

# Bladder Cancer Detection and Monitoring: Assessment of Urine- and Blood-Based Marker Tests

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**Abstract** Bladder cancer is one of the most prevalent cancers worldwide, but the treatment and management of this disease can be very successful if the disease is detected early. The development of molecular assays that could diagnose bladder cancer accurately, and at an early stage, would be a significant advance. Ideally, such molecular assays would be applicable to non-invasively obtained body fluids, and be designed not only for diagnosis but also for monitoring disease recurrence and response to treatment. In this article, we assess the performance of current diagnostic assays for bladder cancer and discuss some of the emerging biomarkers that could be developed to augment current bladder cancer detection strategies.

## 1 Introduction

Bladder cancer (BCa) is among the five most common malignancies worldwide. There are over 70,000 new cases of BCa each year in the United States alone [1]. The most common form of BCa in Western countries is transitional cell carcinoma (TCC), constituting approximately 95 % of all cases [2]. Risk factors associated with the development of BCa include carcinogens in tobacco smoke, and to a lesser extent exposure to chemical compounds in the chemical and rubber industries. The disease has a five times higher prevalence among men than women, and the median age at diagnosis is 65 years.

At diagnosis, the majority (80 %) of cases present with non-muscle-invasive papillary tumors (stages pTa or pT1),

and these have a much more favorable prognosis than those that show evidence of muscle invasion at the time of detection. If detected early, the 5-year survival rate for BCa is approximately 94 %, so timely intervention can dramatically increase the probability of patient survival. Radical surgery is required for muscle-invasive lesions, but non-muscle-invasive BCa can be treated through transurethral resection of the tumor. However, the major clinical problem is that more than 70 % of patients with Ta/T1 BCa will have disease recurrence within 2 years of treatment. Extensive long-term surveillance and repeated surgical intervention is needed to prevent progression of early-stage tumors to the more lethal invasive disease. When the high recurrence rate of BCa is taken into consideration, there are approximately 500,000 cases of active BCa in the US [3], and it is one of the most prevalent cancers worldwide.

For initial clinical diagnosis of BCa, the gold standard remains cystoscopic examination of the bladder coupled with voided urine cytology (VUC), the cytologic examination of cellular material present in the urine [4–6]. Cystoscopy is an uncomfortable and costly invasive procedure that may require anesthetization of the patient. The technique enables visualization of the bladder lining and biopsy of suspicious lesions for histopathological diagnosis and staging. Evaluation by VUC relies on the microscopic visualization of shed cancer cells in voided urine. The technique performs well with high-grade and high-stage tumors (T2–T4), but because Ta–T1 tumors shed fewer cancer cells into the urine, the sensitivity of VUC for the detection of early-stage tumors is low, ranging from only 20 to 40 % [6, 7]. Coupled with the fact that VUC is prone to interobserver variation [8], it is understandably not seen as a standalone test for application in a potential BCa clinical investigation. After initial treatment of non-muscle-invasive tumors, BCa patients are placed under

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continued surveillance, with routine examinations performed by cystoscopy and cytology in order to achieve early detection of new tumor development. The regimen is typically cystoscopy every 3 months for 2 years, then every 6 months for 2 years, and then every year thereafter. Consequently, the development of non-invasive assays that can reduce the need for cystoscopy would be of tremendous benefit to both patients and the healthcare system. For BCa, the accessibility of urine in this context is a major advantage. The sample can be obtained non-invasively (avoiding patient discomfort and potential complications from an invasive procedure), copious amounts of sample can be obtained for analysis, and repeat sampling is easily achievable. While there have been efforts to identify serum-based biomarkers for BCa detection, this is less well developed, and there are currently no blood-based tests available for detection. The analysis of blood-based biomarkers may have more utility in prognosis or therapeutic decisions [9–11]. In this review, we will assess the current molecular tests available for BCa and describe some of the research advances made in biomarker discovery for the non-invasive diagnosis and monitoring of BCa.

## 2 Current Molecular Tests for BCa Detection

A number of commercial molecular tests have been FDA-approved for specific scenarios (Table 1). These tests include the measurement of soluble proteins such as bladder tumor associated antigen (BTA), nuclear matrix protein 22 (NMP22), proteins detected on fixed urothelial cells (ImmunoCyt), and chromosomal aberrations detected by fluorescence in situ hybridization (UroVysion). The reported performance parameters and potential advantages or disadvantages of these tests (based on an assessment of the relevant literature) are discussed below.

### 2.1 Voided Urine Cytology

As it is the gold standard for the detection of malignancy through urinalysis, molecular tests are most often compared to VUC. Actually, VUC is not difficult to outperform given its poor sensitivity, but its established coupling with cystoscopy and its entrenchment as an in-house pathology test means that it remains the go-to test in the majority of health care systems [12]. The lack of sensitivity achieved by VUC is skewed by low-grade and early-stage tumors. These lesions shed relatively few cancer cells into the urine, and as the test relies on microscopic visualization, there is no opportunity to include a signal amplification method to aid the interpretation of minimal samples. However, the detection of high-grade tumors is reported to be over 80 % [4–6]. The VUC test does have a number of advantages, the most obvious of which is the high specificity (i.e., a low rate of false-positive interpretations) [4, 5]. Furthermore, VUC is a morphological test, so it is not impacted by the presence of blood in a sample (hematuria) or by chemical confounders such as pH or salt concentrations that can bias or negate biochemical urinary tests. It is a simple test, so it is also not prone to the amplification artifacts or errors inherent to complex molecular techniques. The combination of its high specificity, the inexpensive nature of the equipment required to perform it, the lack of a need for special patient preparation, and its reasonable in-house costs explain why VUC has been the cornerstone of urine-based BCa detection assays for >50 years. Disadvantages of the test include the fact that not every voided urine specimen contains cancer cells. The collection and analysis of three serial first morning specimens for urinary cytology has been shown to reduce the sampling error associated with voided urinary cytology [6], but the feasibility of this approach is impacted by poor patient compliance. Another problem is the subjectivity of the assay. In accordance with accepted

**Table 1** FDA-approved urinary assays for bladder cancer

Assay	Commercial analyte	FDA clearance/ approval	Assay type
BTA stat	Bladder tumor associated antigen (human complement factor H-related protein)	Diagnosis, monitoring	Colorimetric Ag–Ab reaction (point of care)
BTA TRAK	Bladder tumor associated antigen (human complement factor H-related protein)	Diagnosis, monitoring	Sandwich immunoassay
NMP22	NUMA1 (nuclear mitotic apparatus protein 1)	Diagnosis, monitoring	Colorimetric Ag–Ab reaction (point of care)
NMP22	NUMA1 (nuclear mitotic apparatus protein 1)	Monitoring	Sandwich immunoassay
ImmunoCyt/ uCyt+	High-MW form of glycosylated CEA and MUCIN-like antigens	Monitoring	Fluorescent antibody cytology
UroVysion	Detection of aneuploidy for chromosomes 3, 7, and 17, and loss of the 9p21 locus	Diagnosis, monitoring	FISH (fluorescence in situ hybridization)

nomenclature, VUC results are classified by cytopathologists into four categories: normal, atypical/indeterminate, suspicious, or malignant [12, 13]. Variability in interpretation and indecisive atypical categorizations can lead to unnecessary invasive and costly follow-up procedures [14].

## 2.2 Bladder Tumor Associated Antigen Assays

Two versions of the BTA test (Polymedco Inc., Cortlandt Manor, NY, USA) are available: BTA stat, an immunochromatographic, qualitative point-of-care assay [15], and BTA TRAK, an ELISA test that measures the human complement factor H (cFH)-related protein in a quantitative fashion [16]. These assays are FDA-approved for the detection and surveillance of BCa in urine samples [17, 18]. They are simple to perform and interpret, and the versatility of the point-of-care format enables testing at outpatient clinics. For BCa detection, urinary BTA tests have diagnostic sensitivities ranging from 29 to 91 % and specificities ranging from 56 to 86 % [17–19]. While sensitivities can be better for BTA over cytology, the specificity is impacted by other non-cancerous conditions [8].

Many patients visiting the urological clinic present with some level of hematuria, and this is known to interfere with urinalysis tests, potentially causing false-positive or false-negative diagnostic results [20]. A number of reports have noted that there is often a high correlation between BTA data and hematuria levels [20, 21]. Given the fact that the cFH proteins are serum factors, there is the possibility that BTA tests are monitoring the presence of serum proteins that are introduced through bleeding from the tumor. Strong correlations of BTA levels with hematuria in clinical samples are evident, and urine-spiking models confirm that hematuria can cause false-positive BTA tests at levels that would register as negative or trace hematuria in clinical tests [21–23]. Another concern is the source of the BTA antigen; the large soluble glycoproteins of the cFH family are produced and secreted into the serum by Kupffer cells, hepatocytes, vascular endothelial cells, and platelets [15], and searches of publicly available cancer tissue profiling databases do not support the notion that bladder tumor tissue is a source of cFH.

## 2.3 Nuclear Matrix Protein 22 Assays

Another immunochromatographic assay for BCa detection monitors urinary nuclear matrix protein 22 (NMP22/NUMA1) [24, 25]. NMP22 is a nuclear mitotic apparatus protein that is responsible for the distribution of chromatin to daughter cells during mitosis [26]. As with the BTA tests, the NMP22 tests also come as a quantitative ELISA or a quantitative point-of-care test known as BladderChek

(Matritech Inc., Newton, MA, USA). The POC test is FDA-approved for initial diagnosis, and both are approved for disease surveillance. The advantages of the POC test include low cost, ease of use, and interpretation without the need for a trained pathologist.

The ELISA test performed reasonably well in early studies [18, 20, 24], but has largely been superseded by the POC test, which has been shown to perform at least as well as the original test format with respect to accuracy. In a major study of over 1,300 subjects, the BladderChek NMP22 test achieved a sensitivity of 56 %, while cytology achieved only 16 % in the same cohort. The specificity achieved in the study did not equal that of cytology, but was a respectable 86 % [27]. A follow-up study by the same group evaluated the utility of the NMP22 POC test for the surveillance of 668 patients with a history of BCa. The NMP22 test outperformed cytology as an adjunct to cystoscopy, but the sensitivity and specificity of the test alone were 49 and 87 %, respectively [28]. Another large cohort study enrolled 1,328 consecutive patients to evaluate the impact of risk factors on the BladderChek NMP22 test. Among the 79 patients with malignancy, the overall positive predictive value (PPV) of the NMP22 test was only 20%, but the PPV was higher in men with a history of smoking and/or those presenting with gross hematuria. The negative predictive value (NPV) was >95 %, except in men with gross hematuria [29]. Most recent cohort studies confirm that the NMP22 test outperforms cytology but not cystoscopy for sensitivity, and specificity is similar to cytology. The test does not reduce the need for cystoscopy [30, 31].

The impact of hematuria and infection on NMP22 tests has been noted [24, 31, 32], and experimental models have confirmed the effect of blood by spiking at relatively high levels [22, 33–35]. NMP22 levels can also be elevated in pyuria, urolithiasis, or cystitis, and after instrumentation [24, 32]. The impact of these conditions on the test may be related to the nature of the NMP22 marker itself. NMP22 is a ubiquitous nuclear protein that is expressed in all tissues with an epithelial component, including the normal urothelial lining of the urinary bladder. In fact, normal tissues often appear to express more NMP22 than malignant tumors [35], so its potential as a biomarker must be related to its release from cells rather than its discrete expression or abundance. Proliferating tumor cells do turn over at a high rate, and many undergo apoptosis, and so the test may reflect that phenomenon, but this makes the test susceptible to any condition that results in a higher rate of turnover than normal urothelia, or any condition that causes cellular damage and release of nuclear contents. Such conditions would include nucleated cells from serum incursion, inflammatory conditions, and damage from instrumentation.

## 2.4 ImmunoCyt Test

The ImmunoCyt/uCyt+ assay (Scimedx Corp, Denville, NJ, USA) is designed to augment cytology through the detection of cellular biomarkers on cytology slides using a cocktail of fluorescent monoclonal antibodies [36, 37]. The antigens targeted in the test are a high molecular weight form of carcinoembryonic antigen and two bladder tumor cell-associated mucins. This test is performed under microscopy by a trained cytopathologist, and a relatively large number of exfoliated cells are necessary to perform an accurate test. A test is scored as positive when a single red or green cell is observed, but the manufacturer recommends that all positive cells should be correlated to morphology. Conversely, a cytology slide must contain a minimum of 500 cells for a negative score to be valid. Sensitivities of the ImmunoCyt/uCyt+ when combined with cytology are reportedly in the range of 81.0–89.3 %, a considerable improvement over cytology alone, but the specificities of the combined assays are less than that achieved by cytology alone, in the range of 61.0–78 % [38–43]. Studies also suggest that ImmunoCyt/uCyt+ has a superior sensitivity to cytology for early pathological stage (Ta–T1) and low-grade tumors, and can significantly improve the detection of carcinoma in situ [39, 40, 42]. As it is a cellular assay, ImmunoCyt/uCyt+ is less impacted by hematuria and inflammatory conditions, but the test is subjective and depends on specimen stability and handling as well as interobserver variation [38]. These limitations restrict the ImmunoCyt test to being recommended as an adjunct to cytology, and it is only approved for the surveillance of patients with a history of BCa.

## 2.5 UroVysion FISH Assay

The UroVysion Bladder Cancer Kit (Abbott Molecular Inc., Des Plaines, IL, USA) is designed to detect aneuploidy for chromosomes 3, 7, and 17 as well as loss of the 9p21 locus via fluorescence in situ hybridization (FISH). It is FDA-approved for analysis of urine specimens from subjects suspected of having BCa. Determination of results is conducted by enumeration of four-color fluorescent signals that are indicative of the copy numbers of chromosomes 3, 7, and 17 and of the *p16* gene, through microscopic examination of the nucleus. Results are intended for use in conjunction with (but not in lieu of) current standard diagnostic procedures for the initial diagnosis and surveillance of patients with a history of bladder carcinoma. Analysis requires a fluorescence microscope equipped with appropriate excitation and emission filters. Additional equipment can be used to standardize specimen processing and automate hybridization protocols. Studies that have evaluated the performance

of UroVysion have consistently shown that the test can achieve sensitivities in the range of 75 % [44–48], which is an improvement over cytology, and sensitivity increases for higher-grade tumors. Specificity is equal to or lower than that of cytology (65–96 %) [49], but the overall performance of UroVysion is an improvement over cytology alone. As with the ImmunoCyt assay, the test is dependent on the amount of tumor cells available on a cytology slide, so the detection of low-grade and early-stage tumors can be problematic. Also in common with the ImmunoCyt assay, the accuracy of the UroVysion test can be improved by combining the aneuploidy results with cellular morphology. Automated systems that facilitate this show an increase in accuracy, achieving up to 100 % sensitivity [50].

In some UroVysion-based studies, a number of false positives were reported, but follow-up studies have shown that in up to 50 % of such cases, BCa recurrence occurred within months [45]. This suggests that the chromosomal aberrations are evident before malignant lesions are detectable by cystoscopy or any other standard test. Accordingly, the best clinical utility might be in the surveillance of patients who have had BCa previously, where UroVysion would be used to check for aberrations associated with new lesions [51, 52]. The test takes advantage of the high occurrence of specific chromosomal abnormalities in urothelial cancers, but not all BCa lesions harbor these chromosomal aberrations, so the test can only detect a subset of malignancies. The technical demands of the test regarding sample preparation, specialized equipment, and trained personnel requirements as well as a lack of consensus on the definition of abnormality are reasons why UroVysion has not been approved to supersede standard evaluations, including cytology [44], as a standalone test.

## 2.6 Summary of Current Tests

In studies that have compared current tests in the same cohort, there is a trend that shows more robust performance of the cell-based tests (e.g., cytology, UroVysion, and ImmunoCyt). Because these tests are focused on specific cells in the sample, they are less impacted by confounders such as urinary milieu (e.g., gross hematuria, pH, osmolality), or by conditions caused by infection, or by instrument-induced damage associated with sampling [53, 54]. Of the cell-based assays, cytology remains the test with the best specificity, but UroVysion improves upon sensitivity rates [53, 55, 56]. Combining the tests described above does show some improvement over single tests [53, 55, 57]. For example, in a study of over 2,000 cases (>500 with BCa), the PPVs of the four individual tests—cytology, UroVysion, ImmunoCyt, and NMP22—were improved

upon when they were used in almost any combination, with the combination including all four tests performing the best [55]. However, although combining tests (i.e., monitoring multiple targets) improves accuracy, proprietary issues mean that this currently requires multiple, distinct tests to be performed, which is not technically or economically feasible.

Another way to combine single tests into more accurate evaluations is to include the test result in a nomogram that can predict the presence of BCa [29, 58], but it is clear that the derivation of multiplex molecular assays will be an important step towards overall accuracy rates that reach those of cystoscopy and cytology.

### 3 Investigational Biomarkers for Non-Invasive BCa Detection

#### 3.1 Blood-Based Detection of BCa

There are no blood-based biomarker tests currently in clinical practice for BCa detection or surveillance. Given the availability of urine for bladder disease monitoring, blood-based analyses have disadvantages that include dilution effects, the complexity of the serum milieu and proteome, and the issue of ascribing a blood-based biomarker to its actual source. The analysis of blood-based biomarkers may have more utility in bladder disease prognosis or informing therapeutic decisions. On the research front, a number of serum biomarkers have been reported to be associated with disease status and/or survival. Serum levels of CYFRA21-1 and soluble E-cadherin were found to be elevated in cases with advanced and high-grade tumors [9, 59]. Potential prognostic markers include MMP2 and MMP7 [60–62] and endostatin [63]. Serum levels of TGF- $\beta$ 1, uPA, and E-cadherin have been reported to be predictive for lymph node metastasis, disease-free survival, and cancer-specific survival [59, 64, 65].

Another major field of blood-based detection and monitoring for multiple cancers is the isolation of circulating tumor cells (CTCs). Viable tumor-derived epithelial cells are present in the peripheral blood of cancer patients with primary or metastatic disease. Although extremely rare, CTCs represent a potential alternative to invasive biopsies as a source of tumor tissue for the detection, characterization and monitoring of solid tumors. An in-depth discussion of the CTC field is beyond the scope of this review, but a few studies that have investigated this in BCa are described briefly below.

Given the rarity of CTCs and the difficulty involved in detecting and isolating them, the approach does not offer much for the initial diagnosis of primary disease [66, 67]. There may be more purpose in using this strategy to detect

residual disease after surgical intervention and as an aid to adjuvant therapy decisions, or for monitoring subsequent disease recurrence. Early studies used RT-PCR to detect BCa tumor cells in the patient's peripheral blood [68–70]. Amplification targets that were chosen to identify epithelial cells included CK-20 [68] and uroplakin II [69, 70], survivin [71], and EGFR mRNA [72]. These assays qualitatively determined the presence of CTCs in BCa patients. More recent techniques include immunomagnetic separations and digital imaging. One system that is being used clinically to some extent is the CellSearch assay (Veridex LLC), which employs antibodies targeted at epithelial cell markers (EpCAM and cytokeratins 8, 18, and 19). Using the CellSearch system in a BCa study, CTCs were detected in 44 % of patients with metastatic disease, and the number of detectable cells correlated with the number of metastatic sites [73]. In patients with metastatic cancer, assessment of CTCs may represent an earlier and more reproducible indication of disease status than current imaging modalities. In a clinical follow-up study, the CellSearch system was employed to show that preoperative detection of CTCs, even in patients with non-metastatic disease at the time of surgery, was associated with a worse overall survival compared to CTC-negative patients [74–77]. As technical aspects of CTC detection and analysis improve, the hope is that the characterization of individual cells in the blood will serve as a low-invasive, real-time approach for monitoring molecular changes in cancer occurring in an individual patient, and to track response to therapy. This would enable clinicians to customize treatment strategies as the disease evolves.

#### 3.2 Multi-Analyte Biomarker Assays

Given the limitations of the current tests described above, many investigative teams have proposed novel biomarkers for the non-invasive detection of BCa, primarily via urinalysis. Numerous biomarkers with promise have been reported in preliminary studies. Examples include Aurora kinase [78], CEACAM1 [79], telomerase [80], survivin [81], and hyaluronic acid/hyaluronidase [82, 83] at the protein or mRNA level; at the DNA level, mutations in the *FGFR3* gene [84, 85] have been proposed to be indicative of BCa. The sensitivities and specificities of these markers are encouraging in specific cohorts (reviewed previously, see [86, 87]), but as with the currently used single-marker clinical tests described above, they are necessarily limited by the fact that not all BCas—or even all of the cases in one specific category of lesions (e.g., stage/grade, growth pattern, etc.)—will harbor any particular molecular change [12]. What is needed are multiplex biomarker assays that can be developed into risk scores and nomograms such that an assay can be applicable over a broad range of disease

states and be robust against errors. Below we discuss some of the studies that report biomarker panels and tumor-associated signatures of BCa.

### 3.3 DNA Markers

Common cancer-associated genetic aberrations can be detected accurately by monitoring alterations in microsatellite markers and loss of heterogeneity. Many such aberrations have been documented in BCa, and a number of studies have analyzed microsatellite markers in voided urine samples. An early study monitored 17–20 microsatellite biomarkers across multiple chromosomes in serum voided urine sediment [88]. The most frequent mutations found in the urine samples were detected in chromosomes 8p and 9p. In a study that monitored nine microsatellite markers in patients with recurrent BCa, a panel of six markers achieved an overall accuracy of 86 % [89]. In a study of over 300 patients, Kompier et al. [90] monitored multiple mutations in five genes, including *FGFR3*, in bladder tumors. Mutations in individual genes were not overly prevalent (11–63 %), but mutations in one or more target genes were found in 88 % of primary tumors and 88 % of recurrent tumors. Mutation analyses have been applied successfully to voided urine sediments [91, 92], so multiplex assays are entirely feasible. If a diagnostic score could be derived from a mutation screen, it may have utility in diagnosis, and there is also potential for tumor-specific mutations to be useful in follow-up and surveillance for residual or recurrent disease, but these analyses may be more applicable to the stratification of patients for more tailored therapeutic decisions.

The analysis of DNA methylation in cells obtained from voided urine is also an approach that could query multiple gene targets and identify specific patterns associated with BCa. Early studies used methylation-sensitive PCR to identify methylation of the *DAPK*, *RAR $\beta$* , *E-cadherin*, and *p16* genes as being associated with BCa, and the detection of this panel in urine achieved a sensitivity of 91 % and a specificity of 76 % [93]. A similar approach identified the presence of *DAPK*, *BCL2*, and *TERT* gene methylation in 78 % of BCa patients, but they were found to be absent in age-matched controls [94]. Dumiel et al. [95] achieved 87 % sensitivity and 100 % specificity by monitoring hypermethylation of the *APC*, *RASSF1A*, and *p14* (*ARF*) genes. A quantitative PCR approach showed that the *TWIST1* and *NID2* genes are frequently methylated in urine samples collected from BCa patients. The two-gene panel achieved high sensitivity (90 %) and specificity (93 %) in almost 500 urine samples, significantly outperforming cytology in the same cohort [96]. Through the analysis of nine gene promoters, Hoque et al. [97] found that 69 % of 175 BCa patients had promoter methylation in at least one

of four genes (*CDKN2A*, *ARF*, *MGMT*, *GSTP1*), whereas the control cases had no such methylation detectable. By combining the data from all nine genes, a logistic prediction model was derived that achieved an overall sensitivity of 82 % and specificity of 96 %. Importantly, the majority of these studies showed that the methylation pattern was retained between the primary tumor and urine sediment DNA [97]. Numerous other genes have been proposed as valuable methylation biomarkers in recent studies [98–102]. Scher et al. [100] developed a nested methylation-specific PCR assay to detect BCa in small volumes of patient urine. The genes assayed were *BCL2*, *CDKN2A*, and *NID2*. In a pilot study, this assay achieved a sensitivity of 81 % and a specificity of 86 %. Costa et al. created a candidate list of methylation markers by assessing cell lines and tissue samples. The methylation status was quantified for two selected genes in 318 clinical samples. *PCDH17* and *TCF21* methylation levels in tissue provided a sensitivity rate of 92 % for BCa, but this dropped to 60 % in urine samples [102]. Chung et al. selected ten candidate hypermethylated genes identified in tumor tissue and tested them for detection of BCa in urine sediments using quantitative methylation-specific RT-PCR (qMSP). Using data from a cohort of 128 BCa patients and 110 age-matched control subjects, a multigene predictive model was derived. A methylation model comprising five target genes (*MYO3A*, *CA10*, *NKX6-2*, *DBC1*, and *SOX11* or *PENK*) achieved 85 % sensitivity and 95 % specificity for the detection of BCa [101]. These panels of biomarker targets look promising, but a definitive panel needs to be tested in larger cohorts with appropriate controls. The analysis of DNA from urinary sediment is based on PCR technologies, so it has the advantages of being relatively simple, potentially cost-effective, and quantifiable. As it is an amplification technique, it is also possible to achieve data from minimal sample materials.

### 3.4 RNA Markers

Transcribed, non-protein-coding microRNA (miRNA) molecules are key post-transcriptional regulators of gene expression [103]. To date, over 1,500 human miRNAs have been identified and characterized to some extent. Each miRNA controls the expression of multiple genes, so this molecular family may represent an opportunity to identify biomarkers of a higher order. Array-based profiling and deep-sequencing approaches for miRNA analysis are becoming routine technically, and studies targeting miRNAs as potential diagnostic biomarkers are increasing accordingly. Tumor tissue profiling studies have identified the expression of single miRNA transcripts as being associated with primary BCa or outcome, and some of the candidate biomarkers have been confirmed in urine

samples [104–109]. Encouragingly, more recent studies have derived signatures or panels of miRNA biomarkers with good diagnostic performance for urinalysis. Hanke et al. [110] examined the expression of 157 miRNAs in exfoliated urothelial cells using quantitative RT-PCR and reported that the ratio of miR-126 to miR-182 achieved 72 % sensitivity and 82 % specificity in a cohort of 47 samples. A quantitative PCR study of a panel of 15 miRNAs in 121 urine samples revealed that a combination of three miRNAs (135b/15b/1224-3p) detected BCa with a high sensitivity (94.1 %), but the specificity was lower (51 %) [111]. The combination of miR-222 and miR-452 has been reported to be helpful in tumor stratification and for non-invasive diagnosis [108], and the expression of miR-96 and miR-183 has been shown to augment cytology and to correlate with advancing tumor grade and stage [109]. Serum microRNA has also been investigated in BCa patients. Plasma miRNA was isolated from 20 patients with BCa 18 controls and profiled using arrays. Seventy-nine miRNAs were differentially expressed in patients with or without cancer, and logistic regression modeling was able to predict diagnosis with 89 % accuracy [112]. The miRNAs do hold promise as bladder biomarkers because they are resistant to nuclease degradation and are relatively stable within urine and serum [111, 112].

One of the most promising sources for the derivation of multiplex diagnostic biomarker signatures is the tumor cell transcriptome. Gene expression profiling studies of urological clinical material have focused on the analysis of excised solid tumor tissue. These studies have identified gene signatures that are associated with tumor stage [113, 114], disease recurrence and outcome prediction [113–115], and subtype classification [114]. The fact that follow-up studies have validated some of the biomarkers in independent tissue collections shows the potential utility of microarray profiling of solid tissue source materials [116, 117]; however, the molecular analysis of solid tissue is most applicable to the development of assays that will aid the histological evaluation of biopsy or excised tumor material. Normal tissue is not available for comparison for obvious reasons.

Analysis of naturally shed urothelia has several advantages (described above for existing and investigational assays)—stable material source, low impact of urinary milieu, comparison with morphological and other cellular markers, and availability of samples from the complete range of disease conditions and healthy controls. Through PCR amplification, the analysis can be performed on the often minimal cellular material obtained from naturally voided urine, and the detection methods are accurate, quantitative, and economical. A number of groups have profiled excised tissue, and some of the mRNA targets identified in such studies have been monitored in urine as

potential non-invasive biomarkers. Holyoake et al. [118] also published a study that used molecular profiling of solid tissues to identify genes that were overexpressed in tumor stages Ta, T1, or >T1, relative to non-tumor epithelial tissues and inflammatory cells. Using this strategy, *CDC2*, *MDK*, *IGFBP5*, and *HOXA13* were selected for the development of a quantitative RT-PCR urine assay for TCC detection and disease risk stratification of patients. RNA for the assay was extracted from voided urine samples, but it is not clear whether this extraction would have included urothelial cells or only soluble urinary RNA. The measurement of the combination of mRNA markers detected BCa at a sensitivity of 85 % and a specificity of 80 % across all stages, with the best performance obtained with stages >T1 and tumors >1 cm in diameter [118]. A recent study by the same team compared the performances of assays derived from this biomarker panel and currently under commercial development (uRNA Assay, Pacific Edge Ltd., Dunedin, New Zealand). In a cohort of 485 patients presenting with hematuria, the uRNA test achieved a higher sensitivity (62 %) than NMP22 (ELISA and BladderChek) and cytology at a pre-specified specificity of 85 %, and a modification of the assay derived from the 485-cohort data detected 82 % of BCa cases [119]. Hanke et al. analyzed the expression of a selected panel of mRNAs as biomarkers of BCa in whole urine, cell pellets, and clarified urine. In a cohort of 98 subjects, they found that the ratio of v-ets erythroblastosis virus E26 oncogene homolog 2 (*ETS2*) to urokinase plasminogen activator (*PLAU*) in whole urine facilitated the detection of BCa with a sensitivity of 75 % at 100 % specificity [120]. Other mRNA-based diagnostic urinalyses have targeted *BIRC5* (survivin), *HYAL1*, *KRT20*, and *MUC7* [121–123]. These targets performed similarly at sensitivities between 62 and 90 %, and confirmed that combinations of 2–3 mRNA markers perform better than single-target assays.

The feasibility of transcriptome profiling of the exfoliated urothelia present in urine has recently been shown [124, 125], and the approach has been used to identify tumor-associated profiles with high diagnostic accuracy [125, 126]. In one study, the genome-wide mRNA profiles of over 90 urothelial samples were subjected to advanced feature selection algorithms [127–129] to reveal an optimal gene signature for BCa prediction. A 14-gene signature was able to detect BCa with 100 % specificity at 90 % sensitivity in an independent cohort of 81 cases using quantitative RT-PCR [124, 126]. In comparison, cytological evaluation of this cohort diagnosed only 35 % of tumor cases correctly. In a study utilizing a similar strategy, a panel of 384 genes that were identified in tissue-based analyses was subsequently tested in urothelial samples using quantitative RT-PCR [125]. Analyses identified a 12-gene signature that achieved high accuracy (89 %

sensitivity and 95 % specificity) in identifying BCa cases in a cohort of 211 subjects. Despite significant differences between the studies with respect to the biomarker discovery phase, both groups were able to derive molecular signatures that could accurately classify BCa samples. This demonstrates that a multiplex quantitative RT-PCR test of voided urine samples holds promise as a non-invasive urine-based assay in the evaluation of patients being investigated for BCa. Although a quantitative RT-PCR test has some upfront processing requirements, it has the advantage of being developed into an assay that can be automated and highly standardized for consistency between laboratory sites.

One of the major advantages of multiple biomarker assays is that the results can be input into algorithms that can provide a continuous score for predicting disease status or prognosis. This provides much more useful information than cut-off data, and is being used in clinical tests in the breast cancer prognosis field [130]. Furthermore, algorithms that incorporate clinical data and molecular risk scores into a nomogram can give the physician the most valuable guidance regarding patient management decisions [131, 132].

### 3.5 Protein Biomarkers

Numerous investigations have tested the diagnostic utility of various proteins that have previously been associated with bladder disease status. The majority of studies have tested single biomarkers in diverse cohorts, or combined novel and current tests in combination [133], but increasingly multiplex combinations are being evaluated. A study by Abogunrin et al. evaluated 23 previously reported protein biomarkers for bladder disease in urine from a cohort of 80 patients with BCa and 77 controls. Univariate analysis revealed that nine biomarkers were significantly differentially expressed with respect to cancer burden. Multivariate algorithms that combined demographic information (age and smoking history) with molecular data significantly improved performance compared to demographic information alone. A combination of NMP22, BTA, serum CEA, EGF, and thrombomodulin enabled sensitivities of up to 91 %, and specificities of up to 80 % [132]. Based on the fact that cancer often involves inflammatory processes, Margel et al. [134] monitored a panel of immune modulators in urine to investigate biomarker potential. The panel included 15 heat shock proteins and cytokines, monitored by commercial ELISA assays. The combined urinary concentrations of HSP60 and IL-13 significantly improved the performance over any single factor.

The discovery of novel protein biomarker panels has surged recently due to advances in high-throughput

proteomic technologies. The appropriate use of these approaches has the potential to provide highly efficient biomarkers for BCa detection and monitoring. Protein-based biomarkers have several advantages over nucleic acid targets. Only proteomic profiling enables the evaluation of global changes in gene expression that result from both transcriptional, translation, and post-translation modifications. Although genomics may be more amenable to comprehensive surveys, phenotypic changes can only manifest themselves through altered protein expression, so the identification of protein factors involved in bladder disease can best inform us of tumor biology. Beyond their diagnostic and prognostic value, protein biomarkers provide potential therapeutic targets and represent markers of disease progression, treatment response, and other clinical utilities.

Proteome profiling studies have tended to identify factors that can classify tumors or predict patient outcome or disease recurrence, but biomarkers identified in solid tissue may be subsequently translated into a serum or urinary test, and some have shown promise as urinary diagnostic markers. Examples from proteomic analyses include psoriasin for squamous cell carcinoma [135], TACSTD2 [136], and cystatin B [137], and panels for serum-based diagnosis [138], but direct proteomic analysis of the urine is more likely to reveal promising non-invasive diagnostic biomarkers. Early urinary profiling studies used gel-based technologies to define the urinary proteome and to begin to identify proteins associated with BCa [139–142], but advances in MS technology have been rapidly applied to the profiling of bladder tissues, serum, and urine. Vlahou et al. [143] used SELDI-TOF to compare the proteomic profiles of urine samples from healthy controls and patients with transitional cell carcinoma of the bladder. Multiple protein changes were reproducibly detected in the cancer group, including five potential novel biomarkers and several protein clusters. One of the biomarkers, alpha-defensin, was subsequently shown to be present in bladder tumor cells. The combination of the biomarkers and protein clusters significantly improved the accuracy of patient classification. In a separate cross-validation study by the same authors [144], alpha-defensin monitoring was used to detect BCa with better sensitivity and specificity than commercial tests. Theodorescu et al. [145] used capillary electrophoresis (CE)–mass spectrometry to identify urinary biomarkers for BCa in a training set composed of 46 patients with urothelial carcinoma and 33 healthy volunteers. These were further refined using CE-MS spectra of another cohort of urine samples from healthy volunteers and patients with malignant and nonmalignant genitourinary diseases. Using this two-step approach, a diagnostic biomarker signature of 22 urinary peptides was established. In a validation study, this signature enabled the correct



classification of all urothelial carcinoma patients in a test set containing 31 urothelial carcinoma patients and 138 nonmalignant genitourinary disease patients [145].

Another study used an isobaric tag for relative and absolute quantitation (iTRAQ) technique to discover proteins that were differentially expressed between pooled urine samples and non-tumor controls. This strategy identified 55 candidate biomarker proteins. Orthogonal techniques confirmed that the level of apolipoprotein A-I (APOA1) was significantly elevated in urine samples from BCa patients. Using a commercial ELISA assay, APOA1 was confirmed to have high diagnostic potential in an extended sample set [146]. Using a glycoprotein enrichment strategy to profile urine samples from 100 subjects (54 with cancer), Yang et al. [147] identified a panel of glycoproteins associated with BCa. The most discriminatory protein in that study was alpha-1-antitrypsin (A1AT), also known as SERPINA1. In an independent validation cohort of 70 subjects, A1AT measurement by ELISA achieved a sensitivity of 74 % and a specificity of 80 % [147]. Through integration of proteomic and genomic urine sample profiling data [124, 126, 147, 148], we identified panels of promising biomarkers for inclusion in diagnostic urinalysis assays. Combinations of 2–3 biomarkers were analyzed by ELISA in a series of studies [149–151], and multivariate analysis identified an eight-protein biomarker panel that achieved 92 % sensitivity and 97 % specificity in cohorts of 64 patients with BCa and 63 controls [152]. The performance was far better than current urinalysis tests in the same cohort. Validation of these multiplex biomarker panels in larger, more diverse cohorts is underway. BCa biomarker panels discovered by proteomic profiling have also been derived from serum samples. Schwamborn and colleagues searched for discriminating protein patterns in serum using magnetic bead-based separation followed by MALDI-TOF MS. Multidimensional analyses of serum samples from 105 patients with BCa, 98 healthy controls, and 45 prostate cancer patients generated algorithms capable of distinguishing between cancer patients and healthy individuals. The best algorithm achieved 96 % sensitivity and 86 % specificity [138]. The studies described above show the power of MS-based urinary analysis for the discovery of biomarkers. The latest proteomic technological developments, such as arrays for phosphoproteins, glycoproteins, or phospholipoproteins, can reduce the sample complexity that plagues the proteomic analysis of biological fluids. As these techniques are applied to BCa samples, there will likely be further advances in urinary biomarker discovery.

### 3.6 Metabolomic Biomarkers

The most recent developments in biological fluid analyses have come in the field of metabolomics. The application of urine-based metabolomics using high-pressure liquid

chromatography (HPLC) or nuclear magnetic resonance (NMR) with multivariate analysis can identify specific metabolites or profiles that can aid cancer diagnosis [153]. To date, only a few studies have investigated differential urine metabolite profiles associated with BCa. Using HPLC, Issaq et al. [154] profiled urine samples from 41 patients with BCa and 48 healthy controls. Statistical analyses allowed at least 40 of the 41 BCa cases to be predicted correctly, but the specific metabolites identified in that study have not been confirmed elsewhere. In a similar study format, Pasikanti et al. [155] identified a 15-marker metabolite model that achieved a 100 % detection rate for BCa in 24 patients. Another study of 58 clinical specimens identified 35 metabolites associated with BCa. The metabolic signature distinguished both normal and benign bladder from BCa, and even showed promise in distinguishing tumor stages [156]. There was no overlap between the profiles identified in the studies, and it remains to be seen whether these preliminary urinary studies can be built upon. A recent study used NMR-based metabolomics to investigate differences in serum metabolic profiles associated with BCa. In a study of 67 patients and 25 healthy controls, serum sample profiles from BCa patients suggested perturbed metabolic pathways of aromatic amino acids, glycolysis and the citrate cycle, and lipogenesis [157].

Changes at the metabolite level may be detectable in biological fluids before the appearance of clinical symptoms, making them potentially useful early detection biomarkers, and metabolic profiling can provide insights into bioprocesses perturbed during tumor development and progression; however, there are major problems with urinary metabolomic profiling. The analytes are small metabolites that are not always filtered by the kidney, so confounding factors such as polypharmacy or even recent dietary intake can create large variations between individuals, so such studies require large cohorts and standardization of sample collection and processing.

## 4 Conclusions

Detecting BCa using diagnostic markers remains a challenge. The inadequate power of single markers may partly explain this. The concept that the presence or absence of one molecular marker will aid clinical evaluation has not proved to be the case. This makes sense when one considers the complex interactions between various molecules within a single pathway, the cross-talk between molecular pathways, and the oligoclonality of many tumors. Advances in molecular techniques, especially profiling approaches, have enabled investigators to derive a new generation of compound molecular diagnostic signatures that may

provide assays with the desired clinical utility. Technology is also extending the range of molecular classes that may serve as potential biomarkers. Potential biomarkers that can provide valuable information and are amenable to urinalysis include proteins, mRNA and microRNA, DNA markers that include mutated sequence or epigenetic information, and metabolite concentrations. It may well be that the most efficient diagnostic signatures are multifaceted, including a mix of molecular classes, and instead of trying to replace existing clinical criteria in patient evaluation, combining both clinical and biomarker information in nomograms is a promising strategy.

Multiplex marker systems for BCa diagnosis are still at an early stage compared with the FDA-approved markers. Signatures and panels of markers have been derived and tested on varied cohorts, and require further validation in independent studies. Validation studies that incorporate more diverse cohorts may require the adjustment of combinatorial assay components to match performance data derived from the initial discovery studies, but that flexibility is one of the advantages of multiplex marker assays, and the hope is that they will provide robust tests that can be informative across the broad range of clinical presentation. If fully tested and optimized, multiplex diagnostic assays may enter the clinical setting to augment, or eventually even replace, cystoscopy and/or cytology for diagnosis and recurrence monitoring. Such assays may also guide other aspects of patient management, including therapeutic intervention decisions and monitoring of response to treatment.

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