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Review Article

Urine cytology and adjunct markers for detection and surveillance of bladder cancer

Peggy S. Sullivan[1], Jessica B. Chan[2], Mary R. Levin[1], Jianyu Rao[1]

[1]Department of Pathology and Laboratory Medicine, David Geffen School of Medicine, University of California at Los Angeles, 10833 Le Conte Avenue, CHS A7-149, Los Angeles, CA 90095-1732, USA; [2]Kaiser Permanente, Oakland Medical Center, Department of Pathology, 280 W. MacArthur Blvd, Oakland, CA 94611, USA.

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Abstract: Urine cytology coupled with cystoscopic examination has been and remains the standard in the initial evaluation of lower urinary tract lesions to rule out bladder cancer. However, cystoscopy is invasive and may miss some flat lesions, whereas cytology has low sensitivity in low-grade papillary disease. Additional lab-based or office-based markers are needed to aid in the evaluation of these lesions. Recently, many such markers have been developed for the purpose of improving the cytologic diagnosis of bladder malignancies. In this review, we will first discuss conventional cytomorphologic analysis of urine cytology followed by a discussion of markers that have been developed in the past for detection and surveillance of urothelial carcinoma. We will focus on how these markers can be used in conjunction with urine cytology in daily practice.

Keywords: Bladder cancer, urine cytology, tumor markers

Introduction

Bladder cancer, most commonly urothelial carcinoma, is the 4th most common cancer in males in the United States [1]. However its cost per patient is the highest of all the cancer types, reaching approximately 200,000 U.S. dollars per patient from diagnosis to death [2]. It has been estimated that in 2009 approximately 70,980 new cases of bladder cancer will be diagnosed with 14,330 deaths in this country [1]. Approximately 75% of patients present with superficial disease (Ta and T1) while 20% present with T2 or greater disease. The remaining 5% of patients present with metastatic disease. Overall, 70% of treated tumors recur, with 30% of recurrent tumors progressing to metastatic disease [3].

Roughly 60% of patients with newly diagnosed bladder cancer do not have muscle invasive disease and do not require cystectomy [4]. The majority of these patients have a recurrence after endoscopic resection, thus lifelong surveillance with cystoscopy is recommended. Unfortunately, cystoscopy, which is the "gold standard" for the detection of *de novo* and recurrent bladder cancer, is an expensive and invasive procedure. In addition, it may miss a flat lesion, especially carcinoma in situ which is considered a high grade malignant condition rather than a precancerous lesion as in other organ systems.

Voided urinary cytology is a useful noninvasive adjunct to cystoscopy because of its overall high specificity. Cytology also has a relatively high sensitivity at detecting high-grade lesions. Its sensitivity, however, is anywhere between 20 to 50% for low-grade papillary tumors. Of the non-muscle-invasive lesions, approximately 10% of low-grade papillary tumors subsequently develop muscle-invasive or metastatic cancer whereas roughly a third of high-grade tumors progress, if not already muscle-invasive at the time of diagnosis [4]. Therefore, close monitoring and early detection of all lesions are important for management, and noninvasive tumor markers with high accuracy for the detection of

all grades of urothelial carcinoma will significantly reduce patient cost, anxiety and morbidity.

Urine cytomorphological analysis

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) can be as high as 80 to 90% [6, 7]. As indicated before, however, the main shortcoming of voided cytology is the low sensitivity (approximately 20-50%) for detecting low grade neoplasms including benign papilloma, urothelial carcinoma with low malignant potential (borderline), and low grade papillary urothelial carcinoma (Grade 1 to 2 of 3 of the WHO classification) [3] [4, 8]. There are two main reasons for such low sensitivity. First, tumor cells of the low grade tumors are not routinely shed into the urine because of their cohesive nature. Second, and probably more important, is the fact that low grade tumor cells by definition have similar cytomorphology to normal urothelial cells microscopically. While increased cellularity and presence of papillary fragments in “true” voided urine sample may be a hint for such a low grade lesion, one has to rule out the possibility of urothelial hyperplasia due to various reasons such as lithiasis, infection, and instrumentation.

Probably the most common reason for the presence of increased cellularity or papillary fragments in an otherwise morphologically normal voided urine sample is instrumentation as a result of cystoscopy, since many such samples are collected after the procedure is performed even though the requisition may incorrectly state the specimen is a "voided urine". Thus, caution should be taken and clinical correlation should be advised in such a setting.

Common indications for urinary cytology

Urine cytology, as an "ancient" technique, has been used in following. First, it has been used as a screening tool to detect urothelial cancers in high risk populations, especially in populations exposed to chemical carcinogens through occupational means, for example the Drake cohort [9]. Second, it has been used as an initial test for patients presenting with hematuria to rule out (or rule in) the possibility of urothelial malignancy. Third, it has been used as a monitoring and follow-up tool for patients with a previous diagnosis of urothelial cancer to rule out tumor recurrence. Fourth, it has been used after transurethral resection for assessment of the completeness of tumor removal [10]. Finally, recently it has been applied as a test for detecting inflammation or infection, especially in kidney transplant patients where the presence or absence of polyoma virus infection may have significant clinical implications for rejection [11].

Type of urine samples

The most common type of urine specimen for cytologic analysis is voided urine. Again, keep in mind that although the submitted sample is marked as a "voided urine", it is important to determine whether a cystoscopy has been performed, and if so, whether the sample is collected before or after the procedure. In collecting “true” voided urine, one should avoid a “first morning” specimen and collect the “second morning” voided sample, since the overnight urine often contains many degenerated urothelial cells complicating both morphologic and marker analysis. Although there are data to suggest that three specimens of “second morning” voided urine collected over three consecutive days may optimize the detection of urothelial malignancy [12], this is not a common practice, likely because of cost and convenience.

It is important to remember that, unlike wash or brush samples as discussed below, a true voided urine has the so-called "funnel" effect, i.e., it samples the entire urinary tract system from renal pelves (bilateral) to ureters (bilateral), bladder, and urethra. Considering the fact that urothelial cancer is often a "field" disease, the funnel effect ensures detection of lesions in the entire urinary tract, especially high grade lesions. Thus, at least in theory, voided urine should have a higher sensitivity for detecting urothelial malignancies of the entire urinary tract. However, the trade-off is that often the exact location of the lesion may be difficult to find, especially if the lesion is in the upper urinary tract (ureters and renal pelves). This may result in a so-called “false” false positive urine cytology. Last but not least, in female voided-urine samples, most of the epithelial cells present on the slide are squamous cells contaminated from the female genital tract. Thus, for

any type of molecular marker analysis, especially PCR-based rather than image-based analysis, the contaminated squamous cells will be problematic. Unfortunately, the contamination factor is often ignored in many molecular based studies.

Another common urine sample is catheterized urine, which is usually more cellular than true voided urine but is otherwise identical. Genital contamination may be less of a problem compared to a true voided urine. The wash and brush samples from bladder, ureter, or pelves provide a complement to voided urine samples for the evaluation of urinary tract lesions where cystoscopy (or retrograde ureterocystoscopy) is performed at the same time. Depending on whether a suspicious lesion is seen, a washing or brushing may be performed at the same time as well. The advantages of washing and brushing samples include greater cellularity and a more targeted and homogeneous population of urothelial cells to be analyzed. For low grade lesions, cytomorphologic analysis alone for washing or brushing samples can be extremely challenging. In such a setting, correlating the cytology with the cystoscopy finding is essential. In contrast, for high grade lesions, especially carcinoma in situ, there may be many single or loosely cohesive, highly atypical cells on cytology while cystoscopy may or may not show a visible lesion. Biopsy may show only a few tumor cells on the surface (the "clinging" type of carcinoma in situ). A positive cytology coupled with what appears to be a negative cystoscopy or biopsy is another source of the so-called "false" false positive diagnosis. More discussion on this point will be provided in conjunction with the discussion on tumor markers below.

Other specimen types include ileal conduit or neobladder urine, which are often characterized by the presence of many degenerated columnar epithelial cells and inflammatory cells. On occasion, recurrent urothelial carcinoma may be seen, and diagnosis of such lesions can be extremely difficult since many biomarkers (as discussed below) may not be helpful in such a case.

Cells and other materials

Cells and other materials are summarized in **Box 1**.

Urine sample processing

Sample fixation: The urine specimen should be processed immediately or refrigerated at 4 degrees Fahrenheit for no longer than 24 hours. If a delay of greater than 24 hours is anticipated, the specimen should be fixed with an equal volume of 50% ethanol, or the specimen should be centrifuged and the sediment mixed with an ethanol-based fixative for liquid-based cytology or with 50% isopropyl alcohol or denatured

Box 1

- Cells normally occurring in urine (**Figures 1A-B**):
  - Urothelial cells - basal, intermediate, superficial (umbrella) cells
  - Squamous cells - trigone, distal urethra, vagina, squamous metaplasia
- Other cells in urine:
  - Glandular cells - prostate, endometrium, cystitis glandularis, paraurethra
  - Renal tubular cells
  - Lymphocytes, leukocytes, RBC's
  - Seminal vesicle cells (**Figure 2**)
    - Sporadically seen in urine specimens of older patients
    - Bizarre appearance with greatly enlarged nuclei and foamy fragmented cytoplasm
    - Golden-brown lipofuscin pigment
    - Spermatozoa accompany cells
    - Abnormal DNA ploidy
      - Eosinophilic inclusion bodies - giant lysosomes
    - Hyaline inclusion bodies

- Non-cellular components:
  - Crystals, casts, spermatozoa, corpora amylacea, mucus, fibrin, lubricant, pollen, and rarely *Alternaria* spp. and microconidia

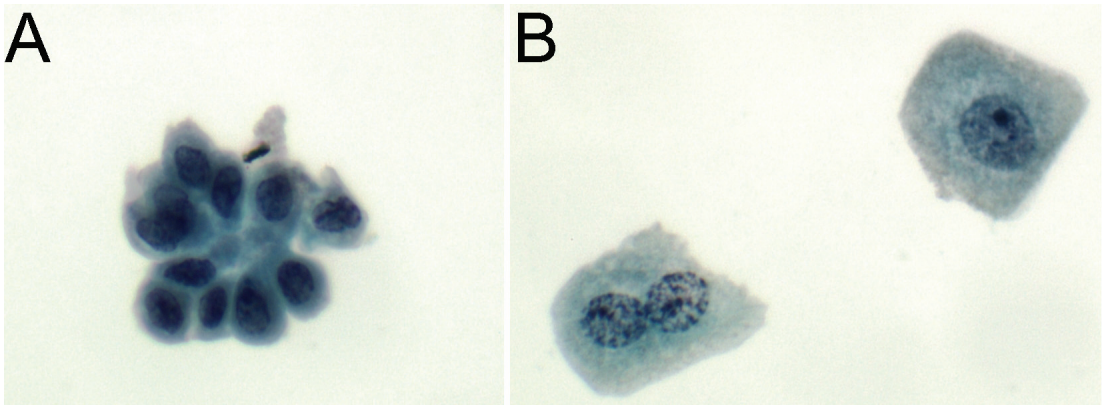

Figure 1. Normal cell components present in urine. (A) Basal urothelial cells have moderate dense cytoplasm with well-defined borders. Nuclei are centrally-placed with small nucleoli and smooth nuclear contours. (B) Superficial urothelial cells (umbrella cells) have abundant granular cytoplasm with rounded and scalloped borders. Nuclei are large, round, frequently multiple, and centrally placed with prominent nucleoli and smooth nuclear borders. (A-B, Papanicolaou stain, 600x)

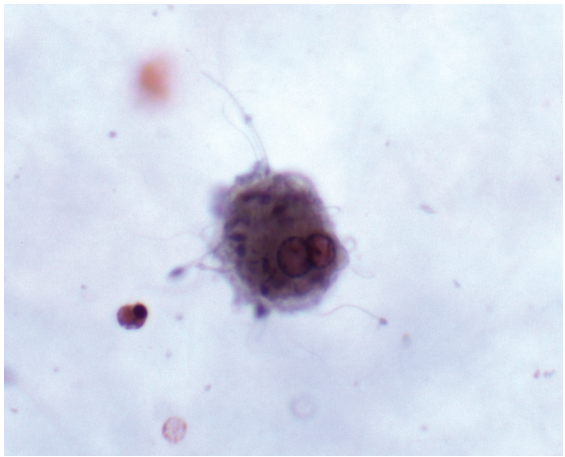

Figure 2. Seminal vesicle cells may occasionally be seen in urine. The cytoplasm may have a golden-brown pigment. Nuclei tend to be hyperchromatic with degenerative chromatin. Note the associated spermatozoa. (Papanicolaou stain, 600x)

alcohol. Low pH appears to favor preservation of urothelial cells.

Specimen processing: Specimens should be processed by cytocentrifugation or by a liquid-based preparation. Fifty milliliters of specimen are transferred to individual centrifuge tubes and spun down at 10 min / 1500 rpm. The supernatant is aspirated off and the sediment is resuspended in a balanced salt solution. Most commonly used slide preparation methods include cytocentrifugation and SurePath or ThinPrep liquid-based techniques. The traditional membrane filter technique is rarely used currently.

Specimen adequacy

Unlike cervical specimens, exact adequacy guidelines for urine specimens have not been established. In general, slides should contain at least fifteen well-visualized basal and intermediate cells to be classified as adequate. Specimens with abnormal cells are by definition satisfactory.

Diagnostic format and categories for urine cytology specimens

The format shown in **Box 2** is recommended for urinary cytology diagnosis.

For samples that are negative for an epithelial cell abnormality, the type of inflammation (acute versus chronic mixed), if present, should be characterized since the information may help the urologist to determine potentially treatable conditions for the patient's urological symptoms. The presence of specific organisms, if observed, should be specified as well. **Figure 3A** shows polyoma virus-infected cells. Occasionally SV40 immunocytochemistry may be added to confirm the diagnosis (**Figure 3B**).

Therapeutic changes are the major source of

Box 2. Diagnostic format and category recommendations for urine cytology specimens:

Adequacy Statement (optional)
- Satisfactory for evaluation
- List any quality factor affecting specimen
- Unsatisfactory for evaluation (give reason)

General Categories
- Negative for epithelial cell abnormality (see descriptive diagnosis)
- Epithelial cell abnormality present (see descriptive diagnosis)

Negative for epithelial cell abnormality:
- Infectious agents
  - Bacterial organisms
  - Fungal organisms
  - Viral changes (CMV, herpes, polyomavirus, adenovirus)
- Nonspecific inflammatory changes
  - Acute inflammation
  - Chronic inflammation
  - Changes consistent with xanthogranulomatous pyelonephritis
- Cellular changes associated with:
  - Chemotherapy/radiation

Epithelial cell abnormality present:
- Atypical urothelial cells (further comment - optional)
  - -Favor reactive
  - -Favor urothelial carcinoma
- Suspicious for urothelial carcinoma
- Low-grade urothelial tumor versus hyperplasia
- High-grade urothelial carcinoma
  - (including invasive carcinoma vs. carcinoma in situ)
- Atypical squamous cells
  - -NOS
  - -HPV related changes/condyloma
  - -Squamous cell carcinoma
- Atypical glandular cells
  - -NOS
  - -Adenocarcinoma, NOS
  - -Adenocarcinoma of prostate
- Other malignant neoplasms (specify type)

Other (Specify)

"true" false positive diagnoses. However, as a general rule of thumb, therapeutic changes are characterized by large cells with abundant cytoplasm, low nuclear-to-cytoplasmic ratio, and smudgy nuclear chromatin (**Figures 4A-C**). Cellular-based markers, especially uCyt+ (ImmunoCyt)™, may be useful in assisting the evaluation.

Needless to say, the category of so-called "atypical urothelial cells" is probably the most controversial diagnosis in terms of patient management. In our experience, about 20 to 25% of all samples will be signed out as "urothelial cell atypia" (this does not include the "suspicious for malignancy" category - unpublished data). This category will probably benefit the most from marker analysis. As discussed below, we have applied marker analysis (mainly uCyt+ and sometimes cytokeratin 20 immunocytochemistry) as a reflex test for cases signed

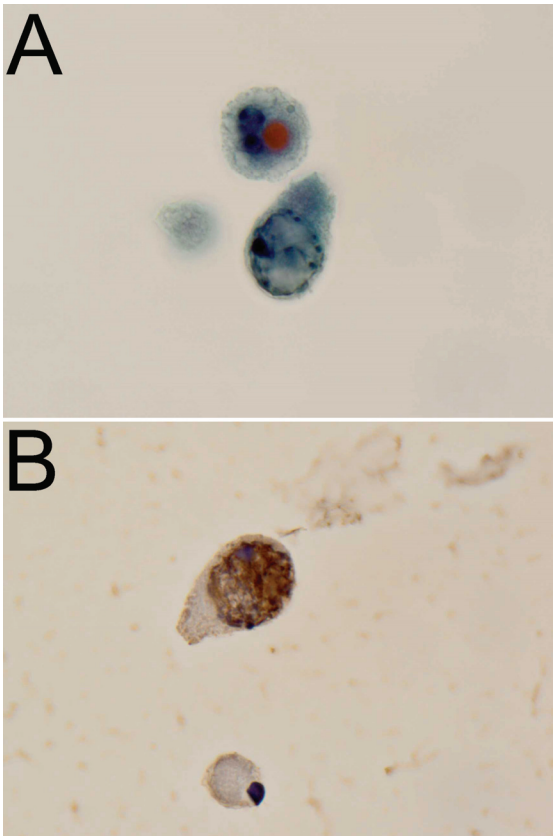

Figure 3. Polyoma virus-infected cells. (A) Infected cells have an increased nuclear to cytoplasmic ratio mimicking carcinoma in situ ("decoy cells"), a ground glass nucleus with marginated chromatin, and occasionally short cytoplasmic tails ("comet cells"). (B) Immunocytochemistry for SV40 is supportive of polyoma virus-infected cells. (A, Papanicolaou stain, 1000x; B, immunoperoxidase stain, 1000x).

out as urothelial cell atypia.

As discussed before, urine samples for low grade lesions (papilloma, borderline, and low grade urothelial carcinoma) are more cellular than normal, with cohesive or papillary fragments and subtle morphologic changes that overlap greatly with hyperplastic urothelial lesions (**Figures 5A-C**). Therefore, these lesions are grouped together.

High grade tumor (including carcinoma in situ) usually shows many atypical single cells and loosely cohesive groups, many of them degenerated. The background may be necrotic, bloody, inflammatory, or clean. It is important to find viable cells and carefully evaluate the nuclear-to

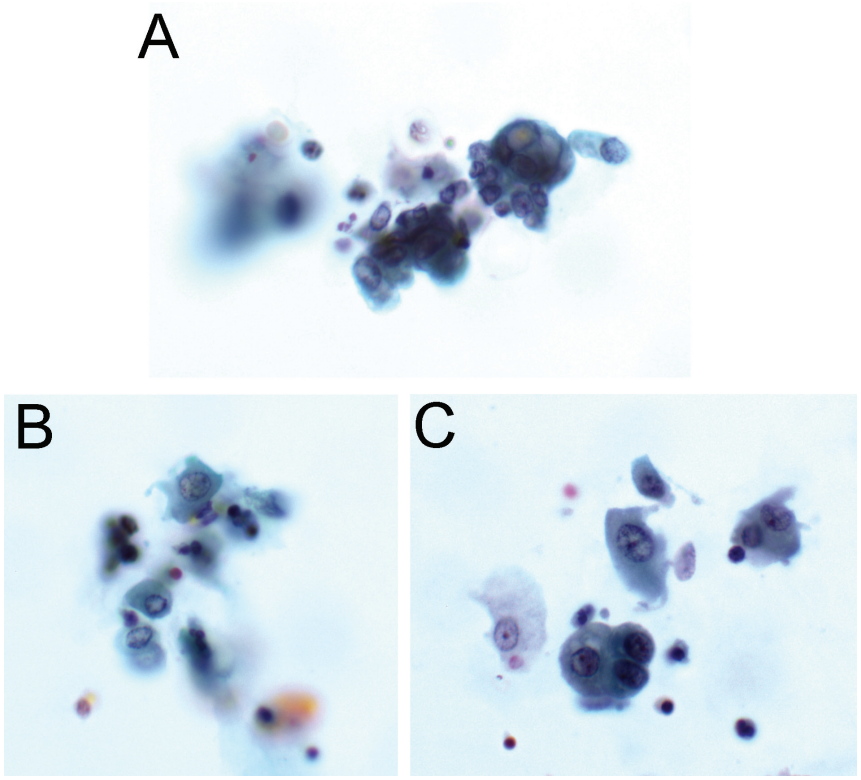

Figure 4. Urothelial cells with therapeutic changes. Bacille Calmette-Guerin (BCG), an attenuated bovine mycobacterium, is used to treat carcinoma in situ. It also induces an inflammatory reaction, especially submucosal granulomas, and urothelial atypia. Urine specimens in patients treated with BCG may have urothelial cells with marked nuclear atypia and hyperchromasia, however, the nuclear to cytoplasmic ratio is not increased and the cytoplasm has a reactive appearance. Histiocytes, including multinucleated forms, are commonly seen. (A-C, Papanicolaou stain, 600x)

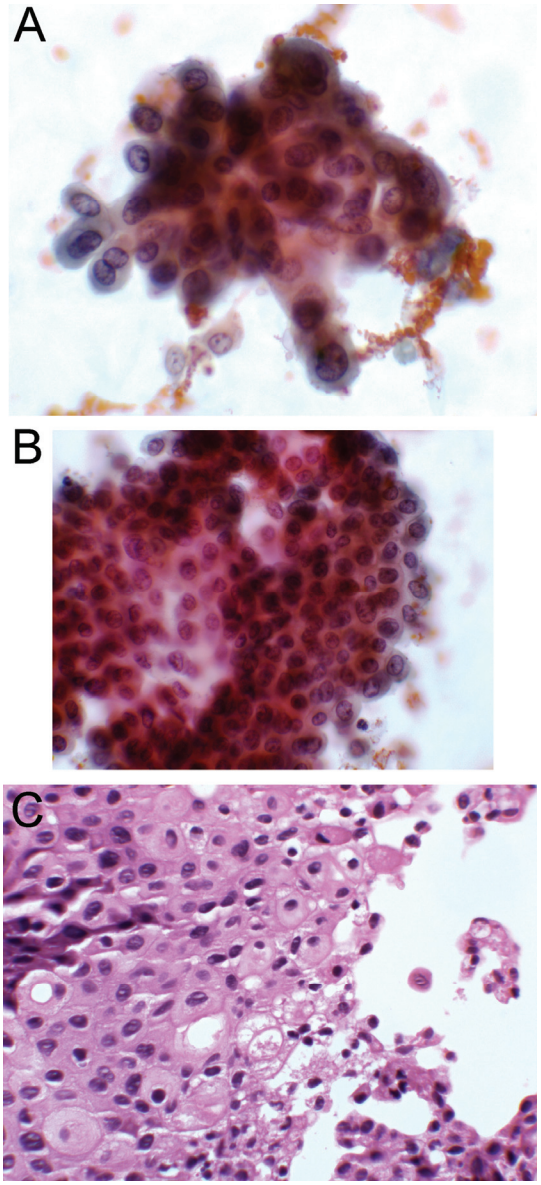

Figure 5. Low grade urothelial neoplasia versus hyperplasia. The distinction may be difficult as both entities form papillary clusters with cellular crowding. The nuclear changes in low grade neoplasms (increased nuclear to cytoplasmic ratio, irregular nuclear membranes, conspicuous nucleoli, haphazard growth pattern) may be subtle. Retained umbrella cells can be seen in both entities. (A-B, Papanicolaou stain, 400x; C, hematoxylin and eosin stain, 400x)

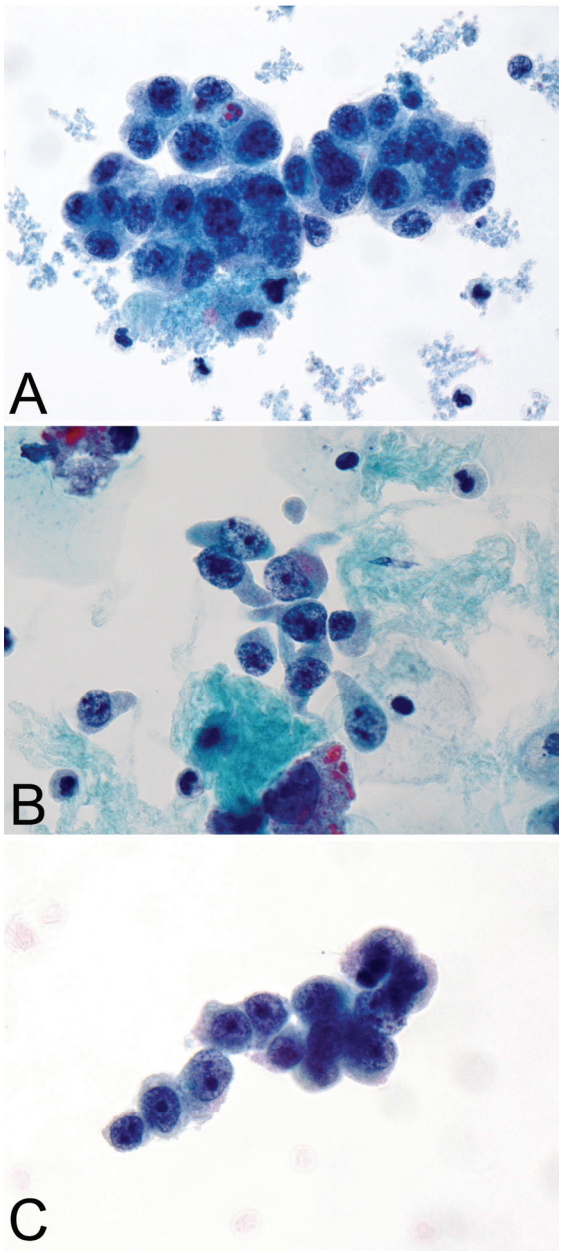

Figure 6. High grade urothelial carcinoma. The cytologic changes are more apparent in high grade lesions and include a very high nuclear to cytoplasmic ratio, dark coarse chromatin, irregular nuclear borders, and occasional prominent nucleoli seen in large, often single, cells. The background may show degenerate cells, necrotic debris, inflammatory cells, and blood. Glandular or squamous cytoplasmic features may be seen. Umbrella cells are mostly absent. (A-C, Papanicolaou stain, 1000x)

-cytoplasmic ratio, nuclear membrane, and chromatin. Examples of high grade urothelial neoplasms are shown in **Figures 6A-C**.

Squamous cell lesions may be seen either with or without human papilloma virus (HPV) effect. The HPV-related changes are relatively rare in

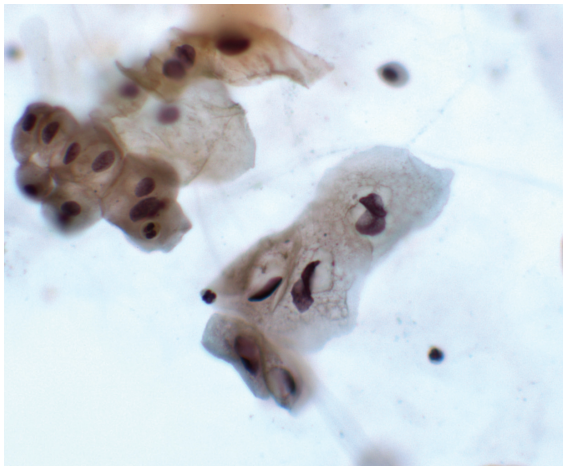

Figure 7. HPV changes of squamous cells in urine. The changes may be seen as primary infection of squamous cells of the urinary tract (for example, urothelial cells with squamous metaplasia or squamous cells lining the distal urethra) or in female urine, as contaminations from the genital area. The features of human papilloma HPV in urinary squamous cells are identical to those seen in cervical smears and include increased nuclear size, irregular nuclear borders, hyperchromasia, and perinuclear halos. (Papanicolaou stain, 400x).

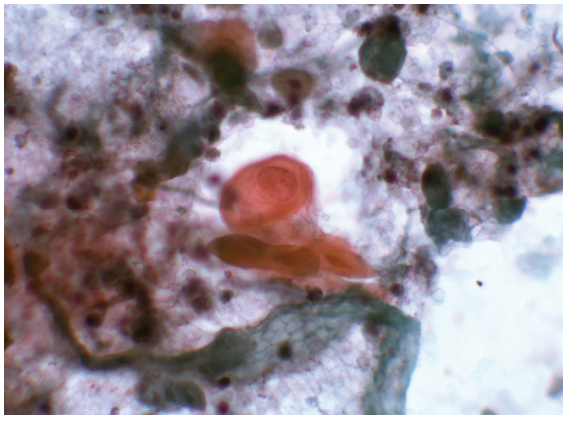

Figure 8. Squamous cell carcinoma. Malignant cells have abundant dense cytoplasm that can be intensely orangeophilic. Nuclei are large and hyperchromatic with irregular nuclear borders. Note the granular, necrotic background. (Papanicolaou stain, 400x)

specimens from male patients and more common in female patients, mostly due to contamination from the gynecologic tract (**Figure 7**). Squamous cell carcinoma (**Figure 8**) in the United States is mostly associated with diver-

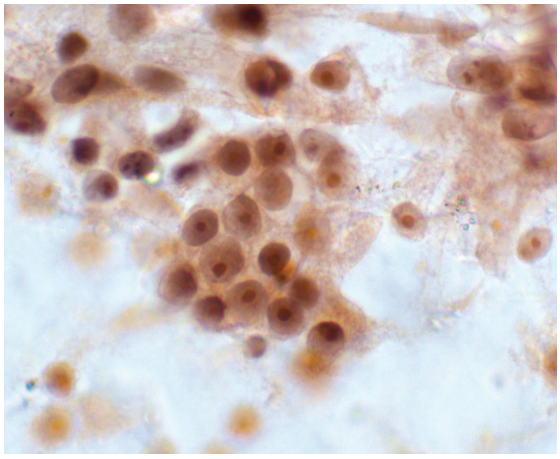

Figure 9. Prostate adenocarcinoma. Sheets or small aggregates of uniform glandular cells have large round nuclei, open chromatin, and prominent single or double nucleoli. (Papanicolaou stain, 600x)

ticulitis while elsewhere, especially in Egypt, it is associated with schistosomiasis.

Adenocarcinoma can be either primary (mostly derived from glandular metaplasia or from urachal remnants) or metastatic. Considering the high incidence of prostate carcinoma, it is rare to see the shedding of prostate cancer cells, characterized by loosely cohesive or single oval-to-low columnar cells with amphophilic cytoplasm and prominent nucleoli into urine (**Figure 9**). This is because most prostate cancers arise from the peripheral zone. Only a large tumor that extends to the urethra or a primary central zone tumor (the so-called ductal/endometrioid type of prostate carcinoma) may shed into urine. Renal cell carcinoma may also be seen in voided urine, although this is uncommon (**Figure 10**). For mucinous tumors, it may be impossible to distinguish between a tumor of primary urothelial origin, urachal origin, or metastatic from the gastrointestinal tract based solely on cytology alone or even on a small biopsy. Currently no specific markers exist to make this distinction, and clinical or radiologic correlation is the only way to determine the origin of the tumor.

Potential sources of misdiagnosis

While it is uncommon to have a false positive urine cytology, especially in the case of low grade lesions, occasionally one may be encoun-

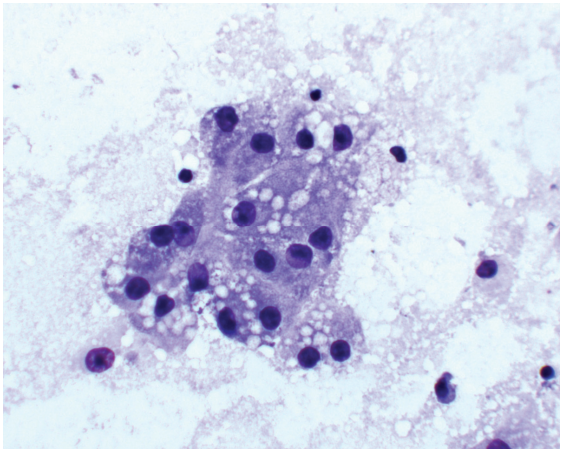

Figure 10. Renal cell carcinoma. Renal cell carcinoma cells rarely shed into urine. This air-dried smear demonstrates the abundant, vacuolated cytoplasm characteristic of renal cell carcinoma. Atypical, centrally-placed nuclei are also noted. (Diff-Quik stain, 600x)

tered. Potential causes include the so-called "false" false positive diagnosis, instrumentation effect (**Figure 11**), stones, therapeutic changes, including chemotherapy and radiation, and viral infection (e.g., polyoma or CMV). The so-called "false" false positive finding is a positive urine cytology with lesions that are not detected clinically at the time of urine examination. Most commonly this is a flat dysplastic or malignant lesion or occasionally an upper tract lesion that may be missed by cystoscopy and biopsy. There has been no specific study to determine the exact proportion of such a lesion in urine cytology.

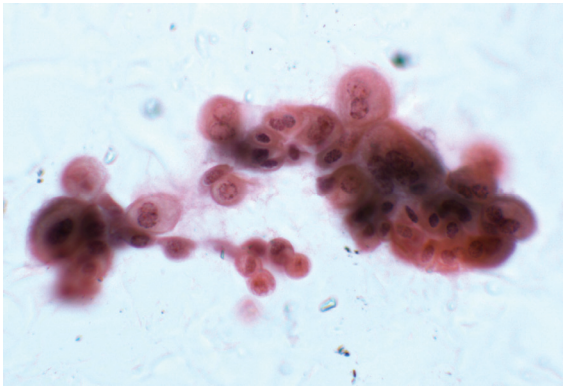

Figure 11. Instrumentation effect. Bladder washing or voided urine after cystoscopy will contain tissue fragments with smaller basal cells surrounded by large binucleated umbrella cells. Note that each cell has a distinct outline and that the tissue fragment boundaries are not smooth. (Papanicolaou stain, 400x)

Adjunct markers to urine cytology

Due to its ease of accessibility, the bladder represents an ideal model for studies in risk assessment, early detection, and the investigation of biomarkers. The ideal biomarker should be noninvasive, provide rapid results, be easy to interpret with little or no variability amongst users, be cost-effective, and most importantly, have a high sensitivity and specificity [3, 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 [8]. The selection of a biomarker depends on whether the objective is prevention, screening, surveillance, or predicting the biological behavior (i.e., risk of progression) of the neoplasm [8].

We will briefly highlight markers that are currently available or under investigation for the detection and monitoring/surveillance of bladder cancer (**Tables 1 and 2**). As such, markers that are useful in predicting recurrence are beyond the scope of this review, although many of the markers herein discussed will cross over into the other categories. We will first briefly discuss markers currently used in clinical practice, some of which have been approved by the Federal Drug Administration (FDA). We will follow with a preview of markers that are more investigational but may potentially be integrated into clinical practice in the near future.

Nuclear matrix protein 22 (NMP-22) – FDA approved

Nuclear matrix proteins (NMP) consist of a three-dimensional web of RNA and proteins that supports the nuclear shape, organizes DNA, and coordinates DNA replication, transcription, and gene expression [3, 13]. NMP-22 is released from the nuclei of tumor cells during apoptosis. NMP released into the urine may be detected by an FDA-approved NMP-22[14] 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 in tumor than normal urothelium [15, 16]. The

Table 1. Adjunct markers for urine cytology

Methods	Sensitivity	Specificity	Comment
Cytology	20-90%	>90%	Low sensitivity for detecting low grade lesions
uCyt™ (Immunocyt)	67%->90%	62-84%	Technically simple, interpretation difficult; FDA-approved
FISH (UroVysion)	30-86%	75->90%	Technically difficult; interpretation difficult; FDA-approved
BLCA-4	89%	>90%	Needs further testing
Telomerase (TRAP)	70-90%	66-88%	Lacks standardization and technically difficult
Cytokeratin 20 (by immunocytochemistry)	65->90%	67->90%	Simple marker; exclude benign conditions to improve specificity
Hyaluronic acid/ Hyaluronidase	83->90%	63->90%	Higher sensitivity for low grade lesions
Survivin	53->90%	88->90%	Needs further testing
Microsatellite instability	58->90%	73->90%	Technically difficult
DD23 (QFIA)	70-85%	55->90%	Needs further testing
Quanticyt	59-69%	68-70%	Low sensitivity, technically difficult
PSCA	80%	85.7%	Needs further testing
DNA methylation	69->90%	60->90%	Needs further testing

Table 2. Strip-based Adjunct Markers for Urine Cytology (all FDA-approved)

Methods	Sensitivity	Specificity	Comment
NMP 22	73%	56->90%	Good sensitivity in low grade lesions
BTA Stat	9.3-89%	50-90%	Benign hematuria lowers specificity
BTA TRAK	56-68%	54-75%	Better sensitivity, specificity still low
FDP	68%	78%	Currently not being produced

enzyme-linked immunoassay uses two monoclonal antibodies to measure the levels of complexed and fragmented forms of the mitotic apparatus in urine [15]. A cut-off of 10 u/ml is endorsed by the manufacturer and initial study for recurrence [17]; however, there is no universally accepted cut-off point. Other studies have suggested cut-offs ranging from 5 to 20 u/ml [17-21].

Some suggest NMP22 may be useful as a screening tool [22]. Surveillance studies either alone or as an adjunct to cytology have estimated sensitivities ranging from 32-100% and specificities from 56-95% [23]. In a 50 study meta-analysis, Lotan et al reported a median sensitivity and specificity of 73% and 80% respectively, superior to a voided urine cytology sensitivity of 34% [24]. Major sources of false positivity are hematuria and pyuria [25]. This is a serious problem since many benign urologic conditions such as stone disease and infection present with hematuria [26]. In general, NMP-22 has a higher sensitivity than cytology, especially in detecting low grade and low stage tumors.

Multiple studies have evaluated the usefulness of NMP-22 as a marker of tumor recurrence. Soloway et al used NMP-22 to predict the likelihood of recurrence after transurethral resection

at subsequent cystoscopy in ninety follow-up patients [17]. Levels less than 10 U/mL were predictive of a low likelihood of recurrence while levels greater than 10 U/mL were predictive of recurrence (overall sensitivity of 69.7% and specificity of 78.5%). Subsequent studies, however, were less impressive. Boman et al reported a sensitivity and specificity of 45% and 65% respectively [27]. Miyanaga et al reported a sensitivity and specificity of 18.6% and 85.1% respectively. Both studies concluded that the low sensitivity was due to the small size of recurrent tumors [18].

A new point-of-care test for NMP22 (BladderChek test) was shown to have sensitivities ranging from 50-85% and specificities ranging from 40-90% [28-32]. Advantages include on-site testing with immediately available qualitative results, making the test an attractive adjunct for cystoscopy.

Overall analysis of the data shows that the NMP-22 test has superior sensitivity over cytology for detection of low grade bladder cancers and may be used to predict increased recurrence risk in patients with elevated levels after transurethral resections. Because of the low specificity, using NMP-22 routinely as a primary detector of bladder cancer is not recommended. The specificity, however, can be improved if patients with benign inflammatory conditions (infections, etc), renal or bladder calculi, foreign bodies (stents or nephrostomy tubes), bowel interposition, other genitourinary cancer, and/or instrumentation are excluded [25, 33].

Bladder tumor antigen (BTA) –FDA approved

The term BTA actually describes three separate tests: 1) BTA, 2) BTA stat, and 3) BTA TRAK. Since the BTA tests depend on the disruption of basement membrane, their sensitivity improves with more invasive cancer [34]. Advantages include increased sensitivity for invasive tumors. Disadvantages include a high rate of false-positive readings secondary to patients with inflammatory conditions secondary to benign prostatic hypertrophy (BPH) and a low overall sensitivity for detection of all bladder tumors.

The original BTA test was a latex-agglutination test that measured levels of basement protein antigen released into urine as a result of tumor invading into the stroma [35]. 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% [20] [36-41].

BTA stat and BTA TRAK detect human complement factor H-related protein (hCFH) which is produced and secreted by several bladder and renal cancer cell lines. The qualitative BTA stat test costs only five dollars and is easily performed in the office with a dipstick format [42]. The overall sensitivity ranges from 9.3% to as high as 89% with higher sensitivity in higher grade tumors [43-50]. The specificity of the BTA stat among healthy individuals is greater than 90%. However, it has low specificity (about 50%) among patients with urinary tract infections, urinary calculi (90% positive using BTA stat [51]), nephritis, renal stones, cystitis, BPH, hematuria, and 2+ to 3+ protein on urine dip stick [49, 52-54]. The low specificity in these conditions is secondary to the test's ability to detect both complement factor H-related protein and complement factor H. Complement factor H is present in human serum at high concentrations and therefore BTA-Stat testing may be falsely positive in benign, hematuria-causing conditions [13].

BTA TRAK is a quantitative test that has a slightly improved sensitivity over its two BTA predecessors [55-57] but has high false positive rates for similar reasons (e.g., inflammation and trauma) which therefore leads to low specificity [57-59]. Moreover, multi-center studies and cohort studies have shown that the sensitivity of the BTA TRAK also varies depending upon the cut-off limit used for the test [57, 59-62].

Overall, the three BTA tests lead to an improved sensitivity compared to cytology but lower specificity due to high false positive rates associated with recent instrumentation, stones, inflammatory conditions, BPH, and hematuria.

Fibrin-fibrinogen degradation products (FDP) – FDA approved

Since bladder tumor cells induce 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) [63]. Thus, patients with bladder cancer may have increased levels

Table 3. Tumor Recurrence in Patients with Negative Cystoscopy [78]

uCyt™	Six Month Follow-up, % (n)	One Year Follow-up, % (n)
Negative Test	13.9 (8/59)	11.9 (7/59)
Positive Test	35.7 (21/59)	47.0 (8/17)

of FDP in their urine. In a review of four series, the sensitivity and specificity of the AccuDx-FDP assay ranged from 68-83% and 68-100%, respectively [38, 64-66]. 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. The test costs about fifteen dollars and takes less than 10 minutes to complete [42]. However, the assay is currently not being produced due to issues regarding test formulation [4].

uCyt+ (ImmunoCyt)™ and tumor associated antigens -FDA approved

uCyt+ (ImmunoCyt)™ is based on the detection of tumor-associated antigens, mostly mucinglycoproteins, in transitional or urothelial carcinoma using monoclonal antibodies. The uCyt+™ assay is the most frequently used immunocytological test today. Three antibodies, fluorescein-labeled M344 and LDQ10 (directed against sulfated mucinglycoproteins), and Texas -red linked antibody 19A211 (directed against glycosylated forms of high molecular carcinomaembryonic antigens, i.e., CEA) are used.

Many studies have evaluated the performance of the test since 1997. Using a threshold of any single cell positive as positive, uCyt+™ has a sensitivity and specificity ranging from 67-100% and 62-84% [67-76] for all tumors and is a promising diagnostic marker for bladder cancer. Since one of the antibodies, M344, appears to be quite sensitive for low grade tumor cells, this test offers an important advantage for detecting low grade tumors. Indeed, a recent split sample study comparing cytology, uCyt+™, and UroVysion™ in 100 urine samples collected from 100 bladder cancer patients (monitoring population) showed that the sensitivity of uCyt+™ outperforms cytology and UroVysion™ for detecting bladder cancer, especially in low grade tumors [77]. The sensitivities of all tumors for UCyt+™, cytology, and UroVysion™ were 76%, 21%, and 13%, respectively. The drawback of the test, however, is low specificity compared to cytology or UroVysion™ (63% for UCyt+™ versus 97% for cytology and 90% for UroVysion™). Piaton et al [78] demonstrated that patients who had a positive UCyt+™ but negative cystoscopy (so called "false" positive) had a much higher risk of tumor detection within a 12 month-follow up (**Table 3**). The findings suggest that many of these so-called "false" positive samples may actually be “false” false positive, i.e., the test detects early lesions, or lesions that may not be seen by cystoscopy at the time of examination. **Figure 12** shows UCyt+™ staining in a urine sample that had atypical urine cytology and subsequent low grade tumor on cytology six months later. Exactly how many of the false positives belong to the "false" false positive category versus the true false positive category due to benign conditions such as urinary tract obstruction is not clear.

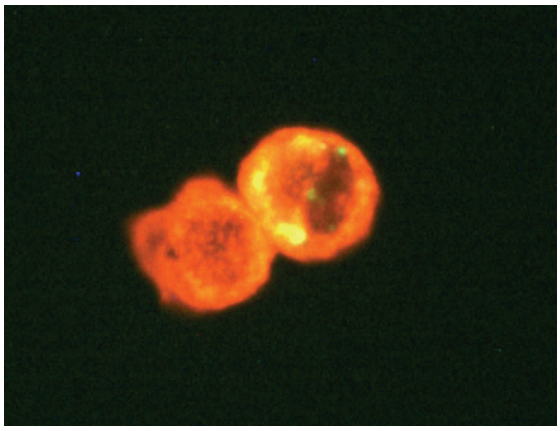

Figure 12. UCyt+™ (ImmunoCyt) in a sample with atypical urine cytology. Fluorescein- and Texas-red-labeled antibodies detect tumor-associated antigens in urothelial carcinoma. Positive tumor cells are both green and red under fluorescence microscopy. (400x)

In addition to a relatively high sensitivity in detecting malignancy, uCyt+™ is technically simple (about a 30 minute incubation on either ThinPrep or filtered slides) and relatively inexpen-

sive. However, the disadvantage is the false positive result occasionally seen associated with urinary tract obstruction due to either stones or BPH. Also, the interpretation of the test using fluorescence microscopy may be difficult for many cytologists, since determination of a positive cell may not be as easy as one might expect in some cases. Further, because the antigens detected are mucinglycoproteins or glycosylated CEA, colonic mucosa cells are positive for the test. As such, the test is not suitable for loop or neobladder urine samples. Another important point worth noting is that the FDA approved this test with a threshold of any single cell positive as positive. However, in our experience (data not shown), and perhaps as expected, samples that have less than or equal to 5 cells positive. Thus, in our report, we not only provide the overall positive or negative finding of the test, but also report samples with 0-5 cells positive as borderline and specify the number of fluorescein- or Texas-red-labeled positive cells.

Multi-target multicolor FISH assay (UroVysion™ test) - FDA approved

Urothelial carcinomas have a number of associated cytogenetic abnormalities involving chromosomes 1, 3, 4, 7, 8, 9 11, 17, etc.[79-81]. These chromosomal abnormalities can be detected by fluorescent *in situ* hybridization (FISH) using DNA probes to chromosome centromeres or unique loci that are altered in tumor cells. Hybridization is detected by fluorescent microscopy. By utilizing multicolored probes (i.e., different DNA probes labeled with different fluorescent dyes), sensitivity is improved compared to using a single probe.

The UroVysion™ test is a multi-target, multicolored FISH assay that utilizes peri-centromeric fluorescent probes for chromosomes 3, 7, 17, and a locus-specific probe to the 9p21 (p16 locus) region. Exfoliated cells from urine specimens are fixed into 12-well slides and incubated with denatured Chromosome Enumeration Probe (CEP) 3 (spectrum red), CEP7 (spectrum green), CEP 17 (spectrum aqua), and Locus Specific Identifier (LSI) 9p21 (spectrum gold). The slides are counterstained and observed under a fluorescent microscope (UroVysion™/Abbott Laboratories). Suggested criteria for a positive assay include finding 5 or more urinary cells with gains of 2 or more chromosomes, or 10 or more cells with gain of a single chromosome (e.g. trisomy 7). Also homozygous detection of 9p21 locus in greater than 20% of epithelial cells is considered a positive test [48, 82]. However, consensus criteria for a positive FISH test have not been determined, and the studies evaluating the sensitivity and the specificity of UroVysion™ utilize varying criteria for positivity.

Initial case-control cohort studies showed that the sensitivity of UroVysion™ to detect bladder cancer was 81-84% [83, 84]. In more recent studies, the sensitivity ranged from 30-86% [85-88]. The assay has increased sensitivity for detecting higher grade and higher stage tumors, however, the sensitivity for detecting low grade tumors is not clear. Low grade tumors are the most difficult to diagnose by cytology. In fact, our split-sample study of 100 bladder cancer monitoring urine samples showed that the sensitivity for UroVysion™ test is substantially lower compared to the uCyt+™ test (13% versus 76%, respectively) [77]. The specificity for UroVysion™, however, is high in our study (90%, similar to cytology) and varies between 75% and 100% by others [48, 85, 89-94]. Notably, the test appears to have high specificity among patients who have a variety of benign genitourinary conditions, including microhematuria, BPH, infections, and inflammation [48, 84, 91].

Some studies also suggest that UroVysion™ can predict recurrence. Skacel et al in a retrospective cohort study reported that 8 out of 9 FISH positive patients with atypical cytology but negative biopsy had biopsy proven bladder cancer within 12 months [95]. In another study, Bubendrof et al reported that 4 of 5 so-called "false-positive" UroVysion™ tests had recurrence within 8 months; none of the true negative cases recurred within 18 months. However, the criteria used for a positive test in this study differed from those suggested by the manufacturer. Moreover, the authors concluded that not all FISH aberrations were equally important [96].

In summary, UroVysion™ seems to have high specificity for the detection of bladder cancer and for the ability to detect bladder tumor recurrence prior to clinical detection. Thus, it may be used as a confirmatory test for either cytology or uCyt+™ test. One major limitation to the assay is the lack of consensus on the criteria used to

evaluate abnormal cells. Additionally, the test has relatively low sensitivity in the detection of low-grade bladder tumors as discussed before and therefore may not improve the sensitivity as an adjunct for cytomorphologic analysis.

BLCA-4

Konety and Getzenberg have described several specific nuclear matrix proteins which are present only in patients with bladder cancer (BLCA 1-6) and three proteins that are present in normal bladder tissue (BLNL 1-3) [97]. One of these markers, BLCA-4, is found throughout the bladder in patients with bladder cancer, including both tumor and normal regions. The marker is hypothesized to reflect a type of "field effect", which has been described by several investigators at the genetic level. Studies by Getzenberg et al have shown that this marker appears to be a transcriptional regulator that may play a role in regulating gene expression in bladder cancer [98]. In initial studies using an indirect enzyme linked immunosorbent assay (ELISA), they report 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 [99]. Subsequent trials utilizing a sandwich-based immunoassay examined BLCA-4 expression in a variety of patients including those with biopsy proven bladder cancer, benign urologic conditions, prostate cancer, and normal individuals. The results of this trial demonstrated a sensitivity of 89% and a specificity of 100% [100].

Similar proteins, namely BLCA-1, have also been found to be potentially useful. Unlike BLCA -4, BLCA-1 is expressed in tumor areas only and is not seen in adjacent normal tissue or tissue from normal individuals. An immunoassay detecting BLCA-1 in urine samples has been developed and demonstrates relatively high sensitivity and specificity [101]. However, additional independent studies will be needed to validate the findings.

Telomerase

Telomeres are nucleotide sequences on the ends of chromosomes that are important in maintaining the integrity of DNA. With each replication cycle, a portion of the telomere is lost, and complete loss of telomeres is associated with cell death. Telomerase is an enzyme that lengthens telomeres; thus, increased levels of telomerase allow tumor cells to maintain immortality [102].

The telomeric repeat amplification protocol (TRAP) assay is a polymerase chain reaction (PCR)-based test that detects increased levels of telomerase secreted into the urine by bladder cancer cells. Other telomerase assays are available that detect human telomerase reverse transcriptase and its RNA component. However, for sake of brevity, discussion will be limited to the TRAP assay. It detects telomerase reaction products in vitro, has a 10 hour turnaround time, and costs around seventeen dollars [4, 102]. Generally, the TRAP assay has better sensitivity than cytology with slightly lower specificity [20, 103]. Recent studies show the sensitivity to range from 70-90% at a 50 arbitrary enzymatic unit cutoff value. Specificity is slightly lower, ranging from 66-88% [13, 20, 104-107]. The lower specificity may be explained by contamination of benign cells with telomerase activity (e.g., lymphocytes). The sensitivity was slightly increased with bladder washings compared to voided urine [103]. In detecting recurrent tumors, however, TRAP has a low sensitivity (35%) [108].

Difficulties associated with the telomerase test have limited its widespread use. Urine must be processed within a 24 hour period [13]. At least 50 cells must express telomerase for the assay to detect telomerase reliably [13, 109]. Finally, false negative results may occur depending on sample collection, processing, and the presence of PCR inhibitors or ribonucleases [110]. Currently the TRAP assay is not recommended in the clinical setting because of complicated laboratory procedures and the lack of standardized sample processing to reduce false positive and false negative results.

Cytokeratins

Cytokeratins (CK) make up a large component of intermediate filaments that are found in epithelial cells [111]. Twenty cytokeratins have been identified in human cells, and their expression varies depending on epithelial cell type and state of differentiation [112]. Expression of cytokeratins 8, 18, 19, and 20 has been evaluated as potential bladder cancer markers.

UBC-Rapid and UBC-ELISA tests (manufactured by IDL Biotech, Börlabger Sweden) detect the presence of cytokeratin 8 and 18 in the urine of

bladder cancer patients. UBC-Rapid is a point-of-care test, and UBC-ELISA is a 2-hour sandwich ELISA test. Several studies show that the sensitivity of the UBC tests to detect both primary and recurrent bladder cancers varies from 12-79% with a specificity ranging from 63-97% [46, 57, 113-121]. Several studies have also reported lower sensitivities for the detection of low grade and low stage tumors. The sensitivity of UBC to detect grade 1, 2, and 3 bladder tumors is 13-60%, 42-79%, and 35-75% respectively [114, 116, 117, 122]. Retrospective studies report a 21-25% sensitivity of UBC- Rapid to detect stage Ta tumors and carcinoma in situ (CIS) and therefore it has insufficient diagnostic value for detecting superficial bladder cancer [46, 122]. Compared to other bladder tumor markers and cytology, UBC tests have generally lower sensitivity.

The expression of cytokeratin 20 is restricted to the superficial and occasionally the intermediate cells of the normal urothelium, but not the basal cells. Aberrant cytokeratin 20 (CK 20) expression is seen in bladder cancer cells [112]. Reverse transcription (RT)-PCR assays have been used to evaluate CK 20 expression in urine samples. Several studies have shown that CK 20 RT-PCR has a 78-87% sensitivity for detecting bladder cancer in urine. The specificity of CK 20 RT-PCR ranges from 55.7% to 98% [111, 123-129]. CK 20 RT-PCR on blood specimens has also been studied for early detection of systemic bladder cancer progression [126]. Overall, 17-29% of bladder cancer patients were positive for CK 20 RT-PCR [130, 131]. The high sensitivity intrinsic to the RT-PCR methodology may also be associated with low specificity [125].

Cytokeratin 20 immunocytochemistry has also been evaluated as an adjunctive marker for atypical cytology. Klein et al reported a sensitivity of 91% and a specificity of 67% in a study of 87 patients [132]. Specimens with false-positive results had cytology consistent with premalignant conditions such as atypia, hyperplasia, or metaplasia [111, 132]. All completely healthy patients had negative CK 20 levels. Lin et al showed that overall sensitivity and specificity of CK20 immunocytochemistry for the detection of urothelial carcinoma were 94.4% and 80.5% respectively [133]. This study demonstrated that CK20 is a useful adjunct marker for urine cytology, that is, analysis of CK20 can be conveniently performed on the same slide after routine morphological evaluation and be used to triage atypical urine cytology into low and high risk categories for clinical follow-up. Golijanin et al also reported a high sensitivity (82%) for CK 20 immunocytochemistry in patients with microhematuria and those with bladder cancer. The specificity in this study was 76% [134]. Although overall sensitivity was high, the Golijanin study also showed that the sensitivity varied depending on tumor grade, with only 56.5% sensitivity for grade 1 tumors. CK 20 staining had a much higher sensitivity with grade 2 and 3 bladder tumors (93% and 92% respectively) [134]. More recent studies have supported these findings, demonstrating sensitivities ranging from 65-86%, specificities from 86-100%, and advantages over urine cytology in the detection of primary, recurrent, stage pT1 and grade 2/3 tumors [135-137]. One of the pitfalls of the CK20 immunocytochemical staining is that often benign umbrella cells are positive. Thus, in our practice, we only use CK20 in samples containing few small basaloid cells and to distinguish whether these cells represent normal basal cells (negative CK20) versus dysplastic or malignant cells (positive CK20).

Cytokeratin 19 (CK 19) is expressed in normal urothelium. CYFRA 21-1 is a soluble fragment of CK 19 that can be measured in the urine when urothelial cells are exfoliated and lysed. There are two commercially available tests that can measure CYFRA 21-1; one is a solid phase sandwich immunoradiometric assay (Cis Bio International, Gif-sur-Yvette, France) and the other is an electrochemiluminescent immunoassay with the Elecsys 2010 system (Roche Diagnostics). CK 19 levels are measured after urinary creatinine is normalized [138]. A retrospective cohort study showed that CYFRA 21-1 levels are increased in bladder cancer patients when compared to patients with other urologic conditions and normal controls. The level of CYFRA 21-1 in patients with bladder cancer, patients with other urologic conditions, and normal controls were 154.4 ng/mL, 22.3 ng/mL, and 2.4 ng/mL respectively [139]. When a cutoff level of 4 ng/mL was applied, the sensitivity and specificity of CYFRA 21-1 for detection of bladder cancer were 96.9% and 67.2% respectively. The low specificity was attributed to high CK 19 levels in patients with urolithiasis and urinary tract infection. Another study reported the sensitivity and specificity of CYFRA 21-1 to be 75.5% and

71% respectively when using the electrochemiluminescent assay [140]. This study showed the sensitivity of detecting bladder cancer increased with higher grade tumors (sensitivities to detect grade 1, 2, and 3 tumors were 54.5%, 66.7%, and 88.2% respectively). However, the study also reported a false positive rate of approximately 33% in patients with various urologic conditions including urolithiasis, stenosis, BPH and urinary tract infections. Subsequent later studies have shown sensitivities ranging from 43-79% and specificities ranging from 68-88% with a 4 ng/mL cut-off [118, 141-143]. Some have reported improved sensitivity over cytology in detecting Grade 1 tumors [141].

In summary, cytokeratin 20 detected by RT-PCR or immunocytochemistry appears to be a useful and simple marker. However the often positive findings of CK20 in normal umbrella cells preclude widespread application of the test as a primary screening tool. Rather, it is better used as a test in specific settings; for example, in samples that contain scant small basaloid cells. The UBC tests appear to have lower sensitivity compared to other tumor markers and currently do not have sufficient diagnostic value for the detection of bladder cancer. Overall, the use of CYFRA 21-1 is promising with some conflicting studies of its benefit over urine cytology.

Hyaluronic acid/Hyaluronidase

Hyaluronic acid (HA) is a glycosaminoglycan that promotes tumor cell adhesion and angiogenesis [13, 144]. Hyaluronidase (HAase) is an enzyme which cleaves HA into fragments; these cleaved fragments then aid tumor growth and propagation by promoting angiogenesis [145, 146]. Initial case control studies measured both HA levels and HAase activity in the urine. The results showed that there was a 2.5 to 6.5-fold increase in HA levels (83% sensitivity and 90.1% specificity) in patients with bladder cancer, regardless of tumor grade [146]. HAase activity levels were also increased 3 to 7-fold in patients with high grade bladder cancers (81.5% sensitivity and 83.8% specificity) [147]. The combination of tests increases the overall sensitivity to 92%.

A prospective study to monitor bladder cancer recurrence showed that the HA-HAase was more sensitive and more accurate than BTA stat (sensitivities of 94% and 61% respectively). The BTA stat test, however, had better specificity than the HA-HAase test (74% and 63% respectively) [49]. Subsequent comparative studies showed that HA-HAase testing had the highest sensitivity in detecting both low grade/low-stage, and high grade/high stage tumors [46, 67]. Passerotti et al compared accuracy of the HA test to UroVysion™, BTA stat, and cytology in a prospective study involving bladder cancer patients with either primary or recurrent tumors. The specificity of the test was determined in patients with a history of bladder cancer not evident at the time of testing and in patients with BPH. HA testing had the highest sensitivity among all the tests (83%) and a slightly higher specificity [148]. Eissa et al examined HAase RNA in urine detected by RT-PCR and found superior sensitivity (90.8%) over cytology (68.9%) and CK 20 (78.1%) with specificities of 93.4%, 98.1% and 80.2%, respectively [149].

In summary, HA-HAase testing is a promising marker for the detection of primary and recurrent bladder tumors. The test has high sensitivity with the ability to detect low grade/low stage and high grade/high stage tumors. The test may be most useful in screening bladder cancer patients for recurrence.

Survivin

Survivin is an inhibitor of apoptosis that extends cell viability in bladder tumors [150, 151]. Survivin is undetectable in most normal adult tissue and correlates with unfavorable disease and shortened overall survival in neuroblastoma, colorectal cancers, and non-small cell lung cancers [152-155]. Gazzaniga et al demonstrated using RT-PCR that Survivin mRNA is expressed in 30% of bladder tumors [156]. Schultz et al found Survivin mRNA expression in 100% of bladder tumors [157].

Smith et al analyzed urine specimens with a polyclonal antibody for Survivin and then validated findings with both western blot and RT-PCR. Survivin was detected in 31 of 31 patients with new onset or recurrent bladder cancer using the polyclonal antibody system and 15 of 15 patients with RT-PCR, giving a sensitivity of 100%. Only 3 of 35 patients with treated bladder cancers and negative cystoscopies tested positive, suggesting Survivin could be used for surveillance. Additional data showed Survivin

was negative in 17 healthy volunteers and 30 patients with non-urothelial genitourinary cancers. There were only 4 false positive results amongst the 30 patients with non-neoplastic urinary tract disease, including 3 with bladder abnormalities on cystoscopy and one patient with an elevated PSA. The overall specificity for Survivin was 95% [151]. Other studies have shown that urinary Survivin levels are higher in patients with recurrence of carcinoma compared to those who achieved remission after treatment with BCG or mitomycin C. The sensitivity and specificity for detecting recurrence were 100% and 78% respectively [158]. Finally, urinary assays detecting Survivin mRNA by RT-PCR have shown sensitivities ranging from 53-94% and specificities from 88-100% [159-162].

In short, Survivin could potentially be a valuable marker for both detection and monitoring of bladder cancer, but its validation awaits further testing.

DNA ploidy and S-phase fraction

DNA ploidy and S-phase fractions can be evaluated from urine samples by either flow cytometry, image cytometry (ICM), laser scanning cytometry (LSC), or fluorescence *in situ* hybridization (FISH) [3]. While an FDA-approved FISH methodology has already been discussed in this article (UroVysion™), other methodologies such as flow cytometry can identify neoplastic cells with increased nuclear size and increased nuclear chromatin ratios and further determine their DNA ploidy (e.g., diploid, tetraploid, or aneuploid). High grade tumors may be detected by the presence of aneuploidy and a higher percentage of cells in the S phase [3]. Sensitivity for high grade urothelial tumors or carcinoma in situ may reach 90% [3, 163-165]. Because this technique is expensive and requires a large number of cells as well as highly trained personnel, flow cytometry has not gained widespread acceptance.

Image cytometry (ICM), especially a fluorescence-based system, allows the measurement of DNA content in each individual cell, making this an attractive alternative to flow cytometry, which requires a large cell population. Hemstreet's group, using a specific platform called Quantitative Fluorescence Image Analysis (QFIA), demonstrated that single cell-based DNA content analysis (by detecting cells over 5c DNA) is more sensitive than cytology or flow cytometry in detecting low grade tumors [166]. Moreover, such a system allows the analysis of multiple other protein (or potentially DNA)-based markers at the same time. Subsequent studies have shown similar findings [92, 167]. Laser scanning cytometry combines the advantages of flow cytometry and ICM by laser-scanning individual cells to quantify fluorescence [168]. While numerous studies have generally shown that imaging-based DNA content analysis is a useful marker for detecting bladder cancer, the need for expensive instrumentation and careful quality control measures for fluorescence quantification precludes the widespread application of this useful technology.

Microsatellite instability assays

Similar to other malignancies, bladder cancer DNA repair mechanisms may be defective, leading to persistent errors in replication, i.e., genomic instability. Microsatellites are inherited tandem repeat DNA sequences that can be analyzed to detect replication errors [169, 170], also known as microsatellite instability. PCR amplification of these tandem repeat sequences can detect microsatellite instability and loss of heterozygosity (LOH) of tumor suppressor genes. While microsatellite instability tends to be found more frequently in advanced bladder cancers, it can be demonstrated in low grade tumors when more microsatellite markers are used. The methodology requires a substantial number of microsatellite markers to achieve high sensitivity [171-174].

Microsatellite analysis has been used to confirm that low grade papillary urothelial carcinoma has instability and/or loss of chromosome 9 and p16 (MTS1) tumor suppressor gene [169, 175]. Regardless of tumor grade and stage, bladder tumors typically have LOH in the 9p region on microsatellite analysis [169, 176]. 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 were also positive [13, 177]. Recent studies by van Rhijn et al showed that activating FGFR3 mutations are detectable in low grade superficial bladder cancers, and, when used in conjunction with microsatellite analysis, the sensitivity for bladder cancer detection increases to 89% (compared to 71% for negative FGFR3 mutations) [178]. Other studies have shown that microsatellite instability may be used to predict

recurrence of urothelial carcinoma. The sensitivity of selected studies ranged from 58% to 95% with specificity ranging from 73-100% [169, 177, 179-185] with some studies showing a prediction of recurrence months before positive cystoscopy. However, large-scale analysis will be needed to determine specificity, especially in symptomatic populations, to understand the true clinical utility of the test.

DNA chips (HuSNP chip) can detect alleles differing by a single nucleotide polymorphism. With this technology, LOH can be detected at 1500 different loci at once. Preliminary results in thirty-one patients show LOH at 24 or more loci, demonstrating the ability of chip technology to detect bladder tumors with 100% sensitivity. Nine control subjects and 4 of 5 patients with hematuria had negative chip findings [186].

Although studies to date show that microsatellite analysis has excellent sensitivity and specificity regardless of tumor grade and stage, tumor multiplicity, or previous history of bladder cancer, the studies are based on relatively small sample sizes. Disadvantages include a potential contamination of non-urothelial cells that may cause either false negative or positive findings, long turnaround time, high equipment cost, and need for trained personnel, rendering this test impractical for routine clinical use. Currently, the testing of urine for microsatellite instability is not recommended for monitoring or for detection of primary tumors.

DD 23

A monoclonal antibody called DD23 resulted from the immunization of a BALB/c mouse with fresh bladder cancer. The antigen recognized by DD23 is identified in 81% of bladder tumors. Testing for this antigen utilizing Quantitative Fluorescence Image Analysis (QFIA) has an 85% sensitivity and a 95% specificity [13, 187]. When used in combination with cytology, the sensitivity is 94%, and the specificity is 85% [3]. UroCor, Inc., (now part of LabCorp Inc.) licensed the DD23 monoclonal antibody, and the analytic method was converted to an alkaline phosphatase immunohistochemical assay. A prospective study evaluating the utility of DD23 immunohistochemistry showed that the overall sensitivity and specificity of DD23 were 81% and 60%, respectively compared to a sensitivity and specificity of 66% and 85%, respectively with cytology alone. The combination of cytology and DD23 had a sensitivity of 85% and specificity of 55% [188]. Another study showed a sensitivity of 70% and specificity of 60% with improved sensitivity in patients with a prior history of intravesical treatment [189]. Overall, DD23 is a promising monoclonal antibody that can be used in the detection of bladder cancer. Fluorescent assays seem to have better overall sensitivity and specificity when compared to immunocytochemistry. When both the fluorescent and the immunocytochemical assays are used in combination with cytology, the sensitivity is increased with a slight decrease in specificity. Further studies are necessary, however, to validate the utility of both methods in larger prospective trials.

Quanticyt nuclear karyometry

Quanticyt is an automated quantitative karyometric cytology system that objectively interprets nuclear features (nuclear shape and DNA content) based on microscopic images. Cytospin preparations of ethanol-polyethylene glycol-fixed bladder wash specimens are made. Light microscopy nuclear images are transferred to a computerized image analysis system. Using an internal lymphocyte standard, mean nuclear shape (MPASS) and DNA content (2c deviation index, or 2cDI) are measured. The samples are then stratified into low, intermediate, or high-risk groups [39, 190-194].

Van der Poel et al reported that Quanticyt test had a 59% sensitivity and a 70% specificity for detecting bladder cancer. Wiener et al reported a sensitivity of 69%. The sensitivity increased for higher grade tumors, with a sensitivity of 85% for grade 3 tumors [39, 195]. Additionally, a 2cDI of $\geq$ 2.00 was a significant predictor of carcinoma in situ, invasive bladder cancer, and progression [190].

The utility of Quanticyt is somewhat limited by the low sensitivity. In one study by van der Poel et al the rate of finding invasive disease was 10% among individuals classified as high-risk by Quanticyt but only after five consecutive samples were collected from the patient [190]. Other studies suggest that Quanticyt also overestimates the risk for bladder abnormalities, and therefore has a lower specificity than bladder wash cytology and voided urine cytology [87, 195].

In summary, Quanticyt is a potential adjunctive test for risk stratifying patients for bladder cancer. However, the test is limited by its low sensitivity, its need for sophisticated instrumentation and technical expertise, and its potential to overestimate the risk of bladder cancer. At the present time, general applicability of this methodology is restricted.

Prostate Stem Cell Antigen

Recent studies have shown expression of prostate stem cell antigen (PSCA), a glycosylphosphatidylinositol (GPI)-anchored cell surface antigen, to be increased in human urothelial carcinoma. PSCA expression is detected in more than 80% of local tumors and in 60 to 100% of metastatic tumors [196-198]. In 2003, a study utilizing PSCA immunocytochemistry as an adjunctive marker for urothelial carcinoma in voided urine samples showed that positive staining with PSCA had increased sensitivity and specificity for detection of urothelial carcinoma when compared to cytology alone. Cheng et al showed that the sensitivity and specificity of PSCA staining alone were 80% and 85.7% respectively compared to cytology (46.7% sensitivity). Of the false positive cases, one had a history of interstitial cystitis and the other had a history of hematuria. The sensitivity and specificity increased when cytology and PSCA immunocytochemical staining were combined in an either/or situation to 83.3% and 85.7% respectively [199]. Wu et al recently conducted a genome-wide association study on 969 bladder cancer cases and 957 controls and identified a missense variant in the PSCA gene consistently associated with bladder cancer in US and European populations [200].

In summary, it appears that PSCA may be a useful adjunctive marker to urine cytology in the detection of urothelial carcinoma in voided urine samples. Initial studies, however, need to be validated with a larger number of samples and in a wider variety of clinical settings before any definite conclusions can be made.

DNA methylation

CpG dinucleotides are present in the promoters of many genes and may become methylated which in turn inhibits gene expression. Alteration in methylation status is a frequent occurrence in cancer where, for example, methylation inactivates a tumor suppressor gene and results in cancer development. Methylation of the p16/CDKN2A gene in bladder cancer was first described by Gonzalez-Zulueta et al [201] and since then many studies have examined various loci and combination methylation marker panels in urine specimens for the detection of bladder cancer.

Dulaimi et al [202] examined the methylation status of APC, RASSF1A, and p14(ARF) tumor suppressor genes and demonstrated an 87% sensitivity in the urine of 45 tumor patients obtained prior to bladder cancer surgery. Others have examined a combination of panels ranging from 2 to 15 separate gene panels and have reported sensitivities and specificities ranging from 69-92% and 60-100%, respectively [203-207]. Renard et al [207] recently identified two genes, TWIST1 and NID2, frequently methylated in bladder cancer including early-stage and low grade tumor. The two-gene panel detected bladder cancer in three separate sets of urine specimens (selection, training, and validation sets) for a total of 496 patients with a sensitivity of 90% and specificity of 93%.

Studies demonstrating the use of DNA methylation for screening and surveillance are promising. Further multi-institutional studies to examine the various proposed methylation panels and validate this methodology are required to better understand its applicability to the management of bladder cancer.

Perspective

Cystoscopy in combination with Papanicolaou [15] cytology remains the most effective means of detecting bladder cancer. However, cystoscopy is an invasive procedure, and while cytology remains a useful method for detecting high grade tumors, its utility in detecting low grade tumors remains limited due to the lack of distinguishing cytologic features between low grade disease and reactive processes. The selection of the ideal biomarker depends on whether the goal is detection/screening, monitoring/surveillance, or predicting progression to invasion or metastatic disease. This article has 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. Most of these markers also have lower specificities when compared to cytology. Furthermore, all of these tests must still be utilized in conjunction with cystoscopy findings. Complete elimination of cystoscopy or cytology to detect bladder cancers does not appear feasible, at least in the near future. One or more of these tests may eventually prove to be a useful adjunct for cytology and cystoscopy, but each and every one of the markers awaits further validation. Currently, with all information on hand, the best approach still-seems to be using cytomorphologic analysis as the initial screen test, using uCyt+™ as a reflex test for atypical cytology, and using UroVysion™ as a confirmatory test for either positive cytology or uCyt+™. Whether such an approach would withstand time remains to be seen.

Please address correspondence to: Peggy S. Sullivan, MD, Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA, Center for the Health Sciences, A3-231L, 10833 Le Conte Avenue, Los Angeles, CA 90095-1732, USA. TEl: (310) 983-1092, Fax: (310) 206-8108, Email: psullivan@mednet.ucla.edu

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