Urine Detection of Survivin and Diagnosis of Bladder Cancer

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Deregulated expression of inhibitors of apoptosis is thought to contribute to cancer by abnormally extending cell viability, favoring the accumulation of mutations, and promoting resistance to therapy.1 A novel modulation of mutations, and promoting cell viability, favoring the accumulation of mutations, and promoting resistance to therapy. Survivin is an inhibitor of apoptosis that is selectively overexpressed in common human cancers, but not in normal tissues, and that correlates with aggressive disease and unfavorable outcomes.

Objective To investigate the potential suitability of survivin detection in urine as a novel predictive/prognostic molecular marker of bladder cancer.

Design, Setting, and Patients Survey of urine specimens from 5 groups: healthy volunteers (n=17) and patients with nonneoplastic urinary tract disease (n=30), genitourinary cancer (n=30), new-onset or recurrent bladder cancer (n=46), or treated bladder cancer (n=35), recruited from 2 New England urology clinics.

Main Outcome Measures Detectable survivin levels, analyzed by a novel detection system and confirmed by Western blot and reverse transcriptase polymerase chain reaction (RT-PCR), in urine samples of the 5 participant groups.

Results Survivin was detected in the urine samples of all 46 patients with new or recurrent bladder cancer using a novel detection system (31 of 31) and RT-PCR (15 of 15) methods. Survivin was not detected in the urine samples of 32 of 35 patients treated for bladder cancer and having negative cystoscopy results. None of the healthy volunteers or patients with prostate, kidney, vaginal, or cervical cancer had detectable survivin in urine samples. Of the 30 patients with nonneoplastic urinary tract disease, survivin was detected in 3 patients who had bladder abnormalities noted using cystoscopy and in 1 patient with an increased prostate-specific antigen level. Patients with low-grade bladder cancer had significantly lower urine survivin levels than patients with carcinoma in situ (P=.002).

Conclusions Highly sensitive and specific determination of urine survivin appears to provide a simple, noninvasive diagnostic test to identify patients with new or recurrent bladder cancer.

JAMA. 2001;285:324-328

CONTEX:

Dysregulation of apoptosis may favor onset and progression of cancer and influence response to therapy. Survivin is an inhibitor of apoptosis that is selectively overexpressed in common human cancers, but not in normal tissues, and that correlates with aggressive disease and unfavorable outcomes.

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METHODS

Urine Specimens

One hundred fifty-eight urine specimens were collected at the urology clinics at Yale-New Haven Hospital and at the Veterans Affairs, New England Health Care Systems, West Haven, Connecticut, Division. Random clean-catch or straight catheter urine samples were obtained from individuals who were categorized into 5 different groups: group 1 included healthy volunteers with a mean (SD) age of 47.6 (20.8) years who were not taking any medication (n=17); group 2 patients had a mean (SD) age of 60.0 (18.1) years with diagnosis of non-
neoplastic urinary tract disease or hematuria (n = 30); group 3 patients had a mean (SD) age of 71.5 (9.9) years with diagnosis of genitourinary cancer, excluding bladder cancer (n = 30); group 4 patients had a mean (SD) age of 69.7 (8.7) years with diagnosis of new-onset or recurrent bladder cancer (n = 46); and group 5 patients had a mean (SD) age of 76.1 (8.9) years and were undergoing treatment or had already received treatment for bladder cancer and had negative cystoscopic findings on the day of urine collection (n = 35). Treatment measures in group 5 included intravesical bacillus Calmette-Guérin, thiotepa, transurethral resection, partial cystectomy, and radiation. Group 4 included patients who, after urine collection, underwent similar treatment measures and/or salvage cystectomy or radical cystectomy.

Urine Detection of Survivin
Urine specimens were filtered onto a nitrocellulose membrane using a microfiltration apparatus in a module providing a 48-wells-lot format. The blot was analyzed for the presence of survivin using a polyclonal antibody. The protocol was as follows: urine was collected and stored at −80°C until analysis. On the day of analysis, urine samples were centrifuged at 20000g for 20 minutes. Meanwhile, the Bio-Dot microfiltration apparatus was assembled with a 0.2-µm nitrocellulose membrane (Bio-Rad Laboratories, Hercules, Calif) and moistened in 20-mmol Tris-buffered saline (pH, 7.5). Then, the urine supernatant (300 µL), along with increasing concentrations of Escherichia coli–expressed recombinant survivin15 as a standard (0.001–1.0 µg/mL) in 300 µL of Tris-buffered saline, were filtered onto the nitrocellulose membrane using a microfiltration apparatus in a module providing a 48-wells-lot format. The blot was incubated with 2 µg/mL of a rabbit antibody to survivin16 for 3 hours at 22°C, incubated with 1 µg/mL of an antibody to survivin16 followed by horseradish peroxidase–conjugated goat antirabbit IgG (Amersham Biotech, Piscataway, NJ) for 1 hour at 22°C. After washes in PBS for 10 minutes, PBS-Tween twice for 5 minutes, and PBS twice for 5 minutes, binding of the primary antibody was detected by enhanced chemiluminescence (Amersham Biotech) and autoradiography. Bands were quantitated by densitometry and a weighted survivin score was calculated on the basis of the antibody reactivity with increasing concentrations of recombinant survivin as follows: 0 for not detectable; 1 for 0.001–0.25 µg/mL; 2 for 0.25 to 1 µg/mL; and 3 for more than 1 µg/mL. Each urine specimen was analyzed at least twice on 2 different occasions and comparable results were obtained.

Western Blot
Urine specimens (100 µL) were centrifuged at 1200g for 10 minutes at 22°C, and the cell pellet was washed twice in Tris-buffered saline and made soluble in 0.5% Triton X-100 (Sigma, St Louis, Mo) in the presence of protease inhibitors for 30 minutes at 4°C. Samples were separated by SDS gel (Bio-Rad Laboratories) electrophoresis, transferred to nylon membranes (Millipore Corp, Bedford, Mass), and further incubated with 1 µg/mL of an antibody to survivin16 followed by horseradish peroxidase–conjugated goat antirabbit IgG and chemiluminescence.

Reverse Transcriptase Polymerase Chain Reaction
Fifty milliliters of clean-catch urine was obtained from 15 patients with new or recurrent urothelial cancer, 2 patients with treated bladder cancer, 1 patient with prostate cancer, 1 patient with non-neoplastic urinary tract disease, and 1 healthy volunteer. Total RNA was isolated from urine pellets using the TriZol reagent (Life Technologies Inc, Gaithersburg, Md). Single-strand complementary DNA (cDNA) was synthesized by random priming of 1-5 µg total RNA using 1 µL of RT (Gibco BRL, Life Technologies Inc) for 1 hour at 43°C. After heating at 70°C for 15 minutes, a first amplification reaction was carried out with survivin primers 5'-CTG CTC TGG CAG CCC CTT TCT - CAA-3' (forward) and 5'AAT AAC CCGT GAAG TG GTG CA-3' (reverse) with denaturation at 94°C for 15 seconds, annealing at 53°C for 15 seconds, and extension at 72°C for 1 minute for 20 cycles, followed by incubation at 72°C for 5 minutes. A 463-base pair fragment of the survivin cDNA was subjected to a second round of amplification with nested survivin primers 5'-CG CATCTGCTACATTCAAGAAC-3' (forward) and 5'-CTTG CCTCT TTCTCTGTCC-3' (reverse), with denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 45 seconds for 30 cycles, followed by incubation at 72°C for 5 minutes. The amplified survivin cDNA of 279 base pair was separated on a 2.0% solution of agarose gel and visualized by ethidium bromide staining. Control reactions were amplified using β-actin–specific primers 5'-AGCGG GAA ATCGT GGTG-3' (forward) and 5'-CAGG GTAT CATG GTGTC C-3' (reverse) with generation of a 309-base pair fragment.

Statistical Analysis
The relationship between urine survivin and patients’ diagnosis was analyzed by a χ2 test. Nonparametric statistical analysis was used to compare the weighted urine survivin score with the grading classification system performed at the Yale-New Haven Hospital. The calculation of predictive accuracy is not appropriate for this study since the diagnosis was known at the time of urine collection.

RESULTS
A representative experiment of detection of urine survivin using the Bio-Dot test is shown in FIGURE 1. Determination of urine survivin with the Bio-Dot method was carried out in 138 of the 158 specimens collected for this study (TABLE 1). Twenty additional urine samples were analyzed for survivin expression by reverse transcriptase polymerase chain reaction (RT-PCR) to independently evaluate the

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SURVIVIN AND BLADDER CANCER

specifcity of the new method. Survivin was not detected in urine of the 16 volunteers, 6 patients with benign pros-

patients with cryoprotective hematuria (weighted survivin score, 2), who presented with a history of retention and dysuria post-transurethral prostate resection, and revealed a trabeculated, irregularly thickened bladder, by cystoscopy (see “Comment” section). One patient with increased prostate-specific antigen levels but without diagnosis of prostate cancer was positive for urine survivin (Table 1). This patient also had a trabeculated, thickened bladder, by cystoscopy. Survivin was not detected in urine specimens of 19 patients with prostate, 8 with renal, 1 with vaginal, or 1 with cervical cancer (Table 1). In contrast, urine survivin was detected in all 31 patients with new-onset or recurrent bladder cancer (Table 1). Histopathologic grading (grades I through IV) of the 31 patients in group 4 analyzed for urine survivin by the novel method included 13 patients with grade II, 7 patients with grade III, and 5 patients with grade IV tumors. Carci-

noma in situ was found in association with the papillary and invasive carcinomas of 5 patients and in association with high-grade urothelial cancer of the ureter in 1 patient. Thirty of 33 patients in group 5 analyzed by the novel system had no detectable urine survivin (Table 1). Five of these 30 patients were re-

ceived bacillus Calmette-Guerin and had completed 3 to 5 treatments, the other 25 were status posttreatment with negative cystoscopy findings. Three patients in group 5 with initial diagnosis of grade I noninvasive bladder cancer had positive test results for urine survivin after undergoing negative cystoscopic examination. One of the 3 patients had urine cytology positive for bladder cancer. Two of the 3 patients were treated with transurethral resection of the bladder tumor and 1 was treated with fulguration.

When normalized for a weighted mean (SD) survivin score, patients with carcinoma in situ had considerably higher survivin score (2.5 [0.5]; n=6) than patients with grade II bladder cancer (1.3 [0.6]; n=13). The correlation between weighted survivin score and histopathology or grading of the various bladder cancer cases is shown in Table 2 and Table 3, respectively. By Western blot, a single survivin band of 16.5 kD was detected in the urine cell pellet from a patient with bladder can-

Table 1. Survivin Detection in 138 Urine Specimens Using a Novel Detection Method

<table>
<thead>
<tr>
<th>Urine Specimens</th>
<th>Total No. of Patients</th>
<th>No. of Patients Survivin-Negative</th>
<th>No. of Patients Survivin-Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (control healthy volunteers)</td>
<td>16</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Group 2 (nonneoplastic urinary tract diseases)</td>
<td>29</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>########################################</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hematuria</td>
<td>6</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Urinary tract infection</td>
<td>6</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Benign prostatic hyperplasia</td>
<td>6</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Increased prostate specific antigen</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Intestinal cystitis</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Renal calculus</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Other†</td>
<td>6</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Group 3 (genitourinary cancers except bladder)</td>
<td>29</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>Prostate</td>
<td>19</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>Renal</td>
<td>8</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Vaginal</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Cervical</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Group 4 (new or recurrent bladder cancer)‡</td>
<td>31†</td>
<td>0</td>
<td>31</td>
</tr>
<tr>
<td>Group 5 (treated bladder cancer)‡</td>
<td>33</td>
<td>30</td>
<td>3§</td>
</tr>
</tbody>
</table>

*Includes 1 patient with papillary necrosis, 2 with prostatitis, 1 with vesicoureteral reflux, and 2 with renal transplant with elevated creatinine.
†Includes 1 patient with urethral cancer of the ureter.
‡Patients had normal cystoscopy results.
§Two of these patients were treated with transurethral resection of the bladder tumor, and 1 with fulguration. One of these patients had urine cytology positive for bladder cancer.

Table 2. Correlation Between Weighted Urine Survivin Score and Bladder Cancer Histopathology

<table>
<thead>
<tr>
<th>Histopathology</th>
<th>No. of Cases Tested</th>
<th>Mean (SD) Survivin Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not determined</td>
<td>3</td>
<td>1.7 (1.2)</td>
</tr>
<tr>
<td>Noninvasive papillary carcinoma</td>
<td>4</td>
<td>1 (0)</td>
</tr>
<tr>
<td>No detrusor muscle invasion</td>
<td>12</td>
<td>1.6 (0.8)</td>
</tr>
<tr>
<td>Muscle invasion</td>
<td>6</td>
<td>1.7 (0.8)</td>
</tr>
<tr>
<td>Carcinoma in situ</td>
<td>6</td>
<td>2.5 (0.5)†</td>
</tr>
</tbody>
</table>

*The weighted survivin score was calculated using a standard curve with increasing concentrations of recombi-

nant survivin as follows: 0, not detectable; 1, 0.001 to 0.25 µg/mL; 2, 0.25 to 1 µg/mL; and 3, more than 1 µg/mL.†Significantly greater than either grade II or noninvasive papillary carcinoma (P<.02).
cancer but not in that from a healthy volunteer (Figure 2).

To independently evaluate the results obtained with the new method, 15 additional patients with new or recurrent bladder cancer were analyzed for urine survivin by RT-PCR. A 279-base pair survivin cDNA was amplified from urine cell pellets of all the 15 new patients with bladder cancer (Figure 3) and data not shown). In contrast, urine cell pellets from 5 additional individuals, 1 with urinary tract infection, 2 with treated bladder cancer and negative cystoscopy results, 1 with prostate cancer, and 1 from a volunteer, had no survivin cDNA (Figure 3). In control experiments, a 309–base pair β-actin–cDNA fragment was indistinguishably amplified from urine of controls and patients with bladder cancer (Figure 3). Histopathologic cases of bladder cancer analyzed by RT-PCR included 5 patients with grade II tumors, 1 patient with grade III, 6 patients with grade IV, and 3 patients with carcinoma in situ. These experiments suggest that exfoliated cancer cells may passively release survivin in the extracellular milieu (ie, urine) during tumor progression.

**COMMENT**

In this study, we describe a simple, antibody-based test to identify the apoptosis inhibitor survivin (2,4) in urine of patients with bladder cancer. Survivin was found in urine samples of all 46 patients with new or recurrent bladder cancer, but not in any of the 17 healthy volunteers, or in any of the 30 patients with other urologic cancers, and only in 4 of 30 patients with nonneoplastic genitourinary disorders. Importantly, of the 3 patients with hematuria who tested positive for urine survivin, 1 had a positive cytology result for bladder cancer and another was diagnosed with bladder cancer within 6 months of survivin detection. Moreover, 32 of 35 patients treated for bladder cancer and achieving cystoscopic remission had negative test results for urine survivin. There is a positive correlation between a weighted urine survivin score and more advanced histopathologic tumor grading.

For its overexpression in cancer but not in normal tissues, (2,4) and its unfavorable predictive and/or prognostic significance in various malignancies, (5-9) survivin may constitute a useful molecular marker in cancer. This may be particularly relevant in bladder cancer, (11,17) in which simple and noninvasive diagnostic means to monitor response to therapy and simplify follow-up protocols are urgently needed. Although regarded as the criterion standard, (18) urine cytology has low sensitivity (30%-40%) in bladder cancer, and fails to detect superficial, low-grade lesions. In this context, several urine

**Table 3. Correlation Between Weighted Urine Survivin Score and Bladder Cancer Grading**

<table>
<thead>
<tr>
<th>Grade</th>
<th>No. of Cases Tested</th>
<th>Mean (SD) Survivin Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>13</td>
<td>1.3 (0.6)</td>
</tr>
<tr>
<td>III</td>
<td>7</td>
<td>1.5 (0.8)</td>
</tr>
<tr>
<td>IV</td>
<td>5</td>
<td>2 (1)</td>
</tr>
<tr>
<td>IV</td>
<td>1</td>
<td>3†</td>
</tr>
</tbody>
</table>

*The weighted survivin score was calculated using a standard curve with increasing concentrations of recombinant survivin as follows: 0, not detectable; 1, 0.001 to 0.25 µg/mL; 2, 0.25 to 1 µg/mL; and 3, more than 1 µg/mL. Histopathological analysis was carried out using the Broader cytologic grading system for the classification of papillary transitional cell tumors, grades I through IV.†One of the 6 patients with associated carcinoma in situ had urothelial cancer of the ureter (grade IV; survivin score, 3).
markers including bladder tumor antigen, nuclear matrix protein, telomerase activity, hyaluronic acid/hyaluronidase, and fibrin degradation products have been characterized for their potential diagnostic/predictive value in bladder cancer.10,20

In this patient series, the sensitivity of the urine survivin test for new or recurrent bladder cancer was 100%, and its specificity for other neoplastic and nonneoplastic genitourinary tract diseases was 95% (P<.02). However, the overall specificity of the test is likely to vary depending on which patient population is the focus of clinical interest. A screening test for group 1 individuals will have a false-positive rate of essentially zero, whereas patients with clinical symptoms in groups 2 and 3 will likely have a combined false-positive rate of 5% to 10%. However, similarly to the 2 patients with hematuria described above, these individuals should be closely followed up because they may subsequently develop bladder cancer. Because of its high specificity, the urine survivin test may be useful to complement cytology and/or other diagnostics markers19,20 to better monitor bladder cancer patients and identify early recurrences or de novo neoplasms. Other potential advantages of the urine survivin test include its simplicity, suitability as a point-of-service procedure, and its cost-effectiveness, using 1-step detection with a single antibody to survivin that has now become commercially available. Analysis of a larger patient series may establish the general suitability of urine survivin detection for monitoring response to therapy and follow-up protocols in bladder cancer.

Author Contributions: Dr Smith participated in study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, provided statistical expertise, obtained funding, and provided administrative, technical, or material support.

Ms Wheeler participated in study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, provided administrative, technical, or material support.

Ms Plescias participated in study concept and design, acquisition of data, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, and provided administrative, technical, or material support.

Dr Colberg participated in study concept and design and acquisition of data.

Dr Weiss participated in study concept and design, acquisition of data, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, obtained funding, provided administrative, technical, or material support, and supervised the conduct of the study.

Dr Altieri participated in study concept and design, analysis and interpretation of data, drafting of the manuscript, and obtained funding.

Funding/Support: Our work was supported by National Institutes of Health grants DK02499 (Dr Smith); DK47548 and DK38311 (Dr Weiss); and CA78810 and CA82130 (Dr Altieri).

Acknowledgment: We thank all the patients for their participation in the study. We also thank Bernard Lytton, MD, Harris Foster, Jr, MD, Hubert Swana, MD, Greg Barne, MD, Ithar Derweesh, MD, and Luke Cho, MD, and Sharyn Jones, PCA, Kathy Olsen, RN, and Claire Pulkkin, RN, for devoting their time for sample collection and their practical perspective of patient care.

REFERENCES