Bladder tumor markers: from hematuria to molecular diagnostics – where do we stand?

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Abstract

Bladder cancer is a common malignancy in the USA. Currently, the detection of initial tumors and recurrent disease is based on evaluation of voided urinary specimens, often followed by cystoscopy. With the high rate of recurrence, cystoscopies are regularly repeated with the aim of halting progression of the disease. For patients, this process is fraught with anxiety, pain and high cost. As a result, intense work is being done in the field of bladder tumor markers with the goal of identifying bladder cancer earlier, both in the initial diagnosis and in recurrences of known tumor. The possibility of identifying a marker that could noninvasively differentiate benign and malignant causes of hematuria, and identify recurrences prior to their pathologic progression is the objective of this area of research. Currently, a large number of tumor markers exist, each scrutinized in both the laboratory and in clinical trials. Here we present many of the most widely used and tested markers. Background details are provided as to the mechanism of detection of malignant cells, the results of recent trials and future directions of study. Some novel modalities for tumor detection are also presented. The next few years will no doubt bring newer markers and lead to the elimination of others. Studies continue to refine the role of these markers in clinical practice, but their ultimate efficacy will need to be borne out in large-scale clinical trials in a multitude of settings.

Keywords

bladder cancer; diagnosis; surveillance; tumor marker

The National Cancer Institute and the American Cancer Society estimate that approximately 67,000 new cases of bladder cancer were diagnosed in 2007, with approximately 14,000

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deaths expected to occur – almost 40 per day [1]. The vast majority of patients are over 55 years of age. The lifetime risk for a man developing this cancer is approximately one in 30, and for a woman, one in 90. Racial differences also exist in bladder cancer epidemiology, with African–Americans having a lower incidence yet more severe forms of the disease compared with Caucasians [2], possibly explained by diagnostic delay in these groups.

For patients with bladder cancer, the extent of the disease at presentation has a significant impact on treatment options and outcomes. At the time of diagnosis, between 16 and 30% of patients will have muscle-invasive bladder cancer. This bodes a much worse prognosis and in many cases the treatment of choice is cystectomy [3,4]. A total of 70–85% of patients will present with nonmuscle-invasive disease of either high or low grade. Often these patients are initially treated endoscopically without the need for radical surgery. Unfortunately for these patients, bladder cancer has the highest recurrence rate of any solid tumor [5]. This often results in extensive follow-up surveillance and testing. Without such close observation, a percentage of patients will recur with muscle-invasive disease, leading to the morbidity of invasive surgery and, possibly, the mortality of metastatic disease [6].

Great strides have been made over the last decades in the treatment of bladder cancer, including the use of chemotherapy [7], novel surgical and bladder-sparing approaches, and intravesical treatments, which have proven very effective in the appropriate clinical scenario. Throughout this time the mainstay of the diagnosis of lower tract urothelial cancer has been cystoscopic and cytologic evaluation of the bladder, most often initiated upon the finding of microscopic or gross hematuria. Given the intermittent nature and specificity of hematuria, the American Urological Association (AUA) currently recommends cytology and cystoscopy (along with upper tract imaging) in high-risk patients, identified as those with a history of smoking, those over 40 years of age, and those with certain chemical exposures among other risk factors [8].

The cost of such testing is extremely high. In 2003, Botteman *et al.* calculated a cost of almost US$200,000 for treating a patient with bladder cancer for a lifetime. This included initial treatment, follow-up with frequent cystoscopic and laboratory analysis, and subsequent treatments of recurrent and metastatic disease [9,10]. Frequent testing also exposes the patient to other risks, including infection from instrumentation of the lower urinary tract, bleeding from minor trauma and contrast reaction from upper tract imaging.

As a consequence of the costly and invasive nature of cystoscopy and the large number of negative evaluations obtained with voided urine cytology, much research is ongoing into the field of bladder tumor markers. The search is underway for a marker or test that can reliably predict those patients at highest risk of bladder cancer and possibly stratify them further by likelihood of having muscle-invasive disease or high-grade tumors [11,12].

The characteristics of an ideal tumor marker have been examined in detail in the literature [12,13]. A brief overview will be presented here. Of primary concern is the sensitivity or specificity of any marker. A decrease in either of these areas puts the patient at risk: a diminished sensitivity will result in tumors present in the bladder not picked up by the testing, with the associated risk of progression; diminished specificity will result in an

unnecessary cystoscopy for a patient without tumor. The marker or test should also be easily and accurately reproducible. Since the goal is to avoid unnecessary cystoscopy, the test should be noninvasive, at most requiring catheterization of the patient but preferably being able to evaluate voided urine samples. As a screening test, an inexpensive cost and ease of use is highly desirable. Yossepowitch *et al.* and Vriesema *et al.* also showed that patients desire an accurate test, being unwilling to forgo cystoscopy based on markers than have less than 90% sensitivity [14,15].

Currently, a number of biomarkers exist for evaluation of bladder cancer (Table 1). They can be broken down into a number of subgroups based on the target of their assessment. These groups include soluble antigens, cellular morphology, cell surface antigens and molecular genetic alterations. Soluble antigens, which include BTA-Stat and BTA/TRAK (Polymedco Inc., NY, USA), NMP-22, BCLA-4, survivin, hyaluronic acid–hyaluronidase (HA–HAase), hematuria and urinary bladder check (UBC), can be detected without tumor cells being present in the sample. These tests use proxy markers initially associated with urothelial cells, which can then be assayed in the urine [11]. Cellular morphology is the evaluation of a voided cytology specimen. This requires direct microscopic visualization of the cells by a pathologist. Tests evaluating cell-based antigens include Ucyt, UroVysion (Abbott Labs, IL, USA), DD23, microsatellite DNA analysis (MSA), cytokeratins, and telomere repeat amplification protocol (TRAP) and h-TERT assays (telomerase). These tests detect tumor cell-associated proteins (Ucyt and DD23) or antigens, chromosomal aberrations (UroVysion and MSA) and ribonucleoprotein enzyme assays (telomerase). Finally, a newer area of intense research aims to evaluate genetic aberrations associated with tumor formation and propagation. Recent advances in the use of tissue microarrays and molecular profiling have allowed a large number of cell-cycle- and angiogenesis-related markers to be identified and evaluated.

Soluble tumor markers

Hematuria detection

No discussion of bladder cancer can begin without an analysis of hematuria. Hematuria is the most common presenting symptom in bladder cancer, seen in approximately 85% of patients with the disease [16]. The origin of hematuria in bladder cancer is from direct hemorrhage of the tumor, however minor it may be. Hematuria may be either microscopic or gross. The AUA defines microscopic hematuria as more than three red blood cells per high-powered field in at least two of three specimens collected at different times [8]. Gross hematuria refers to the patient being able to directly visualize some degree of blood in the urine [17]. However, hematuria can be caused by a variety of benign conditions, including menses, urinary tract infection, trauma and prostatic hypertrophy, and, as a result, the specificity of hematuria to detect bladder cancer is quite low. Since hematuria is intermittent in nature, sensitivity of hematuria, in one-time voided urine specimen, is variable (47–74%) [18]. Repeat testing has shown a better sensitivity of between 90 and 95%. As a result, many authors advocate cystoscopic examination in the event of a specimen with hematuria even when a repeat examination shows none.

Hematuria screening has been widely discussed recently. For example, in their study of home-screening for hematuria, Messing *et al.* found that patients were detected at earlier stages of the disease and had a decreased mortality from bladder cancer [19]. The authors felt that such a screening protocol (weekly in their case) is able to detect cancers destined to become muscle invasive and intervention can occur earlier. As a result, in their series no screened patients died of bladder cancer. There was no significant difference in the percentage of patients from the screened and nonscreened group that died of nonbladder cancer causes. Clearly, no absolute conclusions can be made on the basis of this one study; however, screening shows a great deal of promise in selected populations and further testing will be needed to validate these findings.

Another almost universally used noninvasive screening tool for bladder cancer is urine cytology. This refers to the pathological evaluation of a voided urine sample by light microscopy. Many authors point out that urine cytology is not a point-of-care marker as it needs interpretation of the urothelial cells in the specimen by a trained pathologist, along with significantly time-intensive preparation. This notwithstanding, cytology is very useful in certain situations. In high-grade tumors, in particular carcinoma *in situ*, cytology has been shown to be very specific (close to 100%) and with very few false-negative results. Conversely, the results are less impressive with low-grade disease. Most studies report the sensitivity of cytology in these cases as between 35 and 65% [20,21]. Confounding factors in cytologic evaluation of the urine include tumor load and sampling error. Studies have shown that even in the setting of high-grade tumors, up to 23% of urine samples contain no tumor cells [22]. Most samples evaluated cytologically are voided; however, bladder washing has been shown to increase diagnostic yield of cells [23]. In addition, evidence of atypical cytology may precede cystoscopic confirmation of tumor by varying amounts of time [24]. This can expose the patient to multiple cystoscopies over time and a great deal of anxiety and trepidation.

Given the shortcomings of the widely used methods for non-invasive evaluation of bladder cancer used currently, the search for and evaluation of various bladder tumor markers is intense. Presented here is an overview of the currently tested and used markers, and the state of the latest research. While this is not a complete list, most markers under scrutiny can be found below.

BTA-Stat/BTA-TRAK

These tests detect a bladder tumor antigen now identified as human complement factor H and complement factor H-related protein in the urine [25]. BTA-Stat is a point-of-care test, performed by placing five drops of voided urine samples into the manufacturer-provided test device and qualitatively interpreting the result after 5 min [26]. BTA-TRAK is an ELISA test that measures the levels of these antigens in urine samples. These tests have been used as adjuncts, both in the initial diagnosis of bladder cancer and in surveillance for recurrent disease [27]. In the evaluation of both new and recurrent tumors, Holmang *et al.* found the sensitivity of detection of this test to be 75 and 54%, respectively. In his study, cytology had a greater than 92% sensitivity and specificity [28]. These results echo the findings of a large number of authors who have published data reporting sensitivities between 8 and 89%, and

specificity between 50 and 70% [13,29–31]. In healthy individuals, the specificity is improved, however, in patients with a history of benign urologic conditions; for example, in BPH and nephrolithiasis, it drops precipitously [32,33].

Recently, the results of the FinnBladder group's follow-up studies have been published examining the utility of BTA-Stat in recurrent disease [34]. They note an overall 56% sensitivity and 19.2% specificity for the BTA-Stat test, but much higher sensitivity (85.7%) and specificity (98.3%) for cytology. This is comparable to the cytology values of 85.7% and 98.3%. They found a significant number of false-positive results in patients with a history of Bacille Calmette-Guérin (BCG) instillation and urinary tract infection. The authors conclude that cystoscopy is still required to evaluate recurrent disease, but BTA-Stat may have some utility in the follow-up of patients with low-grade disease.

Summary—BTA-Stat and BTA-TRAK are markers that assay for complement factor H and H-related protein in the urine. It has displayed a lower sensitivity and specificity compared with voided cytology. Its accuracy in high-grade disease is improved; however, the high rate of false-positive results in the setting of nonmalignant urologic conditions makes its clinical utility limited.

NMP-22

NMP-22 is a nuclear matrix protein involved in chromatin distribution during the replication phase of the cell cycle. Expression of this protein is increased in malignant cells, with cell death resulting in release into the urine [35]. Two different tests make use of this tumor marker: the NMP-22 ELISA and BladderChek (Matritech Inc., MA, USA). Various authors have investigated this marker and report sensitivities of 47–100% for the ELISA test. The specificity of the ELISA was reported to be between 60 and 80% [36,37]. These values depend on the concentration of the marker used as the cutoff limit, in order to infer a positive test result [38,39]. Sharma *et al.* identified six categories of conditions that can result in a false-positive result on the NMP22 test and these include benign inflammatory conditions (e.g., infections), renal or bladder calculi, foreign bodies (stents or nephrostomy tubes), bowel interposition, other genitourinary cancer and instrumentation [40]. The NMP test shows differences in sensitivity when applied to primary and recurrent tumors. Multiple authors have reported that sensitivity decreases markedly during detection of bladder cancer recurrence [41,42]. One hypothesis is that this is related to the reduced tumor burden in recurrent disease versus primary disease. However, Stampfer *et al.* reported 49% sensitivity and 92% specificity for NMP-22 to detect recurrent bladder tumors. The authors note that NMP-22 was superior to cytology without the interoperator variation seen in a subjective test. Of note, they state that 6.4 units/ml should be the most appropriate concentration used as a cutoff [43].

The BladderChek is a point-of-care test that requires four drops of urine to be placed on the device, with a result returned within 40 min [44]. Fewer studies have evaluated BladderChek given its recent arrival to the market. Witjes *et al.* reported a sensitivity and specificity for BladderChek of 57.1 and 89.8% compared with 42.9 and 93.2% for cytology, respectively. They conclude that, while convenient and rapid, BladderChek did not enhance urine tests

already available [45]. Recently, Grossman *et al.* reported that the combination of BladderChek and cystoscopy was more effective in detection of bladder cancer recurrence than cystoscopy alone, although, BladderChek showed 49.5% sensitivity, suggesting limited utility of this test as a stand-alone marker [46]. In an earlier study, involving the evaluation of high-risk individuals and of patients exhibiting symptoms often seen in urothelial cancer, Grossman *et al.* also reported that the BladderChek could significantly increase the accuracy of cystoscopy compared with voided cytology. In that study, BladderChek was referred to as the point-of-care proteomic test that measures the nuclear matrix protein NMP22. The sensitivity of BladderChek (55.7%), although higher than cytology (15.8%), was again low for consideration as a standalone marker. The specificity of NMP-22 was 85.7% compared with 99.2% in cytology [47].

Summary—NMP-22 has shown some promise in detection of recurrent tumors. More studies are needed to reach a consensus on cutoff levels of urinary marker in testing. As with other assays, in the setting of other urologic pathology, the utility of this test is limited. While some studies do report a low sensitivity and specificity for the NMP-22 test, the overall published results are variable and work continues to be done evaluating this marker.

Hyaluronic acid–hyaluronidase test

A glycosaminoglycan that has been shown to promote tumor progression and metastasis, HA and the enzyme that cleaves it into further biologically active products, HAase, have been shown to be promising urinary markers for bladder cancer [48,49]. The HA–HAase test is a combination ELISA-like assay for which authors have reported between 83 and 94% sensitivity and 60–80% specificity [13,50–52]. The lower specificity to detect recurrent disease may be related to the test's ability to detect a bladder tumor nearly 5 months before its clinical detection [49]. One area where the HA–HAase test may show a great deal of promise is in the detection of low-grade and low-stage disease. In this scenario, the sensitivity has been reported as between 75 and 100% [53–55]. Given that this group of cancers are the most difficult to detect with conventional methods and yet the most likely to recur, these findings suggest significant strides in noninvasive surveillance in this group. Sensitivity of this test in noninvasive disease (<T2) was found to be 87% and, for invasive disease (T2 disease), 100% [53]. Furthermore, false-positive tests have been shown to portend a four- to tenfold increase in the risk of recurrence of bladder cancer [50]. Work recently carried out by Eissa *et al.* has shown that HYAL1 mRNA detected by reverse transcriptase (RT)-PCR from exfoliated cells has a greater than 90% sensitivity and specificity for tumor detection [56].

Summary—HA–HAase is a promising urinary tumor marker. It is efficacious in detection of low-stage/low-grade disease, an area often underdetected by conventional methods. Further studies will need to be performed in larger cohorts; however, this test has potential for clinical utility in the outpatient setting.

Survivin

Survivin is an anti-apoptotic protein that has been found to be a promoter of bladder cancer progression [57–59]. It is measured using a Bio-Dot microfiltration detection system (Bio-

Rad, CA, USA). In human trials, survivin has been reported to have a sensitivity of 64–100% and specificity of 78–100% [13,60–63]. Recently, Shariat *et al.* reported that survivin expression was associated with bladder cancer recurrence, disease-specific mortality and all-cause mortality [64]. They concluded that the addition of survivin improved the diagnostic accuracy of bladder cancer without requiring additional invasive testing. Weikert *et al.* performed a quantitative analysis of survivin mRNA in bladder tumor and urine specimens and found that survivin mRNA levels correlate with pathologic stage. Furthermore, high survivin mRNA expression correlates with decreased disease-free survival. Based on these results, Weikert *et al.* concluded that survivin mRNA may predict disease-free survival in certain patients, while being more sensitive and specific than cytology in the detection of bladder cancer [65].

Summary—Survivin has been shown to be as sensitive and specific as cytology in the detection of bladder cancer. Some authors currently advocate its use as an adjunct to cytology in the evaluation of primary hematuria or of recurrent disease [66]. More studies are necessary to clearly delineate the utility of this test.

Cytokeratins

Cytokeratins are cytoskeletal intermediate filament proteins found in a large number of cells. Some, such as 8, 18, 19 and 20, have been investigated as tumor markers in bladder cancer after authors had noted increased gene expression in malignant urothelial cells compared with normal cells [67]. Two assays exist based upon cytokeratins 8 and 18: the UBC-Rapid and UBC-ELISA tests. Cytokeratin 19 is detected by the CYFRA 21 – an ELISA that assays for fragments of this filament protein dissolved in the urine. Finally, cytokeratin 20 is detected using RT-PCR, which requires the presence of cells in the specimen. Pariente *et al.* showed that cytokeratin 19 detection has 96% sensitivity and 74% specificity to detect bladder cancer and is superior to cytology (43% sensitivity). They also noted a significant difference in cytokeratin 19 expression among patients with urothelial cancer compared with those with benign urologic conditions and normal controls [68]. One hindrance to the use of cytokeratin-19 is the unacceptably high false-positive rate seen in patients with a history of BCG instillation [69]. The UBC tests initially returned promising results, being more accurate than NMP and BTA-Stat in some early studies [70,71]. The variability of cutoff values between different studies has hindered further comparison of UBC, as has its low sensitivity in the detection of low-stage tumors. Overall, the sensitivity of UBC varies between 13 and 75%, and for Grade 2 or higher tumors, it is between 35 and 79% [13,70,72–74]. Early studies of cytokeratin-20 had demonstrated sensitivity and specificity quite comparable to cytology, but this has been difficult to replicate in follow-up analyses [10,75,76]. Inclusion of HYAL1 (a tumor-derived HAase) mRNA measurement has been shown to improve the accuracy and sensitivity of cytokeratin-20 mRNA measurement to detect bladder cancer, and the performance of the combined marker is superior to the individual markers [56].

Summary—Cytokeratins, a class of proteins found in cellular skeletal components, have shown some promise in early studies of their use in detection of primary and recurrent

bladder cancer. Later studies have not replicated these results and more work is needed to elucidate the role of cytokeratins in the management of bladder cancer.

BLCA-4

A nuclear matrix protein, BLCA-4, has drawn much interest based on early reports that it was found throughout bladder epithelium (both in tumorous and nontumorous regions) in patients with bladder cancer but not seen in those without bladder cancer [77,78]. A role for this protein as a marker for the 'field' effect of pro-oncogenic urothelium was implied by these studies. Authors have reported over 90% sensitivity and over 90% specificity in the ELISA test [78,79]. These promising results have also been confirmed in studies of patients with chronic genitourinary disease, where BLCA-4 was not correlated with a history of urinary tract infection or chronic catheterization [80]. The authors noted that the cutoff value for their assay and optical density units may need to be adjusted based on clinical scenario.

Summary—BLCA-4 shows promise in early studies with a high sensitivity and specificity. It appears to be more resistant to the confounding effects of benign genitourinary disease. More work is needed to evaluate the most appropriate cutoff levels for this test in the setting of malignant and benign disease of the bladder.

Soluble Fas

Soluble Fas (sFas), is generated by alternative splicing of Fas mRNA [81]. Fas is a cell surface receptor for Fas-ligand (Fas-L). Upon Fas–Fas-L interaction on the cell surface, the cells undergo apoptosis via the extrinsic pathway. sFas blocks Fas–Fas-L interaction and, hence, inhibits apoptosis [82,83]. Svatek *et al.* found higher levels of sFas associated with higher tumor stage (T1) and sFas outperformed NMP22 in terms of sensitivity and specificity. They conclude that urinary sFas is an independent predictor of recurrent disease among patients with a history of noninvasive disease, and that potential exists for its use in a wider surveillance regimen [81].

Summary—sFas, an anti-apoptotic protein, is overexpressed in bladder tumor cells and has been shown to be a predictor of bladder cancer recurrence with the ability to detect higher stage tumors. Current research is ongoing into this marker and it shows promise for future clinical utility.

Matrix metalloproteinases & tissue inhibitor of metalloproteinases

Matrix metalloproteinases (MMPs) are a heterogeneous group of enzymes functioning in the degradation of the extracellular matrix. This degradation is one of the most important steps in the progression of urothelial carcinoma, and a requirement for invasive disease of the tumor. Two commonly investigated MMPs known as gelatinases, MMP-2 and MMP-9, have been evaluated as diagnostic markers for bladder cancer, as has tissue inhibitor of metalloproteinases (TIMP)-2, an inhibitor of MMP activity [84]. It is the imbalance of the normally well-regulated MMP and TIMP system that results in the invasive nature of some tumor cells. Investigations are ongoing into the utility of these markers; however, preliminary data have been promising. Recently, in a study involving 154 bladder cancer (including 136 bilharzial) and 90 nonbladder cancer patients, Eissa *et al.* showed that

MMP-2 and TIMP-2 levels inversely correlate with tumor grade, and MMP-9/TIMP-2 levels correlate with bilharziasis [84]. Conversely, Kanayama *et al.* reported higher expression of MMP-2 and TIMP-2 in muscle-invasive bladder tumors than in nonmuscle-invasive tumors. Furthermore, higher MMP-2 and TIMP-2 levels correlated with poor prognosis and reduced survival even after cystectomy [85]. Sier *et al.* reported markedly elevated MMP-2 and MMP-9 activity in the urine of patients with invasive disease compared with those with nonmuscle-invasive bladder cancer [86]. In this study, MMP activity was also found in noninvasive tumors, suggesting a role of these matrix-degrading enzymes in the earlier stages of the disease. Thus, MMP levels probably increase and correlate with invasiveness of bladder tumors, and therefore may have diagnostic and prognostic potential in predicting bladder tumor progression [87,88].

Summary—MMPs and TIMPs have been shown to have increased urinary expression in patients with bladder cancer. There appears to be a relationship between the degree of invasiveness of the tumor and the levels of MMP expressed. Further work elucidating the role of MMPs, especially in predicting invasive and advanced disease, is currently being carried out. Currently, the use of these tests remains limited.

Cell-based antigens as tumor markers

Telomerase

Telomeres are multiply repeated sequences of ribonucleotides that are found at the end of chromosomes [89]. With each successive replication in cell division, these telomeres are lost and, eventually, the lack of telomeres induces chromosomal instability and cellular loss. Telomerase is a ribonucleoprotein that is expressed by tumor cells and that regenerates the telomeres at the end of the replicating chromosomes, essentially creating a cell able to perpetually regenerate [90,91]. The TRAP assay detects telomerase expressed in exfoliated urothelial cells in urine. Also available is a RT-PCR assay that measures a subunit of telomerase, hTERT. Eissa *et al.* evaluated three methods for detecting telomerase efficiency, namely the TRAP assay, RT-PCR of hTR and hTERT mRNA by quantitative RT PCR. They report that the detection of hTERT in urine improved the sensitivity and specificity in diagnosing urothelial cancer compared with the other methods [92]. Recent work by a variety of authors shows a favorable specificity of between 85% and 100% for telomerase tests. Sensitivity, however, varies quite greatly, between 9 and 100%. This variation is probably related to the poor stability of telomerase in the urine sample [92–95]. Bravaccini *et al.* recently reported that in 50% of study subjects, in whom cytology was either negative or inconclusive, telomerase assay had 88% sensitivity and 65% specificity. They also note a high accuracy from samples containing as few as 100 urothelial cells. They conclude that the major advantage of this test is in the case of negative or inconclusive cytology [96]. A confounding factor in the use of these tests is their high false-positive rates in the setting of inflammation, which is probably related to the presence of lymphocytes in the specimen [97,98].

Summary—Telomerase is a promising marker that may have utility in the setting of negative or nondiagnostic cytology. It does have significant limitations, including false-

positive results due to inflammation or infection and the instability of telomerase in the harsh urine environment. Telomerase may ultimately become a useful diagnostic marker; however, the need for very precise handling of specimens must first be addressed.

Fluorescence *in situ* hybridization

The basis of the FISH test, known in the marketplace as UroVysion, is that certain chromosomal aberrations are seen more commonly than others in bladder cancer [99,100]. The UroVysion test is a multitarget FISH assay for detecting the gain of chromosomes 3, 7 and 17, and the loss of chromosome locus 9p21. The cells labeled with chromosome 3, 7 and 17 centromeric fluorescent probes and a 9p21 locus-specific probe are then evaluated microscopically for positive staining, and a 'positive' FISH test is determined by criteria based on numbers of cells and the number of chromosomes (3, 7 and/or 17) gained or lost (9p21). One point of contention in the evaluation of FISH is that there is little widespread agreement on the criteria that distinguish a positive FISH test from a negative one [10]. An area of research where FISH appears to have a significant advantage is in the evaluation and detection of lesions that have not become visually apparent. Skacel *et al.* found that in nine patients with a negative cystoscopy and biopsy, a positive FISH predicted the appearance of urothelial cancer in eight out of nine (89%). They conclude that in patients with an abnormal FISH, increasingly aggressive surveillance is warranted even in the presence of a negative cystoscopy and biopsy at the time of the FISH [101]. Laudadio *et al.* recently reported an overall sensitivity of 73% and overall specificity of 65%. They note that, in their series, FISH detected 95% of high-grade cancers, the majority of which were missed by cytology. The detection of low-grade cancers was also improved over cytology, 56% detection rate versus 32%. They conclude that FISH may be a useful tool in the diagnosis of both recurrent and initial tumors [102]. In a number of studies, the sensitivity for FISH was reported to be between 73 and 87%, and specificity between 90 and 96% [103–106]. A large number of authors advocate more widespread use of FISH given the ease of use, accuracy of detection of cancer and the ability to detect lesions not yet evident on cystoscopy [102,103]. Currently, the most significant barrier to widespread use of FISH remains the high cost [107].

Summary—The FISH test is rapidly becoming utilized in clinical practice in the diagnosis and surveillance of bladder cancer. Its high sensitivity and specificity and increased accuracy compared with cytology alone may make it useful. Further work must be carried out to reach agreement as to the detection standards for abnormal cells in this test and to resolve issues related to false-positive results, which have been encountered since the use of this test in clinical settings. As one of the most expensive bladder tumor markers, decisions must also be made based on the economic impact of this test upon bladder cancer surveillance.

Microsatellite DNA analysis

MSA is a PCR-based assay for microsatellite DNA, the term for the 2–6 base pair short tandem repeats identified throughout the human genome. A multitude of different locations of microsatellite alterations have been identified in bladder cancer, including chromosomes 4p, 8p, 9p and q, and 11p [108,109]. Two distinct alterations take place, which form the basis of detection in MSA: loss of heterozygosity (LOH) and microsatellite instability. In testing, a large number of microsatellites are used over a number of chromosomes to

improve the accuracy of detection. One drawback of this test is that a larger amount of malignant cellular DNA is required to detect LOH compared with microsatellite instability. Over a large number of studies, microsatellite DNA analysis is shown to have a sensitivity of 72–100%, and a specificity of greater than 80% in detecting both primary and recurrent tumors [110–114]. As in FISH, MSA may detect tumor recurrence before it becomes clinically evident. In one study, 79% of patients with an LOH were discovered to have a recurrence between 4 and 31 months after transurethral resection. In this study, Shigyo *et al.* concluded that MSA is a helpful predictor of recurrent disease in which intravesical therapy may be particularly advantageous [115]. The detection of low-grade/low-stage tumors is one area where MSA shows particular promise. As noted above, these tumors are least likely to be detected by conventional methods yet most likely to recur. However, these authors pointed out that microsatellite alterations are reflections of a urothelium with changes that may make tumor development more likely, as opposed to predicting clear tumor formation.

Summary—MSA is a urinary marker with significant potential for clinical utility. It has high sensitivity and specificity, is able to detect low-grade and low-stage tumors well, and is highly predictive of tumor recurrence. A consensus must be reached, however, as to which DNA markers should be assayed for optimal detection with reasonable cost and complexity.

uCyt

uCyt was developed as a test to aid in the detection of malignant cells in cytology. It is an immunofluorescence test that detects carcinoembryonic antigen and sulfated mucin glycoproteins that are overexpressed in bladder cancer cells [116,117]. Multiple studies have reported the sensitivity of the test as between 40 and 95%, with specificity ranging from 60 to 70% [117–120]. A number of authors have reported increased sensitivity compared with cytology in the surveillance of recurrent bladder cancer. Pianton *et al.* studied 694 patients with either gross or microscopic hematuria. They found that sensitivity of uCyt was much higher than that of cytology alone in detection of bladder tumors (80–88% vs 40–47%). However, cytology was more specific in these cases. They conclude that uCyt could be used in place of cytology in the evaluation of gross hematuria [121]. It has been reported that a combination of UCyt and cytology yields best results in the initial diagnosis of bladder cancer, especially low-grade tumors [119,122]. In reports by Lodde *et al.*, sensitivity for G1 tumors increased from 5% with cytology alone to 85% with the addition of uCyt. Similarly, in Ta tumors sensitivity increased from 14 to 86% with uCyt as an adjunct to cytology [123].

Summary—uCyt is a useful addition to cytology in the evaluation of both initial and recurrent bladder tumors. Some of the variability seen in studies of uCyt can be ascribed to the interobserver variability in interpretation of the sample and the experience of the person evaluating specimens.

Oncogenes & DNA methylation

Multiple genetic derangements have been found in bladder cancer. These include presence of mutated oncogenes and methylation of promoters of tumor suppressor genes [124–127]. Promising investigations into these markers have yet to have an impact in clinical practice as testing is expensive and difficult to replicate without specialized equipment, and little

consensus has been reached on specific markers that could be used in an overall assay. Nonetheless, very recent studies have linked hypermethylation of known tumor suppressor genes, such as the retinoblastoma gene and p14/ARF, with the presence of urothelial malignancy. Control subjects with both normal and benign urologic conditions showed none of these changes [128,129].

Summary—The search for presence of mutated oncogenes and tumor suppressor genes in bladder cancer continues to be a very active area of research. A wide variety of targets are being assessed with positive results. Some of these studies have shown as much as a twofold increase in the accuracy of cytology when combined with these markers. Further investigation must be carried out into the most accurate markers for bladder cancer and how best to detect them. At the current time, multiple disadvantages, including cost and complexity, keep this group of markers from having a clinical impact, although this may change with further research.

Newer molecular markers for prognosis

An area of intense research in the last few years is that of molecular markers for bladder cancer detection. Based on the large number of events necessary for cellular transformation of a normal urothelial cell to a malignant one, researchers are actively evaluating markers from each step in this transformation and their clinical significance. Newer techniques of identification and targeting of molecular signaling pathways have allowed this young field to advance quickly. The areas of most active study include cell-cycle genetic mutations, inhibition of apoptotic pathways, proangiogenic factors and a combination thereof.

As mentioned earlier, mutations in the genes that regulate the cell cycle are frequently seen in bladder carcinoma. Many studies have been focused on p21, p27, pRb, cyclins and p53; however, the results have been mixed up to this point. p53 has been the most studied regulatory target, with authors linking genetic alterations to recurrence and progression in a number of studies [130]. Some recent data also suggest that a higher proportion of deranged p53 correlates with increasingly invasive tumors [131]. To date, however, p53 has not been positively linked to disease prognosis in early trials [132,133]. P27, pRB and cyclins continue to undergo intense scrutiny but results for their prognostic implications are mixed [134].

Apoptosis is a vitally important pathway in cell regulation and alterations in this pathway are reported in most cancers. Survivin and sFas are the two most scrutinized molecules implicated in apoptotic inhibition. Other targets, such as caspase-3, have been evaluated and show promise in early studies in predicting stage and, in some studies, survival [135].

Additionally, angiogenesis is a necessary step in tumor progression. Multiple pro-angiogenic markers have been identified, as has downregulation of angiogenic inhibitors in bladder cancer. These have been linked to disease recurrence and progression. Zu *et al.* was able to correlate increased VEGF expression with positive lymph node status and poor prognosis [136].

Summary

Molecular markers are a subject of very active research. Many molecular markers have been identified that play a role in cell cycle dysregulation, inhibition of apoptosis and enhancement of angiogenesis in the tumor field. These markers have been shown to correlate with disease severity (including stage and grade), prognosis, recurrence and even survival. Most authors currently advocate a combination of the above markers, given the poor sensitivity and specificity for each taken individually. Many barriers exist for these markers to have clinical significance in the practice setting; however, it seems likely that once better delineated, molecular markers may have a very important role in bladder cancer.

Expert commentary

The field of tumor markers in bladder cancer is rapidly evolving. A number of markers investigated heavily within the last decade have since been discarded, while newer assays continue to be developed and enhanced. The current mainstay of clinical practice remains cystoscopic and cytologic evaluation of a patient presenting with either hematuria or another risk factor for bladder cancer, or surveillance for recurrent disease. The addition of one or more of the markers examined above has been shown in many cases to enhance the detection of primary or recurrent disease when combined with cystoscopy and cytology. Work is being carried out to examine which patients may be followed noninvasively using a combination of the above markers. A more recent shift has been toward genetic studies evaluating the chromosomal makeup of cells in a bladder with or without obvious tumor and identifying those that have an increased risk of undergoing malignant transformation or recurrence. In many cases this may raise further questions, including appropriate surveillance protocols in low- versus high-risk patients, the role of 'anticipatory testing' and patient selection for appropriate testing. While studies continue to define the role of biomarkers in bladder cancer, their utility in the clinical setting continues to be debated and, as yet, no consensus has been reached.

Five-year view

Due to the opportunity of testing of any biomarker (soluble antigens or cell-based antigens) noninvasively, using voided urine specimens, bladder cancer will continue to dominate as the cancer of choice for developing new diagnostic tests. The new biomarkers most certainly will be developed based on the results of whole genome-wide array, cDNA microarray, micro-RNA and proteomic analyses. However, for such biomarkers to be adopted in standard clinical practice, more traditional tests (e.g., ELISA, point-of-care device or PCR-based assays) will have to be developed for a single marker or a panel of markers identified by the array data mining studies. Traditionally, most bladder tumor markers have better accuracy than many established tumor markers, such as the prostate-specific antigen (PSA) blood test for prostate cancer. However, bladder tumor markers are not routinely used in the diagnosis of bladder cancer because both urologists and patients are reluctant to forgo cystoscopic evaluation of the bladder due to the fear of missing a tumor via noninvasive testing. Thus, it is difficult to predict whether within next 5 years, bladder tumor markers, collectively, will overcome the issues related to their acceptance in standard clinical practice.

Acknowledgments

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Key issue

- Bladder cancer remains a significant cause of mortality and morbidity in the USA.

- The work-up of bladder cancer is generally precipitated by the finding of hematuria.

- The current work-up includes cystoscopic evaluation, radiographic evaluation of the upper urinary tract and urine cytology.

- Extensive work has been performed in the field of biomarkers in bladder cancer.

- The goals of this work are to maximize the diagnostic yield and minimize the invasive nature of the hematuria work-up.

- A number of studies have been conducted to evaluate the utility of these biomarkers in the detection of bladder cancer.

- A wide variability exists in the utility and proposed effectiveness of various markers currently under investigation.

- At the present time, no marker has become widely used clinically either alone or as an adjunct to cystoscopy in the diagnosis of bladder cancer.

- Further work, including widespread clinical trials, will need to be performed to fully evaluate these markers.

- Future tumor marker trends include molecular markers, including genetic mutations in pathways leading to or precluding malignant transformation.

Table 1

Comparison of current bladder tumor markers.

Marker	Sensitivity	Specificity	Company	Advantages	Disadvantages	Future directions
Hematuria	47–74% isolated; 90–95% repeat			Inexpensive	Poor sensitivity	Continued initial screening test
Cytology	80–90% in high grade; 35–65% in low grade	90–100%		Highly specific	Requires pathologic evaluation. Decreased utility in low grade/stage	Continued component of hematuria work-up
BTA-Stat/BTA-TRAK	8–89%	19–70%	Bard Diagnostics, WA, USA	Inexpensive	Poor utility in patients with benign disease. Variable sensitivity in prior studies	US FDA approved, research ongoing
NMP-22	47–100%	60–80%	Matritech, MA, USA	Inexpensive, useful in lesions not seen on cystoscopy	False-positive results in benign urologic conditions	US FDA approved, research ongoing
BladderChek	50–85%	40–90%	Matritech, MA, USA	Inexpensive, may be an adjunct to cystoscopy	False-positive results in benign urologic conditions and low sensitivity	US FDA approved, research ongoing
Hyaluronic acid-hyaluronidase	83–94%	60–90%		High sensitivity, useful in low-grade and -stage disease		Not in widespread use
Telomerase	9–100%	85–100%		High sensitivity throughout grades/stages	Unstable products of evaluation	More research is ongoing
Survivin	64–100%	78–100%	Bio-Rad, CA, USA	High sensitivity		Further research ongoing
FISH	73–87%	90–96%	Abbott Labs, IL, USA	High sensitivity in occult disease	Criteria for positive test not yet established, Significantly higher cost than other markers and cytology	US FDA approved and research ongoing
Microsatellite DNA analysis	72–100%	80–100%		High sensitivity throughout stages/grades	Little consensus on markers used in the test	Further research ongoing
uCyt	40–95%	60–70%	Diagnocure, Quebec, Canada	Sensitive in lower grade disease	Requires pathologic evaluation for testing	US FDA approved with further research ongoing
Cytokeratins	13–79%	36–95%	IDL Biotech, Borlange, Sweden	Some studies show acceptable sensitivity	Diminished sensitivity and specificity in many studies	Limited utility based on current evidence
BLCA-4	90–100%	90–100%		High sensitivity and specificity, Very little confounding by benign disease	Little consensus on levels for test evaluation	Further testing ongoing
Soluble Fas				Initial results are promising	Few studies evaluating this marker	Further research is ongoing