



Urinary extracellular vesicles: a rising star in bladder cancer management

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Abstract: Clinically, the detection of bladder cancer (BCa) typically requires cystoscopy, which is potentially harmful and sometimes accompanied by adverse effects. Thus, new biomarkers are desirable for improving the management of BCa. Recently, “liquid biopsy” has received enormous attentions and has been extensively studied due to its promising clinical implication for precise medicine. Especially, extracellular vesicles (EVs) have attracted strong interest as a potential source of biomarkers. EVs have been reported to be found in almost all types of body fluids and are easy to collect. In addition, EVs tightly reflect the current state of the disease by inheriting specific biomolecules from their parental cells. Urinary EVs have gained great scientific interest in the field of BCa biomarker research since urine is in direct contact with BCa and can contain large amounts of EVs from the tumour microenvironment. To date, various kinds of biomolecules, including noncoding RNAs, mRNAs, and proteins, have been investigated as biomarkers in urinary EVs. In this narrative review, we summarize the recent advances regarding urinary EVs as non-invasive biomarkers in patients with BCa. The current hurdles in the clinical implications of EV-based liquid biopsy and the potential applications of EV research are also discussed.

Keywords: Extracellular vesicles (EVs); bladder cancer (BCa); biomarker; exosome

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Introduction

Bladder cancer (BCa) is the 10th most commonly diagnosed cancer type in the world; it ranks 13th in Japan and 6th in the USA. It occurs approximately four times more frequently in men than in women; therefore, BCa in men is the 8th most commonly diagnosed cancer in Japan and the 4th

most commonly diagnosed cancer in the USA, whereas in women, it ranks 16th in Japan and 11th in the USA (1-3).

Current diagnostic tools for detecting BCa are cytology and cystoscopy. Cytology of the voided bladder urine specimen is a common and non-invasive screening test for BCa. Although cytology is very specific (approximately 80%), its overall low sensitivity (approximately 40%) is a

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clear limitation. Indeed, it has a relatively high sensitivity for high-grade or advanced BCa, whereas it has a low sensitivity for low-grade or early-stage BCa, ranging only 5% to 30% (4-7). Therefore, a positive cytology indicates the presence of BCa; however, negative cytology cannot exclude the presence of BCa. Cystoscopy is the gold standard for cancer diagnosis; however, its effectiveness is operator-dependent, and it is highly invasive and can cause complications, such as frequent urination, pain during urination, visible haematuria, and urinary infection (8,9). In addition, it is sometimes difficult to identify carcinoma in situ (CIS) by cystoscopy. Recently, new biomarker-based urine tests were approved by the Food and Drug Administration (FDA). Three protein-based noninvasive urine tests [bladder tumour antigen (BTA), nuclear matrix protein 22 (NMP22), and ImmunoCyt/uCyt], which are approved by the FDA and commercially available, are not recommended in the current guidelines because of heterogeneous data (10). In addition, UroVysion, which is a fluorescence in situ hybridization (FISH) probe set to detect BCa, is limited by low sensitivity for low-grade BCa (11). Thus, UroVysion has not been approved for the initial detection of BCa but for assessing responses to intravesical Bacillus Calmette-Guerin (BCG) therapy (12). To date, many efforts have been made to establish effective molecular markers for BCa. However, these urine tests are currently not used in daily clinical practice. Further research is required to establish more clinically useful tests, especially to detect low-grade and early-stage BCa.

In the search for a novel biomarker, a number of studies have focused on extracellular vesicles (EVs). EVs can be detected in almost all kinds of body fluids, and as we introduce later, EVs contain a complex mixture of RNAs, DNAs, and proteins that are covered by or localized on a lipid bilayer membrane (13). In this review, we summarize the current knowledge of EVs as biomarkers for BCa and discuss the potential of EVs in BCa management.

Classification of EVs

EVs comprise various types of small vesicles, ranging from approximately 50 to 4,000 nm in size, which are secreted by almost all kinds of cells into the extracellular environment (14). Based on the size and secretion mechanism, EVs are often classified into three main classes: exosomes, microvesicles, and apoptotic bodies (15). Exosomes are nanosized bilayer EVs with diameters of approximately 50 to 100 nm. Exosomes are first

generated by a process of inward and reverse budding of an endosomal membrane of multivesicular bodies and are consequently secreted into the extracellular environment (16,17). Microvesicles, which are larger than exosomes (100–1,000 nm), are directly released from cellular membranes by budding or shedding (18). Apoptotic bodies vary in size (50–4,000 nm) and are released from the cellular membrane during programmed cell death (19). Although the origins of these vesicles are distinct, their characteristics, such as size and density, are similar, and it is impossible to completely separate these vesicles with the currently available technologies. Thus, as the International Society for Extracellular Vesicles (ISEV) has recommended (20), we use EVs as a collective term covering all types of EVs.

Accumulating evidence has shown that EVs are key mediators of cell-to-cell communication through transferring their components, including miRNAs, mRNAs, DNAs, and proteins (13). Especially in the cancer research field, accumulated studies have helped us gain a deeper understanding of the mechanism of tumour progression (21). The role of EVs in tumour progression or metastasis, which is directly related to patient mortality, has repeatedly been reported. In focal tumour progression and systemic cancer metastasis, EVs secreted from cancer cells as well as other cells, such as tumour endothelial cells (TECs), cancer-associated fibroblasts (CAFs), and tumour-educated platelets (TEPs), are reported to have distinct roles (22). Therefore, it is valuable to elucidate how cancer cells educate and communicate with surrounding and distant cells via EVs in their microenvironment.

On the other hand, EVs have great potential as cancer biomarkers. Although a tissue biopsy is required for definitive diagnosis of cancers, it is an invasive procedure for patients. Therefore, it is difficult to perform tissue biopsy frequently. In daily clinical practice, rapid, simple, and less invasive biomarkers are required. Recently, liquid biopsy has emerged as an evolutionary technique that provides novel perspectives on cancer treatment (23,24). In particular, EV-based liquid biopsy shows some advantages compared to tissue biopsy. First, EVs have been repeatedly reported to be stable in almost all types of body fluids, and they are less-invasive to collect (25). Second, EVs reflect the current state of the disease in a timely manner by inheriting specific biomolecules from the parental cells. For these reasons, EV-based liquid biopsy allows us to monitor the disease with multiple minimally invasive snapshots. In the next section, we summarize the recent studies of EVs in BCa and discuss their clinical application.

The potential of urinary EVs in BCa

As BCa is bathed in urine, most EV biomarker studies for BCa use urine specimens. On the other hand, some studies have shown that EVs from BCa are also released into systemic circulation (26,27). Although whether urine or blood is the better source of BCa biomarkers remains to be answered, urine can contain larger amounts of EVs from BCa than blood, and most studies are focused on urinary biomarkers. Thus, in this review, we focused on the potential of EVs in urine specimens as biomarkers for the diagnosis and follow-up of patients with BCa. Among the contents of urinary EVs, noncoding RNAs, mRNAs, and proteins are frequently reported as biomarkers for BCa.

miRNA

miRNAs are small noncoding RNAs of 20–25 nucleotides in length that post-transcriptionally regulate the expression of thousands of genes, affecting various kinds of biological processes (28). miRNAs can be bound to RNA-binding proteins, including Argonaute 2 protein, or packed in EVs and are highly stable in various kinds of body fluids. They can not only exert decisive roles in cell-to-cell communication but also have the potential to serve as biomarkers for various kinds of cancers (29). To date, the majority of the studies of urinary EVs have focused on miRNAs.

First, Armstrong *et al.* investigated the miRNA molecular profile of matched tumour tissue and body fluids, including urinary EVs, from 16 patients with BCa and found that miR-205, miR-2003c-3p, and miR-29b-3p were common to tumour tissue and urinary EVs (30). As muscle invasive BCa (MIBC) is characterized by specific molecular alterations (31), it was expected that miRNAs in urinary EVs also reflected the specific characteristics of MIBC. Baumgart *et al.* selected 9 candidate miRNAs that were deregulated in both invasive BCa cell lines and their secreted EVs compared to non-invasive cells and their EVs (32). However, they could not validate the results of these miRNAs using patients with MIBC and non-MIBC (NMIBC). Therefore, further research is needed to identify clinically important miRNAs in urinary EVs to detect MIBC.

Andreu *et al.* and Matsuzaki *et al.* directly investigated miRNAs in EVs. Andreu *et al.* used microarray analysis of miRNAs to select candidate miRNAs in urinary EVs from 4 patients with high-grade BCa and 4 healthy non-smokers. Subsequently, selected miRNAs were validated in

the different cohorts (9 healthy non-smokers, 16 patients with low-grade BCa, and 18 patients with high-grade BCa). The results showed that miR-375 could identify high-grade BCa, while miR-146a could be used as a biomarker for low-grade BCa (33). In another study, Matsuzaki *et al.* performed microarray analysis of miRNAs of urinary EVs from 6 patients with BCa and 3 healthy donors and selected candidate miRNAs that were enriched in EVs from patients with BCa. They subsequently validated the expression of a miRNA-independent cohort (n=60) and showed significantly higher levels of miR-21-5p in BCa patients (34). As the tumour-surrounding cells can also secrete specific miRNAs in urinary EVs, it might be reasonable to directly investigate urinary EVs without selecting miRNAs on the basis of the results of tumour cells.

Other non-coding RNAs

To date, two kinds of non-coding RNAs, long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs), have been reported to serve as biomarkers in urinary EVs for BCa (35-43). LncRNAs are composed of more than 200 nucleotides. Dysregulation of lncRNA expression has been reported to play district roles in malignant processes, such as tumour progression or development of metastasis (35,36). Recently, lncRNAs in urinary EVs have been isolated as potential biomarkers. Berrondo *et al.* reported that lncRNAs, HOX transcript antisense RNA (HOTAIR), HOX-AS-2, ANRIL, and linc-ROR, were enriched in urinary EVs from BCa patients with high-grade muscle-invasive disease (n=8) compared to healthy volunteers (n=5) (37). In the same study, they also identified additional and novel lncRNAs (HYMA1, LINC0047, LOC100506688, and OTX2-AS1) in urinary EVs, which were upregulated in invasive BCa (n=8) compared to healthy volunteers (n=3), using RNA sequences (37). Zhan *et al.* screened and evaluated the expression of 8 lncRNAs that have been reported to play functional roles in tumorigenesis in a training set and validation set using qRT-PCR. They established a panel of three lncRNAs (MALAT1, PCAT-1, and SPRY4-IT1) for BCa diagnosis in the training set (104 BCa patients and 104 healthy volunteers) and subsequently validated its performance in the validation set (80 BCa patients and 80 healthy volunteers); it had an area under the ROC curve (AUC) of 0.813 (38). In addition, they also revealed that the expression of PCAT-1 could act as a biomarker to predict the recurrence of BCa (38).

CircRNAs are a class of non-coding RNAs with

covalently closed loop structures that are formed from exon direct back splicing of pre-mRNA transcripts (39). Although circRNAs have been thought of as no more than junk RNA molecules, recent studies have shown that they have multiple regulatory roles (40,42). In BCa patients, circPRMT5 was found to promote epithelial-mesenchymal transformation (EMT) by sponging miR-30c, contributing to tumour progression (43). Additionally, circPRMT5 was upregulated in urinary EVs from 18 patients with BCa compared to 14 healthy volunteers, and its expression was associated with tumour metastasis (43).

mRNAs

mRNA is reported to be contained in EVs. mRNAs transferred into recipient cells via EVs can be translated into proteins and provide phenotypical changes (44). Murakami *et al.* performed RNA-seq analysis on urinary EVs from 12 BCa patients and selected 12 mRNA candidates for the detection of BCa. In the validation cohort (n=173) using qRT-PCR, SLC2A1, GPRC5, and KRT17 were confirmed as diagnostic markers. These three mRNAs were able to detect both NMIBC and MIBC and outperformed conventional cytology or BCa biomarkers such as BTA (45). Additionally, a panel of the three mRNAs in combination with urine cytology improved the detection of pT1 and higher stage BCa compared to that with urine cytology alone (AUC 0.93). Furthermore, they revealed that the panel of the three genes could be useful for assessing patients with negative cytology results (45). On the other hand, Perez *et al.* screened urinary EVs using an mRNA microarray (n=11) and validated that LASS2 and GALNT1 were only detected in urinary EVs from BCa patients, while ARHGEF39 and FOXO3 were only detected in urinary EVs from non-cancer controls using PCR (n=4) (46). These genes were not detected or differentially expressed in the RNA-seq data from the study of Murakami *et al.* (45). The differences in these findings may have resulted from the limited number of samples used in RNA-seq or microarrays; therefore, further research will be necessary to evaluate these results using a large number of samples.

Proteins

Proteins are one of the most investigated molecules in EV biomarker studies. Based on proteomic analysis, several reports have shown the usefulness of proteins in urinary EVs. However, the proteins identified by proteomic

analysis differ among studies (47-49). On the other hand, other studies focused on specific proteins in urinary EVs and evaluated their diagnostic or prognostic value in BCa management (50-54). Lin *et al.* developed matrix-assisted laser desorption ionization time-of flight (MALDI-TOF) platform and enrolled 129 urothelial carcinoma (UC) patients and 62 participants without UC for determining diagnostic markers. In this study, they identified alpha 1-antitrypsin and H2B1K in urinary EVs, and these proteins could be diagnostic and prognostic biomarkers for BCa. Verification by immunohistochemistry revealed that these proteins were significantly upregulated in BCa tissue compared with normal tissue (50). Silvers *et al.* reported that four proteins (HEXB, S100A4, SND1, and TALDO1) were enriched in urinary EVs from patients with BCa compared to healthy controls (51). In another study, Chen *et al.* proposed that TACSTD2 in urinary EVs has diagnostic value as a biomarker for patients with BCa (52).

In addition, several reports have focused on the aggressiveness of BCa and proteins in urinary EVs. Beckham *et al.* showed that epidermal growth factor-like repeats and discoidin 1-like domain 3 (EDIL-3) in EVs derived from BCa cells facilitate angiogenesis and promote the migration of urothelial and endothelial cells *in vitro*. In the same study, EDIL-3 in EVs from urinary EVs from 12 MIBC patients was significantly higher than that in EVs from 12 healthy volunteers, suggesting its effectiveness as a biomarker for MIBC (53). In another study, Silvers *et al.* found that MIBC cells secrete EVs containing periostin and that the transfer of periostin activates ERK oncogenic signals, promoting cell aggressiveness in recipient cells. They also found that the expression of periostin in urinary EVs was higher in 4 patients with MIBC than in 4 healthy controls and 3 patients with NMIBC following transurethral resection of bladder tumour (TURBT) (54). The different technical approaches and small series of patients are thought to be the main reasons for the heterogeneity of the results between the studies (Table 1).

The current hurdles towards clinical application

Although urinary EVs appear to be a valuable source for the establishment of novel biomarkers in BCa, there are several points to discuss regarding their clinical application. In this section, we summarize the advantages and disadvantages of EV-associated noncoding RNAs and proteins.

The advantage of targeting noncoding RNAs in EVs is that we can repeatedly and readily evaluate their expression

Table 1 Potential use of urinary extracellular vesicles in bladder cancer

Biomolecules	Role	Marker	Isolation methods	Type assay	Ref
miRNAs	Diagnostic	miR-205, -2003c-3p, 29b-3p	Urine Exosome RNA Isolation Kit	nanosttring microRNA assay, droplet digital PCR	(30)
	Diagnostic (high grade bladder cancer)	miR-375	Ultracentrifugation	microarray, qRT-PCR	(33)
	Diagnostic (low grade bladder cancer)	miR-146a	Ultracentrifugation	microarray, qRT-PCR	(33)
	Diagnostic	miR-21-5p	Ultracentrifugation	microarray, qRT-PCR	(34)
	Diagnostic	Combination of miRNAs	No isolation method	microarray	(32)
lncRNA	Diagnostic (invasive bladder cancer)	HOTAIR, HOX-AS-2, ANRIL, linc-RoR	Ultracentrifugation	RNA sequence, RT-PCR	(37)
	Diagnostic (invasive bladder cancer)	HYMA1, LINC0047, LOC100506688, OTX2-AS1	Ultracentrifugation	RNA sequence, RT-PCR	(37)
	Diagnostic and prognostic	MALAT1, PCAT-1, SPRY4-IT1	Urine Exosome RNA Isolation Kit	qRT-PCR	(38)
circRNA	Diagnostic and prognostic	circPRMT5	Ultracentrifugation	circRNA array, qRT-PCR	(43)
mRNA	Diagnostic	SLC2A1, GPRC5, KRT17	Exosome isolation tube	RNA sequence, qRT-PCR	(45)
	Diagnostic	LASS2, GALT1	Ultracentrifugation	microarray, RT-PCR	(46)
Protein	Diagnostic and prognostic	alpha 1-antitrypsin H2B1K	Ultracentrifugation	mass spectrometry (LC-MS/MS)	(50)
	Diagnostic	HEXB, S100A4, SND1, TALDO1	Ultracentrifugation	western blotting	(51)
	Diagnostic	TACSTD2	Ultracentrifugation	mass spectrometry (LC-MS/MS, LC-MRM/MS), ELISA	(52)
	Diagnostic (invasive bladder cancer)	EDIL-3	Sucrose/D ₂ O cushion ultracentrifugation	mass spectrometry (LC-MS/MS), western blotting	(53)
	Diagnostic (invasive bladder cancer)	Periostin	Ultracentrifugation	mass spectrometry (LC-MS/MS), western blotting	(54)

by using real-time PCR with a small number of samples. In addition, as noncoding RNAs in EVs are covered by lipid bilayer membranes, their stability and repeatability are guaranteed. On the other hand, to establish a platform that accurately evaluates the expression level of noncoding RNA, a standardized method to collect EVs is required. To date, ultracentrifugation and density gradient-based isolation are conventional gold standard methods to isolate EVs, but these procedure require a lot of time and effort (55). Commercial EV isolation kits are fast and simple methods; however, they may be a relatively rough isolation procedure with contaminating soluble proteins (56). Recently, to overcome these drawbacks, microfluidics has gained attention. Microfluidic approaches have the potential to revolutionize and realize high-throughput EV analysis. To

date, these approaches have successfully demonstrated the isolation of EVs (57-60). For example, in urinary EVs, Yasui *et al.* established a nanowire-based method for evaluating urine EV-encapsulated miRNAs (60). This device was able to extract much larger kinds of miRNAs than conventional ultracentrifugation or a commercial EV isolation kit from a smaller sample volume and over a shorter period of time (60). Therefore, the establishment of novel devices like these to purify EVs is required for the clinical application of urinary EV-associated noncoding RNAs. Moreover, there is no consensus on which small RNA is suitable for use as an internal control, which presents an additional problem. For EV-associated miRNAs, spike-in external controls, such as non-human (*Caenorhabditis elegans*) miRNAs, are commonly used in normalization. However, it is difficult to

precisely manage the amount of the external controls added to different samples. The use of unchanged miRNAs as endogenous controls might be ideal, but no consensus has been reached to date (29).

The advantage of focusing on proteins on EVs is that we can directly assess the EVs without EV isolation steps, such as ultracentrifugation. Ultracentrifugation requires a large amount of time and is not suitable for the clinical setting. Therefore, targeting the proteins on urinary EVs has great potential for establishing feasible biomarkers. To date, several detection methods using antibody-based assays have been used to characterize the proteins expressed on EVs (61–66). For instance, Yoshioka *et al.* established a method called the ExoScreen assay, which can detect circulating EVs in serum based on an amplified luminescent proximity homogeneous assay using photosensitizer beads and two specific antigens residing on EVs. In this study, they revealed that the ExoScreen assay detected colorectal cancer more exactly than the conventional tumour marker CA19-9 (61). However, these detection methods were examined in a relatively small number of patients. In addition, as the proteins on EVs are sensitive to collection and preservation conditions (67,68), the examination should be performed under strict protocols. Furthermore, the expression of protein on EVs in urine can be affected by urinary concentration. Urinary concentration can be easily influenced by the amount of water intake. Therefore, to normalize this, several studies reported that the creatinine concentration of undiluted urine is highly correlated with the particle counts of EVs in the urine specimen and can be used for normalization in urinary EVs (34,69–71).

In the studies of both urinary EV-associated noncoding RNAs and proteins, the difference in target molecules among the studies can be a hurdle to clinical application. To validate the usefulness of each molecule in urinary EVs, large-scale and inter-laboratory research should be performed. In addition, the combination of these molecules would be valuable for establishing a useful urinary EV panel for BCa management.

Although several issues remain, urinary EV-associated noncoding RNAs and proteins could be novel and useful liquid biopsy tools for the management of BCa.

Points of view from clinicians in BCa management

There are several topics that can directly influence daily clinical practice in BCa management. In this section, we

highlight recent clinical concerns in BCa and discuss the potential application of urinary EVs.

While radical cystectomy remains the gold standard strategy for invasive localized BCa or BCa with a high risk of dissemination, it is an invasive surgery with a severe impact on the quality of life of patients (72). First, to diagnose MIBC, TURBT is performed in clinical practice. However, a useful biomarker for predicting MIBC prior to TURBT does not exist. In patients with invasive BCa, the tumours easily spread into lymphatic vessels and blood vessels; therefore, the amount of EVs released into systemic circulation will also be increased. Indeed, in gastric cancer, there is a report that the number of plasma EVs was correlated with the stage of cancer development (73). Therefore, in BCa, the amount of circulating EVs from blood samples or the ratio of urinary EVs to EVs in blood might be an important biomarker for predicting invasive BCa.

Recent whole genome characterization of primary BCa elucidated that it is a heterogeneous disease that can be grouped into several molecular subtypes. This classification advanced our understanding of the biology of BCa (74). Specific genomic alterations are enriched in particular molecular mutations and copy number aberrations that underline progression patterns and biological and clinical properties. Intriguingly, several studies reported that the responses to chemotherapy and immunotherapy depend on the molecular subtypes of BCa (75–78). In prostate cancer (PCa), AR-V7, which is the most well-known AR splice variant, is associated with resistance to androgen-receptor axis targeted (ARAT) agents in metastatic castration-resistant PCa (mCRPC) and has the potential to be a good prognostic marker (79,80). Indeed, AR-V7 in plasma EVs is reported to be a biomarker to predict resistance to ARAT agents in patients with mCRPC (81). To date, there is no report to show the relationship between the characteristics of urinary EVs and the molecular subtypes of BCa. However, the contents of urinary EVs can be drastically influenced by primary BCa; therefore, they can also be good candidates for predicting the molecular subtype of BCa.

Until recently, the therapeutic strategy for BCa had seen little progress. For approximately thirty years, clinicians had the same, limited range of cisplatin-based chemotherapy, such as methotrexate, vinblastine, doxorubicin and cisplatin (MVAC) or gemcitabine-cisplatin (GC), and unfortunately, 5-year survival rates remained the same (82,83). However, thanks to the introduction of immune checkpoint inhibitor drugs, the overall survival of patients with advanced BCa is prolonged, and some patients with advanced BCa who were

given only months to live are now in durable remission (84). PD-1, which is highly expressed on activated T cells, and its ligand PD-L1, which is expressed on tumour cells or immune cells, comprise one of the main immune checkpoint pathways that downregulates immune activity in the tumour microenvironment. Blockade of this immune checkpoint pathway has demonstrated clinical activity in several types of solid cancers, including BCa (85,86). Logically, the expression level of PD-L1 on tumours could be an effective marker to predict the response to immune checkpoint inhibitors in BCa; however, a validated predictive biomarker of response has yet to be defined (86). Therefore, selecting patients who will respond to immune checkpoint inhibitors remains a pressing clinical issue. Another specific issue is that sequential tissue biopsy is an invasive procedure; thus, less-invasive liquid biopsy would be more ideal. To date, several studies have reported that EVs have great potential as biomarkers for immune checkpoint inhibitors. The expression level of PD-L1 on EVs has been demonstrated to be associated with disease progression and immunotherapy response in patients with head and neck squamous cell carcinoma (HNSCC) and melanoma (87,88). In addition, the expression of PD-L1 mRNA in EVs has also been reported to be associated with the response to anti-PD-1 antibodies in patients with melanoma and non-small cell lung cancer (NSCLC) (89). Furthermore, recently, the roles of PD-L1 on EVs from cancer cells were reported. Chen *et al.* revealed that metastatic melanoma cells secrete high levels of PD-L1 on EVs, which bind to PD-1 on CD8 T cells, suppress their functions, and promote tumour progression. In this study, they also revealed that PD-L1 on EVs in patients with melanoma can be a biomarker for predicting the response to anti-PD-1 therapy (90). Poggio *et al.* revealed that PD-L1 on EVs from metastatic PCa cells is a major regulator of tumour progression by suppressing T cell activation. In the same study, using a PCa syngeneic model, they also showed that resistance to immunotherapy can be caused by PD-L1 on EVs from cancer cells and concluded that PD-L1 on EVs could be a novel target for overcoming resistance to current immunotherapy approaches (91).

Future perspectives for EV-based therapy

As we mentioned previously, EVs facilitate cell-cell communication to promote tumour development by transferring their contents. Indeed, in BCa, several reports have shown that EVs play key roles in tumour

dissemination, e.g., tumorigenesis and progression (53,92), angiogenesis (53), and cancer-associated fibroblasts formation (93). Therefore, inhibition of transmission of EV cargo may provide a potential novel therapeutic strategy for BCa patients.

To date, three strategies of EV-based therapies (inhibition of EV secretion, elimination of circulating EVs, and disruption of EV absorption) have been proposed (21). Among these three strategies, inhibition of EV secretion and elimination of circulating EVs were mainly reported.

Kosaka *et al.* reported that the knockdown of neutral sphingomyelinase 2 (nSMase2) reduced EV secretion from breast cancer cells, resulting in the suppression of lung metastasis *in vivo* (94). However, nSMase2 is also expressed in normal cells (95). Additionally, the downregulation of nSMase2 did not inhibit the secretion of EVs in PCa cells (96); therefore, the suppression of nSMase2 will not provide the same effect on the secretion of EVs in all kinds of cancers. To overcome this dilemma, the mechanism of cancer-specific EV secretion should be investigated. Recently, we established a screening assay to comprehensively screen the regulator genes of EV secretion, which can be carried out in a short period of time and can be applied to all kinds of cancer (97,98). This high-throughput screening method can contribute to revealing the mechanism involved in EV secretion and contribute to the establishment of EV-based therapy targeting EV secretion.

Human epidermal growth factor receptor 2 (HER-2) localized on EVs has been shown to interfere with trastuzumab, an anti-HER-2 monoclonal antibody, and contribute to cancer progression (99). Marleau *et al.* applied this mechanism to establish an EV-targeting strategy for absorbing circulating EVs (100). They developed the hemofiltration system, which can selectively absorb circulating EVs derived from breast cancer cells by targeting HER-2 on the surface of EVs (100). Nishida-Aoki *et al.* demonstrated that administration of antibodies against CD9 and CD63, which are enriched on the surface of EVs, decreased circulating EVs and inhibited cancer progression in an *in vivo* mouse model (101).

Although there are still many obstacles to overcome before clinical implementation, the reduction of cell-to-cell communication via EVs has great potential as a novel therapeutic strategy in cancers. As the concepts of these strategies can be applied to BCa, EV-based therapies may add additional value to existing therapeutic methods, such as surgery, radiation therapy, and chemotherapy for patients

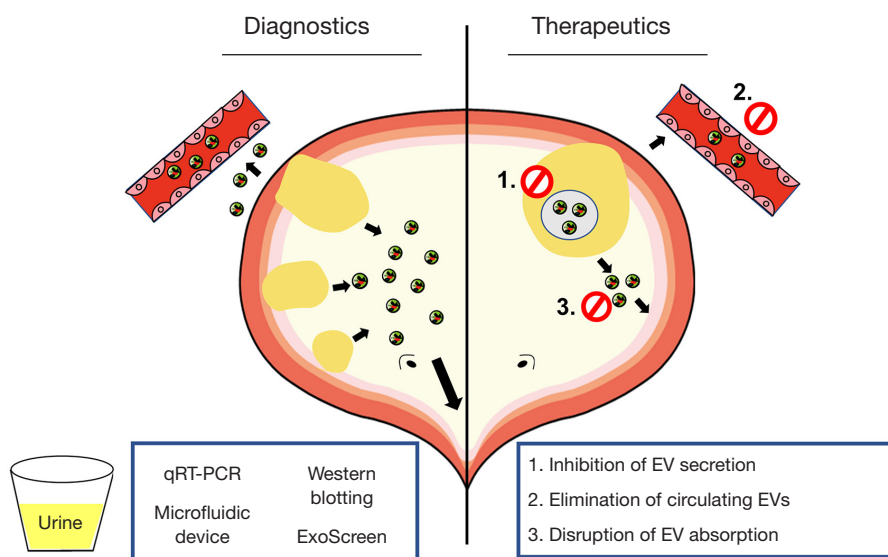


Figure 1 Clinical application of extracellular vesicles (EVs) in bladder cancer management. Urinary EVs are likely to be clinically useful biomarkers for bladder cancer. EV-associated biomolecules can be examined using several modalities, including real-time PCR, western blotting, and ExoScreen assays and microfluidic devices. Three therapeutic applications have been proposed: 1. inhibition of EV secretion, 2. elimination of circulating EVs, and 3. disruption of EV absorption. These EV-targeted strategies may add additional value to existing therapeutic methods.

with BCa in the near future (*Figure 1*).

Conclusions

This review demonstrated the high potential of urinary EVs as biomarkers for patients with BCa. Although none of the EV-based biomarkers has been used in daily clinical practice to date, the speed of EV research development is astonishing; therefore, novel technologies will overcome the present hurdles. We believe that urinary EVs will be used as clinically useful biomarkers in BCa management.

Additionally, EVs are multifunctional and potent cell-to-cell communicators that can contribute to changes in local and distant microenvironments to promote the survival, progression, and invasion of tumour cells. Therefore, EV research has the potential to unveil a novel mechanism of disease progression and to also provide an opportunity for the development of EV-targeted therapeutics. Further research is required; however, EVs could be used in every aspect of cancer treatment from early diagnosis to treatment of advanced stage cancer patients. We enthusiastically hope that the findings of EV research will clinically contribute to the treatment of BCa patients.

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