New Biomarkers for the Pathologic Diagnosis of Urologic Neoplasia

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Abstract
Since its description in 1945 by Papanicolaou and Marshall, urine cytology has been the mainstay of screening and surveillance for urothelial neoplasia. It provides a convenient noninvasive means for observing patients with histories of bladder cancer, for evaluating patients with microscopic hematuria or irritative symptoms, and for screening high-risk populations. As a gold standard, urine cytology is undeniably inadequate and is plagued by low sensitivity and subjective diagnostic criteria for the majority of lesions. Whether urine cytology possesses the optimal combination of sensitivity and specificity to retain consideration as a screening modality depends upon the perceived goals of the clinical practice. Medical technology companies have seized the opportunity to make significant changes in the way we perform this test. Several promising bladder tumor biomarkers have been investigated as potential screening and surveillance tools. Efforts have focused on improving the sensitivity for detecting urothelial neoplasia, utilizing new biomarkers using both “on-slide” and “off-slide” technologies. Designed for the practicing pathologist, this workshop will integrate evolving science of new technologies with practical use in a clinical setting. The workshop will present an assessment of each FDA-approved test with discussion of methodology, appropriate use of the test, accuracy, quality assurance, regulatory compliance, billing and coding, cost analysis, utilization, information management, and results reporting. The workshop will be interspersed with clinical cases, with examples of the performance, interpretation and reporting of molecular assays.

Objectives
1. Recognize the current limitations of cytologic examination for urologic neoplasia.
2. Evaluate the potential effectiveness of new technologies in attempting to increase the accuracy of urologic cytology testing
3. Understand the practical aspects of managing the integration of new technologies into the laboratory
4. Review interpretation and reporting of selected molecular assays.
Introduction

Clinical epidemiology of urothelial carcinoma
- 60,240 (ACS est) new cases of bladder ca (2004); Increased by 36% between 1984 and 1993.
- 12,710 deaths annually (ACS est. 2004)
- 4th most common ca in men (3% of all cancer deaths); 6th most common ca in women.
- Most are superficial tumors (non-invasive), 60% will recur, and 42% risk of tumor progression (stage and grade) over 10 years.
- Definitive proof of bladder Ca requires invasive cystoscopic examination.
- Main issues for clinicians is detection, recurrence, and progression. The clinician requires assistance from the pathologist for continued surveillance, follow-up and management of these so-called “nuisance tumors”
- The new case rate plus high rate of recurrence cause a burden to the medical system. (about 1 million patients currently). Is it clinically necessary or cost-effective to detect all recurrent lesions?
- Detection of “new” low-grade lesion may be of secondary importance to the early detection of disease progression.

Carcinogenesis of urothelial carcinoma
- Mostly industrialized nations. Related to cigarette smoking and occupational exposure (rubber industry and organic solvents).
- Abnormalities on chromosome 9 most common finding. Also, p53 mutations.

Grading and Staging of Urothelial carcinoma.
- 90% of bladder tumors are TCC. Either “papillary” low-grade, non-invasive or “flat” in situ or invasive tumors. Majority are superficial.
- >75% Ta/T1; 20% invasive (>T1), 5% metastatic at presentation.
- Current classification strategy used for histopathology and cytopathology is 1998 WHO/ISUP scheme. Urothelial carcinoma (low-grade or high-grade), PUNLMP (papillary urothelial neoplasm of low malignant potential), and Papilloma.
- Clinically, divided into low-risk and high-risk lesions, based upon pathologic stage, pathologic grade, risk of recurrence, single vs. multiple lesions, and presence of CIS.

Treatment of Urothelial Carcinoma
- TUR-BT
- Cystectomy
- Fulguration
- Chemotherapy
- BCG (intravesicle agents)
Surveillance and Follow-up for Urothelial Carcinoma

- Pathologic Stage:
  - Ta – 4% progress, 52% recur
  - T1 – 30% progress, 77% recur
  - Tis – 60% progress

- Histologic Grade:
  - Grade 1: 2-10% progress, 63% recur
  - Grade 2: 11-19% progress, 67% recur
  - Grade 3: 35-45% progress, 71% recur

- Main method for surveillance and followup is cystoscopy and voided urine cytology. Costly and involves multiple invasive procedures each year. (?for life)

- Cystoscopy:
  - Q3 months X1-2 years
  - Q6 months X 2 years
  - Q12 months X10 years.

- Estimated need for 500,000 cystoscopies annually for surveillance in US.

Current Methodologies to Detect Urothelial Neoplasms

Cystoscopy

- Sensitivity .70 to .90 (depending upon series). Limitations are due to small, flat, inaccessible lesions.

- Pros: Provides information about multifocality, appearance, size, ability for resection, and submission of specimens for histopathology examination.

- Cons: invasive, costly, not cost effective for screening (e.g. hematuria work-up).

- Goal: reduce need for surveillance cystoscopy.

Urinary Cytopathology

- Assay of choice, adjunct to cystoscopy; most specific assay.

- Sample: Voided urine cytology (VUC) (most often used), bladder washing, upper urinary track brushings, upper urinary track washings.

- Use: follow-up treated TCC, presence of recurrence, evidence of early progression in stage and grade, diagnosis of new ca, screen for TCC.

- Regulatory:

- Methodology: routine cytomorphologic examination, cytopsins, filters, thinlayers, smears; widely available; Prone to high inter-observer variability, requires expert personnel for screening, interpretation, and reporting, not automated, manual method, time consuming

- Sensitivity: .25-.40, especially for low-grade and low-stage tumors. Can be improved when experts use vigorous cytomorphologic criteria to separate low-grade lesions from benign urothelial cells.

- Specificity: >.93
Utility: positive VUC is highly predictive of presence of TCC

The invasive nature of cystoscopy and the lack of consistency of cytology have led to an aggressive search for a urine-based maker for bladder cancer. A plethora of markers have been identified and are currently undergoing evaluation. Because cytology is relatively accurate at identifying high-grade TCC and CIS, the key advantage of most of the new markers is their ability to identify patients with low-grade TCC.

FDA-Approved Biomarkers
- BTA-Stat
- BTA-Track
- NMP22 (2)
- Accu-DX Test AuraTek FDP (no longer available)
- ImmunoCyt/uCyt+
- UroVysion

Morphology-Based Biomarker Assays

Immunocyto/ImmuCyte/uCyt+ (Diagnocure, Sainte-Foy, Quebec, Canada)
- Manufacturer: Diagnocure, Inc. (direct distributor in US) ([www.diagnocure.com](http://www.diagnocure.com))
- Principle: Panel of monoclonal antibodies expressed on urothelial tumor cells
- Sample: Voided urine – 2\(^{nd}\) AM void preferred. Mix 1:1 with 50% ETOH.
- Use: slide based immunofluorescent assay, used in combination with routine cytology
- Clinical data shows reduced need for cystoscopy (40%) to a q6-12 schedule
- Method: immunocytochemistry to M344, LDQ10, and 19A211
  - LDQ10/M344 is cytosolic mucin, expressed in 70% Ta/T1, 25% Tis, and 15% invasive ca.
  - 19A211 is a sialoglycoprotein (HMW CEA) expressed in 70% of Ta/T1, 60% Tis, and 50% of invasive ca.
- Sensitivity: 38-100%; mean=74% best used in combination with VUC.
- Specificity: 73-84%; mean=80%
- Cost: $2,000/kit (kit=50 tests) 2 hrs lab time for 10 tests. 12 min per slide reading.
  - Use filters or TP slides ok (spray fix with 50% isopropyl alcohol).
- Codes: CPT 88346 (X2) + 88112 or 88108. charges $358

Immunocyto/Cell adhesion molecules(E-cadherin/CD44)
- Manufacturer: varies
- Principle: Loss of expression of E-cadherin associated with high histologic grade, advanced pathologic stage, and adverse outcomes. Elevated E-cadherin in urine associated with papillary lesions (low-grade) (e.g. up-regulated). E-cadherin is down-regulated when these lesions progress.
Aberrant CD44 activity detected at ELISA, nucleic acid, and protein level have been reported in exfoliated cancer cells in patients with bladder cancer.

Sample: urine
Use: bladder ca detection
Regulatory: IUO/RUO
Method: immunocytochemistry
Utility:

**Immunocytology/Blood Group Antigens**

- Manufacturer: varies
- Principle: loss of cellular expression of blood group antigens are makers of urothelial neoplasia. Lewis X blood group expression indicator of recurrent TCC
- Sample: urine
- Use:
- Regulatory: IUO/RUO
- Method: Lewis X expression by immunocytochemistry. 486p3/12 or BG7 antibody.
- Sensitivity: .85
- Specificity: .85
- Costs: unknown
- Utility: not associated with stage or grade. 51% of “reactive” urothelia will express Lewis X.
- “No significant improvement over VUC”

**Fluorescence cytology**

- Manufacturer: varies
- Principle: Photodynamic diagnosis plus urine cytology
- Sample:
- Use: bladder ca detection
- Regulatory: n/a
- Method: 5-aminolevulonic acid or hypericin instillation into bladder (invasive!); examine cell sediment with fluorescent scope
- Sensitivity: “high”
- Specificity: “high”
- Costs: expensive
- Utility: Not very practical. Does NOT solve the problem of too many invasive cystoscopies.

**DNA Ploidy and S-Phase Analysis** (Flow cytometry, image capture (ICM), laser scanning cytometry, FISH)

Initial enthusiasm has diminished because of marginal increases in sensitivity and specificity. However, with newer flow cytometry techniques, the detection of
low-grade tumors has increased to .86. One-third of “diploid” tumors will progress to muscle invasion.

Cytometry and DNA Aneuploidy

- Manufacturer: Quanticyt (Gentian Scientific Software, Niawier, the Netherlands)
- Sample: routine urinary cytology samples (bladder wash)
- Use: differentiate tumor (diploid vs. aneuploid) from reactive atypia (always diploid).
- Regulatory: IUO/RUO
- Method: digital image analysis system
- Sensitivity: 59-69%; mean=58%
- Specificity: 72-93%; mean=80%
- Costs: expensive
- Utility: accurate for high-grade lesions mostly, difficult to perform, requires highly trained experts, equipment. Need highly volume of urine to obtain adequate numbers of cells.

Fluorescence in situ hybridization (FISH)/UroVysion

- Manufacturer: Vysis, Inc. (Downers Grove, Il)
- Principle: detect chromosomal aneusomy and locus specific abnormalities. Sensitivity depends upon number of chromosomal centromeric probes.
- Sample: VUC (also bladder wash, upper track samples)
- Use: Surveillance of patients with prior history of biopsy proven TCC
- Regulatory: FDA-approved (510-k) 2002; (follow-up TCC) ??? anticipatory positive claim; modified with new cytoperservative (PreservCyt) and approved 510(k) in 2004.
- Method: multiprobe fluorescence in situ hybridization
- Sensitivity: 73-81%; mean=77%
- Specificity: 96-100%; mean=98%
- Costs: expensive, time consuming; expertise required 88271 x4 DNA Probe, each; 88274 Interphase; 88291 Interpretation and report. Charges $475.
- Utility: monitor TCC pts (especially G3 tumors)

Biochemical Markers

Nuclear Matrix Protein/NMP22

- Manufacturer: Matritech, Inc. (Newton, MA) NMP22™ Test Kit; Cytogen, (Princeton, NJ) NMP22™ BladderChek Test
Principle: Nuclear mitotic apparatus protein involved in distribution of chromatin in daughter cells. Released from nuclei of tumor cells during apoptosis. Patients with bladder Ca have 25X increase levels in urine as compared to normals.

Sample: urine

Use: Detection of occult or rapidly recurring TCC after resection. Adjunct to cystoscopy in the diagnosis of TCC. However, not intended as a stand alone replacement.


Method: Monoclonal antibody - immunoassay – quantitative microplate immunoassay (NMP22™ Test Kit) or qualitative dip-stick immunoassay (NMP22™ BladderChek Test).

Cut-off at 6 to 12 IU/mL. >6 for recurrence and >10 for screening.

Pos/neg for BladderChek test

Sensitivity: .48-.81 mean = 74% (NPV>86%)

Specificity: .60 - .86; mean=74%

Costs: HCPCS code S3701; CPT 86294 or 86316. charges $125 to $150 (NMP22™ Test Kit) or $10-$30 (NMP22™ BladderChek Test)

Utility: might be more sensitive for new TCC than recurrent TCC. false positive problems; gross hematuria, prostate Ca.

Bladder tumor antigen (BTA)

BTA Test (not available) No better than VUC

BTA STAT

Manufacturer: Polymedco (Courtlandt Manor, NY)

Principle: bladder tumor antigen is human complement factor-H related protein (hCFHrp). Similar to human complement factor-H(hCFH). In vivo, confers a selected growth advantage to cancer cells by allowing them to evade the host immune system. hCFHrp-BTA interrupts the complement cascade avoiding lysis of cells “foreign” to the host.

Sample: voided urine

Use: “Point-of-care” rapid immunoassay

Regulatory: FDA-approved 1998; CLIA “waived”


Sensitivity: .67 - .87; mean=68%

Specificity: .40 - .70 ; mean=73%

Costs: CPT is 86294. $149/box of 10; $340/box of 30.

Utility: Superior to VUC, false positive problems including genitourinary trauma (including cystoscopy), stone disease, UTI, other genitourinary malignancies
(e.g. prosate CA), gross hematuria, BCG therapy. Recent studies have shown low positive predictive value of this test.

**BTA TRAK**
- Manufacturer: Polymedco
- Principle: Same
- Sample: voided urine
- Use: The BTA stat test is an in vitro immunoassay intended for the qualitative detection of bladder tumor associated antigen in urine of persons diagnosed with bladder cancer. This test is indicated for use as an aid in the management of bladder cancer patients in conjunction with cystoscopy.
- Regulatory: FDA-approved 1997
- Sensitivity: 17-77%; mean=61%
- Specificity: 50-88%; mean=71%
- Costs: charges $175
- Utility: must be sent off to reference lab. False positives due to UTI, urolithiasis, recent instrumentation

**Cytokeratins**
- Manufacturer: CIS Bio International (Gifsur-Yvette, France)
- Principle: Differential cytokeratin (CK20) expression by epithelial neoplasms.
- Sample: urine
- Use: detect bladder Ca
- Regulatory: IUO/RUO
- Method: electrochemiluminescent assay for CK20 fragments
- Sensitivity: 71-94% ; mean=82%
- Specificity: 36-96%; mean=73%
- Costs: unknown
- Utility: most promising biochemical assay. Sensitivity of CK19 in one study was .91 vs. .51 for VUC. Combined assay (CK8/18/19) or CK20 alone also has been studied. Lower specificity with BCG therapy.

- Manufacturer: CIS Bio International (Gifsur-Yvette, France) **ELSA-CYFRA 21-1 Test**
- Principle: Differential cytokeratin (CK19) expression by epithelial neoplasms.
- Sample: urine
- Use: detect bladder Ca
- Regulatory: IUO/RUO
- Method: electrochemiluminescent assay for CK19 fragments
- Sensitivity: 69-80% ; mean=74%
- Specificity: 88-94%; mean=91%
- Costs: unknown
Utility: most promising biochemical assay. Sensitivity of CK19 in one study was .91 vs. .51 for VUC. Combined assay (CK8/18/19) or CK20 alone also has been studied. Lower specificity with BCG therapy.

Urine Bladder Cancer (UBC) ELISA Test
- Manufacturer: IDL Biotech AB (Bromma, Sweden)
- Principle: differential cytokeratin (CK8/18) expression by human epithelial malignancies
- Sample: urine
- Use: detect urothelial ca
- Regulatory: IUO/RUO
- Method: ELISA or IRMA. 2hr assay, 2-step, solid phase,
  - Sensitivity: 48-70%; mean=62%
  - Specificity: 64-95%; mean=80%
- Costs:
  - Utility: different investigators use different cut-off values. Lower sensitivity for low-grade TCC. May be non-specific, especially with other malignancies.

Urine Bladder Cancer (UBC) Rapid Test™
- Manufacturer: IDL Biotech AB (Bromma, Sweden)
- Principle: differential cytokeratin (CK8/18) expression by human epithelial malignancies
- Sample: urine
- Use: detect urothelial ca
- Regulatory: IUO/RUO
  - Sensitivity: .66
  - Specificity: .90
- Costs: unknown
- Utility:

Tumor-associated hyaluronic acid and Hyaluronidase
- Manufacturer:
- Principle: Hyaluronic acid (>500 ng/mg) involved in tumor adhesion and migration. Pts with high-grade TCC have elevated levels of hyaluronidase (>10 mU/mg) in urine
- Sample: urine
- Use: Marker of disease progression?
- Regulatory:
- Method: ELISA based test, competitive binding. 4hrs
  - Sensitivity: 1.0 (high-grade lesions only)
  - Specificity: .89
- Costs:

Fibrinogen degradation products
- Manufacturer: Accu-Dx (Intracel Corp) AuraTek FDP PerImmune,(Rockville, Md) NOT AVAILABLE OR MARKETED IN US
- Principle: Focal DIC associated with bladder neoplasia
- Sample: urine
- Use:
- Regulatory: FDA-approved
- Sensitivity: .81
- Specificity: .75
- Costs: $15.00/test

Fibrinolysis markers
- Manufacturer:

Metalloproteinases

DD23
Manufacturer: Urocor
Principle: “novel” antibody to “bladder tumor-associated antigen”
Sample: urine
Use: unknown
Method: quantitative fluorescence image analysis. monoclonal antibody
Sensitivity: .85
Specificity: .95
Costs: unknown
Utility: – must be used in combination with VUC

Molecular Pathology

Telomerase
Manufacturer: Oncor, others
Principle: Ribonucleoprotein reverse transcriptase enzyme that adds TTAGGG repeats to telomers, maintaining telomere length and chromosomal stability, and conferring ‘immortality’. Telomeres are short ends of chromosomes that degrade with age. Inactive in normal tissue, active in germ cells (rapidly dividing) Telomerase detected in 91% of urine samples from bladder cancer pts and 23% of normals.
Sample: urine or tissue
Use: bladder cancer detection
Regulatory: RUO/IUO
Method: TRAP (Telomerase Repeat Amplification Protocol). TTAGGG amplified by PCR. PCR product visualized by DNA banding or ELISA. Test Kit available TRAPeze™ Oncor Appligene®. Other assays include using human telomerase RNA template assay, and human telomerase reverse transcriptase expression assay.
Sensitivity: .70-.95
Specificity: .6-.99
Costs: expensive, but superior combination of time and cost effectiveness compared to FISH or TRAP assay
Utility: outperforms VUC. Presently cumbersome to perform. False positive problems due to telomerase activity in inflammatory cells and “stem” cells. Future developments include “on-slide” ISH or IPOX assay. MOST SENSITIVE ASSAY FOR TCC. Specificity of newer assay (RT-PCR method for hTERT) comparable to VUC.

Microsatellites
Manufacturer:
Principle: Inherited short random repeat DNA sequences unique to individuals, with low mutation rate. The assay is looking for abnormality in microsatellite region, either mutational copy error or deletions of gene loci (LOH). Instability and loss of parts of chromosome 9 (near 9p21 – the p16 locus) associated with papillary low-grade neoplasms of the bladder. There are also abnormalities found on chromosomes 11,13,3,4,8,17,18. Most frequent assays detect LOH in the 9p21 region in low-grade tumor.
Sample: urine
Use: detect bladder cancer
Regulatory: RUO/IUO
Method: PCR; Microsatellite Instability Assay; LOH 9p21 Microsatellite Instability Assay
Sensitivity: .83 -.95
Specificity: 1.0
Costs: automated, low-cost analysis is possible
Utility: Twice as sensitive than VUC. “Positive” before cystoscopy.

Hypermethylation

Need more data

Mitochondrial DNA mutations

Need more data

Oncogenes
(c-erb-B2, c-ras, c-myc, c-jun, mdm2)
**Cell Cycle Regulation Proteins**

*(p16, p21, p53, pRb)*

**P53**

Manufacturer:  
Principle: tumor suppressor gene, cell cycle inhibitor at G1 to S check point, apoptosis regulation. Mutant p53 associated with deeply invasive, non-papillary, high-stage disease. Associated with decrease survival, decrease response to therapy, and tumor progression.

Sample:  
Use: Prognostic biomarker

Regulatory: IUO/RUO

Method: PCR or immunohistochemistry

Sensitivity:  
Specificity:  
Costs:  

**Other Markers of Potential Usefulness**

**Mini Chromosome Maintenance 5 (Mcm5) –**

- immunofluorometric protein assay

**Survivin**

**Clinical Trials**

- Ancillary tests measured prospectively against cytology
- Added costs vs. reduced cystoscopy and reduced morbidity and mortality from bladder Ca
- Understand conditions that cause false negatives and false positives

**Biomarker Characteristics**

- Fast, cheap, easy to find
- High sensitivity and specificity
- Office-based (point of service) or self-administered: BTA Stat, NMP22 BladderChek, UBC Rapid Test
- Screening of high-risk populations
- Evaluation of patients with microscopic hematuria or irritative voiding symptoms
- Surveillance of patients with history of TCC
Clinical Applications (take home points)

- Preliminary studies indicate that judicious incorporation of a combination of urine-based biomarkers in the diagnostic strategy for bladder cancer may reduce costs by almost 50% (J Urol 2003; 169:917-920)
- Given the multitude of markers that will be available, the method to maximally exploit all their strengths would be to use a panel of markers in each patient.
- Using decision tree analysis (J. Urol 2002;167:75-79) urinary bladder cancer biomarkers can be effectively incorporated into a surveillance protocol as long as the marker cost is less than $264 (assuming 20-80% yearly recurrence rate and 4-40% progression rate)
- Most of the office-based point-of-service markers available today cost less than this.
- Integration into laboratory – pay attention to price and place of service.
- They could be used to supplement the standard surveillance approach or could be periodically used during surveillance in patients who could self-administer them between cystoscopies.
References

Textbooks

Bladder Cancer

Urine Cytopathology

Review Articles

ImmunocytoLOGY/IMMUNOCYTe

FISH/UroVysion

**Nuclear Matrix Protein/NMP22**


**Bladder Tumor Antigen/BTA**


Biochemical Markers

Molecular Pathology