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Emerging Roles of Urine-Derived Components for the Management of Bladder Cancer: One Man's Trash is Another Man's Treasure

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Abstract

Urinary bladder cancer (UBC) is the most common malignancy of the urinary tract in humans, with an estimated global prevalence of 1.1 million cases over 5 years[1]. Due to high rates of recurrence and resistance to chemotherapy, UBC is one of the most expensive cancers to treat, resulting in significant health care costs. There is, therefore, a critical need to develop innovative molecular and cellular tools to refine patient stratification and help predict response to treatment. Urine is an underused resource of biological components shed from bladder tumors, such as exfoliated cells and extracellular vesicles, that could serve as molecular fingerprints and provide valuable biological insights into tumor phenotype and mechanisms of resistance to chemotherapy. Additionally, characterization of urine-derived extracellular vesicles and cells could be used as reliable biomarkers for prediction of response to neoadjuvant therapy.

Introduction

Urinary bladder cancer (UBC) is a common urogenital malignancy causing approximately 80,000 new cases and 18,000 deaths each year in the United States alone[2]. Urothelial carcinoma accounts for 90% of bladder cancers and can be categorized into non-muscle invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC) subtypes; although the majority of UBC present as NMIBC, the MIBC subtype is associated with the highest risk of developing metastases. Overall, 75% of patients diagnosed with high-risk bladder cancer will experience tumor recurrence, advancement of cancer, or decease within 10 years of their diagnosis[1]. Transurethral resection (TUR) of all visible lesions is a standard treatment for NMIBC but is associated with a high recurrence rate[3]. Intravesical chemotherapy and immunotherapy have demonstrated significant benefit in delaying disease recurrence in patients with NMIBC[4]. In MIBC patients, neoadjuvant chemotherapy with platinum-based drugs has been offered prior to local definitive treatment and has been associated with lower rates of recurrence and survival benefits[5,6]. Recently, a myriad of clinical trials has been launched to investigate the efficacy of immune checkpoint inhibitors combined with neoadjuvant therapy[7,8,9]. The outcome of these clinical trials may significantly change the therapeutic landscape of MIBC patients as half of MIBC patients are not eligible to receive platinum-based neoadjuvant chemotherapy[10]. In patients receiving treatment with neoadjuvant therapy, pathological complete response (pCR, pT0N0) rates have been observed in 20% to 50% of cases[6,7,9]. While there is still room to develop more effective neoadjuvant therapies and increase pCR rates, avoiding surgery in bladder cancer patients who completely respond to neoadjuvant therapy is a continuing challenge faced by many urologic oncologists. Additionally, disease recurrence has been reported in a subset of patients initially diagnosed with pCR highlighting the need to identify patients who present with occult metastasis at the time of surgery, as they could benefit from active surveillance and additional therapy to prevent disease recurrence. There is a critical need to identify those patients who can safely avoid surgery following neoadjuvant therapy, as well as those who need follow-up and additional therapy[11,12,13]. In this review we will discuss several emerging platforms that have strong potential to address these needs. First, we will describe challenges and clinical opportunities of *ex vivo* patient-derived tumor systems including urine-derived tumor organoids as preclinical drug testing platform for patients

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diagnosed with bladder cancer. Second, we will provide an overview of urine-based liquid biopsies, in particular with tumor-derived extracellular vesicles that can help monitoring response to treatment and identify complete responders.

Current precision medicine-approaches for the treatment of bladder cancer are promising but have significant drawbacks

To date, bladder cancer management decisions have been based on conventional histological features including tumor stage, lymph node status, histology variant at the time of diagnosis. Half of patients treated with neoadjuvant therapy, however, do not respond to treatment, highlighting our current inability to accurately predict those patients who will respond to chemotherapy[6,7,9]. Pathological factors have been evaluated for their predictive value in the context of muscle-invasive bladder cancer[14]. Specifically, patients with pure urothelial carcinoma have ~11 times more chance to experience pathological complete response post-NAT compared to tumors with histological variants or mixed tumors. While pure urothelial carcinoma constitutes ~70% of cases of bladder cancer, the remaining cases contains a histologic variant or mixed histological features[15]. This intratumor heterogeneity is a significant hurdle to any clinical decision-making involving best choice of treatment for patients with UBC[19].

Recent technological advances have allowed for efficient deep molecular profiling of bladder cancer tumors to support prediction of clinical outcomes and responses to therapy[17,18,21]. Transcriptomic profiling of biopsy and cystectomy specimens has, for instance, revealed distinct molecular subtypes of bladder cancer[16,18,20,22,23]. Similar to histology, molecular classification reveals important tumor heterogeneity with co-existence of luminal and basal subtypes within the same tumor in ~30% of cases[24,29]. However, while studies agree on gene expression signatures that identify each molecular subtype, they have shown conflicting results with regards to prediction of response to chemotherapy. Two recent studies, including one meta-analysis of 16 transcriptomic datasets, showed no significant difference in response rates to chemotherapy between tumor subtypes[16,20]. Overall, these findings collectively support the fact that UBC is a multifactorial disease whose genomic, transcriptomic and epigenomic diversity represent a significant challenge in treatment decision-making. Additionally, the high cost of such molecular analyses and the relatively long turn-around time for data collection and downstream bioinformatic interrogation, are further obstacles for personalized medicine applications[25,26]. These limitations underscore the need to develop additional biological resources that can improve patient stratification and better predict response to chemotherapy.

Preclinical 2D and patient-derived xenograft models bring value to drug discovery but have limited bedside applications

The increasingly recognized complexity and heterogeneity of bladder cancer has posed a major challenge to predicting treatment response. New tumor models generated from patient's tumor specimens, such as primary cell lines and patient-derived xenografts, have recently gained attention for preclinical drug testing. Conventional two-dimensional (2D) culture of UC cells[28] has traditionally been previously used for prediction of chemotherapeutic efficacy but the relative lack of primary bladder cancer cell lines that have been successfully established *ex vivo*[27] constrains the impact of this approach.

Although 2D cell lines can expand rapidly and offer the possibility for high-throughput drug screening but they do not faithfully reproduce the 3-dimensional nature and cellular diversity of native bladder cancer. Compounding this, cancer-derived 2D cell lines typically exhibit genetic drift after multiple passages[27]. These limiting factors likely contribute to failure in predicting *in vivo* drug response in cancer patients using 2D cell lines.

Patient-derived xenografts (PDX) is an approach whereby patient tumor fragments are implanted into immunocompromised mice to generate tumors that recapitulate genomic and phenotypical features of patient's original tumor[30,31]. PDX have value in both better understanding tumor biology and evaluating the efficacy of FDA-approved anticancer therapies or novel targeted treatments[37]. Although PDX models present an exciting opportunity for improving predictive value of preclinical studies, there are several hurdles to their translation into the clinic. The lack of an immune system in the immunocompromised host makes PDX models inadequate for modeling immune response and testing immunotherapies. Further, engraftment rates tend to positively correlate with tumor grade, meaning that low-grade patient tumors may not lead to a high yield of viable mouse tumors[94,95]. Finally, engrafted tumors can take several months to grow. This is a critical drawback for their application in translational medicine as, in the neoadjuvant setting, treatment is usually initiated within 3-4 weeks from the time of diagnosis. An ideal tumor model would combine the rapid growth and high-throughput potential of 2D models with the faithful recapitulation of host tumor microenvironment provided by PDX platforms.

Patient-derived tumor organoids: a preclinical platform for individualized prediction of drug response

Patient-derived tumor organoids represent a novel and superior model to identify and evaluate the efficacy of anticancer drugs. Tumor organoids are *ex vivo* mini tumors grown from patient's tumor fragments. By maintaining the original cellular composition of tumors, tumor organoids better reflect the physiology of tumor growth compared to conventional models such as two-dimensional primary cell lines[26]. Therefore, UBC-derived organoids have the potential to provide an *ex vivo* model of bladder cancer that can functionally predict treatment responses[32]. Moreover, many of the strategies outlined above rely on the invasive collection of large tissue specimens through cystectomy. We propose that culture of patient-derived organoids for chemo-sensitivity drug screening be performed on non-invasively obtained urine samples, as previously described in dogs[33]. This constitutes a significant innovation and advantage over currently established methods in the context of precision medicine.

Organoids can be propagated from bladder cancer cells derived from urine[32,33,35,36] or bladder biopsies[34]. Urine and biopsy-derived organoids have been shown to recapitulate molecular subtypes and heterogeneity[32,33,35,36]. Specifically, steady expression of urothelial cell markers (e.g. CK7, CK20, UPK3A, and CD44) has been reported on the luminal side of UBC organoids along with that of the proliferation marker Ki67[32,34]. In addition, xenografted urine-derived organoids were able to cause tumorigenesis in immunodeficient mice demonstrating their ability to maintain oncogenic properties *ex vivo*. Characterization of organoids can be done through an array of cellular and molecular techniques including immunohistochemistry, RNA-Seq, proteomics, and

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others[37,38,39]. Leveraging our expertise in the culture and maintenance of canine organoids, our consortium has successfully cultured MIBC organoids from urinary samples of 6/6 human patients (**Fig. 1**)[37,38,39]. Immunohistochemistry staining for Gata 3, p63 and Pax 8 (Gata 3+, p63+, Pax 8-) confirmed the urothelial origin of 3D organoids (**Fig. 2**). Of note, Pax 8 positivity is a sensitive and specific marker for both benign and malignant renal epithelial cells, which is used in clinical practice to separate urothelial carcinoma from renal cell carcinoma[40]. The malignant nature of urinary PDOs was finally assessed by cytomorphological evaluation, along with immunostaining for uroplakin-2 (urothelium-specific protein overexpressed in bladder cancer) (**Fig. 2**). Current research using organoids to study cancer biology offers promising preliminary results. Melanoma derived organoids, for example, have shown to be responsive to immune checkpoint inhibitors such as PD-1 and CTLA-1 antibodies[41]. This is a significant advantage over other model systems because we can now study the interaction of immune and tumor cells, through their co-culture, to refine prediction of drug response *ex vivo*. In addition, organoids have been shown to provide detailed information on the tumor microenvironment and stroma[42].

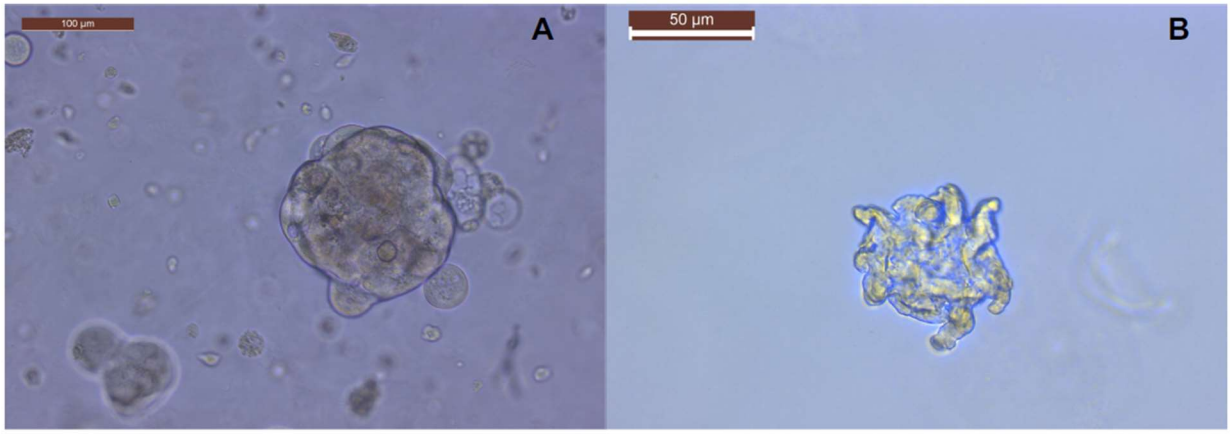

Figure 1. Growth and maintenance of urinary patient-derived organoids from a MIBC patient. (A) Typical cystic appearance of urinary PDO in Matrigel at Day 4. (B) Differentiation into larger budding structures on Day 12.

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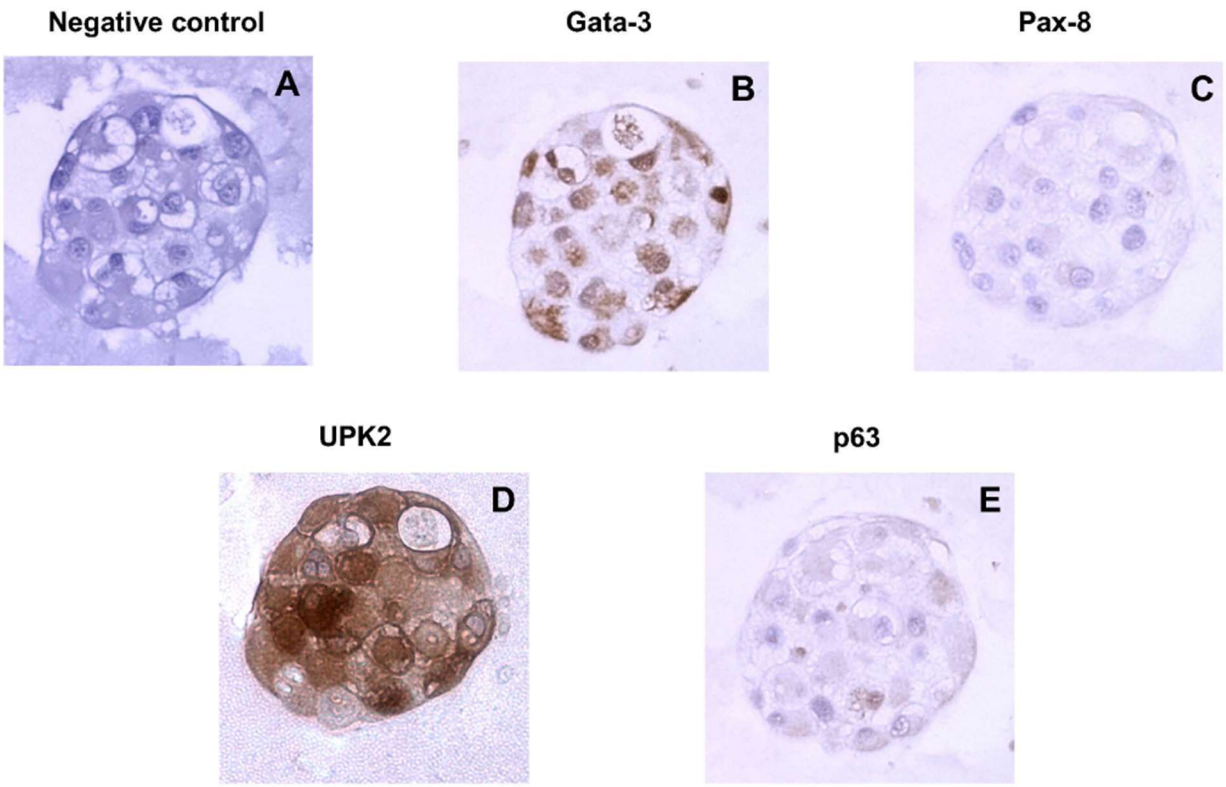

Figure 2. Immunohistochemistry staining of urinary patient-derived organoids from a MIBC patient. (A) Negative control (primary antibody only). (B) Positive immunohistochemistry (IHC) staining for Gata-3 (marker for luminal UC). (C) Negative IHC staining for Pax-8 (renal epithelial marker). (D) Strongly positive staining for UPK2 (upregulated in UC). (E) Faintly positive staining for p63 (marker for basal UC).

Dogs with bladder cancer are a highly relevant model for MIBC.

Although murine models have been extensively used for the study of bladder cancer, they typically do not reflect the biological behavior of MIBC in human patients[43]. First, the urothelium of mice is inherently refractory to developing cancer and tumors typically do not metastasize in mice as they do in humans[43,44]. Second, genetically modified mouse models do not effectively mimic the heterogeneity of the human patient population[43]. Even human xenograft models are not ideal for all purposes, as the tumors are transplanted into immunocompromised animals[43] Dogs, on the other hand, are a well-recognized, natural disease model of human MIBC, with very similar molecular features, tumor heterogeneity and subtypes, metastatic behavior as well as treatment responses[45,46,47,48]. Importantly, our group has recently been successful in propagating canine MIBC organoids from voided urine samples using established protocols in our laboratory[39]. Our preliminary data show a high degree of redundancy in marker expression between canine organoids and their parent tumors (see **Fig. 3**). In addition, some markers associated with MIBC and poor prognosis in human bladder cancer patients (Keratin 7 and CD44) were upregulated in canine organoids, consistent with the notion that dogs present most commonly with MIBC[32,47,49]. Furthermore, we have used MIBC-derived organoids to perform proof-of-concept drug screening assays using doxorubicin (see **Fig. 4**).

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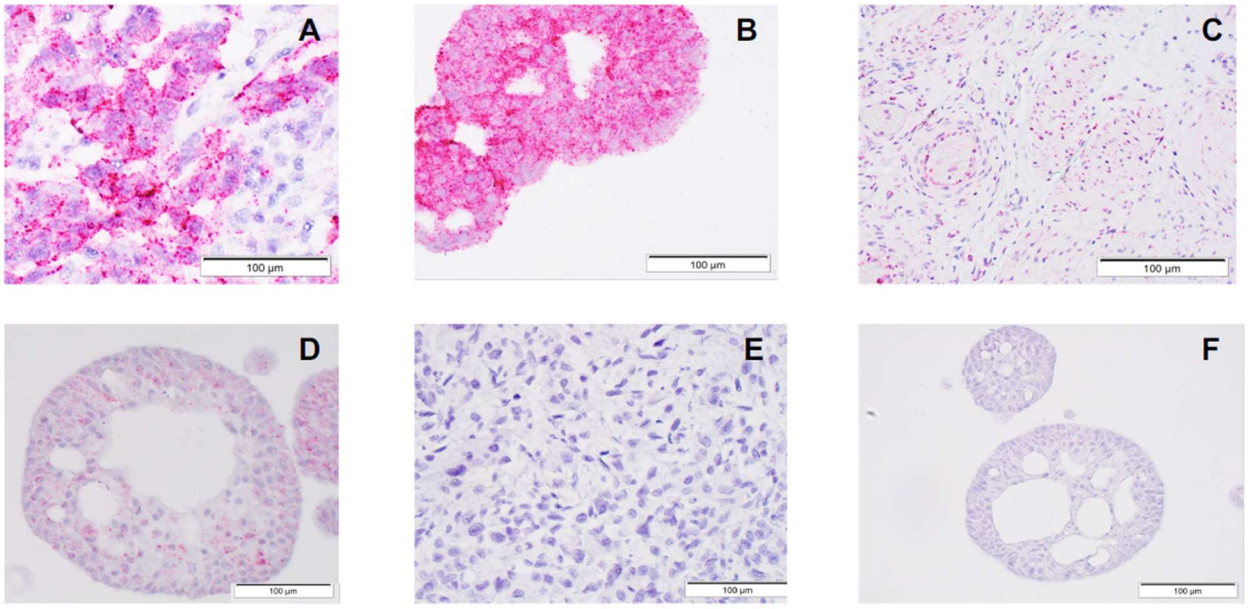

Figure 3. RNA *in situ* hybridization staining of canine MIBC organoids and their parent tumors. KRT 7 staining of MIBC tumor (**A**) and organoid (**B**) CD44 staining of MIBC tumor (**C**) and organoids (**D**); FOXA1 staining of MIBC tumor (**E**) and organoids (**F**). Consistent with a an MIBC phenotype, expression of urothelial and stem cell markers is present (KRT7 and CD44), while expression of luminal markers (FOXA1) is minimal to absent. Scalebar = 100um. Ubiquitin probe was used as positive control for all RNA ISH experiments.

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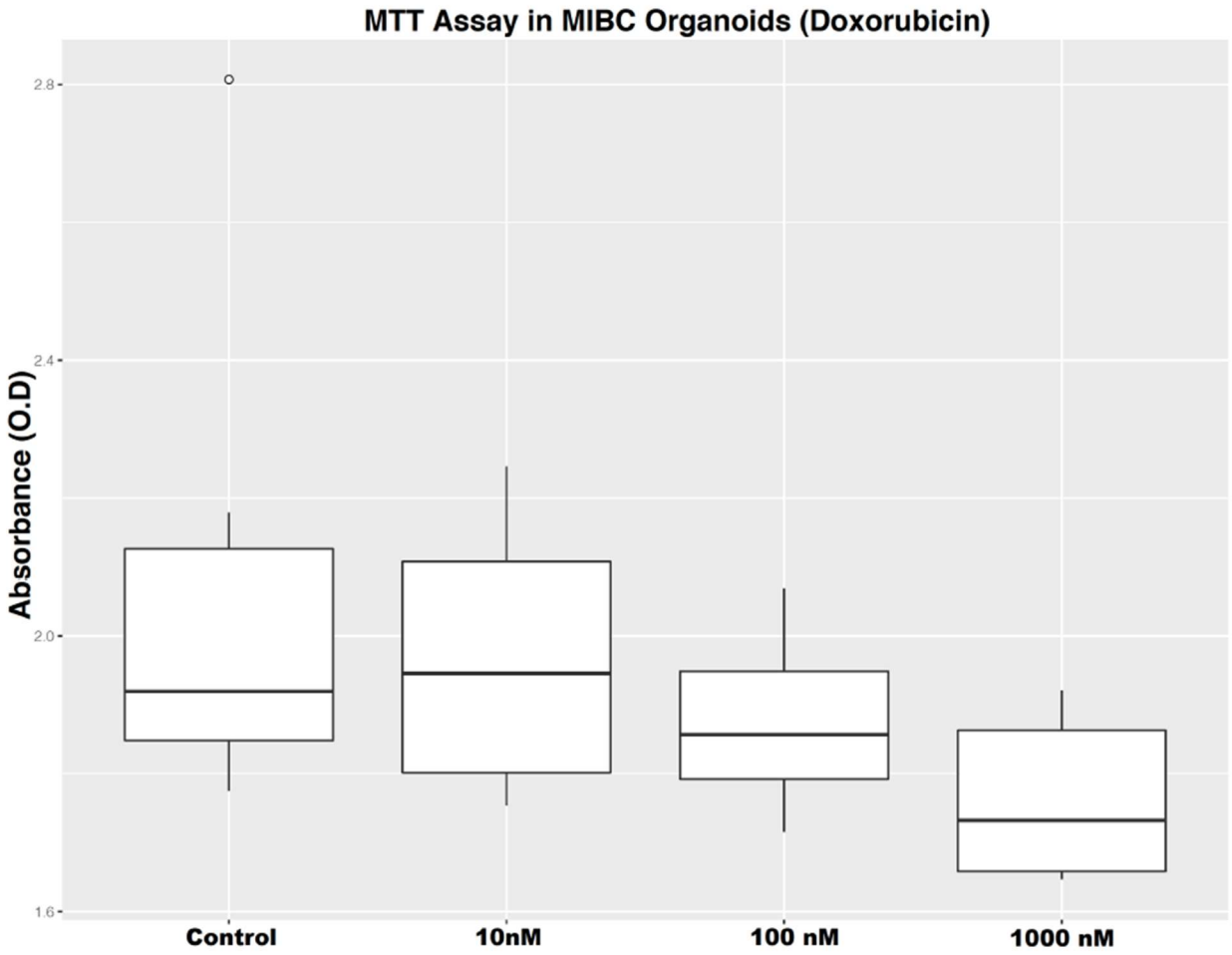

Figure 4. MTT cell viability assay on canine MIBC organoids after 48 hours exposure to doxorubicin. On Day 1-4 after passage, organoids were incubated with MMC for 48 hours. Cytotoxicity was determined using 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) at a final concentration of 0.5 mg/mL for 1.5 hr. After medium removal, 200uL/well DMSO was used to dissolve the formazan dye crystals and absorbance was read at 570 nm using a plate reader (SpectraMax 190, Molecular Devices).

Other research groups are currently investigating the value of using canine bladder cancer organoids for the prediction of drug response and combination[32]. Cell viability assays can determine the lethal drug combination needed to effectively treat for UBC. Herein, we propose to use a spontaneously occurring, analogous disease which constitutes a highly relevant model for UBC in people. Noteworthily, the FDA requires preclinical safety and efficacy data from rodent *and non-rodent animal models* (commonly dogs) prior to testing of novel drug candidates in human clinical trials. Therefore, we propose that future therapeutic leads for UBC be screened *ex vivo* using canine organoids to select the most promising drug candidates. Subsequently, these novel therapeutics could be tested *in vivo* in dogs with bladder cancer prior to formal clinical testing in human patients with UBC. The establishment of canine organoids as an *ex vivo* model in combination with the ability to test new candidate drugs in preclinical trials in dogs may therefore represent a *quantum leap* in comparative oncology for the faster development of viable treatment options for MIBC. Importantly, while dogs are an excellent for modeling

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disease phenotypes in humans, access to human UBC organoids would allow for greater research and testing into drug resistance of human UBC subtypes.

Tumor-derived extracellular vesicles for the monitoring of bladder cancer treatment response

Monitoring response to chemotherapy and identifying complete responders are two common challenges in urology oncologist daily practice. The close contact with the urothelium makes urine an attractive approach to detect the presence of exfoliated tumor cells and tumor derivatives (including soluble proteins and other factors with diagnostic potential). As such, collection of urine specimens offers distinct advantages over tissue biopsies due to the non-invasive nature of the method and the ability to perform longitudinal sample collection during the 6-to-8-week course of NAT administration. Extracellular vesicles (EVs) are one tumor derivative with recognized emerging diagnostic potential[50]. EVs are nano-scale (<1,000 nm) membrane bound structures released by all living cell types, including tumor cells[51]. EVs contain diverse molecule cargo (extracellular DNA, RNA, lipids and proteins) and surface molecules reflecting their parental cells and can be isolated from an array of biofluids including urine[52,53]. Originally characterized as professional “garbage bags” carrying waste cellular products[54,55,56], subsequent research has shown that EVs are key facilitators of intercellular communication by mediating cargo transfer between cells[57]. As EVs can be found in relative abundance, their enumeration in biofluids offers quantitative advantages over the paucity and short half-life of other tumor biomarkers such as circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA)[41,59,60]. Since RNA and DNA are packaged within EVs and protected from degradation by a phospholipid bilayer, their analysis may provide additional diagnostic and prognostic value, and prove useful for monitoring of treatment response[61]. In addition, EVs are shed from metabolically active cells which provide a more accurate reflection of tumor burden, while ctDNA is derived from apoptotic cells[62]. As of October 1st 2020, 24,002 publications with the search key “extracellular vesicles” have been indexed on PubMed, with 75% of these publications being released within the last seven years. Strikingly, more than 50 biotechnology companies focusing on diagnostic and therapeutic applications of EVs have emerged in the last decade. These numbers reflect the translational potential of EVs for clinical application in biomedical sciences.

Molecular composition of urine-derived EVs in bladder cancer patients

A rich body of literature has begun to describe the molecular composition of urine-derived EVs in bladder cancer patients vs. healthy individuals. These studies show that RNA (also referred to as extracellular RNA or exosomal RNA) is one of the most abundant molecules found in EVs. Among RNA subclasses, miRNA and ribosomal RNA represent more than 80% of total RNA composition of EVs[63]. Specifically, miRNAs from the miRNA-200 (miR-141-3p/5p, miR-200a/b/c-3p/5 and miR-205-3p/5p) family have been isolated from urine exosomes[26]. These miRNAs are associated with epithelial-to-mesenchymal transition (EMT) and reflect highly invasive tumor cell types due to loss of epithelial proteins leading to reduced cell adhesion[25]. Several other miRNAs have shown promise as potential biomarkers. For instance, miR-375 was found in EVs of patients with high-grade bladder cancer, while miR-146a was found in EVs of patients with low-grade tumors[64]. The long non-coding RNA (lncRNA) HOTAIR (HOX transcript antisense RNA) has also been found in urinary exosomes from patients with UBC[65]. HOTAIR is known to

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expedite tumor initiation and assist in tumor progression in many different cancers[70,71,72,73,74]. Specifically, Berronodo et al. (2016) showed that knocking out HOTAIR in UBC cell lines resulted in reduced cell migration and invasion, demonstrating a potential therapeutic use for UBC[65]. Other lncRNAs found in urinary EVs include LINC00355, UCA1-203, and MALAT1. Interestingly, all three of these lncRNAs have significantly higher expression in UBC-derived vesicles compared to healthy controls[67]. Exosomal DNA isolated from urine-derived EVs could be another potential biomarker for UBC. Indeed, using deep sequencing, exosomal DNA was found to have somatic mutations that are commonly found in UBC cells and provide insights into the genetic abnormalities of UBC tumors[66].

Alongside with RNA sequencing of urine-derived EV content, several research groups have conducted proteomic profiling of EVs[67,68,69,75,76,77]. Tumor Associated Calcium Signal Transducer 2 (TACSTD2) was found in high association on the outside of UBC urine isolated EVs and could therefore be used as another potential biomarker for UBC[78].

Opportunities for translating research on extracellular vesicles from bench to bedside

One of the major limitations of previous studies focusing on the molecular characterization of UBC-derived EVs lies in the use of total urinary EVs for RNA and proteomic profiling. Urinary EVs may originate from non-malignant cells of the urinary tract including the prostate, kidneys and the upper urinary tract[68]. Therefore, it is important to discriminate UBC-derived EVs from the heterogeneous population of urinary non-tumor-derived EVs, including those produced by the healthy urothelium. Reliable isolation of UBC-derived EVs is dependent on recognizing markers exclusively and consistently expressed on the surface of EVs released by tumor cells. These cell-surface markers can be utilized to collect UBC-derived EVs through magnetic bead-based immunocapture or fluorescence activated particle sorting across multiple platforms[96,97]. In the absence of tumor-specific antigens, isolation of bladder-specific EVs (from both tumors and normal cells) can be acceptable as a way to enrich the diagnostic EV pool with UBC-derived EVs and to minimize confounding urinary EVs with other cellular origins.

As an example of these strategies, uroplakins are attractive candidates for isolating bladder cancer-derived EVs. Uroplakins are a family of four highly glycosylated cell-surface proteins (1A, 1B, 2, 3) involved in urothelium plaque formation and permeability[79]. Uroplakins are not expressed by non-urothelial tissue, have limited expression in normal urothelium but high expression in bladder cancer[80,81]. Uroplakin 2 is routinely used for immunohistochemical diagnostic of urothelial carcinoma, with more than 80% of patients having detectable levels of uroplakins[82,83] and uroplakins have been found in urinary EVs isolated from patients with bladder cancer[68]. These proteins may represent suitable markers by which it is possible to isolate urinary EVs specifically released by the urothelium and bladder cancer tumor cells.

One of the major challenges in the development of EV-based liquid biopsy strategies has been the lack of reliable and standardized isolation and detection techniques. EV isolation typically requires time-consuming and labor-intensive methods that are not convenient or practical in the clinical setting. Recent improvements include a clinical-grade EV-based assay that benefits from a limited time for sample preparation, automated direct assessment of EV concentration and/or composition, with results

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obtained in a timeframe that is consistent with the current standard of care[84]. While no such assay has been developed for bladder cancer yet, recent advances have been made in other genitourinary cancers such as prostate cancer. The ExoDx Prostate IntelliScore (EPI) from Exosome Diagnostics (a Bio-Techne brand) is a urine-based test that combines ultrafiltration-based bulk EV isolation and RT-qPCR to measure expression of three genes highly upregulated in prostate cancer: PCA3 (prostate cancer antigen 3), ERG (V-ets) erythroblastosis virus E26 oncogene homologs) and SPDEF. A risk score (0-100) is calculated based on gene expression profiles that can predict the presence of clinically-significant prostate cancers (Grade Group ≥ 2)[85,86]. Following two successful clinical studies, the EPI test has been included in the NCCN guidelines for early detection in men for both initial and repeat biopsy. Another EV-based assay, the ClarityDx from Nanostics, is currently investigated to refine prostate cancer risk stratification[87]. Unlike the EPI test, the ClarityDx relies on direct enumeration of circulating prostate cancer-derived EVs from a simple blood draw using microflow cytometry[88,89]. Prostate cancer-derived EVs are characterized as positive for the following three markers: (1) PSMA (prostate-specific membrane antigen), (2) polysialic acid (PolySia) and (3) ghrelin receptor (GHSR). Flow cytometry quantification of EVs positive for these markers combined with machine learning-assisted data analysis provides a diagnostic accuracy of 0.81 (AUC) with 95% sensitivity and 97% negative predictive value for Grade Group ≥ 3 prostate cancer. Microflow cytometry is a state-of-the-art technology allowing for multiparametric phenotyping and enumeration of EVs at an unprecedented resolution. More importantly, it does not require any isolation/purification step and rapid enumeration of EVs from a very small volume of sample provides an excellent opportunity for characterization and quantification of EVs in body fluids. Despite the need for standardization and validation, this technology holds a lot of promise for clinical studies and it provides great potential for prediction of therapy response and improvement of patient outcome[90-93]. The EPI and the ClarityDx assays rely on distinct biofluid sources and different analytical platforms but they demonstrate the clinical value of EV-based liquid biopsies for the management of cancer patients. Upon prospective studies with large population cohorts, such assays will likely see the light in patients with bladder cancer.

Conclusion

Urinary bladder cancer, especially in its muscle-invasive form, is associated with an extremely poor survival rate. As of today, many of the underlying mechanisms of UBC remain unknown, making it difficult to diagnose early and treat effectively. Additionally, UBC exhibits many heterogeneous subtypes and a broad range of disease phenotypes, such that therapeutic response to conventional chemotherapy is extremely variable among patients. The development of new molecular and cellular tools, such as UBC-derived EVs and organoids, provide an opportunity to streamline the diagnosis and characterization of UBC tumor subtypes. Additionally, 3D tumor organoids have been shown to retain their oncogenic like properties when cultured *ex vivo*, and could be used as a platform for drug screening purposes prior to clinical evaluation in patients with UBC.

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