Review of non-invasive urinary biomarkers in bladder cancer

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Abstract: Bladder cancer (BC) is the sixth-most prevalent cancer. The standard diagnostic tool of BC is cystoscopy, whereas cystoscopy has several disadvantages in terms of symptomatic invasiveness and operator-dependency. The urinary markers are attractive because the testing is non-invasive and cost-efficient, and sample collection is easy. Urinary marker is thereby a good tool to detect exfoliated tumor cell in the urine samples for the diagnosis and therapeutic surveillance of BC to supplement the limitations of the cystoscopy. However, they are not recommended as a population-based screening tool because of the low rate of BC prevalence. Although both cystoscopy and urine cytology improve BC diagnostic power, the field still needs additional non-invasive, cost-effective, and highly sensitive and specific diagnostic tools. Various urinary markers with different mechanisms and different targets have been developed and under investigation in these days. However, the accuracy of the urinary marker including its sensitivity and specificity is the most important factor for the diagnosis and surveillance in cancer that this review deals with multiple FDA-approved and non-FDA approved commercialized urinary markers with their accuracy in different purposes for BC. We then discuss more about the potential candidate targets for the future urinary markers in BC

Keywords: Bladder cancer (BC); urine; marker; sensitivity; specificity

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Introduction

Bladder cancer (BC) is the sixth-most prevalent cancer, with age-standardized incidence rates of 9.6 and 2.4 per 100,000 for men and women, respectively (1). The current gold standard for diagnosing BC is cystoscopic visualization of bladder tumors, which has an overall sensitivity of 62–84% and specificity of 43–98% (2). However, cystoscopy has a limited ability to diagnose small papillary BC and BC carcinoma *in situ* (CIS) and is cost-intensive, invasive, and operator-dependent.

The degradation of cells, DNA, and RNA in urine samples depends on time and temperature, resulting in a variable quantity and quality of cell and DNA molecules and leading to highly variable sensitivities and specificities, which causes overestimations of the performance characteristics of urinary biomarker tests (3-9). However, urinary biomarkers are attractive because the testing is non-invasive and cost-efficient, and sample collection is easy. Urine cytology, the most common urinary biomarker test used for BC diagnosis and screening, involves examining voided urine and detecting exfoliated urothelial BC cancer cells that enter the urine through contact with the urinary tract (10).

Many current urinary biomarker tests have various applications. For example, clinicians analyze urinary biomarkers before and after cystoscopy or transurethral resection surgery to detect recurring or progressing BC after intravesical instillation or systemic therapy (11). Additionally, tumor characteristics during surgery provide information about potential tumor aggressiveness and invasiveness. This information is used to decide upon the

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appropriate resection margin, surgical depth, and whether to include the detrusor muscle, especially for high-grade tumors and CIS (12,13).

Postoperative surveillance of urinary biomarkers is a potential non-invasive replacement for cystoscopy after transurethral resection surgery (14). Urinary biomarkers can be used for therapeutic surveillance after either neoadjuvant chemotherapy, adjuvant chemotherapy, or intravesical instillation (15,16). However, they are not recommended as a population-based screening tool because of the low rate of BC prevalence (11). Although both cystoscopy and urine cytology improve BC diagnostic power, the field still needs additional non-invasive, cost-effective, and highly sensitive and specific diagnostic tools. Here, we discuss and compare several potential urinary biomarkers.

Urinary biomarkers

FDA-approved urinary biomarkers

Urine cytology

Urine cytology is the most reliable test among urinary biomarker tools for detecting exfoliated tumor cells in urine, with an overall sensitivity and specificity of up to 48% and 86%, respectively (17,18). It complements both primary diagnosis and recurrence surveillance of high-grade or CIS BC treated with intravesical therapy, with 38–90% sensitivity and 98–100% specificity. However, its sensitivity for low-grade BC is only 4–31%, and the 12% false-positive rate reflects a poor ability to screen out patients with inflammation, atypical urothelial cells, or a history of other cancer therapies (17-20).

Urine cytology also has limitations in terms of discrepancies between cancer types and inter-observer variability, especially for patients with recurrent inflammation or previous immunotherapy. In addition, the interpretation of cystoscopy results is more reproducible than that of urine cytology results; therefore, when atypical urothelial cells are detected using urine cytology, the cytology results should not stand alone as a diagnostic replacement for cystoscopy.

Bladder Tumor Antigen (BTA) stat and BTA TRAK (Polymedco Inc., Cortlandt, NY, USA)

The BTA stat is a commercially available protein biomarker test that uses an immunochromatographic assay to detect BTA in previously diagnosed BC patients for the purpose of surveillance (15). The BTA stat and BTA TRAK tests detect the human complement factor H and complement factor H-related proteins that are involved in cancer cell growth and evasion of the host immune system; these levels increase during the invasion of BC cells (21-23). The sensitivity and specificity of BTA stat are 57–83% and 60–92%, respectively, while those of BTA TRAK are 73–77% and 45–81%, respectively (6,24,25).

For surveillance purposes, the overall sensitivity of BTA stat is superior to that of urine cytology, but the specificity is inferior (26). BTA TRAK also has a sensitivity superior to that of urine cytology and a higher sensitivity for high-grade and low-grade (Ta and T1) BC. However, BTA tests cannot replace urine cytology because of their inferior specificity (27) and high false positive rate caused by detection of benign diseases such as hematuria, urinary tract infection, stones, and *in situ* ureteral stenting (4,25,28-30).

NMP22 kit and NMP22 BladderChek (Alere, Waltham, MA, USA)

The NMP22 kit and NMP22 BladderChek are both ELISA tests that target the NMP22 protein, which is more prevalent in BC cells than in the normal urothelium released in urine during apoptosis of urothelial cells (31, 32). The overall sensitivity and specificity of the NMP22 kit, which is used for surveillance (33), are 52–59% and 87–89%, respectively (33-35). The sensitivity and specificity of NMP22 BladderChek, which is used for diagnostics, are 62–75% and 70–83%, respectively (33). The limitation of these tests is the high false positive rate caused by detection of benign conditions (29,36).

Compared with urine cytology, the NMP22 test has a higher diagnostic sensitivity for microscopic hematuria (3,31) and better detection power for low-grade, low-stage, and high-grade BC due to the increasing rates of cell apoptosis present in these disease conditions (37). However, the NMP22 test cannot replace urine cytology because it has a lower specificity (38).

ImmunoCyt/uCyt+ assay (Scimedx Inc., Denville, NJ, USA)

The ImmunoCyt/uCyt+ assay is the only test available for therapeutic follow-up and applies a combination of urine cytology and immunohistochemical staining using fluorescent-labeled monoclonal antibodies. The assay detects exfoliated BC cells using the carcinoembryonic antigen and two BC-associated mucins (LDQ10 and M344). An overall specificity ranging from 62% to 84% and

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sensitivity ranging from 67% to 100% have been observed during use of the assay for diagnostic and surveillance purposes (28).

The high sensitivity of this assay for detecting increased pathological grades as well as low-grade, low-risk BC (33,39-43) is sufficient to make cystoscopy unnecessary (14). Limitations include the necessity for special laboratory equipment and skillful, experienced assay interpreters (44) and a lower specificity than that of urine cytology. It also has a high false positive rate caused by detection of benign diseases and an especially decreased specificity (~67%) for hematuria (45).

UroVysion (Vysis, Abott Molecular Inc., Chicago, IL, USA)

The UroVysion uses a multi-target fluorescence *in situ* hybridization assay to detect aneuploidy in chromosome copy numbers 3, 7, 17, and 9p21 and identify loss of the P16 tumor-suppressor gene (46). The test has a sensitivity (57.1–84%) and specificity (78–92%) superior to those of urine cytology, especially for low-grade BC (14,47,48). This test is especially useful for detecting BC in high-risk patients with equivocal cystoscopic findings and atypical cytology (14,49), confirming Bacillus Calmette-Guerin (BCG) responsiveness in non-responders (50-53), and detecting BC recurrence in non-muscle invasive bladder cancer (NMIBC) patients with negative cystoscopy but suspicious cytology results (54).

Limitations include the necessity for laboratory equipment and test reading experience as well as time-consuming specimen processing, which results in high costs (44,55). There is a high false positive rate, especially for patients tested within 12 months of a bladder biopsy. The presence of other primary tumors with chromosomal aberrations also contributes to the false positive rate (56).

Summary

Currently, cystoscopy is the standard BC diagnostic tool. None of the urinary biomarkers can replace cystoscopy because they fail to diagnose 18–43% of BCs and yield a 12–26% false positive rate. However, urinary biomarkers are the best tool for overcoming cystoscopy's limitations and enhancing diagnostic accuracy. Compared to the popular urine cytology, the BTA stat, ImmunoCyt, and Urovision tests present higher sensitivities when used as diagnosis and surveillance tools, although they all have lower specificities, especially for low-grade BC, than cytology.

Non-FDA approved urinary biomarkers

ADXBLADDER (Arquer Diagnostics LTD, Sutherlands, UK)

ADXBLADDER, a protein biomarker assay, uses ELISA to detect mini-chromosome maintenance protein 5 (MCM 5), which is present during DNA replication and overexpressed in hematuric BC patients (8,57). The overall sensitivity and specificity are estimated to be 76% and 69%, respectively, although the sensitivity increases up to 95–97% for high-risk and muscle-invasive BC. The negative predictive value (NPV) is 97.1% overall and 99.7% for high-risk BC (58). Therefore, this biomarker might be either a potential replacement for or an adjunctive to urine cytology as a BC diagnostic tool. The advantage of this test is that its diagnostic accuracy is not influenced by any benign conditions.

UBC® Rapid test and UBC ELISA kit

The UBC® Rapid test is a point-of-care ELISA assay that uses an immunochromatographic method to detect soluble fragments of cytokeratin 8 and 18 of the urinary BC antigen, which relate to tumor invasion. This test detects high-grade NMIBC during diagnostics and follow-up more readily than it detects low-grade tumors, with a sensitivity of 50–75% and specificity of 82–93.8% (11,55,59,60). It is standardized and calibrated and thus independent of user, batch, and study site, and has the potential to be a sensitive and specific urinary protein biomarker test for identifying patients with high-grade tumors difficult to detect with cystoscopy.

Uromonitor (U-Monitor, Porto, Portugal) and uromonitor-V2

Uromonitor, a DNA biomarker test, uses real-time PCR to detect *TERTp* and *FGFR3* alterations in exfoliated tumor cells. The Uromonitor-V2 test has 100% sensitivity, 83.3% specificity, 66.7% positive predictive value (PPV), and 100% NPV for surveillance of NMIBC (59). With an overall sensitivity of 73.5% and specificity of 73.2%, Uromonitor performs similarly to cystoscopy across all stages and grades during recurrence surveillance after transurethral bladder resection (61), serving as an alternative test for patients ineligible for cystoscopy. Uromonitor achieves 100% sensitivity and 88.6% specificity when combined with cystoscopy (the sensitivity of cystoscopy combined with

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cytology is 86.7%) (61). The detection rate increases from 62.5% for recurrent low-grade BC to 75% for recurrent high-grade BC. The Uromonitor test is also independent of the presence of inflammation or benign diseases.

UroSEEK

UroSEEK, a DNA biomarker assay, is more sensitive than cytology in both surveillance (71% *vs.* 25%) and diagnostics (95% *vs.* 43%) and detects genetic alterations in *TERT*, *FGFR3*, *PIK3CA*, *TP53*, *HRAS*, *KRAS*, *ERBB2*, *CDKN2A*, *MET*, *MLL*, and *VHL* (62,63). For primary BC diagnosis, high-risk patients and patients with atypical urine cytology are suitable test candidates. For surveillance, a sensitivity of 74–96%, specificity of 72–88%, and an NPV of 53–99% were reported (63). The limitations of this test are the low sensitivity of the next-generation sequencing (NGS) technique used to detect mutations and the poor performance during follow-up tests of previously diagnosed BC patients and patients with upper urinary tract urothelial carcinoma (3).

CxBladder assay (Pacific Edge, NZ)

CxBladder, a laboratory-developed transcriptomic panel assay, measures five mRNAs (IGFBP5, HOXA13, MDK, CDK1, and CXCR2) associated with BC and one mRNA associated with nonmalignant conditions to reduce the false positive rate, with an overall sensitivity of 82% (64). The CxBladderTriage has the advantage of profiling individualized risk by collecting clinical factor data, with a 100% detection rate for high-grade BC with gross hematuria (65). It also has a higher sensitivity for low-grade BC than urine cytology (68% for pTa), with a specificity of 85%. A sensitivity of 93% extends to all other evaluative tests across all stages and grades [sensitivity for low-grade: 86%, sensitivity for high-grade: 95%, NPV: 97%, false negative rate: 1.5% (64)]. Therefore, cystoscopy can be avoided during follow-up of NMIBC cases (66). In comparison to the currently approved FDA tests, the sensitivity and NPV of CxBladder Monitor (91%, 96%) was significantly higher than those of urine cytology (22%, 87%), the NMP22 BC test (26%, 87%), and NMP22 BladderChek (11%, 86%) (49).

The XPERT BC Monitor (Cephaid, USA)

XPERT BC Monitor, a transcriptomic panel test for five mRNAs (ABL1, CRH, IGF2, UPK 1B, and ANXA 10), has an overall sensitivity of 73% (superior to that of urine cytology) and an insignificant differential overall specificity of 90–91%, comparable to that of urine cytology (67). The advantages of this test are its superior sensitivity (84% *vs.* 33%, P<0.001) and NPV (93% *vs.* 76%, P<0.001) for low-grade (77%) and pTa disease (82%) compared to urine cytology (13% for low grade, 21% for pTa) (67).

EpiCheck

EpiCheck, an epigenetic DNA methylation biomarker test, targets 15 altered DNA methylation biomarkers to identify NMIBC recurrence. Its probability algorithm, EpiScore, has a sensitivity of 68.2%, specificity of 88.0%, and an NPV of 95.1%. EpiCheck has a higher sensitivity (62.3–68.2% *vs.* 33.3%) but a lower specificity (86.3–88% *vs.* 98.6%) than urine cytology (68). The advantages of this test are its high sensitivity for high-grade BC, with a 82.9% NPV (69), and that the presence of inflammation in the urinary tract has no influential effect (68,69). The disadvantages are a high cost and technically challenging procedure (68,69).

BC UroMark

BC UroMark, a next-generation sequencing assay, is an epigenetic DNA methylation biomarker test targeting bisulfite. It uses a 150 CpG loci biomarker panel and has 98% sensitivity, 97% specificity, and 97% NPV (70).

AssureMDx (MDx Health, USA)

The AssureMDX panel assay is a somatic DNA methylation biomarker test using next-generation PCR sequencing for *OTX1*, *ONECUT2* and *TWIST1*. It measures the mutational load of the *FGFR3*, *TERT*, and *HRAS* genetic panel with 93–97% sensitivity, 83–86% specificity, and 99% NPV (9,71) for high-risk patients with hematuria. The sensitivities are 81% and 57% for low-grade BC diagnosis and surveillance, respectively, and 94% and 72% for high-grade, respectively. The specificity for low-grade is 59%; that for high-grade is 55%, for surveillance. (71). This test produces a potential 77% reduction in unnecessary diagnostic cystoscopies (9,72).

TaqMan® Arrays

This 12 + 2 gene-set panel is based on a qRT-PCR assay for BC detection and has a sensitivity of 98% and specificity of 99%. For predicting BC aggressiveness, this test has a sensitivity of 79% (*vs.* 86% in the control) and specificity of 92% (*vs.* 80% in the control) (73,74). With a sensitivity of 81.48% and specificity of 91.26%, the gene signature composed of two genes (GS_D2) performs equally to or better than cytology (75).

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Investigational potent targets for urinary biomarkers

Proteolytic region of cytokeratin-19 (CYFRA 21-1)

The CYFRA 21-1 can be detected as a soluble molecule in serum and other body fluids and has been recognized as a non-specific tumor biomarker for several neoplastic diseases, including BC, in terms of diagnosis, prognosis, follow-up, and prompt recognition of recurrence. The CYFRA 21-1 test uses the ELISA method and has a sensitivity and specificity of 70–90% and 73–86%, respectively (76).

BLCA-4

BLCA-4, a member of the NMP transcription factor family, is observed during the early stages of BC. The sensitivity and specificity are high, at 93–96% and 97–100%, respectively, but its role as a diagnostic biomarker for BC still requires further validation (60). Its potential is further strengthened by the absence of high BLCA-4 levels in patients with various benign conditions. Nevertheless, novel methods not requiring urine precipitation analysis are necessary to include the BLCA-4 assay in clinical practice.

Soluble FAS (sFAS)

sFAS, an anti-apoptotic protein, protects cancer cells from anti-tumor activity. The urinary level of sFAS, measured using ELISA tests, is associated consistently with increasing tumor grade, stages ≥ T1, high NMP22 levels, and positive urine cytology results (77). The sensitivity and specificity are 88.03% and 89.19%, respectively. sFAS produces more positive results than urine cytology, particularly for low-grade, early-stage disease cases.

Hyaluronic acid (HA)

HA is involved in cell adhesion and proliferation. During tumor metastasis, the hyaluronidase enzyme (HAase) catalyzes HA reactions to facilitate cellular proliferation and motility (78). The HA test for NMIBC has a sensitivity and specificity of 87–100% and 89–98%, respectively (5).

Telomeres

Urinary telomerase has a protective role in cancer cell chromosomes and is associated with the recurrence of NMIBC. The combination of telomerase activity measurement and cytology produces an increased sensitivity of 60–87% and a specificity of around 65–90% (66). The PPV is 83.3% for superficial stages, 42.1% for invasive stages, 83.3% for grade 1 tumors, 66.7% for grade 2 tumors, and 40.0% for grade 3 tumors; thus, telomerase activity correlates with lower grades and stages of BC.

Bladder tumor fibronectin (BTF)

BTF is a chemiluminescent immunometric test used for NMIBC surveillance after transurethral resection surgery, with a 91% sensitivity, 88% specificity, 93% NPV, and 73% PPV (79). BTF is more sensitive to high-grade and high-stage tumors, with a positivity rate of 93.8% for invasive tumors versus only 73.1% for superficial tumors. Similar to our findings, several studies (12,15) have reported that BTF sensitivity is between 81.6% and 100% for infiltrating tumors and between 70% and 73.3% for noninvasive tumors, this test has higher sensitivity and specificity than FDA-approved tests.

Cytokines and chemokines

The GM-CSF, IFN-alpha, IL-2, IL-6, IL-8, IL-10, IL-18, survivin, TNK-alpha, and TRAIL have also been used as urinary targets for predicting the therapeutic response to intravesical BCG therapy (48,60). PDL-1 and CTLA-4 are also effective immunotherapy targets. Tumor-associated macrophages (TAMs, also known as M2 macrophages) are recruited by chemokines such as interleukin (IL)-4 and IL-13 and promote tumor growth. A high density of tumor-infiltrating TAMs is associated with poor outcomes in various types of cancer, including BC.

IGF2 and MAGE-A3

IGF2 is a glycoprotein receptor on the cell membrane that relates to the PI3K-AKT pathway in BC. Melanoma-associated antigen 3 (MAGE-A3) is found in 43% of BCs as well as in the testes and placenta (74,80-83). In combination, IGF2 and MAGE-A3 have 81% sensitivity, 91% specificity, 87% PPV, and 88% NPV (80).

Topoisomerase-II alpha (TopoIIA)

Expression of the TopoIIA mutation is significantly higher in hematuric patients and MIBC patients than in patients with NMIBC and is significantly associated with higher NMIBC recurrence and progression rates (84). Recent studies reported a sensitivity of 73.8%, specificity of 68.3%, PPV of 64.2%, and NPV of 78.6% using urinary real-time PCR (85).

Future urinary biomarkers

Advances in sequencing technology have enabled researchers to catalogue the spectrum of somatic mutations

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associated with urothelial BC (3) and substantially change therapeutic guidelines for the selection of molecular target therapies (4). Based on BC genomic information and given the advancements in highly sensitive PCR-based technology, various small, obtainable amounts of DNA from urine samples have become ideal candidates for biomarkers that enable early BC detection, as they feature small, cancer-specific alterations (86). The assembly of multiple urinary genetic biomarkers enhances the power to detect recurrence and to evaluate the therapeutic response of each patient (7,87).

Studies over the past decade have consolidated the major molecular taxonomies and demonstrated potentially treatable gene mutations and their fusion proteins. These include the FGFR3-TACC3 fusion protein (16,17) in pathways such as the mitogen-activated protein kinase (MAPK) pathway, BRAF, MAP2K1, MAP2K2 and the phosphoinositide 3-kinase (PI3K) pathway (for example, PIK3CA) in 61% of tumors (13-15) and the receptor tyrosine kinase–RAS pathway (39% of metastatic tumors), including FGFR3 (14%) and ERBB3 (13%), and in the PI3K-RACα serine/threonine-protein kinase (AKT)-mechanistic target of rapamycin (mTOR) pathway (38%), including PIK3CA (16%) and AKT3 (12%) (16).

Potential DNA-methylated urine-based genetic biomarkers include the *ANF154*, *VIM*, *TWIST*, *SFRP1*, *SALL3*, *RUNX3*, *RASSF1*, *RARB*, *POU4F2*, *PCDH17*, *p16INK4A*, *p14ARF*, *ONECUT2*, *NID2*, *HOXA9*, *GSTP1*, *GDF15*, *EOMES*, *DAPK*, *CDH1*, *CCNA1*, *BCL2*, and *APC* genes (7). The *ZNF154* and *POU4F2* genes have the greatest sensitivity for diagnosing BC (>80%) and the combined *TWIST1*, *OTX1*, and *ONECUT2* genes have been used in a commercialized product, called the AssureMDx test, for hematuric patients (88). The most exact combined urine panel for BC detection is that of the *SOX1*, *TIP2*, *MYOID*, *HOXA9_1*, *HOXA9_2*, *VAMP8*, *CASP8*, *SPP1*, *IFNG*, *CAPG*, *HLADPA1*, and *RIPK3* genes, with 100% sensitivity and 100% specificity (89).

cfDNA, long-noncoding RNA (lncRNA), and miRNA are also detectable in urine and are valuable tools for risk stratification and initial disease diagnoses (90,91). For example, levels of ctDNA from the *FGFR3* and *PIK3CA* genes can be detected in urine and correlate with prognoses for recurrence and overall survival after intravesical therapy or cystectomy (90,91). In voided urine, good targets for BC detection (92) include miR-137, miR-124-2, miR-124-3, miR-126, miR-152, miR-148b-3p, miR-182, and miR-199a, miR-3187-3p, miR-15b-5b, miR-27a-3p, miR-30a-5p, miR-324-5p, miR-4738-3p, FBJ murine osteosarcoma viral oncogene homolog B mRNA, Regulator of Calcineurin 1 mRNA (with 99.3% sensitivity and 98.9% specificity), and lncRNA miR-497-HG (with 78.3% sensitivity) (93-95).

Extracellular vesicles (EVs) with multiple cellular components such as proteins, DNA, and mRNA can also be found in urine and have a low degradation rate, due to the similarity between their membrane and that of the origin cell and their ability to avoid immune system detection (96). An innovative methodological device improved the detection of E*Vs*. in urine, with a sensitivity of 81.3% and specificity of 90.0% for BC diagnosis (97).

Limitations of urinary biomarkers

There are several limitations to be aware of when assessing urinary biomarkers, including the time-dependent degradation during storage of collected urine samples, urinary content processing time, and specialized detection tools like sedimentation PCR methodology. The immediate storage of urine samples at temperatures between -20 and −80 ℃ for four weeks has been recommended for maintenance of DNA material (98). Another strategy for preventing testing bias is co-sedimenting normal cells, crystals, and other substances during downstream PCR analyses (99). When using NGS to detect tumor DNA in leukocyte-rich urine, the inconsistency of the concentrations of cells and DNA should be taken into consideration despite the higher sensitivity of cfDNA, because the ratio of tumor DNA to wildtype DNA is higher than that of DNA to urine sediments. Various alternative approaches that enrich the tumor DNA yield have been developed, such as size-based cell selection, in which a filter captures smaller-sized normal cells along with tumor cells (100,101). Repeated urine sampling is another way to increase testing sensitivity. Pooled urine samples collected from low-grade BC patients after at least 24 h have a sensitivity of 100%, compared to only 75% when a single sample is analyzed (102).

Conclusions

This review presents many FDA-approved biomarkers and potential biomarker candidates. However, due to limited prospective and large longitudinal clinical trials of new urinary biomarker tests, the current international guidelines still do not recommend testing for other urinary biomarkers prior to performing cystoscopy. However, newer approaches focusing on molecular, genomic, and transcriptomic

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aberrations have promising accuracies, and these biomarkers may provide additional molecular information to guide individualized surveillance and therapy strategies in the future. This review may be used to inform the scope of future clinical trials.

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