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Prominence of urinary biomarkers for bladder cancer in the COVID-19 era: From the commercially available to new prospective candidates

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Molecular markers detected in urine may improve our understanding of the evolution of bladder cancer (BCa) and its micro- and macroenvironment. Detection of such markers will identify disease earlier, allow stratification of patients according to risk, and improve prognostication and prediction of outcomes, thereby facilitating targeted therapy. However, current guidelines have yet to embrace such markers for routine management of BCa, and most research studies have focused on urine-based tumor markers. In this review, we summarize known urinary biomarkers for BCa and highlight newly identified molecules. We then discuss the challenges that must be overcome to incorporate these markers into clinical care.

Keywords: Biomarkers; DNA; Protein; RNA; Urinary bladder neoplasms

INTRODUCTION

Bladder cancer (BCa) is a common disease worldwide, with high morbidity [1,2]; however, developments in medical technology mean that it is no longer fatal in many cases. Despite this, mortality rates for those with muscle-invasive bladder cancer (MIBC) or those who do not receive optimal treatment remain high [3]. One of the hallmarks of BCa is its heterogeneity, which makes it difficult to manage [4]. This heterogeneity is due to genetic, transcriptomic, epigenetic, and/or phenotypic changes, which result in a molecularly heterogeneous tumor comprising cancer cells with diverse molecular signatures [4,5]. Therefore, tumor heterogeneity is the major barrier to successful management of BCa, including early identification of non-muscle-invasive bladder cancer (NMIBC) or MIBC. Appropriate and early identification will enable suitable treatment planning and assessment of prognosis. Consequently, identification of suitable and reliable tumor biomarkers is essential for diagnosis, prognosis, and treatment planning [6].

Diagnosis and follow-up of BCa are dependent on cystoscopy. This is a highly invasive procedure, which itself can cause complications such as infection or hematuria. Moreover, the high recurrence rate of BCa, along with the frequent requirement for surveillance, place huge economic and quality-of-life burdens on patients [7]. Voided urine cytology (VUC), which has been applied to BCa diagnosis, is a noninvasive option; however, its use is restricted by relatively low

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sensitivity (particularly for diagnosing low-grade tumors); indeed, a previous study reported sensitivity of only 25% [8]. Thus, efforts have been made to explore cost-effective and noninvasive alternatives to cystoscopy. In particular, delivering cancer care during the present COVID-19 pandemic is challenging given the competing risks of cancer-specific death vs. a potentially lethal coronavirus infection. This highlights an urgent need to develop a guide to pragmatic management of BCa.

Clinical decision-making in a pandemic requires a balance between the probable benefits and risks; patients should attend hospitals only when strictly necessary, but care must be given to those most in need. Given this, the European Association of Urology developed guidelines suggesting a traffic-light surveillance pathway based on primary tumor grade and the presence of hematuria [9,10]. This guideline recommends that patients with low-risk or intermediate-risk tumors, and who are asymptomatic, wait a further 6 months for cystoscopy [11]. Although the guidelines are adaptable to the current situation, some patients are happy to defer this often costly and painful process; however, others would rather undergo the procedure quickly than worry about the ambiguity of their disease status.

This period of uncertainty requires timely action and innovation. Urinary biomarkers would enable early detection of cancer, particularly in patients for whom cystoscopy has been deferred in accordance with the up-to-date guidelines. In this review, we summarize the urinary biomarkers used for BCa diagnosis and discuss research and development of new advanced biomarkers.

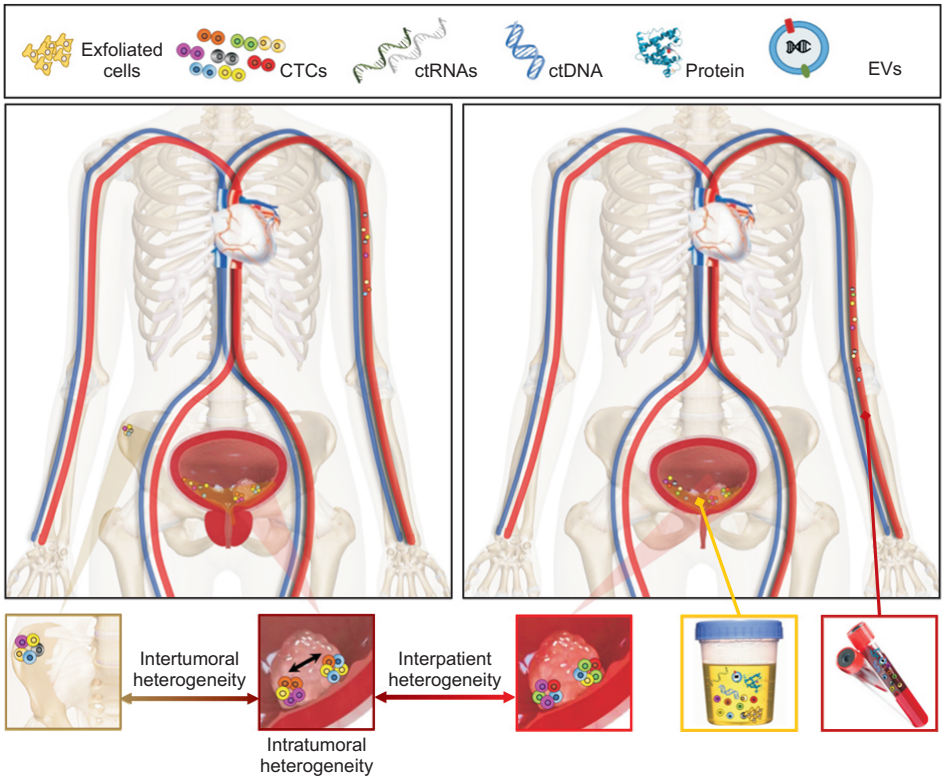
SIGNIFICANCE OF BIOMARKER RESEARCH

1. Bladder cancer markers: what for?

Tumor markers are molecules secreted directly by tumor cells or indirectly by other cells in response to a tumor [12]. Biomarkers can be used for screening, diagnosis, monitoring/surveillance, and prognosis. However, there are no currently accepted biomarkers for BCa screening; therefore, the gold standard tests (a combination of VUC and cystoscopy) are still used for diagnosis in practice [13]. The main role of surveillance markers is to reduce the need for invasive cystoscopy; however, like diagnostic markers, they are not sufficiently reliable for routine clinical use [13,14]. Prognostic markers can be used to stratify patients according to clinical outcome (e.g., recurrence or progression), thereby helping clinicians decide which treatments are most beneficial in a particular case [15]. Consequently, the main goal for those developing biomarkers is to identify relevant molecules or tests that can improve clinical decision-making in a cost-effective way.

2. Biomarker sources: liquid biopsy vs. tissue biopsy

Tissue biopsy is the traditional approach used for cancer diagnosis. The analysis of biopsy samples detects abnormal tumor cells in tumor-like tissue and surrounding tissue. However, this procedure is highly invasive, painful, expensive, and time-consuming. In addition, it requires the intervention of a skilled clinician owing to difficulties in obtaining the right sample for analysis [16]. In addition, the heterogeneity of BCa is a critical limitation in that a tissue biopsy may not always reflect the entire tumor landscape. The tumors within a bladder vary with respect to morphology, gene expression profile, and mutations. This heterogeneity takes several forms: (1) patient-to-patient (interpatient) heterogeneity; (2) spatial region-to-region variation within a tumor in the same patient (intra-tumoral heterogeneity); and (3) tumor-to-tumor variation, including primary tumor to primary tumor, primary tumor to metastatic site, and metastatic site to metastatic site in the same patient (intertumoral heterogeneity) [4]. Consequently, tissue obtained from different areas of the same tumor, from different sites (primary/metastatic) within the same patient, or from different patients may harbor radically different mutations and gene expression patterns [17]. Thus, the use of minimally invasive procedures such as liquid biopsies is gaining traction [18]. Liquid biopsies, which work by measuring circulating tumor-derived material such as circulating tumor cells (CTCs), circulating cell-free tumor DNA (ctDNA), circulating cell-free tumor RNA (ctRNA), proteins, and extracellular vesicles (EVs) in body fluids, have great potential to overcome the limitations inherent to tissue sampling [18]. Because liquid biopsies are minimally invasive, the risk of complications and pain is reduced. Most importantly, liquid biopsies may better represent tumor heterogeneity and allow monitoring of changes in real time. Although liquid biopsies require minimal medical skills and surgical facilities, they still require specialized laboratory equipment and qualified personnel. Another limitation of liquid-biopsy-based research is that it lacks standard protocols, and the low concentrations of materials may complicate interpretation of the results [19]. Nevertheless, the ease of sample collection and the possibility of time-independent analysis are tremendous assets. Ideally, use of reliable urinary biomarkers for BCa will facilitate patient management and could even provide an at-home service. Tissue and liquid biopsies are depicted and



Tissue biopsy	Liquid biopsy
Highly-invasive	Minimal/non-invasive
Painful	Less painful
High risks for complication	Low risks for complication
Time-consuming	Quick & anytime
Need skilled clinicians	Need specialized laboratory equipment & personnel
Influenced by tumor heterogeneity	Represent tumor heterogeneity

Fig. 1. Comparison of tissue and liquid biopsies. Heterogeneity of BCa exists not only between patients (interpatient heterogeneity) but also within the same patient. Intratumoral heterogeneity is caused by variations within regions of the same tumor, whereas intertumoral heterogeneity refers to differences between multiple tumors or metastases within a single patient. Liquid biopsies (which comprise mainly blood and urine) may better represent these heterogeneities. Analysis of liquid biopsies detects alterations in levels of circulating tumor proteins, ctDNA, ctRNA, CTCs, and tumor/normal cell-derived EVs, giving them several advantages over conventional tissue biopsies. BCa, bladder cancer; CTCs, circulating tumor cells; ctDNA, circulating cell-free tumor DNA; ctRNA, circulating cell-free tumor RNA; EVs, extracellular vesicles.

compared in Fig. 1.

URINARY BIOMARKERS FOR BLADDER CANCER

1. Urine as a source of biomarkers

The majority of tumor markers are secreted into blood and can be measured in blood; however, they can also be measured in other types of liquid biopsy (e.g., saliva, urine or seminal plasma, and tissues). Some markers are specific to a single type of cancer, whereas others are associated with several types of cancer [12,20]. Unfortunately, unlike the prostate, which secretes prostate-specific antigen into serum, the bladder secretes no organ-specific markers. The urinary bladder is a hollow muscular organ that stores urine (the capacity is about 300–500 mL). It is a small organ, and most BCa tumors are usually less than several centimeters in size; thus, markers secreted by the tumor may not be easy to detect in blood. However, because bladder tumors are in direct contact with urine, many studies focus on identification of urinary biomarkers of BCa. To date, six urine-based methods have been approved by the U.S. Food and Drug Administration (FDA) for clinical use; however, they must still be used in combination with cystoscopy, and their use remains controversial [21,22]. These FDA-approved markers/tests are the Nuclear matrix protein 22 (NMP22) quantitative and qualitative tests, the bladder tumor antigen (BTA) STAT/TRAK (Polymedco, New York, NY, USA), the ImmunoCyt/uCyt+ assay (DiagnoCure Inc, Quebec, QC, Canada), and the UroVysion bladder cancer kit (Abbott Molecular Inc, Chicago, IL, USA). Most studies of urine-based biomarkers for BCa are of emerging biomarkers to be applied in the future.

2. FDA-approved urinary biomarkers for bladder cancer

NMP is a nonchromatin structure responsible for regulating DNA replication, transcription, and RNA processing [23-25]. Expression of NMP22 is increased in urothelial tu-

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mors, and shedding of apoptotic tumor cells into the urine enables detection of this protein in body fluid. However, NMP22 is also present in normal urothelial cells. NMP22 tests include a quantitative enzyme-linked immunosorbent assay (ELISA; the NMP22 test; Abbott) and a qualitative point-of-care (POC) test (NMP22 BladderChek test; Abbott), which are designed to detect the NMP22 antigen in urine, thereby assisting both BCa diagnosis and monitoring of BCa recurrence. The tests are painless and noninvasive assay and provide a positive or negative result within 30 minutes, and the cost is less than half that of cytology. A previous study showed that the sensitivity of the NMP22 ELISA for primary BCa ranges from 44% to 100%, with a specificity of 60% to 95% [26]. Another study reported that the sensitivity in a cancer cohort (comprising patients with primary and recurrent BCa) was 40% (the sensitivity in the primary and recurrent groups alone was 42% and 34%, respectively). These results are not as good as those for the POC assay, which showed a sensitivity of 59%, 63%, and 48% in these same groups. Both assays showed a specificity of 100% in healthy individuals, while the NMP22 ELISA was 99% specific and the POC test was 93% specific in patients with benign disease [27]. A meta-analysis of 19 studies demonstrated that the pooled sensitivity and specificity of the NMP22 POC test was 56% (95% confidence interval [CI], 52%–59%) and 88% (95% CI, 87%–89%), respectively; indeed, the test showed a good ability to detect BCa in both Asian and White populations [28]. Another study compared the NMP22 biomarker with VUC and found that NMP22 was more sensitive for detecting BCa than VUC, especially among patients with microscopic hematuria (60% vs. 35%). However, the specificity of NMP22 was lower than that of VUC (78% vs. 97%, respectively) owing to the presence of NMP22 in normal urothelial cells [29]. Consequently, false-positive results are common in patients with stones, inflammation, and hematuria [30].

The BTA STAT and BTA TRAK tests target human complement factor-H related protein (hCFHrp), which is found in BCa cells and inhibits the complement cascade to prevent cell lysis. BTA STAT is a qualitative POC immunochromatographic assay, whereas BTA TRAK is a quantitative ELISA. Both have been approved by the FDA for monitoring BCa recurrence, but only as adjuncts to cystoscopy. These tests are more sensitive than VUC; a meta-analysis showed that the sensitivities of the POC and ELISA tests are 64% (95% CI, 58%–69%) and 65% (95% CI, 54%–75%), respectively [31], with specificities of 77% (95% CI, 73%–81%) and 74% (95% CI, 64%–82%), respectively. Generally, the sensitivity of the POC test ranges from 57% to 82%, with a specificity of 68% to 93% [32-34], whereas the ELISA has a sensitivity of 66% to 77% and a specificity of 50% to 75% [35,36]. However, similar to NMP22, the BTA assay also exhibits a higher false-positive rate in patients with hematuria, urolithiasis, inflammation, and other genitourinary malignancies, and in those undergoing intravesical bacille Calmette–Guérin (BCG) therapy [37].

The UroVysion bladder cancer kit is a multicolor fluorescent *in situ* hybridization (FISH) assay designed to estimate aneuploidy of chromosomes 3, 7, and 17, or loss of the 9p21 locus. Its performance with respect to diagnosis and surveillance for BCa has been approved by the FDA. The sensitivity of this test ranges from 69% to 87%, with a specificity between 89% and 96% [38,39]. Similarly, the sensitivity and specificity from a recent meta-analysis (11 studies) was 63% (95% CI, 50%–75%) and 87% (95% CI, 79%–93%) [31]. Another study showed that this kit detected almost twice as many NMIBC tumors as VUC, and identified 88% of invasive tumors (32% of these tumors were missed by VUC) [40]. This test is superior to the NMP22 and BTA tests owing to its high specificity. The assay is not affected by hematuria, inflammation, or other conditions that may result in false-positive readings. Thus, it could be used as an adjunct to VUC, thereby increasing sensitivity while maintaining specificity [41]. Moreover, a preponderance of evidence suggests a role for the UroVysion test for predicting responses to intravesical immunotherapy and BCG treatment [42,43].

The ImmunoCyt assay (also marketed as uCyt+) uses three fluorescently labeled monoclonal antibodies to detect high-molecular-weight forms of carcinoembryonic antigen (CEA) and two bladder tumor cell-associated mucins (LDq10 and M344) that are expressed on urothelial cells shed by tumors. This is the only commercially available test that can be used for BCa follow-up. This fluorescent test has an overall sensitivity of 40% to 100% and a specificity of 62% to 84% [44-49]. A previous case series reported a sensitivity of 74% to 87% and a specificity of 62% to 78% [50], whereas a meta-analysis of 14 studies reported a sensitivity of 78% (95% CI, 68%–85%) and a specificity of 78% (95% CI, 72%–82%) [31]. Another meta-analysis based on data from seven studies reported a pooled sensitivity and specificity of 72.5% (95% CI, 68.3%–76.5%) and 65.7% (95% CI, 62.9%–68.5%), respectively, while the pooled sensitivity and specificity of cytology were 56.6% (95% CI, 52.1%–61.1%) and 90.6% (95% CI, 88.7%–92.3%), respectively [51]. A key advantage of the ImmunoCyt fluorescent test over the NMP22, BTA, and UroVysion assays is the great improvement in sensitivity for low-grade tumors; indeed, the sensitivity increases from 63% for pTa tumors to 80% for pT1 tumors [37]. The sensitivity of the NMP22, BTA, and UroVysion assays for low-grade tumors is poor [30,52,53].

In common with other protein-based assays, the ImmunoCyt fluorescent test is also significantly affected by urinary tract infections, urolithiasis, and benign prostate hyperplasia, which may lead to false-positive results. The detailed characteristics of these assays are described in Table 1.

3. Non-FDA-approved urinary biomarkers for bladder cancer

Here, we summarize proposed DNA-based, RNA-based, and proteomic/peptidomic markers of BCa.

1) DNA-based urinary biomarkers

DNA tests used for surveillance usually detect loss of heterozygosity, gene methylations, and mutations in tumor cells. DNA methylation and mutations are important for the etiology and pathogenesis of many cancers [54-56], including BCa, which is a highly heterogenetic disease [57]. Thus, recent studies have explored molecular classification of both NMIBC and MIBC based on these alterations [58,59]. Alterations in DNA methylation patterns are hallmarks of cancer. Hypomethylation events may result in abnormal activation of genes, which are commonly repressed by DNA methylation. However, hypermethylation of CpG dinucleotides in the promoter regions of tumor suppressor genes can inhibit their transcription in human cells, which gives cancer cells tremendous benefits [59,60]. Thus, methylation status is one of the most studied biomarkers in the follow-up scenario; it is also used to predict treatment responses because it is both chemically stable and quantifiable in liquid biopsies [61].

The Bladder EpiCheck urine test is an *in vitro* diagnostic device produced by Nucleix, Ltd (San Diego, CA, USA). The test analyzes a panel of 15 DNA methylation patterns to detect BCa and has a CE mark, meaning that it is available commercially in Europe. It is effective for monitoring BCa recurrence, thereby minimizing the need for invasive cystoscopy [62]. A validation study of 222 NMIBC patients undergoing surveillance showed 90% sensitivity, 83% specificity, and a negative predictive value (NPV) of 97% [63]. In another study, Witjes et al. [62] designed a blinded, single-arm, prospective multicenter study to evaluate the performance of the EpiCheck urine test for detecting NMIBC recurrence. The overall sensitivity, specificity, and NPV were 68.2% (95% CI, 52.4%–81.4%), 88.0% (95% CI, 83.9%–91.4%), and 95.1% (95% CI, 91.9%–97.3%), respectively. Remarkably, the test could discriminate the absence of high-grade NMIBC with an NPV of 99%; by contrast, it detected the presence of high-grade NMIBC with a sensitivity of 92% [62]. D'Andrea et al. [64] published another multicenter and independent study based on data from 357 NMIBC patients. They showed that the urine test had an overall sensitivity of 67% (95% CI, 52%–80%), a specificity of 88% (95% CI, 84%–91%), and an NPV of 94.4% (95% CI, 91%–97%). The sensitivity, specificity, and NPV for high-grade and low-grade cancers were 89% (95% CI, 65%–99%) vs. 40% (95% CI, 19%–64%); 88% (95% CI, 84%–91%) vs. 88% (95% CI, 84%–91%); and 99% (95% CI, 97%–100%) vs. 96% (95% CI, 93%–98%), respectively [64]. Such consistent results make this urine test an attractive choice for use in clinical decision-making. The high NPV means that clinicians can have high confidence that a negative result rules out tumor recurrence. Accordingly, application of this test could reduce the current burden of repeat cystoscopy and cytology tests. Moreover, the results are consistent under the presence of inflammation in the urinary tract. However, the test is not simple to perform because a dedicated technician and an equipped laboratory are needed; in addition, it is expensive [62,65].

Recently, Nucleix announced the launch of its BE Safe @Home project, which provides an informatics service for NMIBC patients under surveillance with the Bladder EpiCheck urine test. In consultation with world-leading urologists, the project was implemented in Israel, Spain, and the Netherlands with a view to making surveillance more convenient during the COVID-19 pandemic and beyond.

Other markers related to methylation status are also used to follow-up BCa. Zuiverloon et al. [66] developed a methylation detection assay (based on voided urine) for specific detection of recurrence in patients with NMIBC. A logistic regression model based on methylation of a four-gene panel that combines the *APC_a* (APC regulator of WNT signaling pathway), *TERT_a* (telomerase reverse transcriptase), *TERT_b*, and *EDNRB* (endothelin receptor type B) genes correlated with BCa recurrence, providing a sensitivity and specificity of 63.3% and 58.3%, respectively, in the test cohort, and of 72.3% and 55.2%, respectively, in the validation cohort [66]. Another study showed a considerably higher sensitivity and specificity (80% and 97%, respectively) using a model based on hypermethylation of *SOX1* (SRY-box transcription factor 1) and *IRAK3* (interleukin 1 receptor-associated kinase 3) and hypomethylation of a specific LINE1 element in *MET* (mesenchymal epithelial transition) in urine from BCa patients [67]. Methylation of *TWIST1* (twist homolog 1) and *NID2* (nidogen 2) is linked to BCa [68-70]. One study showed that under adjusted thresholds, methylation of *TWIST1* and *NID2* has a sensitivity and specificity of 75% and 71%, respectively [69]. More recently, a multi-institutional study reported comparable results using these two methylated genes; this study showed a sensitivity of 58% to 67% and a specificity of 61% to 69%. However, the article noted that prior BCG

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Table 1. FDA-approved urinary biomarkers

Test name	Biomarker	Assay	Assessed substance	Purpose	Sensitivity (%)	Specificity (%)	Reference	Advantages	Limitations
NMP22 test (Abbott)	NMP22	Quantitative test: ELISA	Peptides	Follow-up	44–100 34–40	60–95 99–100	[26] [27]	Noninvasive Higher sensitivity than VUC	Low sensitivity in low-grade tumor Lower specificity than VUC High false-positive rate Need trained laboratory technicians
NMP22 BladderChek (Abbott)	NMP22	Qualitative test: POC	Peptides	Diagnosis, follow-up	48–63 52–59 60	93–100 87–89 78	[27] [28] [29]	Noninvasive Quick Higher sensitivity than VUC	Low sensitivity in low-grade tumor Lower specificity than VUC High false-positive rate
BTA TRAK (Polymedco)	hCFHrp	Quantitative test: ELISA	Proteins	Diagnosis, follow-up	54–75 66–77	64–82 50–75	[31] [35,36]	Noninvasive Higher sensitivity than VUC	Low sensitivity in low-grade tumor Lower specificity than VUC High false-positive rate Need trained laboratory technicians
BTA STAT (Polymedco)	hCFHrp	Qualitative test: POC	Proteins	Diagnosis, follow-up	58–69 57–82	73–81 68–93	[31] [35,36]	Noninvasive Higher sensitivity than VUC	Low sensitivity in low-grade tumor Lower specificity than VUC High false-positive rate
UroVysion (Abbott)	Chromosomes 3, 7, and 17, and 9p21	FISH	DNA (aneuploidies)	Diagnosis, follow-up	69–87 50–75	89–96 79–93	[38,39] [31]	Noninvasive Higher sensitivity than VUC High specificity	Low sensitivity in low-grade tumor Need trained laboratory technicians
ImmunoCyt/uCyt+ (DiagnoCure)	CEA, LDq10, and M344	Immunofluorescence	Antigens/metabolites	Diagnosis, follow-up	40–100 74–87 68–85 68.3–76.5	62–84 62–78 72–82 62.9–68.5	[44-49] [50] [31] [51]	Noninvasive Higher sensitivity than VUC (especially in low-grade tumor)	Lower specificity than VUC High false-positive rate Need trained laboratory technicians

BTA, bladder tumor antigen; CEA, carcinoembryonic antigen; ELISA, enzyme-linked immunosorbent assay; FDA, U.S. Food and Drug Administration; FISH, fluorescent *in situ* hybridization; hCFHrp, human complement factor-H related protein; NMP, nuclear matrix protein; POC, point-of-care; VUC, voided urine cytology.

treatment for NMIBC reduced the accuracy [70]. These findings are promising, despite the limited sensitivity and specificity. However, these markers were identified by research institutions with limited cohorts; thus, large validation tests and methodologic improvements are needed to achieve more accurate results.

Gene mutations are related to carcinogenesis of BCa. *FGFR3* (fibroblast growth factor receptor 3) is one of the most studied genes; mutations in this gene are found in over 80% of patients with low-grade BCa and are related to a good prognosis [71]. Another well studied gene is *TERT* (telomerase reverse transcriptase), which has been investigated as a prognostic marker for NMIBC recurrence [72,73]. Allory et al. [74] found that the sensitivity of detecting NMIBC relapse was 19% for *FGFR3*, 42% for *TERT*, and 50% for *FGFR3* and *TERT* combined; for comparison, the specificities of mutations in *TERT*, *FGFR3*, and a combination of the two, were 73%, 90%, and 71%, respectively.

The Uromonitor urine-based test (Uromonitor, Porto, Portugal) is an ultra-sensitive assay capable of detecting trace amounts of *TERT* promoter and *FGFR3* mutations in tumor cells shed into urine [75]. Screening of targeted alterations is based on a highly sensitive multiplex competitive allele-specific discrimination PCR that allows clear interpretation of results. Compared with Sanger sequencing, this test can detect a very small number of altered cells in a large pool of unaltered cells. In addition, it is superior to next generation sequencing (NGS)-based assays in terms of cost and time. A multicenter validation study revealed that the Uromonitor urine-based test has a sensitivity of 73.5% and a specificity of 73.2% for detection of NMIBC recurrence [76]. Higher sensitivity and specificity were reported in a study based on a cohort of 72 patients first diagnosed with BCa and under surveillance for NMIBC; the data suggest that the sensitivity, specificity, and NPV of the Uromonitor urine-based test are 100%, 96.3%, and 88.9%, respectively [75]. Concerning low-grade and high-grade recurrence-positive patients, the test showed a detection rate of 62.5% and 75%, respectively. Also, the presence of inflammation or other benign lesions in the urinary tract did not affect results. Thus, routine use of this urine-based test plus cystoscopy could be very cost-effective. Indeed, the sensitivity and specificity are similar to those of cystoscopy (which were 79.4% and 73.2%, respectively) in one study, which suggests that the Uromonitor urine-based test is an appropriate option when cystoscopy cannot be performed or is not available routinely [76].

The Uromonitor-V2 urine-based assay (Uromonitor) added the *KRAS* (kirsten rat sarcoma 2 viral oncogene homolog) hotspot mutation to the Uromonitor kit. The Uromonitor-V2 assay showed 100% sensitivity, 83.3% specificity, and an NPV of 100% for evaluating NMIBC recurrence in a multicenter study of 122 patients [76]. Another recent study reported a sensitivity, specificity, and NPV of 93.1%, 85.4%, and 95.3%, respectively, for detecting BCa recurrence, whereas VUC showed a sensitivity, specificity, and NPV of 26.3%, 90.9%, and 68.2%, respectively. Thus, the Uromonitor-V2 urine-based assay is a promising test for surveillance of BCa [77].

The UroSEEK urine-based molecular assay is another noninvasive commercially available test (although not approved by the FDA or the European organization). The assay is designed to detect alterations in 11 genes, including 10 typical mutations associated with BCa (*FGFR3*, *TERTp*, *TP53* [tumor protein p53], *ERBB2* [erb-b2 receptor tyrosine kinase 2], *CDKN2A* [cyclin-dependent kinase inhibitor 2A], *KRAS*, *HRAS* [v-ha-ras harvey rat sarcoma viral oncogene homolog], *MET*, *PIK3CA* [phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha], *MLL* [mixed-lineage leukemia 1], and *VHL* [von Hippel-Lindau tumor suppressor]) plus detection of aneuploidy [78]. Springer et al. [78] found that this test could detect recurrence with a sensitivity of 68% and a specificity of 80%. Another study revealed that in the setting of early detection, the sensitivity and specificity were 96% and 88%, respectively, with an NPV of 99%; however, the results from a surveillance cohort were less robust (sensitivity of 74%, specificity of 72%, and NPV of 53%) [79]. Nevertheless, the UroSEEK molecular assay was more sensitive than cytology both in the surveillance cohort (71% vs. 25%, respectively) and in the primary detection cohort (95% vs. 43%, respectively); however, the specificity of cytology was superior in the detection cohort (100% vs. 93%) [78]. Accordingly, this test does not show excellent performance for the follow-up of patients with a prior diagnosis of BCa.

All these findings strongly suggest that mutations or methylation status of several genes are promising biomarkers for BCa; thus, a combination of genetic and epigenetic markers for BCa diagnosis and surveillance is both logical and appealing.

The combination of *FGFR3* mutations and methylation biomarkers has been tested, with promising results. Beukers et al. [80] investigated the performance of *FGFR3* and *TERT* mutations combined with *OTX1* (orthodenticle homeobox 1) methylation in 977 patients with NMIBC. They reported that the sensitivity for detecting NMIBC recurrence was 57%, with a specificity of 59% [80]. Similarly, a 3-plex methylation (combination of *OTX1*, *ONECUT2* [one cut homeobox 2], and *OSR1* [odd-skipped related transcription factor 1] methylation) assay combined with the *FGFR3* mutation assay detects recurrent NMIBC in voided urine with a sen-

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sitivity of 79% and a specificity of 77% [81]. Another study combined *FGFR3* mutation with methylation of a set of DNA markers (*HS3ST2* [heparan sulfate-glucosamine 3-sulfotransferase 2], *SEPTIN9*, and *SLIT2* [slit guidance ligand 2]) and reported a sensitivity of 94.5% for discriminating recurrent tumors. The specificity and NPV for this assay were 75.9% and 98.5%, respectively [82]. Recently, MDxHealth SA (Euronext: MDXH.BR; Herstal, Belgium) announced the commercial launch of its AssureMDx laboratory-based test in the United States, which combines methylation (*OTX1*, *ONECUT2*, and *TWIST1*) and mutation (*FGFR3*, *TERT*, and *HRAS*) biomarkers to identify BCa in patients with hematuria [83]. A multicenter study verified a sensitivity of 93% and a specificity of 86% for BCa diagnosis [84].

Thus, combined analyses of DNA mutations and DNA methylation markers could be used for risk stratification of patients with BCa and for surveillance, forming the foundation for a promising noninvasive urine test.

2) RNA-based urinary biomarkers

RNA-based urinary biomarkers are less well studied than DNA markers. One commercially available RNA test is the Cxbladder Monitor (Pacific Edge Diagnostics, Dunedin, New Zealand). This test evaluates the expression of five urinary mRNAs (insulin-like growth factor binding protein 5 [IGFBP5], homeobox protein Hox-A13 [HOXA13], midkine [MDK], cyclin-dependent kinase 1 [CDK1], and chemokine receptor type 2 [CXCR2]) and incorporates them into a mathematical algorithm that also includes clinical variables, such as primary vs. recurrent BCa and time since tumor occurrence, to generate a score to give a positive or negative result [85]. Lotan et al. [85] compared the Cxbladder Monitor with current FDA-approved urine tests; they examined 1,036 urine samples from 803 patients undergoing surveillance for recurrent BCa and found that the Cxbladder Monitor test significantly outperformed the other tests. The Cxbladder Monitor showed a sensitivity of 91% (95% CI, 86%–95%) when monitoring a BCa population. This is obviously higher than that of other tests: 22% for cytology, 26% for the NMP22 ELISA test, and 11% for NMP22 BladderChek. The NPV of the Cxbladder Monitor was also superior (96%) to that of cytology (87%), the NMP22 ELISA (87%), and NMP22 BladderChek (86%) [85]. In addition, the Cxbladder Monitor showed a sensitivity of 93% (95% CI, 85%–97%) and an NPV of 94% (95% CI, 88%–97%) in patients undergoing routine surveillance for recurrent BCa [85,86]. By contrast, cytology, the NMP22 ELISA, and NMP22 BladderChek showed sensitivities of 22%, 29%, and 8%, and NPVs of 83%, 83%, and 81%, respectively. After seeing the evidence, New Zealand's public healthcare providers have integrated the Cxbladder Monitor into their routine clinical surveillance of BCa patients. A recent study demonstrated that the Cxbladder Monitor accurately detected about 77.8% of recurrence-free patients per year who could avoid unnecessary cystoscopy [87]. During the COVID-19 lockdown, two of New Zealand's public healthcare providers started using the Cxbladder Monitor for in-home testing as an out-patient solution for BCa monitoring; this is especially useful for older patients at high risk for COVID-19.

Another commercially available RNA test is the Xpert Bladder Cancer Monitor, which measures five mRNAs (v-abl Abelson murine leukemia viral oncogene homolog 1 [ABL1], corticotropin-releasing hormone [CRH], insulin-like growth factor 2 [IGF2], uroplakin 1B [UPK1B], and annexin A10 [ANXA10]) that are frequently overexpressed in BCa. This test provides qualitative monitoring of BCa recurrence within 90 minutes. Wallace et al. [88] developed this urine-based test using 450 urine specimens collected from 18 multinational sites and obtained an overall sensitivity of 73%, with specificities of 90% and 77% in hematuria and surveillance patient populations, respectively. In another study, Pichler et al. [89] examined 140 patients with a history of NMIBC who were undergoing routine surveillance and reported for the first time that the Xpert Bladder Cancer Monitor outperforms VUC in terms of sensitivity (84% vs. 33%, respectively) and NPV (93% vs. 76%, respectively), even in those with low-grade and Ta tumors; however, the specificity of the two tests was similar (91% vs. 94%, respectively). Another multicenter study compared the Xpert Bladder Cancer Monitor, VUC, and the UroVysion bladder cancer kit to determine their follow-up performance in patients previously diagnosed with NMIBC. The Xpert Bladder Cancer Monitor showed a higher sensitivity and NPV (74% [95% CI, 94%–99%] and 93% [95% CI, 89%–96%]), respectively, than did VUC or the UroVysion bladder cancer kit. Moreover, the sensitivity and NPV for high-grade tumors were 83% (95% CI, 64%–93%) and 98% (95% CI, 94%–99%), respectively [90]. The improved NPV of this test in patients under follow-up for BCa suggests that the Xpert Bladder Cancer Monitor is a promising tool for excluding BCa and reducing the need for cystoscopy. However, D'Elia et al. [91] indicated a lower overall sensitivity: 46.2% for detecting NMIBC recurrence. In addition, results from the Bladder Cancer Italian Active Surveillance project, which enrolled 106 patients with low-grade NMIBC who developed recurrence during follow-up and underwent active surveillance, suggest the need to optimize the cutoff value [92]. Thus, further research on larger populations is mandatory before this test can be used routinely in clinical

practice.

In addition to these mRNA-based biomarkers, a number of urinary microRNA (miRNA) biomarkers are emerging. miRNAs interact with their target mRNAs to modulate their expression, thereby controlling many physiologic processes, including carcinogenesis [93]. Most miRNA-based studies have focused on the diagnostic performance of the miRNAs (one special miRNA or miRNA panels) that are differentially expressed in BCa urine [8,94,95]. As a prognostic marker for predicting NMIBC recurrence, Kim et al. [96] found that urinary *miR-214* was down-regulated in NMIBC patients who experienced recurrence during surveillance, with a hazard ratio of 2.011 (95% CI, 1.027–3.937), when compared with those without recurrence. Sapre et al. [97] examined the potential of a urinary miRNA panel for predicting the presence of BCa in NMIBC patients undergoing surveillance. They found that a combination of six miRNAs (*miR16*, *miR200c*, *miR205*, *miR21*, *miR221*, and *miR34a*) yielded an area under the curve (AUC) of 0.85 for distinguishing NMIBC patients with recurrence from those without in the discovery cohort, and they showed high sensitivity (88%) and adequate specificity (48%) (AUC=0.74) in the validation cohort; these data suggest that cystoscopy rates in the validation cohort would have been reduced by 30%.

Lately, another group of noncoding RNAs, named long noncoding RNAs (lncRNAs), which are longer than miRNAs, have emerged as an informative tool for the management of BCa [98]. The advent of high-throughput technology, such as RNA-seq, has identified more than 10,000 unique lncRNAs and clarified their biological functions. lncRNAs play a crucial role in BCa tumorigenesis by modulating cellular pathways involved in cell transformation [99-101]. Zhang et al. [102] investigated the potential application of an lncRNA called urothelial cancer associated 1 (UCA1), which is found in BCa patients' urine. They identified that UCA1 showed high sensitivity and specificity (84.4% and 92.4%, respectively) for BCa (AUC=0.898). Moreover, the study highlighted the role of UCA1 as a prognostic biomarker for NMIBC patients who may progress to MIBC (sensitivity, 86.4%; specificity, 92.3%) [102]. Similarly, Eissa et al. [103] showed that UCA1 has great sensitivity (91.5%) and specificity (96.5%) for detecting BCa. Indeed, urinary UCA1 was more accurate than VUC in NMIBC patients [103]. Therefore, accumulation of UCA1 in urine may be a prospective marker for BCa diagnosis and surveillance. HOX antisense intergenic RNA (HOTAIR) is a recently discovered lncRNA that plays an important role in BCa. A study revealed that HOTAIR expression has prognostic value for BCa progression, recurrence, and survival [104]; recurrent NMIBC tumors showed significantly higher HOTAIR expression than did nonrecurrent tumors. Kaplan–Meier analysis revealed that patients with higher HOTAIR expression exhibited significantly earlier recurrence and earlier progression after recurrence. Another pilot study detected lncRNA H19 in urine sediment from 90.5% of BCa patients and 25.9% of healthy controls, making it a supplemental tool for BCa diagnosis [105]. Although expression of other lncRNAs correlates with BCa, most studies were based on tissues or cell lines; therefore, the results require validation in urine samples to confirm their practical applicability as noninvasive BCa biomarkers [106-108]. In addition, urine-based research mostly relies on urine sediment, and different lncRNA targets are detected in urine cells. Thus, results may vary because of the paucity of genitourinary-derived cells in urine [109,110], the presence of urinary crystals [111], and the concentration of inhibitors in urine sediment [112,113]. A recent study reported a 16 cell-free urinary lncRNA-based panel, which showed differential expression between NMIBC and urocystitis patients [114]. Among them, the AUCs for four biomarkers (UCA1-201, HOTAIR, HYMA1, and metastasis associated lung adenocarcinoma transcript 1 [MALAT1]) were higher than 0.80, suggesting superior diagnostic performance in differentiating NMIBC from urocystitis. Next, machine learning was used to train the four lncRNA panel as a predictive model; the panel made good predictions in the validation phase, showing a sensitivity of 93.3% and a specificity of 96.7% for discriminating NMIBC from urocystitis.

3) Proteomic/peptidomic urinary biomarkers

The UBC urinary bladder cancer test (IDL Biotech, Borläbger, Sweden) is a commercially available test that measures soluble fragments of cytokeratin 8 and 18 (CK8 and CK18) in urine samples for the purpose of diagnosis and monitoring of BCa. The UBC test is available in ELISA or POC formats. The sensitivity and specificity vary from 12% to 88% and from 77% to 92%, respectively [115]. Pichler et al. [116] found a sensitivity of 61.3% and 64.5%, and a specificity of 77.3% and 81.8%, for the qualitative and quantitative UBC tests, respectively; thus, the assay is more sensitive than VUC (sensitivity, 25.8%) and NMP22 (sensitivity, 12.9%). A multicenter study demonstrated another interesting outcome: the sensitivity for high-grade NMIBC was greater than that for low-grade NMIBC (75.0% vs. 38.8%, respectively), suggesting the potential of UBC as a clinically valuable urinary protein biomarker for detection of high-grade NMIBC [117]. Babjuk et al. [118] reported that the sensitivity and specificity of the UBC ELISA test were 12.1% and 97.2%, respectively, for detecting NMIBC in patients under

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surveillance. However, when setting a new cutoff to reach a sensitivity of 90%, the specificity declined to 20.4%, indicating that individually installed cutoffs are of no benefit [118]. Concerning the issue of an appropriate cutoff for the UBC test, a study compared the uncorrected and corrected cutoff values of the UBC test for distinguishing BCa patients with and without a recurrence. The overall sensitivity, specificity, and NPV of the uncorrected UBC test were 20.7%, 84.7%, and 72.6%, respectively; those for the corrected UBC test were 20.7%, 79.2%, and 71.3%, respectively. Nonetheless, the receiver operating characteristic analyses showed no statistical significance (both $p>0.05$), indicating that the UBC test has no diagnostic value [119]. In contrast to other markers, the UBC test is rapid, with results available within 10 minutes; however, the clinical utility of the UBC test for follow-up of BCa patients remains unconvincing.

The ADXBLADDER *in vitro* diagnostic test (Arquer Diagnostics, Sunderland, UK) is an up-to-date commercial urine test that detects mini-chromosome maintenance complex component 5 (MCM5), which is a marker of cells that are replicating (or that still have the capability to replicate). In July 2020, the UK National Health Service approved the use of the ADXBLADDER test to help with diagnosis and surveillance of BCa [11]. The test was superior to VUC for detecting BCa recurrence [120]. The ADXBLADDER MCM5 test has sensitivities of 44.1% and 58.8% for low- and high-grade recurrence, respectively, which is more accurate than VUC (sensitivity of 17.6% for both low-grade and high-grade recurrence); thus, this test could be a reliable alternative to VUC for follow-up monitoring. Similar results were reported in a multicenter prospective, blinded study carried out from August 2017 to July 2019 at 21 European Union centers. The study demonstrated that the ADXBLADDER test excluded the presence of high-grade recurrence in 97.8% of cases (compared with 97.1% with VUC). Meanwhile, the sensitivity of the test was 51.9%, which was much higher than that of VUC (16.7%) [121]. The test demonstrated an impressive NPV of 92% to 99%, using a standard ELISA and with a rapid 2-hour turnaround time. Despite having advantages over VUC, the overall performance of the ADXBLADDER test remains relatively low. The clinical implementation of these biomarkers for the follow-up of BCa must be investigated further in prospective randomized trials in patients with low-grade as well as high-grade tumors.

Cytokeratin fragment 21.1 (CYFRA 21.1) is an ELISA-based assay that measures the concentration of a soluble fragment of cytokeratin 19, the levels of which in urine samples differ between healthy persons and those with BCa (sensitivity, 82%; specificity, 80%) [122]. An extensive meta-analysis study reported not only that the CYFRA 21.1 level has diagnostic value for BCa, but also that CYFRA 21.1 levels are higher in those with metastatic BCa than in those with locally invasive disease, inferring a role for detecting metastases [123]. Moreover, Nisman et al. [124] showed that CYFRA 21.1 detected 100% of carcinoma *in situ* cases, 92.8% of MIBC cases, and 91.9% of grade 3 tumors; also, the assay detected 65% of recurrent tumors and 71% of primary tumors missed by VUC. Unfortunately, like other protein markers in urine, CYFRA 21.1 has a high false-positive rate in patients with urinary tract infections, stones, or a history of pelvic radiotherapy, urethral catheterization, or BCG intravesical instillation within the 3 previous months.

The characteristics of non-FDA-approved but commercially available biomarkers are shown in Table 2.

4. Limitations of current urinary biomarkers

Although newly developed molecular biomarkers for BCa management show superior sensitivity to VUC, few are included in clinical guidelines. Most studies concur that the currently FDA-approved biomarkers do not perform well in real clinical practice as they lack sensitivity or specificity for detecting BCa, or the studies suffered from high false-positive rates. Indeed, a systemic meta-analysis of 57 studies concluded that the false-positive rates of FDA-approved markers range from 44% to 78% [31]. This review also showed that these urinary biomarkers missed 18% to 43% of patients with BCa. These poor performances suggest that limited sensitivity, specificity, and high false-positive rates are the greatest challenge to application of urinary biomarkers in clinical practice.

Most studies of these markers reported "promising" results and initially "positive" observations, which may contribute to early clinical application. However, these preliminary findings lose their glory when tested in real clinical practice; such differences in performance may be related to study design (i.e., selection of suitable cohorts, sufficient number to achieve statistical significance, inadequate follow-up, and poor validation of results). Moreover, the definition of an ideal marker contains the words *easier*, *faster*, *better*, and *cheaper* [125]. *Easier* allows performance in a clinical environment; *faster* means a rapid turnaround; *better* means that it must at least be equal to currently clinically available alternatives and provide information that is helpful to clinicians with respect to management of the disease; and *cheaper* means a reduced economic burden on patients and health services. Nevertheless, many urine tests require highly skilled personnel and specialized laboratory equipment, which increases both time and cost. Initially, the cost of

Table 2. Commercially available (non-FDA-approved) urine tests for bladder cancer

Test name	Biomarker	Assay	Assessed substances	Purpose	Sensitivity (%)	Specificity (%)	NPV	References	Advantages	Limitations
DNA-based assays										
Bladder EpiCheck (Nucleix)	15 DNA methylation	Real-time PCR	Urinary tumor DNA	Follow-up	Overall: 67–90 High-grade: 89–92 Low-grade: 40	Overall: 83–88 High-grade: 88 Low-grade: 88	Overall: 94.4–97 High-grade: 99 Low-grade: 96	[62-64]	Noninvasive Higher sensitivity than VUC High NPV Provide in-home testing service	Low sensitivity in low-grade tumor Lower specificity than VUC High false-positive rates Need a dedicated technician and an equipped laboratory
Uromonitor (Uromonitor)	DNA[a] mutations	Real-time PCR	Urinary tumor DNA	Follow-up	Overall: 73.5–100 High-grade: 75 Low-grade: 62.5	Overall: 73.2–96.3	Overall: 88.9	[76,77]	Noninvasive Higher sensitivity than VUC High NPV Lower false-positives than other tests	Low sensitivity in low-grade tumor Lower specificity than VUC Need a dedicated technician and an equipped laboratory
Uromonitor-V2 (Uromonitor)	DNA[a] mutations	Real-time PCR	Urinary tumor DNA	Follow-up	Overall: 93.1–100	Overall: 83.3–85.4	Overall: 95.3–100	[77,78]	Noninvasive Higher sensitivity than VUC High NPV No confusions from other benign diseases	Need a dedicated technician and an equipped laboratory
UroSEEK	10 gene mutations[b]	NGS	Urinary tumor DNA	Diagnosis, follow-up	95–96 68–74	88–100 72–80	99 53	[79,80]	Noninvasive Higher sensitivity than VUC (especially for early detection of BCa) High specificity and NPV for BCa early detection	Lower sensitivity, specificity and NPV for BCa follow-up surveillance Need a dedicated technician and an equipped laboratory Complicated methods
AssureMDx (MDxHealth)	DNA[c] methylation+ DNA[d] mutation	Real-time PCR	Urinary tumor DNA	Diagnosis	93	86	NA	[85]	Noninvasive Higher sensitivity than VUC	Need more validation results Need a dedicated technician and an equipped laboratory
RNA-based assays										
Cx Bladder Monitor (Pacific Edge Diagnostics)	5 mRNA[e] expression	Real-time PCR	Urinary tumor RNA	Diagnosis, follow-up	91 93	85	96 94	[86,87]	Noninvasive Higher sensitivity Higher NPV Provide in-home testing service	Need more validation results Need a dedicated technician and an equipped laboratory
Xpert Bladder Cancer Monitor	5 mRNA[f] expression	Real-time PCR	Urinary tumor RNA	Follow-up	Overall: 46.2–84 High-grade: 83	90–91	Overall: 77–93 High-grade: 98	[89-92]	Noninvasive Qualitative monitoring of BCa recurrence in 90 minutes Higher sensitivity than VUC (Especially in high-grade tumor)	Need an optimization of its cutoff Need further research on larger population

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Table 2. Continued

Test name	Biomarker	Assay	Assessed substances	Purpose	Sensitivity (%)	Specificity (%)	NPV	References	Advantages	Limitations
Proteomic/Peptidomic assays										
UBC Rapid and ELISA (IDL Biotech)	CK8, CK18	POC/ELISA	Peptide	Diagnosis, follow-up	12–88 12.1	77–92 97.2	NA	[117,119]	Noninvasive Quick test in 10 minutes	Need an optimization of its cutoff Need further research on larger population Clinical utility in the follow-up needs more convincing results
ADXBLADDER (Arquer Diagnostics)	MCM5	ELISA	Peptide	Diagnosis, follow-up	High-grade: 51.9–58.8 Low-grade: 44.1	NA	92–99	[121,122]	Noninvasive Superior to VUC for detecting recurrence of BCa No confusions from other benign diseases	Inadequate sensitivity for clinical utility Need further research on larger population

ABL1, v-abl Abelson murine leukemia viral oncogene homolog 1; *ANXA10*, annexin A10; BCa, bladder cancer; *CDK1*, cyclin-dependent kinase 1; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; *CRH*, corticotropin-releasing hormone; *CXCR2*, chemokine receptor type 2; ELISA, enzyme-linked immunosorbent assay; *ERBB2*, erb-B2 receptor tyrosine kinase 2; FDA, U.S. Food and Drug Administration; *FGFR3*, fibroblast growth factor receptor 3; *HOXA13*, homeobox protein Hox-A13; *HRAS*, v-ha-ras harvey rat sarcoma viral oncogene homolog; *IGF2*, insulin-like growth factor 2; *IGFBP5*, insulin-like growth factor binding protein 5; *KRAS*, kirsten rat sarcoma 2 viral oncogene homolog; MCM5, mini-chromosome maintenance complex component 5; *MDK*, midkine; *MET*, mesenchymal epithelial transition; *MLL*, mixed-lineage leukemia 1; NA, not available; NGS, next generation sequencing; NPV, negative predictive value; *ONECUT2*, one cut homeobox 2; *OTX1*, orthodenticle homeobox 1; PCR, polymerase chain reaction; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; POC, point-of-care; *TERT*, telomerase reverse transcriptase; *TP53*, tumor protein p53; *TWIST1*, twist homolog 1; VHL, von Hippel-Lindau syndrome; VUC, voided urine cytology; *UPK1B*, uroplakin 1B.

[a]:*TERT* promoter and the *FGFR3*.

[b]:*FGFR3*, *TP53*, *CDKN2A*, *ERBB2*, *HRAS*, *KRAS*, *PIK3CA*, *MET*, *VHL*, *MLL*, and *TERT* promoters.

[c]:*OTX1*, *ONECUT2*, and *TWIST1*.

[d]:*FGFR3*, *TERT*, and *HRAS*.

[e]:*IGFBP5*, *HOXA13*, *MDK*, *CDK1*, and *CXCR2*.

[f]:*ABL1*, *CRH*, *IGF2*, *UPK1B*, and *ANXA10*.

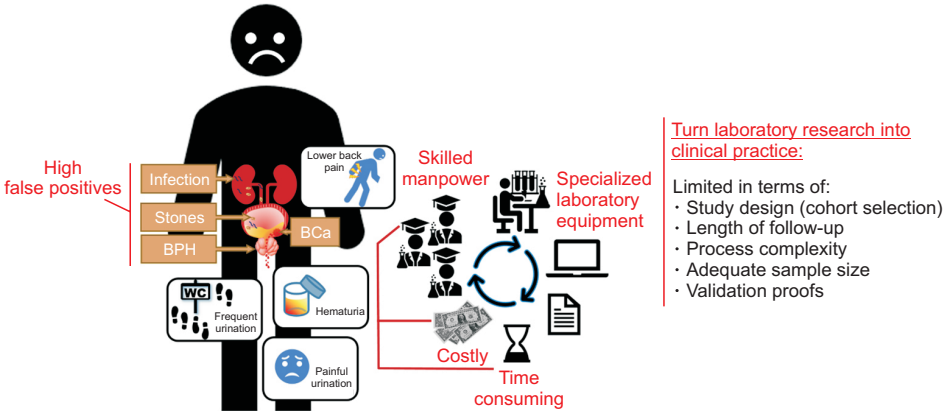

Fig. 2. Limitations of current urine tests. Lack of disease-specific symptoms means that BCa can be confused with other benign diseases such as infection, stones, and BPH, all of which can cause false-positive results in tests based on BCa diagnostic markers. Currently developed markers are more suitable for laboratory research than for use in clinical practice. There are several reasons for this. Studies in the field of biomarker research are limited with respect to appropriate cohort selection. Also, recent studies comprise too few samples, and follow-up is too short for meaningful statistical analysis. Additionally, laboratory-derived biomarkers are still relatively new and need to be validated in larger cohorts. Moreover, tests and assays often require skilled technicians and specialized equipment; thus, they are time-consuming and expensive. Although the biomarker market has grown rapidly, many problems await solutions. BCa, bladder cancer; BPH, benign prostatic hyperplasia; WC, water closet.

laboratory research is covered by the sponsor of the research project; however, these costs are then passed on to patients and hospitals when the tests enter clinical use.

Owing to the lack of disease-specific symptoms, diagnosis and follow-up of BCa are challenging. Noninvasive urine tests are designed to diagnose BCa, but it remains unclear how they can be integrated effectively into clinical decision-making, particularly with respect to discrimination of BCa from other diseases presenting with similar signs and symptoms. This pertains largely to patients with gross hematuria, non-urinary tract infection-related irritative voiding symptoms, and those with microscopic hematuria found on routine urinalysis. These variables should be taken into account during study design, although they are often neglected. The majority of studies are case-control trials comparing artificially composed study cohorts, in which the prevalence of the cancer frequently exceeds 50%. This high disease prevalence is not common in urological practice, and such evaluations lead to an overly optimistic calculation of positive predictive value. Finally, poor study design with respect to selection of patient cohorts and endpoints, and statistical considerations, is one of the reasons for the limited incorporation of novel BCa urinary markers into clinical decision-making. This may be connected to the lack of coherent and comprehensive processes (pipelines) for biomarker development. Therefore, to improve and standardize BCa marker development, Goebell et al. [126] proposed a stepwise procedure comprising four phases (analogous to therapeutic trials). Shariat et al. [127] placed emphasis on this four-phase process and stressed the importance of statistical considerations when conducting research into clinical biomarkers for BCa. It is crucial that researchers are aware of the complexity and poor success rate of biomarkers trying to enter the clinical arena. Fig. 2 describes the limitations of the current urine tests.

THE MICROBIOME AS A SOURCE OF BIOMARKERS

Mounting evidence indicates that the microbiota plays an important role in carcinogenesis and response to treatment. The dogma that urine is sterile has been discredited, and dysbiosis of the urinary microbiota is linked to urological disorders [128-132]. Developed techniques, including high-throughput sequencing, single-cell transcriptomics, and mass spectrometry, offer precise characterization of single enteric, neoplastic, and immune cells, and culture assays enable detection of microbes throughout the urinary system [131,133-135]. A recent study demonstrated an innovative mechanism underlying intravesical immunotherapy with BCG, a live attenuated strain of *Mycobacterium bovis*. The study suggests that the effects of BCG on BCa may be related to the action of certain microbiomes. The local microbiota may inactivate BCG directly in the bladder or may modulate urothelial sensitivity to BCG through competitive binding to fibronectin [136]. All these advances have focused BCa research on an attempt to understand the relationship between the commensal urinary microbiome and BCa development, as well as its impact on treatment efficacy through modulation of the anti-cancer immune response. The exact nature and role of the most applicable microbes remain unclear, but

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their potential involvement in BCa is apparent. A study by Xu et al. [137] undertook 16S sequencing analysis of voided urine from six healthy adults and eight BCa patients. They reported that the genus *Streptococcus* was enriched in the BCa patients [137]. Similarly, another study compared voided urine from 12 BCa patients with that from 11 healthy adults using 16S sequencing. They identified a known colorectal cancer-related genus, *Fusobacterium*, and showed that it was enriched in the BCa group; however, they found no significant differences in microbial diversity or overall microbiome composition between the groups [138]. Wu et al. [139] compared 31 male BCa patients with 18 healthy control subjects using 16S sequencing of midstream voided urine; they found that enrichment of the genera *Acinetobacter*, *Anaerococcus*, and *Sphingobacterium* was associated with BCa. In addition, greater bacterial richness was also present in urine from NMIBC patients with a high risk for recurrence or progression, indicating that higher bacterial richness may be a potential indicator of NMIBC prognosis. More recently, Chipollini et al. [140] performed 16S ribosomal RNA sequencing in voided urine samples from 10 noncancerous controls, 12 NMIBC patients, and 15 MIBC patients and discovered that the noncancerous group had less species variation and phylogenetic diversity than both the NMIBC and MIBC groups (all $p<0.05$). The authors also found that *Bacteroides*, *Lachnoclostridium*, and *Burkholderiaceae* species were significantly enriched in noncancerous samples, whereas *Bacteroides* and *Faecalibacterium* species were enriched in MIBC samples [140].

Unfortunately, these studies have a critical limitation in that they all analyzed the microbiome in voided urine. Voided urine is not representative of the bladder microbiome because the bacterial DNA detected in midstream voided urine differ substantially from the DNA detected in transurethral catheterized urine, which may be free from external contamination [141,142]. Moreover, although 16S sequencing is a powerful and sensitive tool for microbiome research, there are limitations. First, the results of 16S rRNA gene sequencing are relative rather than absolute; this is because the actual quantity of a particular bacteria is uncertain. Second, the outcomes can be biased due to fluctuating PCR amplification frequency and incomplete reference databases used for sequence analysis. Third, it does not determine cause-and-effect relationships. Fourth, it cannot determine whether bacteria were alive or dead, thereby requiring further urine culture for confirmation [143]. Connections between the urinary microbiome and BCa seemed real, and the possibility of using it as a noninvasive biomarker is highly intriguing. Further investigations should be conducted to improve the studies in this intriguing area.

CONCLUSIONS

Urinary biomarkers have been disregarded because of a perceived lack of sensitivity, a high rate of false positivity, and a paucity of independent validation studies, even though they have great potential as biomarkers of BCa owing to their noninvasive and easy sampling methods. For this reason, substantial improvements in this area have been made in the past few years. In addition, the current COVID-19 pandemic, which has caused inevitable delays in diagnosis, has highlighted the value of quick, efficient, easy, and contactless methods that can be used in clinical practice. Thus, much effort should be devoted to translating potential urinary biomarkers into clinical practice to alleviate the backlog of patients awaiting diagnostic procedures. However, current screening and surveillance urine tests are hampered by a low disease prevalence, which results in a high cost-to-benefit ratio. Although preliminary reports suggest that urinary biomarkers are feasible, there is insufficient detailed information from large study populations to make a final judgment. We urge the scientific and clinical communities to join hands to tackle these problems by modifying protocols and conducting prospective trials to provide a basis for integrating molecular markers into clinical practice. Guidelines on the use of biomarkers would also be welcome. Also, there should be direct head-to-head comparisons of urinary biomarkers to determine the best combination that provides the greatest sensitivity and specificity. The limitations of urinary biomarkers are solvable, albeit time-consuming; however, such markers will be of great benefit to BCa patients around the world. Accordingly, we can use them to improve care for patients with BCa during the COVID-19 pandemic and beyond.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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AUTHORS' CONTRIBUTIONS

Research conception and design: Xuan-Mei Piao. Data acquisition: Xuan-Mei Piao and Howon Kang. Statistical analysis: Xuan-Mei Piao. Drafting of the manuscript: Xuan-Mei Piao. Critical revision of the manuscript: Wun-Jae Kim and Seok Joong Yun. Obtaining funding: Howon Kang and Seok Joong Yun. Approval of the final manuscript: Wun-Jae Kim and Seok Joong Yun.

DATA AVAILABILITY STATEMENT

The data presented in this study are available from the corresponding author upon reasonable request.

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