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Review Article

Urinary biomarkers in bladder cancer: A review of the current landscape and future directions

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Abstract

Aim: This narrative review aims to describe established and emerging urinary biomarkers in the diagnosis and surveillance of non-muscle invasive bladder cancer. It provides a comprehensive account of classical, FDA-approved protein biomarkers and discusses their limitations. Further, we discuss the role that epigenetic, genetic, and exosomal markers can play to enhance sensitivity and specificity of the available tests.

Background: The initial diagnosis and surveillance of bladder cancer involves a combination of cystoscopy, upper urinary tract imaging, and urine cytology. Despite high specificity, cytology is limited by low sensitivity. There are currently 6 urinary assays approved by the FDA to enhance diagnosis and surveillance of bladder cancer. While these have improved diagnosis and surveillance when combined with cytology, these tests are still not sufficiently sensitive and false positives often occur in benign conditions which result in inflammation of the urinary tract. Advancements in laboratory techniques have produced significant advancements in epigenetic and genetic markers, as well as extracellular vesicles, with DNA- and RNA-based markers dominating the research in this area in recent years.

Methods: We identified relevant published data, using the PubMed/ Medline search engines as well as Google Scholar. We performed an online search using the terms "bladder cancer", "non-muscle invasive bladder cancer" in combination with "urine biomarkers" and limited articles in English published up to February 2020. This review consolidated on all available narrative and systematic reviews published in the 5 years in this field, while also reviewing the original data of each clinical trial or observational study which led to the development of the biomarkers.

Conclusion: The development of laboratory techniques and understanding urine-based biomarkers in BC has fuelled the use of noninvasive liquid-based biomarkers to complement urine cytology. Nonetheless, none are sufficiently effective when used in isolation, and cytology remains the gold standard in many practices. Future efforts will be focused on using these markers in combination as a predictive signature, and moving on to validating them for use in everyday clinical practice.

Keywords: Biomarkers; Bladder cancer; Urinary biomarkers

1. Introduction

Bladder cancer (BC) is the 8 most common cancer worldwide, with over 550,000 cases diagnosed worldwide in 2018 [1]. Eighty percent of patients with BC present with non-muscle invasive bladder cancer (NMIBC), with the remainder presenting as muscle-invasive BC (MIBC). Up to 50% of NMIBC cases eventually recur despite radical treatment, and up to 30% of them experience disease progression to an MIBC [2]. Due to its high recurrence rate, surveillance cystoscopy is recommended at an interval dictated by the initial grade and stage of the disease. In cases of high-grade disease, cystoscopy may be required up to 3-monthly intervals [3].

The initial diagnosis and surveillance of BC usually requires a combination of cystoscopy, upper urinary tract

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imaging, and urine cytology. Cystoscopy and imaging have limited sensitivity in the detection of small lesions of the urinary tract. In these cases, there is a reliance on urine cytology, the most widely used noninvasive test for the detection and surveillance of BC. Despite its high specificity (approximately 86%), the utility of cytopathology is hindered by low sensitivity (48%) as well as interobserver variation [4], limiting its use especially in low-grade tumor [5,6].

The reliance on invasive procedures as well as the limited sensitivity and specificity of current investigation modalities represent a clinical unmet need both in the diagnosis and surveillance of patients with BC. In addition, the requirement for cystoscopy represents a significant cost to healthcare services in diagnosing BC [7]. Urinary biomarkers for BC represent an area of considerable research tested in both patients presenting with hematuria and patients with NMIBC requiring surveillance cystoscopy. There are currently 6 urinary assays approved by the US Food and Drug Administration (FDA) for clinical use in conjunction with cystoscopy. NMP22 enzyme-linked immunosorbent assay (ELISA), NMP22 BladderChek, and UroVysion have FDA approval for diagnosis and surveillance; immunocyte (UCyt+), BTA-TRAK, and BTA-STAT have been approved only for bladder surveillance following the diagnosis of a primary tumor. This review will summarize the current data on all FDA-approved and commercially available assays and cover a range of emerging biomarkers for detection and surveillance of BC, as depicted in Fig. 1. In this era of precision medicine, the performance of any single biomarker is limited by methodological issues, and therefore none of them are approved for diagnosis or surveillance when used in isolation. This review will not cover biomarkers in relation to screening for BC, which is a distinct topic in its own right.

2. Urine collection and processing

Urine specimens demonstrate a high degree of intra- and inter-individual variability [8]. This includes variation in protein concentrations, total protein excreted, and pH. These differences could be due to individual biological factors, such as variability in urine components due to health, age and diet, or proteolysis while the urine is stored in the bladder. Alternatively, variability could be due to degradation of collected urine samples upon storage [9].

There are many clinical and testing aspects which may affect processing and interpretation of urine biomarkers, particularly the currently FDA-approved ones. There are a myriad of potential urine samples with different advantages and disadvantages, such as spot urine, 24 hour urine, or first morning urine. First morning urine demonstrates the least variability in protein concentration [10]. Second morning urine or a random spot sample may demonstrate higher variability but suffers from less proteolysis from time spent in the bladder. Twenty four hour urine collections may provide a reflection of the protein type and concentration across a period of time, but are awkward for the patient and may lead to degradation and contamination of the urine sample prior to processing.

A complete review of urine sample collection, storage, processing and its associated challenges are beyond the scope of this review. However, for uniformity of results, it is imperative that all clinical and laboratory facilities

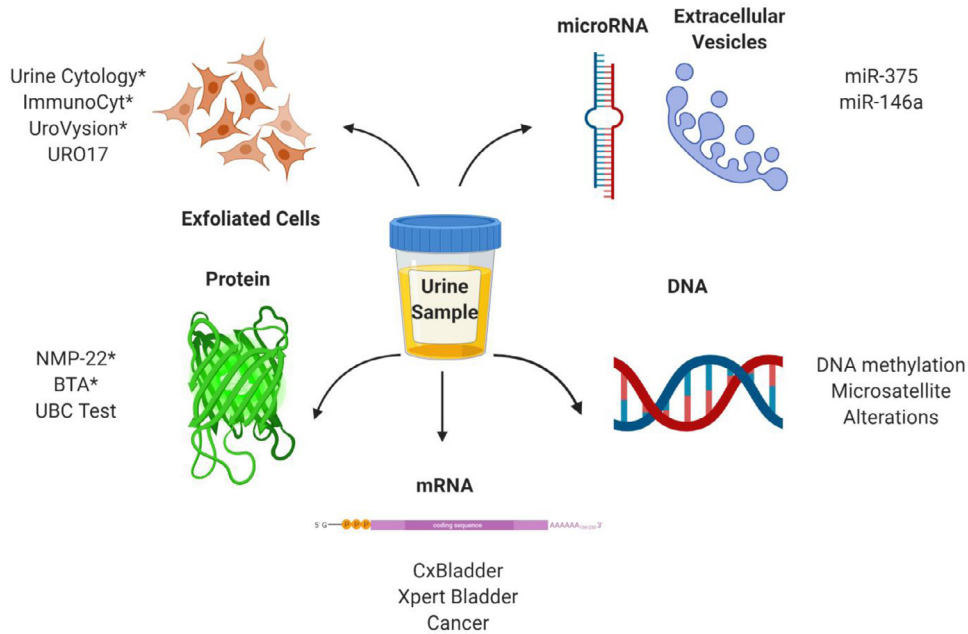

Fig. 1. Potential of Urine Based Liquid-Biopsy Biomarker Testing. Selected representative examples denoted for each category of testing. *represent FDA approved assays. mRNA = messenger RNA, miRNA = micro RNA, NMP22 = Nuclear Matrix Protein 22, UBC = Urinary Bladder Cancer, BTA = Bladder Tumor Antigen.

abide by an agreed standard operating procedure. These collection and processing procedures will need to be adjusted based on the clinical hypothesis, analytical platform, and/or parameter for normalizing data.

3. Classic FDA-approved biomarkers

3.1. Nuclear matrix protein 22

Nuclear matrix proteins (NMPs) are a family of proteins that play a crucial role in the structure of the nucleus and are involved in every step of its function, ranging from DNA replication to regulation of gene expression. Several of the NMPs are overexpressed in urothelial tumor and are released into the urine upon apoptosis of the tumor cells. Of these, NMP22 has been the most extensively investigated, and assays for the antigen are used both in the context of diagnosis and monitoring for cancer recurrence.

NMP22 Bladder Cancer ELISA-Test and NMP22 BladderChek tests have been approved by the US FDA. The former is often referred to as the quantitative NMP22 test which is performed in a laboratory, while the qualitative BladderChek test is a point-of-care (POC) test. These tests have approval both in the context of diagnosis and surveillance. The performance of the NMP22 assays has been evaluated in several meta-analyses. In 2015, Chou et al. performed a meta-analysis on NMP22, demonstrating a sensitivity of 69% and a specificity of 77% for the quantitative ELISA test. The corresponding value for the POC test was 58% for sensitivity and 88% for specificity, although notably only 4 studies were considered for this qualitative test [11]. In 2017, Wang et al. conducted a separate meta-analysis of 19 studies looking at NMP22 POC test encompassing 5291 patients. It demonstrated a sensitivity of 52%−59% and a specificity of 87%−89% [12].

NMP22 remains one of the most well-studied biomarkers to date. While relatively specific, most meta-analyses concur that the NMP22 assay is insufficiently sensitive when used in isolation. Like many available biomarkers, the test has a particularly lower sensitivity to detect low-grade tumor [13]. NMP22 assays measure the cellularity or amount of cell turnover that may be introduced into the urine by a variety of conditions, which includes surface shedding from bladder tumor. Hence, false-positive results are common in patients with benign bladder conditions such as infection, stones, inflammation, and hematuria.

3.2. Bladder tumor antigen (BTA) assays

BTA Stat/BTA TRAK test are in vitro immunoassays, which detect the presence of human complement factor H-related protein in the urine of patients with BC. BTA-stat is a qualitative bedside POC assay with results available within 5 minutes, whereas BTA-TRAK is a specialized quantitative ELISA. These tests have been approved by the FDA only for monitoring BC recurrence in combination with cystoscopy.

In a meta-analysis of 13 studies of BTA Stat, Guo et al. [14] found that the test had a higher sensitivity (67%, 95% confidence interval 64%−69%) than urine cytology (43%, 95% confidence interval 40%−46%), but the specificity, likelihood ratios and area under the curve were inferior to urine cytology. Like other biomarkers, BTA Stat was found to have a much higher sensitivity for high-grade tumor (74%) than low grade tumor (25%), with a specificity of 77% [15].

In general, the sensitivity of BTA Stat ranges from 57% to 82%, with a specificity of 68%−93% [16−18], whereas BTA TRAK has a sensitivity of 66%−77% and a specificity ranging from 5% to 75% [19,20]. These figures generally point towards a higher sensitivity than cytology, but like NMP22, the BTA assays suffer from a higher false positive rate in patients with inflammatory disease in the urinary tract.

3.3. UroVysion

The UroVysion test is a multicolour fluorescent in situ hybridisation assay which detects aneuploidy of chromosomes 3, 7, or 17 or loss of the 9p21 locus. It has received FDA approval for urothelial BC diagnosis and surveillance. The criteria set for detecting BC by UroVysion are at least one of the following [21]:

a. ≥4 cells (of 25) with gains of ≥2 chromosomes in the same cell.
b. ≥10 cells with a gain of a single chromosome.
c. ≥10 cells with tetrasomic signal patterns.
d. Homozygous deletion of the 9p21 locus in 20% or more cells.

The sensitivity of this test ranges between 69% and 87% with a specificity between 89% and 96% [22,23]. The UroVysion test has demonstrated excellent sensitivity to detect Carcinoma In Situ and high-grade tumor, with sensitivities ranging between 83% and 100% [21]. It is also a useful adjunct to cytology as it maintains the specificity of this test but simultaneously increases sensitivity [24,25] (45.8% vs. 72.2%). A key advantage of this test is its high specificity, as the assay is not affected by haematuria, inflammation, and other conditions which may give false-positive readings with some other tumor markers. There is data suggesting its use for monitoring patients with NMIBC for response to intravesical therapy [26].

3.4. ImmunoCyt test

The ImmunoCyt assay (also marketed as uCyt+) uses three fluorescently labelled monoclonal antibodies to detect carcinoembryonic antigen and sulphated mucin glycoproteins that are expressed on most BC cells, but not on normal cells. The sensitivity of this assay varies widely among

studies, ranging from 60% to 100%, with a specificity of 75%−84% [27−29]. In a meta-analysis, uCyt+ showed the highest sensitivity at evaluating symptoms and for surveillance [11]. It is approved for bladder surveillance following diagnosis of a primary tumor.

However, uCyt+ has been shown to be significantly affected by urinary tract infections, urolithiasis, and benign prostate hyperplasia. Other difficulties causing the low uptake of this test is the need for technical expertise, substantial interobserver variability, and a high rate of test failure due to inadequate specimen cellularity.

As of early 2020, the uCyt+/ImmunoCyt test is currently off the market due to the unavailability of the antibody. However, this immunocytological testkit has been unique in employing a cytology-only strategy, and may warrant reinstating into the market, perhaps with a newer, more BC-specific antibody.

4. Limitations with current urinary biomarkers

The FDA-approved biomarkers are collectively the most studied biomarkers to date, with multiple meta-analyses to support their clinical utility. The results of the meta-analyses must be interpreted with caution due to interstudy heterogeneity between the study populations. Selected meta-analyses have also failed to take key confounding factors influencing test performance into consideration, such as the proportion of subjects who smoked in the NMP22 BladderChek meta-analyses reported by Wang et al. [12]. Many of the meta-analyses described here have also limited their analysis to English language articles, with additional variability as to whether they took nonpeer reviewed meeting abstracts into account.

Nonetheless, most of these studies concur that currently FDA-approved biomarkers suffer from a high rate of false positive cases by nature of its assay design. Urinary biomarkers may yield false-positive results in 12%−26% of patients without BC. This is coupled with its limited sensitivity when used in isolation, leading up to a missed diagnosis in up to 43% of patients with bladder [11]. A consideration of the patient's pretest probability, assimilating the patient's clinical history and investigations where necessary (especially cystoscopy and cytology), will be required to put the results of these tests in context.

Considering the high false-positive and false-negative rates of the approved markers, multiple biomarker assays have been studied to provide additional molecular information to guide individualized surveillance and therapy. These will be described in the remainder of the review. While the mechanism of detection of recurrence or diagnosis is novel, the majority has had variable consistency at detecting cancer and are lacking in high quality studies and meta-analyses.

5. Additional protein markers detectable in the urine

Several immunological assays have been developed to detect the presence of cytokeratin fragments in the urine. Cytokeratins form part of the cytoskeleton of epithelial cells, and urothelial cytokines are released into the urine after cell death and can be predictive of the presence of cancer. Cytokeratins 8,18,19, and 20 have been associated with BC [30].

For instance, Urinary Bladder Cancer (UBC) ELISA and UBC immunoradiometric assay have been developed to detect the presence of fragments of cytokeratin 8 and 18 in the urine [31]. CYFRA21-1 is an ELISA which measures soluble fragments of CK19 in the urine. While a standardized cut-off is unavailable, studies usually employ normalization to urine creatinine. Detection sensitivities of cytokeratin immunoassays for low-grade bladder tumor could be as low as 13 percent, and the specificity can be particularly low in individuals where urinary tract infections are present [32].

However, these assays still have a relatively low sensitivity for detecting low-grade disease. The mean sensitivities for Grade 1/Grade 3 diseases are 53.4%/77.4% for NMP22 and 51.4%/87.5% for BTA for the FDA-approved biomarkers. The equivalent values are 48.5%/76.0% for UBC and 55.7%/91.9% for Cyfra 21-1 [33]. While the sensitivities for low-grade disease remain higher than urine cytology (albeit less specific), their performance is inferior to cystoscopy in the context of both specificity and sensitivity.

Recently, URO17 urine test for BC utilizing another member of cytokeratin family, Keratin 17 (K17), was shown to be a promising urine test for BC. A study by Babu et al. [34] used immunocytochemistry to detect presence of K17 in 112 urine specimens. The results showed that K17 was significantly elevated in BC specimens with a sensitivity of 100% and specificity of 96% in BC detection from urine samples. Analysis of histological tissue sections showed that K17 is elevated in both low-grade and high-grade tumor, and urothelial cancer. Significance of elevated level of K17 in cancer cells was described in another study that showed that K17 binds to $p27^{kip1}$ in the nucleus and aid in transporting $p27^{kip1}$ to cytoplasm where it is degraded [35]. Degradation of $p27^{kip1}$ allows the cancer cell to bypass G_1-S phase cell cycle control thus leading to cell proliferation which could explain specific association of K17 elevation and BC and high sensitivity and specificity of URO17 test. Interestingly, the current data suggest that URO17 could be a sensitive and specific test to detect PUNLMP and both papillary and nonpapillary carcinomas which could potentially providing diagnostic utility in cases where it could help identify lesions that can be easily missed by traditional urine cytology. Furthermore, the data also showed that URO17 test was able to detect BC in renal pelvis that was missed by urine cytology and cystoscopy which suggest that URO17 test could be used to augment and

increase the accuracy of cystoscopy and traditional urine cytology in monitoring patients for recurrence.

Two transcription factors, BLCA-1 and BLCA-4, have also shown promise as biomarkers. They are protein components of the nuclear matrix which are present in the urothelium of patients with bladder tumor. BLCA-1 is not expressed in nonmalignant urothelium [36], whereas BLCA-4 is expressed in both the tumor and adjacent benign areas of the bladder, but not in malignant bladders [37]. BLCA-4 may represent the field effect observed at the molecular level in normal tissues adjacent to tumor. The reported sensitivity of BLCA-4 is in the order of 89%−96% with specificity 95%−100% [38]. These markers appear to show a degree of promise as an adjunct to diagnosing early tumor, and further validation is warranted.

In addition, the CellDetect assay is a novel histochemical staining platform which allows for the discrimination between normal and malignant cells on the basis of color and morphological discrimination—based on the higher metabolic activity in cancer cells [39]. An Israeli study across 9 hospitals employing urine smears found that the overall sensitivity of this test was 84%, and the specificity was also 84% for patients undergoing routine surveillance by cystoscopy [40]. This test is currently gaining importance by using a cell based assay in clinical practice.

6. Epigenetic alterations

6.1. DNA methylation

The most well characterized epigenetic phenomenon is DNA methylation. Hyper- and hypomethylated regions of DNA are identified in BC and in premalignant lesions. DNA methylation status can be assessed in cell free DNA fragments and tumor cells shed in urine. A significant prevalence of methylated genes, for example, APC and cyclin D2, was elevated compared to benign cases [41]. Hypermethylation of selected genes, including GSTP1, APC, and RARb2 have been identified in patients with urothelial BC [42].Table 1 summarizes some of the key DNA-based urine biomarkers investigated in recent years, along with their accompanying sensitivities and specificities. Although the specificities of these markers are highly encouraging, the molecular genetic techniques required to detect these are expensive, time consuming, and highly specialized.

6.2. Histone tail modifications

Histone modifications represent a diverse set of epigenetic markers involved in both dynamic cellular processes and the stable maintenance of chromatin. In BC, the levels of histone methylation are lower in advanced tumor and correlated to poor survival. For instance, high levels of H3K27me3 correlated with poorer prognosis postcystectomy in pT1-3 and node negative patients with BC [50].

Table 1
Tumor-derived DNA methylation status as urine biomarker of urothelial bladder carcinoma diagnosis and/or surveillance

Gene	Sensitivity	Specificity
Diagnosis of bladder cancer		
GSTP1, RARb2, APC [42]	62	89
TWIST1 and NID2 [43,44]	79	63
POU4F2 and PCDH17 [45]	90	94
CFTR, SALL3/TWIST1 [46]	84	68
HDAC3 [47]	89	63
Surveillance of bladder cancer recurrence		
SOX-1, IRAK3, and Li-MET [48]	86	89
HS3ST2, SEPTIN9, and SLIT2/FGFR3 [49]	98	85
Delineation of bladder cancer grade and stage		
APC/Cyclin 2 [41]	55	100

7. Genetic alterations

7.1. DNA mutational analysis

Analysis of tumor-derived DNA via cell-free DNA can reveal mutations and serve as noninvasive biomarkers. Amongst the mutations which have been analyzed include urinary telomerase reverse transcriptase (TERT) promoter mutations, FGFR3 and telomere length. TERT maintains the integrity of telomeres and mutations in the TERT promoter are frequent in BC. Descotes et al. [51] reported that an assay analyzing the TERT promoter mutation in urine showed an overall sensitivity of 80.5% and specificity of 89.8% in diagnosis of BC, and that TERT mutations significantly predicted recurrence of NMIBC ($P<0.0001$). TERT, in combination with FGF3 and OTX1 also showed high sensitivity of diagnosis of NMIBCs as well as in pT1 tumor [52]. Mutations in FGF-3 are seen in approximately half of BC patients, with an elevated incidence (60%−70%) in low-grade tumor. Recent studies have suggested that partial replacement of cystoscopy with FGFR3 mutational analysis during surveillance can be safe and cost effective [53].

7.2. Microsatellite analysis

Microsatellites are polymorphic repeating units of 1−6 base pairs in length in human DNA. Microsatellite analysis is a Polymerase Chain Reaction (PCR) analysis of DNA in exfoliated urine cells. One of the most common genetic changes in BC is loss of heterozygosity in chromosome 9 [54]. Chromosomes 4p, 8p, 9p, 11p, and 17p also often display Loss of Heterozygosity in patients with BC. Generally, the sensitivities of these markers range from 72% to 97% and the overall specificity between 80% and 100% [55,56].

8. Urinary tumor RNAs

8.1. MicroRNAs

MicroRNAs (miRNA) are small 21−23 nucleotide long nonprotein coding RNAs that regulate gene expression by

pairing to the 3' untranslated region of their target mRNAs. They can be found in body fluids as free circulating miRNAs, bound to ribonucleoprotein complexes or in extracellular vesicles (EVs) such as exosomes [57]. Changes in miRNA expression in cancer tissues exhibit tissue specificity with a high level of stability and detectability. Due to their short length, miRNAs are less vulnerable to degradation than mRNA chains and can be stored for up to 48 hours at room temperature [58]. Hence miRNA expression analysis is considered a potential biological marker for both detection and surveillance.

Urinary miRNA can be derived from a range of specimens—voided urine, urine sediment, or supernatant. In a systematic review, Kutwin et al. showed that miRNA from urine supernatant have the greatest sensitivity (78.4%) followed by urine sediment (75.6%) and voided urine (74.3%). Urinary supernatant also has the highest specificity amongst the 3 at 79.4% [59].

To date, 12 studies have reported the diagnostic performance of miRNA. Of the miRNA panels, four have a sensitivity and specificity above 80% or more, and employed miRNA arrays or next generation sequencing to identify targets. MiRNA was then quantified by real time PCR.

8.2. Urinary-based mRNA assays

Circulating messenger RNAs (mRNAs) reflect the status of intracellular processes. Despite the majority of them being degraded by RNAses, they are still detectable in the urine of BC patients and may represent potential biomarkers. For instance, the Urine Ubiquitin Conjugating Enzyme E2C and isoleucine glutamine motif-containing GTAase-activating proteins mRNA levels are higher in BC patients than in controls [60,61]. In practical terms, commercially available mRNA-based urine biomarkers combine multigene panels, which are described below (Table 2).

8.3. Multigene panels

Several groups have investigated the utility of multigene panels in the detection of BC from urine samples. Of these, Cxbladder, which quantifies mRNA biomarkers is the most well-known. The test suite includes assays to potentially rule out the presence of BC in low-risk patients with haematuria (Cxbladder Triage), complement cystoscopy for BC detection in the presence of haematuria (Cxbladder Detect), and complement cystoscopy for surveillance in the context of recurrence (Cxbladder Monitor). Other tests, along with their accompanying sensitivity and specificity as well as validation studies (wherever relevant) are found in Table 3.

9. Extracellular vesicles and exosomes

Exosomes are membrane vesicles secreted at an elevated level in cancer patients—they participate in intercellular communication through transferring biologically active molecules (including RNA, DNA, and proteins) [71]. EV enrichment has been observed in the urine of patients with BC, and analysis has demonstrated specific patterns of protein and miRNA. For instance, an interesting micro-fluidic chip-based system has been employed to analyze EVs from patients with and without BC, demonstrating that the concentration of EVs in urine from patients with BC was significantly higher compared to healthy controls. This technique depicted a sensitivity of 81% and specificity of 90% for accurately diagnosing BC [72].

In addition to evaluating concentration of EVs, a parallel research strategy has been to categorize the cargoes contained within the EVs to determine whether there is a profile predictive of BC. One proteomic analysis of urinary EVs identified 2 proteins—alpha-1-antitrypsin and H2B1K, which are enriched in EVs isolated from patients with BC [73]. There has simultaneously been focus on the genetic cargo, specifically long-noncoding RNAs (lncRNAs), in urinary exosomes, although these have predominantly been explored in the MIBC patient cohort.

A separate study sought to analyze the profile composition of miRNAs and proteins associated with urinary EVs in patients with BC [74]. Using a microarray platform of >850 different miRNAs, the authors aimed to investigate dysregulation of particular miRNA and its association with the presence of BC. They found that 26 miRNAs were dysregulated in patients with high-grade BC. Real-time PCR analysis indicates that miR-375 is a biomarker for high-grade BC while miR-146a could identify low grade patients.

Table 2
Study characteristics and diagnostic accuracy of urinary miRNA for the diagnosis of bladder cancer: Selected multi-miRNA studies have sensitivity and specificity of 80% or more

Author	Marker	Specimen	Proportion of Low Grade (%)	Sensitivity	Specificity	PPV	NPV	AOC
Mengual et al.[87]	6 miRNAs: miR-187 + miR-18a + miR-25 + miR-142-3p + miR-140-5p + miR204	Not specified	38	85	87	88	83	0.92
Zhang et al.[88]	miR-99a + miR-125b	Urine supernatant	30	87	81	92	71	0.88
Eissa et al.[89]	miR-96 + Cytology	30–60ml void	80	87	87	86	80	
Urquidi et al.[90]	25 target diagnostic miRNA signature	Mid-stream void	16	87	100			

Table 3
Multigene panels in the diagnosis and surveillance of bladder cancer involving DNA, mRNA and epigenetic targets

Commercial Test	Genes Involved	Sensitivity	Specificity	Additional Notes
		mRNA tests		
Cxbladder [62]	IGFBP5, HOXA13, MDK, CDK1, and CXCR2	82% in patients with haematuria	90%	Large study comparing Cxbladder with FDA approved markers showed superior sensitivity and NPV [63]
XpertBC [64]	UPK1B, IGF2, CRH, ANXA10, and ABL	46.2%	77%	Study of 140 patients showed Xpert BC outperforms cytology at sensitivity and NPV even in low grade tumor, with no reduction of specificity [65].
		DNA-based tests		
Assure MDx [66]	FGFR3, TERT, and HRAS in combination with methylation analysis of OTX1, ONECUT2, and TWIST1	97%	83%	Follow-up validation study demonstrated 93% sensitivity and 86% specificity [67]
UroSEEK [68]	Mutations in 11 genes or presence of abnormal number of chromosomes	96%	88%	
Uromonitor [69]	FGFR3 hotspot and TERT promoter mutations	73.5%	93.2%	
		DNA methylation assays		
EpiCheck [70]	15 proprietary DNA methylated genes	68.2%	88.0%	

Although EVs represent an interesting source of biomarkers, the lack of accurate isolation and detection affects their uptake in clinical practice. However, the diverse exosome cargo represents a rich source of biomarkers, and the development of more sensitive capture platforms will increase its incorporation into clinical practice.

10. The practical value of urinary biomarkers

From a practical standpoint, the variety of test systems can be broadly categorized into 2 distinct characteristics. Two different approaches could be employed in laboratory test marketing − (1) the specialized system, where test systems employ complex techniques and elaborate pre-analytics that have high test qualities, but are limited to specialized centers and expensive, or (2) easy to perform assays that are cheaper, but test results are of limited value as less specific. The value of urinary biomarkers and its clinical utility depends on the clinician's ability to estimate pretest probability of the disease, the importance to patients (and their treating clinician) of relatively small changes in the probability of BC, and the acceptable threshold and clinical consequences of missed or delayed diagnoses and false-positive results.

The potential benefit of urinary biomarkers depends on the situation in which it is employed. For instance, a urinary biomarker used as a diagnostic tool in a patient with haematuria will require a high negative predictive value and high specificity. Patients with hematuria should be categorized by gross and microscopic hematuria, with the former receiving cystoscopy. For patients with only microscopic hematuria, urinary markers can be an important adjunct to nomograms leading to more accurate evaluation of their disease status [75].

The clinical applicability of urinary biomarkers in the context of surveillance is arguably more complex, and dependent heavily on the initial tumor grading. Following a transurethral tumor resection, markers may be a useful surveillance tool reducing the frequency of cystoscopies in a low-grade tumor. Due to the low probability of recurrence, an acceptable threshold for recurrence can be agreed with the individual patient to allow urinary markers and sonography to guide follow-up investigations. The UroFollow trial, which studies the use of noninvasive marker-based follow-up with standard of care, will provide some answers for patients with pTa G1-2/low-grade NMIBC [76].

In the context of high-grade tumor, it is unlikely that urologists will rely on biomarkers solely (in isolation or in combination) in the near future, and are likely to instead fall on more conventional methods like cystoscopy and cytology. In this subgroup of tumor, the added value of urinary biomarkers is the assessment of tumor aggressiveness to help guide treatment intensification and planning. In the first study investigating the combined use of urine markers to predict aggressiveness, Todenhofer et al. [77] demonstrated that the presence of simultaneously positive urine cytology and NMP22 was associated with a 20-fold risk for G3/CIS. From a genetic perspective, a 12 + 2 gene-set panel based on qRT-PCR developed by Mengual et al. has demonstrated ability to predict tumor aggressiveness. With a sensitivity of 79% and specificity of 91% in voided tumor samples, they devised and validated a panel of molecular markers that could help guide the intensity of a follow-up schedule for patients [78,79]. In aggressive tumor with a higher number of genetic mutations, urinary markers could indicate the need to switch from receiving intravesical therapy to an early cystectomy.

Another area of clinical unmet need is the development of independent prognostic urinary biomarkers. A urinary

biomarker which remains elevated postradical treatment has often been described as a poor prognostic marker but is likely to just reflect the presence of residual disease. Most biomarkers discussed in this study generally increase with stage and grade and can broadly be defined as prognostic. However, few studies have independently studied the relationship between the level of a biomarker and likelihood of relapse or survival. Three studies investigated carcinoembryonic antigen as an independent prognostic marker [80–82] but they were all performed in the 1980s. More recently, single studies have explored the utility of EGFR, EpCAM, BTA, MMP2, Tenascin-C, and Cystatin B as independent prognostic markers [33]. None of these markers have been taken for independent validation. The development of prognostic or predictive markers may influence decision-making in the context of organ-preservation versus radical approaches of treatment. Further, they may help risk-stratify patients to receiving treatment intensification with adjuvant immunotherapy in the future [83].

11. Discussion and conclusion

Urine cytology is useful and remains the current standard for the detection of high-grade tumor. Most of the other available markers are characterized by low positive predictive values that limit their application in routine clinical practice [84]. The FDA-approved biomarkers almost uniformly suffer from high false positive rates as a result of benign inflammatory conditions. While the novel genetic markers have shown initially promising results, the enthusiasm is often dampened by similar shortcomings. For instance, urinary DNA methylation markers produced many false positive results in symptomatic men with sexual infections [85]. This low specificity remains one of the greatest limitations of urine biomarkers in clinical practice.

The UroFollow study, by nature of its multipanel design, will hopefully guide de-intensification of follow-up for low-grade tumor through noninvasive monitoring methods. For high grade tumor, urine cytology (and cystoscopy) is likely to remain common practice in the near future. The question then is how we can best combine the array of available biomarkers, taking into consideration their different utilities and limitations, to help guide surveillance and treatment. A comprehensive systematic review by Tan et al. [86] reinforces that single target assays have limited value regardless of their "-omics" class. Only 4 single target urinary biomarkers achieved a sensitivity and specificity of 90% or more, that is, the protein markers orosomucoid 1 (ORM1) and HtrA1, the epigenetic marker POU Class 4 Homeobox 2, and the transcriptomic marker long noncoding RNA urothelial carcinoma associated-1. There is an increasing appreciation that the use of multitarget biomarkers is increasing and that these biomarkers have better diagnostic performance. At present, despite an expanding field of urinary biomarkers, none of these reported have displaced cystoscopy as the gold standard for diagnosis and surveillance. The lack of field testing, validation studies, diverse thresholds of normal ranges, and complex interplay of different "omics" each present a range of challenges in biomarker development and validation.

Whereas established test systems often employ common features of cell degeneration or proliferation for detection (eg, cytokeratins), modern assays already use BC specific features—though these have to undergo larger studies to validate their utility. Therefore, we propose that the requirements of an optimal BC urine assay include (a) an assay that may detect BC-specific features (exclusive from normal urothelium), (b) expansion of the gold-standard cytology technique with these BC-specific features, thereby combining modern developments while maintaining the important contribution of microscopy.

Conflict of Interest

Regarding the work under consideration for publication, there are no conflicts of interest for any of the authors. Regarding relevant financial activities outside of the submitted work, there are the below declarations: Anand Sharma and Nikhil Vasdev – no conflicts of interests. Kenrick Ng has received honoraria and travel grants from TESARO and GSK. Kenrick Ng is currently funded by Cancer Research UK under a research grant under a Training Fellowship (Award Number 549580). Arnulf Stenzl has received fees from Ipsen Pharma, Janssen, Alere, Bristol-Myers-Squibb, Stebabiotech, Synergo, Ferring, CureVac, Astellas, Amgen, and Sanofi Aventis. Arnulf Stenzl has also received grants from Johnson and Johnson, Roche, Cepheid, Amgen, Bayer AG. Immatics Biotechnologies GmbH, GenomeDX Biosciences, Novartis AG, and Karl Storz AG. Regarding intellectual property, patents and copyrights, there are no conflicts of interest for any of the authors. No authors declared any other relationships or activities that readers could perceive to have influenced, or that give the appearance of potentially influencing, what was written in the submitted work

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