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BLADDER CANCER DETECTION WITH URINARY SURVIVIN, AN INHIBITOR OF APOPTOSIS

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1. ABSTRACT

The current "gold standard" for the diagnosis of bladder cancer is cystoscopy and urine cytology. Cystoscopy, a naked eye assessment of the bladder, is invasive, uncomfortable and costly while cytology has high specificity but low sensitivity (40-60%) particularly for low-grade lesions. Therefore, there is a need for a molecular tumor marker assay that is simple to perform and sensitive, particularly for low-grade lesions. By looking to the pathophysiology of bladder cancer, we identified survivin, an inhibitor of apoptosis that is not generally expressed in fully differentiated adult tissue and is highly expressed in bladder cancer. Survivin is detected in whole urine of patients with TCC using a simple antibody based test. The sensitivity of survivin testing for new or recurrent bladder cancer is 100% while the specificity for other neoplastic and non-neoplastic genitourinary disease is 95%. The high sensitivity of this simple, noninvasive test is well suited to bladder cancer, a disease with high rates of recurrence.

2. BLADDER CANCER: AN OVERVIEW

In the United States, bladder cancer is the fourth most common cancer in men and the eighth most common cancer in women. Each year 54,400 Americans are newly diagnosed with bladder cancer, primarily after presenting to physicians with painless hematuria or irritative voiding symptoms. Bladder cancer can occur at any age, but it is generally a disease of the middle-aged and elderly populations with a median age at diagnosis of 70 years. Bladder cancer is approximately two times more common in Caucasian-American males than in African-American males, and is one-and-a-half times more common in Caucasian-American females than in African-American females (1). The known risk factors for bladder cancer are cigarette smoking, occupational exposure to dyes, dietary exposure to nitrates, chronic inflammation of the bladder, the use of cyclophosphamide, and pelvic irradiation (2). Family history of bladder cancer, however, has not been implicated as a risk factor for developing disease.

Like other malignancies that arise in later life, bladder cancer is the result of acquired alterations in DNA; these alterations may manifest as induction of oncogenes, loss of tumor-suppressor genes, impairment of normal DNA repair mechanisms, or interruption of cell cycle homeostasis. One aspect of normal cell turnover is programmed cell death, or apoptosis, which will be considered more comprehensively later on (3).

Under histopathological examination, most bladder cancers prove to be transitional cell carcinomas. These tumors exhibit the entire spectrum of biologic behavior, from relatively benign, superficial, low-grade papillary lesions to the highly malignant and aggressive anaplastic carcinomas. Seventy to eighty percent of patients are found to have low grade, non-aggressive tumors confined to the superficial urothelium (4). While these tumors are generally amenable to treatment with local excision and selective intravesical immunotherapy or

Table 1. Comparison of detection methods in bladder cancer

Marker	Sensitivity	Specificity	Comments	References
Cytology	40-60% (range,16-60%)	90-95%	Better for CIS and high grade lesions vs low grade disease	10-14, 16
Hematuria	67-90%	Low for GU disease		17-20
BTA	57-83%	46-73%	Confounded by hematuria	12, 14, 21-26
NMP-22	60-70% (range, 47-100%)	60-70%	Performance similar for all grades and stages	12-14, 21-23, 27-34
HA-HAase	92%	84%		35-38
Telomerase	70-86% (range, 7-100%)	60-90%		17, 23, 39

chemotherapy, the recurrence rate is up to seventy percent. Ten to fifteen percent of these recurrences will have progressed to a more aggressive or invasive tumor type (5).

The overall mortality for bladder cancer is 12,500 deaths per year. It has been established that patients with high-grade, muscle-invasive tumors at primary diagnosis or recurrence have the poorest prognosis. Fifty percent of these patients develop distant metastases within two years and sixty percent die within five years of initial treatment. Thus, the questions: How do we diagnose patients early in the disease process, and how do we best monitor patients for recurrence and progression of disease?

3. DIAGNOSIS OF PRIMARY AND RECURRENT BLADDER CANCERS

3.1. Cystoscopy and cytology: the current gold standard

The overall prevalence of bladder cancer is too low to make general screening practical or effective and diagnosis is largely made as a result of symptomatic manifestations. Traditionally, patients presenting with hematuria, or irritative voiding symptoms (in the absence of infection) undergo upper urinary tract radiographic imaging, cystoscopy, and urine cytology to rule out malignancy. In addition, patients who have been previously diagnosed and treated for bladder cancer are monitored for recurrence via cystoscopy and urine cytology as often as every three months. The frequency of follow-up depends on the grade and stage of the initial tumor and the patient's history of remission or recurrence.

Cystoscopy and cytology are currently considered the gold standard for diagnosis and follow up. Their primary goal is to recognize flat, superficial lesions prior to invasion, as well as the ten- percent of papillary lesions that are destined to invade.

Cystoscopy involves insertion of a hollow, tubal instrument called a cystoscope through the urethra to the bladder. A light source and camera are then place through the instrument. This allows for a full visual inspection of the bladder mucosa. Additionally, cutting and cautery devices can be inserted through the cystoscope to biopsy suspicious areas of erythema or mucosal irregularity, or resect tumors.

A recent study of endoscopic procedures concluded that the sensitivity of traditional white-light cystoscopy ranged from 47.2% to 53% (6). Being essentially a naked-eye assessment, there is a great deal of inter- and intra-personal variability in cystoscopic results, which may account for the paucity of information on the sensitivity and specificity of what is generally considered a valuable diagnostic tool. Cystoscopy can be performed in the urologist's office and carries a low risk of complications, but it is invasive, uncomfortable, and costly.

Urine cytology has an estimated sensitivity of 40% - 60%, but a positive cytology is highly predictive of transitional cell carcinoma, and may even precede cystoscopic diagnosis. Specificity is estimated at 95% (7-14). Urine cytology is extremely effective in the detection of high-grade lesions, making it an important screening and surveillance tool. Overall sensitivity is variable largely because cytology is inconsistent in the detection of low-grade lesions. The application of strict criteria during analysis can improved sensitivity up to 85% in some cases (15). Still, the difficulty remains with cytology specimens signed out as "atypical urothelial cells; cannot rule out a low-grade lesion".

4. MOLECULAR MARKERS FOR BLADDER CANCER

Numerous efforts have been made to identify tumor markers that could improve overall survival through both the early, accurate diagnosis of primary and recurrent tumors, and through the identification of tumor features that could guide selection of therapy. An ideal bladder tumor marker would be extremely sensitive, particularly to lesions amenable to local resection, but also would be very specific, to reduce the amount of unnecessary follow-up testing for patients with benign disease (8). The method for detecting this marker should be simple, noninvasive, and relatively inexpensive. To this end, a number of tumor-related substances in bladder cells and in urine have been investigated, including: bladder tumor antigen (BTA, a human complement factor-H related protein), nuclear mitotic apparatus protein (NMP22), fibrinogen-fibrin degradation products, telomerase, and hyaluronic acid-hyaluronidase (HA-HAase).

Though all of these markers may be clinically useful, the search for the ideal marker has not yet identified a single diagnostic or prognostic marker capable of supplanting cystoscopy and urine cytology as the gold standard (8, 9). Table 1 summarizes the sensitivities and specificities for these markers.

5. INHIBITORS OF APOPTOSIS

Each year, the average adult produces and eradicates a mass of cells approximately equal to his or her body weight. Apoptosis, or programmed cell death, is a complex process essential to tissue homeostasis. Unlike cell death caused by trauma, infection, or infarction, apoptosis is a coordinated cascade of protease activity that ultimately leads to membrane-bound cell fragmentation and phagocytotic clearance of cellular constituents without the ravages of inflammation or scarring.

Defects in this highly regulated mechanism can abnormally extend cell life, favoring the accumulation of genetic mutations and the tendency towards neoplastic transformation. In addition, failure of the apoptotic mechanisms can promote resistance to cytotoxic drugs and radiation therapy used to combat malignancy (40).

5.1. The IAP family

The inhibitors of apoptosis protein family (IAPs) were first characterized in 1993 in baculovirus and metazoans as a novel group of apoptosis suppressors conserved throughout phylogenic and physiologic differentiation. Since then, IAPs have been shown to block a broader range of apoptotic triggers than any other group of apoptotic inhibitors. This includes the bcl-2 family, a group of pro- and anti-apoptotic proteins implicated in the landmark studies of apoptosis in malignancy (41).

5.2. Survivin, a novel apoptosis inhibitor

Survivin, first described in 1997 as a structurally unique IAP, is present during fetal development but is generally absent in fully differentiated adult tissues (42). Currently, there are two small exceptions; survivin is expressed in normal human endometrium during the proliferative phase (43), and in prostatic neuroendocrine cells (44).

Survivin is a 16.5 kD cytoplasmic protein whose gene is located on chromosome 17q25. Survivin is expressed at the G2/M regulatory point in the cell cycle, and is thought to associate with microtubules of the mitotic spindle in a specific and saturable reaction that is regulated by microtubule dynamics. The caspase cascade of the interleukin-1b-converting enzyme family of proteases is a major apoptosis execution pathway. Survivin has been shown, *in vitro,* to inhibit the effector cell death caspases-3 and -7 (45). Thus survivin may play a role in maintaining cell viability at mitosis via the coupling of apoptosis control with cell division.

5.3. Survivin in common human cancers

Survivin is over-expressed in a number of common human cancers *in vivo*, including those of lung, colon, pancreas, prostate, and breast. In colorectal cancer (46), breast cancer (47), non-small-cell lung cancer (48), diffuse large B-cell lymphoma (49), esophageal cancer (50), and neuroblastoma (51), survivin expression correlates with unfavorable prognosis and poor survival. Survivin expression also has been shown to have negative correlation with response to conventional chemotherapy. A recent *in vitro* study has targeted survivin with an antisense oligonucleotide prior to chemotherapy, with encouraging results in malignant pre-B cell lines (52). In gastric carcinoma, survivin expression also was found to correlate significantly with bcl-2 expression and mutant-type p53 (53). Pediatric renal tumors express abnormal levels of both pro-apoptotic receptors, such as fas, and survivin; a high ratio of survivin:fas mRNA is of prognostic value for recurrence, independent of clinical stage or histopathological type (54).

6. BLADDER TUMOR CONTENT OF SURVIVIN AND RATES OF RECURRENCE

In 1999, Swana, *et al* (55), used immunohistochemical (IHC) staining to examine expression of survivin, bcl-2, and p53 in bladder specimens from thirty-six patients with primary, localized bladder cancer. Subjects included twenty-five men and eleven women, average age sixty-six years (range, 51 to 78). IHC was preformed with rabbit polyclonal IgG using the standard avidin-biotin-peroxidase complex technique as outlined previously (53).

Survivin was detected in 78% of the tumors (28 of 36). Segregation of results according to the historic grading classification system of the American Joint Committee on Cancer revealed that survivin was present in 90% (9 of 10) of grade II/III tumors and 100% (6 of 6) of grade III/III tumors, compared to 65% (13 of 20) of grade I/III tumors. Although expression of bcl-2 and p53 did not correlate with disease recurrence, mean time to recurrence among patients with grade I survivin-negative tumors was thirty-six months (SD=16 months) versus twelve months (SD=6 months) for patients with grade I survivin-positive tumors ($P<0.001$).

7. URINE DETECTION OF SURVIVIN

Preliminary studies which examined the suitability of urine survivin as a method of detecting bladder cancer was published in January, 2001, by Smith, *et al* (56). Urine specimens from random clean-catch or catheter samples were collected from five different groups: healthy volunteers (n=16); patients with benign genitourinary disease, such as hematuria, benign prostatic hypertrophy, urinary tract infection, renal calculi, or interstitial cystitis (n=29); patients with prostate, renal, vaginal, or cervical malignancies (n=29); patients with new or recurrent bladder cancer (n=31); and patients with treated or partially treated bladder cancer (n=33).

Urine supernatant aliquots were filtered onto a nitrocellulose membrane using a dot-blot microfiltration apparatus (Bio-Rad Laboratories, Hercules, California). Increasing concentrations of *Escherichia coli*-expressed recombinant survivin were also filtered onto the blot as a standard. The membrane was incubated with an anti-survivin antibody, then with a horseradish peroxidase-conjugated antibody. Binding of the primary and secondary antibodies was detected by enhanced chemiluminescence (Amersham Biotech) and

Table 2. Urine survivin results compared to diagnosis

	Urine survivin Negative	Urine survivin Positive
HEALTHY CONTROLS	16	0
BENIGN GENITOURINARY DISEASE		
• Hematuria	2	3
• Urinary tract infection (UTI)	6	0
• Benign prostatic hypertrophy (BPH)	6	0
• Elevated PSA (without formal diagnosis)	0	1
• Interstitial cystitis	2	0
• Renal calculi	3	0
• Other	6	0
GENITOURINARY CANCER (other than bladder)		
• Prostate Cancer	19	0
• Renal Cancer	8	0
• Vaginal Cancer	1	0
• Cervical cancer	1	0
• Bladder Cancer (Primary Or Recurrent)	0	31
• Treated Bladder Cancer	30	3

From Smith, *et al*. (56)

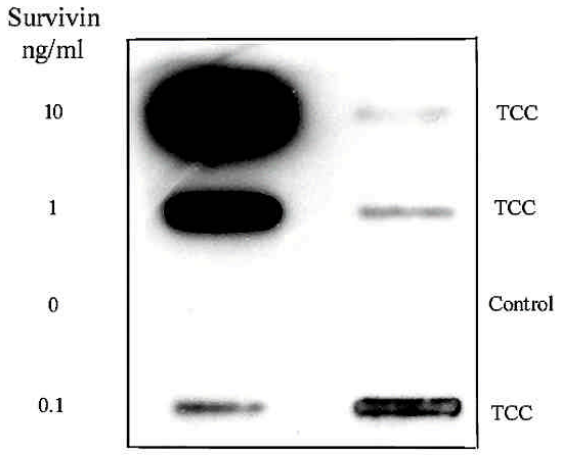

Figure 1. Representative survivin dot blot immunodetects recombinant survivin protein (0.1-10 ng/ml) and survivin in the urine of three subjects with TCC, but not in a healthy control subject.

autoradiography. A representative dot-blot is shown in Figure 1. Qualitative results were independently evaluated by Western Blot and RT-PCR on twenty samples representing each of the five study groups.

Results for the different study groups are summarized in Table 2. Sensitivity for new or recurrent bladder cancer was 100% (31 out of 31 by dot-blot, 15 out of 15 by Western blot and RT-PCR), and specificity for other neoplastic and nonneoplastic genitourinary disease was 95% ($P < 0.02$). The three patients with treated bladder cancer who tested survivin-positive, and who had a negative cystoscopy at time of specimen collection, one had a positive cytology and another was diagnosed with recurrent bladder cancer within six months of the study.

The greatest advantage of the dot-blot method is that it does not depend on cellular recovery from urine. Moreover, this test involves relatively few processing steps and utilizes an antibody to survivin that is currently commercially available.

8. CONCLUDING REMARKS

The high sensitivity of this simple, noninvasive, point-of-service test is well suited to bladder cancer, a disease with high rates of recurrence and current reliance on invasive and subjective methods of evaluation. The specificity indicates an estimated five to ten percent false positive rate in symptomatic patients; though these patients would undergo further testing, this method still represents a reduction in the number of individuals subjected to unnecessary cystoscopy.

Survivin detection in the urine appears to represent a clinically useful test, especially for frequent surveillance of post-treatment patients. Urine survivin levels may prove to have prognostic significance, whether alone or in conjunction with other tumor markers. Furthermore, this test could improve identification of patients who would benefit from more aggressive therapy at the time of initial diagnosis.

There are a number of questions that warrant further investigation. Further analysis of a larger patient series, including patients in apparent remission from bladder cancer, will further elucidate the potential of urine survivin as a diagnostic and prognostic marker. In addition, long term follow-up of patients found to be survivin positive in the absence of known disease should be carried out. Presence or absence of invasion into the urine collection system may explain discrepancies between immunohistochemical detection of survivin and urine detection of survivin in patients with prostate cancer; still, examination of tissue and urine samples from the same individuals would allow for more meaningful discussion.

The investigation of survivin should be watched with anticipation. As more patient populations are included

for investigation and long-term follow up, the role of this protein in health and disease will be revealed.

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