

Bladder cancer exosomes: Getting the message across

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Abstract

Bladder cancer is the seventh most common cancer in men and the seventeenth most common in women. It is also the most expensive cancer to treat over the lifetime

of a patient, partially due to the necessity of frequent cystoscopy to monitor for tumor recurrence. There have also been no new developments for the treatment of bladder cancer in the last several decades. Exosomes are small, secreted, membrane-bound vesicles representative of the donor cell. Increasing understanding of the role of exosomes in cancer biology has inspired interest in their potential use as a non-invasive diagnostic tool, prognostic markers and/or indicator of recurrence of bladder cancer, and even for use in the treatment of bladder cancer. Exosomes can be readily isolated from urine. Several groups have already demonstrated differences in the protein and micro RNA content of exosomes in bladder cancer patients compared to normal healthy volunteers. Furthermore, cancer cell-derived exosomes mediate tumor progression through the delivery of their biologically active content to recipient cells. Exosomes may be useful for the delivery of targeted molecules for the treatment of bladder cancer.

Key words: Bladder cancer; Cystoscopy; Exosome; Biomarker; Urine

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Core tip: Exosomes are small membrane-bound vesicles representative of their donor cell. There is growing interest in understanding the function of exosomes in diseases such as cancer. Because of their unique properties, there is developing interest in using exosomes as biomarkers, and as a therapeutic modality in cancer. There is a critical need for affordable, non-invasive methods for diagnosis and monitoring for recurrence of bladder cancer as well as novel therapeutic options. Exosomes have the potential to meet these needs. In this review, we explore what is currently known about exosomes and their role in bladder cancer.

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INTRODUCTION

The first reliable documentation of exosomes was in the 1970's with the observation of the release of membrane-bound vesicles into the extracellular space after fusion of an endosome with the plasma membrane^[1,2]. For many years, exosomes were thought to be a method of disposing of cellular content. In the late 1990's, a few publications emerged demonstrating that exosomes may have a role in intercellular communication. Raposo *et al.*^[3] demonstrated that exosomes released by B cells could stimulate CD4⁺ T cells *in vitro*. Zitvogel *et al.*^[4] found that exosomes released by human dendritic cells which were tumor peptide-pulsed could suppress or eradicate established murine tumors. These findings led to an increased interest in understanding the role of exosomes in both the normal state, and eventually disease processes.

Exosomes are formed from the endocytic pathway. Early endosomes recognize and incorporate ubiquitinated proteins^[5]. This process leads to the formation of an intraluminal vesicle giving rise to multivesicular bodies (MVBs). The MVBs then fuse with the plasma membrane releasing the contents into the extracellular space. Exosomes are the resulting 30-150 nm membrane-bound extra cellular vesicles with a typical density of 1.15 to 1.19 g/mL^[5]. Exosomes are secreted by many different cell types, and are found in most body fluids including blood, urine, semen, saliva and breast milk. Exosomes are representative of their cells of origin^[5-8]. They contain cytoplasmic and membrane proteins, micro RNA (miRNA), messenger RNA (mRNA), long non-coding RNA (lncRNA), and even short DNA segments. Exosomes are also enriched in certain lipids including cholesterol, ceramide, sphingolipids and phosphoglycerides with long saturated fatty acids^[6-8].

Exosomes have many biological functions which are dependent on their cells of origin. Examples include modulation of the immune system, programmed cell death, angiogenesis, inflammation and coagulation. Exosomes also have roles in many pathologic conditions such as infectious diseases, neurodegenerative disease, cardiovascular disease, and cancer^[5,7,9].

EXOSOME ISOLATION

There are multiple isolation methods available to the exosome researcher. The investigator must decide what the downstream application of the exosome preparation is intended for in order to determine the most reasonable approach. Isolation by ultracentrifugation is considered the "gold standard" for exosomes isolation in the literature. However, due to the time-commitment associated

with this method, several other approaches have been investigated and applied. Each of the following methods can be used for isolation of exosomes from several different sample sources including cell culture medium and urine.

Isolation by ultracentrifugation

Serial centrifugation with or without filtration followed by ultracentrifugation is the most widely accepted method for exosome isolation^[6,10-14]. Several protocols have been used in the literature with varying speed, duration and number of low-speed centrifugation and ultracentrifugation steps. The process begins with a series of low speed centrifugation steps to remove cells and larger vesicles. The supernatants are then collected and subjected to ultracentrifugation. This method alone is insufficient to isolate pure exosomes, but the addition of filtration and the use of a 30% sucrose cushion or 5%-30% sucrose gradient step increases the purity of the isolation. The use of a sucrose cushion or gradient allows the low-density exosomes to be separated from other higher-density vesicles, contaminating particles and protein complexes. This isolation strategy maintains the exosome structure, but is time-consuming^[11,12,15-17]. If one wishes to use exosomes for functional studies, purification by ultracentrifugation with sucrose cushion or sucrose gradients yield the most purified exosomes at the lowest cost.

Immunoaffinity

In this method, antibodies to surface proteins on the exosomes are used for isolation. Antibodies are associated with beads or other matrices to immobilize them. The target exosome surface proteins bind covalently to the antibodies allowing isolation from other particles. Immunoaffinity allows for specific exosome isolation. However, it requires a clean-up procedure to remove proteins that are bound in a nonspecific manner. This process is often inefficient, yielding very few exosomes for downstream functional studies, and is very expensive^[11,12,15-17].

Ultrafiltration

Ultrafiltration isolates exosomes based on their size using polyethersulfone nanomembrane concentrators. This method has a lower limit of sample volume, and is faster and easier to execute. However, because proteins in the sample can obstruct the filter, this method leads to decreased isolation efficiency^[11,12,17]. Serum and urine have abundant levels of albumin and Tams-Horsfall proteins making this technique feasible only in combination with ultracentrifugation or other isolation techniques.

Size-exclusion chromatography following ultracentrifugation

Size-exclusion chromatography also uses exosome size as a principle for isolation. Heteroporous beads are constructed from a neutral, cross-linked polymeric support

placed in a column creating pores of varying sizes. As a solution passes through the column, molecules are separated by their size with the smaller molecules taking longer to pass through the pores. The major advantage of this method is that it excludes high abundant proteins. However, it is time consuming and labor-intensive^[11,15-17].

Commercial kits

Several commercial kits for exosome isolation are available. The exact mechanism by which these reagents isolate exosomes is usually not disclosed by the companies. Typical steps in these protocols include incubation with a polymer, precipitation of the mixture, and centrifugation. Kits are simple, fast, and allow for processing of large volumes of sample. However, they tend to yield low quantities of exosomes and can be cost prohibitive^[11,12,15-17].

QUALITY CONTROL AND QUANTIFICATION

Western blotting using known exosome marker proteins such as CD63, Alix or Tsg101 can be used to confirm the presence of exosomes in isolated samples. Commercially available Micro Bicinchoninic Acid protein assay kits can be used to quantify total exosome protein concentration^[11,16,17].

Electron microscopy can be used to identify exosomes based on size and morphology. Transmission electron microscopy in combination with immune-gold staining outlines more detail and establishes the presence of specific exosome markers^[11,12,16,17].

Nanoparticle tracking analysis (NTA) uses an ultra-microscope and laser illumination to calculate the mean velocity of particles suspended in a solution on the basis of Brownian motion. The velocity is then used to determine the particle size and concentration. NTA requires careful optimization and modification and setting adjustments, but is promising as a method for confirming the presence and determining the quantity of particles in the size range of exosomes after isolation by any method^[12,15-17]. Western blotting and electron microscopy often accompanies this technique to provide sufficient evidence for the presence of exosomes.

BLADDER CANCER EXOSOMES

Exosomes are representative of the cells from which they originate. They contain miRNA, mRNA, lncRNA and protein. Exosomes play a role in several processes crucial for cancer progression, invasion and metastasis. Cancer cell-derived exosomes have altered content and composition which leads to altered biology. Several studies have demonstrated that exosomes from patients with bladder cancer contain discrete proteins and miRNA not found in the exosomes isolated from healthy volunteers^[18,19]. The process of formation and

secretion of exosomes as well as the mechanisms by which they influence tumorigenesis is not fully understood. However, there is significant evidence that cancer cell-derived exosomes influence the phenotype of recipient cells through several different mechanisms including angiogenesis, cytotoxicity, cell proliferation, migration, invasion and inhibition of apoptosis^[10,13,14,19,20].

Beckham *et al.*^[10] previously demonstrated that bladder cancer exosomes are involved in tumor progression as exosomes isolated from bladder cancer cell lines or the urine of bladder cancer patients were shown to facilitate angiogenesis, migration and invasion. They demonstrated that exosomes isolated from bladder cancer cell lines contain hundreds of proteins such as epidermal growth factor like repeats and discoidin-1 like domains or EDIL-3. Knockdown of EDIL-3 in the high-grade bladder cancer cell line TCC-SUP resulted in exosomes that could not facilitate migration or angiogenesis, demonstrating an important role for exosome driven tumor progression in bladder cancer^[10].

Yang *et al.*^[20] demonstrated that T24 bladder cancer cells (a high-grade bladder cancer cell line) treated with varying concentrations of exosomes have increased levels of bcl-2 and Cyclin-D1, reduced levels of Bax and caspase-3, and resulted in activation of Akt and ERK tumor cell apoptosis. Zhang *et al.*^[21] demonstrated that T24 bladder cancer cell exosomes can promote the anti-tumor effect of cytotoxic T lymphocytes *in vitro*.

A recent publication revealed that metastatic bladder cancer cells are dependent on RAB27 to secrete miR23b, miR224 and miR921 *via* exosomes. They also determined that silencing RAB27A or RAB27B halted the secretion of miR23b and miR921 and reduced cellular invasion^[22].

BLADDER CANCER EXOSOMES AS BIOMARKERS

Several properties of exosomes make them desirable as biomarkers. As previously discussed, exosomes contain protein, miRNA, mRNA, lncRNA representative of the cell from which they originate. Exosomes are extremely stable, and RNA contained within exosomes is protected from degradation^[23,24]. Additionally, they can be isolated from almost every body fluid, including urine. These characteristics give exosomes the potential to be used as a non-invasive biomarker for diagnosis, prognosis and recurrence of bladder cancer.

Bladder cancer tumors contain a high number of variable mutations, and tumors are frequently heterogeneous. Due to this, biomarker discovery will likely include a panel of molecules including bladder cancer specific proteins, miRNA, mRNA and lncRNA rather than a single marker. Welton *et al.*^[25] discovered several proteins in HT 1376 bladder cancer cell line exosomes using in-depth proteomic analysis. Jeppesen *et al.*^[26] completed proteomics on fractionated membranes compared to luminal contents of exosomes of metastatic and

non-metastatic bladder cancer cell lines and discovered several proteins important in epithelial-to mesenchymal transition.

Several groups have identified unique proteins and miRNA in the urine of bladder cancer patients which are not found in the urine of healthy volunteers. Chen *et al.*^[18] identified 22 discrete proteins in the exosomes of bladder cancer patients compared to healthy volunteers. In addition, they identified 7 proteins found differentially enriched patients. Smalley *et al.*^[27] found 9 exosomes proteins with differential enrichment in bladder cancer patients compared to normal healthy controls. Weber *et al.*^[19] identified 2 miRNA (miR-200a and miR449b) present in the urine of bladder cancer patients not present in the urine of healthy pregnant women. They also found other miRNA either enriched or reduced in bladder cancer samples compared to normal control samples. The authors speculate that the extracellular miRNA in the urine is transported in urinary exosomes. They also make the observation that quantification and normalization of miRNA is difficult due to the lack of housekeeping gene equivalents.

FUTURE DIRECTIONS

Although there are promising results for bladder cancer biomarker discovery with the use of exosome-derived proteins and miRNA, there are limitations. Microarray for miRNA has been unable to discover novel miRNA signatures for bladder cancer. High-depth RNA-sequencing may be necessary to reveal bladder cancer markers and multi-institutional cooperation may be necessary to overcome the cost barrier.

Further directions in biomarker discovery in bladder cancer may include the use of exosomal mRNA and/or lncRNA. To date, there are no published data on the function or biomarker potential of bladder cancer exosome mRNA or lncRNA. In an attempt to fill this gap, our group recently completed deep RNA-sequencing of 8 bladder cancer patient tumors, distal normal tissue and corresponding urinary exosomes, and the urinary exosomes of 7 normal healthy controls. We identified the enrichment of several mRNA and lncRNA in the bladder cancer patient exosomes compared to healthy normal controls. We used quantitative real-time reverse transcriptase polymerase chain reaction to confirm two lncRNAs enriched in the urine exosomes of bladder cancer patients compared to healthy controls. Further work in an appropriate population will be needed for validation of these transcripts for biomarker use^[28].

In addition to their use as biomarkers in bladder cancer, urinary exosomes have the potential to aid in the diagnosis and monitoring of a variety of diseases. Exosome have been proposed as biomarkers for a wide-range of benign and malignant diseases. Detection of biomarker exosomes in the urine could provide a non-invasive option for the diagnosis of an assortment of conditions.

The functional roles of exosomes in cancer continue to be revealed over time. There is evidence that bladder cancer exosomes are involved in angiogenesis, cytotoxicity, cell proliferation, migration, invasion and inhibition of apoptosis. Additional functions of exosomes have been described in other cancers. For example, melanoma-derived exosomes have been shown to prime the metastatic niche^[29]. There is also evidence in lung and breast cancer that exosomes may be instrumental in transferring chemotherapeutic drug resistance to chemosensitive cells^[14]. There are no published data on the role of bladder cancer exosomes in priming of the metastatic niche or regulating the response to chemotherapeutics. This is another gap left to be filled in the understanding of bladder cancer exosomes.

Urinary exosomes may also be useful in predicting response of patient with bladder cancer to adjuvant therapy. Bladder cancer cells that are likely to respond to therapy may have unique (or differential expression of) proteins, mRNA, miRNA and lncRNA compared to cells that are not likely to respond to therapy. Exosomes are representative of their cells of origin in content and these differences can potentially be identified in urinary exosomes. If these differences can be identified, then urinary exosomes could potentially be used to help predict response to treatment.

There is growing interest in using exosomes in the treatment of cancer. One potential use for exosomes is as a vehicle for targeted therapy delivery in the form of cellular components of pharmaceuticals. A study by Alvarez-Erviti *et al.*^[30] used exosomes derived from the dendritic cells of bone marrow in mice to deliver siRNA in a targeted fashion to the brain. Another option for treatment, is in the therapeutic removal of exosomes from the circulatory system, but further work is needed to determine whether or not this method may be effective^[31]. The observation that tumor-derived exosomes can activate a specific cytotoxic response has been used to initiate Phase I and Phase II clinical trials in the use of exosomes for the treatment of advanced stage non-small cell lung cancer^[5]. There are currently no publications regarding the use of exosomes in the treatment of bladder cancer.

Exosomes show promise as a means for treatment of a variety of cancers. However, there are some limitations. Thus far, investigation into exosomes as therapeutics has been limited by technical and financial difficulties. In addition, there is concern for the safety in using exosomes for treatment. Exosomes for therapeutic use would likely be produced from cell lines grown in animal serum-containing medium. Although exosomes have been shown to be non-immunogenic, exosomes can conceivably carry theoretically harmful particles such as viruses, parasites, prions and transposons^[32-34]. One potential advantage in the treatment of bladder cancer is the ability to deliver therapeutics intravesically, potentially avoiding risks associated with the treatment delivered systemically.

CONCLUSION

Exosomes are desirable as biomarker for disease because of their unique properties, including stability, protein and nucleic acid content representative of their donor cell, and presence in most body fluids. Several studies have demonstrated their potential use as biomarkers for bladder cancer. In these studies, both proteins and miRNA unique to bladder cancer patients were found in urinary exosomes. These studies use pooled data, highlighting the importance of developing a reliable panel of biomarkers for bladder cancer diagnosis and monitoring for recurrence of disease. And finally, there is some data to support the use of exosomes in cancer treatment.

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