial cultures have shown the heterogeneity of response to current and novel treatments including autophagy^[20,26], necrosis^[27], cell differentiation^[44,45], apoptosis, DNA damage^[15,27], cell cycle arrest and senescence^[20] [Figure 6]. Several of these can act as a crossroads for a cell resulting in cell survival or cell death and if we are able to predict which response may occur then we may be able to manipulate it towards cell death. It is not appropriate to totally rely on endpoint assays; we cannot be satisfied with a 90% reduction in cell viability without questioning what is happening in the other 10% of cells, and indeed characterizing these cells relative to the whole population. If we can identify mechanisms of resistance in bulk populations as well as rare cell populations we will be more able to design biologically relevant combination treatments. In addition, there shouldn't be too much reliance on a single model. All models have their advantages and limitations; the important thing is to acknowledge them rather than to ignore them. In terms of primary prostate cultures, heterogeneity provides an advantage to testing therapies rather than a confounding factor. If we are able to use techniques such as QPI to measure each individual cell as a data point we should be able to tease apart the variation in cell responses to different treatments as well as identifying and characterizing resistant cells. Ultimately, the hope is that this could lead to more targeted use of current drugs as well as better testing of novel treatments prior to clinical trials.

DECLARATIONS

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Authors' contributions

Conceived the overall study and wrote the manuscript: F.M. Frame

Designed the graphics in Figures 1 and 6 and executed the work in Figure 2: F.M. Frame

Conceived and executed the experiments in Figures 4 and 5: F.M. Frame, A.R. Noble

Provided technical expertise integral to completion of the experimental works: H.F. Walker

Conceived and carried out the data in Figure 3: S. Klein

Provided technical expertise for the Quantitative Phase Imaging work: R. Suman, R. Kasprovicz

Arranged permission, collection and delivery of patient samples: V.M. Mann

Provided patient material and be the liaison between the laboratory and the clinic: M.S. Simms

Oversaw all work and was awarded the funding for this study: N.J. Maitland

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Conflicts of interest

RK and RS are employees of PhaseFocus Ltd. This company has provided the VL21 microscope for use by FMF and AN. RK and RS have provided technical knowledge and support.

Patient consent

Patients gave informed consent and all patient samples were anonymized.

Ethics approval

Patient samples were collected with ethical permission from Castle Hill Hospital (Cottingham, Hull) (Ethics Number: 07/H1304/121). Use of patient tissue was approved by the Local Research Ethics Committees.

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Topic: How does the prostate cancer microenvironment affect the metastatic process and/or treatment outcome?

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Membrane-type matrix metalloproteinases: expression, roles in metastatic prostate cancer progression and opportunities for drug targeting

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ABSTRACT

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The membrane-type matrix metalloproteinases (MT-MMPs), an important subgroup of the wider MMP family, demonstrate widespread expression in multiple tumor types, and play key roles in cancer growth, migration, invasion and metastasis. Despite a large body of published research, relatively little information exists regarding evidence for MT-MMP expression and function in metastatic prostate cancer. This review provides an appraisal of the literature describing gene and protein expression in prostate cancer cells and clinical tissue, summarises the evidence for roles in prostate cancer progression, and examines the data relating to MT-MMP function in the development of bone metastases. Finally, the therapeutic potential of targeting MT-MMPs is considered. While MT-MMP inhibition presents a significant challenge, utilisation of MT-MMP expression and proteolytic capacity in prostate tumors is an attractive drug development opportunity.

INTRODUCTION

Several decades of research have established the matrix metalloproteinases (MMPs) as key players in the progression of cancer, largely through alterations observed in the tumor microenvironment. Indeed, MMPs have been considered as potential diagnostic

and prognostic biomarkers for many types and stages of cancer^[1]. Despite their potential as therapeutic targets, however, a clinically useful agent has yet to materialise, despite several MMP inhibitors having been developed and evaluated in clinical trials^[2]. More recently, attention has turned to the potential utility of MMPs as activators of targeted prodrug therapies^[3-5].



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Table 1: MT-MMP nomenclature

Gene	Protein
<i>MMP-14</i>	MT1-MMP
<i>MMP-15</i>	MT2-MMP
<i>MMP-16</i>	MT3-MMP
<i>MMP-17</i>	MT4-MMP
<i>MMP-24</i>	MT5-MMP
<i>MMP-25</i>	MT6-MMP

MT-MMP: membrane-type matrix metalloproteinases

The MMP family of proteolytic enzymes comprises over 26 structurally similar zinc-dependent endoproteases. The wider family comprises two major subgroups: (1) the soluble or secreted MMPs and (2) the membrane-type MMPs (MT-MMPs). The MT-MMPs are further sub-classified by their cell surface association, either by a transmembrane domain (as is the case for MT1-, MT2-, MT3-, and MT5-MMP) or by a glycosylphosphatidylinositol anchor (in MT4- and MT6-MMP). The nomenclature for the MT-MMPs is outlined in Table 1: the gene and protein names are generally used interchangeably. The structures of the MT-MMP family have been well described^[6]. They are each synthesised as inactive pre-pro enzymes in the Golgi apparatus, with cleavage of the signal peptide and pro-domain required before transport to the cell surface.

The roles played by many MMPs in wider cancer initiation, progression and metastasis have been extensively explored, with pivotal roles described in prostate cancer in particular^[7]. Expression and the roles played by the MT-MMP family in prostate cancer are less well studied, however, and it is this area that is the focus of this review. The available evidence for expression of MT-MMPs in prostate cancer cells and clinical tissues will be examined first, followed by consideration of their roles in prostate cancer function and in metastasis to the bone.

EXPRESSION OF MT-MMPs IN PROSTATE CANCER CELL LINES

A significant body of literature exists concerning the expression of MT1-MMP in prostate cancer cell lines, albeit limited almost exclusively to the widely studied PC3, DU145 and LNCaP cell lines. Prostatic adenocarcinoma cells PC3 (derived from a bone metastasis) and DU145 (derived from a brain metastasis) are androgen-insensitive and metastatic (PC3 being the more aggressive of the two), while LNCaP cells (prostatic adenocarcinoma cells derived from a lymph node metastasis) are androgen-sensitive cells and non-metastatic^[8].

MT1-MMP gene expression has been consistently

reported in the androgen-insensitive, more metastatic cell lines PC3 (and sublines PC3-M and PC3-MM2^[8]) and DU145. Expression has also been reported in TSU-Pr1 cells^[9,10], also androgen-insensitive. Meanwhile, the less aggressive androgen-sensitive cell line LNCaP (and sublines LNCaP-C4 and LNCaP-C4-2^[8]) exhibit low or an absence of *MT1-MMP* gene expression^[8,10-13]. Daja *et al.*^[8] explain that despite these differences in gene expression, active MT1-MMP protein expression was identified in both LNCaP and PC3 cells and their sublines. Furthermore, Jennbacken *et al.*^[14] demonstrated that transformation of LNCaP into an androgen-independent cell line (i.e. LNCaP-19^[15]) was accompanied by increased aggressiveness (growth and migratory capacity) and by upregulation of both MT1-MMP gene and protein expression. The influence of the tumor microenvironment, specifically fibroblasts (WPF5), was investigated on PC3 and DU145 cells in a study by Coulson-Thomas *et al.*^[16]. An increase in MT1-MMP gene and protein expression was reported in co-cultures of WPF5 and PC3 or DU145 cells. Protein was localised at the cellular projections of all cell lines. When considered together with changes in vimentin distribution and an up-regulation of integrin $\alpha 5 \beta 1$ expression, this is indicative of a more invasive phenotype.

Information regarding the remaining members of the MT-MMP family is more scarce. Interestingly, in contrast to the picture observed with *MT1-MMP*, *MT2-MMP* gene expression has been reported as more significant in LNCaP cells and sublines than in PC3 cells. The pattern of *MT3-MMP* gene expression is the opposite to this, and thus similar to that observed for MT1-MMP^[8]. Protein levels were similar in both cell lines, however. Processed MT-MMPs, indicative of latent MMP activation, were observed to be increased in the more aggressive sublines. Jung *et al.*^[11] described significant gene expression of MT2-MMP and MT5-MMP in both cell types. MT3-MMP expression was observed in LNCaP cells with negligible expression in PC3 or DU145 cells. Meanwhile, MT4-MMP expression was observed in PC3 and DU145, with negligible expression in LNCaP cells.

EXPRESSION OF MT-MMPs IN PROSTATE CANCER CLINICAL TISSUES

The clinical expression of secreted MMPs in prostate cancer has been well reviewed by Gong *et al.*^[7], with expression of MMP-2, -3, -7, -9 and -13 gene and protein each identified in serum and tumor tissue of patients with prostate cancer, and correlation with progression and metastasis observed. Interestingly,

MMP-1 expression has been associated with lower grade prostate tumors. In this section, we have summarised the clinical expression data available for MT-MMPs in prostate cancer tissues.

Trudel *et al.*^[17] examined tissues from 189 prostate cancer patients who had undergone surgery (radical prostatectomy). MT1-MMP expression and its effects on disease-free survival were examined immunohistochemically, differentiating cancer, stromal, and benign epithelial cells. This study showed that in 167 (88.8%) cases, MT1-MMP was expressed by benign epithelial cells and MT1-MMP was expressed by cancer cells in 171 (90.5%) cases. The expression in cancerous tissue was mostly observed in cells at or near the tumor front. Overall the expression was described as heterogeneous though cancer cells at the tumor margin were seen to always express MT1-MMP. The expression of MT1-MMP in benign epithelial cells was somewhat unexpected, given what is generally understood about MMPs in other cancers. The authors did raise the issue of the quality and specificity of commercial antibodies for MT1-MMP, and did further suggest that active MT1-MMP could be cleaved by other MMPs resulting in soluble fragments, which may have been detected. It is the potential effect of prostate cancer cells on the local microenvironment that is perhaps the key, however. The authors speculated as to whether the presence of MT1-MMP in benign epithelial cells (near cancerous tissue) from these patients might in fact be induced by the cancer. A study by Paterson *et al.*^[18] in bladder cancer was cross-referenced, which had further suggested that the so-called “benign” epithelial cells in those patients were in fact not benign at all, but genotypically abnormal. These findings have been encountered by others and are worthy of further study.

Discrepancies between gene expression (higher MT1-MMP expression in benign prostate hyperplasia and prostate cancer tissues, when compared to normal prostate) and protein expression (lower expression in prostate cancer tissues compared to normal prostate and benign prostate hyperplasia) were noted by Neuhaus *et al.*^[19]. These results contrast with most other published studies, which led the authors to speculate that cells of the prostate interstitium (included in assessment of total immunofluorescence due to MT1-MMP) may have increased protein expression and may account for the apparent levels of protein expression seen in epithelial cells.

Cardillo *et al.*^[20] analysed 38 paraffin-embedded samples from prostate cancer patients (who had undergone radical prostatectomy) by

immunohistochemistry and compared prostate intraepithelial neoplasia (PIN) and its normal adjacent prostate (NAP) counterpart. MT1-MMP was observed to be more strongly expressed in tumor tissue than in PIN and NAP tissue, with the expression of MT1-MMP reaching its highest levels in the most aggressive prostate tumors with high Gleason scores (Gleason scores are used to grade prostate cancer, with a score above 7 indicative of aggressive, metastatic disease). Once again, expression was detected in surrounding stroma and epithelia, backing up the findings of Trudel *et al.*^[17]. The authors speculate that stromal and tumor cells could co-operate in facilitating tumor cell invasion, hence the requirement for MT1-MMP expression, and that transition from benign epithelium via PIN to cancer is associated with changes in localisation of MT1-MMP in the prostate epithelium^[20].

In a further 40 patients, Reis *et al.*^[21] also monitored tissue inhibitor of matrix metalloproteinase 1 (TIMP-1) expression together with that of MT1-MMP. TIMPs inhibit some MMPs and other protease enzymes, but not MT1-MMP^[22]. The loss of TIMP-1 protein expression was correlated to cancer progression, with MT1-MMP protein expression being identified in the majority of prostate cancer specimens. Once again, a positive correlation with Gleason score was observed^[21]. This reinforced an earlier study by the same group (79 prostate cancer patients), which indicated that MT1-MMP expression was higher in patient samples with Gleason scores ≥ 7 , though that dataset only exhibited marginal statistical significance^[23].

Upadhyay *et al.*^[24] investigated the relationship between the MT1-MMP and MMP-2 expression, with immunohistochemistry confirmed by western blotting and gelatin zymography. A significant correlation between the pattern of MMP-2 and MT1-MMP expression within the epithelial components of individual specimens was observed. Differential staining was seen between benign epithelia, high-grade PIN, and prostate cancer. In benign glands, the greatest expression for MT1-MMP was in basal cells (BCs), whereas secretory cells were rarely positive. Conversely in high-grade PIN, secretory cells showed consistent cytoplasmic staining. In cancer cells, staining was heterogeneous and varied from no staining to very intense staining in select glands^[24].

High expression of insulin-like growth factor-1 receptor (IGF-1R) in prostate cancer was identified by Sroka *et al.*^[25], who suggested that using MT1-MMP localization and IGF-1R expression may serve as a predictive biomarker of aggressive disease. MT1-MMP expression was high in the apical regions of the

luminal cells in PIN and prostate cancer cells, though less intense in the basolateral regions of benign tissues. IGF-1R was expressed primarily in the basal cells of normal glands and highly expressed in prostate cancer tissues^[25].

As is the case with studies on cell lines, the majority of clinical reports focus on expression of MT1-MMP. Both Jung *et al.*^[11] and Riddick *et al.*^[26] compared a wider range of MT-MMP family member gene expression in paired tissue samples from non-malignant and malignant parts of the same prostate cancer patient biopsies, using real-time polymerase chain reaction (PCR). Interestingly, Jung *et al.*^[11] identified a significant down-regulation for all investigated MT-MMPs except for MT2-MMP in malignant tissue and did not detect a correlation between tumor classification and MT-MMP expression. Riddick *et al.*^[26] investigated *MT-MMP* gene expression in 44 prostate cancer cases and 23 benign prostate hyperplasia specimens, also by real-time PCR. This study additionally found increased gene expression of MT2-MMP, MT5-MMP and MT6-MMP in malignant tissue compared to benign prostate tissue, and suggested that these proteases are likely to participate directly in prostate tumor invasion. It is important to note that MT6-MMP was primarily expressed by the epithelial cancer cells rather than stromal cells. The lack of difference in MT1-MMP expression between malignant and local non-malignant tissue perhaps provides further evidence for the conclusions previously discussed^[17,18,20].

The evidence for MT-MMP expression, and particularly MT1-MMP expression, in prostate cancer cells and tumors from patients is compelling. The picture is complicated by the involvement of the tumor microenvironment, in which MT-MMP expression is influenced, perhaps initiated, by the development of cancer. In the following section, the roles of individual MMPs are considered, along with links to other pathways known to be important in prostate cancer progression and metastasis.

FUNCTIONS OF MT-MMPs IN PROSTATE CANCER PROGRESSION AND METASTASIS

It is widely accepted that MT-MMPs play key roles in the metastatic process^[27]. With regards prostate cancer, MT-MMPs have been identified as contributing towards apoptosis, angiogenesis, proliferation and metastasis^[7]. MT-MMPs have been shown to be involved in various molecular processes in prostate cancer progression and metastasis, which will now be considered here, together with information regarding

potential regulatory pathways. Some of the molecular events associated with MT-MMP expression and function in tumor cell migration, angiogenesis and vascular signalling are summarised in Figure 1^[28].

MT1-MMP plays a role in epithelial-to-mesenchymal transition (EMT). EMT is an important process in the metastatic cascade, involving multiple oncogenic drivers^[29]. Cao *et al.*^[30] initially used DNA microarray database mining to reveal upregulation of MT1-MMP in human primary and metastatic prostate cancer samples. Using 3D cell culture models, the study additionally demonstrated that transformation of LNCaP cells with MT1-MMP induced morphological changes and modulation of epithelial and mesenchymal markers consistent with EMT, and thus metastatic transformation. Further experiments demonstrated that these MT1-MMP-induced phenotypic changes were linked to Wnt5a, also associated with EMT^[31]. These findings are supported by the aforementioned study by Jennbacken *et al.*^[14], which similarly described E-cadherin downregulation and N-cadherin upregulation (both consistent with EMT) in the androgen-independent LNCaP-19 cell line, accompanied by increased MT1-MMP.

Degradation of the extracellular matrix (ECM), and specifically laminin-10 (Ln-10), was explored by Bair *et al.*^[32]. Laminins are key glycoprotein components of the ECM: providing structural support to the basal lamina in both normal prostate and malignant tissue. The authors point to evidence previously published describing upregulation of Ln-10 as prostate cancer progresses from normal to PIN through to invasive cancer, suggesting a role for MT1-MMP in the invasion of prostate cancer^[33]. Here, recombinant MT1-MMP (and MT1-MMP-expressing cells and tissues) was shown to cleave the $\alpha 5$ chain of purified human Ln-10 from its 350-kDa form into specific fragments. This cleavage was shown to decrease cell adhesion to purified Ln-10, and to increase transmigration of DU-145 cells through cleaved Ln-10 and thus the basal lamina. Increased invasion mediated by MT1-MMP was also observed by Wang *et al.*^[34]. Using cells engineered to overexpress MT1-MMP (namely PC3-LN4), invasion into type-I collagen gels *in vitro* was observed, through activation of pro-MMP2. PC3-LN4 cells additionally proliferated at a faster rate than mock-transfected control cells when grown subcutaneously in nude mice.

Endo180 (uPARAP, urokinase-type plasminogen activator receptor-associated protein) regulates collagen remodelling and chemotactic cell migration through cooperation with MT1-MMP. A study by Kogianni *et al.*^[35] describes how Endo180 is

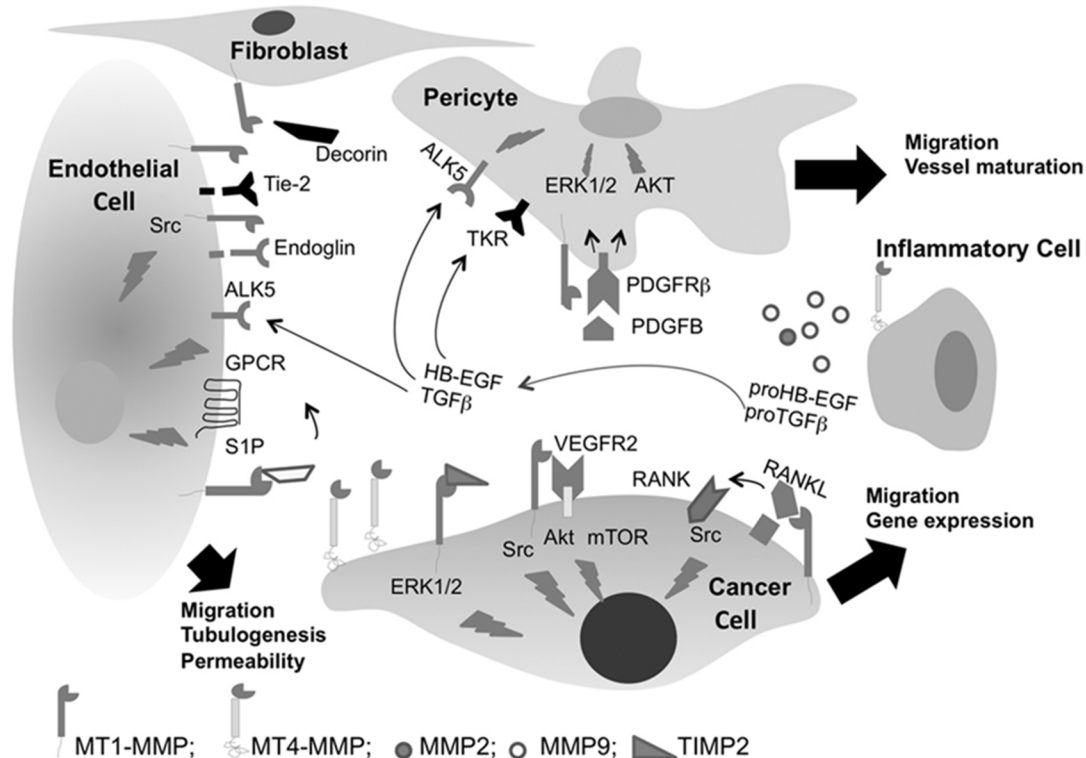


Figure 1: Membrane type-MMPs regulate cell signalling in cancer. MT1-MMP regulates cell migration through both ECM proteolysis and non-proteolytic-dependent TIMP-2 activation of ERK1/2 pathway. MT1-MMP regulates VEGF gene expression through Src, Akt, and mTOR activation and stimulates tumor angiogenesis. Roles for MT-MMP expression are also associated with TGFβ, Tie-2 and PDGFRβ. Figure was originally published by Sounni *et al.*^[28]. MT-MMP: membrane-type matrix metalloproteinases; ECM: extracellular matrix; VEGF: vascular endothelial growth factor

positively correlated with prostate cancer clinical risk and suggests that it is a stronger predictor of Gleason score than serum PSA. Endo180 disrupts epithelial cell contact and plays a potential role in EMT in prostate cancer. Endo180 and MT1-MMP co-expression was identified as strongly upregulated in the stroma of prostate cancer with low clinical risk, indicating that tumor-associated stromal fibroblasts can acquire the ability for effective collagen degradation and internalisation at the early stages of tumor development. These findings are interesting and add further support to prostate cancer cells influencing MT1-MMP expression in their surrounding microenvironment.

The transcription factor p53 is known to play significant roles as a tumor suppressor in cancer progression. Wang *et al.*^[36] considered the involvement of p53 in prostate cancer cell invasion and metastasis, using DU145 cells in which p53 was silenced by siRNA. Increased invasion and metastasis were observed in a series of *in vitro* experiments, including increased MT1-MMP expression and activity, along with that of MMP-2 and MMP-9. These findings are also consistent with the studies associating MT1-MMP with EMT, as demonstrated by reduced E-cadherin,

increased N-cadherin and enhanced vimentin staining. The authors additionally provide evidence that these effects are mediated via FAK-Src signalling.

A study by Sankpal *et al.*^[37] provides evidence for regulation of MT1-MMP by specificity protein 1 (Sp1). Sp1 is expressed in a number of different cancers, and plays key regulatory roles in processes associated with prostate cancer progression and metastasis. DU-145 cells were reported to express constitutively phosphorylated ERK, while PC3 and PC3N cells express constitutively phosphorylated AKT/PKB and c-Jun NH2 terminal kinase (JNK). Interestingly, both MT1-MMP and Sp1 levels were decreased in PC3 cells when PI3K and JNK were inhibited, and MT1-MMP levels were decreased in DU-145 cells when MEK was inhibited. These results suggest Sp1-mediated transcriptional regulation of MT1-MMP in prostate cancer cell lines via differential signalling control^[13,37]. Sroka *et al.*^[25] additionally considered the relationship between IGF-1R and MT1-MMP in prostate cancer cells and tissues, the expression data for which were discussed earlier. IGF-1R has been identified to play a role in prostate cancer metastatic progression, through PI3K, MAP kinase and ERK signalling^[38]. Interestingly, decreased MT1-MMP expression at mRNA and

protein level resulted from inhibition of IGF-1R (using picropodophyllin) in PC3N cells. Increased IGF-1R, when activated by IGF-1, led to increased MT1-MMP expression and activity following treatment of LNCaP cells with synthetic androgen R1881.

Reversion-inducing cysteine-rich protein with Kazal motifs (RECK), originally found to suppress transformation caused by the oncogene KRAS, is a glycoprotein tumor suppressor which inhibits metastasis and angiogenesis^[39]. Previous studies have identified RECK as an inhibitor of various MMPs, including MT1-MMP^[40]. Rabien *et al.*^[41] identified RECK expression in prostate cancer cell lines and tissue and observed a significant decrease in malignant tissue. Significantly, RECK overexpression led to a dramatic reduction in tumor cell invasion and a decrease of pro-/active MT1-MMP expression (up to 53% of control).

Filiz and Dass^[42] demonstrated decreased expression of MT1-MMP associated with reduced pigment epithelium-derived factor (PEDF). The authors noted that PEDF had been previously found to be downregulated in prostate cancer patients (specifically in high-grade PIN, the most likely precursor of prostate cancer)^[43]. PEDF was examined for effects on PC3 cells, with increased adhesion to ECM protein collagen-I and decreased expression of phosphorylated FAK observed. Tumor cell invasion through collagen-I was also reduced. These findings were attributed to the decreased expression of MT1-MMP.

Increased expression of both MT1-MMP and LIM kinase 1 (LIMK1) in prostate tumor tissues was reported by Tapia *et al.*^[44]. LIMK1 is a downstream effector of Rho signalling, modulates actin dynamics and is overexpressed in prostate cancer cells, where it promotes invasion and metastasis. Results showed that treatment with ilomastat (broad-spectrum hydroxamate-based MMP inhibitor) reduced LIMK1-induced invasion of benign prostate epithelial cells (BPH-1 cells) suggesting that the process is mediated by MMPs, notably MT1-MMP. Increased MT1-MMP expression in cells overexpressing LIMK1 was also reported, along with transcriptional activation and localisation of protein to the plasma membrane. LIMK1 was shown to physically associate with MT1-MMP and to co-localise with it in Golgi vesicles, thereby enabling transport of MT1-MMP to the cell surface^[44].

FGFR4 expression and polymorphism has been linked to prostate cancer progression and drug resistance^[45]. In particular, a single nucleotide polymorphism (SNP)

in codon 388 of the human *FGFR4* gene has been linked to poor prognosis in prostate cancer patients. This SNP results in Gly388 being transformed to Arg in the transmembrane domain of the receptor, leading to prolonged FGFR4 receptor activation^[46]. MT1-MMP and FGFR4 were found to be co-expressed in the tumor edges and prostate carcinoma: MT1-MMP upregulation was observed in cancer cells (9 of 14) and/or reactive stroma (9 of 14), whereas FGFR4 expression was mainly found in the tumor cells. FGFR4-R388 was shown to enhance MT1-MMP-mediated prostate cancer cell invasion. FGFR4 was thus also identified as playing a role in MT1-MMP-dependent ECM degradation and tumor progression involving EMT *in vivo*^[47].

MT1-MMP has additionally been associated with oxidative stress in prostate cancer cell lines. Nguyen *et al.*^[48] described how expression of MT1-MMP increased oxidative DNA damage via reactive oxygen species (ROS) in LNCaP and in DU145 cells, causing oxidative stress. The study confirmed the findings of others in demonstrating that MT1-MMP is associated with a more aggressive phenotype as illustrated by increased cell migration, invasion and anchorage-independent cell growth. Use of the scavenger *N*-acetylcysteine to block ROS activity inhibited the MT1-MMP-mediated increase in cell migration and invasion. The authors additionally suggested a role for β 1-integrins in facilitating cell adhesion to matrix proteins, and that this was necessary for induction of ROS in MT1-MMP-expressing prostate cancer cells.

PTEN (phosphatase and tensin homologue deleted on chromosome ten) is a phosphatase enzyme involved in regulation of PI3K-Akt pathway signalling and thus cancer progression. Most metastatic prostate cancers exhibit loss-of-function mutations or deletions of this key tumor suppressor^[49]. Kim *et al.*^[50] considered the role of PTEN inactivation on MT-MMP expression in prostate cancer. Mouse PTEN null cells exhibited up-regulation of MT1-MMP and MT3-MMP gene expression (and the associated increased migration and invasion), and increased MT1-MMP protein expression *in vivo*. Interestingly, the MT1-MMP displayed by PTEN null cells exhibited a slow rate of turnover, which was thought to be due to differential O-glycosylation of the MT1-MMP hinge region modulating enzyme stability. MT1-MMP expression in PTEN null cells was additionally determined to be regulated by PI3K/Akt but not MAPK signalling, as determined by inhibitors of those pathways. A role for downstream pathway mTORC1 (positively regulates translation thereby promoting protein synthesis^[51]) was predicted, given the upregulation of MT-MMP protein

expression, but intriguingly inhibition by rapamycin (an mTOR inhibitor) actually upregulated MT1-MMP protein expression in PTEN null cells further, an effect that was reversed by Akt inhibition. The authors discuss this potential side effect of rapamycin, and note that similar observations have been reported elsewhere^[50].

As was the case with expression data, information regarding the roles of the other members of the MT-MMP family is somewhat limited. Delassus *et al.*^[12] identified a series of prostate cancer progression modulators and engineered overexpression in prostate cancer cells (PC3) and others. Changes in gene expression of MT1-, MT3- and MT6-MMP were then evaluated. Over-expression of activator protein-2 α , interleukin 4 and p16^{INK4a} had no effect on MT-MMP expression. Fibulin1D led to down-regulation of MT1- and MT3-MMP (no reliable data for MT6-MMP). Supporting the work of Wang *et al.*^[36], p53 over-expression led to reduced MT1-MMP expression (no reliable data for the other MT-MMPs evaluated). Over-expression of PTEN produced no change in MT1-MMP expression, which is at odds with the data of Kim *et al.*^[50], with the caveat that cells in that study were mouse prostate cancer cells. Upregulation of MT3-MMP was observed, however. Furthermore, raf kinase inhibitor protein over-expression led to increased MT1-MMP, with no change detected for the others. Finally, over-expression of E-cadherin led to reduced expression of all three MT-MMPs. This finding thus reinforces the observations discussed earlier regarding the role of MT-MMPs in EMT (i.e. a phenotypic shift from E- to N-cadherin expression)^[14,30,36].

Lin *et al.*^[52] undertook genomic association studies to identify genetic variants, i.e. SNPs utilising data from the Cancer Genetic Markers of Susceptibility dataset, which includes data from 1,151 prostate cancer patients. The authors explain how SNP-SNP interactions, rather than studying individual SNPs, potentially have greater impact on unravelling the underlying mechanisms of complex disease^[52]. Three important SNP-SNP interactions were identified, linking MT3-MMP to ROBO1, CSF-1 and EGFR. ROBO1 is a member of the roundabout immunoglobulin superfamily, and has been identified as playing key roles in prostate cancer progression^[53]. It is cleaved by MMPs and translocates into the nucleus of cancer cells, which perhaps suggests a signalling role. The authors suggest that as no specific MMP has to-date been linked to ROBO1 cleavage, these data may have uncovered the potential for such a role for MT3-MMP. Colony stimulating factor-1 has been associated with increased tumor angiogenesis^[52]. Epidermal growth

factor receptor (EGFR) plays an important role in regulating cancer cell growth and function, not least in prostate cancer, and is therapeutically important^[54]. More recently, a much wider study by the same group using the prostate cancer PRACTICAL consortium data with approximately 21,000 patients, identified four key SNP-SNP interactions found to be associated with prostate cancer aggressiveness. Of relevance here, this study again linked MT3-MMP and EGFR^[55].

In addition, several studies point to an important role for MT-MMPs, and MT1-MMP in particular, in the processes associated with metastatic spread to bone in prostate cancer. This data will be considered in the following section.

EXPRESSION AND ROLES OF MT-MMPs IN PROSTATE CANCER BONE METASTASIS

It is suggested that more than 80% of patients with disseminated prostate cancer will present with metastasis to the bone^[56,57]. Skeletal complications are thus one of the leading causes of morbidity and mortality in prostate cancer patients. The normal equilibrium between osteoblastic and osteolytic activity in bone is disturbed in prostate cancer, leading to changes that are likely to provide a favourable microenvironment for metastatic colonisation. Given the established roles for MMPs in normal bone remodelling, a role for MMPs in prostate cancer bone metastasis has long been proposed^[58]. With this in mind, it is perhaps surprising that relatively little research has been directed to the MT-MMPs in this area to-date. MT1-MMP knock-out mice exhibit severe skeletal abnormalities, confirming a role in normal bone maintenance and development^[59]. Given these roles in normal bone health, the expression of MT-MMPs in prostate cancer cells and tissues led Bonfil *et al.*^[58] to speculate as to the existence of a selective process in which prostate cancer cells may have a greater propensity to metastasise to bone, or whether the microenvironment within the bone itself may induce MMP expression in prostate cancer cells, after their arrival at the bone.

Nemeth *et al.*^[60] evaluated MT-MMP expression in clinical samples and the role of MMP activity in prostate cancer that had metastasised to the bone, using a preclinical mouse model of bone metastasis employing PC3 xenografts. MT1-MMP protein expression (as identified by immunohistochemical staining) was consistently observed in the 18 core bone biopsy samples from prostate cancer patients. In preclinical studies, single human foetal bone fragments were implanted subcutaneously in immunodeficient

mice. After an interval of 4 weeks, PC3 cells were then injected directly into some of the implants, with/without initiation of daily treatment with broad-spectrum MMP inhibitor batimastat for 2 weeks. MT1-MMP expression was subsequently identified in PC3 bone tumors, localised primarily to tumor cells, with some stromal expression noted. The PC3 bone tumors were mostly osteolytic in nature, and MMP inhibition by batimastat reduced the number of osteoclasts per millimetre in these implants. The authors concluded that MT1-MMP activity in prostate cancer cells appears to be crucial in bone matrix turnover. This, together with metastatic tumor growth, appeared to be linked in cycle that is disrupted by MMP inhibition^[60].

A further correlation between MT1-MMP expression in prostate cancer cells and bone metastasis was also reported by Bonfil *et al.*^[61]. MT1-MMP expression was abundant and consistent in tumor cells identified in paraffin sections of bone metastases from 20 prostate cancer patients (androgen independent disease). It should be noted that MT1-MMP expression was noted in endothelial cells, osteocytes, osteoblasts and stroma in matched normal bone samples, consistent with a role in bone development, albeit at expression levels which appear significantly lower than those exhibited by the tumor cells. Preclinical models were utilised to examine the role of MT1-MMP in metastatic bone colonisation of prostate cancer cells. MT1-MMP was introduced into LNCaP cells, while it was silenced (using siRNA for MT1-MMP) in DU145 cells. MT1-MMP over-expression enhanced bone tumor growth (via intra-tibial injection) and associated osteolysis, while not affecting cell proliferation *in vitro* or subcutaneous tumor growth *in vivo*. This led the authors to conclude that MT1-MMP contributes a unique stimulatory effect on tumor growth in the bone microenvironment. Further studies utilising orthotopic models which better replicate the normal disease dissemination process are required to confirm these findings, but the authors nevertheless suggest the possibility that MT1-MMP activity may be worthy of pursuing as a therapeutic target for prostate cancer bone metastases. Furthermore, a role for RANKL was suggested: RANKL (receptor activator of NF- κ B ligand) is a regulator of osteoclastogenesis, and its release in the bone microenvironment was linked to MT1-MMP activity. siRNA knockdown of MT1-MMP inhibited bone tumor growth of DU145 cells and simultaneously led to osteogenesis, a phenomenon for which mechanistic information was not obtained. It was suggested that MT1-MMP inhibition may have shifted the balance toward bone formation simply by inhibition of osteolysis/osteoclastogenesis^[61].

Sabbota *et al.*^[62] subsequently followed up their initial findings and provided further evidence for a link between RANKL shedding and MT1-MMP protein expression, summarised in Figure 2. In this study, conditioned media from LNCaP cells expressing both RANKL and MT1-MMP was shown to enhance cell migration of LNCaP-C4-2b cells, which are MT1-MMP deficient. This was inhibited by osteoprotegerin (soluble decoy receptor of RANKL) and selective MT1-MMP inhibitor MIK-G2. The authors hypothesised that these findings indicated that MT1-MMP enhances tumor cell migration through initiation of an autocrine loop requiring RANKL shedding in prostate cancer cells. Evidence was also provided for a role for Src as a downstream mediator of RANKL^[62].

The importance of cadherin-11 in prostate cancer bone metastasis was considered by Huang *et al.*^[63]. Cadherin-11 is an osteoblast cadherin, identified as being aberrantly expressed in prostate cancer cells derived from bone metastases^[64]. LNCaP-C4-2B4 cells in which expression of cadherin-11 had been engineered demonstrated increased spread and intercalation into an osteoblast layer *in vitro* and exhibited enhanced migration and invasion. Downregulation of cadherin-11 in PC3 cells, which naturally express cadherin-11, decreased cell migration and invasion. A possible role for MT2-MMP was suggested, following gene array analysis of the LNCaP-C4-2B4 cells. Several genes related to invasion and metastasis were identified as upregulated, among which MT2-MMP was a prominent finding^[63]. Interestingly, *IGF-1* gene expression was also reported as upregulated, which supports the evidence provided by Sroka *et al.*^[25] relating to a role for IGF-1R in MT-MMP expression and activity, as discussed earlier.

MT-MMPs clearly play important roles in the development of metastatic bone deposits. Given that most patients with prostate cancer ultimately succumb to metastasis, a strategy to specifically target MT1-MMP-expression on tumor cells may prove an attractive means by which to address prostate cancer metastasis to bone.

OPPORTUNITIES FOR DRUG TARGETING

The expression of MT-MMPs in prostate cancer and associated bone metastases suggests an opportunity for targeted therapy. As an example, the Bonfil group focused on MT1-MMP expression in prostate cancer bone metastases, and suggested that the increased MT1-MMP activity was worthy of pursuing as a therapeutic strategy. The group later

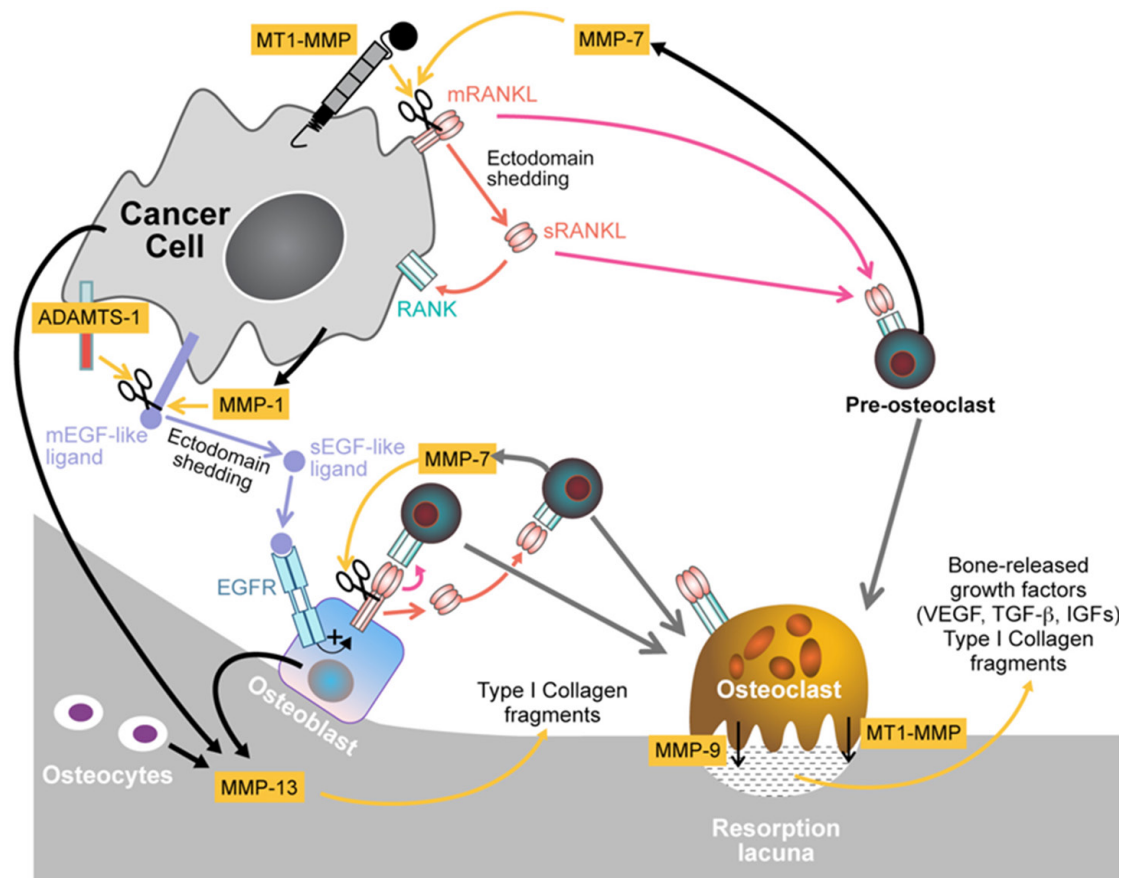


Figure 2: Diagram representing the most representative roles of MT1-MMP at bone metastatic sites. MT1-MMP expression by cancer cells results in proteolytic cleavage of tumor-associated membrane-bound RANKL (mRANKL), generating a soluble form of RANKL (sRANKL) that activates RANK favouring migration of tumor cells and osteoclastogenesis, respectively. Roles for MMP-7, MMP-9 and MMP-13 were also described. Reprinted by permission from Macmillan Publishers Ltd.^[78]. MT-MMP: membrane-type metalloproteinases

went on to propose a combination of Src inhibition with RANKL and/or selective MT1-MMP inhibition as a strategy, particularly for prostate cancer patients with bone metastases. Other strategies are worthy of investigation, utilising the knowledge that has been gained in understanding key pathways and how they interact with MT1-MMP activity, as have been discussed. Further study is required to fully understand the expression and roles of MT-MMPs in normal cells in the tumor microenvironment, however, given the possibility that tumor cells may induce expression in benign epithelial cells. Furthermore, very few studies contain data from healthy individuals for true “normal” tissue comparison.

MMP inhibition is an area that has been well explored in the past by big pharma, but has yet to fulfil its clinical potential. This is due to the complex roles of individual MMPs and their inter-connected compensatory mechanisms, poor clinical trial design, and drugs lacking exquisite selectivity^[2,65]. Utilising MMP expression and proteolytic activity, however,

is perhaps a more attractive approach^[5,66]. This is especially true given the role of MMPs in angiogenesis and in maintenance of tumor vasculature^[67,68]. While not specifically studied in prostate cancer, evidence for other cancers is compelling, particularly for MT1-MMP^[66,67].

The need for novel therapeutics in metastatic prostate cancer is clear. The burden of drug toxicity endured by many patients, often elderly and frail, means that an emphasis must be placed on targeted agents with minimal side effects. Small molecule chemotherapeutics remain central to prostate cancer therapy, but despite some considerable recent advances, these new agents still suffer from a lack of selectivity and dose-limiting toxicities. There is therefore considerable interest in the development of prodrugs to tailor the pharmacokinetics of molecules in favour of tumor-selective drug targeting, thereby decreasing these dose-limiting side effects and enhancing therapeutic index^[69]. Multiple reviews have been published covering a huge range of approaches for cancer drug delivery, but it is fair to say that

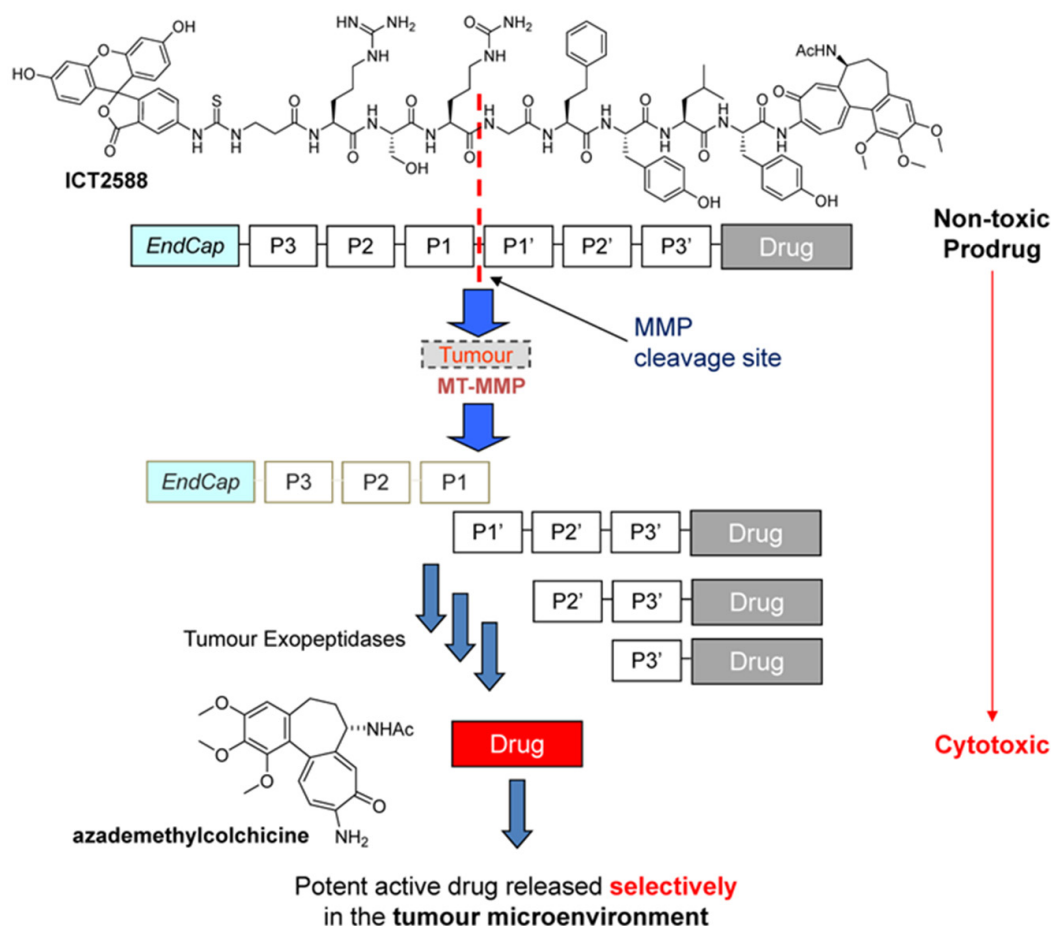


Figure 3: Structure of ICT2588 and MT-MMP targeting concept. Peptide conjugation renders the drug (azademethylcolchicine) pharmacologically inactive. Following initial MT-MMP recognition and cleavage, the remaining amino acids are metabolised to release the toxic drug selectively in the tumor microenvironment. MT-MMP: membrane-type matrix metalloproteinases

prodrug approaches specifically developed for prostate cancer are limited. MMP-activated prodrugs in prostate cancer are considered by Barve *et al.*^[70]. Here the concept of attaching an MMP-recognition peptide to a drug is discussed. Peptide conjugation renders the drug inactive (thereby creating the prodrug) until such time that tumor-expressed MMPs recognise and cleave the peptide to release the drug. This area has considerable potential. Further examples are provided by Choi *et al.*^[3] and Law and Tung^[4].

ICT-2588 is a prodrug of azademethylcolchicine (an analogue of colchicine^[71]) and is a potent anti-vascular agent [Figure 3]. Activated by MT1-MMP, ICT-2588 has shown promise in preclinical studies, successfully achieving enhanced therapeutic index^[72], an absence of cardiotoxicity^[73], and activity in a range of tumor types, not least prostate cancer (activity in PC3 xenografts in mouse models)^[73]. This potential for the treatment of prostate tumors led the authors to apply the same technology to paclitaxel, yielding ICT-3205^[74]. This agent provides for enhanced

tumor delivery of paclitaxel in preclinical studies (PC3 xenograft in mice), realising 10-fold increases in tumor concentrations (as measured by *in vivo* pharmacokinetics studies) while decreasing the exposure of drug to normal tissues, and associated toxicities. Given the findings of the STAMPEDE prostate cancer trial supporting earlier use of taxanes in treatment of metastatic prostate cancer^[75-77], this approach is particularly timely.

While our understanding of the roles of MT-MMPs in prostate cancer and metastatic disease is growing all the time, much is still to be learnt. The potential for exploiting the proteolytic capacity of these enzymes is without question, but it remains to be seen whether a clinically useful drug molecule will emerge.

DECLARATIONS

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Authors' contributions

R.A. Falconer and P.M. Loadman contributed equally to writing this review.

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RAF and PML declare that they are founding shareholders of Incanthera Ltd.

Patient consent

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Topic: How does the prostate cancer microenvironment affect the metastatic process and/or treatment outcome?

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Androgen-AR axis in primary and metastatic prostate cancer: chasing steroidogenic enzymes for therapeutic intervention

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ABSTRACT

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Androgens play an important role in prostate cancer (PCa) development and progression. Although androgen deprivation therapy remains the front-line treatment for advanced prostate cancer, patients eventually relapse with the lethal form of the disease. The prostate tumor microenvironment is characterised by elevated tissue androgens that are capable of activating the androgen receptor (AR). Inhibiting the steroidogenic enzymes that play vital roles in the biosynthesis of testosterone (T) and dihydrotestosterone (DHT) seems to be an attractive strategy for PCa therapies. Emerging data suggest a role for the enzymes mediating pre-receptor control of T and DHT biosynthesis by alternative pathways in controlling intratumoral androgen levels, and thereby influencing PCa progression. This supports the idea for the development of multi-targeting strategies, involving both dual and multiple inhibitors of androgen-metabolising enzymes that are able to affect androgen synthesis and signalling at different points in the biosynthesis. In this review, we will focus on CYP17A1, AKR1C3, HSD17B3 and SRD5A, as these enzymes play essential roles in all the three androgenic pathways. We will review also the AR as an additional target for the design of bifunctional drugs. Targeting intracrine androgens and AKR1C3 have potential to overcome enzalutamide and abiraterone resistance and improve survival of advanced prostate cancer patients.

INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed cancer in men and the second leading cause of death^[1]. Androgens, which regulate normal

prostate growth and function by interacting with the androgen receptor (AR), drive PCa growth and play a central role in PCa progression^[2]. Individuals diagnosed with high-risk PCa are typically treated with surgery or a combination of radiation and androgen



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Table 1: Therapies and approved drugs for PCa treatment according to its progression

PCa progression	Therapy	Mechanisms		Drugs	Structure or number in the text
Localised disease	Surgery				
	Radiation				
	ADT	GnRH agonists and antagonists		Buserelin	Synthetic peptide
				Goserelin	Synthetic peptide
				Leuprolide	Synthetic peptide
Advanced PCa	ADT	AR antagonist	Steroidal Non steroidal	Triptorelin	Synthetic peptide
				Degarelix	Synthetic peptide
				Cyproterone	50
				Flutamide	51
				Nilutamide	52
CRPC	ADT	AR antagonist	Non steroidal	Bicalutamide	53
				Enzalutamide	54
				Abiraterone	2
	Chemotherapy	Androgen synthesis inhibitors (CYP17 inhibitors)			
				Docetaxel	Taxane
				Cabazitaxel	Taxane
				Alpharadin	Radium-223
		Inductors of microtubule stabilization		Sipuleucel-T	-
				Denosumab	-

PCa: prostate cancer; ADT: androgen deprivation therapy; AR: androgen receptor; GnRH: gonadotropin-releasing hormone analogues; CRPC: castration-resistant prostate cancer

deprivation therapy (ADT) via chronic administration of gonadotropin-releasing hormone analogues, anti-androgens or a combination of these drugs [Table 1]. ADT is considered the standard choice of treatment for men with *de novo* or recurrent metastatic disease^[3]. Initially, ADT provides palliation of symptoms, but the therapeutic effects of castration are usually short lived, with 70% of patients developing signs of disease progression within 2 years despite very low levels of circulating testosterone (T)^[4,5]. Many patients will inevitably relapse and ultimately develop castration-resistant prostate cancer (CRPC), which is responsible for the vast majority of PCa mortalities. Although the mechanisms of resistance are multifactorial, the androgen axis still plays a major role^[6]. Evidence accumulated over the past decade clearly indicates that castration-resistant growth, to a large extent, is driven by continued AR signalling, despite castration resulting in only low levels of T in the serum. Emerging literature indicates a complex network of molecular players linked in part with amplification or mutations in androgen receptors allowing activation by progesterone, estrogens and androgen antagonists, generation of alternative splicing variants or with androgen neo-synthesis within the prostate tumour or adrenals^[7-10]. Accordingly, both the management of PCa patients and complete abolition of androgens are difficult to achieve. Direct measurement of androgen levels in clinical samples from patients with CRPC reveal residual T (0.2-2.94 ng/g) and dihydrotestosterone (DHT, 0.36-2.19 ng/g) levels in tissue samples, respectively; nonetheless these levels are considered more than sufficient to activate the

AR machinery and support tumour cell growth and survival^[11]. Additionally, a number of studies have indicated several enzymes are able to facilitate the intratumoral neo-synthesis or conversion of circulating adrenal androgen precursors to the active AR ligands^[12].

This review is focussed on outlining and discussing the key players in the steroidogenic pathway that is tightly linked with the AR activation.

THE STEROIDOGENIC CASCADE INVOLVED IN PCA

Under normal physiological conditions about 60% of androgens produced in the prostate come from circulating T synthesised from cholesterol in the testis. The remainder derives from dehydroepiandrosterone (DHEA) synthesised in the zona reticularis of the adrenal glands [Figure 1]. The prostate itself contributes to androgen anabolism by reducing testicular T to the more potent AR ligand DHT and converting DHEA to T and DHT [Figure 2]. The enzymes converting T to DHT are type 1 or 2 5 α -reductase (SRD5A), the type 2 being the predominant isoform in prostate. This mechanism of production of DHT presumably allows the prostate to maintain constitutive levels of AR that are sufficient for activity in the luminal epithelium. The adrenal DHEA taken up by prostate cells as the sulphate derivative is reduced to androstenedione (AD) by a 3 β -hydroxysteroid dehydrogenase type 1 (HSD3B1)

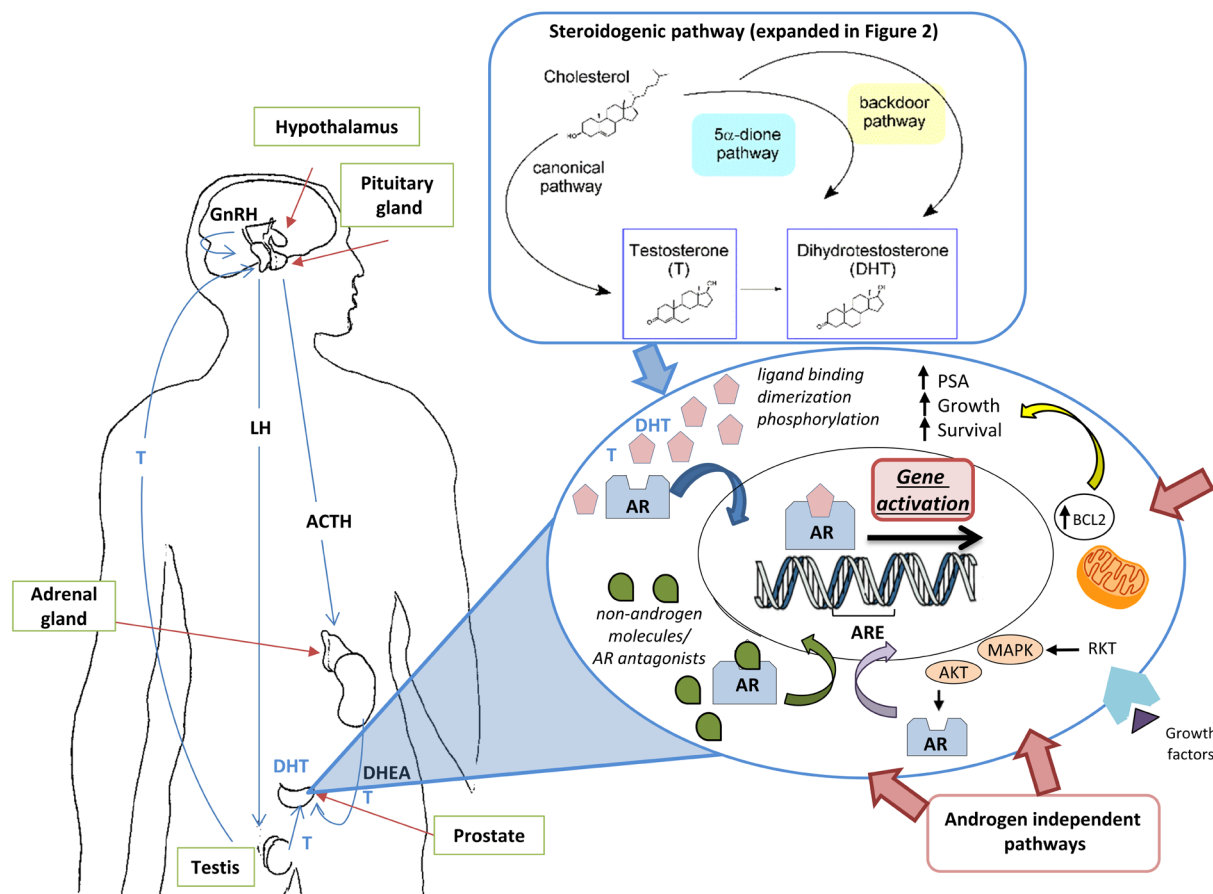


Figure 1: The production of androgens is regulated by the hypothalamic-pituitary-gonadal-adrenal axes. AR activation (dimerisation and phosphorylation) is regulated by both androgen-dependent (blue arrows) and androgen-independent pathways (red arrows). In the androgen-dependent pathway, T and DHT production is catalysed by the steroidogenic enzymes and occurs through the canonical, 5 α -dione and backdoor pathways^[24]. The androgen-independent pathway includes: (1) AR gain-function mutations; (2) activation by non-androgen steroids or androgen antagonists; (3) activation by non-steroid growth factors (receptor tyrosine kinases are activated and both AKT and MAPK pathways, producing a ligand-independent AR); and (4) increase of AR co-regulators. A parallel survival pathway, involving the anti-apoptotic protein BCL-2, also induces the cancer cell proliferation via bypassing the AR^[183,184]. AR: androgen receptor; GnRH: gonadotropin-releasing hormone analogues; T: testosterone; DHT: dihydrotestosterone; ARE: androgen response element; DHEA: dehydroepiandrosterone; LH: luteinizing hormone; ACTH: adreno-cortico-tropic-hormone

expressed in prostate basal epithelial cells. This is followed by AD conversion to T by 17 β hydroxysteroid dehydrogenase type 5 (HSD17B5). This enzyme is a member of the aldo-ketoreductase family, also known as AKR1C3 (aldo-keto reductase family 1, member 3), is somewhat different to the 17 β reductases that are derived from the family of SDRs (short-chain dehydrogenase/reductase). By contrast, the synthesis of T in the testis mediated by a SDR enzyme, named HSD17B3. In the normal prostate, AKR1C3 has been identified in stromal, endothelial and perineural cells, where its significance appears to be related to the ability to reduce prostaglandin D2 to F2 rather than to the synthesis of T, which can be assumed from the circulation.

Intracellular levels of DHT are also regulated by phase I (reducing) and phase II (conjugating) enzymes that

mediate DHT catabolism: AKR1C1 and AKR1C2 (reductive 3 α -HSDs) convert DHT to 3 α -androstenediol and 3 β -androstenediol respectively, which are then glucuronidated by UDP glycosyltransferase UGT2B15 or UGT2B17^[13]. 3 α -androstenediol can be oxidised back to DHT by HSB17B6, which is expressed in prostatic stromal cells. In PCa patients that have received ADT, the presence of low levels of androgens, relative to high levels of T and DHT, can be maintained by intraprostatic synthesis, which essentially can occur through three putative synthetic pathways: the principal pathway is the classical or “canonical” *de novo* synthesis that initiates from cholesterol or other intermediates and results in T production. The two alternative pathways, “5 α -dione” pathway and the “backdoor” pathway, allow direct synthesis of the AR ligand DHT without the requirement of T as intermediate.

The canonical pathway

This biosynthetic pathway is similar to that occurring in the testes. Androgens are known to be synthesised *de novo* starting from a number of precursor molecules absorbed from the circulation, including cholesterol, progesterone and adrenal DHEA. Starting from cholesterol, the first step is the conversion to C21 pregnenolone by the cholesterol aliphatic side-chain specific metabolic activity of mitochondrial CYP11A1. The next steps lead to the synthesis of AD by two different pathway branches where the intermediates progesterone or DHEA are formed by CYP17A1 or HSD3B respectively. The subsequent reduction of AD to T is catalysed by AKR1C3. T is further reduced to DHT by SRD5A enzymes. In patients with CRPC, DHEA, derived from the adrenal gland, is the predominant T precursor implicated in this pathway. The strongest evidence for the action of the canonical pathway in the prostate comes from a study published by Fankhauser *et al.*^[14] which is focussed on the incubation of cultures of prostate samples from patients with benign prostatic hyperplasia (BPH), androgen-naïve and/or hormone-refractory PCa with various precursor substrates including cholesterol, progesterone, AD, DHEA, and T. The results show the prominence of the conversion of AD to T, suggesting that the canonical pathway is the most pertinent T/DHT synthesis pathway in patients with PCa. These conclusions are supported by findings that expression of the HSD17B isoenzymes, and in particular AKR1C3, key enzymes responsible for the conversion of AD to T, are upregulated in tumour biopsy samples from patients with CRPC^[15-17].

The 5 α -dione pathway

The 5 α -dione pathway allows PCa cells to generate the potent signalling androgen DHT without the need for T as a substrate. In this pathway, the order of reactions is reversed compared to the canonical biosynthesis: AD is initially 5 α -reduced to 5 α -androstenedione by SRD5A1 and then further reduced to DHT by HSD17B3^[18,19]. In contrast, in the canonical biosynthesis AD is the substrate of HSD17B3 that is reduced to T which is then further reduced to DHT by SRD5A. The 5 α -dione pathway was first described in 2011, and as such, fewer studies supporting this model are currently available compared with the other two models of androgen synthesis, although indirect evidence is available and supports the clinical relevance of this pathway too^[15,17].

The backdoor androgen synthesis pathway

This biosynthetic pathway was originally identified in Tammar wallabies in 2003^[20]. It was the first report to demonstrate that in the prostate the “backdoor”

pathway was contributing to the synthesis of androgens without the need for androgenic precursors. Here, the progesterone produced by the same reactions as in the canonical pathway, is converted to androsterone by CYP17A1, SRD5A, and AKR1C2. These enzymes are responsible for converting AD to T in a similar manner to HSD17B3 and AKR1C3 in the canonical pathway, resulting in the conversion of androsterone to 5 α -androstane-3 α ,17 β -diol. The final step of the pathway leading to DHT is catalysed by retinol dehydrogenase type 5 (RDH5)^[21,22]. This enzyme, upregulated in mice with castration resistance^[17,22], mediates a key step in DHT biosynthesis and is one of the few steroidogenic enzymes acting at a single point in the biosynthetic pathway. Studies in LNCaP xenografts indicate that the backdoor pathway might be dominant when tumours are treated with inhibitors of androgen synthesis, including ketoconazole and finasteride, which inhibit CYP17A1, and SRD5A2, respectively^[23].

All these pathways (“canonical”, “backdoor” and “5 α -dione” pathways, Figure 2), ultimately aim at generating the potent signalling androgen DHT. Involving mainly the same enzymes, they differ in terms of substrate preference and/or reaction sequence. Their occurrence and relative importance in the development and progression of PCa remains controversial, since the experimental evidence comes mainly from preclinical cell culture models, where different results are obtained depending on the cell lines studied or where more clinically-relevant biopsy samples have been used for analysis. The current understanding of androgen synthesis and the evidence for its role in castration resistance, either supporting or rebutting the relevance of each pathway to patients with PCa were recently extensively reviewed by Stuchbery *et al.*^[24].

Inhibiting these steroidogenic enzymes that play vital roles in the biosynthesis of T and DHT seems to be an attractive strategy for the development of therapies for the treatment of PCa. The existence of alternative pathways in PCa leading to the synthesis of T and DHT supports the idea for the development of multi-targeting strategies, involving both dual and multiple inhibitors of androgen-metabolising enzymes that are able to affect androgen synthesis and signalling at different points in the biosynthesis. Therapeutic strategies aimed at more efficiently targeting the steroidogenic pathway could involve the concomitant use of inhibitors targeting two different enzymes or a unique dual-targeting inhibitor able to modulate more than one enzyme in the steroidogenesis pathway. A potential variation of this strategy involves the modulation of an androgen-metabolising enzyme

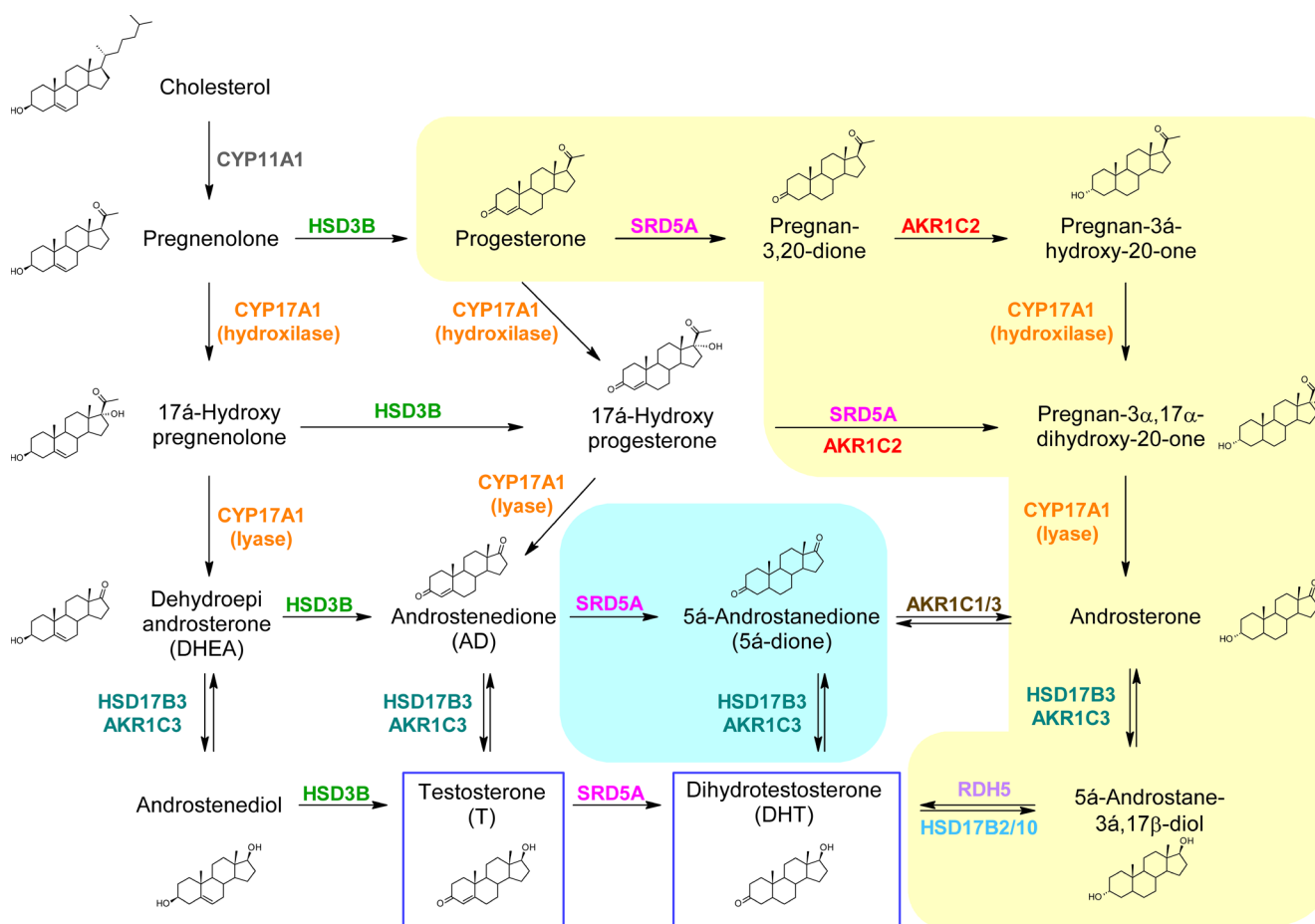


Figure 2: The principal and the two alternative androgen biosynthetic pathways: the canonical pathway is shown on white background, the backdoor pathway is shown on yellow background and the 5 α -dione pathway on light blue background. In the squares, production of T and DHT in the development of PCa are shown. PCa: prostate cancer; T: testosterone; DHT: dihydrotestosterone; CYP17A1: cytochrome P450 17A1; HSD3B: 3 β -hydroxysteroid dehydrogenase; HSD17B2/3/10: 17 β -hydroxysteroid dehydrogenase type 2/3/10; SRD5A: steroid 5 α -reductases; AKR1C1/2/3: aldo-keto reductase family 1, member 1/2/3; RDH5: retinol dehydrogenase type 5; AD: androstenedione

and the AR by a bifunctional targeting molecule. In the following chapters, we will focus on CYP17A1, AKR1C3, HSD17B3 and SRD5A, as these enzymes play essential roles in all the three pathways mentioned above. In Table 2, their different expression level during the progression of PCa is described. Finally, we will review the AR as an additional target for the design of bifunctional drugs.

CYP17A1

Cytochrome P450 17A1 (CYP17A1, P450c17) plays a major role in the steroidogenic pathway that produces androgens and estrogens. It is expressed principally in the adrenal gland and gonads. In humans, the expression of CYP17A1 is driven by a complex interaction of different transcription factors (TFs) and, differently from rodents^[25], it appears not directly influenced by epigenetic regulation^[26,27]. Indeed, CpG islands, the sites of the epigenetic methylation, are absent in human *CYP17A1* gene^[25]. Indirect

epigenetic control is however suggested by studies on the inductive effect of 5 α za-dC on TFs required for CYP17A1 expression^[28]. This membrane-bound protein has both 17 α -hydroxylase and a 17,20-lyase activity. The 17 α -hydroxylase activity is important for the production of the glucocorticoid cortisol, whereas the 17,20-lyase activity leads to androgen production^[29]. The lyase activity is stimulated in a concerted fashion by cytochrome b5 and appears to be an allosteric function rather than via conventional electron transfer mechanism of this co-enzyme^[30]. CYP17A1 is required in the three parallel pathways to catalyse the hydroxylation of the steroid ring carbon 17 of pregnenolone to form 17 α -hydroxypregnenolone and progesterone to form 17 α -hydroxyprogesterone (major product, Figure 2) and 16 α -hydroxyprogesterone (minor product). The resulting metabolites undergo the 17,20-lyase reaction by the same enzyme involving the cleavage of the side-chain of the steroid nucleus in order to obtain DHEA and AD, respectively. The androsterone, precursor of DHT in the backdoor

Table 2: Different expression level of CYP17A1, AKR1C3, HSD17B3 and SRD5A enzymes during progression of PCa

Enzyme	Presence	Ref.
CYP17A1	Expressed in all PCa and upregulated in CRPC	[15]
AKR1C3	Expressed 10-16 fold higher in several PCa cell lines with respect to healthy prostate cells and up to 3 fold in androgen responsive and androgen independent PCa cell xenografts upon androgen deprivation	[9]
HSD17B3	Upregulated in CRPC, both within the tumor microenvironment and in soft-tissue metastasis	[15-17,52,176,177]
	Expressed almost exclusively in the testis, there are some reports of its over-expression in PCa tissues. HSD17B3 mRNA was increased over 30 fold in PCa biopsies and the enzyme has been shown to be upregulated 8-fold in LuCaP-23 and LuCAP-35 PCa cell lines, obtained from metastatic tissues of a patient resistant to castration therapy	[15,22]
SRD5	A1 During PCa development its expression increases. A 2-4 fold increase of SRD5A1 expression, induced by activation of AR, has been observed in three androgen-responsive PCa cell lines	[88,89,178-181]
	A2 Predominant isoform expressed in the normal prostate. During PCa development, its expression decreases. AR represses SRD5A2 expression	
	A3 Overexpressed in hormone-refractory PCa tissues	[182]

PCa: prostate cancer; AR: androgen receptor; CRPC: castration-resistant prostate cancer

pathway is formed by CYP17A1 via metabolism of pregnan-3 α -hydroxy-20-one. The need for this enzyme in all the metabolic pathways that allow and maintain the activation of the AR in the prostatic cells makes CYP17A1 one of the most important therapeutic targets in the biosynthesis pathway.

To date, there are eight co-crystal structures of CYP17A1 complexed with an inhibitor or substrate and revealing the characteristic cytochrome P450 fold^[31]. The crystal structure of CYP17A1 bound either to abiraterone [Figure 3A] or to galeterone (TOK-001), two clinically trailed CYP17A1 inhibitors (2 and 3, Figure 4), show that both inhibitors bind the haem iron at a 60° angle above the haeme plane while aligning their chemical structures against the central helix with the 3 β -OH interacting with Asn 202 in the F helix^[32].

More recently the co-crystal structure of CYP17A1 mutant Ala105Leu in complex with hydroxylase substrates pregnenolone [Figure 3B], progesterone, 17,20-lyase substrates 17 α -hydroxyprogesterone and 17 α -hydroxypregnenolone, showed that the general orientation of all physiological substrates in the active site is quite similar to the one observed for abiraterone. Each substrate is aligned in a position that allows the formation of a hydrogen bond with the Asn202 side chain. The 17 α -hydroxypregnenolone, a substrate of lyase activity, could also assume a second pose, that is closer to the catalytic iron and further away for Asn202, hence preventing the formation of a hydrogen bond as observed in the first position^[33]. This observation could explain the substrate selectivity of the lyase reaction and the increased 17,20-lyase activity after the allosteric binding of cytochrome b5. NMR studies have already established that b5 binds differently to CYP17A1 depending on whether the substrate is pregnenolone or 17 α -hydroxypregnenolone^[34]. Cytochrome b5 could alter the positioning of

17 α -hydroxypregnenolone to the second position, thus increasing the rate of the lyase reaction. These structural studies provide a rationale to increase our understanding of this enzyme's dual hydroxylase and lyase activity and facilitate the design of inhibitors that may specifically interact with the androgen-generating lyase activity, ultimately leading to novel therapeutics with improved efficacy.

Several well-characterised CYP17A1 inhibitors have been discovered over the years for the treatment of advanced PCa [Figures 4 and 5] and several excellent reviews have been published on this topic^[35]. Only abiraterone (2, Figure 4) has been approved for clinical use for the treatment of CRPC. Abiraterone, administered as an acetate prodrug, consists of a steroidal scaffold with a pyridin-3-yl moiety in position 17 that inhibits CYP17A1 through coordination to the haem iron^[32]. This coordination obstructs the binding of endogenous substrates, leading to the competitive inhibition of CYP17A1. Recently, the steroidal CYP17A1 inhibitor galeterone (3, TOK-001)^[36], has been shown to be three times more potent than abiraterone in CYP17 enzyme activity assays^[37].

Together, the steroidal scaffold and the aromatic nitrogen-containing ring give to abiraterone a promiscuous profile with affinity toward steroid receptors and other CYP enzymes, which are likely to contribute to the undesirable side effects observed in patients receiving abiraterone treatment including liver dysfunction, characterised by elevated total bilirubin, aspartate aminotransferase and alanine aminotransferase^[38].

Thus, these potential adverse effects of steroidal drugs triggered the efforts to develop nonsteroidal CYP17A1 inhibitors. Combinatorial synthesis programmes have been initiated by pharmaceutical companies to identify non-steroidal inhibitors to avoid the side

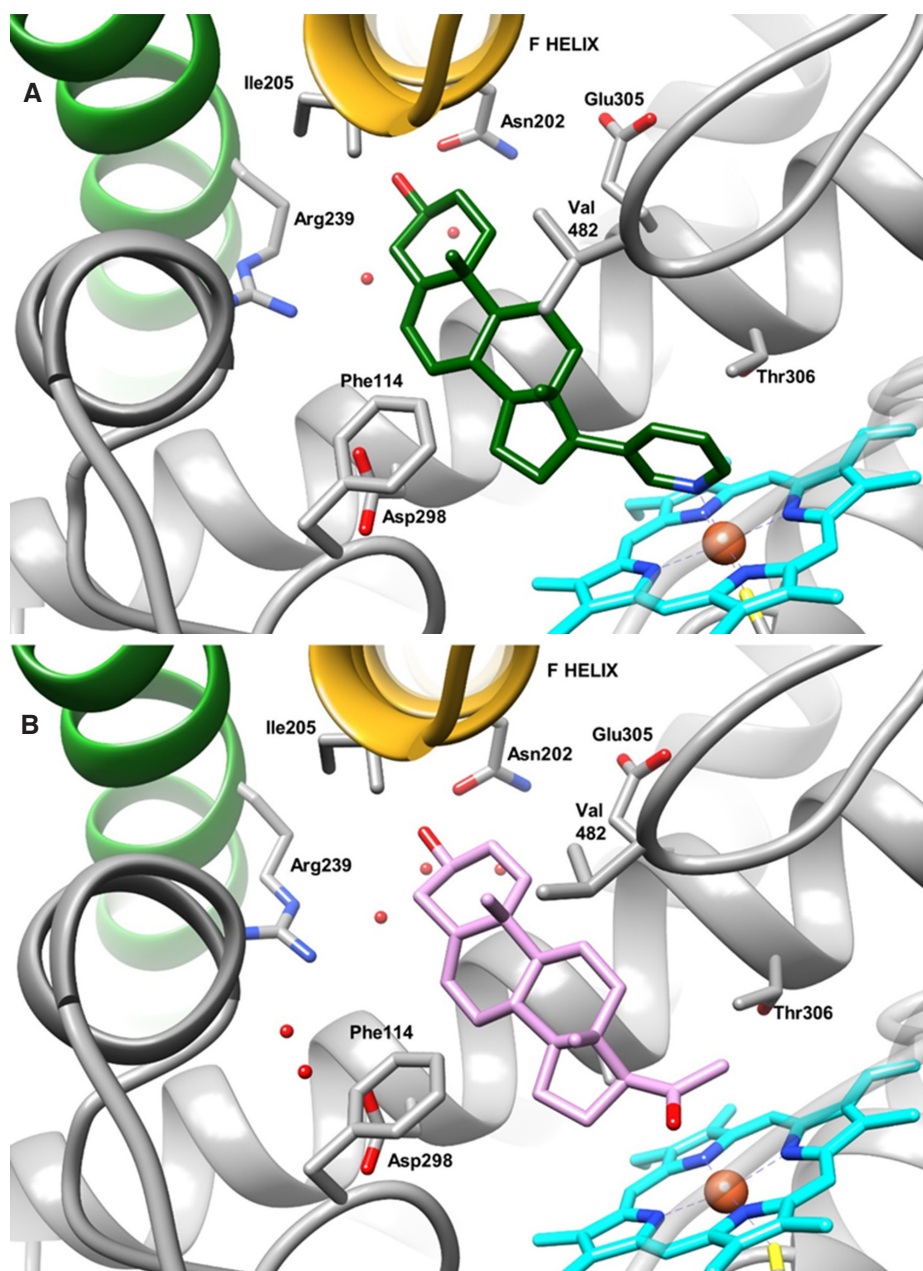


Figure 3: Structures of (A) CYP17A1 in complex with abiraterone (PDB ID 3RUK) and (B) CYP17A1 mutant A105L with substrate pregnenolone (PDB ID 4NKW). Abiraterone is depicted in green, pregnenolone in pink. Carbon atoms of the protein are depicted in grey, the haeme prosthetic group is coloured in blue. Nitrogen, oxygen and sulphur atoms are depicted in blue, red and yellow, respectively. Relevant water molecules are represented by red points

effects associated with the steroidal scaffold and two such compounds, orteronel (4) and seviteronel (5), have been developed and are subject to clinical trials. Although, orteronel (TAK-700), an oralimidazole based inhibitor^[39] had a 5-fold selectivity for 17,20-lyase activity in comparison with the 17 α -hydroxylase activity of CYP17A1, it failed to increase overall survival in CRPC patients (NCT01193257).

Seviteronel (VT-464), an orally administered nonsteroidal CYP17A1 lyase inhibitor, is at the present

under clinical development^[40]. Similar to galeterone, seviteronel works downstream of abiraterone to inhibit CYP17A1 lyase and does not cause the same degree of mineralocorticoid production. This agent can therefore be administered without concomitant glucocorticoid administration, resulting in lack of associated toxicities (such as muscle wasting, skin friability, cushingoid features, and decreased bone mineral density).

Recently, Larsen and collaborators identified two

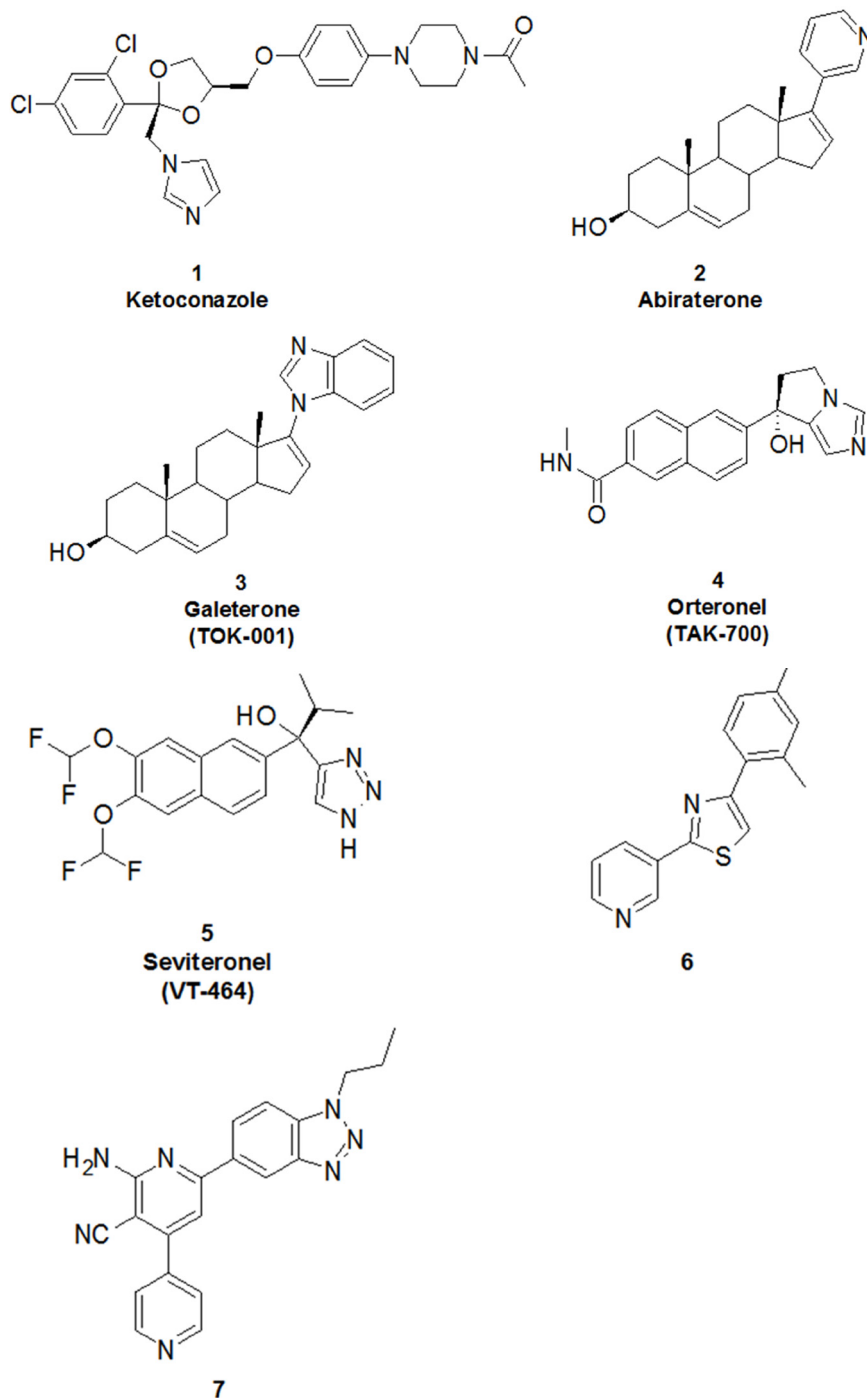


Figure 4: Chemical structures of selected CYP17A1 inhibitors

novel non-steroidal and selective CYP17A1 inhibitors by virtual screening and reported the structural optimisation of one of these inhibitors, identifying compound 6 [Figure 4]^[41].

Compound 6, which like abiraterone also contains a pyridin-3-yl moiety, inhibited CYP17A1 with IC_{50} values of 230 and 500 nmol/L for the 17α -hydroxylase and $17,20$ -lyase reactions, respectively. The binding

mode of compound 6 was determined by docking experiments, further refined by QM/MM optimisation. Compound 6 is a relatively non-polar compound with no hydrogen-bonding possibilities and, accordingly, no polar enzyme-inhibitor interactions were observed^[41].

Subsequently, the combination of a structure-based virtual screening approach with density functional theory calculations was used to suggest newnon-

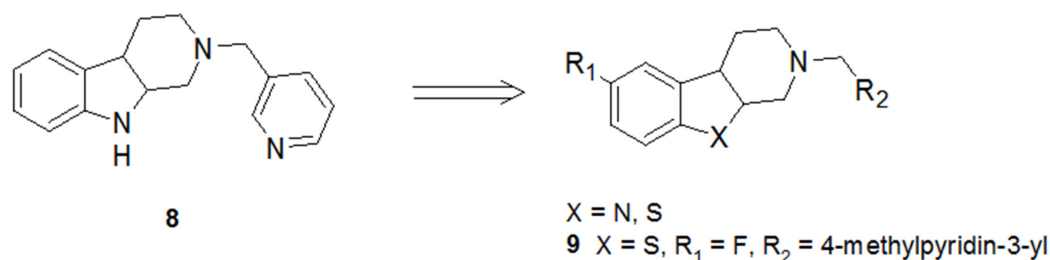


Figure 5: Design strategy of metal-binding inhibitors of CYP17A1

steroidal compounds selective for CYP17A1^[42]. This second study afforded the discovery of compound 7. *In vitro* assays in human H295R cells demonstrated that compounds 6 and 7 selectively inhibited CYP17A1 17 α -hydroxylase (IC₅₀ values of 830 and 52 nmol/L, respectively) and 17,20-lyase (IC₅₀ values of 94 and 7.4 nmol/L, respectively) activities. Strong coordination of compound 7 to the haem iron is likely to be responsible for inhibition of both reactions. These compounds do not bind selected drug-metabolising cytochrome P450 enzymes or the steroidogenic CYP21A2, suggesting a reduced risk for undesirable side effects, especially on the corticosteroid production, consistent with data observed *in vitro*. Taken together, these data recommend compounds 6 and 7 as promising tools for the continued development of new drugs against PCa^[42].

Structural analysis of the reported CYP17A1 inhibitors reveals that most of the inhibitors consist of two structural features. One is the metal-binding group that binds to the haem iron and the second is the scaffold that binds to the substrate pocket of CYP17A1. Based on this observation, recently Wang *et al.*^[43] conducted a screen of compounds from an in-house metalloenzyme inhibitor library and identified compound 8 [Figure 5] to selectively inhibit rat CYP17A1 lyase with sub micromolar activity.

A preliminary modelling study indicated that compound 8 could fit nicely into the CYP17A1 binding pocket and maintain the key interactions with the residues of CYP17A1. The nitrogen of the pyridine and the tetrahydro- β -carboline core formed a coordination bond and hydrophobic interactions with haem group (iron atom) and hydrophobic pocket respectively. Since authors showed that there was unfilled space on the pyridine part in the active site cavity, they introduced substituent onto the pyridine ring to occupy this space and enhance the potency. These efforts led to the design and synthesis of a series of compounds bearing different substituted pyridine and pyrimidine moieties and evaluated their CYP17A1 activity. Of

these analogues, the most potent compound was 9 [Figure 5], showing 1.5 fold greater potency against rat and human CYP17A1 protein than abiraterone. In NCI-H295R cells, the inhibitory effect of compound 9 on T production was also more potent than that of abiraterone at a concentration of 1 μ mol/L. Further, it was shown that 9 reduced plasma T level in a dose-dependent manner in Sprague-Dawley rats and may be a lead compound for further preclinical studies.

AKR1C3

AKR1C3, also named HSD17B5, is a soluble enzyme member of the aldo-ketoreductase family, highly expressed in testes and extragonadal tissues such as basal cells of the prostate, adrenals and liver. Principally, it catalyses the NADPH dependent reduction of AD to T but is known to be involved with 3 α -HSD, 20 α -HSD, dihydrodiol dehydrogenase and prostaglandin synthase activities^[44]. Compared to other HSD17B isoforms, AKR1C3 was the most abundant isoform expressed in several PCa cells and its expression is upregulated in CRPC [Table 2]. AKR1C3 plays a key role in producing DHT in each of the three pathways, since it can lead to the synthesis of DHT starting from AD and DHEA in the canonical pathway, from 5 α -androstenedione in the 5 α -dione pathway, and from androsterone in the backdoor pathway [Figure 2]. Elevated levels of expression of AKR1C3 in CRPC provide a mechanism to divert trace androgens that remain after ADT to the potent AR ligand DHT via these three pathways intratumourally and may indirectly also impact on CYP17A1 inhibitor or AR antagonist resistance mechanisms^[9]. Furthermore, AKR1C3 has also been discovered to play a role in resistance to radiation therapy^[45].

Because of its structural differences with HSD17B3, an enzyme belonging to SDR family and catalysing the same reaction of AKR1C3 in testis^[46], AKR1C3 could be a good target for selective inhibition.

At present, there are more than 40 crystal structures of AKR1C3 in the 2017 International Union of Crystallography Protein Data Bank. The first crystal

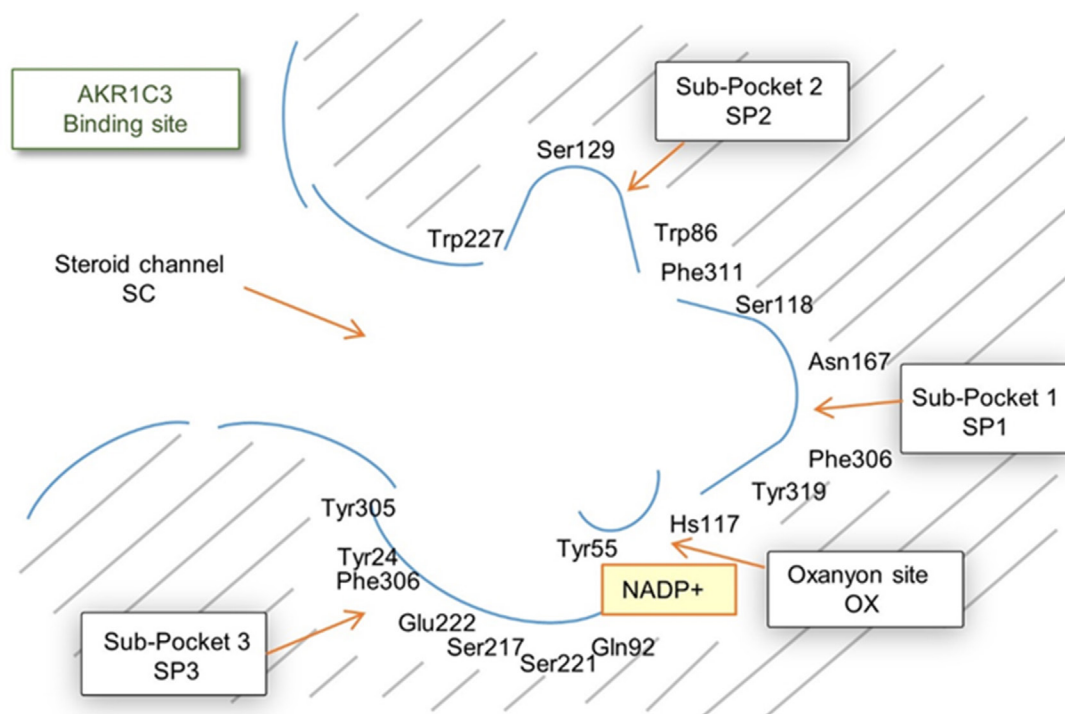


Figure 6: Close-up view of the AKR1C3 ligand-binding pocket. Illustrating the different compartments (the oxyanion site, the steroid channel and subpockets SP1, SP2 and SP3) that can be targeted with small molecules. NADP⁺ molecule is represented by a yellow square

structure of AKR1C3 was reported by Lovering *et al.*^[47] and revealed AKR1C3 as a typical aldo-keto reductase structure, with a catalytic pocket consisting mainly of loops A (116-143), B (217-238) and C (298-323). The ligand-binding pocket of AKR1C3 can be divided into five compartments as follows: an oxyanion site, a steroid channel SC and subpockets SP1, SP2 and SP3 [Figure 6].

The oxyanion site consists of the cofactor NADP⁺ and the catalytic residues Tyr55 and His117, which are conserved among all AKR1C enzymes. The steroid channel is formed by Tyr24, Leu54, Ser129 and Trp227 and is open to solvent, guiding substrates into the oxyanion site. The SP1 pocket is located inside the ligand-binding pocket and is surrounded by Ser118, Asn167, Phe306, Phe311 and Tyr319. In contrast, the SP2 pocket is located in a shallow region surrounded by Trp86, Leu122, Ser129 and Phe311, while the SP3 pocket is located near the phosphate moiety of NADP⁺ and is surrounded by Tyr24, Glu192, Ser221 and Tyr305^[48].

The structure of human AKR1C3 has been determined in complex with different substrates and inhibitors, which has enabled an excellent basis for the design of specific inhibitors. Selectivity is even more necessary

with respect to AKR1C1 and AKR1C2, enzymes that have more than 86% of identity with AKR1C3, but inactivate DHT to 3 β -androstenediol and to 3 α -androstenediol respectively^[49-51], thus decreasing the androgenic signalling. Between AKR1C3 inhibitors, several nonsteroidal anti-inflammatory drugs have been demonstrated to be very potent in inhibiting this enzyme. Some of them also exhibited good selectivity for the C3 isoform, e.g. indomethacin (10, Figure 7) and their binding mode within the ligand pocket has been investigated through X-ray crystallography^[48].

Discussion as to the use of AKR1C3 inhibitors to treat CRPC has been described in excellent reviews in 2011 and 2013^[51,52]. Since that time, several groups have reported on the discovery of hit and lead compounds, and these will be briefly reviewed here.

Among natural inhibitors, Skarydova *et al.*^[53] investigated the possible inhibitory effect of diverse types of isoquinoline alkaloids isolated from plant sources against the recombinant form of AKR1C3. Nineteen isoquinoline alkaloids were examined for their ability to inhibit AKR1C3 and as a result, stylopine (11, Figure 7) was demonstrated to be the most potent inhibitor among the tested compounds, demonstrating moderate selectivity towards AKR1C3.

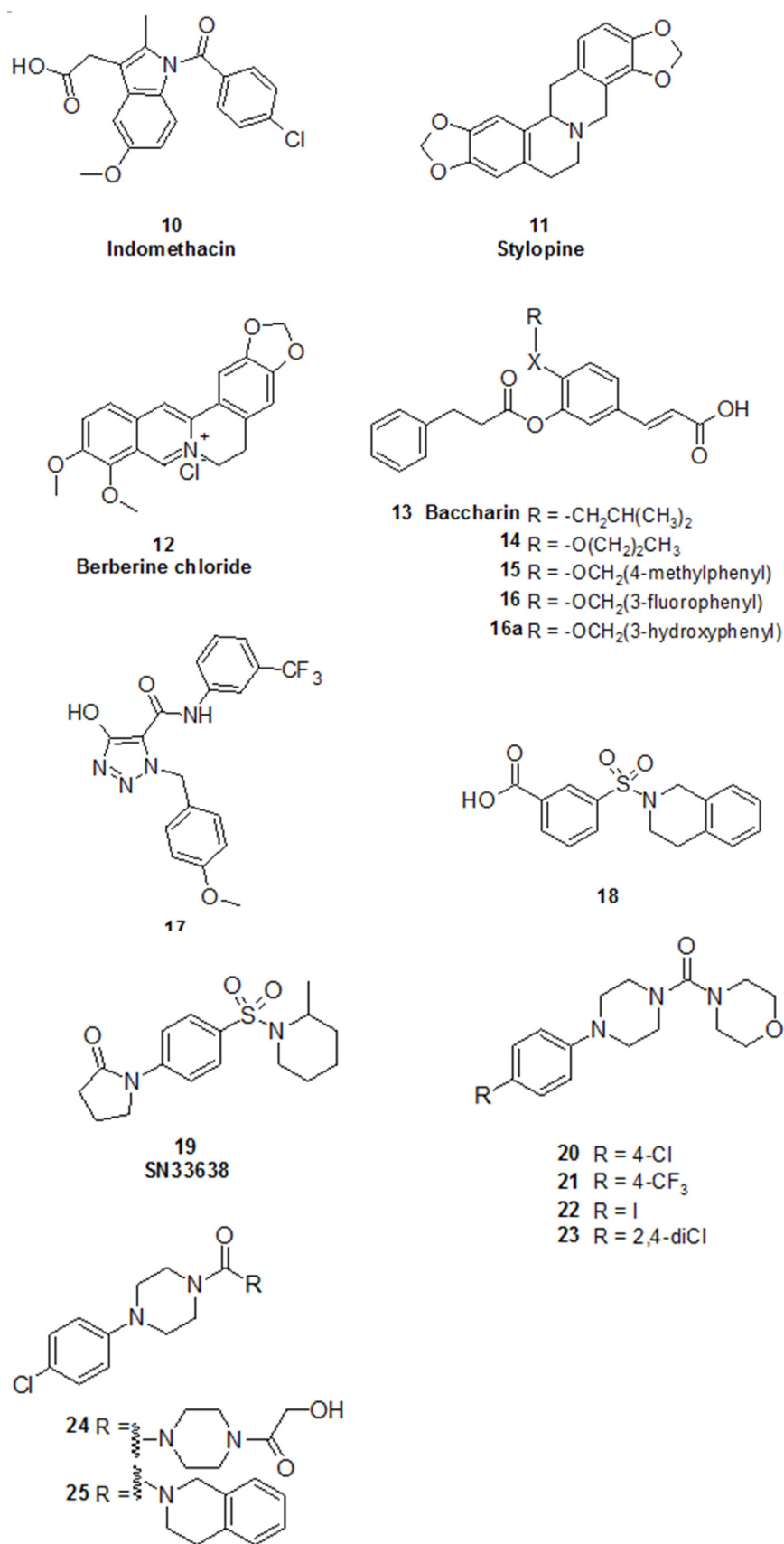


Figure 7: Examples of AKR1C3 inhibitors based on different chemical scaffolds

In an attempt to identify potential AKR1C3 inhibitors based on known natural-based pharmacophores, Tian *et al.*^[54] studied the blocking mechanism of berberine (2,3-methylenedioxy-9,10-dimethoxyprotoberberine chloride; 12). This isoquinoline alkaloid screened from a traditional Chinese medicine monomer library, was shown to prevent AKR1C3-mediated intratumoral steroidogenesis in castrated nu/nu mice bearing subcutaneous LNCaP xenografts. The authors found that berberine inhibited AKR1C3-expressing 22Rv1 PCa cell proliferation and decreased cellular T formation in a dose-dependent manner, provided the experimental basis for the use of berberine as the lead compound for the further design, research, and development of AKR1C3 inhibitors.

Baccharin (3-prenyl-4-(dihydrocinnamoyloxy)cinnamic acid, 13) is a constituent in the ethanol extract of Brazilian propolis^[55], which is a natural resinous substance collected by honeybees and has been used in alternative medicine to treat inflammation, liver disorders, and stomach ulcers. Recently Endo *et al.*^[56] found that baccharin is a selective and potent inhibitor of AKR1C3, correlating with the antiproliferative effect of baccharin against human PC3 PCa cells. Baccharin was shown to exhibit a 900-fold selectivity for AKR1C3 over the other three AKR1C isoforms. Due to its high inhibitory selectivity, baccharin represented a promising lead for the development of more potent and specific agents targeting AKR1C3. The structure activity relationship (SAR) of propolis-derived cinnamic acids suggested that the 3-prenyl moiety of baccharin is responsible for the selective binding to AKR1C3^[56]. Endo *et al.*^[46] also reported on the commercially available 3,4-dihydroxybenzaldehyde derivatives configured with 3-aliphatic and aryl ethers instead of the 3-prenyl moiety. Within the series of aliphatic ethers, AKR1C3 inhibition was shown to decrease proportionally with increase in the aliphatic chain lengths. Compound 14, possessing an n-butyl ether, showed the highest inhibitory potency. Within the series of aromatic ethers, two benzyl ether derivatives, 15 and 16, showed an equivalent inhibitory potency to baccharin. The molecular docking of 15 in the crystal structure of AKR1C3 informed the design of a novel baccharin-based inhibitor (16a) with improved potency (K_i 6.4 nmol/L), which may be due to the introduction of a new interaction between the 3-hydroxyl group of the benzyl moiety of 16a and Tyr24 of the enzyme. The inhibitory selectivity of 16a for AKR1C3 over other human AKR1C isoforms was comparable or superior to that of baccharin. Additionally, 16a significantly decreased the cellular metabolism by AKR1C3 at much lower concentrations than baccharin.

Since carboxylic acids are likely to be transported into cells by carrier-mediated processes rather than passive diffusion^[57], there are potential advantages in finding non-carboxylate inhibitors^[58,59]. Following this rationale, we have applied a scaffold hopping strategy replacing the benzoic acid moiety of flufenamic acid with an acidic hydroxyazolecarbonylic scaffold^[60]. In particular, differently N-substituted hydroxylated triazoles were designed to simultaneously interact with both subpockets 1 and 2 in the active site of AKR1C3, larger for AKR1C3 than other AKR1Cs isoforms. Through computational design and iterative rounds of synthesis and biological evaluation, novel compounds were reported, sharing high selectivity (up to 230-fold) for AKR1C3 over 1C2 isoform and minimal COX1 and COX2 off-target inhibition. A docking study of compound 17, the most interesting compound of the series, suggested that its methoxybenzyl substitution has the ability to fit inside subpocket 2, being involved in π - π stacking interaction with Trp227 (partial overlapping) and in a T-shape π - π stacking with Trp86. This compound was also shown to diminish testosterone production in the AKR1C3-expressing 22RV1 prostate cancer cell line while synergistic effect was observed when 17 was administered in combination with abiraterone or enzalutamide.

Heinrich *et al.*^[61] also reported on a non-carboxylate inhibitor class of phenylpyrrolidin-2-one derivatives, obtained modulating 18, an inhibitor deriving from a high-throughput screen^[62]. This modulation afforded compound 19, named later as SN33638, that inhibited AKR1C3 without forming a direct interaction with the oxyanion hole in the active site. Furthermore, in a cell-based assay, 19 was shown to be more potent than the carboxylic acid analogue 18 (ratio $IC_{50}(\text{enz})/IC_{50}(\text{cell})$ was 0.48 for 18 vs. 8.5 for 19), suggesting a pharmacological disadvantage for the acids in PCa cells^[61]. The authors explored the role of the sulphonamide substituent and probed its affinity within the enzyme hydrophobic pocket bound by residues Met120, Asn167, Tyr216, Phe306, Phe311, Tyr317, Pro318 and Tyr319 [Figure 8]^[61]. SAR studies of potent and selective non-carboxylate AKR1C3 inhibitor 19 showed that while the sulphonamide function was still as critical as in 18^[62], there was much more tolerance for the sulphonamide substituent, with a range of monocyclic six-membered ring analogues retaining activity and AKR1C selectivity. Crystal structure studies show that the 2-pyrrolidinone was located in the SP3 pocket but did not bind to the oxyanion site, and variations in the position, co-planarity or electronic nature of the pyrrolidinone ring abolished or severely diminished activity. The effectiveness of compounds at inhibiting AKR1C3 activity in cells broadly correlated

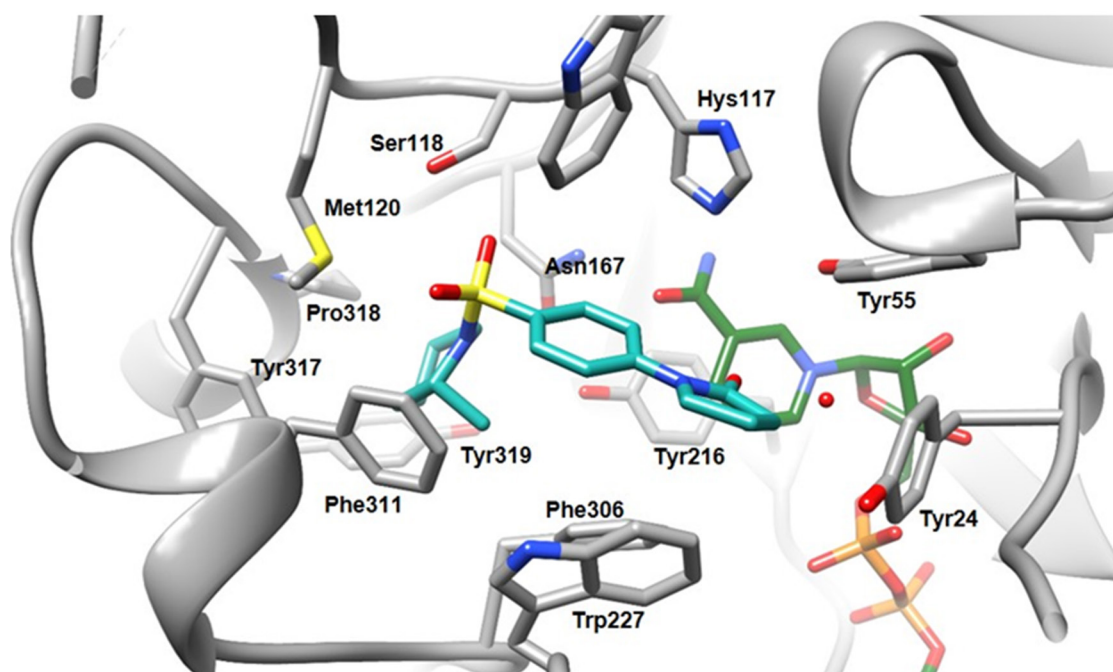


Figure 8: Structure of AKR1C3 in complex with SN33638 (19, PDB ID 4H7C). Carbon atoms of 19 are colored in blue, carbon atoms of cofactor NADP⁺ in green, carbon atoms of the protein are grey. Nitrogen, oxygen, sulphur and phosphorous atoms are depicted in blue, red, yellow and orange respectively. Relevant water molecules are represented by red points

with their enzyme inhibitory activity^[61].

Equipped by this powerful compound SN33638, Yin *et al.*^[63] treated a panel of CRPC and ER-positive breast cancer cell lines, in the presence of hormone or prostaglandin precursors, prior to evaluation of cell proliferation and levels of 11 β -prostaglandin F₂ α (11 β -PGF₂ α), T production and PSA expression. Although SN33638 was shown to inhibit 11 β -PGF₂ α formation, its ability to prevent T and 17 β -estradiol production and their roles in CRPC and ER-positive breast cancer progression was limited due to AKR1C3-independent steroid hormone production. This is except in LAPC4 AKR1C3 cells, where the majority of T production was AKR1C3-dependent. These results suggested that inhibition of AKR1C3 is unlikely to produce therapeutic benefit in CRPC and ER-positive breast cancer patients, except possibly in the small subpopulation of CRPC patients with tumours that have upregulated AKR1C3 expression and are dependent on AKR1C3 to produce T required for growth. The study by Yin *et al.*^[63] provided a valuable framework for future preclinical or clinical studies aimed at verifying this hypothesis that AKR1C3 inhibition suppresses tumour formation only in a selected population of CRPC patients expressing high levels of AKR1C3^[64].

From the same high-throughput screen that enabled the discovery of SN33638 also came

morpholino(phenylpiperazin-1-yl)methanone 20 [Figure 7], which was identified as a novel potent (IC₅₀ = 100 nmol/L on isolated enzyme) AKR1C3-selective inhibitor without carboxylic function^[65]. SAR studies of the new class of morpholino(phenylpiperazin-1-yl)methanone AKR1C3 inhibitors derived from 20 showed that these compounds bind selectively to AKR1C3 via the carbonyl oxygen of the central urea linker. This activity is favoured by lipophilic electron-withdrawing substituents on the phenyl ring (e.g. compounds 21-23) that probe specific regions of the SP1 pocket and H-bond acceptors on the other terminal ring and this was also supported by a QSAR study. Furthermore in the crystal structure of 20 bound to AKR1C3, the morpholine oxygen is within hydrogen bonding distance to a structured water molecule (HOH556), which is part of a network located in the SP3 pocket. The importance of it was examined via studying a series of derivatives in which the authors modulated the morpholine ring bound to urea. In this second series the complete inactivity of compounds bearing cyclohexane or phenylic ring suggested that there is a requirement for a secondary aliphatic nitrogen (i.e. a urea moiety) and an H-bond acceptor. It is possible also to replace the morpholine ring with larger substituents; e.g. compounds 24 and 25 derived from 20 were found active within this second series.

The discovery of AKR1C3 inhibitors with clinical potential has also been pursued by the pharmaceutical

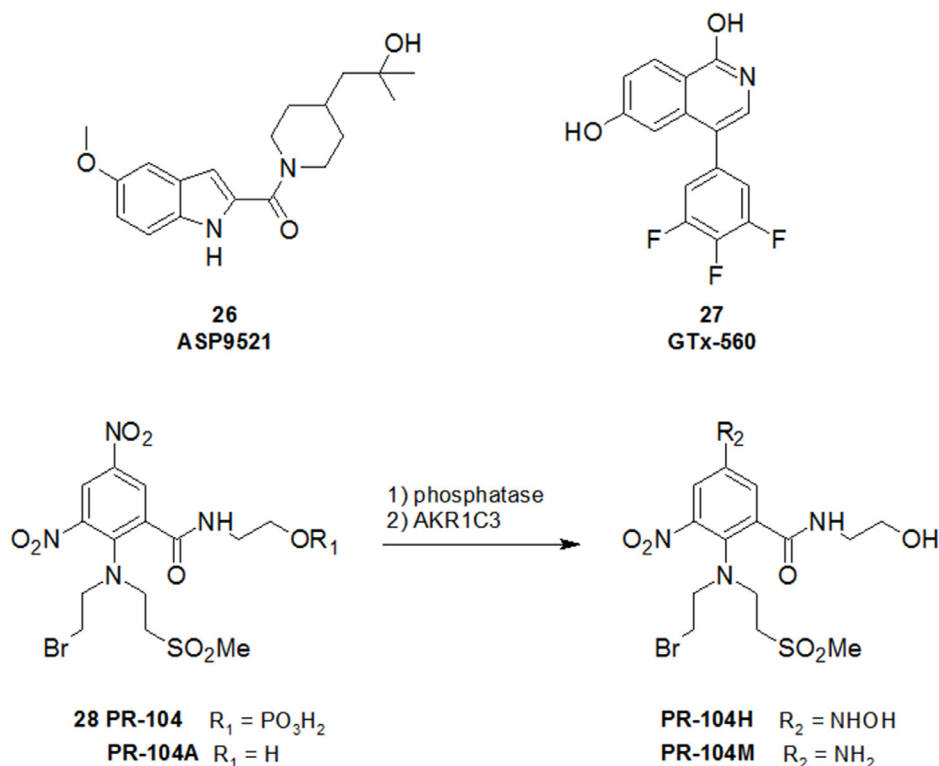


Figure 9: Examples of chemical structures of AKR1C3 inhibitors

sector, including Astellas Pharmaceuticals and GTx-therapeutics. Both companies designed *in vivo* active compounds, namely ASP9521 (26, Figure 9) and GTx-560 (27), respectively^[66,67]. However, only ASP9521 has so far been the subject of clinical evaluation. In a multi-centre phase I/II study, the compound was found to be orally bioavailable and well tolerated, but disappointingly without efficacy^[68]. It is noteworthy, however, that 6/13 mCRPC patients discontinued treatment before the end-trial and patients were not preselected for AKR1C3 status. Also, none of the patients in the study had received prior treatment with abiraterone, so AKR1C3 expression may have been insufficient to observe significant inhibitory effects by ASP9521 in these patients.

ASP9521 remains the first and only rationalised AKR1C3-specific inhibitor to reach clinical evaluation. In addition, the non-selective AKR1C3 inhibitor indomethacin (10, Figure 7) has been used in combination with both enzalutamide^[69] and abiraterone^[70] in two different phase II clinical trials (NCT02935205 and NCT02849990, respectively).

The catalytic capacity of AKR1C3 has also been exploited in prodrug design and some work has focussed on the clinically evaluated bioreductive prodrug PR-104 (28, Figure 9)^[71]. This prodrug that

was originally designed to be bioreduced under hypoxic conditions to generate a DNA alkylating agent, has also been shown to be bioactivated by AKR1C3 in a hypoxia-independent manner to active species PR-104H and PR-104M. It is possible that a sub-population of patients with AKR1C3-expressing tumours could benefit by PR-104 treatment and hence expand the CRPC armamentarium of drugs^[72].

HSD17B3

17 β -hydroxysteroid dehydrogenase type 3 (17 β -HSD3 or HSD17B3) is a microsomal enzyme member of the group of the NAD(P)(H) dependent oxidoreductases that catalyse the redox of hydroxyl/keto groups at position C17 of androgens and estrogens and in this manner regulate intracellular availability of steroid hormone ligands to their nuclear receptors. The 17 β -hydroxysteroid dehydrogenases (HSD17Bs) belong to the short-chain dehydrogenase/reductase (SDR) superfamily, with the exception of HSD17B5 (AKR1C3), which is part of the aldo/ketoreductase family as already discussed.

Though HSD17B3 is expressed almost exclusively in the testis, there have been some reports of its over-expression in PCa tissues [Table 2]. In the testes, this enzyme catalyses the last step in the biosynthesis of T, by stereoselectively reducing the C17 ketone

of AD using NADPH as cofactor. In PCa, HSD17B3 may operate like AKR1C3 and participate in all the three putative biosynthetic pathways [Figure 2]. In the canonical pathway, in addition to the reduction of AD to T, it could also reduce DHEA to androstenediol, while in the 5 α -dione and the backdoor pathways it could also lead directly to DHT by reducing 5 α -androstanedione or androsterone respectively [Figure 2]^[24]. A better understanding of what governs HSD17B3 and AKR1C3 in the PCa microenvironment could improve efforts to more effectively target these key enzymes in the steroidogenic biosynthetic pathway.

Due to the exclusive expression of HSD17B3 in the testes, selective inhibitors exerting effects equivalent to chemical castration may have potential as therapeutics for the treatment of PCa, and may be superior to the existing endocrine therapies based on a potential reduction in off-target effects. In addition, combination with an AKR1C3-selective inhibitor could possibly lead to more effective inhibition of the biosynthetic pathway and subsequent AR binding. Due to HSD17B3 being a membrane-bound protein, a crystal structure of HSD17B3 is not yet available. Nonetheless, as a member of the SDR family, HSD17B3 could share some highly conserved structural features, including the Rossmann fold, the cofactor binding site and the wide and easily accessible catalytic active site already demonstrated for the other six members of this family.

Several research groups have reported potent selective steroidal and nonsteroidal inhibitors as promising leads^[73-76]. Recently, a review describing HSD17B3 as a target in hormone-dependent PCa therapy has been published^[77], which described the main structure, function and reporting only a few examples of steroidal and non-steroidal inhibitors of HSD17B3. Here we provide a discussion of the most active nonsteroidal inhibitors developed to-date.

To aid structure-based drug design, some homology models of HSD17B3 have been developed^[78-81]. A series of compounds based on the dibenzazepine scaffold was discovered in 2006 and compound 29 [Figure 10] was initially identified as promising hit compound and used as a lead to discover compound 30, which exerted picomolar activity in enzymatic as well as cellular (stably expressing 17 β -HSD3 MDA-MB453 cells) assays^[78]. This compound was very useful in helping the design of the subsequently discovered 17 β -HSD3 inhibitors. In fact, Vicker *et al.*^[79] built a homology model of 17 β -HSD3 and used 30, as well as some structures described in Schering-Plough patents^[82] (e.g, 31, Figure 10), to construct

a pharmacophore and identified a potent compound STX2171 [32, IC₅₀~200 nmol/L in the whole-cell 293-EBNA(HSD3) assay], that had only negligible activity against 17 β -HSD2 (the enzyme that catalyses the reverse reaction) and was inactive against 17 β -HSD1^[83].

STX2171 and 31 (later named STX1383) were also tested in a hormone-dependent PCa LNCaP(HSD3) xenografts, which were established in castrated male mice and using AD to stimulate tumour proliferation^[83]. Both compounds were able to inhibit the proliferation of androgen-dependent prostate tumours (when stimulated by AD) and to reduce but not completely inhibit plasma T levels. An explanation of the incomplete abolition of plasma T levels can be found in the fact that also AKR1C3, prevalently expressed in the prostate, performs the conversion of AD to T.

In 2010, high-throughput screening led to the identification of 4-methylumbelliferone (4-MU, 33) as an inhibitor of HSD17B3^[84]. The authors studied new 7-hydroxycoumarin derivatives of 4-MU and observed the most potent compounds carried substituents in the 4-position. Structures 34 and 35 exhibited low nanomolar inhibitory activity in HeLa cells expressing human 17 β -HSD3 and selectivity versus other HSD17B isoenzymes and nuclear receptors.

Schuster *et al.*^[81] rationalised the potential therapeutic opportunity of the concomitant inhibition of HSD17B3 and HSD17B5 because of their partly overlapping functions. They developed pharmacophore models for HSD17B types 3 and 5 and found interesting HSD17B 3/5 dual-targeting inhibitors with different selectivity profiles, although some of them were affected by weaker off-target activity against other HSD17B enzymes. For example, structure 36 [Figure 10] was able to reduce HSD17B3 and HSD17B5 activity by 56% and 58% at 2 μ mol/L, respectively. Unfortunately, this compound was shown to also inhibit HSD17B1 by 20% at the same concentration. Although research for a dual inhibitor of HSD17B 3/5 enzymes needs deeper exploration, this approach could yield better compounds with clinical potential.

In 2012, Harada *et al.*^[85] developed a phosphate ester prodrug 37 as an orally bioavailable HSD17B3 inhibitor. The potency of the active molecule (IC₅₀ = 12 nmol/L in HeLa cells expressing human HSD17B3) rendered 37 capable of reducing plasma LHRH-induced T levels in a dose-dependent manner when administered orally to male Sprague-Dawley rats.

Interestingly, some environmental chemicals like

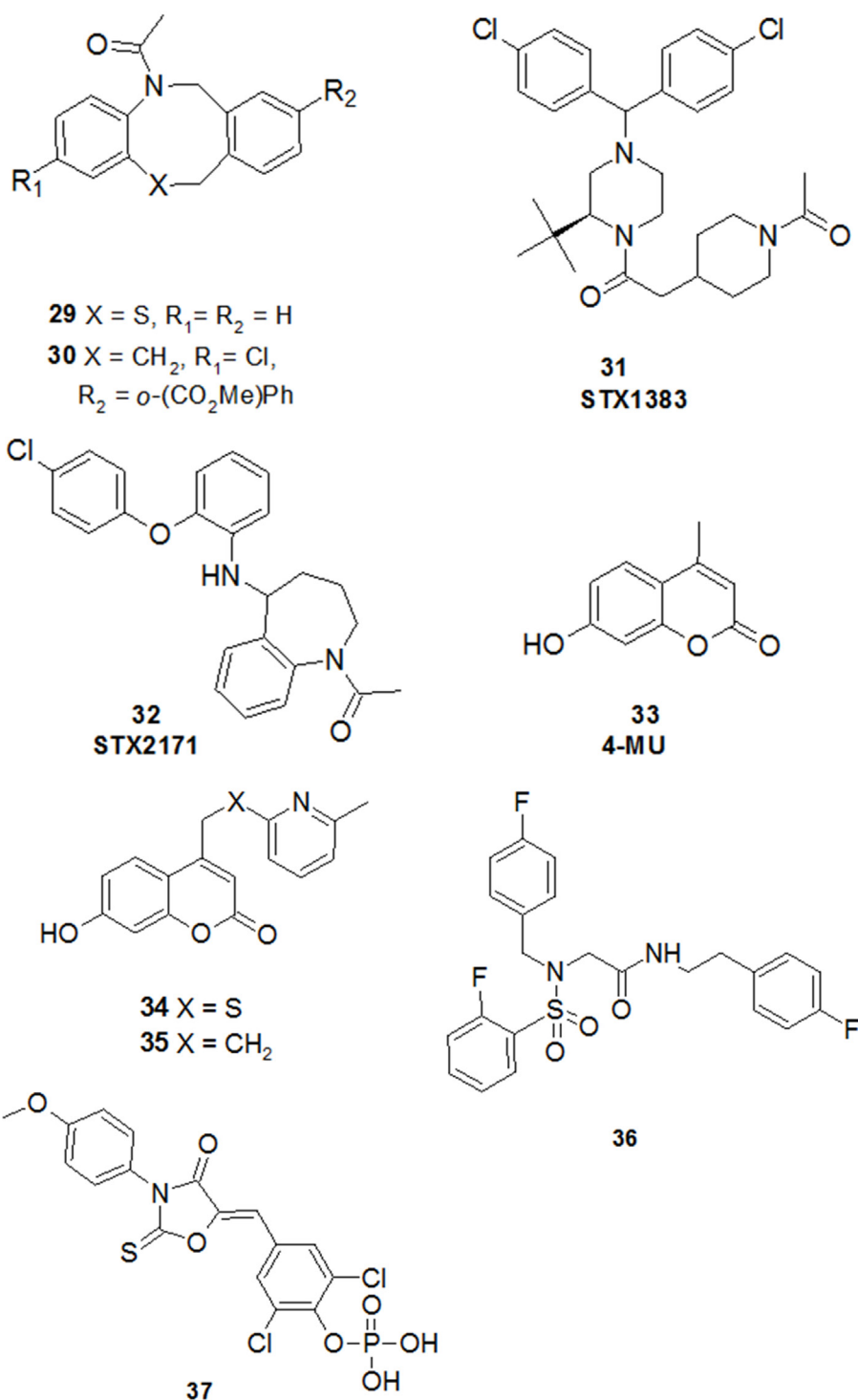


Figure 10: Structures of some nonsteroidal 17 β -HSD3 inhibitors

benzophenone-1^[80], tributyltin chloride and triphenyltin chloride^[86] have been identified as HSD17B3 inhibitors, but their use is considered harmful to normal sexual development, since this enzyme plays an essential role in that process.

In the last decade, several steroidal and non-steroidal

inhibitors of HSD17B3 have been designed and developed, but none of them has reached the clinic. One reason for this might be the difficulty in identifying an appropriate species to conduct the functional assays. Due to little sequence homology between human and other species isoforms, very potent inhibitors of the human enzyme show little

activity toward HSD17Bs of other species, especially rodents^[73,75,77]. Moreover, as AKR1C3, predominantly expressed in the prostate, performs the same biochemical conversions of HSD17B3, the *in vivo* HSD17B3 inhibition alone is not sufficient to completely abolish T levels in plasma. Additionally, the complexity and versatility of the steroidogenic pathways could bypass HSD17B3 inhibition *in vivo*, rendering these inhibitors not sufficiently efficacious in blocking tumour progression when tested alone.

SRD5A

Steroid 5 α -reductases (SRD5A) are membrane-associated (microsomal) enzymes that catalyse the 5 α -reduction of 3-oxo (3-keto), Δ 4,5 C19/C21 steroids. The reaction involves a stereospecific, irreversible breakage of the double bond between carbons 4 and 5 with the aid of cofactor NADPH and the insertion of a hydride anion to the α face at carbon C-5 and a proton to the β face at position C-4. Examples of substrates are T, progesterone, AD, epitestosterone, cortisol, aldosterone and deoxycorticosterone.

Three isoenzymes of SRD5A, which are encoded by different genes (SRD5A1, SRD5A2, and SRD5A3), have been identified. All are involved in the conversion of T into DHT in the canonical pathway. In addition, SRD5A enzymes are also involved in the reduction of AD and progesterone or 17 α -hydroxyprogesterone respectively to 5 α -androstenedione and pregnan-3,20-dione or pregnan-3 α ,17 α -dihydroxy-20-one. Therefore, these reductases appear to be key enzymes for the activation of both 5 α -dione and backdoor pathways [Figure 2].

Even though these three isozymes are intrinsic membrane-bound proteins that catalyse the same reaction, they only share a limited degree of homology in protein sequence and possess distinctive biochemical properties. SRD5A1 is expressed in low levels in the prostate, and is relatively insensitive to finasteride, a 4-azasteroid enzyme inhibitor (38, Figure 11). SRD5A2 is expressed in high levels in the prostate and in many other androgen-sensitive tissues and is sensitive to finasteride. Instead, it remains controversial whether SRD5A3 enzyme activity is inhibited by finasteride or dutasteride, the latter a 4-azasteroid derivative effective at inhibiting both SRD5A1 and SRD5A2^[87].

The inter- and intra-individual variability, the type and stages of progression of PCa and the methods used could explain the differences in the expression of the 3 isoenzymes observed in numerous reports [Table 2]. A summary of many studies that have discussed

the distribution of SRD5A1-3 in different human tissues is tabulated in the review of Azzouni *et al.*^[88], which extensively examines the basic biology of the SRD5A isoenzyme family. The different expression levels of SRD5A isoenzymes may confer response or resistance to 5 α -reductase inhibitors and thus may have importance in PCa prevention. The mechanisms underlying androgen regulation of expression of the three different SRD5A isoenzymes in human prostate cells has been investigated by Li *et al.*^[89]. The authors found that androgens regulate the mRNA levels of SRD5A isoenzymes in a cell type-specific manner, with regulation occurring at the transcriptional level and dependent on the AR. In addition, AR seems to be recruited to a negative androgen response element (nARE) at the promoter of SRD5A3 *in vivo* and directly binds to the nARE *in vitro*.

Due to the unstable nature of these enzymes during purification, the crystal structures of both SRD5A1 and SRD5A2 are still unresolved. Despite this, a large number of molecules has been developed as SRD5A inhibitors over the past 40 years. Finasteride (38, Figure 11) and dutasteride (39) are the only two clinically used drugs, having been approved by the FDA for the treatment of BPH in 1992 and 2002, respectively^[90,91]. Finasteride is a potent inhibitor of SRD5A2 with only weak *in vitro* activity versus SRD5A1 having IC₅₀ value of 9.4 and 410 nmol/L on the isolated enzyme, respectively^[92,93]. Dutasteride on the other hand, is a dual inhibitor of both SRD5A1 and SRD5A2 isozymes, with IC₅₀ of 2.4 and 0.5 nmol/L respectively^[94]. Both drugs are time-dependent competitive inhibitors and belong to the 4-azasteroids class of steroidal SRD5A inhibitors.

A detailed review of each category of inhibitors synthesised was published in 2010^[93], and aids understanding of the structural features required for SRD5A inhibitory activity. The review discussed the need for more potent and less toxic inhibitors of SRD5A and suggested the use of molecules outside the steroidal template, as they can decrease the potential interaction with an additional enzyme or receptor of the steroidal endocrine system. Since 2010, only a few molecules have emerged as nonsteroidal SRD5A inhibitors^[95]. In 2011, a hybrid molecule (40) was derived by merging structural features of finasteride and epristeride (41) as an inhibitor of SRD5A2. As epristeride behaves as an uncompetitive inhibitor^[93,95,96], a hybrid compound from these two molecules could have propensity for exploiting both mechanisms. However, the relative potency of 40 was only 0.49 (compared with finasteride), and the authors concluded, from observing docking poses of

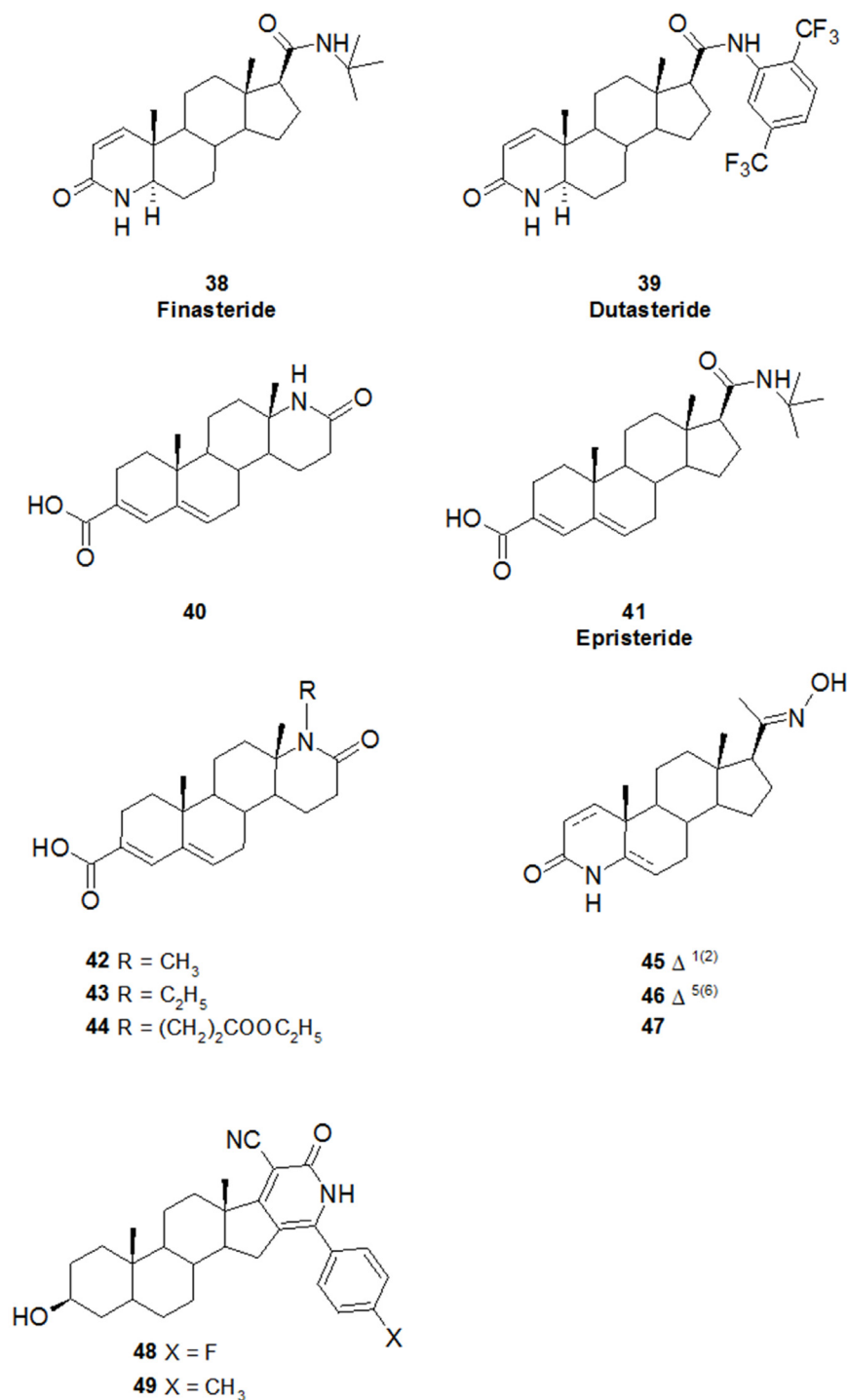


Figure 11: Structures of some 5 α -reductase inhibitors

40 in the AKR1D1 active site, a surrogate of SRD5A2, that its inhibitory mechanism was the same as that of finasteride^[97].

Aggarwal *et al.*^[98] studied similar steroidal molecules (structures 42-44) able to inhibit the type II enzyme in the same range of concentration as finasteride.

The carboxylic group at position-3 provided selective inhibition of SRD5A2, as all the compounds of this series showed minimal inhibition against the type I enzyme. 4-azasteroid-2-oximes (structures 45-47) were reported to be active against the SRD5A enzymes present on rat ventral prostate extract (both type 1 and the type 2 isozymes were present)^[99].

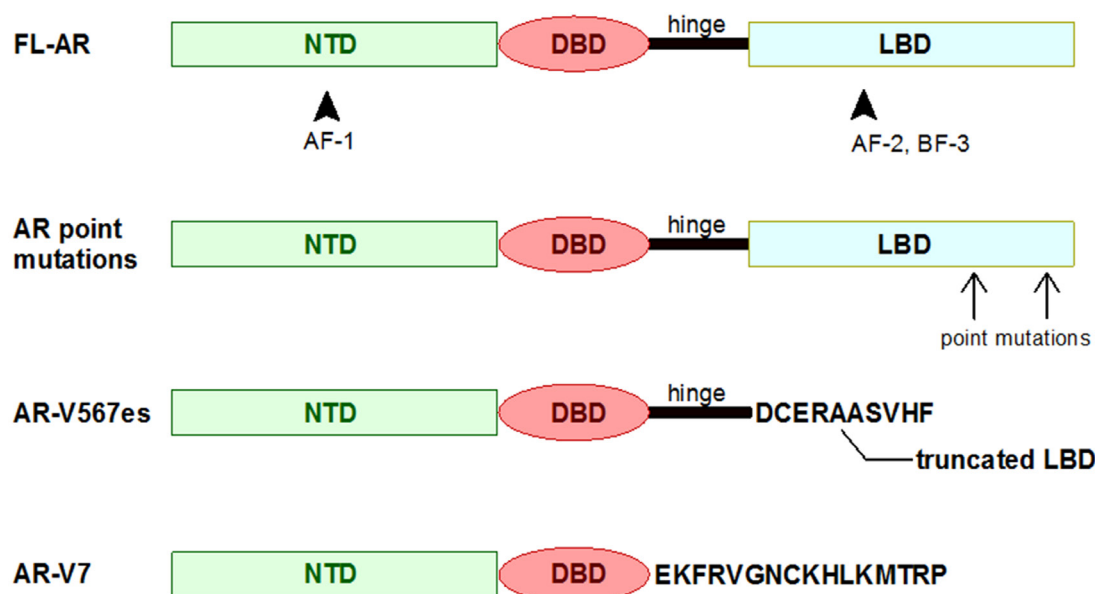


Figure 12: Domain organisation of full length AR (FL-AR) and some AR splice variants (AR-Vs). The four functional domains are indicated as follows: the N-terminal domain (NTD, green rectangles), the DNA-binding domain (DBD, red circles); ligand-binding domain (LBD, blue rectangles) and the “hinge region”, separating the LBD from the DBD (black lines). For AR-Vs lacking the LBD and/or the hinge region, the amino acids outside the previously described domains are listed. AR: androgen receptor

Recently, Al-Mohizea *et al.*^[100] prepared and performed pharmacological screening, including SRD5A inhibitory activities and antitumour properties (e.g. in LNCaP and PC-3 PC cell lines), of several steroids with a cyanopyridone heterocycle fused with its D-ring. The authors reported that these compounds had potent SRD5A inhibitory properties (*in vivo* assay with Sprague-Dawley rats). The best results were observed for the cyanopyridone structures with an oxygen bound to C3 (compounds 48-49, $IC_{50} = 210$ and 270 nmol/L, respectively when measured against rat SRD5A)^[95,100].

Finasteride and dutasteride^[90,91] have additionally been discussed in context of PCa prevention. Two clinical trials performed in the early 2000s in men at risk of developing PCa showed that the PCa incidence was significantly decreased in the treatment group, but the patients treated who were diagnosed with PCa had higher-grade tumors^[101]. A retrospective study rejected the results of these clinical trials on PCa prevention and hence these drugs have not been FDA-approved yet for the prevention of PCa^[102]. Subsequently, two clinical trials, (in phase II and IV, respectively) showed dutasteride to decrease the incidence of histopathological progression in patients with low-grade PCa and delay biochemical progression in patients who underwent radical prostatectomy or radiation therapy for localised PCa, respectively^[103,104].

Androgen receptor

The AR is crucial for normal functioning of the prostate. As a member of the steroid hormone receptor family of

ligand-activated nuclear transcription factors, it consists of four distinct functional domains, a poorly conserved N-terminal domain (NTD) with transcriptional activation function; a highly conserved deoxyribonucleic acid (DNA)-binding domain (DBD); and a moderately conserved ligand-binding domain (LBD). A short amino acid sequence called the “hinge region” separates the LBD from the DBD and also contains a part of a bipartite ligand-dependent nuclear localisation signal (NLS) for AR nuclear transport [Figure 12]^[105]. NTD contains a transactivation AF-1 region, with two transcription activation units (TAU1 and TAU5) and two motifs involved in protein-protein interactions and AR N/C interactions^[6]. NTD contains a co-regulator binding surface, the disruption of which reduces the androgen-dependent proliferation and migration of PCa cells^[106]. The LBD contains an activation AF-2 region, which is responsible for agonist-induced activity and androgen binding to induce conformational changes, which facilitates intra- and intermolecular interactions between the N-terminal and C-terminal domains and subsequently AR homo-dimerisation and nuclear translocation [Figure 12]^[107,108]. In the nucleus, ligand bound AR binds to specific recognition sequences known as “androgen response elements” (AREs) in the promoter and enhancer regions of target genes and recruits co-activators and co-repressors, which then modulate transcription of androgen-dependent proteins^[109]. Under physiological conditions, both T and DHT can bind to and activate AR signalling [Figure 1]^[110,111]. Deregulated AR signaling is common during PCa development and CRPC progression. The

ARs in tumour cells exposed to ADT undergo selective alterations that result in aberrant AR reactivation, which ultimately allows the AR pathway to remain active despite the shortage of androgenic ligands. AR amplification leads to AR overexpression, which is present in approximately 30% of CRPCs. Mutations in the AR gene occur in approximately 20% of CRPCs. Most significant AR mutations occur in the LBD, which increase the sensitivity and decrease the specificity of ligand binding^[112]. Constitutively active splice variants (AR-Vs) are detected in PCa cell lines (e.g. LNCaP95, VCaP and 22Rv1) and in CRPC tissues. More than 20 AR-Vs have been reported, but only ARv567 and AR-V7 are considered to be clinically relevant because their levels of expression are correlated with CRPC and poor survival rates [Figure 12]^[6]. AR-V7, like other AR variants lacks an LBD, and via its nuclear localisation binds DNA independently, without androgen activation, regulating a unique set of target genes that facilitate mitosis in addition to the regular androgen-dependent genes that are activated by full-length ARs that promote disease progression^[113].

As other steroid hormone receptors, also AR appear to be regulated by epigenetic mechanisms^[27]. The first evidence of epigenetic regulation of AR came from comparing the hypermethylation of AR promoters in AR-deficient and AR-expressing cell lines (hyper- and hypomethylated, respectively)^[114]. In human prostate cancer, a significant role of hypermethylation of AR genes has been suggested: AR hypermethylation was observed both in primary cell lines from PCa patients (20%) and in hormone-refractory prostate cancers (28%)^[115]. Also other epigenetic mechanisms, such as histone acetylation/deacetylation, seem to participate in the regulation of AR-driven genes^[116].

The literature is abundant with research articles and reviews concerning the development of AR-based therapy for PCa. The AR is a validated therapeutic target for PCa and five molecules have already been approved by the FDA (cyproterone acetate 50, flutamide 51, nilutamide 52, bicalutamide 53, enzalutamide 54, Figure 13)^[117] while several others are currently under preclinical/clinical development. Among papers of considerable interest on AR, we recommend the following for further reading: the report of Lu *et al.*^[118] that describes the mechanism of function of AR and its targetable domains, the review by Imamura and Sadar^[6], which focuses on AR-related mechanisms of resistance and AR antagonist therapeutic agents undergoing clinical trials, and finally the review by Martinez-Ariza and Hulme^[117], that encompasses non-ligand-binding protein modulators of the AR.

Unfortunately, most CRPC patients treated with AR-antagonist therapy will eventually develop resistance and succumb to the disease. Mechanisms of resistance to these drugs include modification of the AR, AR gain-of-function point mutations, truncated AR isoforms and constitutively-active AR splice variants^[6]. Accordingly, new agents to target these alternative ARs through novel mechanisms of action should lead to intensified research in the PCa community and lead to new drugs with clinical potential.

AR-antagonists can be classified based on their ability to interact with different domains of the AR. Approved drugs and similar structures under development show affinity for the LBD. These molecules, also named traditional AR antagonists, compete with androgens in binding the AR and prevent formation of the AF-2 (activation function-2) hydrophobic groove inside the LBD and its interaction with co-regulators. However, in some cases, the AR can still dimerise and become nuclear, as observed with enzalutamide using confocal micrographs^[119]. Apalutamide (55, Figure 13) and darolutamide (56, OMD-201) are two molecules under evaluation in phase 3 clinical trials in patients with non-metastatic CRPC (NCT01946204 and NCT02200614, respectively). Apalutamide shows high structural similarity to enzalutamide, but achieves the same therapeutic response as enzalutamide at a lower dose in a LNCaP xenograft mouse model and does not induce AR nuclear translocation or DNA binding^[120]. Darolutamide is characterised by a different chemical scaffold from its cognate antagonists, and is able to antagonise AR mutants F876L, W741L and T877A^[121].

Seviteronel (5, Figure 4), a non-steroidal CYP17A1 inhibitor with 17,20-lyase selectivity (see above), has been found to show AR-antagonist activity independent of CYP17A1 enzyme inhibition, with evidence of direct binding to the AR LBD^[40]. Similarly, also galeterone (3, Figure 4) is a competitive AR antagonist mediated by binding of the drug to the steroid-binding pocket of AR and concomitantly inhibiting T biosynthesis through inhibition of CYP17A1 lyase activity^[36]. In addition, galeterone targets the LBP of mutated T878A AR. The authors also reported on PSA reduction in LNCaP and VCaP cell lines, an effect that was partially reversed upon addition of DHT in a dose-dependent manner^[122,123].

The SAR for AF-2 targeting have been extensively studied^[124-126], and X-ray structures of AR-LBD in complex with T (PDB: 2AM9), *R*-bicalutamide (PDB codes: 1Z95 and 4OJB, Figure 14) or hydroxyflutamide (PDB: 2AX6) and other ligands have been resolved^[127]. Essentially, the compounds consist of three structural parts: the first part is usually an

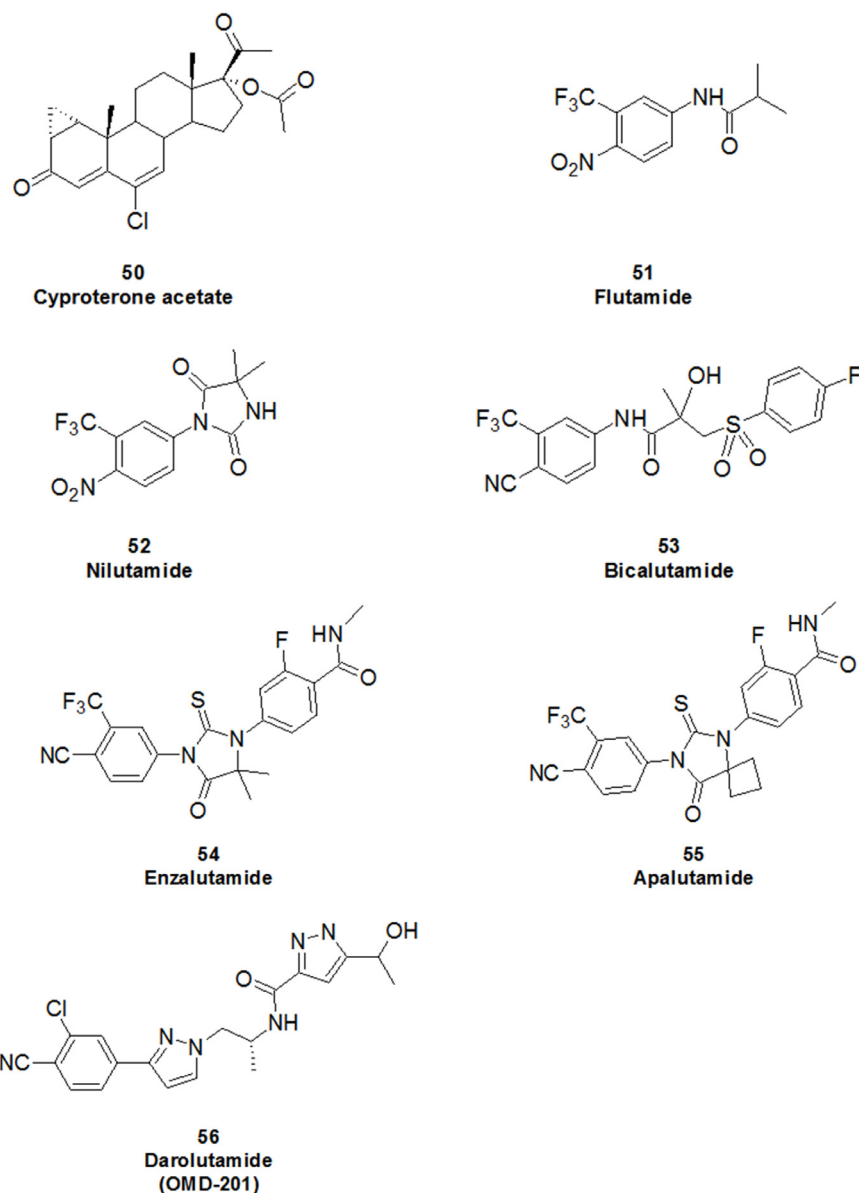


Figure 13: Androgen receptor ligand-binding protein antagonists approved by FDA or in clinical trials

aromatic ring substituted with a nitrile and an electron withdrawn group interacting with Arg752, Phe764, and Gln711 [Figure 14], the second part is a nitrogen-containing moiety such as an amide function or a heterocyclic ring, and the third part, often connected to the second through a short linker, is different in nature in various antagonists but nonetheless important in suppressing the agonistic effect^[118].

Most of these agents are derivatives of already approved non-steroidal AR antagonists, but there is a need to discover broader chemotypes, distinct from known scaffolds to avoid cross-resistance with these compounds. In this regard, Kandil *et al.*^[128] synthesised umbelliferone derivatives merging two independent *in silico* pharmacophores based on virtual screening

studies. Putative binding modes of compound 57 [Figure 15], the most active of the series ($IC_{50} = 0.93$ in 22Rv1 cells), within the antagonistic AR-LBD showed hydrogen bond interactions with key amino acids Arg752, Gln711 (with the lactone carbonyl group), Thr877 (with the terminal carbonyl group) and Asn705 (with the methylene group). Another interesting example of an innovative structure is represented by the molecule synthesised by Johnson *et al.*^[129] (compound 58), though only the PSA luciferase assay was conducted to evaluate biological activity. BMS-641988 (59) is a non-steroidal compound disclosed in 2015 with high binding affinity for the AR ($K_i = 1.7$ nmol/L), efficacious in a CWR22-BMSLD1 PCa xenograft model with superior efficacy to bicalutamide. Due to its acceptable preclinical safety profile both *in vitro* and

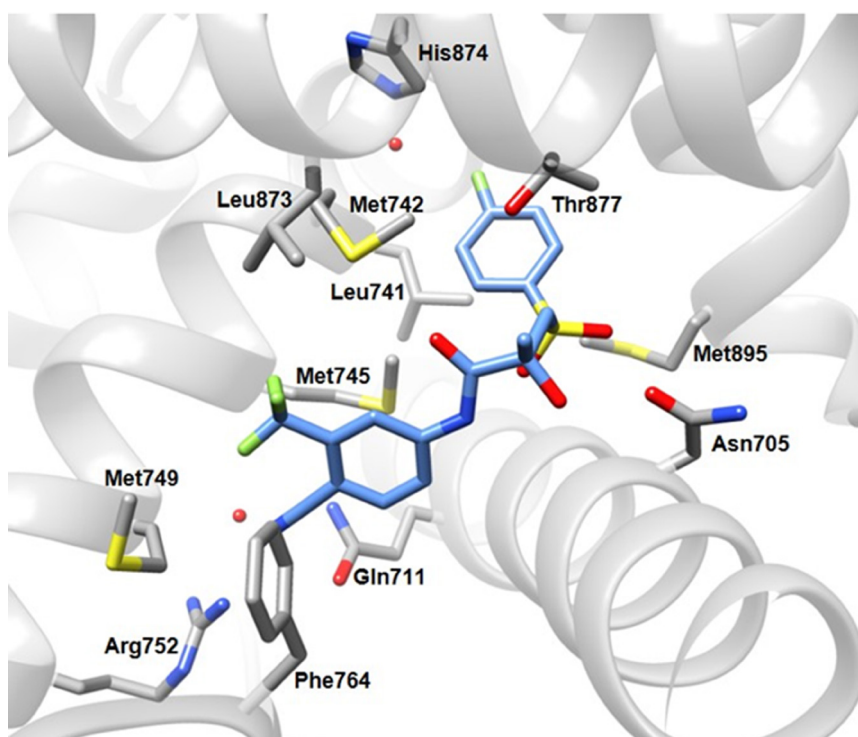


Figure 14: Crystal structure of Trp741Leu AR-LBD in complex with R-bicalutamide (PDB ID 4OJB). Carbon atoms of R-bicalutamide are coloured in blue, the AR is grey. Nitrogen, oxygen, sulphur, atoms are depicted in blue, red and yellow respectively. Relevant water molecules are represented by red points. AR: androgen receptor; LBD: ligand-binding domain

in vivo, it was selected for clinical development and the outcomes of two Phase I studies in patients with CRPC (NCT00644488 and NCT00326586) have been published^[130].

Recently, new derivatives^[131] of DIMN (60), a potent and well characterised AR antagonist interacting with the LBD were designed and synthesised^[132]. Some of these derivatives exhibited higher AR antagonistic activity than DIMN itself and bicalutamide, even with DHT co-treatment, and higher inhibitory effects on LNCaP cells proliferation. Compounds 61 and 62 bear long, linear and hydrophobic side chains on the tetrahydroisoquinoline moiety, while 63 carries an additional bulky group such as a phenyl ring [Figure 15]. Their potency in inhibiting LNCaP cells (IC_{50} range: 0.35-1.01 $\mu\text{mol/L}$) was shown to be superior to DIMN ($IC_{50} = 4.46 \mu\text{mol/L}$); this indicates that the occupation of a cone-shaped cavity, located near Thr877 (interacting with ethereal oxygen from docking studies proposed by the authors) increases bioactivity of the series.

Recently, a new class of AR modulators bearing the triazole core has been proposed, which are able to exert antiproliferative effects on LNCaP-AR cells and on CW22Rv1 cells, which constitutively expresses high

levels of AR-V7. Compound 64 was the most effective compound of this series and was also evaluated *in vivo* using CW22Rv1 xenografts, demonstrating superior activity to enzalutamide in this model^[133].

BF-3 is another targetable binding domain located at the surface of the AR, where it controls the allosteric modulation of AF-2^[134]. Notably, the mutation which occurred in the AF-2 of LBD will not alter or weaken the binding of antagonists in the BF-3 site. Interestingly, flufenamic acid (65, Figure 16), that has also the ability to inhibit AKR1C3, can bind BF-3 with moderate affinity (range of activity: 10-50 $\mu\text{mol/L}$)^[135]. Among small molecules inhibiting this domain and described in recent reviews^[117,118,125,126], compound 66 displays excellent anti-androgen potency, antiproliferative activity against androgen-sensitive (LNCaP) and enzalutamide-resistant (MR49F) PCa cell lines, and effective inhibition of tumour growth *in vivo*, in both LNCaP and MR49F xenograft models^[136]. The data are very promising in highlighting the therapeutic relevance of the BF-3 groove in AR function. Recently, Zhang *et al.*^[137] designed a conjugate of thiosalicylamide and the BF-3 binding small molecule tolfenamic acid. This molecule, named YZ03 (67), enhanced acetylation of endogenous AR at Lys720 residue, critical for protein-protein interaction with the FXXLF coactivator peptide binding.

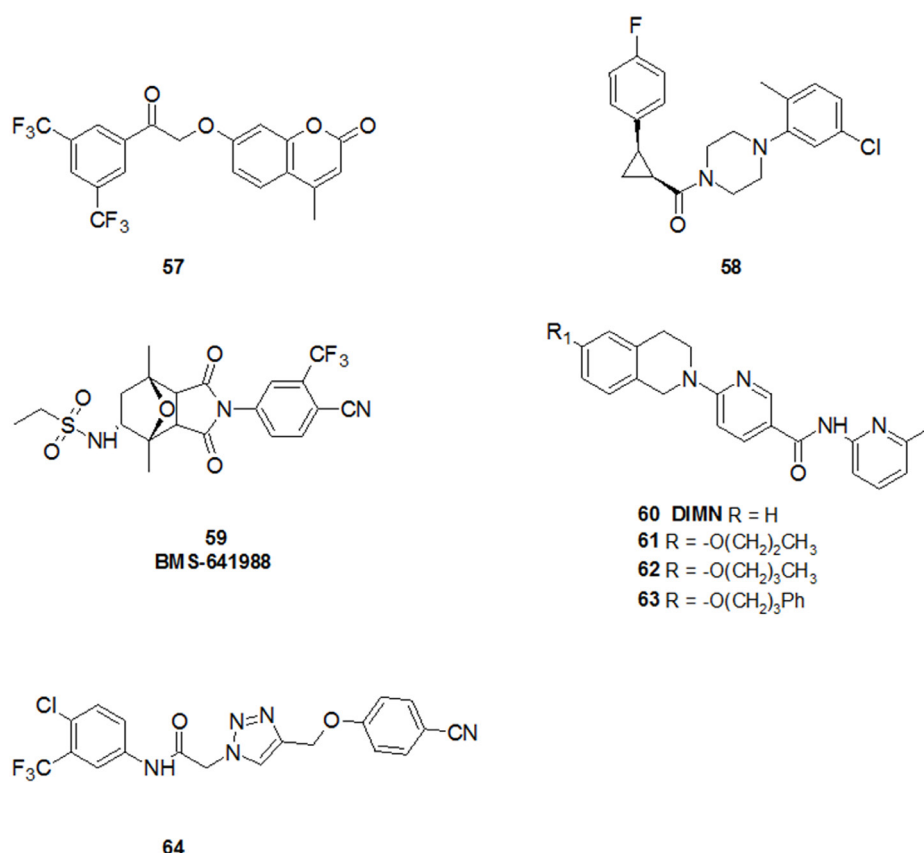


Figure 15: Selected competitive hormone antagonists under development

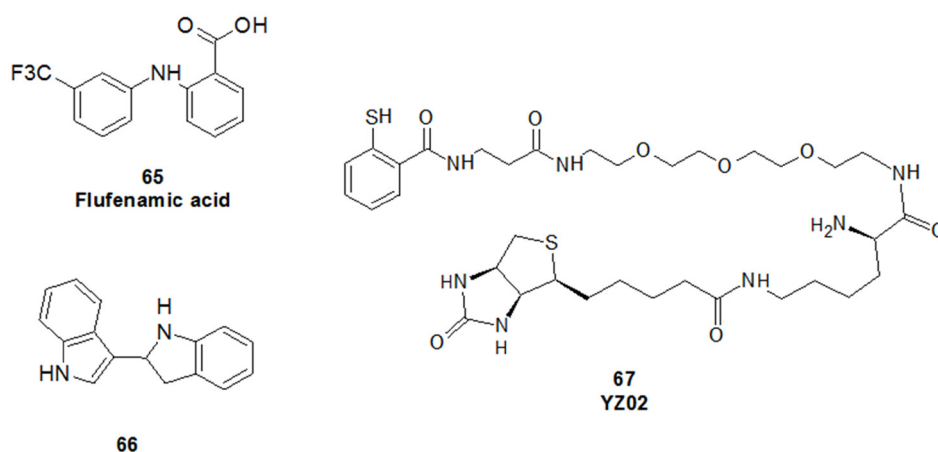


Figure 16: Selected examples of BF-3 site modulators

The AR NTD contains amino acids 1-558 and is an intrinsically disordered region. Activation function-1 (AF-1) is a protein binding domain known to bind different co-regulators and its low sequence identity with other nuclear receptors renders it an appealing target for selective small molecule inhibitors^[117]. The most studied AR NTD modulators are EPI compounds that have been shown to be bound covalently to AF-1 and to inhibit AR nuclear

translocation. Importantly, also constitutively-active AR splice variants lacking LBD are inhibited by EPI analogues where traditional AR antagonists are ineffective^[138]. EPI-001 (68), discovered by Andersen *et al.*^[139] by functional assay screening of marine sponge extracts, is shown in Figure 17; its stereoisomers EPI-002-EPI-005 have also been isolated and evaluated later. The potential therapeutic benefits of EPI have been demonstrated using

different human PCa cell lines and xenograft models in castrated male mice^[140]; a small molecule belonging to this class (EPI-506, a prodrug analogue of EPI-002^[141]) is now under clinical evaluation in a phase I/II study in men with mCRPC that have disease progression after enzalutamide and/or abiraterone treatment (NCT02606123).

The DBD is responsible for mediating interactions with AREs. Encouragingly, the 3D crystal structure of the rat AR DNA-binding domain has been obtained (PDB: 1R4I)^[142]. Some structures able to interact with this domain and prevent its interaction with DNA and are shown in Figure 17. Pyrvinium (69), an anthelmintic (and its hydrogenated analogue 1,2,3,4-tetrahydropyrvinium^[143]), was able to bind at the interface of the DBD dimer (inhibiting also AR splice variants lacking the LBD) and inhibit cell lines derived from both bone and prostate^[144]. Although the results are promising, some doubts have been expressed about the binding site on the DBD involved in the AR inhibition by 69^[117,145].

Insights into AR DBD inhibition were provided by Li *et al.*^[145] through the study of two molecules (70 and 71) identified through a virtual screening campaign and subsequent medicinal chemistry investigations. Both compounds exhibited nanomolar potency against the AR and effectively inhibited the growth of enzalutamide-resistant cells. Their binding modes were corroborated by mutagenesis experiments, confirming interaction of these inhibitors with residues Gln592 and Tyr594 of AR DBD. Compound 71 was also evaluated in LNCaP xenografts in mice, causing comparable reductions in tumour volume to enzalutamide^[146].

In summary, much effort has gone into AR-targeted drug design and in particular the LBD has been pursued as a target. However, mutated forms of AR, especially constitutively active AR variants, need to be considered as an aspect of AR-related resistance mechanisms and, for this reason, targeted with novel inhibitors. Accordingly, we look with particular interest to molecules targeting co-regulator binding regions and include AF-2 and BF-3 pockets (still within LBD, but their inhibition is able to overcome gain-of-function point mutations induced by AF-2 inhibitors), the NTD and the DND, or molecules able to inhibit AR through an indirect mechanism.

Degradation of all forms of ARs are emerging as an advantageous therapeutic paradigm for the more effective treatment of PCa in the context of AR mutations that confer resistance to second-generation AR antagonists. The compounds able to degrade the

AR are classified as Selective Androgen Receptor Degraders (SARD). A first-in-class non-steroidal SARD, AZD3514 (72, AstraZeneca, Macclesfield, UK, Figure 17) was developed and was shown to downregulate the AR^[147]. AZD3514, which binds the AR LBD and prevents its ligand-driven nuclear translocation, promotes down-regulation of AR levels. It has been clinically evaluated, but disappointingly only moderate anti-tumour activity in patients with advanced CRPC was observed; it was also shown to be poorly tolerated, with nausea and vomiting being the main toxicities^[148]. In the 2012, Yamashita *et al.*^[149] identified that ASC-J9 (73), also named as dimethylcurcumin, functioned as an AR degradation enhancer for full length AR and AR splicing variants. ASC-J9 is the first anti-AR compound discovered that selectively degrades AR in selective cells^[150].

Niclosamide (74), an anthelmintic drug, has been foundable to inhibit AR-V7 transcription activity and downregulate its protein expression. Both *in vitro* and *in vivo* PCa tumour growth were reduced upon treatment with 74 and it has been suggested that it acts by promoting AR-V7 degradation via a proteasome-dependent pathway^[151]. Furthermore, the combination of niclosamide and enzalutamide resulted in significant inhibition of enzalutamide-resistant tumour growth, suggesting that niclosamide enhances enzalutamide therapy and overcomes enzalutamide resistance in CRPC cells^[151]. A phase I study of niclosamide in combination with enzalutamide in men with CRPC is currently under investigation (NCT02532114).

Also galaterone (3, Figure 4), a known CYP17 inhibitor and AR antagonist, promotes the proteasomal degradation of both AR and its ligand-independent variants AR-V7 and Arv567es^[152,153].

Hydrophobic tagging technology has also been employed to degrade the AR. Bradbury *et al.*^[154] showed that molecules containing hydrophobic regions linked to small-molecule AR ligands induce AR degradation, reduce expression of AR target genes and inhibit proliferation in androgen-dependent PCa cell lines. By appending the alkylfluoroyl chain of fulvestrant onto DHT, a selective SARD compound was discovered. As a parallel strategy to the rational design of a SARD, Gustafson *et al.*^[155] appended an adamantyl moiety to the AR agonist RU59063 (75) via a short PEG linker to create SARD279 and SARD033 (76 and 77, Figure 17). They found that this addition switched the agonist into a pure antagonist capable of degrading AR protein (half-maximal degradation at 1 μ mol/L; maximal degradation of 95%). Moreover, this SARD was also able to inhibit proliferation of a

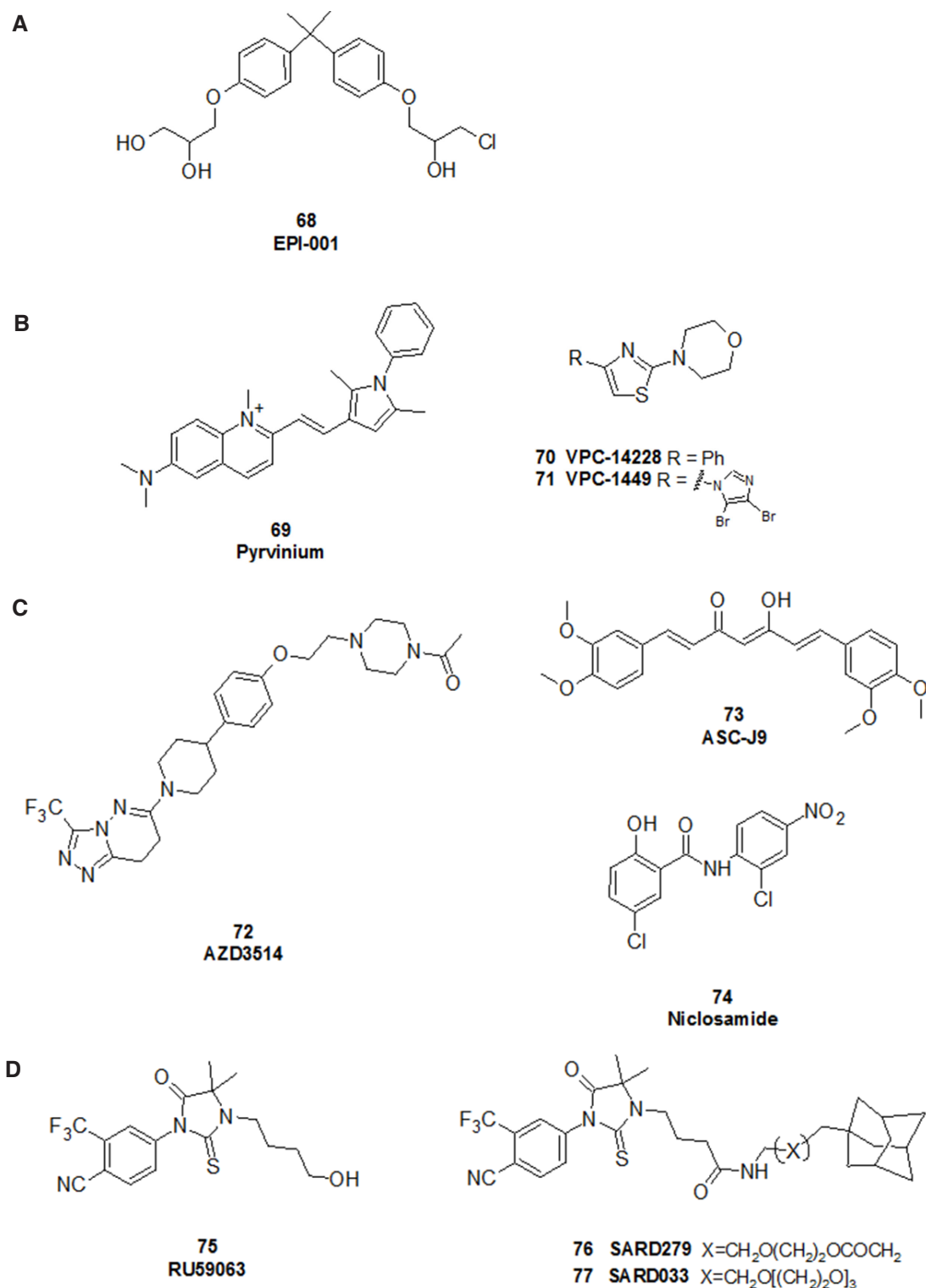


Figure 17: Selected compounds that act as: (A) NTD modulators; (B) DBD inhibitors; (C) indirect inhibitors of AR and AR variants; and (D) selective androgen receptor downregulator (SARD) compounds. AR: androgen receptor; DBD: DNA-binding domain; NTD: N-terminal domain

model castration-resistant PCa cell line resistant to enzalutamide. These results suggest that selective AR degradation may be an effective therapeutic prostate

tumour strategy in the context of AR mutations that confer resistance to second-generation AR antagonists^[156].

CO-ADMINISTRATION OF INHIBITORS AND ANTAGONISTS TARGETING THE AR AXIS

Androgen biosynthesis prevention and AR signalling inhibition, should in principle produce blockade of the AR axis. As these pathways are implicated in the progression of CRPC, these concurrent therapeutic actions should both reduce the incidence of resistance and increase therapeutic efficacy. Such a potential powerful combination strategy could replace the current PCa treatment paradigm of sequentially adding agents at the time of disease progression. Many drug combinations targeting the AR axis are described in literature. Unfortunately, the use of dutasteride in combination with bicalutamide for advanced PCa has not been as successful. Dutasteride plus bicalutamide in patients with progressive non-metastatic PCa did not delay further progression compared to only bicalutamide^[157] while dutasteride in combination with abiraterone as well as enzalutamide is currently in a phase II clinical trial where the outcome is pending at the time of writing^[158]. Recently Liu *et al.*^[159] showed overexpression of AKR1C3 to confer resistance to enzalutamide. Furthermore, the combination of indomethacin, an AKR1C3 inhibitor, and enzalutamide resulted in significant inhibition of enzalutamide-resistant tumour growth. These results suggested that AKR1C3 activation is a critical resistance mechanism associated with enzalutamide resistance. Accordingly, the dual targeting of intracrine androgens and AKR1C3 promises to overcome enzalutamide resistance and improve survival of advanced PCa patients. Subsequently, the same research group reported that treatment of abiraterone-resistant cells with indomethacin overcomes resistance and enhances abiraterone therapy both *in vitro* and *in vivo* by reducing the levels of intracrine androgens and diminishing AR transcriptional activity^[160]. Furthermore, these studies provide preclinical proof-of-principle for starting clinical trials focussed on investigating the combination of using indomethacin with enzalutamide, or with abiraterone for advanced PCa^[69,161].

BIFUNCTIONAL INHIBITORS AND ANTAGONISTS TARGETING THE AR AXIS

Despite the highly significant therapeutic relevance of combination therapies, potential advantages of a targeted therapy based on a single drug that modulates the activity of multiple targets over combination therapy are: (1) a more predictable pharmacokinetic profile; (2) a lower probability of developing target-based resistance^[162]; (3) a superior safety profile; and (4) a minimised risk of adverse effects^[162,163].

One of the first bifunctional non-steroidal small molecules studied by Chen *et al.*^[164] as therapeutic leads for CRPC was an N-(aryl)amino-benzoate inhibitor (77, Figure 18). The authors exploited the observation that some flufenamic acid analogues with AKR1C3 inhibitory activity also acted as AR antagonists^[165] and subsequently synthesised a second generation of AKR1C3 inhibitors in which the key features were the inclusion of an additional ring on the phenylamino ring. The derivative 3-[(4-nitronaphthalen-1-yl)amino] benzoic acid (77, Figure 18A) retained nanomolar potency and selective inhibition of AKR1C3 but also acted as an AR antagonist. It inhibited 5 α -dihydrotestosterone-stimulated AR reporter gene activity with an IC₅₀ = 4.7 μ mol/L and produced a concentration-dependent reduction in AR levels in PCa cells. The *in vitro* and cell-based effects of compound 77 makes it a promising lead for the development of dual acting agent for CRPC.

As mentioned above, the CYP17A1 inhibitor galeterone not only inhibits the enzyme but is also a competitive AR antagonist and causes degradation of the AR and its variants AR-V7 and Arv567es^[153,166-168]. Furthermore, galeterone also impaired AR binding to DNA and selectively up-regulated degradation of the mutated T878A AR protein^[122,123]. For its multifunctional activity, three different clinical studies have been initiated with galeterone. A phase I clinical trial has been completed, while a phase II clinical trial (ARMOR2) is still ongoing in CRPCa patients. A phase III clinical trial (ARMOR3-V7) has started recruiting CRPCa patients that specifically express AR-V7. Unfortunately ARMOR3-SV was terminated as it failed to meet its primary endpoint of demonstrating an improvement in radiographic progression-free survival (rPFS) for galeterone versus enzalutamide in AR-V7 positive mCRPC^[169].

Another interesting polyfunctional drug is D4A, a metabolite of abiraterone. Li *et al.*^[170] recently showed that abiraterone is converted to D4A in mice and patients with PCa [Figure 18B]. D4A inhibits CYP17A1, HSD3B and SRD5A, which are required for DHT synthesis. In particular, D4A is approximately 10-fold more potent than abiraterone at blocking the conversion of [3H]DHEA to AD by 3 β -HSD in LNCaP and VCaP cells.

D4A and abiraterone similarly block conversion of [3H] pregnenolone to DHEA by CYP17A1, as shown by a study in cells expressing CYP17A1^[170]. To determine the effect of D4A on endogenously expressed SRD5A, LAPC4 cells, which exhibit robust SRD5A enzymatic

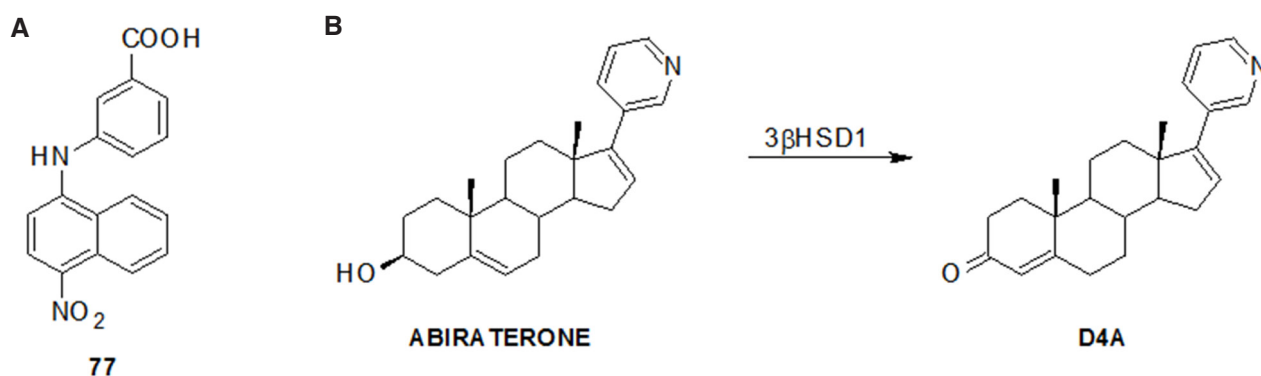


Figure 18: (A) Compound 77, analogue of flufenamic acid; (B) metabolism of abiraterone to D4A^[170]

activity^[18], were treated with D4A, abiraterone or enzalutamide respectively and cultured in the presence of [3H]AD (the preferred natural substrate of SRD5A1)^[18]. D4A (10 mmol/L) almost completely blocked conversion of AD to 5 α -androstenedione and other 5 α -reduced androgens, whereas abiraterone and enzalutamide had no detectable effect, even at a concentration of 100 mmol/L. Abiraterone has been reported to have modest affinity for AR, particularly in the presence of mutations in the ligand-binding domain^[171]. To determine how conversion from abiraterone to the 3-keto structure of D4A affects drug affinity for AR, Li *et al.*^[170] performed a competition assay. The affinity of D4A for mutant (expressed in LNCaP) and wild-type (expressed in LAPC4) AR is greater than that of abiraterone and comparable to that of enzalutamide, and greater than bicalutamide. D4A also has more potent anti-tumour activity against xenograft tumours than abiraterone. These findings suggest that direct treatment with D4A potentially could be more clinically effective than abiraterone treatment.

CONCLUSION

Inter-patient heterogeneity and distinct patterns of abnormal expression and regulation of steroidogenic enzymes contribute to PCa patient relapse. As discussed in this review, the many enzymes involved in the steroidogenic pathway provide obstacles and opportunities for researchers engaged in developing better drugs. Currently, hormone therapy remains first choice for patients with advanced PCa, either as alone or in combination with chemotherapy. Androgen-dependent and independent production is central to fuelling PCa growth, and the biosynthetic steroidogenic pathway plays a vital role in the former. Great progress in PCa biology and drug design have enabled effective therapies to be used clinically, while several promising preclinical strategies are underway. Newer drugs such as abiraterone have performed well in several clinical

studies, but although able to extend overall survival rates, it rarely has curative power. The lack of long-lasting therapeutic effects of abiraterone and ADT may in part be linked with androgen-independent pathways and crosstalk to signal transduction pathways and in part to mutations to the AR^[172]. An ever-increasing number of studies over the past decade have provided insight into prostate cancer biology and it is becoming apparent that new chemotypes and new drug combination strategies are required to target the heterogeneous prostate microenvironment more effectively. Accordingly, future drug discovery should be focussed on multi-targeting agents that inhibit several steps in the biosynthetic steroidogenic pathway, or degrader drugs that eradicate the AR (normal or mutated) to prevent it fuelling PCa growth. Whereas the former requires multifactorial drug design and appropriate multifunctional *in vitro* models, the latter may be a strategy to be implemented in the clinic within a shorter timeframe. In parallel efforts, an attractive route to better therapeutic outcomes is to conduct clinical trials that explore the possibility of using certain types of drugs at a much earlier disease state. Indeed, this is the thinking behind the STAMPEDE trial, which tests a number of additional therapies, given alongside first-line ADT and is discussed by Malcolm Mason in another review "Getting better at treating prostate cancer: what clinicians should want from scientists" in this themed prostate cancer issue. PCa patients who have suffered relapse with bone metastasis currently have poor overall survival rates, with only bisphosphonates available for palliative treatment. Obstacles in obtaining bone biopsies have halted our understanding of how we can effectively treat PCa patients suffering from bone metastases. Generally, the PCa microenvironment is known to be under oxidative stress and indeed this might have a significant impact on how steroidogenic enzymes respond within the bone microenvironment. Evidence points to the intra-tumoural synthesis of T and DHT is minimal, yet high

expression levels of certain steroidogenic enzymes (SRD5A1, AKR1C2, AKR1C3, and HSD17B10) have been detected in a smaller number of bone metastases when compared to non-malignant prostate and primary prostate tumor tissue^[173]. Furthermore, in a recent study AR amplification was not observed in bone metastases from previously untreated PC patients, but was detected in about half of metastatic samples from patients with CRPC. This AR amplification was associated with increased AR and AR-V7 expression and a particularly poor prognosis^[174].

Steroidogenic enzymes are dependent on NAD/NADH co-factor activity and hence better knowledge of the Warburg effect in the bone microenvironment might also be important. Additionally, enhanced understanding of how other cytochrome P450 or aldehyde dehydrogenase isoforms exist and cooperate in retinoic acid production, stem cell maintenance or in inflammatory response may also help to understand significant differences between localised and metastasised PCa. As osteoblasts regulate the intratumoral steroidogenesis of CRPC in bone, targeting osteoblasts may therefore be important in the development of new therapeutic approaches^[175]. To improve on our strategies for therapeutic intervention, we need better models for evaluating new compounds, including co-culture systems, multicellular spheroids, patient-derived xenografts and organoids. Discovery of highly selective chemical probes to investigate steroidogenic/metabolic pathways should yield new drugs that more effectively target the AR axis, which can be employed in combination with other drugs employed in PCa management.

DECLARATIONS

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There are no conflicts of interest.

Patient consent

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Treatment of liver metastases in patients selected for cytoreductive surgery and hyperthermic intraperitoneal chemotherapy for colorectal peritoneal carcinomatosis

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ABSTRACT

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Cytoreductive surgery (CS) and hyperthermic intraperitoneal chemotherapy (HIPEC) have gained increasing consensus in treatment of peritoneal carcinomatosis from colorectal cancer. The presence of liver metastases is generally considered a contraindication for CS + HIPEC, as hepatic involvement no longer represents a loco-regional aspect of disease. Despite this, liver resection (LR) has been tested in selected cases in combination with CS + HIPEC for treatment of peritoneal carcinomatosis with liver metastasis. Relevant studies on this topic were identified through a search in the electronic PubMed database, using the appropriate keywords. CS + HIPEC + LR allows similar outcomes in terms of survival and morbidity with respect to CS + HIPEC, especially in patients with low tumor load. CS + HIPEC + LR represents a reasonable approach for patients with peritoneal carcinomatosis and liver metastases from colorectal cancer. Patients should be selected in high volume tertiary centres, preferably in the context of a prospective trial.

INTRODUCTION

Peritoneal carcinomatosis (PC) from colorectal carcinoma is present in about 10% of patients at the time of diagnosis, and appears in 25% of patients during follow-up^[1,2]. Median survival of patients affected by colorectal peritoneal carcinomatosis

varies between 5 and 7 months^[3]. The introduction of systemic chemotherapy treatment regimes, based on oxaliplatin and irinotecan alone or associated with vascular endothelial growth factor (VEGF)-inhibitors (in various combinations and treatment sequences), has enabled a significant improvement in outcome for patients with metastatic colorectal cancer (MCC), where in some trials a median survival greater than



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20 months has been obtained^[4]. However, all the data presently available refer to patients with un-specified metastatic disease (liver, lung, peritoneum) and the role of modern systemic chemotherapy for treatment of isolated PC has been adequately investigated in only a few retrospective studies^[5-7].

The role of radical surgery for MCC has gained increasing attention in the oncological community and has been proposed in selected cases for liver^[8], lung^[9] and peritoneal metastases^[10]. Although the role of surgery in presence of multiple sites of MCC is almost un-explored, the combined and/or sequential resection of liver, lymph nodes, lung metastases in various combinations has been tested with encouraging outcomes in very selected cases^[11]. Radical surgery for concomitant liver and extra-hepatic MCC has shown results that seem comparable to those obtained for isolated hepatic metastases^[12-15]. For colorectal liver metastases (LM), radical surgery offers a chance of cure in at least 17% of patients and liver resection (LR) has become the standard treatment for patients with resectable disease^[16]. In isolated PC, the role of surgery is less defined and is generally restricted in the context of the multimodal approach of cytoreductive surgery (CS) + hyperthermic intraperitoneal chemotherapy (HIPEC), which appears as the only chance of cure in selected patients^[17-20]. CS + HIPEC has been shown to be superior over systemic chemotherapy in one randomized controlled trial^[17] and in several uncontrolled studies that reported a median overall survival of 33 months and 5-year survival of 43%^[7]. Patients with low tumor load and in whom a complete cytoreduction (CCR-0) is obtainable are those who benefit most from CS + HIPEC^[18,19], but research for further selection criteria (clinical and biological) is still ongoing.

Until a few years ago, the presence of peritoneal carcinomatosis with concomitant liver metastases was considered an absolute contraindication to CS + HIPEC and these patients were referred for systemic palliative treatment. The coexistence of liver metastases (haematogenous metastases) and peritoneal metastases (loco-regional metastases) was considered not amenable to curative surgical treatment due to the spread of the disease and to the complexity of a combined surgical approach. In the last decade a growing number of publications have reported patients treated with CS + HIPEC and LR, but it is still not clear which patients should be selected for this surgical approach.

SURGERY FOR PERITONEAL AND LIVER MCC

The introduction of oxaliplatin/irinotecan and VEGF inhibitors based chemotherapy has improved response rate and prolonged overall survival of patients with MCC^[4]. Despite that the absolute survival benefit obtained with modern systemic chemotherapy has been constantly increasing and the expected median survival of more than 20 months is predictable, surgical approach to MCC has gained increasing interest and LR has become the only chance of cure for resectable liver metastases^[16]. Although early experience has identified patients with LM associated with extra-hepatic disease as a group with a poor prognosis, surgery for limited and stable disease has been frequently offered to patients with lung, peritoneal and other site metastases^[11]. Small series of patients suggest that resection of the liver combined with other sites can offer a survival benefit, but the argument is still under investigation and the results of the few available studies are influenced by a high selection bias.

Data on surgery for peritoneal and liver MCC are very limited. Although, in one study, patients who underwent liver and extra-hepatic disease resection seem to have a worse prognosis^[14], a single center experience reported a 28% 5-year survival rate in patients who underwent an R0 resection of extra-hepatic disease simultaneously with hepatectomy for colorectal liver metastasis^[12]. Unexpected peritoneal disease at time of planned liver resection was estimated in 3% of cases in a large single center experience^[21]. After multivariate analysis, risk factors for peritoneal implants in this subset of patients were previous peritoneal carcinomatosis, T4 primary tumor and bilobar LM. In patients with completely resected and limited PC (PCI less than 2), the 5-year overall survival was 18% with a median survival of 42 months, regardless of the extent of LM. These findings suggest that an accurate pre-operative radiological evaluation not only of the liver but also of the peritoneal cavity is warranted, based on the risk of peritoneal implants (primary T4, resected PC or bilobar LM). Laparoscopic evaluation before surgery for colorectal LM combined with PC should be considered after a complete and accurate radiologic work-up, considering that the occurrence of unresectable disease is 5% in patients selected for liver resection only^[22]. Diagnostic laparoscopy to discover peritoneal implants should be selectively considered in groups undergoing surgery for LM, as the efficacy to discover peritoneal disease is very high^[23]. This allows the selection of patients for the

Table 1: Comparative studies of CS + HIPEC ± LR for peritoneal carcinomatosis and liver metastases from colorectal cancer

Studies	Study period	Study sample (liver metastasis)	Peritoneal load (PCI)	Liver metastases (median and range)	Major hepatectomy (n)	Ablative techniques (n)
Wake Forest University Winston-Salem, USA ^[29]	1991-2007	14/142 (9%)	NR	1 (1-7)	1	6 thermal cryo ablation, 4 cryotherapy, 2 radiofrequency
Uppsala University, Sweden ^[30]	1994-2010	11/22 (50%)	13 vs. 13	1 (1-3)	2	-
Gustave-Roussy Institute, France ^[20]	1995-2010	37/156 (23.7%)	10 vs. 11	2 (1-16)	12	7 radiofrequency
St George Hospital, Sidney, Australia ^[31]	1997-2008	16/55 (29%)	8 vs. 12^	2 (1-7)	2	4 cryotherapy
Université Catholique de Louvain, Belgium ^[32]	2007-2015	25/77 (32.5%)	6 vs. 10	NR	2	1 radiofrequency

^Mean. CS: cytoreductive surgery; HIPEC: hyperthermic intraperitoneal chemotherapy; LR: liver resection; PCI: peritoneal cancer index; NR: not reported

more appropriate treatments and directs the group with limited peritoneal carcinomatosis to a more appropriate approach with HIPEC.

PATIENT SELECTION FOR CS + HIPEC + LR

For colorectal peritoneal carcinomatosis, cytoreductive surgery, normally adopted in the context of a multimodal approach of surgery followed by intraperitoneal chemotherapy, offer a significant survival advantage in selected patients treated in a high volume tertiary center^[24]. Surgery, which represents the first step of the procedure, was standardized twenty years ago and consists of visceral resections and peritonectomies in various combinations^[10]. The main surgical goal is to obtain an optimal cytoreduction with a macroscopic residual disease of less than 2.5 mm. Intraperitoneal drug is delivered intra-operatively under hyperthermic conditions after completion of CS (HIPEC)^[25]. The more important prognostic factor for patient selection for CS + HIPEC are the grade of cytoreduction and the peritoneal tumor load^[19,20]. Although a locoregional approach, CS + HIPEC has been considered contraindicated in the presence of systemic disease and patients with peritoneal carcinomatosis and liver metastases were usually deemed not suitable for treatment and referred to oncologist for systemic chemotherapy.

The encouraging data on the curative role of surgery for liver metastases made CS + HIPEC combined with liver resection a less stringent contraindication^[18,20]. Over the last few years, an increasing number of studies investigated the role of LR in patients selected for CS + HIPEC^[26-28]. In some cases, the treatment of LM was done after intra-operative finding^[28], but in the majority of cases resection was planned with respect to pre-operative staging. In a few cases, liver metastases and peritoneal carcinomatosis treatment

were sequential, performing liver resection after CS + HIPEC.

Patients selected for CS + HIPEC + LR presented with limited liver disease, as suggested by the median number of nodules (in general between 1 and 2), the rare occurrence of major resection and the frequent use of ablative techniques (cryotherapy, radiofrequency)^[20,29-32] [Table 1 and Figure 1]. On the other hand, peritoneal load probably reflects the same tumor diffusion of patients selected for CS + HIPEC only, where a PCI index less than 20 is considered by the majority of referral centers as the preoperative cut-off value in selecting patients for treatment.

Analysing the prognostic factor, peritoneal and liver tumor load seems to have the most important impact on outcome. These data are in concordance with previous reports on surgical treatment of multiple sites MCC; the presence of multiple extra-hepatic site and more than five liver metastasis were the only two variables correlated with survival^[14]. More recently a tumor load-based nomogram have been proposed for patients with potentially resectable synchronous peritoneal and liver metastases^[20]. Although not yet prospectively validated, this simple nomogram combines as prognostic predictors the number of LM and the PCI and represents an interesting decision-making tool that could aid clinicians during multidisciplinary discussion to evaluate the most appropriate treatment.

ASSESSMENT OF MORBIDITY AND MORTALITY

An important issue arises from the potential increase in morbidity and mortality of combining CS + HIPEC to LR, both considered two surgical procedures

Table 2: Studies comparing outcomes of CS + HIPEC alone vs. CS + HIPEC + LR in peritoneal carcinomatosis with or without liver metastases from colorectal cancer

Studies	Follow-up (months)	2-year survival (%)	3-year survival (%)	4-year survival (%)	5-year survival (%)	Median survival (months)	Morbidity (%)	Mortality (%)	Prognostic factors
Wake Forest University, Winston-Salem, USA ^[29]	20.7 vs. 13.4	43.3 vs. 26.8	NR	14.4% vs. 17.4%	NR	23.0 vs. 15.8	40.1 vs. 57.1	7.1 vs. 7.7	NR
Uppsala University, Sweden ^[30]	45 vs. 57	NR	47 vs. 30	NR	NR	34.0 vs. 15	27 vs. 27	9.0 vs. 0	R1 resection
Gustave-Roussy Institute, France ^[20]	62.4	NR	54 vs. 36.5	NR	43.6 vs. 26.4	NR	17 vs. 41	4.2 vs. 8.1	PCI, number of LM, type of surgery
St George Hospital, Sidney, Australia ^[31]	19	65 vs. 68	NR	NR	NR	22.1	38.5 vs. 12.5	0	NR
Université Catholique de Louvain, Belgium ^[32]	34.2	89.5 vs. 70.2	NR	NR	NR	59.2	15.4 vs. 32	0 vs. 4	NR

CS: cytoreductive surgery; HIPEC: hyperthermic intraperitoneal chemotherapy; LR: liver resection; PCI: peritoneal cancer index; LM: liver metastases; NR: not reported



Figure 1: Colorectal peritoneal carcinomatosis and liver metastases (VII segment). Computed tomography performed 3 months after cytoreductive surgery + hyperthermic intraperitoneal chemotherapy combined with radiofrequency ablation

associated with a high risk of complications. The overall postoperative morbidity rate ranged from 14.8% to 58%^[33]. Due to improved surgical techniques, newer dissectors, increased anesthesiological skills and, most of all, refined selection criteria, the mortality and morbidity rates after CS + HIPEC have decreased during the last decade and postoperative outcomes are similar to a major gastrointestinal surgery^[4]. In the same way, the mortality rates for liver resection, which was traditionally considered as one of the surgical procedures with the highest risk of death, have been lowered even more: for instance, recent series reported

mortality rates less than 2%, comparable to those observed after surgery for rectal cancer^[34,35]. CS + HIPEC + LR does not seem to give an additional risk of complication as the morbidity and mortality seems similar with respect to CS + HIPEC alone^[20,29-32] [Table 2]. No study has reported a clear distinction between complication/mortality rate related to the liver resection or to the cytoreductive surgery. It should be underlined, however, that the reported LR combined with CS + HIPEC are classified as minor procedures, normally associated with lower risk of complications. Although no liver specific complications (biloma, bleeding, abscess) are generally reported, a higher risk of systemic toxicity has been noted after major LR^[36]; the hypothesis that transient liver failure leads to a decreased drug inactivation and therefore a higher systemic toxicity should be further tested.

SURVIVAL OUTCOME

In retrospective studies investigating the role of CS + HIPEC + LR, the procedure resulted in a median survival between 15 and 23 months, a little worse with respect to the one reported for CS + HIPEC alone^[20,29-32]. Also, in a comparative retrospective analysis of single centers, the 2-year overall survival seems lower when liver metastases are treated at the time of CS + HIPEC, ranging 43.3-89.5% for CS + HIPEC alone, and 26.8-70.2% for CS + HIPEC combined with LR^[30-32] [Table 2]. However, in the most relevant comparative study, no significant difference is detected between the treated groups^[20].

These findings appear in contradiction with the anatomic basis of colorectal cancer spread:

peritoneal and liver metastases arise from different ways of diffusion (loco-regional vs. haematogenous), probably reflecting a different cancer cell genetics or epigenetic alterations; as previously hypothesized, a curative surgical resection for cancer is only cytoreductive surgery: surgery removes all detectable disease, but does not completely eradicate all tumor cells, since micro-metastases and circulating tumor cells are present in the blood of colorectal cancer patients after radical primary tumor resection in one third of all patients^[36]. The fact that survival after HIPEC is not lowered by concomitant liver resection adds an important piece of evidence supporting this hypothesis that needs to be confirmed by more evidence.

CONCLUSION

The reported data confirmed that CS-HIPEC allows the achievement of long-term survival in patients usually referred for palliative systemic treatment. Although an advantage of HIPEC in terms of oncological outcome over systemic treatments has been not yet clearly proven, patients with no macroscopic residual disease, low peritoneal tumor load and few liver metastases seem to be the best indicated for CS + HIPEC + LR. CS + HIPEC associated with LR have mortality and morbidity rates similar to that observed with CS + HIPEC alone, although major hepatectomy has rarely been performed. For these reasons patients with peritoneal carcinomatosis and liver metastases from colorectal cancer should be referred to a tertiary center for selection of the more appropriate treatment, possibly within clinical trials.

DECLARATIONS

Authors' contributions

A. Sommariva contributed solely to the paper.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

The patient of the computed tomography scan of Figure 1 gave his consent for publication.

Ethic approval

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AUTHOR INSTRUCTIONS

1. Submission Overview

Before you decide to publish with us, please read the following items carefully and make sure that you are well aware of Editorial Policies and the following requirements.

1.1 Topic Suitability

The topic of the manuscript must fit the scope of the journal. Please refer to Aims and Scope for more information.

1.2 Open Access and Copyright

The journal adopts Gold Open Access publishing model since its establishment and has been distributing contents under Attribution 4.0 International License since October 2017, whereas Attribution-NonCommercial-ShareAlike 3.0 Unported had been adopted by then. Please make sure that you are well aware of these policies.

1.3 Publication Fees

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All submissions are required to be presented clearly and cohesively in good English. Authors whose first language is not English are advised to have their manuscripts checked or edited by a native English speaker before submission to ensure the high quality of expression. A well-organized manuscript in good English would make the peer review even the whole editorial handling more smooth and efficient.

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If an accepted manuscript was funded by National Institutes of Health (NIH), the author may inform editors of the NIH funding number. The editors are able to deposit the paper to the NIH Manuscript Submission System on behalf of the author.

2. Submission Preparation

2.1 Cover Letter

A cover letter is required to be submitted accompanying each manuscript. It should be concise and explain why the study is significant, why it fits the scope of the journal, and why it would be attractive to readers, *etc.*

Here is a guideline of a cover letter for authors' consideration:

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In the second paragraph: concisely explain what was done, the main findings and why they are significant;

In the third paragraph: indicate why the manuscript fits the Aims and Scope of the journal, and why it would be attractive to readers;

In the fourth paragraph: confirm that the manuscript has not been published elsewhere and not under consideration of any other journal. All authors have approved the manuscript and agreed on its submission to the journal. Journal's specific requirements have been met if any.

If the manuscript is contributed to a special issue, please also mention it in the cover letter.

If the manuscript was presented partly or entirely in a conference, the author should clearly state the background information of the event, including the conference name, time and place in the cover letter.

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There is no restriction on the length of manuscripts, number of figures, tables and references, provided that the manuscript is concise and comprehensive. The journal publishes Original Article, Review, Meta-Analysis, Case Report, Commentary, *etc.* For more details about paper type, please refer to the following table.

Manuscript Type	Definition	Abstract	Keywords	Main Text Structure
Original Article	An Original Article describes detailed results from novel research. All findings are extensively discussed.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Review	A Review paper summarizes the literature on previous studies. It usually does not present any new information on a subject.	Unstructured abstract. No more than 250 words.	3-8 keywords	The main text may consist of several sections with unfixed section titles. We suggest that the author includes an "Introduction" section at the beginning, several sections with unfixed titles in the middle part, and a "Conclusion" section in the end.
Case Report	A Case Report details symptoms, signs, diagnosis, treatment, and follows up an individual patient. The goal of a Case Report is to make other researchers aware of the possibility that a specific phenomenon might occur.	Unstructured abstract. No more than 150 words.	3-8 keywords	The main text consists of three sections with fixed section titles: Introduction, Case Report, and Discussion.
Meta-Analysis	A Meta-Analysis is a statistical analysis combining the results of multiple scientific studies. It is often an overview of clinical trials.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Systematic Review	A Systematic Review collects and critically analyzes multiple research studies, using methods selected before one or more research questions are formulated, and then finding and analyzing related studies and answering those questions in a structured methodology.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Technical Note	A Technical Note is a short article giving a brief description of a specific development, technique or procedure, or it may describe a modification of an existing technique, procedure or device applied in research.	Unstructured abstract. No more than 250 words.	3-8 keywords	/
Commentary	A Commentary is to provide comments on a newly published article or an alternative viewpoint on a certain topic.	Unstructured abstract. No more than 250 words.	3-8 keywords	/
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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Opinion	An Opinion usually presents personal thoughts, beliefs, or feelings on a topic.	Unstructured abstract (optional). No more than 250 words.	3-8 keywords	/
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

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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.

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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.

2.3.2.3 Results

This section contains the findings of the study. Results of statistical analysis should also be included either as text or as tables or figures if appropriate. Authors should emphasize and summarize only the most important observations. Data on all primary and secondary outcomes identified in the section Methods should also be provided. Extra or supplementary materials and technical details can be placed in supplementary documents.

2.3.2.4 Discussion

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

Anyone who contributed towards the article but does not meet the criteria for authorship, including those who provided professional writing services or materials, should be acknowledged. Authors should obtain permission to acknowledge from all those mentioned in the Acknowledgments section. This section is not added if the author does not have anyone to acknowledge.

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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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Organization as author	Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. <i>Hypertension</i> 2002;40:679-86. [PMID: 12411462]
Both personal authors and organization as author	Vallancien G, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1,274 European men suffering from lower urinary tract symptoms. <i>J Urol</i> 2003;169:2257-61. [PMID: 12771764 DOI: 10.1097/01.ju.0000067940.76090.73]
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Books	Sherlock S, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub; 1993. pp. 258-96.
Book chapters	Meltzer PS, Kallioniemi A, Trent JM. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. <i>The genetic basis of human cancer</i> . New York: McGraw-Hill; 2002. pp. 93-113.
Online resource	FDA News Release. FDA approval brings first gene therapy to the United States. Available from: https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm . [Last accessed on 30 Oct 2017]
Conference proceedings	Harnden P, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer; 2002.
Conference paper	Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. <i>Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming</i> ; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. pp. 182-91.
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Tables should be provided in editable form like DOC or DOCX format (picture is not allowed);

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General italic words like *vs.*, *et al.*, *etc.*, *in vivo*, *in vitro*; *t* test, *F* test, *U* test; related coefficient as *r*, sample number as *n*, and probability as *P*; names of genes; names of bacteria and biology species in Latin.

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