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Clinical Significance of Urine Heparanase in Bladder Cancer Progression[1]

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Abstract

Heparanase is an endo-β-glucuronidase capable of cleaving heparan sulfate (HS), an activity implicated in tumor metastasis. Heparanase expression is upregulated in primary human tumors, correlating with reduced post operative survival and elevated microvessel density. An ELISA method was used to quantify heparanase in urine from 282 individuals. Urine was collected from healthy volunteers ($n = 41$), patients diagnosed with noncancerous pathologic disorders ($n = 90$), and bladder cancer patients ($n = 92$). Fifty-nine bladder carcinoma patients after transurethral resection (TUR) with no evidence of disease (NED) were also included. Heparanase levels were significantly elevated in urine from bladder cancer patients compared with healthy controls ($P < .001$) and with noncancerous urinary disorders ($P < .05$). Heparanase elevation strongly correlated with tumor grade ($P < .001$) and stage ($P = .027$). An optimal cutoff value of 154 pg/ml was determined. Of 199 individuals enrolled (59 patients after TUR and 24 patients with recurring disease were excluded), 65 had heparanase levels above 154 pg/ml. Only 3 of 65 (4.6%) were healthy individuals. In contrast, 52.3% (34 of 65) of individuals with heparanase levels above 154 pg/ml were bladder cancer patients. The results indicate that urine heparanase levels are elevated during bladder cancer progression, suggesting that the ELISA method may be applied for bladder cancer diagnosis.

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Introduction

Bladder carcinoma is the primary malignancy of the urinary tract system with nearly 60,000 new cases and 13,000 deaths annually in the United States [1]. Disease mortality rate has not been significantly changed during the past decade [1,2]. Surgical resection remains the primary and most effective treatment for carcinoma *in situ* (CIS; accounts for 80% of all new cases), although recurrence rates are high, and patients are required to undergo periodic cystoscopic examination as frequently as once every 3 months [3]. The prospective for metastatic bladder cancer (20%) remains poor. Treatment for refractory superficial tumors or muscle-invasive tumors is radical cystectomy with urinary diversion. Neoadjuvant chemotherapy, with or without adjuvant chemotherapy, has also shown promising results in preventing disease progression and tumor recurrence in patients with adverse clinical or pathologic features [4]. Diagnosis of the disease is usually preceded by hematuria and cytologic evaluation, although sensitivity is relatively low. Additional diagnostic markers include bladder tumor antigen, nuclear matrix protein (NMP)-22, telomerase, fluorescence *in situ* hybridization, and others, yet these are mostly applied for posttreatment surveillance rather than for diagnosis [3,4]. Thus, better understating of the biologic nature of the disease is required for the establishment of new molecular markers for early diagnosis and more efficient treatment modalities.

Abbreviations: CIS, carcinoma *in situ*; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; NED, no evidence of disease; NMP, nuclear matrix protein; ROC, receiver operating characteristic; TUR, transurethral resection; UTI, urinary tract infection

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Heparanase is an endo-β-glucuronidase, which cleaves heparan sulfate (HS) side chains of heparan sulfate proteoglycans (HSPGs) in a distinct manner [5], an activity that is strongly implicated in cell dissemination associated with tumor metastasis, inflammation, and angiogenesis [5–7]. Cleavage of HS, a major constituent of the extracellular matrix (ECM) and basement membranes, is considered an important step for breaking down the ECM barrier and penetrating the blood vessel basement membrane required for tumor cell metastasis [8]. This notion gained further support by employing siRNA and ribosome technologies, clearly depicting heparanase-mediated HS cleavage and ECM remodeling as critical requisites for inflammation, angiogenesis, and metastatic spread [9–11]. More recently, heparanase upregulation has been documented in a large number of primary human carcinomas [12,13]. In many cases, heparanase upregulation correlated with reduced postoperative survival rates, increased lymph node and distant metastasis, tumors bigger in size, and higher microvessel density, collectively providing a strong clinical support for the prometastatic and proangiogenic nature of the enzyme and positioning heparanase as an attractive target for the development of anticancer drugs [14–18]. In addition, HS side chains can bind a variety of biologic mediators such as growth factors, enzymes, cytokines and chemokines [19], thus forming a readily available reservoir that can be liberated on local or systemic cues, including heparanase availability [20–22]. Extracellular retention of heparanase is, therefore, kept tightly regulated [13,23–26]. Heparanase upregulation by primary human carcinomas and the secreted nature of the protein predict that the enzyme may be found in body fluids such as plasma and urine, the latter being most relevant for bladder carcinoma. We have recently developed a sensitive ELISA method capable of determination and quantification of heparanase and demonstrated that heparanase levels are elevated in the urine and plasma of cancer patients [27]. Moreover, we have found decreased heparanase levels in the plasma of pediatric cancer patients following anticancer treatment [28]. Here, we used the recently established ELISA method to determine heparanase levels in urine samples collected from a large number of bladder cancer patients in comparison with heparanase levels in urine collected from patients diagnosed with noncancerous pathologic disorders and from healthy volunteers. We provide evidence that heparanase levels are elevated three- to four-fold in the urine of bladder carcinoma patients, correlating with tumor grade and stage. Moreover, high levels of heparanase were found in the urine of patients with active disease compared with patients with no evidence of disease (NED) following transurethral resection (TUR), suggesting that heparanase originates primarily from the tumor mass. An optimal cutoff value of 154 pg/ml was extracted from a receiver operating characteristic (ROC) curve, and heparanase diagnostic potential was revealed. Of 199 individuals enrolled, 65 had heparanase levels above 154 pg/ml of which only 3 (4.6%) were healthy individuals. In striking contrast, 52.3% (34 of 65) of the individuals with heparanase levels above 154 pg/ml were bladder cancer patients, suggesting that the ELISA method may be applied for bladder cancer diagnosis.

Materials and Methods

Experimental Design

A total of 282 individuals were enrolled in this study and included healthy control participants (group I, *n* = 41), patients diagnosed with noncancerous symptoms such as hematuria, irritative voiding symptoms, urinary tract infection (UTI), and urinary stones (group II, *n* = 90), and patients with primary and recurrent bladder carcinoma (group III, *n* = 92). A group of 59 patients with a history of superficial bladder carcinomas who were under surveillance after TUR and who had NED at the time of sample collection was also included (group IV). All patients in groups II and III, except those with urolithiasis and UTI, underwent cystoscopy. Tumors were graded according to the World Health Organization grading system and were staged according to the TNM classification system [29]. The study protocol was approved by the Hadassah Hospital Institutional Review Board. Informed consent was obtained from every patient.

Urine samples were collected from each patient before cystoscopy, centrifuged (1500*g* for 10 minutes) to remove cells and cell debris, and the supernatant was kept at −20°C until analysis. All patients were diagnosed at the Oncology and Urology Departments of the Hadassah-Hebrew University Medical Center, Jerusalem [30].

Heparanase ELISA

Urinary heparanase was analyzed by heparanase ELISA method, essentially as described [27,28]. Briefly, wells of microtiter plates were coated (18 hours at 4°C) with 2 μg/ml of anti–heparanase 1E1 monoclonal antibody in 50 μl of coating buffer (0.05 M Na_2CO_3, 0.05 M $NaHCO_3$, pH 9.6) and were then blocked with 1% BSA in PBS for 1 hour at 37°C. Samples (200 μl) were loaded in duplicates and incubated for 2 hours at room temperature, followed by the addition of 100 μl of anti–heparanase polyclonal antibody 1453 (1 μg/ml) for an additional 2 hours at room temperature. Horseradish peroxidase–conjugated goat anti–rabbit IgG (1:20,000) in blocking buffer was added (1 hour at room temperature), and the reaction was visualized by the addition of 50 μl of chromogenic substrate (tetramethylbenzidine) for 30 minutes. The reaction was stopped with 100 μl of H_2SO_4, and absorbance at 450 nm was measured with reduction at 630 nm using an ELISA plate reader. Plates were washed five times with a washing buffer (PBS, pH 7.4, containing 0.1% (v/v) Tween 20) after each step. As a reference for quantification, a standard curve was established by a serial dilution of recombinant single-chain (GS3) active heparanase ranging from 187 pg/ml to 5 ng/ml [27,28].

Statistical Analysis

The distribution of heparanase values in urine was asymmetric; therefore, nonparametric analyses were applied. Kruskal-Wallis one-way analysis of variance (ANOVA) was performed to test for overall homogeneity. Pairs of groups were compared using the Mann-Whitney test once ANOVA was significant. Correlations between numerical variables were analyzed by linear nonparametric (Spearman) correlations. Associations between categorical variables were evaluated with chi-square test. For the analysis of sensitivity–specificity relation of the assay, an ROC curve was constructed, and the area under this curve was calculated [30,31]. A P value of $< .05$ was considered significant.

Results

Heparanase Elevation in Noncancerous Urinary Tract Disorders

We evaluated the level of heparanase in urine obtained from noncancerous and bladder carcinoma patients (Figure 1; Table 1). The average level of heparanase in the urine of healthy donors was

61.6 ± 10.5 pg/ml. Patients diagnosed with noncancerous urinary tract disorders exhibited elevated levels of heparanase (141.7 ± 17.4; Figure 1*A*), significantly higher than control values (61 ± 10; $P = .05$). The variance among the four subgroups of patients with noncancerous urinary tract conditions (group II; Table 1) was significant (Kruskal–Wallis test; $P = .0291$). Paired comparison of the various groups revealed that heparanase elevation was most prominent in patients with hematuria (177 ± 37; Table 1) ($P < .05$), likely due to the release of heparanase by activated platelets [32], whereas other noncancerous patients did not significantly differ in their heparanase levels from the control group.

A substantial increase in heparanase levels was quantified in the urine of carcinoma patients (210 ± 25; Figure 1*A*; Table 1), significantly higher than noncancerous urinary tract disorders ($P < .05$). These results indicate that elevation of urinary heparanase levels is common in noncancerous and cancerous patients, the latter being more prominent (Figure 1*A*).

Urine Heparanase Levels Correlate with Bladder Cancer Grade and Stage

To further characterize heparanase levels in bladder carcinomas, the cancer patient group was subdivided into low- ($n = 54$) and high- ($n = 38$) grade tumors, and the respective heparanase levels were compared to those found in the urine of healthy donors ($n = 41$) (Figure 1*B*; Table 1). A three-fold increase in heparanase levels was observed in the urine of low-grade bladder cancer patients (192.6 ± 42 pg/ml) and a further increase was measured in urine collected from high-grade bladder cancer patients (286.5 ± 52.5 pg/ml), differences that are statistically highly significant (healthy *vs* low-grade: $P = .045$; healthy *vs* high-grade: $P = .001$; low-grade *vs* high-grade; $P = .0001$). Next, we determined urine heparanase levels at distinct morphologic stages of the disease (Figure 1*C*; Table 1). Increased amounts of heparanase were already detected in urine collected from noninvasive low-grade lesions (pTa, $n = 60$; 175 ± 24.9 pg/ml) and was highest in patients with high-grade intraurothelial *neoplasia* (CIS, $n = 14$; 300 ± 69; Figure 1*C*), differences that are statistically highly significant (healthy *vs* pTa: $P = .036$; healthy *vs* CIS: $P = .001$; pTa *vs* CIS: $P = .027$) (Table 1). Thus, urine levels of heparanase correlate with bladder cancer grade and stage. Furthermore, patients with active disease exhibited a four-fold increase in heparanase levels (210 ± 26), significantly higher than those of patients with NED following TUR (162 ± 31; $P = .0175$; Table 2). These findings indicate that urine heparanase originates primarily from the tumor mass.

Receiver Operating Characteristic Curve and Heparanase Specificity

Because elevated levels of heparanase were detected in urine obtained from cancer and noncancerous patients, an ROC curve was constructed (Figure 2). The curve delineates a graphical plot of the sensitivity *versus* the specificity of the system and is typically used to determine an optimal cutoff value [31]. The area under the ROC curve was 0.61 ± 0.04; an optimal cutoff value of 154 pg/ml was extracted, corresponding to a sensitivity of 51.1%, specificity of 69.7%, and accuracy of 62.8%. The heparanase assay at this cutoff exhibited sensitivity of 52.7% and 48% for primary and recurrent bladder cancer, respectively.

Next, we used the cutoff value of 154 pg/ml to examine the significance of urine heparanase levels for bladder cancer diagnosis (Table 3). Of 199 individuals enrolled [excluding patients already diagnosed (59 patients under surveillance after TUR and 24 patients with recurrent disease)], 65 had heparanase levels above 154 pg/ml of

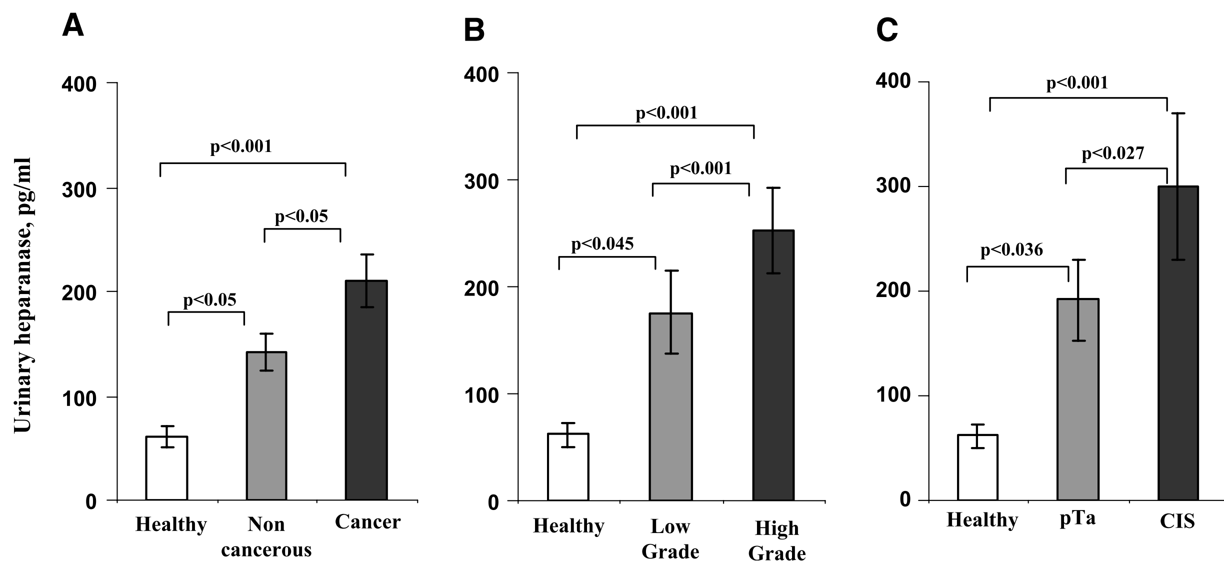

Figure 1. Heparanase levels in the urine of bladder cancer and noncancerous patients. (A) Urine was collected from healthy donors ($n = 41$; group I), patients exhibiting noncancerous pathologic disorders ($n = 90$; group II), and patients with bladder carcinoma ($n = 92$; group III). Heparanase levels were determined using the heparanase ELISA method, as described under the Materials and Methods section. The variance among the patients in the three groups is highly significant ($P < .0001$; Kruskal-Wallis test). Paired comparisons between different groups showed significantly higher urine heparanase values in group III, compared with group II ($P < .05$) and group I ($P < .001$). The difference between group II and group I was also significant ($P < .05$). Patients with bladder carcinoma were subdivided according to tumor grade (B) and stage (C) (see Table 1 for more details) and urine heparanase levels were similarly compared to those determined in healthy control donors.

Table 1. Heparanase Levels in the Urine of Patients with Noncancerous Urinary Tract Disorders and Bladder Carcinoma.

	Urine Heparanase (pg/ml)				Patients with Heparanase Levels above 154 pg/ml
	No. of Patients	Mean	Median	SEM	No. of Patients (%)
Group I: Healthy control participant	41	61.6	32.5	10.5	3 (7.3)
Group II: Noncancerous disorders	90	141.7	101.1	17.4	28 (30.2)
Hematuria	33	177.0	125.0	37.1	12 (36.4)
Irritative voiding symptoms	21	139.5	125.0	25.6	8 (38.1)
Urolithiasis	13	122.6	75.0	44.7	4 (30.8)
Urinary tract infection	23	103.8	82.0	24.6	4 (17.4)
Group III: Bladder tumors	92	210.2	145.8	25.9	45 (48.9)
Primary tumors	68	232.0	154.0	33.7	34 (50.0)
Recurrent tumors	24	148.4	128.7	24.8	11 (45.8)
By stage					
pTa	60	175.0	120.8	24.9	26 (43.3)
pT1–4	18	257.2	116.7	87.1	8 (44.4)
CIS	14	300.3	275.0	69.6	11 (78.6)
By grade					
Low	54	192.6	116.7	42.0	22 (40.7)
High	38	286.5	204.0	52.5	23 (60.5)
Group IV: Follow-up (under surveillance after TUR)	59	162.5	91.7	31.6	17 (28.8)

which only 3 (4.6%) were healthy individuals, indicating a high degree of specificity of the ELISA assay (Table 3). In striking contrast, 52.3% of the individuals with heparanase levels above 154 pg/ml were bladder cancer patients (34 of 65), compared with 18.5% (12 of 65) with hematuria, 12.3% (8 of 65) with irritative voiding symptoms, and 6.2% (4 of 65) with UTI (Table 3). Thus, urinary heparanase may be considered as a possible parameter for the diagnosis of bladder cancer.

Discussion

Bladder cancer is compliant to biomarker development, because tumor cells and molecules are shed into the urine and can thus be diagnosed and monitored. Approximately 75% of bladder tumors are low-grade and low-stage (i.e., stage Ta), and these tumors rarely progress [33]. However, approximately 25% of bladder tumors are high-grade, and early detection of these tumors once still superficial (Ta or T1) could improve patient's prognosis [33]. Heparanase upregulation was observed in essentially all primary human tumors examined, including bladder carcinoma [34]. Most often, heparanase induction correlates with reduced postoperative survival rate, likely due to increased local and distant metastasis [12,35], thus positioning heparanase as a valid target for the development of anticancer drugs [14–16,18]. Here, we provide evidence that urine heparanase levels correlate with bladder cancer progression. To the best of our knowledge, this is the first systematic study examining urinary heparanase and its diagnostic significance. Importantly, elevated heparanase levels were detected already in the urine of noninvasive, pTa stage patients, and further increase in heparanase levels was measured in the urine of CIS patients (Figure 1*C*). Whereas the increase in urinary heparanase at advanced stages of the disease nicely resembles its elevation at the protein and mRNA levels [34], noninvasive pTa biopsies rarely stained for heparanase [36]. Thus, it appears that our ELISA method is highly sensitive and detects heparanase even at very low levels, suggesting its possible application for primary bladder cancer diagnosis. To further verify this aspect, we constructed an ROC curve and determined an optimal cutoff value. The majority of cases (52.3%) with heparanase levels above 154 pg/ml are bladder cancer patients, followed by 18.5% of noncancerous hematuria patients (Table 3). Whereas the latter subgroup is most likely derived from platelet [32], the urinary heparanase in the cancer patients is primarily tumor-derived, a notion that is further substantiated by comparing heparanase levels in the urine of patients with active disease *versus* NED following

Table 2. Heparanase Levels in the Urine of Bladder Cancer Patients.

	No. of Patients	Mean (pg/ml)	Median (pg/ml)	SEM	*P*
Healthy control participant	41	61.6	32.5	10.5	
					< .0001*
Active disease	92	210.2	145.8	25.9	= .022†
NED	59	162.5	91.7	31.6	= .0013

*Relative to healthy controls.
†Relative to NED.

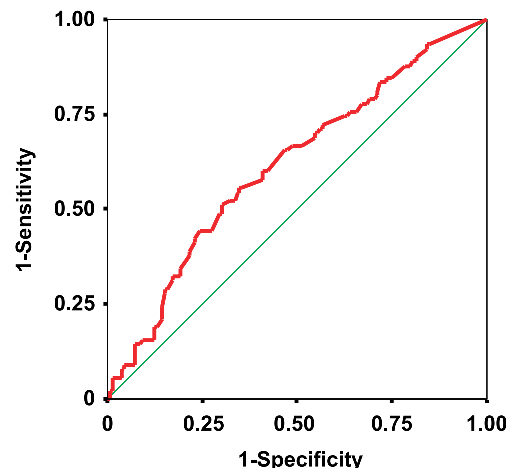

Figure 2. Receiver operating characteristic (ROC) curve of urinary heparanase. An ROC curve was extracted using all patients (groups I, II, III, and IV). The curve provides an optimization of the sensitivity–specificity ratio of the assay. The area under the curve is 0.6 ± 0.04, yielding an optimal heparanase cutoff value of 154 pg/ml.

Table 3. Number of Patients above the Cutoff Value.*

Subgroup	Number of Patients with Heparanase Levels above the Cutoff Level of 154 pg/ml
Healthy control participants	3/65 = 4.6%
Bladder tumors	34/65 = 52.3%
Hematuria	12/65 = 18.5%
Irritative voiding symptoms	8/65 = 12.3%
Urolithiasis	4/65 = 6.2%
UTI	4/65 = 6.2%

*Excluding patients after TUR and patients with recurrent tumor.

TUR (Table 2). This notion is in agreement with recent findings, demonstrating reduced plasma heparanase levels following anticancer treatment [28].

The low percentage of false-positive cases (4.6%; Table 3) encourages heparanase employment for bladder cancer diagnosis, with the following limitations: 1) Overall, only 48.9% of bladder cancer patients were above the calculated cutoff value (45 of 92), leaving approximately 50% of the cases (47 of 92) undetected. This relatively high level of false-negative value decreases dramatically as the tumor progresses to CIS, where the false-negative value drops to 21.4% (Table 1). 2) Elevation of urinary heparanase was also noted in noncancerous disorders. Thus, a positive result in the ELISA method reflects a pathologic disorder, but not necessarily cancer, and requires determination of additional cellular and molecular markers for accurate diagnosis. A combination of methods has become, in fact, the established means for bladder cancer diagnosis, as no single assay is sufficiently sensitive and accurate [37]. For example, microscopic hematuria can be fairly accurate, yet up to 25% of patients with bladder cancer will not develop hematuria [38]. Cytology and cytoscopy are widely used for bladder cancer diagnosis and staging, but flat lesions, in particular CIS, and low-grade or Ta superficial bladder cancer cases are more difficult to detect [37]. False-negative cytoscopy was estimated at the range of 10% to 40% [38], thus urging the development of additional diagnostic tools. A number of urinary markers for bladder cancer have been investigated in recent years, and several, such as bladder tumor antigen, NMP-22, and fibrinogen degenerative product, have been approved by the Food and Drug Administration [33,37,38]. Neither of these markers is, however, sufficiently accurate by itself. For example, NMP-22 has a false-positive rate of 25% or even higher (33%–50%) among patients with urolithiasis and inflammation [33]. Furthermore, NMP-22 has a false-negative rate of 45% to 55% at the initial diagnosis [39,40], although sensitivity increases with tumor size, grade, and stage [33], very similar to the performance of heparanase (Figure 1, *B* and *C*; Table 1). In contrast, the combination of NMP-22 and cytoscopy increases the overall sensitivity to 99% [40], clearly illustrating the need for a combination of methods for accurate diagnosis. An optimal combination of urine markers has not been investigated, probably due to the high cost of such a study. NMP-22 also has a lower sensitivity for detecting recurrent tumors compared to primary tumors because recurrent tumors are often smaller [33]. The feasibility of the heparanase ELISA method for bladder cancer surveillance is yet to be resolved and is currently being investigated.

Taken together, bladder tumors have a strong tendency for recurrence, resulting in high overall disease prevalence. Early detection of high-grade lesions and surveillance following TUR is required to improve patient's prognosis, an objective that largely depends on a sensitive and reliable diagnosis. Our results suggest that urine heparanase levels, combined with other diagnostic means, may prove beneficial for monitoring bladder cancer progression and thereby improving patients' outcome.

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