Tumor-derived Hyaluronidase: A Diagnostic Urine Marker for High-Grade Bladder Cancer

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ABSTRACT

The detection of high-grade bladder tumors prior to invasion is crucial for a good prognosis. We recently found that the levels of hyaluronic acid (HA), a glycosaminoglycan, are elevated in the urine of bladder cancer patients, and small angiogenic HA fragments are present in the urine of high-grade bladder cancer patients. Hyaluronidase is an enzyme that degrades HA into small angiogenic fragments. We compared the urinary hyaluronidase levels of normal individuals and patients with bladder cancer or other genitourinary conditions, using a substrate (HA)-gel technique and an ELISA-like assay. Among the 139 specimens analyzed, the urinary hyaluronidase levels in patients with G2/G3 tumors (33.4 ± 4.5 milliunits/mg protein) are 5-8-fold higher than those in normal individuals (4.2 ± 1.2 milliunits/mg protein) and those in patients with G1 tumors (6.5 ± 1.7 milliunits/mg protein) or other genitourinary conditions (7.4 ± 1.4 milliunits/mg protein; P < 0.001). Urinary hyaluronidase measurement shows a sensitivity of 100% and a specificity of 88.9% to detect high-grade bladder (G2/G3) tumors. Thus urinary hyaluronidase measurement is a simple, noninvasive yet highly specific and sensitive method for high-grade bladder cancer detection. The increase in urinary hyaluronidase levels is due to the secretion of a tumor-associated hyaluronidase into the urine because the hyaluronidase levels in G2/G3 tumor tissues are also higher (6-7-fold) than those in normal bladder and G1 tumor tissues (P < 0.001). The bladder tumor-associated hyaluronidase activity is distinct from other hyaluronidases, has a pH optimum of 4.3, and is attributed to two proteins with molecular masses of 65 kD (p65) and 55 kD (p55).

INTRODUCTION

TCCs of the bladder account for the majority of bladder tumors (1). These tumors are characterized by the heterogeneity in their ability to invade and metastasize (1-4). The two most important prognostic factors for TCC are grade and stage (which indicates the depth of invasion; Ref. 5). Low-grade (G1) tumors are mostly confined to the mucosa (stage Ta) and have a <2% chance of progression (1, 6). Intermediate-grade (G2) tumors range from being noninvasive (Ta) to being invasive (stages T1-T4; Refs. 1 and 6). The G2, Ta tumors have ~11% chance of progression (1). With the exception of CIS, most high-grade tumors are initially detected at least at stage T1 (invading lamina propria) and are thus invasive (6, 7). Muscle invasion (stage T2) by the tumor is ominous because 50% of these patients develop distant metastasis within 2 years of diagnosis despite radical surgery, and 60% of them die within 5 years, however they are treated (6, 8). Due to the malignant nature of high-grade TCCs, their early detection, prior to muscle invasion, is crucial for a favorable prognosis. Extracellular matrix-degrading enzymes are known to regulate tumor invasion/metastasis and angiogenesis (9). Hyaluronidase is a class of extracellular matrix-degrading endoglycosidases that degrade HA, a free nonsulfated glycosaminoglycan (9, 10). The limited degradation of HA by hyaluronidase results in the generation of HA fragments of specific lengths (~3-25 disaccharide units) that are angiogenic (12). In vertebrates, hyaluronidases can be categorized into two classes, those active at neutral pH (pH optimum 5.0), and those active at acidic pH (pH 3.5-4.0; Refs. 11-16). For example, the testicular hyaluronidase is of neutral type (14, 16), whereas the liver hyaluronidase is of acidic type (14, 16).

MATERIALS AND METHODS

Urine Specimens. Voided (clean-catch) urine specimens were collected from 139 individuals under a protocol approved by the Institutional Review Board of University of Miami. The individuals were categorized into three groups. Group 1: normal (healthy) age-matched (30-70 years) individuals (n = 20). Group 2: patients with other GU conditions (n = 48), such as advanced prostate cancer (n = 10), BPH (n = 8), kidney stones (n = 5), cystitis (n = 12), urinary tract infections (n = 8), prostatitis (n = 2), episidymitis (n = 1), and renal trauma (n = 2). Group 3: patients with G1 (n = 22; stage Ta), G2 (n = 9; stages Ta-T2) or G3 (n = 40) bladder tumors. The G3 subcategory included 34 individuals with G3 tumors (stages T1-T4) and 6 individuals with CIS. CIS is a subclass of high-grade tumors that are flat and superficial (confined to the urothelium). All specimens were collected and stored at ~20°C until assayed.

Tissue Specimens. Normal bladder tissues from adults (21-50 years) were obtained from organ donors. Tissue procurement was performed according to relevant state and federal regulations. Bladder tumor tissues were obtained from patients (41-72 years) undergoing cystectomy or transurethral resection of the tumor. Tissue hyaluronidase levels were analyzed in three groups of patients. Group 1: normal bladder (n = 6). Group 2: low-grade TCC (G1; n = 6). Group 3: high-grade TCC (G2, n = 2; G3, n = 6). To evaluate the grade, each tumor specimen was split and the mirror image segment was fixed in formalin, embedded in paraffin, and analyzed histologically.

Tissue Extracts. Tissue specimens (~0.5-1 g) were homogenized in a buffer containing 5 mM HEPES, pH 7.2, and 1 mM benzamidino-1-HCl. The homogenates were centrifuged at 40,000 x g for 30 min, and the clear extracts were assayed.

Substrate (HA)-Gel Assay. Urine samples (~20 μg of protein) were electrophoresed under either nondenaturing conditions on a 7.5% polyacrylamide gel or under denaturing conditions on a SDS-polyacrylamide gel; both gels contained 0.17 mg/ml human umbilical cord HA (Sigma Chemical Co., St.

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3 The abbreviations used are: TCC, transitional cell carcinoma; HA, hyaluronic acid; CIS, carcinoma in situ; GU, genitourinary; BPH, benign prostate hyperplasia.
RESULTS

Detection of Urinary Hyaluronidase Activity

Substrate Gel Assay. Hyaluronidase activity in urine samples was detected using a sensitive substrate (HA)–gel technique (21, 23). As shown in Fig. 1, little or no HA digestion was observed in lanes containing urine specimens of normal individuals (Lanes 1 and 2) and patients with low-grade TCC (Lanes 3 and 4). However, a broad band of HA digestion was observed in specimens from high-grade TCC patients (Lanes 5 and 6), suggesting that these samples contain significant amounts of hyaluronidase activity.

Quantitative Determination of Urinary Hyaluronidase Activity by an ELISA-like Assay. Accurate quantitation of the hyaluronidase levels in urine samples (n = 139) was accomplished by an ELISA-like assay (21). The variation in hydration status and urine output of individuals was corrected by normalizing the hyaluronidase levels (milliunits) to urinary protein concentration (mg). The hyaluronidase levels were normalized to urinary protein concentrations rather than creatinine because the normalization to protein was found to be less influenced by hematuria, a condition commonly found in bladder cancer patients (3). We compared the urinary hyaluronidase levels between normal individuals (n = 20) and those with bladder cancer (e.g., G1, G2, and G3 tumors and CIS; n = 71). As controls, the enzyme levels of patients with GU conditions other than TCC (e.g., BPH, prostate cancer, kidney stones, bacterial infections and cystitis, renal trauma, prostatitis, and epididymitis; n = 48) were also included in the study. As shown in Fig. 2A, the distribution of urinary hyaluronidase levels among normal individuals and patients with either low-grade (G1) bladder tumors or other GU conditions is very similar. Furthermore, the enzyme levels of the majority of the individuals included in these three groups are <10 milliunits/mg. However, the hyaluronidase levels are elevated among all patients with intermediate (G2) to high-grade (G3) bladder tumors and are >10 milliunits/mg (Fig. 2A).

The comparison of the mean urinary hyaluronidase levels among various groups is shown in Fig. 2B. The mean urinary hyaluronidase levels among normal individuals (4.2 ± 1.2 milliunits/mg), those with other GU conditions (7.4 ± 1.4 milliunits/mg), or G1 tumors (6.5 ± 1.7 milliunits/mg) do not vary significantly. However, the levels are significantly elevated among patients with G2 tumors (32 ± 6.1 milliunits/mg) or G3 tumors (34.3 ± 3.1 milliunits/mg). The mean urinary hyaluronidase levels of all patients with G2 or G3 tumors combined (33.4 ± 4.5 milliunits/mg) are 5–9-fold higher than those in normal individuals and patients with other GU conditions or G1 bladder tumors. It is important to note that the urinary hyaluronidase levels are elevated 6–11-fold (46 ± 5.9 milliunits/mg) in all patients with CIS (a subclass of high-grade bladder TCCs that are superficial and flat). These results show that all high-grade bladder cancer patients have elevated urinary hyaluronidase levels prior to the occurrence of an invasive disease.

The statistical significance of the observed differences in the mean hyaluronidase levels among various categories of patients was assessed using the Tukey-Kramer multiple comparison test. As shown in Table 1, the differences between the mean hyaluronidase levels of normal individuals and patients with G1 bladder tumors or other GU conditions are not statistically significant (P > 0.05; Table 1). But those differences between normal individuals or other GU patients and patients with either G2 or G3 tumors are statistically significant.
Fig. 2. Quantitative determination of urinary hyaluronidase activity by an ELISA-like assay. The hyaluronidase activity was measured as described in “Materials and Methods.” A, scatter diagram of individual hyaluronidase activities. The mean activity was calculated from the individual hyaluronidase activities among various categories. The mean activity was calculated from the individual hyaluronidase activities presented in A. Columns, milliunits of mean hyaluronidase activity per mg of protein; bars, SE.

Table 1  Tukey-Kramer multiple comparison test for comparing mean urinary hyaluronidase levels in normal individuals and bladder cancer patients

The data presented in Fig. 2 were analyzed statistically using the Tukey-Kramer multiple comparisons test. If the q value is greater than 3.916 then the P value is less than 0.05.

Comparison | Mean difference | q  | P value |
---|---|---|---|
Normal vs. GU | -3.142 | 1.156 | >0.05 |
Normal vs. G1 | -2.175 | 0.709 | >0.05 |
Normal vs. G2 | -27.725 | 7.126 | <0.001 |
Normal vs. G3 | -30.033 | 10.834 | <0.001 |
GU vs. G1 | 0.967 | 0.398 | >0.05 |
GU vs. G2 | -24.583 | 7.210 | <0.001 |
GU vs. G3 | -26.891 | 13.163 | <0.001 |
G1 vs. G2 | -25.550 | 6.915 | <0.001 |
G1 vs. G3 | -27.858 | 11.189 | <0.001 |
G2 vs. G3 | -2.308 | 0.668 | >0.05 |

(P < 0.001; Table 1). The differences in the mean hyaluronidase levels among patients with G1 tumors and G2 or G3 tumors are also statistically significant (P < 0.001; Table 1). Nevertheless, the differences in the mean enzyme levels among patients with either G2 or G3 tumors are not statistically significant (P > 0.05). Therefore, these results show that the urinary hyaluronidase levels are elevated in all intermediate to high-grade cancer patients.

The data on urinary hyaluronidase levels were further analyzed to determine the specificity and sensitivity of the ELISA-like assay for detecting high-grade TCC. As shown in Table 2, the overall specificity of this ELISA-like assay, using 10 milliunits/mg as a minimum cutoff limit, is 88.8%. At the same cutoff limit, the sensitivity of this assay to detect high-grade TCC was 100% (i.e., not a single high-grade tumor was missed). The analysis shows that false positive and false negative outcomes from this assay were 11.2% and 0%, respectively.

Table 2  Determination of sensitivity and specificity for the ELISA-like assay to detect high-grade bladder cancer

The data on urinary hyaluronidase levels shown in Fig. 2 were analyzed for sensitivity and specificity calculations using 10 milliunits/mg enzyme level as a minimum cutoff limit. The sample included 49 high-grade TCC (G2 and G3 tumor) patients and 90 individuals with no disease (n = 20), other GU conditions (e.g., cystitis, bacterial infections, BPH, kidney stones, pros tate cancer, and so forth; n = 48) or low-grade TCC (G1 tumor; n = 22).

<table>
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<th>Outcome</th>
<th>Urinary hyaluronidase level</th>
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<tr>
<td>Sensitivity</td>
<td>100% (49/49)</td>
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<tr>
<td>Specificity</td>
<td>88.8% (80/90)</td>
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<tr>
<td>False-positive</td>
<td>11.2% (10/90)</td>
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<tr>
<td>False-negative</td>
<td>0% (0/90)</td>
</tr>
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* Sensitivity, true positive results/total number high-grade TCC patients. Specificity, true negative results/total number of patients without high-grade TCC. False-negative rate, false-negative results/total number of patients with high-grade TCC. False-positive rate, false-positive results/total number of individuals without high-grade TCC.

Examination of Hyaluronidase Activity in Bladder Tissue Extracts

Because our studies show that urinary hyaluronidase levels are elevated in high-grade bladder tumor patients, we hypothesized that this increase is a result of the secretion of a tumor-derived hyaluronidase(s) in the urine. Therefore, we tested for the presence of hyaluronidase activity in tissue extracts prepared from normal bladder, low-grade TCC (G1 tumors), and high-grade TCC (G2 and G3 tumors) using the ELISA-like assay. As shown in Fig. 3A, the hyaluronidase levels in normal bladder and in G1 tumor tissues are very similar. However, the G2 and G3 tumor tissue extracts show significantly elevated hyaluronidase levels (Fig. 3A). The mean hyaluronidase levels present in the G2 and G3 tumor tissues (13.2 ± 1.2 milliunits/mg) are indeed 6–7-fold higher than those present in normal bladder (1.9 ± 0.35 milliunits/mg) and G1 tumor (2.7 ± 0.61 milliunits/mg) tissues (Fig. 3B). The statistical analysis of these data by the Tukey-Kramer multiple comparison test shows that the observed differences in the mean hyaluronidase activity between the normal and G2 and G3 tumor tissues (P < 0.001), but not those between normal bladder and G1 tumor tissues (P > 0.05), are statistically significant (Table 3). Furthermore, differences in the mean enzyme levels between G1 tumors and G2 or G3 tumors are statistically significant (P < 0.001; Table 3). Thus, the elevation in both urine and tissue hyaluronidase levels is associated with intermediate to high-grade TCC of the bladder.

Characterization of the Bladder Tumor-associated Hyaluronidase Activity. The pH activity profile of the bladder tumor-associated hyaluronidase activity was determined using the ELISA-like assay. As shown in Fig. 4, the hyaluronidase activity present in the urine and tumor (G3) tissue of a high-grade TCC patient has a distinct pH optimum, 4.3, for HA degradation. The pH optimum for bladder tumor-associated hyaluronidase activity is different from those reported for the normal bladder (1.9 ± 0.35 milliunits/mg) and for the urinary bladder (2.7 ± 0.61 milliunits/mg) hyaluronidase enzymes.
normal vs. low-grade ICC—0.080 0.7946 >0.05
normal vs. high-grade TCC—11.37 12.074 <0.001
low-grade vs. high-grade TCC 10.57 11.225 <0.001

Table 3 Tukey-Kramer multiple comparison test for comparing mean tissue hyaluronidase levels in normal individuals and bladder cancer patients

The data presented in Fig. 3 were analyzed statistically using the Tukey-Kramer multiple comparison test. If the q value is greater than 2.65 then the P value is less than 0.05.

<table>
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<tr>
<th>Comparison</th>
<th>Mean difference</th>
<th>q</th>
<th>P value</th>
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<tr>
<td>Normal vs. low-grade TCC</td>
<td>-0.080</td>
<td>0.7946</td>
<td>&gt;0.05</td>
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<tr>
<td>Normal vs. high-grade TCC</td>
<td>-11.371</td>
<td>12.074</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Low-grade vs. high-grade TCC</td>
<td>-10.571</td>
<td>11.225</td>
<td>&lt;0.001</td>
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Fig. 3. Determination of hyaluronidase activity in bladder tissue extracts. The hyaluronidase activity in tissue extracts was determined by the ELISA-like assay as described in "Materials and Methods." A, scatter diagram of individual hyaluronidase activities. B, hyaluronidase activity among normal and bladder TCC tissue extracts. Columns, mean; bars, SE.

Fig. 4. Determination of the pH activity profile of bladder tumor-associated hyaluronidase. A urine specimen and a tumor tissue extract obtained from a patient with a high-grade bladder tumor were incubated on HA-coated wells at different pH. Following incubation, HA remaining on the wells was estimated as described in "Materials and Methods." The results were calculated as described in "Materials and Methods."
URINARY HYALURONIDASE LEVELS IN BLADDER CANCER

Fig. 5. Molecular mass determination of bladder-tumor derived hyaluronidase by substrate (HA) SDS-PAGE. A, substrate (HA) SDS-PAGE analysis of urine specimens. Urine specimens (~40 μg of protein) were separated on a 9% SDS-PAGE mini gel together with the Bio-Rad broad-range prestained molecular weight markers. Following electrophoresis, the gel was processed as described in "Materials and Methods." Lane 1, urine specimen from a patient with G1 tumor; Lane 2, urine specimen from a patient with G2 tumor; Lane 3, urine specimen from a patient with G3 tumor; Lane 4, urine specimen from a normal individual. B, SDS-PAGE analysis of total urinary proteins. Urine specimens (~20 μg of protein) from a patient with G3 tumor was separated by 12% standard size SDS-PAGE together with Bio-Rad prestained broad-range molecular weight markers. Following electrophoresis, the gel was silver stained. C, substrate (HA) SDS-PAGE analysis of tissue extracts. Extracts (40 μg of protein) prepared from tissue specimens of a G3 tumor and a normal bladder were separated on a 12% standard size SDS substrate (HA) gel together with Bio-Rad prestained broad-range molecular weight markers. Following electrophoresis, the gel was processed as described in "Materials and Methods." Left Lane, G3 tumor tissue extract; right Lane, normal bladder tissue extract.

note that only a single hyaluronidase protein is expressed in other tissues (20, 21, 26, 27).

DISCUSSION

The rapid progression of high-grade bladder cancer is the cause for its poor prognosis. Most patients with high-grade TCC have invasive disease at the time of clinical presentation (e.g., hematuria, irritative voiding symptoms, and so forth; Ref. 3). Therefore, early detection of these tumors is crucial for a better prognosis (6, 8). The data presented here show that urinary hyaluronidase levels are significantly (5-9-fold) elevated in patients with high-grade (G2 and G3 tumors) bladder TCC and indicate that hyaluronidase may be a useful urine marker for detecting high-grade bladder tumors.

The increase in both the urinary and tissue hyaluronidase levels suggests that a tumor-derived hyaluronidase(s) may be secreted into the urine. Our preliminary data show that the hyaluronidase(s) is secreted by bladder tumor epithelial cells.5 These observations are consistent with our previous report that in prostate cancer, the high-level secretion of the hyaluronidase activity is a property of the tumor epithelial cells (21).

The association of hyaluronidase with tumor biology is relatively new (20, 21). We recently showed that hyaluronidase levels are significantly elevated in prostate cancer tissues as compared to those in normal adult prostate and BPH tissues; the increased hyaluronidase levels in these tissues correlate with the aggressiveness of the tumor (21). However, it is important to note that hyaluronidase levels in the urine of prostate cancer patients are not elevated (n = 10; 3.1 ± 0.6 milliunits/mg; Fig. 2). Therefore, increased urinary hyaluronidase levels in high-grade bladder cancer patients are very likely a result of the direct secretion of a tumor-derived enzyme into the urine. Similarly, urinary hyaluronidase levels are elevated in children with Wilms' tumor, in whom, again, the tumor comes in contact with urine (20).

The hyaluronidase class of enzymes is present in normal tissues (e.g., liver, kidney, and testis; Refs. 13, 15, 20, and 27), tumor tissues (e.g., prostate; Ref. 21), and serum (26). The expression of these enzymes appears to be tissue specific. For example, the hyaluronidases found in liver, serum, testis, kidney, and prostate are different from each other with respect to pH optimum, molecular mass, and primary amino acid sequence (16, 20, 21, 26, 27). Our data show that bladder tumor-associated hyaluronidase activity is attributed to two proteins, p65 and p55 (Fig. 5), and has a pH optimum that is distinct from other hyaluronidases (13, 15, 26, 27). It is interesting to note that only a single hyaluronidase has been detected in other sources, such as testis (~55 kDa; Ref. 16), liver (~60 kDa; Ref. 15), prostate (~55 kDa; Ref. 21) and serum (~60 kDa; Ref. 26). Thus, the bladder tumor-derived hyaluronidases may represent two new members of this growing family of enzymes. The relationship between p65 and p55 remains to be established.

The function of the bladder tumor-associated hyaluronidases may be to degrade HA into small angiogenic fragments (12, 18, 28). Because the interstitial microenvironment of malignant tumors is known to be acidic, the bladder tumor-associated hyaluronidases may degrade HA present in tumor matrix (29). In an accompanying study (22), we have demonstrated that HA fragments are present in the urine of high-grade bladder cancer patients and that some of these fragments induce a mitogenic response in human endothelial cells. These observations strongly suggest that the bladder tumor-associated hyaluronidases are active in vivo and may promote tumor angiogenesis by generating small HA fragments. In addition, the high molecular mass HA present in tumor tissues may support tumor cell migration and adhesion and hence aid in tumor invasion (17, 18, 22, 28, 30). Thus, an intricate microenvironment in high-grade bladder tumor tissues that maintains a fine balance of HA and hyaluronidase concentrations may be conducive to both tumor metastasis and angiogenesis. How-

5 V. B. Lokeshwar, unpublished results.
ever, it is yet unclear which of the two events, i.e., an increase in HA concentration or an increase in hyaluronidase level, occurs first and whether these two events regulate each other.

The current mode of bladder cancer detection relies generally on the patients who present with hematuria (3). However, in dealing with high-grade bladder cancer, this mode of detection is often inadequate because the clinical presentation is frequently accompanied by the presence of muscle invasive disease (6, 8, 31). Early detection and follow-up of high-grade cancer patients would be greatly facilitated if a simple noninvasive test to detect these tumors was designed (32). The noninvasive tests that are currently available for bladder cancer detection are either not specific (e.g., home hematuria screening; Refs. 33 and 34) or not sensitive (e.g., Bard BTA latex agglutination assay; Ref. 35) in detecting bladder cancer and high-grade bladder tumors in particular. For example, Sarosdy et al. (35) have shown that the sensitivity of both urine cytology and the BTA test to detect high-grade bladder tumors (G3 including CIS) is only 49–56%. More recently, Soloway et al. (36) have shown that NMP22 urine test detects recurrent high-grade bladder tumors with high sensitivity (100%). However, this study involved only six high-grade patients (36). The overall sensitivity of the NMP22 test to detect recurrent bladder tumors of all grades is ~70% (36). In our study of a total of 139 specimens (49 of which were from high-grade bladder cancer patients), the sensitivity and specificity of the ELISA-like assay to detect high-grade bladder tumors were 100 and 88.8%, respectively. Because urinary hyaluronidase measurement detects CIS (preinvasive high-grade bladder tumors) as well as G2, Ta tumors, it potentially can be a better noninvasive method for the early detection of high-grade bladder tumors. In addition, the method described here is a simple ELISA-like assay that uses a HA-binding protein. This protein can be obtained in large quantities using a well-established purification procedure (24).

In an accompanying paper (22), we have shown that the urinary HA levels are useful in detecting bladder cancer, regardless of the tumor grade (22). The urinary HA levels are measured by a similar ELISA-like assay that also requires the same HA-binding protein for detection (22). Thus, a combination of urinary HA and hyaluronidase measurement by these two very similar ELISA-like assays can be used as a noninvasive yet highly sensitive and specific test (“HA-HAase urine test”) both to detect and to identify the grade of bladder tumors. The HA-HAase test may also be useful in monitoring treatment outcome and recurrence.

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