Sensitivity and Specificity of Urinary Hyaluronic Acid and Hyaluronidase in Detection of Bladder Transitional Cell Carcinoma

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Purpose: To the assess sensitivity and specificity of urinary levels of hyaluronic acid (HA) and hyaluronidase (HAase) as an individual or a combined test to diagnose bladder transitional cell carcinoma (TCC).

Materials and Methods: One hundred and ninety-four urine specimens were collected from individuals between July 2007 and March 2008. The urinary level of hyaluronic acid (HA) was measured by Enzyme-linked immunosorbent assay. Thereafter, the urinary levels of HA and HAase were normalized to urinary creatinine level and expressed as ng/mg and μ/mg.

Results: Eighty percent of patients with bladder cancer had urinary HA level < 500 ng/mg, and 90% of controls showed HA level < 500 ng/mg ($P < .001$). The mean urinary levels of HA in controls did not vary significantly ($P < .05$), whereas they significantly increased (2.5 to 6.5 folds) in all grades of TCC. More than 80% of patients with grades 2 and 3 TCC had urinary HAase level < 10 μ/mg and over 80% of controls showed HAase level < 10 μ/mg ($P < .05$). Hyaluronidase levels increased in patients with grades 2 and 3 bladder TCC.

Conclusion: Measurement of urinary levels of HA and HAase (with 89% sensitivity and 83% specificity) appears to be a highly accurate and non-invasive method for detecting bladder TCC and evaluating its grade.

Keywords: hyaluronic acid; diagnostic errors; urinary bladder neoplasms; transitional cell; neoplasm grading.

INTRODUCTION

Genitourinary cancers are among the most common cancers in men and the fifth most common one in women.[1] Transitional cell carcinoma of the bladder (TCC) is the second most common malignancy of the urinary tract.[2] Approximately, 54 000 new cases of bladder cancer are diagnosed annually in the United States.[3] Despite successful treatment of the initial tumor, bladder tumors frequently recur; hence, close follow-up of patients is mandatory.[4] Therefore, early detection of bladder cancer affects prognosis of patients with bladder cancer.

Current standard methods for detection and follow-up of the bladder cancer consist of cystoscopy, urine cytology, and biopsy from the suspicious area.[5] The gold standard method is the combination of cystoscopy and biopsy, but it is invasive and expensive.[6] Urine cytology is easy to perform, but is particularly insensitive in detection of grade 1 (G1) and grade 2 (G2) tumors.[7] Biochemical measurement of soluble markers in urine, such as nuclear matrix protein 22 (NMP22), bladder tumor antigen (BTA), urinary bladder cancer (UBC), and fibrinogen degradation product (FDP) were also investigated, but they cannot replace cystoscopy. Such measurements are non-invasive and can be performed frequently, but could not be useful except in combination with cystoscopy.[8-10]

Hyaluronic acid (HA) is an unsulfated anionic linear glycosaminoglycan polymer composed of a repeating glucuronic acid and N-acetylglucosamine disaccharide motif.[11] It is a substrate of cell adhesion and originally stimulates angiogenesis. As a result, HA plays a key role in promoting of tumor invasion.[12] Some prostatic histopathologies indicated that HA content of the stroma increased in benign prostatic hyperplasia.[13] Hyaluronidase (HAase) is an endoglycosidase enzyme that predominantly degrades HA.[14] Some studies show that HAase is involved in tumor growth, muscle infiltration by tumor, and tumor angiogenesis.[15,16] In this study, we simultaneously measured urinary levels of HA and HAase to examine the sensitivity and specificity of these markers as an individual or combined test to detect bladder cancer (TCC) and evaluate its grade.

MATERIALS AND METHODS

Urine Specimens

In this cross-sectional study, 194 voided urine specimens were collected from individuals between July 2007 and March 2008. The study was approved by the medical ethics committee of Tehran University of Medical Sciences, and a written informed consent was obtained from each participant. Samples were obtained using clean-catch method and stored at 20ºC.

The samples were divided into two groups as follows: group 1 (cases, n = 97), which included patients with bladder cancer; group 2 (controls, n = 97), which was subdivided into three groups, normal individuals (n = 19), those with other genitourinary diseases (n = 51), and patients with a history of TCC, but without active tumor (n = 27). Characteristics of patients and controls are demonstrated in Table 1. Table 2 shows clinicopathological characteristics of patients with TCC.

Tissue Extraction

Fresh tissue specimens were obtained from individuals undergoing cystoscopy. Transurethral resection-biopsy was performed on patients with bladder tumor.

Enzyme-Linked Immunosorbent Assay

The urinary level of HA was measured by enzyme-linked immunosorbent assay. With this method, plates coated with 200 µg/mL HA were incubated with using serial dilutions of urine specimens in hyaluronidase assay buffer at 37°C for 16 to 18 hours. Following incubation, the degraded HA was washed off and HA remaining in the wells was quantitated using a biotinylated HA-binding protein. Thereafter, the urinary levels of HA and HAase were normalized to urinary level of creatinine and were expressed as ng/mg and µ/mg.

Statistical Analyses

Data are presented as mean ± SD. Data were analyzed with SPSS software (the statistical package for the social sciences, version 10.0, SPSS Inc., Chicago, Illinois, USA). Sensitivity, specificity, and accuracy were calculated as follows:

Sensitivity: test positive/total number of patients with TCC

Specificity: test negative/total number of individuals without bladder cancer

Accuracy: number of true positive + number of true negative/total number of studied individuals

RESULTS

Urinary levels of HA were very similar in normal individuals (206 ± 28 ng/mg), patients with genitourinary diseases (317 ± 87 ng/mg), and those with history of bladder cancer (377 ±

Table 1. Characteristics of study subjects.

Group	Male, no. (%)	Female, no. (%)	Total, no. (%)	Average ,age years
Cases	68 (70)	29 (30)	97 (100)	63 (34-91)
Controls				
Normal	13 (13.4)	6 (6.2)	19 (19.6)	59.7 (51-69)
History of TCC	19 (19.6)	8 (8.2)	27 (27.8)	63 (50-76)
Other GU disease				60 (35-85)
BPH	12 (12.3)		12 (12.3)	
Renal Stone	5 (5.2)	8 (8.2)	13 (13.4)	
Interstitial Cystitis		**1 (1)**	1 (1)	
Ureterocele		**1 (1)**	1 (1)	
Urethral stricture	3 (3.1)		3 (3.1)	
Renal cell carcinoma	5 (5.2)	**2 (2.1)**	7 (7.3)	
Prostate cancer	3 (3.1)		3 (3.1)	
Bladder diverticulum	2 (2.1)		2 (2.1)	
Tuberculosis	1 (1)		1 (1)	
Bladder stone	4 (4.1)	**1 (1)**	5 (5.1)	
UPJO	1 (1)	**2 (2.1)**	3 (3.1)	

Key: TCC, transitional cell carcinoma; BPH, benign prostate hyperplasia; UPJO, ureteropelvic junction obstruction.

37 ng/mg) ($P < .05$). Cut-off limit was set at 500 ng/mg for the HA test for detection of bladder cancer. In the majority of individuals in these three groups, the urinary level of HA was less than 500 ng/mg. However, it increased in patients with the bladder cancer (1119 ± 127 ng/mg), regardless of the tumor grade (i.e., G1, G2, and G3) ($P < .05$). The mean urinary levels of HA in control group do not vary significantly ($P < .05$), whereas they significantly increased (2.5 to 6.5 folds) in patients with all grades of TCC (Table 3). Eighty percent of patients with the bladder cancer had urinary HA level < 500 ng/mg, and 90% of controls showed HA level < 500 ng/mg (Tables 4 and 5). The differences in the mean HA levels in patients with TCC (G1 to G3) (1119 ± 127 ng/mg) and in controls were statically significant ($P < .001$).

Table 2. Distribution of TCC with respect to tumor grade and stage (%).

Grade	Ta	CIS	T1	T2	T3
G1	24.8	0	1	4.1	0
G2	9.3	7 (21.2)	0	0	0
G3	5.2	5.2	6.2	11.3	16.5

Key: TCC, transitional cell carcinoma.

Ten μ/mg was set as cut-off point for the HAase test for detecting TCC G2 and G3. Distribution of urinary levels of HAase among normal subjects (3.4 ± 1.8 μ/mg), patients with genitourinary diseases (20.3 ± 2.1 μ/mg), patients with history of bladder cancer (6.7 ± 2.1 μ/mg), and patients who had TCC G1 at the time of recruitment (7.3 ± 1.4 μ/mg) was very similar. Furthermore, HAase levels in majority of individuals in this category were less than 10 μ/mg. However, they increased (3 to 7 folds) in patients with TCC G2 (22.1 ± 5.3 μ/mg) and G3 (28.1 ± 4.3 μ/mg). More than 80% of patients with TCC G2 and G3 had urinary HAase level < 10 μ/mg and more than 80% of controls showed HAase level < 10 μ/mg ($P < .05$). The data obtained by HA and HAase tests for each study specimen were combined and analyzed as a "combined HA-HAase test" for detecting TCC. The cut-off points for the combined HA-HAase test were the same as an individual one. Any individual with urinary level above the mentioned cut-off point (separately or in combination) was considered positive on the combined HA-HAase test. The data showed that more patients with TCC had positive HA-

Table 3. Mean concentrations of HA and HAase in each group

Category	HA (ng/mg)	HAase (μ/mg)
TCC	1119 ± 127	20.3 ± 2.1
G1	893 ± 105	7.3 ± 1.4
G2	1177 ± 95	22.1± 5.3
G3	1238 ± 115	28.1± 4.3
TCC history	377 ± 37	6.7 ± 2.1
Normal	206 ± 28	3.4 ± 1.8
Other GU disease	317 ± 87	6.1± 2.9

Key: TCC, transitional cell carcinoma; HA, hyaluronic acid; HAsae, hyaluronidase; GU, genitourinary.

HAase test than on individual HA and HAase tests. These results indicated that HA-HAase test was more sensitive and less specific than individual tests alone (Table 6).

DISCUSSION

Measurement of urinary levels of HA and HAase (HA-HAase test) appears to be a highly accurate and non-invasive method for detecting bladder TCC and evaluating its grade. Furthermore, none of them require complex technical skills or equipments and small quantity of urine specimen (50 mL urine) is adequate for both HA and HAase tests. An interesting finding was that 3 patients with G1 showed positive HAase test, but a negative HA test. Although these were considered as "false-positive" on HAase test, 2 of them developed G2 tumor 3 to 6 months later. Tumor volume also can affect the results. For example, a large-volume tumor would ensure to secrete much amount of any marker in urine, but this investigation suggests that outcome of HA-HAase test is not influenced by the tumor volume. For instance, sensitivity of HA-HAase test to detect carcinoma in situ (CIS) is 80%, but carcinoma in situ seldom presents with high volume tumor.

Our finding that HAase test preferentially detect G2 and G3 tumors is consistent with previous studies demonstrating that HAase secretion is associated with invasive/metastatic potential of tumor cells.[14]

Combined test can detect both TCC G1-Ta and CIS with high sensitivity.[7] In a study, the urinary levels of HA and HAase were measured in 513 urine specimens. The HA test showed 83.1% sensitivity, 90.1% specificity, and 86.5% accuracy to detect bladder cancer, regardless of the tumor grade and the HAase test demonstrated 81.5% sensitivity, 83.8% specificity, and 82.9% accuracy in detecting G2 and G3,[17] which

Table 4. Sensitivity of HA and HAase regard to grade and stage of TCC

Grade and Stage, no. (%)	HA test , no. (%)	HAase test , no. (%)	HA-HAase test , no. (%)
G1	(23/29) 79.3	(7/29) 24.1	(24/29) 82.7
G2	(23/25) 92	(22/25) 88	(23/25) 92
G3	(34/43) 79.1	(38/43) 88.4	(40/43) 93
CIS	(4/5) 80	(4/5) 80	(4/5) 80
Ta	(30/38) 78.9	(14/38) 36.8	(32/38) 84.2
T1	(8/11) 72.8	(9/11) 81.8	(10/11) 90.9
T2	(19/21) 90.5	(20/21) 95.2	(20/21)95.2
T3	(19/22) 86.4	(20/22) 90.9	(21/22) 95.4
TCC *	(80/97) 82.5	(60/68) 88.2**	(87/97) 89.7

Key: TCC, transitional cell carcinoma; HA, hyaluronic acid; HAsae, hyaluronidase.

*Sensitivity in all grades and stages of TCC.

** Denominator of fraction is the sum of G2 + G3

Table 5. Specificity of HA and HAase in case and control groups.

	HA test , no (%)	HAase test , no (%)	HA-HAase test , no (%)
TCC	(87/97) 89.7	(103/126)* 81.7	(81/97) 83.5
Normal	(18/19) 94.7	(18/19) 94.7	(18/19)94.7
History of TCC	(23/27) 85.2	(21/27) 77.8	(21/27) 77.8
BPH	(11/12) 91.7	(10/12) 83.3	(10/12) 83.3
Renal stone	(12/13) 92.3	(11/13) 84.6	(11/13)84.6
Other GU disease	(23/26) 88.5	(21/26) 80.8	(21/26) 80.8

Key: TCC, transitional cell carcinoma; HA, hyaluronic acid; HAsae, hyaluronidase; GU, genitourinary.

*Denominator of fraction is number of G1 control group.

Table 6. Sensitivity, specificity and accuracy of HA, HAase, HA-HAase tests on detecting TCC.

Category	HA test , no. (%)	HAase test , no. (%)	HA-HAase test , no. (%)
Sensitivity	82.5	88.2	89.7
Specificity	89.7	81.7	83.5
Accuracy	86.1	84	86.6

Key: TCC, transitional cell carcinoma; HA, hyaluronic acid; HAsae, hyaluronidase.

is consistent with our study. Lokeshwar and colleagues reported that urinary HA measurement has a sensitivity and specificity of 91.9% and 92.8% to detect bladder cancer, respectively.[18] Therefore, urinary HA measurement is a simple, non-invasive, yet a highly sensitive and specific method for detecting the bladder cancer.

CONCLUSION

Results of this study suggest that urinary levels of HA and HAase are very sensitive and specific as TCC markers. With over 89% sensitivity and 83% specificity, HA-HAase test would have a practical application for post-treatment surveillance prior to clinical diagnosis (ie, cystoscopy) and a "False-negative" result may signal a future recurrence. However, further studies are needed to replicate our results.

CONFLICT OF INTEREST

None declared.

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