Urine Detection of Survivin and Diagnosis of Bladder Cancer

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Dysregulated 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 modulator of the cell death/viability balance in cancer was recently identified as survivin,2 a member of the inhibitor of apoptosis gene family.3 Undetectable in most normal adult tissues, survivin becomes the top fourth transcript expressed in common human cancers,2,4 in which it correlates with unfavorable disease and abbreviated overall survival.5-9

Urothelial (transitional cell) carcinoma of the bladder is the fourth most common cancer in men and the eighth most common cancer in women in the United States, accounting for more than 54,000 new cases and 11,200 deaths every year. Recurrences of bladder cancer occur in up to 80% of patients and constitute a formidable obstacle to long-lasting remissions, frequently anticipating muscle invasion, and disseminated disease.10 Despite considerable efforts to develop safe, reliable, noninvasive screening strategies for bladder cancer, the identification of a single predictive/prognostic molecular marker of bladder cancer has remained elusive.11 Consistent with a proposed role of deregulated apoptosis in urothelial cancer,12,13 survivin was found in 78% of bladder cancers, but not in normal urothelium, and its expression correlated with accelerated recurrences.14 Because of its expression in cancer but not in normal tissues, we investigated the potential suitability of survivin as a new molecular marker for detection of bladder cancer.

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 (n=17); group 2 patients had a mean (SD) age of 60.0 (18.1) years who were not taking any medication (n=17); group 3 patients had a mean (SD) age of 47.6 (20.8) years who were not taking any medication (n=17); group 4 patients had a mean (SD) age of 47.6 (20.8) years who were not taking any medication (n=17); group 5 patients had a mean (SD) age of 47.6 (20.8) years who were not taking any medication (n=17).

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.

Methods

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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.

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SURVIVIN AND BLADDER CANCER

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-Guerin, 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 is 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 survivin as a standard (0.001-1.0 µg/mL) in 300 µL of Tris-buffered saline, were filtered onto the membrane. After filtration, the membrane was dried then blocked in 5% dried milk plus 0.01% sodium azide in phosphate-buffered saline (PBS) (pH, 7.4) for 12 hours at 4°C. After washing in PBS-Tween 20 (0.25%), the membrane was incubated with 2 µg/mL of a rabbit antibody to survivin for 3 hours at 22°C, washed in PBS-Tween, and incubated with 1:1000 dilution of horseradish peroxidase-conjugated donkey 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, the urine supernatant was transferred to nylon membranes (Millipore Corp, Bedford, Mass), and further incubated with 1 µg/mL of an antibody to survivin 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 CCT GG CAG CCG CTT TCT-CAA-3′ (forward) and 5′-AAT AAC CCTTGAAAGTGGCA-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′-CGGCATCTCTACATCAAGAAC-3′ (forward) and 5′-CTTGCTCTCTTTCTGTCC-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′-AGCGGGAAATCGTGCGT-3′ (forward) and 5′-CAGGTTACATGGTGTCGCC-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

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

tative hyperplasia, 2 with interstitial cystitis, 3 with renal calculi, 6 with urinary tract infection, or 6 with other nonneoplastic urinary tract disease (Table 1). Urine survivin was detected in 3 of 5 patients with cryp
togenic 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 receiving 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 II 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>Group 1 (control healthy volunteers)</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total No. of Patients</td>
<td>No. of Patients Survivin-Negative</td>
<td>No. of Patients Survivin-Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy Volunteers</td>
<td>16</td>
<td>16</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benign Prostatic Hyperplasia</td>
<td>29</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Renal Cell Carcinoma</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prostate Cancer</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bladder Cancer</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bladder Cancer in Remission</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interstitial Cystitis</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Renal Calculi</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td></td>
<td></td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Urine Specimens</th>
<th>Group 3 (genitourinary cancers except bladder)</th>
<th>Group 4 (new or recurrent bladder cancer)</th>
<th>Group 5 (treated bladder cancer)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total No. of Patients</td>
<td>No. of Patients Survivin-Negative</td>
<td>No. of Patients Survivin-Positive</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>Bladder Cancer Receiving Treatment</td>
<td>31†</td>
<td>0</td>
<td>31</td>
</tr>
<tr>
<td>Bladder Cancer Receiving Treatment</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 urothelial 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 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.
†Signiﬁcantly greater than either grade II or noninvasive papillary carcinoma (P<.02).
**SURVIVIN AND BLADDER CANCER**

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 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, and its unfavorable predictive and/or prognostic significance in various malignancies, survivin may constitute a useful molecular marker in cancer. This may be particularly relevant in bladder cancer, 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, urine cytology has low sensitivity (30%-40%) in bladder cancer, and fails to detect superficial, low-grade lesions. In this context, several urine

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**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 Broadert 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.19,20

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 3% 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 diagnostic markers9,20 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, and provided administrative, technical, or material support. Ms Plescia participated in study concept and design, acquisition of data, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, and obtained funding. Dr Colberg participated in study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, and obtained funding. Dr Altieri participated in study concept and design, analysis and interpretation of data, drafting of the manuscript, and obtained funding.

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REFERENCES