Mini-Review

Emerging Role of Extracellular Vesicles in Prostate Cancer

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Abbreviations: AR, androgen receptor; EMT, epithelial to mesenchymal transition; EV, extracellular vesicle; FABP5, Fatty Acid Binding Protein 5; Enz, enzalutamide; PCa, prostate cancer; PSA, prostate-specific antigen.

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Abstract

Prostate cancer (PCa) is the second most common cancer among men in the United States. While the use of prostate-specific antigen has improved the ability to screen and ultimately diagnose PCa, there still remain false positives due to noncancerous conditions in the prostate gland itself and other prognostic biomarkers for PCa are needed. Contents within extracellular vesicles (EVs) have emerged as promising biomarkers that can give valuable information about disease state, and have the additional benefit of being acquired through noninvasive liquid biopsies. Meaningful communication between cancer cells and the microenvironment are carried by EVs, which impact important cellular processes in prostate cancer such as metastasis, immune regulation, and drug resistance.

Key Words: Extracellular vesicles, exosomes, biomarkers, prostate cancer

Extracellular Vesicles

Extracellular vesicles (EVs) are now recognized as important components of intercellular communication in their transfer of proteins, lipids, and nucleic acids (1). These EVs represent a heterogeneous mix of cell-derived membranous vesicles, subclassified as exosomes, microvesicles, and apoptotic bodies that range in size, protein composition, and mechanism of release from cells. Exosomes are the smallest type of EVs, generally ranging from 30 to 120 nm in diameter and are released from the inward budding of endosomal membranes. Microvesicles, also known as ectosomes, can overlap in size with exosomes being 100 to 1000 nm in diameter, but are released from the cell by budding and pinching outward from the plasma membrane (2). Apoptotic bodies range from 500 to 2000 nm in diameter and are released in the final steps of apoptosis during plasma membrane blebbing (3). While there is much yet to be discovered about the role of apoptotic bodies, this subtype seems to have less of a role in cell–cell communication (4). As such, our review will focus on the exosome and microvesicle subpopulations, and we will refer to both simply as EVs. EVs can be found in common bodily fluids such as saliva (5), blood (6), and urine (7). As the content of EVs reflect their cell of origin (8), this gives EVs potential for use as biomarkers derived from noninvasive liquid biopsy techniques. As intercellular communicators, they have
been shown to induce phenotypic changes in recipient cells (9) and have multiple roles in cancer progression, including metastasis, immune cell regulation, and drug resistance.

**Brief Summary on Prostate Cancer**

Prostate cancer (PCa) is the second leading cause of cancer death among men in the United States with approximately 1 in 8 men at risk of developing this disease (10). PCa is a heterogeneous disease as those who are diagnosed vary in age and face different clinical severities. This further complicates the etiology of PCa; however, several risk factors may contribute, including but not limited to age (mortality of PCa correlates with increasing age), race (African Americans have the highest rates of PCa), and a family history of PCa (11-13).

Starting in the early 1990s, PCa incidence spiked to all-time highs and has since plateaued. The spike in incidence has been attributed to the use of prostate-specific antigen (PSA) testing (14). PSA testing was approved by the U.S. Food and Drug Administration in 1994 in conjunction with a digital rectal exam to test asymptomatic men for PCa. While this test is the gold standard for screening men with PCa, PSA levels can increase through noncancerous clinical conditions, such as inflammation, infection, trauma, prostate manipulation, and benign prostatic hypertrophy (15). The positive predictive value for PSA is approximately 25% to 40% (16) and it is known that PSA testing does contribute to an increase in false-positive PCa cases. Additional markers alongside PSA to improve specificity of diagnosing PCa would be beneficial in preventing overdiagnosis and overtreatment. Multiple studies have recognized significant differences in the content of EVs between noncancerous patients and those with PCa (17-19), indicating that EVs have the potential to be clinically relevant in detecting PCa. However, to date there has been little overlap of the nominated biomarkers, and determining the robustness of such biomarkers will be the primary challenge in using EVs in a clinical setting.

**Nominated EV biomarkers for prostate cancer**

**RNA**

Much work has been done to characterize the RNA cargo in EVs and has identified many mRNA and miRNAs, although other RNA types such as tRNAs and rRNAs have also been found (20). The role of miRNAs in the initiation and progression of PCa (21) has made this type of cargo of particular interest for biomarker development. Here we discuss several miRNAs and mRNAs of interest in the context of their potential use for biomarkers as well as their functional relevance to PCa (Fig. 1). See Table 1 for additional notable RNAs that are upregulated in EVs of PCa.

Oncogenic miRNAs, such as miR-21, miR-141, and miR-375, were found to be upregulated in EVs from urine in PCa patients compared with healthy controls (22). These 3 miRNAs have been previously suggested to be useful diagnostic markers for PCa, having also been found to be upregulated in serum of PCa patients (23). Both miR-21 and miR-141 are involved in the regulation of osteogenesis (24), and miR-21 can activate the immune system through toll-like receptors, which contributes to tumor growth and metastasis (25).

Another miRNA that has been recognized as a potential biomarker is miR-26a, which has been shown to be downregulated in PCa tissue (26). Recently, a study done by Urabe et al. demonstrated that miR-26a is involved in the secretion of EVs in PCa cells, and that miR-26 suppresses the expression of SHC4, PFDN4, and CHOC21 which were shown to be increased in PCa tissues and are involved in the biogenesis of EVs (27). Forced expression of miR-26a in PCa cells inhibited cell proliferation (26) and halted tumor progression in in vivo mouse studies (27), likely through the restoration of regulating EV secretion.

The activation of telomerase (hTERT) is one of the hallmarks of cancer (28), and has been of interest as a potential biomarker for detection of cancer initiation and progression. The prevalence of hTERT in serum has been noted for PCa as well as many other types of cancer including breast, colon, and hepatocellular carcinoma (29-33). A pan-cancer study that included cases with prostate cancer measured the concentration of EV-derived hTERT mRNA in the sera of patients and found that 62% of patients with solid tumors expressed hTERT mRNA in their EVs, 60% with hematological malignancies exhibited hTERT mRNA in their EVs, while no signs of hTERT mRNA were detected in the healthy control group (34).

**Proteins**

The implementation of mass spectrometry has identified thousands of proteins from EVs (35). These include proteins involved in EV structure, such as the tetraspanins CD9, CD63, and CD81, which are frequently used to identify EVs (36), and also the protein cargo that EVs carry. Here we discuss both structural and cargo proteins that may have utility as biomarkers in PCa (Fig. 1).

For PCa, the activity of androgen receptor (AR) is of particular importance for tumor progression. The truncated splice variant AR-v7 generates a transcript that lacks the C-terminal ligand binding domain, leading to ligand-independent activity (37). Expression of AR-v7 is a known biomarker for resistance to androgen-targeted treatments enzalutamide and abiraterone (38-40). Notably, both full length AR and AR-v7 have been identified in PCa EVs (41, 42), and both of these receptors have been shown to be
transferred to AR-null cells and stimulate AR signaling (43). This suggests that AR-v7 expression in EVs may be useful in predicting or monitoring resistance to androgen-targeted therapies.

A study by Fujita et al. demonstrated that EVs isolated from urine containing Fatty Acid Binding Protein 5 (FABP5) were significantly associated with Gleason score 7 and higher. The receiving operator characteristic curve analysis in the study showed that the area under the curve for the prediction of Gleason score ≥7 by FABP5 was 0.856 (95% CI 0.708-1.00, \( P = .002 \)), whereas the area under the curve value for prediction by serum PSA was 0.511 (95% CI 0.280-0.757, \( P = .87 \)) (44). FABP5 is an intracellular lipid-binding protein that transports fatty acids, and it is known that overexpression of FABP5 in PCa tissues correlates with poor patient survival (45, 46). That FABP5 is also secreted into EVs and therefore capable of being monitored via liquid biopsy makes it a potentially promising prognostic biomarker for advanced or aggressive PCa.

Proteomic analyses of EVs from the PC3 prostate adenocarcinoma cell line identified CDCP1 and CD151 as promising PCa biomarkers due to their specificity in expression compared with epithelial cells (47). The tetraspanin CD151 regulates cell migration through its interaction with matrix metalloproteinases, and is critical in the early steps of forming metastatic lesions (48, 49). Expression of CD151 in PCa tissue increases during disease progression, is associated with poor prognosis, and has a better predictive value for the clinical outcome of low-grade PCa patients than traditional histologic grading methods (47). It is worth exploring if CD151 in PCa EVs also has a similar predictive value as a biomarker in this clinical setting. CDCP1’s main function is as an anti-apoptotic molecule.
that facilitates tumor cell survival during metastasis (50). In PCa, the inhibition of CDCP1 through the monoclonal antibody 25A11 inhibited metastasis of the PC3 cell line in a subcutaneous mouse xenograft model (51). The expression of CDCP1 in EVs was significantly higher in PC3 cells than in benign prostate or nonmetastatic cells (52), suggesting that levels of CDCP1 could be useful as a marker of metastatic potential.

While individual proteins found in EVs may be useful biomarkers as described above, it is more likely that a panel of EV proteins will provide greater specificity and sensitivity than a single EV protein. Using targeted proteomics on EVs isolated from urine, the combination of adseverin and transglutaminase was able to differentiate benign and PCa, including both low and high risk (53). Additionally, the 5-panel combination of CD63/GLPK5/SPHM/PSA/PAPP was able to discriminate between low-risk and high-risk PCa, and interestingly the expression of all 5 proteins was lower in the high-risk PCa cohort (53). This study speaks to the great potential of EV proteins as versatile biomarkers for many stages of PCa progression.

DNA
A pioneering study in 2011 demonstrated that EVs contain DNA; it was previously thought that only proteins and RNAs were present (54). While it is now well accepted that genomic DNA fragments, including mutations from the cell of origin, are contained in EVs derived from PCa patients (55-57), this is considered to be a little studied area for this cancer type. It must be noted that studies have shifted to analyzing circulating tumor DNA (ctDNA) or cell-free DNA (cfDNA) vs EV-containing DNA. However, ctDNA/cfDNA data would likely include EV-containing DNA, and results of these studies may implicate the important role of EVs when assessing ctDNA/cfDNA. Targeted sequencing of ctDNA from over 800 metastatic PCa samples observed that the most frequent genetic aberrations were from the genes BRCA2, ATM, and CDK12 and that loss of BRCA2 and CDK12 but not ATM was associated with poor prognosis (58). These results may be extrapolated to EVs where ctDNA data and other DNA alteration data could be used as possible biomarkers for PCa. Also, it has been observed that oncosomes, which are large vesicles (1-10 µm), (59) contain more genomic DNA content than exosomes and microvesicles (60) and so may be of more use in developing DNA biomarkers than the types of EVs that we have discussed in this review.

Functional Roles of EVs
While EVs have potential to be clinically useful as biomarkers for several stages of PCa progression, there is much yet to be discovered about the functional roles EVs may have. We now understand EVs as important mediators of cell to cell communication and not simply exporting “junk” cargo. Here we discuss the impact EVs can have on important aspects of cancer progression such as metastasis, immune activation, and drug resistance (Fig. 2).

Technical challenges
A significant barrier in the study of EVs is the heterogeneity of the subpopulations and the lack of ability to easily distinguish between them. Exosomes and microvesicles overlap in particle size, which is the most common type of characterization method, and there are not yet reliable protein content markers that differentiate between these two groups. Currently, techniques such as flow cytometry, dynamic light scattering, and nanoparticle tracking analysis are used for EV analysis (61). However, flow cytometers are unreliable due to their limited resolution, and thus EV-specific assays have been developed (62). Dynamic light scattering is a more reliable technique except when variable sizes of EVs are present in the suspension, which is a frequent occurrence. Smaller EVs remain undetected in the presence of larger sized EVs (63) and are lost from analysis. Nanoparticle tracking analysis can identify a range of EV sizes, but only when the sample has been diluted sufficiently (64). Additionally, most of these techniques are only capable of analyzing the size of the EVs after isolation. The primary difference between exosomes and microvesicles is how they are secreted from the cell (65), but currently we don’t have any markers to distinguish between exosomes and microvesicles after they are secreted and isolated from fluid.

Metastasis
PCa metastasis is a critically lethal stage of cancer progression and most metastatic tumors arise in the bone, as well as the liver and lungs. While several studies have shown EVs as a possible biomarker for metastasis in general, there is also evidence to suggest EVs may be playing a functional role in this development.

It has been shown that EVs are capable of inducing epithelial to mesenchymal transition (EMT) in normal prostate epithelial cell lines, in which the addition of EVs isolated from PCa patients led to increased expression of EMT markers vimentin and N-cadherin and decreased expression of the epithelial marker E-cadherin (66). This suggests that EVs can alter the transcriptional profile of receiving cells and, specifically, genes involved in EMT, a precursor to metastatic progression. This was corroborated in a study that applied EVs derived from a mesenchymal variant of the 22RV1 cell line to the androgen sensitive VCAP PCa cell line (67), and similarly found an induction of vimentin, N-cadherin, and other EMT markers.
As inhibition of androgen signaling and an increase in transforming growth factor-β signaling is known to induce the EMT transition in PCa (68-70), the researchers investigated the effect of EVs on these signaling networks and found inhibition of AR, PSA, and ERG and an increase in transforming growth factor-β1 and phosphorylated SMAD2. A cluster of miRNAs carried inside the EVs known to interact with AR may have facilitated this mechanism of EMT transition (67). Additionally, changes in expression of tetraspanin proteins CD9 and CD151 in EVs have been found to influence invasion and migration of the normal prostate epithelial cell line RWPE-1 (71). Reduction in CD9 expression and increased CD151 expression are frequently seen in metastatic PCa (72, 73). EVs that have reduced levels of CD9 or overexpression of CD151 promoted invasion in RWPE1 cells, while only CD151 overexpression increased migration. Interestingly, the altered expression of CD9 and CD151 modified the proteome of the EVs (71), though the mechanism related to how these tetraspanins can affect such changes is unclear.

An important reversal of the EMT process is the mesenchymal to epithelial transition which gives rise to secondary tumor sites. EVs appear capable of preparing the premetastatic niche for cancer cells, likely through promoting inflammation in recipient cells (74, 75). Repeated injections of EVs from a breast cancer cell line that primarily metastasized to the lungs led to enrichment of EVs localized to the lungs and seemed to “train” the tumor microenvironment. When breast cancer cells were later injected, there were significantly more cancer cells that localized to the lungs, from both the breast cancer line that the EVs were derived from as well as a secondary cell line that primarily metastasized to bone (76). Protein analysis by mass spectrometry identified integrin expression profiles that appear capable of distinguishing the tissue-specific homing. Integrins ITGa6, ITGb4, and ITGb1 were abundant in EVs that homed to the lungs, whereas EVs that homed to the liver had more abundance of ITGb5 and ITGa4 (76). While it is unclear if there are integrin patterns that predict homing to bone, EVs derived from PCa cell lines seem to preferentially target bone marrow stromal cells (77). The protein transfer of pyruvate kinase M2 from EVs to these recipient cells led to increased levels of the chemokine CXCL12 in a HIF-1α-dependent mechanism (77), which likely contributed to an inflammatory response (78) that prepared the premetastatic niche. Additionally, the introduction of EVs derived from the enzalutamide-resistant PCa cell line CW-R1 resulted in NF-κB signaling and enhanced osteoclast differentiation in bone marrow stromal cells (79), which is an initial step in reprogramming the bone microenvironment to allow for tumor cell proliferation and metastasis (80).

### Immune response

Immune checkpoint inhibitor therapy targeting the PD-L1/PD-1 axis has shown effectiveness in several cancer types including melanoma, non-small-cell lung cancer, and renal cancer, but the responses in PCa with these therapies remains elusive (81, 82). The mechanisms for why PCa remains largely unresponsive to immunotherapy remain unknown. It is possible that the low mutational burden impedes antitumor immunity, or the presence of suppressive cell populations such as regulatory T-cells (83) or myeloid-derived suppressor cells (84).

EVs may drive aspects of immune escape through immune suppression. EVs derived from the LNCaP cell line model were shown to be capable of not only inhibiting proliferation of T-cells but also cause apoptosis (85). EVs have also been shown to suppress the function of natural killer cells (86) and inhibit differentiation of dendritic cells (87). Undifferentiated dendritic cells are incapable of presenting antigens to T-cells, leading to a suppressed immune response. Manipulating the activation of dendritic cells is an area of interest in increasing the effectiveness of immunotherapy (88), and EVs isolated from mature dendritic cells have been used in a phase II clinical trial with non-small cell lung cancer patients to stimulate the immune response. Unfortunately, this trial did not see a significant effect on T-cell responses though natural killer activity was enhanced (89).

Importantly, EVs have been shown to activate the PD-L1/PD-1 axis. PD-L1 (CD274) on the surface of tumor cells binds its receptor PD-1 on effecter T-cells which serves to suppress T-cell activity. PD-L1 has also been found on the surface of EVs in several cancer types (90-92) and shown to be capable of binding PD-1 on T-cells (93). Notably, work done by Poggio et al. showed that PD-L1 from EVs derived from PCa cells were capable of suppressing T-cell activation. Knocking out PD-L1 and thereby removing PD-L1-containing EVs inhibited tumor growth. This inhibition was reversed when EVs containing PD-L1 were reintroduced into the mouse model (94). Additionally, these researchers showed that knocking out the genes Rab27a and nSMase2, which are involved in the biogenesis of EVs, in combination with anti-PD-L1 treatment was capable of extending mouse survival. This offers a potential avenue of increasing effectiveness of immunotherapies in PCa where both cell surface and EV PD-L1 can be targeted.

### Drug resistance

There are multiple avenues in which EVs can contribute to drug resistance in cells, such as transferring drug efflux pumps, apoptotic modulators, and the drugs themselves (95). In PCa, it has been shown that EVs from docetaxel-resistant PCa cell lines can confer that resistance to docetaxel sensitive lines (96). Comparison of EV proteins between
docetaxel sensitive or resistant DU145 PCa cells nominated a potential predictive signature (97) including increased expression of multidrug resistance genes MDR1 and MDR3, but there has been no functional follow-up to this finding.

PCa cell lines that are resistant to enzalutamide (Enz) were found to exhibit higher secretion of EVs than their parental Enz-sensitive lines. Additionally, inhibiting EV secretion by small molecule inhibitors or siRNA knockdown of syntaxin 6 significantly reduced the viability of Enz resistance lines (98). These data suggest that EVs can contribute to cell proliferation in drug resistant lines, though it is unclear in this study if inhibiting EV secretion is the main contributor to resistance or the lack of EV signals from surrounding resistant cells.

Conclusions

The intercellular interactions that EVs mediate have been repeatedly shown to play important roles in PCa initiation and progression. Remodeling the microenvironment to prepare a premetastatic niche, suppressing the immune system, and facilitating resistance to therapies are key functions that contribute to cancer cell survival. Understanding not only the mechanism behind these functions but also how to combat these effects are important considerations for future studies. Blocking these abilities of EVs is a potential avenue to significantly improve current therapies.

EVs may also be a therapy in and of themselves. EVs derived from a fibrosarcoma cell line were engineered to encapsulate the chemotherapeutic doxorubicin and homed to the cancer cell of origin, successfully delivering the drug and inhibiting tumor growth in mouse models (99). That EVs can function as shuttles back to their parent cancer cell of origin opens up new ways to deliver therapies with specificity, including treatments with RNAi. EVs have been modified to include siRNAs or shRNAs targeting the mutated form of KRAS, a driver in many cancer types. KRAS has been historically difficult to target, however, the first KRAS-targeted therapy, sotorasib, was recently approved by the FDA for non–small-cell lung cancer (100). These modified EVs, known as iExosomes, were able to successfully inhibit mutant KRAS signaling in a pancreatic cancer mouse model and increase survival (101). This success has led to a clinical trial using iExosomes in metastatic pancreatic cancer patients (NCT03608631). For PCa, perhaps EVs can offer a new mechanism to deliver agents that specifically target androgen receptor signaling that drive the majority of PCa cases.

The cargo that EVs carry have been shown to have phenotypic impacts whereby cancer cells can utilize this cargo to aid in either proliferation and metastasis or by researchers for targeting and inhibition. But, no matter the function, the contents of EVs can also be used as a noninvasive means to monitor the disease state. Several RNA and protein biomarkers have been nominated as being able to help diagnose PCa or distinguish a subtype. To move forward and positively impact patient care, we need to identify robust markers that are repeatable across several research groups. Ideally, the markers would be able to overcome the variety of ways to isolate and evaluate EVs. That EVs can offer information about tumor progression through a noninvasive liquid biopsy is of huge benefit for PCa.

Some or all data generated or analyzed during this study are included in this published article or in the data repositories listed in References.
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