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TUMOR MARKERS FOR THE EARLY DETECTION OF BLADDER CANCER

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1. ABSTRACT

Several urine markers have been and are currently being investigated for the diagnosis and prognostication of bladder malignancies. While cystoscopy and urine cytology remain the gold standard in the detection of bladder cancer, cystoscopy is invasive and cytology yields low sensitivities in low-grade disease. The availability of a non-invasive, accurate, office-based test would be ideal. In this review, we discuss markers that are useful in the prevention and detection of transitional cell carcinoma of the bladder. In general, each of the markers has better sensitivities than cytology, but lower specificities. Furthermore, each of these markers must still be used in adjunct with cystoscopy.

2. INTRODUCTION

2.1. Background

Transitional cell carcinoma (TCC) is the second most common malignancy in the genitourinary tract in the United States. Approximately 53,200 cases of bladder cancer were diagnosed with 12,100 deaths in 2000(1). Approximately 75 % of patients present with superficial disease (Ta and T1), while 20 % present with T2 or greater disease. The remaining 5 % present with metastatic disease. Overall, 70 % of treated tumors recur, with 30 % of these recurrent tumors progressing to metastatic disease (2).

2.2. Rationale For Tumor Markers

Due to its ease of accessibility, the bladder represents an ideal model for studies in risk assessment, early detection, and the investigation of biomarkers(3). Currently, the treatment and monitoring of patients with bladder cancer imposes a heavy economic burden on the health care system. While urinalysis, cytology, and cystoscopy remain established modalities for the detection and monitoring of TCC, these tests are either too invasive or lack sensitivity and/ or specificity(2). Furthermore, long-term surveillance is inconvenient to the patient. These shortcomings have led to the impetus to develop histologic, molecular, and genetic markers that will aid in the early detection, screening, chemoprevention, and perhaps the therapeutic targeting of bladder cancer.

The ideal biomarker should be noninvasive, provide rapid results, be easy to interpret with little or no variability amongst users, cost-effective, and most importantly, have a high sensitivity and specificity (2,4). Potential roadblocks in identifying the ideal marker include the need to obtain consistent samples, to standardize methods of fixation, to assure quality control of assay methods, and to optimize interpretation of the data in the context of the clinical question at hand (3). The selection of a biomarker depends on whether the objective is prevention, detection/screening, surveillance, or predicting the biological behavior (ie risk of progression/spread) of the neoplasm (3).

In this review, we briefly highlight markers that are currently available or under investigation for the detection of bladder cancer. As such, markers that are useful in monitoring/surveillance and predicting progression/ recurrence are beyond the scope of this review, although many of the markers herein discussed will cross over into the other categories. We will begin by briefly summarizing markers that are currently used in clinical practice, some of which have been approved by the Federal Drug Administration (FDA). We follow that with a preview of markers that are more investigational but may potentially be integrated into clinical practice in the near future.

3. CYTOLOGY

Urinary cytology identifies malignant cells that have been exfoliated from the urothelium into the urine. The specificity of cytology is greater than 90 % (5), while the sensitivity for high-grade disease and carcinoma-*in-situ* (cis) is about 80 – 90 % (6,7).

Shortcomings of voided cytology include a low sensitivity of approximately 20-40 % for low grade disease (2). Low grade or well-differentiated TCC cells are not routinely shed into the urine, are very difficult to differentiate from normal cells microscopically, and are cohesive.

In short, cytology is ideal for detecting and following high grade tumors, including carcinoma-*in-situ* but lacks distinguishing histologic findings with regards for low grade tumors, yielding low overall sensitivity rates (3,4).

4. NUCLEAR MATRIX PROTEIN 22 (NMP-22) – FDA APPROVED

The nuclear matrix protein (NMP) consists of a three-dimensional web of RNA and proteins which supports the nuclear shape, organizes DNA and coordinates DNA replication, transcription, and gene expression (2,8) NMP released into the urine may be detected by a FDA approved NMP-22 (9) enzyme-linked assay kit (Matritech, Newton, Mass).

NMP-22 is a 238-kDa protein that may be detected at up to 25-fold greater concentration when compared to normal urothelium (10,11). The enzyme-linked immunoassay uses 2 monoclonal antibodies to measure the levels of complexed and fragmented forms of the mitotic apparatus in urine (10). In a review of over 1100 patients from seven series, Konety and Getzenberg reported an overall sensitivity of 70.5 % and a specificity of 75.2 % (8,12-16). A major source of false positivity is hematuria and pyuria (17). This is a serious problem since many benign urological conditions such as stone disease and infection present with hematuria (18).

Soloway *et al* used NMP-22 to predict likelihood of recurrence after transurethral resection (12). Levels less than 10 U/mL were predictive of low likelihood of recurrence while levels greater than 10 U/mL were predictive of recurrence.

In short, NMP-22 has an overall higher sensitivity when compared to cytology and may be used to predict increased recurrence risk in patients who levels are greater than 10 U/mL after transurethral resections.

5. BLADDER TUMOR ANTIGEN (BTA) –FDA APPROVED

The term BTA actually describes three separate tests: (1) BTA (2) BTA stat (3) and BTA TRAK. The original BTA test consisted of a latex-agglutination test which measures levels of basement protein antigen which are released into urine as a result of tumor invading into the stroma (19). Since the BTA tests depend on the disruption of basement membrane, its sensitivity improves with more invasive cancer (20).

In a review of over 1000 patients (seven series), the sensitivity of the original BTA test was only 52.3 %, while the specificity was 84.6 % (13,21-26).

Advantages include increased sensitivity for invasive tumors. Disadvantages include a high-rate of false-positive readings secondary to patients with inflammatory conditions such as benign prostatic hypertrophy and a low overall sensitivity for detection all bladder cancers.

BTA stat and BTA TRAK detect human complement factor H (hCFH). The qualitative BTA stat test costs only $ 5 dollars and is easily performed in the office with a dipstick (27). Overall sensitivity is 65 % and specificity is 65 % (8). BTA TRAK is a quantitative test which has improved sensitivities over its two BTA predecessors (28,29), but has high false positive rates in patients with stones, inflammatory conditions, benign prostatic hyperplasia (BPH), and trauma; this leads to low specificity rates (31).

Overall, the three BTA tests lead to an improved sensitivity compared to cytology, but lower specificities due to high false positive rates associated with recent instrumentation, stones, or inflammatory conditions (i.e. BPH).

6. FIBRIN-FIBRINOGEN DEGRADATION PRODUCTS (FDP) – FDA APPROVED

Since bladder tumor cells have increased vascular permeability, cellular proteins such as plasminogen and fibrinogen leak into the urine. Urokinase subsequently converts plasminogen into plasmin which then converts fibrinogen into fibrin-fibrinogen products (FDP) (32). It follows then that patients with bladder cancer may have increased levels of FDP. In a review of four series, combined sensitivity was 68 % and combined specificity was 78% (23,33-35).

The test costs about $ 15 dollars and takes less than 10 minutes (27). However, the AccuDx-FDP is not currently being produced due to issues regarding test formulation (4). Advantages include high-yield with invasive tumors presumably because of increased leakage of FDP. Disadvantages include poor sensitivities for low-grade disease and poor specificities due to reasons previously mentioned in association with BTA tests.

7. BLCA-4

Konety and Getzenberg have recently described several specific nuclear matrix proteins which are present only in patients with bladder cancer (BLCA 1-6) and 3 proteins which are present in normal bladder tissue (BLNL 1-3) (36). They reported that BLCA-4 levels were significantly higher than those found in normal controls and that 53 of 55 (96 % sensitivity) samples had BLCA-4 expression (37). From their early results, Konety concluded that BLCA-4 may be more accurate that NMP-22 and that it may be more specific because it is not present in any other tissue or tumors. They are currently conducting a large multicenter prospective clinical trial to confirm whether BLCA-4 may be a bladder cancer specific marker that is not falsely elevated by other tumors or other benign bladder conditions which have lowered the specificities of other tests such as BTA and NMP.

8. TELOMERASE

Telomeres are nucleotide sequences that are important in maintaining DNA stability of cells. Loss of telomeres is associated with each cycle of DNA replication. Telomerase is an enzyme which protects telomeres from degradation enzymes; increased levels of telomerase subsequently allows tumor cells to maintain immortality (38).

Increased levels of telomerase secreted into the urine by bladder cancer cells are detected with the telomeric repeat amplification protocol (TRAP) assay, which is based on PCR. This test takes about 10 hours and costs about $ 17 (4,38). A review of three series (over 200 patients) series confirmed the sensitivity to be about 74 % with a specificity of almost 79 % (8,13,39,40).

Difficulties associated with the telomerase test have limited its widespread use. Urine must be processed within a 24 hour period.(8)At least 50 cells must express telomerase for the assay to reliably detect telomerase (8,41). Finally, contaminants such as ribonuclease may render the test falsely negative (42).

9. CYTOKERATINS

Cytokeratins (CK) make up a large component of intermediate filaments that are found in epithelial cells (43). While there are many urine-based tests that detect cytokeratins, we will focus only CK 20 because it has been shown to be expressed only in bladder cancer cells, while the others are non-specific (43,44).

Klein *et al* reported a sensitivity of 91 % and a specificity of 67 % in a study of 87 patients. There was no correlation with grade (44). Specimens with false-positive results had cytologies consistent with premalignant conditions such as atypia, hyperplasia, or metaplasia (43,44). Completely health patients all had negative CK 20 levels.

Using a immunocytochemical approach, recent analyses on archived urine slides showed that overall sensitivity and specificity for CK20 for the detection of urothelial carcinoma were 94.4% and 80.5%, respectively. Cytokeratin 20 is a novel early detection immunocytochemical marker for transitional cell carcinoma (TCC) in archived urine slides (45). This study demonstrated that CK20 analysis is a useful adjunct marker for urine cytology, in which analysis of CK20 can be performed conveniently on the same slide after routine morphological evaluation. This marker could be used to triage atypical urine cytology into low and high risk categories so that different follow up modalities can be carried out.

Drawbacks to CK's may include the possibility that they may be positive in other types of epithelial cancers. In total, CK 20 has very low false-positive rates in completely healthy patients. It not only predicts bladder cancers of all grades with high sensitivities (91 %), but also may possess the potential to detect premalignant conditions such as atypia and metaplasia.

10. HYALURONIC ACID/HYALURONIDASE

Hyaluronic acid (HA) is a glycosaminoglycan that promotes tumor cell adhesion and angiogenesis (8,46). Hyaluronidase (HAase) is an enzyme which cleaves HA into fragments; these cleaved fragments then aid tumor growth and propagation by promoting angiogenesis (47,48). Increased levels of HAase have been associated with high-grade disease (49). HA by itself has a 92 % sensitivity and 93 % specificity for all tumor grades (50).

11. SURVIVIN

Survivin is a recently discovered inhibitor of apoptosis which allows bladder cancer to extend cell viability (51,52). Survivin is undetectable in most normal adult tissue and correlates with unfavorable disease and shortened overall survival in neuroblastoma, colorectal and non-small cell lung cancers (53-56).

Smith *et al* have recently published the results of survivin and its connection to bladder cancer.(52) They surveyed urine specimens from five groups: (1) 17 healthy volunteers, (2) 30 patients with nonneoplastic disease, (3) 30 patients with genitourinary cancer, but not TCC, (4) 46 patients with new onset or recurrent bladder cancer, and (5) 35 patients whose bladder cancer had been treated.

Urine specimens were analyzed with a polyclonal antibody and then validated with both western blot and RT-PCR. Survivin was detected in 31 of 31 bladder cancer patients using the polyclonal antibody system and 15 of 15 bladder cancer using RT-PCR, giving a sensitivity of 100 %. Only 3 of 35 patients with treated bladder cancers and negative cystoscopies tested positive for survivin, meaning survivin could potentially be used for surveillance as well (52). Or, put another way, if a patient's survivin is negative after TURBT, the interval between cystoscopies could potentially be lengthened as a negative survivin could be predictive of a lack of bladder cancer.

Survivin was also negative in the 17 healthy volunteers and the 30 patients with genitourinary cancers unrelated to TCC. There were only 4 false positives in the group of 30 patients with nonneoplastic urinary tract disease. One patient had an elevated PSA, while the other three had abnormal cystoscopies (i.e. after TURP with trabeculated bladders secondary to BPH). The overall specificity for survivin was 95 % (52).

In short, survivin identified all 46 patients with bladder cancers and became negative in 32 or 35 patients once the bladder cancer was treated. Out of the 4 false-positives, one patient actually developed bladder cancer 6 months later. Survivin could be potentially a valuable marker for both detection and monitoring, but its validation awaits further testing.

12. DNA PLOIDY AND S-PHASE FRACTION

DNA ploidy and S-phase fractions can be evaluated from urine samples by either flow cytometery, image cytometry (ICM), laser scanning cytometry (LSC), and fluorescence *in situ* hybridization (FISH) (2).

Flow cytometry of a voided urinary sample can be used to determine DNA ploidy and to estimate S-phase fraction (DNA synthesis). Since neoplastic cells have increased nuclear size and increased nuclear; chromatin ratios, flow cytometry will identify cells as diploid, tetraploid, or aneuploid. High grade tumors may be detected by the presence of aneuploid populations and a higher percentage of cells in the S phase (2). Sensitivities for high grade TCC or carcinoma-*in-situ* may reach 90 % (2,57,58). Because this technique requires a large number of cells, highly trained personnel and is expensive, flow cytometry has not gained widespread acceptance.

Image cytometry (ICM) uses a fluorescence microscope to measure the DNA content in each cell making this an attractive alternative to flow cytometry, which requires a large cell population. ICM is more sensitive than cytology or flow cytometry in detecting low grade TCC (59).

Laser scanning cytometry combines the advantages of flow cytometry and ICM by laser scanning to quantify the fluorescence of individual cells (60).

13. TUMOR-ASSOCIATED ANTIGENS

Monoclonal antibodies can be used to identify several TCC-associated antigens which are normally absent in healthy urothelium. M 344, 19 A 211, and DD23 are some of these tumor markers that are currently being investigated as potential markers for TCC.

13.1. M 344

M 344 has been shown to be detectable in about 70 % of low grade (Ta and T1) tumors, but its expression drops to 25 % for carcinom-*in-situ* and 15 % for invasive disease (2,61). A recent study involving a cohort of Chinese workers demonstrated that it is a very specific marker for the detection of bladder cancer at the early stage (62).

13.2. 19 A 211

This antigen is found in up to 25 % of normal umbrella cells. Similar to M 344, it is better for predicting grade, with 70 % expression in Ta and T1 tumors, but dropping to 50 % expression in invasive tumors (2,63).

13.3. DD 23

This antigen is identified in 81 % of bladder tumors and has a 85 % sensitivity with quantitative fluorescence imaging with a 95 % specificity (8,64). When used in combination with cytology, the sensitivity for detecting bladder cancer is 94 % with a specificity of 85 % (2).

14. MICROSATELLITE INSTABILITY ASSAYS

Similar to other malignancies, bladder cancer DNA repair mechanisms may be defective, which lead to persistent errors in replication. Microsatellites are inherited tandem repeat DNA sequences with low mutation rates that can be analyzed to detect these replication errors (65,66). Polymerase chain reaction (PCR) amplification of these tandem repeat sequences can identify loss of heterozygosity and can be used to map tumor suppressor genes (67-70).

Microsatellite analysis has been used to confirm that low grade papillary TCC has instability and / or loss in parts of chromosome 9 and p16 (MTS1) tumor suppressor gene (65,71). Low stage tumors typically have loss of heterozygosity (LOH) in the 9p region on microsatellite analysis (65,72). Using microsatellite analysis and PCR, Mao *et al* were able to identify 19 of 20 patients (95 % sensitivity) with genetic alterations, however, 2 of 4 samples with inflammatory atypia also showed positive findings in Mao's analysis (8,73).

Other studies have shown that microsatellite assay may be used to predict recurrence of TCC. The sensitivities of four studies ranged from 83 % to 95 %

(65,73-75). Some of these studies reported that the assays were able to predict a recurrence months before cystoscopic confirmation of recurrence. However, large scale analysis will be needed to determine the specificity of this test, especially in symptomatic populations, before one can evaluate the true clinical utility of the test.

15. BIOMARKER RISK ASSESSMENT

Up to this point we have mainly focused on markers that are used for the early detection of bladder cancer. A review on bladder cancer detection would not be complete without mention of risk assessment. The mean time from initial carcinogen exposure to the development of transitional cell carcinoma may be up to 20 years. Thus, the best form of detection may be actually predicting which patients will eventually develop bladder cancer by assessing risk factors.

In a landmark study, Hemstreet *et al* examined three biomarkers in an effort to risk stratify a population of Chinese workers that had been exposed to benzidine, a well know bladder carcinogen (62). In this prospective study (6 years), 1788 exposed Chinese workers and 373 nonexposed workers had their voided urine specimens assayed for DNA ploidy (expressed as 5 C exceeding rate [DNA 5 CER]), the bladder tumor-associated antigen p300, and a cytoskeletal protein (G-actin).

28 out of 1788 exposed workers developed bladder cancer, while two out of 373 nonexposed subjects also developed bladder cancer. If any of the exposed workers were positive for either DNA 5 CER or p 300, their risk of developing bladder cancer was 19.6 times higher than in workers who were negative for both markers. Workers who were positive for both markers had an 81.4 times greater risk of developing bladder cancer. G-actin was a poor marker (62). Based on their findings, patients at risk (such as those with a smoking history) can be stratified, screened, monitored, and diagnosed based on predefined markers.

16. PERSPECTIVE

Cystoscopy in combination with Papanicolaou (PAP) cytology remains the most effective means of detecting bladder cancer. However, cystoscopy is an invasive procedure and while cytology remains a useful marker for high grade tumors, its utility in detecting low grade tumors remains limited due to its lack of distinguishing features in low grade disease. The selection of the ideal biomarker depends on whether the goal is prevention, detection/screening, monitoring/surveillance, or predicting progression to invasion or metastatic disease. In this review, we have focused on markers that are currently used or are being investigated for detection purposes, keeping in mind that many of the markers can also be used for other objectives.

Most of the current markers in use have higher sensitivities than cytology, especially when used to identify low grade disease. However, most of these markers also have lower specificities when compared to cytology. Furthermore, all of these tests must still be confirmed in association with cystoscopy. Complete elimination of cystoscopy to detect bladder cancers does not appear feasible, at least in the near future. One or more of these tests may eventually replace cytology as the adjunct to cystoscopy, but each and every one of the markers awaits further validation.

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