Review Article

Extracellular vesicles for personalised therapy decision support in advanced metastatic cancers and its potential impact for prostate cancer¹

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Abstract

In recent years, the use of circulating tumour cells (CTCs) and circulating extracellular vesicles (EVs), such as exosomes, as liquid biopsy-derived biomarkers for cancers has been investigated. CTC enumeration using the CellSearch based platform provides an accurate insight on overall survival where higher CTC counts indicate poor prognosis for patients with advanced metastatic cancer. EVs provide information based on their lipid, protein and nucleic acid content and can be isolated from biofluids and analysed from a relatively small volume, providing a routine and non-invasive modality to monitor disease progression. Our pilot experiment by assessing the level of two subpopulations of small EVs, the CD9 positive and CD63 positive EVs, showed that the CD9 positive EV level is higher in plasma from patients with advanced metastatic prostate cancer with detectable CTCs. These data show the potential utility of a particular EV subpopulation to serve as biomarkers for advanced metastatic prostate cancer. EVs can potentially be utilised as biomarkers to provide accurate genotypic and phenotypic information for advanced prostate cancer, where new strategies to design a more personalised therapy is currently the focus of considerable investigation.

Keywords:

biomarker, prostate cancer, exosome, extracellular vesicle, Circulating Tumour Cells (CTCs)

Introduction

Cancer management has evolved over the years. Whilst in the past the search for better and more accurate biomarkers was focused on improving early diagnosis, more recently the focus has shifted to the development of predictive markers that can guide personalized therapies for cancer patients. This is driven by an understanding that pathways which drive the progression and survival of cancer cells are complex, and that cancer cells are able to adapt upon treatment¹⁻³. Variability in the adaptability of cancer cells could explain the wide spectrum of clinical responses observed in similar patients towards a given treatment. Thus, a new strategy that can differentiate between responsive and non-responsive patients is urgently needed.

Prostate cancer accounts for 6.6% of male mortality with 1.1 million cases recorded worldwide in 2012⁴. The prostate specific antigen (PSA) test was introduced 30 years ago as a serum marker to monitor response to treatment, but recognition that serum levels were generally higher in patients with cancer compared to benign pathology meant it was rapidly adopted for prostate cancer diagnosis^{5,6}. In general, an increasing serum PSA level is

associated with increasing tumour grade and volume ⁷. Although PSA measurement has helped to detect early cases of prostate cancer over the years and has transformed clinical management of the disease, it has a number of significant limitations. For instance, it is well recognised that clinically significant cancers can be present in a patient with a serum PSA within the normal range ⁸, even in cases with high grade high volume disease ⁹. In addition, although limited studies have demonstrated that PSA doubling time and velocity could be used to predict the incidence of prostate cancer bone metastasis ^{10,11}, this may be limited to patients with high PSA levels ¹². Other studies have also reported that PSA is not a suitable biomarker to evaluate response to novel systemic therapies, as the association between treatment effect, PSA end point and overall survival (OS) is low in men with advanced prostate cancer ^{13,14}.

Prostate cancer metastasises predominantly to osteoblast rich areas of the bone, and can be detected in 90% of patients with advanced prostate cancer ^{15,16}. The presence of prostate cancer cells in the bone environment disrupts the bone structure by altering the balance between osteoblast and osteoclast cell activation, leading to chaotic new bone formation that can be detected radiologically ¹⁶⁻¹⁸. The median survival for men with bone metastatic prostate cancer is 2-3 years ¹⁹. Androgen deprivation therapy (ADT), introduced in 1941 by Huggins and Hodges, ²⁰ remains the treatment of choice for patients with bony metastatic disease. However, ADT is associated with multiple side effects including hot flushes, decreased libido, erectile dysfunction, gynecomastia, abdominal obesity, and osteoporosis among others ¹⁹. Furthermore, whilst ADT is initially effective in treating metastatic disease, even with a combination of secondary hormonal therapy, secondary pathways develop that allow the androgen receptor (AR) signalling axis to remain active. As AR signalling controls prostate cancer cell proliferation and survival, this leads to the progression despite castrate levels of serum testosterone (castration resistant prostate cancer, CRPC)²¹.

Detection of bone metastasis can be performed by plain x-ray, computed tomography or nuclear scintigraphy, by identification of osteoblastic lesions in the former, and the increased uptake by radiolabelled diphosphonate by latter ²². Bone scans, the most commonly used imaging modality for staging, however cannot detect the reduction of bone mass caused by prostate cancer progression nor the early response of cancer upon treatment ²³. In addition, although ADT has been shown to reduce PSA levels, rate of response does not reflect eventual response, with one study reporting that a faster time to reach a PSA nadir was actually associated with a shorter survival duration in men with metastatic hormone

responsive prostate cancer ²⁴. Given this, the development and validation of new biomarkers which can indicate treatment response for advanced prostate cancer remains an intense area of research. Such biomarkers will provide prognostic information needed by clinicians for a future cancer management where therapy can be tailored to suit the biological background of an individual patient.

Circulating Tumour Cells as prognostic biomarkers for metastatic diseases

Circulating tumour cells (CTCs), found in peripheral blood, are thought to originate from primary tumour or metastatic sites. CTCs have been observed at low frequencies with one CTCs detected per 10^6 – 10^8 blood cells ²⁵. CTCs express a specific set of epithelial proteins; these molecular characteristics have been exploited to isolate CTCs from peripheral blood using antibodies against those markers. A number of studies have supported the use of CTC enumeration and characterisation as a marker for cancer management, as a relatively non-invasive liquid biopsy approach for personalised therapy in patients with cancer, including prostate cancer ^{25,26}. The methodologies for CTC enumeration have been developed to capture CTCs by utilising their physical properties or biological characteristic. The most studied method for CTC enumeration is by employing the CellSearch platform (Veridex). Using this platform, CTC enumeration can be performed using approximately 20 ml blood, where CTCs are captured by ferromagnetic nanoparticles coupled with EpCAM antibodies, and characterised by assessing their expression of cytokeratins (CTC markers) and the lack of expression of CD45, a marker for leukocytes ^{25,27,28}. Prostate cancer cells are thought to metastasise through the vein to the lumbar spine as well as via the vena cava ¹⁵, suggesting that there is a transition process from localised prostate cancer cells to CTCs which mediates metastasis ²³. This provides the biological relevance of CTCs as a biomarker for advanced prostate cancer.

In clinical trials for the treatment of metastatic prostate cancer, various studies have investigated the relevance of CTCs as a prostate cancer biomarker ²⁹⁻³³. Prostate cancer patients were stratified using a cut off ≤ 5 and > 5 CTCs/7.5 mL blood ³⁴ and a higher CTC number was reported to associate with worse survival ²⁹. CTCs can be detected at levels as low as 2 CTCs/7.5 mL blood in 75% of patients with CRPC ²⁹. In a seminal finding reported by De Bono *et al.*, CRPC patients with > 5 CTCs/7.5 mL blood (unfavourable) detected using the CellSearch platform have a shorter OS in comparison with men with ≤ 5 CTCs (favourable, 11.5 vs. 21.7 months respectively, $p < 0.0001$). Patients who experienced a change from favourable to unfavourable CTC enumeration also have a significantly worse

survival when compared to those with favourable CTC counts after treatment (>26 vs. 9.3 months, $p < 0.0001$), while patients who had a favourable CTC count after treatment show a 15-month improvement in OS compared to those with continuously unfavourable CTC counts³⁴. CTC counts also result in a better prediction of OS compared to PSA levels³⁴. A CTC cut off of 3 cells per 7.5 mL was reported to predict the progression to CRPC³⁵.

CTC enumeration using the CellSearch platform has been approved by the Food and Drug Administration (FDA) as a prognostic marker to improve overall survival of cancer patients, including prostate cancer^{34,36,37}. It has been noted however that, the EpCAM expression is only found in 71% of cases of prostate cancer carcinoma³⁸. During metastasis, the expression of EpCAM on the cell surface of CTCs may change in the process of epithelial to mesenchymal transition (EMT), leading to undetected EpCAM negative CTCs³⁹. The change of EpCAM expression in prostate cancer cells under a particular treatment has not been thoroughly tested and as such the CTC enumeration alone by this platform is thought to be insufficiently informative for determining whether patients may respond towards treatment^{23,26,40,41}.

Physical enrichment by a method of filtration has also been assessed to isolate CTCs from peripheral blood without relying on the use of EpCAM expression, as CTCs are relatively larger than leucocytes⁴². This method, while potentially giving a more complete biological and physiological understanding of CTCs, has also been contested. This is due to the understanding that CTCs in prostate cancer can be similar in size to leucocytes in average total cell area ($89.03 \mu\text{m}^2 \pm 53.77 \mu\text{m}^2$) found in 98% of CRPC patients^{43,44}. Other methods to isolate CTCs by exploiting their physiological characteristic, such as the use of dielectrophoresis, microcrescent and high speed laser scanning are currently being clinically evaluated to assess whether CTCs isolated using these methodologies could provide a better understanding on the biology of CTCs and be used as predictive and prognostic biomarkers in various cancers^{45,46}.

The association between the molecular drivers of prostate cancer (AR, PTEN and ERG) and CTCs in CRPC, the effect of treatment on CTC counts and/or AR status and PSA levels, and comparison between isolation methods has been reviewed extensively^{23,47,48}. CTCs have been genetically sequenced and one study has indicated that there are shared genomic alterations between tumours and CTCs⁴⁹, but molecular characterisation of CTCs while possible, has been challenging due to the limited amount of material and low purity of isolated CTCs⁵⁰. Additional CTC analysis is necessary to improve the use of CTCs as a predictive biomarker for personalised therapy.

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Do extracellular vesicles (EVs) have a potential as prognostic and/or predictive biomarkers for cancers?

Extracellular vesicles (EVs) are vesicles released by cells into the extracellular environment to mediate communication between cells ^{51,52}. EV secretion is conserved across species (bacteria ⁵³, plants ^{54,55} and animals ⁵⁶). EVs were initially thought of as cellular debris, however current evidence confirms that EVs play an important role in cellular, physiological and pathophysiological functions ^{57,58}.

Cells secrete heterogeneous populations of EVs with different sizes and intracellular origins. The exosomes are small EVs (<200 nm), formed at the endosome/multivesicular bodies. Others originate from plasma membrane such as the ectosome/microvesicles, or apoptotic vesicles ⁵¹. Several studies have shown that EVs can be secreted by cells as a response towards stimuli such as hormones ², growth factors ⁵⁹, stress ⁶⁰, drugs ⁶¹⁻⁶³, calcium ⁶⁴ or a certain class of lipids such as ceramides ⁶⁵. Recent reports also show that EV sub-populations were observed ^{2,66}. EVs are comprised of a lipid bilayer and apart from their protein content; EVs carry genetic messages such as mRNA, miRNA and other small RNAs. As the content of EVs is reflective of the cell of origin and EVs can be isolated from biofluids such as blood plasma, urine and saliva, circulating EVs have been investigated as potential disease biomarkers ^{67,68}.

Circulating small EVs, often referred to as exosomes based on isolation methods and/or vesicle size, have also been assessed in clinical studies. Small EVs concentrations are significantly higher in cancer patients such as shown in lung adenocarcinoma and ovarian cancer ^{69,70}. In oesophageal cancer, small EVs which were quantified by acetylcholinesterase (AChE) activity also showed a higher levels when compared to non-malignant patients ⁷¹.

Several EVs-derived proteins have been investigated as potential cancer biomarkers in clinical settings. Alpha-1-antitrypsin and histone H2B1K were identified as potential biomarkers for diagnosis in urothelial carcinoma. The expression of both proteins in EVs also resembled their expression in tumour tissue. Further, the presence of circulating EVs-derived H2B1K indicates an increased risk of recurrence and progression ⁷². Another strategy employing the use of a panel of EVs-derived proteins was reported in pancreatic cancer where 95% of patients showed positive expression of CD44v6, Tspan8, EpCAM and CD104 in their serum, while healthy control and patients with non-malignant diseases were negative ⁷³. In ovarian cancer, TGF-beta1 and MAGE3/6 were found exclusively in the plasma-derived EVs from patients ⁷⁰. Another study designed to identify childhood haematuria, utilised a panel of urine-derived exosomes containing aminopeptidase N, vasorin precursor,

alpha-1-antitrypsin, and ceruloplasmin which can differentiate early IgA nephropathy and thin basement membrane nephropathy ⁷⁴.

The EV-derived RNA has also been assessed for their potential as cancer biomarkers in clinical samples. In colorectal cancer, small EVs-derived miR-19a levels in serum was significantly increased in patients compared with healthy individuals; this is also associated with poorer prognosis in comparison with the low expression group ($p < 0.001$)⁷⁵. MicroRNA-21 (miR-21) was highly expressed in EVs-derived from oesophageal cancer patients ⁷⁶. Other EV-derived RNA can also help diagnosis of cancer as was shown with the concentration of serum-derived EVs lncRNA CRNDE-h which indicates a lower overall survival rate (34.6% vs. 68.2%, $P < 0.001$) and significantly correlated with lymph node and distant metastasis of colorectal cancer patients ⁷⁷. Similarly, serum-derived EVs miR-1246, miR-4644, miR-3976 and miR-4306 were found at higher levels in 83% of pancreatic cancer patients ⁷³. Further, plasma-derived EVs were reported as a suitable surrogate biomarker in glioma where total EVs-derived protein and mRNA expression levels show association with immunological and clinical responses in patients treated with antitumor vaccines. This study also identified IL-8 and TGF-beta mRNA as predictive towards patient response to immunotherapy ⁷⁸. In prostate cancer, plasma vesicles isolated using the precipitation based ExoQuick method identified miR-1290 and miR-375 as potential prognostic biomarkers in CRPC as their level correlates with poorer overall survival ($p < 0.004$) ^{79,80}. Another paper has reported survivin as a plasma-derived EVs biomarker, through isolation of total EVs using the ultracentrifugation based method for prostate cancer ⁸¹.

For prostate cancer, the expression of the androgen receptor splice variant V7 (AR-V7) RNA in CTCs, was identified as a predictive marker for response to enzalutamide (anti-androgen) and abiraterone (anti-androgen and CYP17 inhibitor)⁸². Whether the AR-V7 transcript could also be measured in plasma EVs and serve as a biomarker, was investigated by Del Re *et al.* ⁸³. Using the exoRNeasy purification method, EV-RNA was extracted and the AR-V7 transcript detected, again preferentially in patients resistant to enzalutamide or abiraterone treatment. This and other studies show the biomarker potential of plasma-derived EV-RNA and the advantages with respect to costs, ease of workflow, likelihood that all (heterogeneous) tumours are represented by EV population and use of historic biorepositories as compared to tissues and CTCs.

A few studies reported the use of other biofluids that contain small EVs-derived biomarkers. The cerebrospinal fluid (CSF), while not as easily accessible as blood or urine, could provide important insight into diseases, and biological insight for brain cancer and

other cancers which metastasise to the brain. A study on CSF-derived EVs miRNA, discovered that miR-21 was found in patients with recurrent glioma ⁸⁴, indicating the potential to use this EV biomarker to predict patient progression. Another approach using miRNA profiling strategies as well as mRNA and long coding RNA (lncRNA) of CSF-derived EVs has been reported in Parkinson's diseases and Alzheimer diseases ⁸⁵.

Our study on the role of EV in prostate cancer progression demonstrated that the secretion of CD9 positive (CD9+) small EVs is increased by physiological androgens such as dihydrotestosterone (DHT). This effect was not seen in other EVs markers tested, i.e., TSG101 and Alix. Furthermore, we found that knockdown of CD9 significantly downregulates AR and PSA, implicating the role of CD9 in the AR signalling axis ². CD9 is reported to regulate pathways involved in oncogenesis, such as MYC ⁸⁶, Akt ⁸⁷, PI3K ⁸⁷, VEGFA ⁸⁸, TGF alpha ⁸⁹ and EGFR ⁹⁰. The role of CD9 in cancer cellular proliferation is somewhat contradictory. CD9 can activate EGFR signalling pathways and mediate proliferation in gastrointestinal cancer cells ⁹⁰, but it is anti-proliferative in human colon carcinoma ⁹¹. In prostate cancer, CD9 expression is increased in tumours of patients in which prostate recurred in 5 years (**Figure 1A**, Holzbeierlein microarray datasets ⁹²), indicating the role of CD9 in the progression of recurrent advanced prostate cancer. While sample size for the prostate cancer cohort is limited, CD9 tissue expression in other endocrine tumors such as breast and ovarian cancer is also a marker for poor prognosis (**Figure 1B and 1C**), indicating a more universal role of CD9 in cancer progression and a causal correlation between CD9 and endocrine cancer progression.

Figure 1. A. CD9 expression is higher in tumours from a cohort of patients which recurred in 5 years (n=6; in comparison with patients with no observed recurrence of prostate cancer (n=9, *p=0.009⁹²). Kaplan Meier plot on **B.** ovarian cancer (high risk n=717, low risk n=718, p=0.014⁹³) and **C.** breast cancer (high risk n=1975 vs. low risk n=1976, p=0.01⁹⁴).

We further reported that the level of CD9+ EVs in plasma is upregulated in prostate cancer patients in comparison with those with benign prostate hyperplasia², using the established immunocapture and immunodetection method⁹⁵; however the CD63+ EVs level does not show a significant difference between the two groups². The levels of CD9+ and CD63+ EVs in plasma derived from a metastatic prostate cancer cohort show that higher CD9+ EVs were evident in CTC positive prostate cancer patients compared to CTC negative patients as measured by the Cell Search System (Veridex, Figure 2A, p = 0.0073). As expected the PSA value was also significantly higher in CTC positive metastatic prostate cancer patients (Figure 2C, p = 0.0014). In contrast, the CD63+ EV level in plasma did not show significant differences between the cohorts (Figure 2B, p = 0.21). Taken together with our previous report², this evidence suggests that investigating a subpopulation of EV can

provide an informative biomarker for prostate cancer. It is thus important to investigate in more detail which subpopulations of circulating EV would be beneficial as prostate cancer biomarkers for diagnosis, prognosis or prediction of treatment response.

Figure 2. CD9 (A) and CD63 positive EV assays (B), as well as PSA levels (C) in plasma samples with detectable CTC (CTC positive n=17) or undetectable CTC (CTC negative, n=35, Soekmadji C, unpublished results). EV measurement was performed using highly sensitive Time Resolved-Fluorescence Immuno Assay (TR-FIA) plates in duplicates as described in Soekmadji *et al.*, 2016 ². Plasma from men with advanced prostate cancer (n = 52) was isolated at the Princess Alexandra Hospital by Australian Prostate Cancer Collaboration Bioresource (Ethics approval no. 1000001165). Plasma samples were collected at the time of day most convenient to the patient. For inclusion in the study, patients have histologically proven adenocarcinoma of the prostate and plans to monitor progression without further therapy, for definitive ADT, or for continuing ADT with definitive docetaxel treatment. All patients had detectable bony or visceral metastatic disease with no prior history of other malignancies. Variance was estimated with the standard error of means for each group (*p < 0.01).

Conclusions

Elevated CTC counts have been shown to provide an indicator for cancer progression and shorter OS in breast cancer ⁹⁶, while in CRPC, CTC enumeration was able to indicate patient response toward treatment at an earlier time point in comparison with PSA ³⁴. CTC counts and PSA however could not be used to molecularly track diseases progression ²⁶, which is the basis of designing personalised therapies, to help clinicians and patients to

choose which treatment will likely benefit the individual patient. As such, further research focused on investigating CTCs to provide genotypic or phenotypic information on patients is needed.

Circulating EVs provide a novel approach to develop much needed biomarkers required for personalised therapy. EVs can be sampled in a relatively non-invasive way and detected in a much smaller volume of biofluid, as low as 100 μ l plasma. EVs can also provide indicators based on various molecules (lipids, proteins and RNA) which are naturally contained inside EVs. Furthermore, EVs may be able to provide informative insight into metastatic disease as EVs in biofluids will represent all advanced metastatic cancer cells whether these cells are in the systemic circulation or from cells found at the site of metastasis. A routine test of EVs could also be easily integrated into clinical settings where EVs can potentially provide patients with more accurate prognostic information on their treatments. We are now beginning to understand how EV subpopulations may contribute toward disease progression. Future studies on subpopulations of EVs in biofluids are warranted to validate the use of EVs as prognostic or predictive biomarker for advanced metastatic cancers, including prostate cancer.

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Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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