

Bladder Cancer

Urinary Levels of Soluble E-Cadherin in the Detection of Transitional Cell Carcinoma of the Urinary Bladder

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Abstract

Objective: To test the hypothesis that elevated urinary levels of soluble E-cadherin (sE-cadherin) would aid in the detection of transitional cell carcinoma (TCC) of the urinary bladder.

Methods: We performed sE-cadherin staining of one murine (MBT2) and four human (RT4, 5637, T24, and TCCSUP) bladder cancer cell lines. sE-cadherin levels were also determined in voided urine of 188 consecutive subjects at risk for TCC recurrence, 31 patients with other uro-pathologic conditions, and 10 healthy subjects using a commercially-available ELISA kit. sE-cadherin was analyzed continuously and categorically on the basis of its median distribution.

Results: Moderately and poorly differentiated bladder cancer cell lines had decreased cellular E-cadherin expression, whereas RT4, a well differentiated cell line, had preserved expression. All cell lines had measurable sE-cadherin levels in their conditioned media. The area under the ROC curve of sE-cadherin for the detection of TCC was 0.719 (95%CI, 0.637–0.801; $p < 0.001$). Higher levels of sE-cadherin were associated with positive cytology results ($p = 0.012$) and muscle invasive tumor stage ($p = 0.009$). Urinary sE-cadherin was more sensitive, but less specific than urinary cytology for the detection of bladder TCC. In a multivariable logistic regression analysis, higher sE-cadherin and positive cytology were both associated with an increased risk of bladder TCC ($p = 0.048$ and $p < 0.001$, respectively). Combination of cytology and sE-cadherin allowed categorization of patients into three significantly different risk groups for bladder cancer. Adjustment of sE-cadherin for urinary creatinine levels did not affect any of the outcomes.

Conclusions: Urinary level of sE-cadherin may add information to cytology in the detection of bladder TCC.

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1. Introduction

E-cadherin is a calcium-dependent cell-to-cell adhesion molecule expressed exclusively by epithelia [1,2]. Loss of or decreased expression of E-cadherin in bladder cancer tissue has been associated with advanced stage, higher grade, metastasis and a higher probability of cancer progression and death [3–7]. sE-cadherin is detected as a proteolytic cleavage product of the E-cadherin peptide from the cell surface

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[8]. sE-cadherin is found in the circulation of normal individuals but it is particularly elevated in patients with various malignancies [9–12] including transitional cell carcinoma (TCC) of the urinary bladder.13,14 Banks and colleagues first reported the presence of sE-cadherin in urine.15 In a follow-up study they showed that urinary levels of sE-cadherin were elevated in bladder cancer patients compared to healthy controls and patients with non-neoplastic urologic diseases [16].

We sought to confirm that urinary levels of sE-cadherin may aid in the detection of bladder TCC. In order to demonstrate that sE-cadherin is released by bladder cancer cells, we first evaluated the expression of E-cadherin in five established, stable bladder cancer cell lines, and measured the levels of sE-cadherin in their conditioned media. Then, we assessed the association of urinary levels of sE-cadherin with bladder cancer presence and characteristics in a case-control study of 229 consecutive subjects with past history of Ta, T1, and/or CIS TCC stages.

2. Materials and methods

2.1. Bladder cancer cell lines and immunohistochemistry

We measured sE-cadherin in the conditioned media of four human and one murine bladder cancer cell lines. RT4, 5637, T24 and TCCSUP were obtained from American Type Culture Collection (Rockville, Maryland, USA) and the murine cell line MBT2 was a kind gift from Dr. Tim Ratliff (University of Iowa). All cell lines were maintained in the recommended growth medium. Cell blocks established for each cell line were fixed in buffered formalin at room temperature, and then dehydrated and paraffin embedded. Immunohistochemical staining and scoring methods have been described previously [3,4]. Briefly, the cell line slides were incubated with anti-E-cadherin monoclonal antibody (Transduction Labs) diluted 1:25 in blocking solution. Secondary antibody (Vector Labs) was applied at a dilution of 1:400 followed by avidin-biotin complex immunoperoxidase system using diaminobenzidine as the chromogen and methyl green and Alcian blue as the counterstain. The positive control was bladder tissue known to possess 100% preserved E-cadherin expression. The negative control was serial sections processed without incubation of the primary antibody. E-cadherin expression was classified as normal (90–100% positive cells with preserved cell border staining) and abnormal, which included negative (0–10% positive cells) and various degrees of heterogeneous expression (11–89% positive cells). All experiments were run in quintuplicate.

2.2. Patient population

All studies were performed after approval by a local Human Investigations Committee. Informed consent was obtained from each subject. The study comprised (1) 188 consecutive patients with history of bladder cancer presenting for surveillance cystoscopy; (2) 31 patients with other uro-pathologic conditions such as benign prostatic hyperplasia, urinary tract infection, urinary retention, incontinence, urolithiasis, and non-cancer related

hematuria; and (3) 10 healthy subjects. We collected a voided urine sample for measurement of sE-cadherin and creatinine prior to cystoscopy in all patients. We also collected bladder washout samples during cystoscopy for cytology in a subset of 191 subjects (93 had bladder cancer at cystoscopy and 98 did not have bladder cancer at cystoscopy). There were 153 (67%) males and 76 (33%) females, and the median age was 71.0 years (range 21 to 94). Overall, 122 patients (53%) were found to have a bladder tumor. The 107 patients without bladder tumor belonged to three different categories: patients with past history of bladder cancer but without tumor evidence at cystoscopy ($n = 66$), patients with urological pathology other than bladder malignancy, and healthy volunteers.

2.3. sE-cadherin measurements

We used a commercially-available quantitative ELISA assay (Takara Shuzo, Japan) for measurement of sE-cadherin levels in urine of the study subjects and the conditioned media of bladder cancer cell lines. Each cell line conditioned media was collected two days after last passage with 90–100% cell confluence. Samples were centrifuged and stored at -80°C . Before analysis, samples were slowly thawed and gently mixed. This assay is based on the capture of sE-cadherin using a solid-phase adsorbed monoclonal antibody followed by subsequent detection using a labeled second monoclonal antibody to E-cadherin. Every sample was run in duplicate and the mean calculated for data analysis. Differences between the two measurements were minimal, as shown by the mean intra-assay precision coefficient of variation (\pm standard deviation) of $7.3 \pm 6.9\%$.

2.4. Pathologic examination and cytology grading

All histologic slides were reviewed without knowledge of clinical data. Bladder tumors were staged according to the 1997 TNM classification and assigned a grade according to the WHO classification. Cytological findings were grade 0 (no atypical cells), 1 to 2 (low-grade atypia), and 3 (high-grade atypia). Only high-grade atypia was considered positive.

2.5. Urinary creatinine measurements

We measured the levels of creatinine (Olympus America Inc., Melville, NY) in the voided urine from 170 of the original 229 patients. All samples were run in duplicate and the mean utilized for data analysis. Differences between the two measurements were minimal, as shown by the intra-assay precision coefficient of variation of only $5.8 \pm 8.7\%$.

2.6. Statistical analysis

Spearman correlation coefficients were used to examine the correlation between sE-cadherin levels, creatinine levels, and patient age. sE-cadherin was analyzed either as continuous variable or categorically on the basis of its median distribution in the case and control subjects combined. The association between categorical data was tested using the Fisher's exact test. Differences in continuous variables between dichotomous categories were tested using the Mann-Whitney U test. Discordances between two related dichotomous variables were tested using the non-parametric McNemar test. Non-parametric receiver operating characteristics (ROC) curves in which the value for sensitivity is plotted against false positive rate (1-specificity) were generated. Areas under the curves (AUC) were compared using non-parametric Mann Whitney U -statistics [17]. Univariable and multivariable logistic regression models were used to calculate odds ratios and 95%CI. Age had a skewed distribution and therefore was modeled with a logarithm-

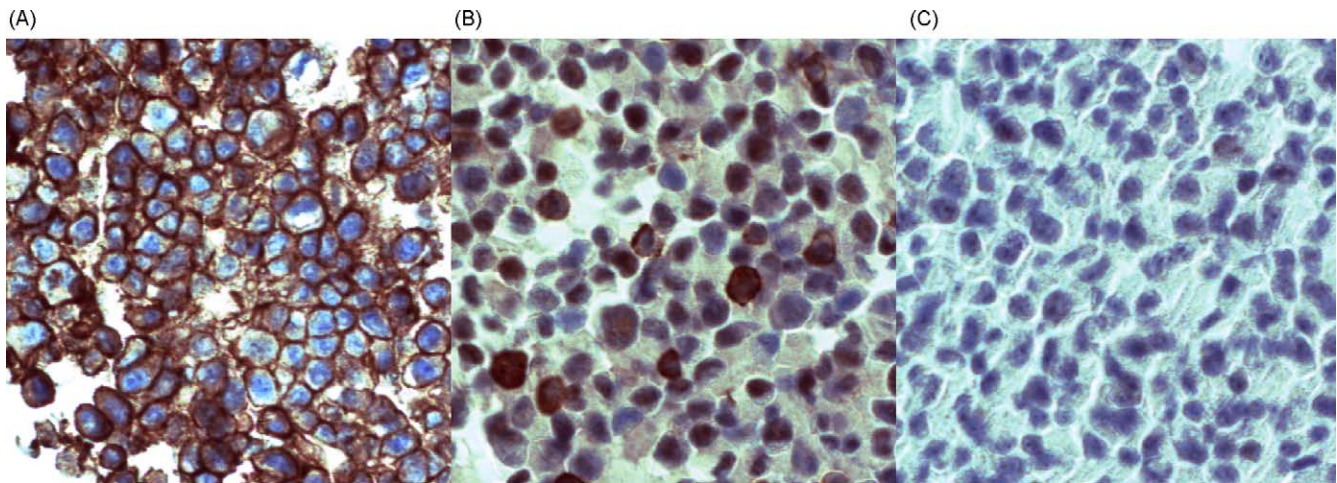


Fig. 1. E-cadherin immunohistochemical staining of bladder cancer cell lines with (A) preserved cell border expression (cell line RT4), (B) heterogeneous expression (cell line TCCSUP), and (C) absent expression (cell line 5637).

mical transformation. Tumor stage was stratified by Tis, Ta, and T1 versus T2 and above; tumor grade was stratified by grade 1 and 2 versus grade 3. Statistical significance in this study was set as $p < 0.05$ and all reported p values were two-sided. All analyses were performed with SPSS statistical package (SPSS version 11.0 for Windows).

3. Results

3.1. sE-cadherin expression in bladder cancer cell lines and release in conditioned media

RT4, a well differentiated cell line, was the only cell line to exhibit preserved E-cadherin expression (Fig. 1A). TCCSUP exhibited heterogeneous E-cad-

herin staining (Fig. 1B). T24, 5637, and MBT2 did not express E-cadherin (Fig. 1C). All cell lines had measurable sE-cadherin levels in their conditioned media.

3.2. Association of urinary levels of se-cadherin, cytology, and age with bladder cancer presence and characteristics

Association of urinary sE-cadherin, cytology, and age with cancer presence is shown in Table 1. sE-cadherin levels were higher in patients with bladder cancer than those in control subjects when analyzed as continuous variable and dichotomized variable split by the median ($p = 0.002$ and $p = 0.005$, respectively). Abnormal urinary cytology was associated with blad-

Table 1

Association of urinary levels of sE-cadherin and patient characteristics with transitional cell carcinoma of the urinary bladder

	No. Subjects (%)	Case Subjects ($n = 122$)	Control Subjects ($n = 107$)	p
Age (median, range)	229	73.1 (40.2–94.2)	69.9 (21.0–86.3)	$<0.001^\dagger$
Urinary creatinine (median, IQR; mg/dL)	170	66.0 (61.0)	65.1 (56.9)	0.691
Gender (No Pts, %)				
Female	76 (33)	34 (28)	42 (39)	
Male	153 (67)	88 (72)	65 (61)	0.091 ‡
Urinary cytology* (No Pts, %)				
Positive	60 (31)	50 (54)	10 (10)	
Negative	131 (69)	43 (46)	88 (90)	$<0.001^\ddagger$
Urinary sE-cadherin				
Continuous (median, IQR; ng/mL)	229	1606.9 (1826.9)	904.2 (1226.8)	0.002 †
Dichotomous (No Pts; %)				
Below median	114 (50)	50 (41)	64 (60)	
Above median	115 (50)	72 (59)	43 (40)	0.005 ‡

IQR = interquartile range.

* Wash urinary cytology was available in 191 patients only.

† Mann-Whitney U test.

‡ Fisher's exact test.

Table 2

Distribution of urinary levels of sE-cadherin across selected patient characteristics

	No. Pts (%)	s-Ecadherin				
		Continuous variable		Dichotomous variable		
		Median levels (IQR)	p^{\ddagger}	Below median (No. Pts, %)	Above median (No. Pts, %)	p^{\S}
Gender						
Female	76 (33)	756 (1511)		48 (63)	28 (37)	
Male	153 (67)	1606 (1768)	0.002	66 (43)	87 (57)	0.005
Cytology*						
Positive	60 (31)	1640 (2284)		23 (38)	37 (62)	
Negative	131 (69)	1028 (1150)	0.012	76 (58)	55 (42)	0.013
Pathologic Stage [†]						
Tis, Ta, or T1	104 (85)	1429 (1425)		48 (46)	56 (54)	
T2 or higher stage	18 (15)	2689 (1931)	0.009	2 (11)	16 (89)	0.008
Pathologic Grade [†]						
1 or 2	85 (70)	1532 (1611)		35 (41)	50 (59)	
3	37 (30)	1659 (8995)	0.948	15 (40)	22 (60)	1.000

IQR = interquartile range.
 * Wash urinary cytology was available in 191 patients only.
[†] Pathologic stage and grade were available in the 122 patients who underwent surgery for bladder cancer.
[‡] Fisher's exact test.
[§] Mann-Whitney *U* test.

der cancer ($p < 0.001$). Patients with bladder cancer were older than those without ($p < 0.001$).

Association of urinary levels of sE-cadherin analyzed as continuous variable and dichotomous variable split by the median with patient characteristics is shown in Table 2. Males had significantly higher sE-cadherin levels than females. Patients with positive urinary cytology assay results had significantly higher sE-cadherin levels than those with negative assay results. Bladder cancer patients with muscle-invasive disease had significantly higher sE-cadherin levels than those with Tis, Ta, or T1 tumor stage. All five patients with carcinoma *in situ* had urinary sE-cadherin levels above the median (data not shown). There was no correlation between urinary sE-cadherin levels and patients' age at time of specimen collection ($r = 0.173$, $p = 0.101$). There was no difference in age between patients with sE-cadherin levels above versus those with sE-cadherin levels below the median level ($p = 0.195$).

Age was higher in patients with positive cytology assay result than in those with negative assay result ($p = 0.023$). Abnormal wash urinary cytology was associated with both invasive tumor stage, (positive in 25 of the 64 Ta or Tis patients versus 25 of the 29 \geq T1 patients, $p < 0.001$) and higher tumor grade (positive in 27 of the 69 Grade 1 or 2 patients versus 23 of the 24 Grade 3 patients, $p < 0.001$). However, cytology was not associated with gender ($p = 0.736$).

Forty-three patients with bladder cancer had negative cytology. sE-cadherin levels were above the median in 31 (72%) of these patients.

3.3. Diagnostic performance of urinary sE-cadherin as continuous and dichotomous variable for prediction of bladder cancer presence

The ability of sE-cadherin levels to predict cystoscopic findings was analyzed using non-parametric ROC analyses. sE-cadherin was more accurate than guessing (null hypothesis; area under the null hypothesis = 0.5) for predicting the presence of bladder cancer in all patients ($p < 0.001$; Fig. 2) and in patients with negative cytology (AUC = 0.640, 95%CI, 0.568–0.712; $p = 0.003$).

In a multivariable logistic regression analysis (Table 3), higher sE-cadherin levels and positive cytology result were both significantly associated with an increased risk of bladder cancer presence after adjusting for the effect of age.

3.4. Diagnostic performance of urinary sE-cadherin and cytology as combined variable for prediction of bladder cancer presence

We combined the test results of urine cytology and sE-cadherin into two categories: both negative versus any positive. The sensitivity, specificity, PPV, NPV, and accuracy of the combined test results of cytology and sE-cadherin for the detection of bladder TCC are

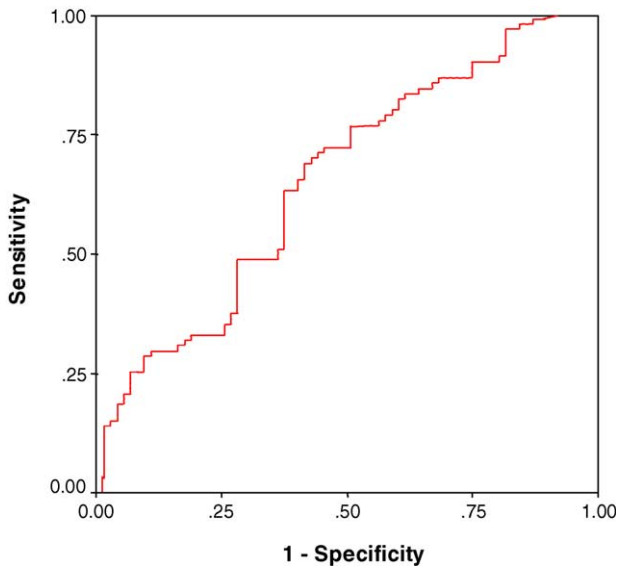


Fig. 2. Receiver operating characteristics curve for detection of bladder transitional cell carcinoma according to soluble E-cadherin levels measured in urine. Note: AUC (95%CI): 0.719 (0.637–0.801); sECD median: Sensitivity(71%); Specificity (65%).

shown in Table 4. The sensitivity of the combined cytology and sE-cadherin for the detection of bladder cancer was significantly higher than those of cytology or sE-cadherin alone (p -values < 0.001).

We then combined the test results of urine cytology and sE-cadherin split by the median into three categories: both negative *versus* one positive *versus* both positive. In multivariable logistic regression analyses (Table 3), the combined sE-cadherin and cytology variable was associated with an increased risk of bladder cancer when adjusted for the effect of age. Patients could be stratified into low, intermediate, and high risk for cancer based on the combined status of cytology and sE-cadherin.

3.5. Adjustment of sE-cadherin for urinary creatinine levels

The mean urinary level of creatinine level was 73.1 ± 46.4 mg/dL in all patients (median 65.2, range 9.8 to 236.1). Urinary sE-cadherin levels were weakly

Table 3

Multivariable logistic regression analyses of urinary levels of sE-cadherin, urinary cytology, and age for the prediction of transitional cell carcinoma of the bladder

	Odds ratio	95% CI	p
Model 1			
Age*	3.628	0.539–24.425	0.185
sE-cadherin ^{*,†}	1.283	1.008–1.633	0.048
Cytology	8.312	3.523–19.613	<0.001
Model 2			
Age*	2.998	0.487–18.458	0.236
sE-cadherin [†] and cytology combined			
Both negative	1.000	Referent	–
Either positive	2.812	1.396–5.664	0.004
Both positive	11.819	3.949–35.377	<0.001
Test for trend			<0.001

* Age and sE-cadherin had a skewed distribution and therefore were modeled with a logarithmical transformation.

[†] sE-cadherin was analyzed categorically on the basis of its median distribution in the case and control subjects combined.

but significantly correlated with urinary creatinine levels ($r = 0.315$, $p < 0.001$). Median urinary creatinine levels in patients with sE-cadherin levels above the median were higher than those in patients with sE-cadherin below the median (75.8, IQR 60.3 versus 51.9, IQR 58.3; $p < 0.001$). There was no difference in urinary creatinine levels between patients with positive versus negative cytology assay result ($p = 0.758$).

sE-cadherin/Creatinine ratio was analyzed either as continuous variable or categorically on the basis of its median distribution in the case and control subjects combined. We found that the sE-cadherin/Creatinine ratio was higher in patients with bladder tumor compared to controls ($p < 0.001$). Patients with sE-cadherin/Creatinine ratio above the median were more likely to have bladder cancer than those with sE-cadherin/Creatinine ratio below the median ($p < 0.001$). There was no correlation between age at time specimen collection and urinary creatinine

Table 4

Diagnostic performance of wash urinary cytology and urinary sE-cadherin for the detection of bladder cancer

	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Accuracy (%)
Cytology*	53.8	89.8	83.3	67.2	72.3
sE-cadherin [†]	71.3	65.0	67.2	69.2	68.1
Combined cytology [*] /sE-cadherin [†]	77.4	56.1	66.5	72.4	62.6

* Wash urinary cytology was available in 191 patients only.

[†] sE-cadherin was analyzed categorically on the basis of its median distribution in the case and control subjects combined.

levels ($r = -0.138$, $p = 0.078$). sE-cadherin/Creatinine ratios were higher in patients with positive urine cytology assay result than in those with negative assay result ($p = 0.009$). sE-cadherin/Creatinine (AUC = 0.646, 95%CI, 0.575–0.717) was more accurate than guessing ($p = 0.016$) but not different from sE-cadherin alone ($p = 0.982$) for predicting the presence of bladder cancer.

In a multivariate logistic regression model, higher urinary sE-cadherin/Creatinine ratio and positive cytology were associated with the presence of bladder cancer ($p = 0.041$ and $p < 0.001$, respectively), when adjusted for the effects of age.

4. Discussion

The sequence of events in cancer progression is characterized in part by the ability of a tumor cell to overcome cell-cell adhesion and to invade the surrounding tissue. E-cadherin is the primary mediator of calcium-dependent cell-cell adhesion in epithelial tissues [18,19]. Loss of E-cadherin mediated cell adhesion coincides with the transition from well-differentiated adenoma to invasive carcinoma supporting its role as a rate-limiting step in the progression to invasive disease [20]. In addition re-establishment of the functional cadherin complex in tumor cell lines resulted in suppression of invasiveness [21,22]. We found that moderately and poorly differentiated bladder cancer cell lines had decreased or absent cellular E-cadherin expression, whereas RT4, a well differentiated cell line, had preserved E-cadherin expression. However, sE-cadherin was measurable in the conditioned media of all bladder cancer cell lines.

We confirmed that urinary levels of sE-cadherin are significantly higher in bladder cancer patients compared to control subjects [15,16]. In addition, we found that higher urinary levels of sE-cadherin were associated with positive urinary cytology assay results and muscle invasive bladder cancer stage. Moreover, sE-cadherin was more sensitive but less specific than cytology for the detection of recurrent bladder cancer. Most importantly, we found that urinary sE-cadherin was an independent predictor of the presence of bladder cancer in a large set of consecutive patients. Since there is no biologically or statistically validated cut point for urinary sE-cadherin, we performed all analyses using sE-cadherin either as continuous variable or as categorical variable stratified on the basis of its median distribution in the case and control subjects combined. We found no differences in the statistical significance of any of the outcomes between using sE-

cadherin as continuous or categorical variable. We found that the combination of cytology and sE-cadherin stratified by the median allowed a more optimal categorization of patients who are likely (at high risk) or unlikely (at low risk) to have bladder cancer. However, while increasing the sensitivity for detection of bladder cancer, the combination of sE-cadherin with cytology reduced the specificity and resulted in an increased number of false positive results. Finally, we adjusted sE-cadherin for the effect of urine concentration by dividing urine levels of sE-cadherin by urine levels of creatinine. We found that adjustment for creatinine levels did not affect any of the statistically significance of any of the outcomes.

Higher urinary levels of sE-cadherin were associated with presence of bladder TCC after adjustment for the effects of urine cytology and patient age. Seventy two percent of the 43 bladder cancer patients who had a negative cytology had urinary sE-cadherin levels above the median. Although urinary cytology has excellent sensitivity and specificity in bladder cancer patients with high grade lesions, it has low sensitivity for the detection of intermediate and low grade tumors. In our study, an expert cytopathologist examined all washout cytologies and considered only high grade disease as positive. This taken together with the independent predictive value of sE-cadherin for bladder cancer suggests that the additional evaluation of urine sE-cadherin may help improve bladder cancer detection over cytology alone.

Interestingly, we found that all our five patients with CIS had urine sE-cadherin levels above the median. CIS is associated with the loss of intercellular cohesiveness causing cells or clusters of cells to detach from the surface epithelium, as reflected by the high incidence of positive urine cytology in patients with CIS of the bladder. In addition, E-cadherin plays an essential role in maintaining cell adhesion in epithelium [23]. We have previously shown that tissue expression of E-cadherin is decreased or absent in 32% of patients with CIS [3]. While sE-cadherin is considered to be the degradation product of intact E-cadherin from the cell surface generated by proteolytic movement, [8] the small sample size of patients with CIS in our study limits conclusions on a possible association between urinary sE-cadherin and CIS.

We found that urinary sE-cadherin levels were higher in males compared to female subjects. To our knowledge, there are no studies showing any association between gender and local or circulating E-cadherin levels. In addition, E-cadherin expression has not been reported to be regulated by sex hormones. In absence of a clear explanation for this

association, it could be that E-cadherin expression is related to the male preponderance of patients who had cancer or this association may simply be unique to this particular patient population.

Various sources may contribute to the total level of sE-cadherin found in the urine of patients with bladder TCC. In addition to direct release from cells in the urinary epithelium, the urine levels of sE-cadherin may result from blood levels filtered through the kidney. We previously found that plasma levels of sE-cadherin were significantly higher in bladder cancer patients than in healthy controls [13]. In addition, within bladder cancer patients undergoing radical cystectomy, preoperative plasma sE-cadherin levels were independently associated with an increased risk of metastases to lymph nodes and of disease progression. However, Griffiths et al. did not find an association between decreased tissue expression of E-cadherin and elevated serum levels of sE-cadherin [14]. In addition, the glomerular capillary wall functions as a filter that allows the passage of small molecules but almost completely restricts the passage of molecules larger than albumin. Molecular radii less than about 2 nm are filtered as freely as inulin (5 kD, molecular radius 1.4 nm), whereas molecules larger than 5 nm are normally excluded from filtration. Since sE-cadherin has a molecular weight of 80 kD and sE-cadherin in urine is not fragmented, it is unlikely that urinary sE-cadherin is filtered by the kidney. This together with the findings that bladder urothelium expresses E-cadherin and that these levels are associated with poor outcome in patients with TCC suggests that the sE-cadherin in urine originates from and reflects the biology of the urinary epithelium [3–7]. A better understanding of the biological mechanisms underlying the altered levels of circulating, urinary, and tissue levels of sE-cadherin in patients with bladder TCC would potentially lead to more effective clinical management as well as provide new target pathways for therapy in these patients.

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We confirmed the strong independent value of urinary cytology for bladder cancer detection and staging. The high sensitivity of cytology in our study is due to the fact that all urine samples were evaluated by a single expert cytopathologist [24]. In addition, we used bladder washout specimens, which have been shown to yield a higher sensitivity than voided urine specimens. In contrast to the high sensitivity, the specificity of cytology in our study was lower than that reported previously. All ten patients who had a presumed false positive urinary cytology had a past history of bladder cancer. Five of the ten patients underwent multiple random biopsies of the bladder without evidence of cancer and remained tumor-free at follow-up. These patients remain at high risk for TCC and must be aggressively followed, and upper tract tumors and prostate urethra CIS must be ruled out. Recent data from the Southwest Oncology Group trial of maintenance BCG demonstrates that cytology may convert to normal up to six months or longer after a single induction course of BCG [25]. Two of the five patients that were not biopsied later developed bladder cancer. The first patient developed Ta grade 3 TCC two years later, and the second patient developed a Ta grade 1 TCC only seven months after inclusion in the study. Interestingly, his urine was positive for both cytology and sE-cadherin supporting the potential role of these urine markers for predicting the subsequent occurrence of bladder cancer. Possibly, this patient may also represent a “false negative” of cystoscopy. Although considered as the gold standard for diagnosis, cystoscopy has a false negative rate up to 20%, due either to operator error or to small areas of carcinoma *in situ*, which may be difficult to detect [26].

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