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European Association of Urology

Bladder Cancer

CEACAM1: A Novel Urinary Marker for Bladder Cancer Detection

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Article info

Article history:
Accepted May 15, 2009
Published online ahead of print on May 28, 2009

Keywords:
Bladder cancer
Tumor markers
Urine
Angiogenesis

Abstract

Background: Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein; CEACAM1) is expressed in normal bladder urothelium and in angiogenically activated endothelial cells, where it exhibits proangiogenic properties.
Objective: The aim of this study was to evaluate the value of urinary CEACAM1 for detection of urothelial carcinoma of the bladder (UCB).
Design, setting, and participants: This prospective study included 175 patients.
Measurements: Immunohistochemistry for CEACAM1 was performed on UCB sections of 10 patients. Enzyme-linked immunosorbent assay (ELISA) for CEACAM1 was performed on urine specimens of healthy volunteers ($n = 30$), patients with benign prostatic hyperplasia (BPH; $n = 5$), severe cystitis ($n = 5$), non–muscle-invasive UCB ($n = 72$), muscle-invasive UCB ($n = 21$), or past history of UCB without evidence of disease ($n = 42$). Western blot analysis was performed on a subgroup of these subjects ($n = 53$).
Results and limitations: CEACAM1 immunostaining in normal urothelium disappears in non-invasive UCB but appears in endothelial cells of adjacent vessels. Western blotting revealed presence of CEACAM1 in the urine of no healthy volunteers, of 76% of noninvasive UCB patients, and of 100% of invasive UCB patients. ELISA analysis confirmed that urinary CEACAM1 levels were significantly higher in UCB patients compared with control subjects (median: 207 ng/ml vs 0 ng/ml; $p < 0.001$). The area under the curve for UCB detection was 0.870 (95% confidence interval [CI]: 0.810–0.931). In multivariable logistic regression analyses that adjusted for the effects of age and gender, higher CEACAM1 levels were associated with cancer presence (hazard ratio [HR]: 2.89; 95% CI: 2.01–4.15; $p < 0.001$) and muscle-invasive cancer (HR: 5.53; 95% CI: 1.68–18.24; $p = 0.005$). The cut-off level of 110 ng/ml yielded sensitivity of 74% and specificity of 95% for detecting UCB. Sensitivity was 88% for detecting high-grade UCB and 100% for detecting invasive-stage UCB. Larger studies are necessary to establish the diagnostic and prognostic roles of this highly promising novel marker in UCB.
Conclusions: Urinary CEACAM1 levels discriminate UCB patients from non-UCB subjects. Moreover, urinary levels of CEACAM1 increased with advancing stage and grade.

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0302-2838/$ – see back matter doi:10.1016/j.eururo.2009.05.040

1. Introduction

Urothelial carcinoma of the bladder (UCB), the fourth most common cancer in men and the ninth most common cancer in women, results in significant morbidity and mortality [1]. At initial diagnosis, about 70% of patients have cancers confined to the epithelium or subepithelial connective tissue. These cancers are usually managed with endoscopic resection and selective use of intravesical therapy. The recurrence rate for these tumors ranges from 50% to 70%, and 10–15% progress to muscle invasion over a 5-yr period [2,3]. This high rate of disease recurrence requires lifelong surveillance, consisting of serial cystoscopy and cytology. These tests are invasive and expensive, with considerable interuser and interinstitutional variability [4–6]. Additionally, although urine cytology has a reasonable sensitivity for the detection of high-grade UCB, it lacks sensitivity to detect low-grade tumors [7]. Therefore, there is a need for new urine markers that may help in UCB detection and surveillance [8,9].

Tumor growth and progression depend on angiogenesis, which is regulated by angiogenic activators and inhibitors [10,11]. In the intricate angiogenesis system, cell adhesion molecules play an important role in vascular morphogenesis and endothelial signaling [12]. Human carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein; CEACAM1) is such a molecule with proangiogenic activity [13]. We have previously observed that CEACAM1, which is ubiquitously expressed in the luminal surface of normal bladder urothelium, is downregulated in bladder cancer cells while it is concurrently upregulated in endothelial cells of adjacent blood vessels [14]. This differential switch in CEACAM1 expression is accompanied by an upregulation of proangiogenic and prolymphangiogenic factors [15–17]. Interestingly, the upregulation of CEACAM1 during this angiogenic activation of endothelial cells is detectable in both the endothelial cells in a membrane-bound form and in the supernatant of these cells [13]. This presence suggests that soluble CEACAM1 forms might be released into body fluids during angiogenic activation. Based on these findings, the aims of this study were to assess whether CEACAM1 is detectable in urine and whether its level could help differentiate bladder cancer patients from healthy subjects.

2. Patients and methods

2.1. Patient population

We obtained approval of the institutional review board of the University Hospital Grosshadern of Ludwig-Maximilians-University Munich. All participants gave written informed consent. Voided urine samples from 175 subjects were collected prospectively in the morning and frozen at −20 °C within 30 min until their analysis. The urine samples were obtained from 30 healthy volunteers, 5 subjects with biopsy-proven benign prostatic hyperplasia (BPH), 5 subjects with severe cystitis, 42 subjects with a history of non–muscle-invasive UCB but without current disease, and 93 patients with actual UCB (17 with pTis, 43 with pTa, 12 with pT1, and 21 patients with pT2–pT4). To exclude renal insufficiency as a reason for CEACAM1 in the urine, no patients with diabetes mellitus or renal insufficiency were included in this study.

2.2. Immunohistochemistry

Paraffin-embedded tissue blocks of human urinary bladder ($n = 10$) were obtained from the Department of Pathology of Ludwig-Maximilians-University Munich. Normal urothelium and primary cancer areas from each patient with non–muscle-invasive ($n = 8$) and muscle-invasive tumors ($n = 2$) were stained according to a previously published protocol [15,18]. Briefly, we used mouse monoclonal antibody (mAb) 4D1/C2 against CEACAM1 (50 ng/ml). Immunostaining was visualized by a modified nickel-enhanced glucose oxidase and peroxidase technique, and sections were counterstained with Calcium Red.

2.3. Western blot analysis

Voided urine samples of a subgroup of the patients (10 healthy volunteers, 3 subjects with biopsy-proven BPH, 5 with severe cystitis, 10 with a history of non–muscle-invasive UCB but without current disease, and 25 with UCB) were analyzed using 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under nonreduced conditions. Volume of loaded sample was 20 μl of the urine sample with 5 μl of 5x Laemmli's loading buffer. CEACAM1 isolated from human granulocytes was used as the positive control [13]. Proteins were transferred to a nitrocellulose membrane (Schleicher & Schell, Germany) and incubated with the antibodies 4D1/C2 and T84.1. Subsequently, the membrane was exposed to the secondary antibody (goat antimouse IgG; Sigma, Germany) conjugated with horseradish peroxidase. The antigen–antibody complex was detected by enhanced chemiluminescence using ECL reagents (Amersham-Pharmacia, Freiburg, Germany) and visualized by autoradiography. The x-ray films were digitized for further use.

2.4. Sandwich enzyme-linked immunosorbent assay for CEACAM1

We measured CEACAM1 levels in all 175 voided urine specimens. Maxisorp enzyme-linked immunosorbent assay (ELISA) immunoplates (Nunc, Wiesbaden, Germany) were coated with 50 μl of 5 μg/ml polyclonal rabbit antihuman carcinoembryonic antigen (CEA; Dako, Glostrup, Denmark; antibody specifically binds to CEACAM1, CEACAM3, CEACAM4, CEACAM5, CEACAM6, CEACAM7, and CEACAM8). Remaining binding sites were blocked with 5% bovine serum albumin (BSA)–phosphate buffered saline (PBS) solution. Plates were then incubated with 50 μl urine diluted with 50 μl PBS containing 3% BSA. The standards were prepared using purified native CEACAM1-Fc protein in different concentrations [19]. Bound CEACAM1 was labeled by incubating overnight with 100 μl mouse mAb anti-CEACAM1 (clone 4D1C2). Plates were washed and incubated with peroxidase-coupled AffiniPure F(ab′)2 fragment goat antimouse antibody (Jackson ImmunoResearch, West Grove, PA, USA). The enzyme reaction was visualized using 150 μl tetramethylbenzidine (Sigma) as substrate. Absorbance was detected at 450 nm in a Sunrise ELISA reader (Tecan, Männedorf, Switzerland). Samples were measured in triplicate. The reproducibility of our ELISA was affirmed by measuring linearity, intrarun precision, interrun precision, analytical sensitivity, dilution verification, and reference range.

2.5. Statistical analysis

The associations between categorical data were tested using the Fisher exact test or the χ^2 test. Spearman rank correlation coefficients were used to examine the correlations between continuous variables. Differences in continuous variables across categorical variables were

tested using the Mann-Whitney test or the Kruskal-Wallis test. Nonparametric receiver operating characteristic (ROC) curves in which the value for sensitivity is plotted against false-positive rate (1-specificity) were generated. Univariable and multivariable logistic regression analyses were used to calculate odds ratios and 95% confidence interval (CI). CEACAM1 had a skewed distribution and therefore was modeled with a logarithmic transformation for logistic regression analyses. Statistical significance in this study was set at $p < 0.05$, and all reported p values were 2-sided. All analyses were performed with SPSS v.16 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. CEACAM1 expression shifts from normal urothelium to endothelial cells of newly formed blood vessels

In line with our previously published findings, immunohistochemical analyses of normal bladder tissue revealed that CEACAM1 was expressed in umbrella cells of normal urothelium (Fig. 1A). There was no CEACAM1 staining in blood vessels. Conversely, in nearly all noninvasive UCB cases, urothelial CEACAM1 expression disappeared (Fig. 1B), while CEACAM1 appeared in tumor-associated blood vessels (Fig. 1C). The proportion of CEACAM1 expression in tumor-associated blood vessels increased with advancing tumor stage, with all patients with invasive UCB exhibiting CEACAM1 expression in tumor-associated blood vessels (Figs. 1D and 2).

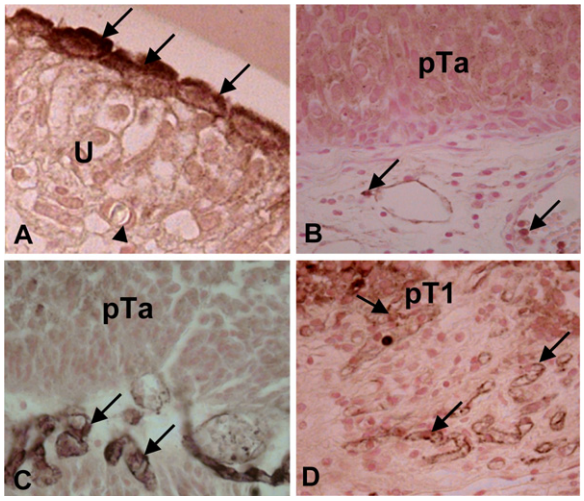


Fig. 1 – Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein; CEACAM1) immunostaining in human bladder tissue. (A) In normal bladder urothelium (U), only umbrella cells (arrows) are positive for CEACAM1, while blood vessels within the lamina propria (arrowhead) are negative for CEACAM1. (B) In a part of pTa tumors, only granulocytes (arrows) within the adjacent blood vessels exhibit CEACAM1 staining, while (C) in another part of pTa, endothelial cells of adjacent blood vessels (arrows) are now positive for CEACAM1. (D) In pT1 tumors in which tumor cells invade the lamina propria, small blood vessels (arrows) are positive for CEACAM1. All of the sections are counterstained with Calcium Red (×450).

3.2. CEACAM1 in voided urine samples using western blot analysis

To assess whether soluble CEACAM1 is secreted into the urine of UCB patients, we performed western blot analysis. Urinary CEACAM1 was undetectable in urine samples from healthy volunteers or subjects with BPH. Two of five patients with severe cystitis exhibited CEACAM1 expression. They expressed a pattern of bands, except the 75-kilodalton (kDa) band. The related urothelium in these two patients was classified as severe erosive cystitis and partly showed reactive changes, which were hard to discern from dysplasia (flat neoplasia) (Fig. 3C). All patients with invasive UCB (≥pT1 UCB) exhibited detectable urinary CEACAM1 bands at 120, 75, 50, and 24 kDa (Fig. 3A and B). In contrast, only a part of urine samples (76%) of early noninvasive tumor stages such as pTa were positive for CEACAM1 while others were negative (Fig. 3A and B). Two patients with a history of UCB but without current disease exhibited CEACAM1-positive urine. Both of these patients had histologically confirmed tumor recurrence (pTa) after 3 months.

We confirmed the pattern of bands detected using the 4D1/C2 antibody with the T84.1 antibody, which recognizes CEA and CEACAM1, with the exception of an additional band at 180 kDa corresponding to CEA (data not shown).

3.3. CEACAM1 in voided urine samples using enzyme-linked immunosorbent assay analysis

Urinary CEACAM1 levels were undetectable in the large majority of patients without UCB (Fig. 4A). Median urinary CEACAM1 levels were significantly higher in patients with

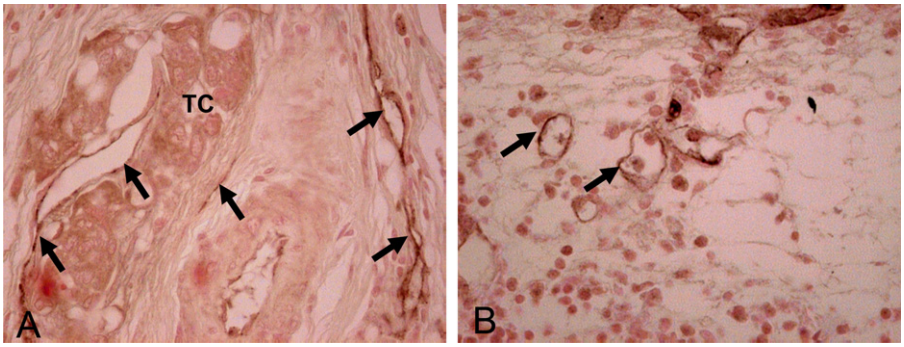


Fig. 2 – Immunostaining for carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein; CEACAM1) in muscle-invasive bladder tumors. (A) Clear staining for CEACAM1 is visible in endothelial cells of tumor vessels. (B) Small vessels in stroma surrounding the tumor cell cluster are positive for CEACAM1. Sections are counterstained with Calcium Red (×450).

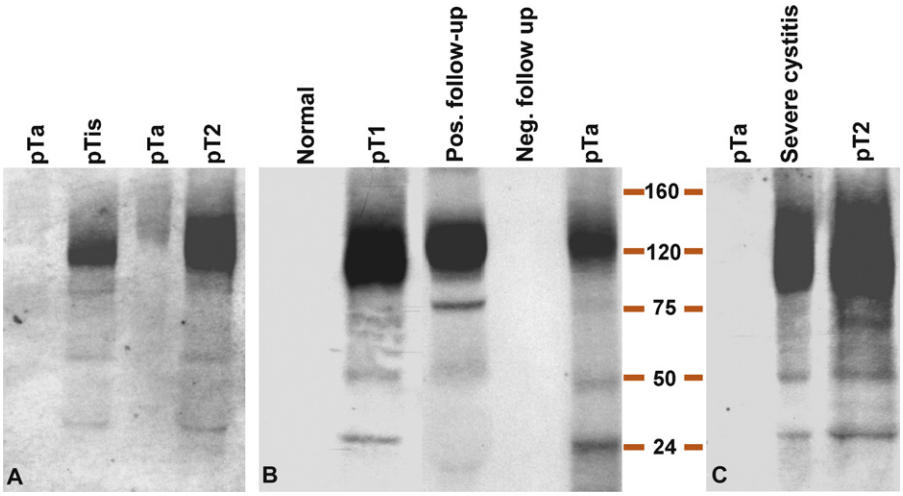
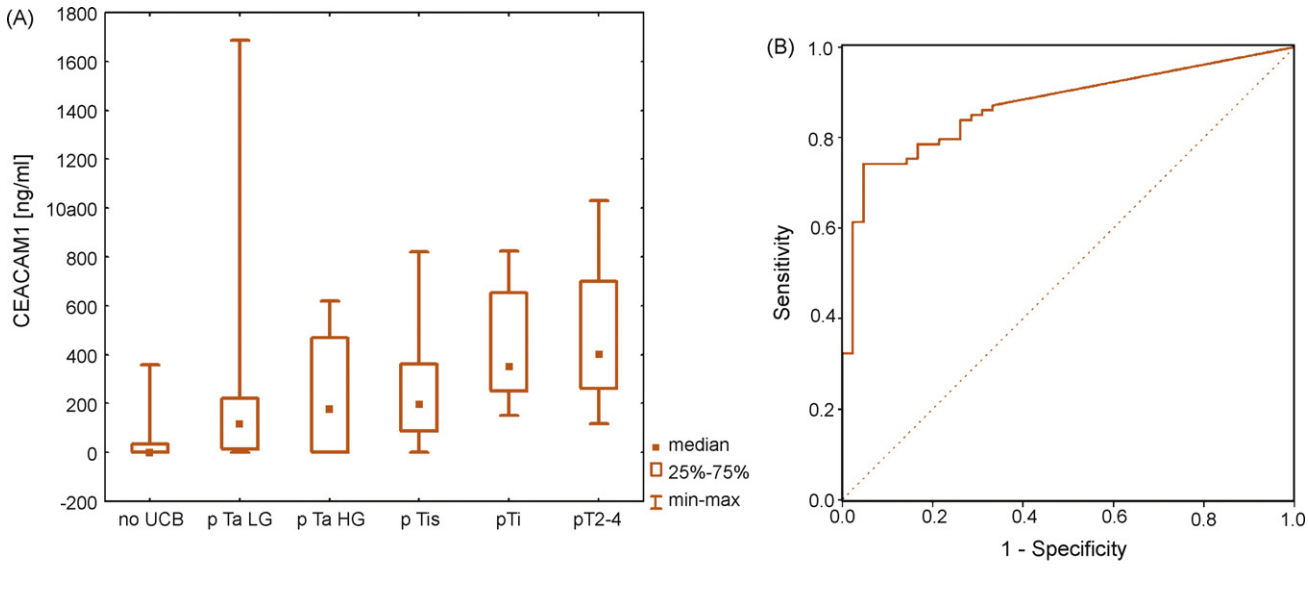


Fig. 3 – Western blot analyses of urine samples of 53 subjects. (A–C) Urine samples of all healthy subjects and all cases of benign prostate hyperplasia are negative for carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein; CEACAM1); in all tumor cases classified as pT1–pT4, the soluble CEACAM1 forms at the sizes of 120 kilodaltons (kDa) and 50 kDa are found. Additionally, two new bands of 75 kDa and 24 kDa are detected. (A, B) Only a part of urine samples from early noninvasive tumor stages such as pTa are positive for CEACAM1, mostly the 120-kDa form. (B) In two cases with a history of urothelial carcinoma of the bladder but without current disease, CEACAM1 is found. (C) Western blot analyses reveal the soluble CEACAM1 forms in urine samples of two cases of severe erosive cystitis, except the 75-kDa band.



(C)

	CEACAM1 <110	CEACAM1 >110	
UCB	24	69	93
No UCB	40	2	42
Total	64	71	135

X^2 test: $p < 0.0001$
Sensitivity (69/93): 74%
Specificity (40/42): 95%
PPV (69/71): 97%
NPV (40/64): 63%

Fig. 4 – Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein; CEACAM1) level in urine samples of patients with nonmalignant bladder tissue and of patients with histologically confirmed urothelial carcinoma of the bladder (UCB). (A) The median expression is shown (square). (B) Diagnostic efficacy of the enzyme-linked immunosorbent assay for urinary CEACAM1 in the absence of an arbitrary cut-off value was visualized using a receiver operating characteristic (ROC) curve. Based on this ROC, a cut-off of 110 ng/ml was determined, reaching an overall sensitivity for CEACAM1 of 74% and an overall specificity of 95% (χ^2: $p < 0.001$). (C) The positive predictive value (PPV) was 97%, and the negative predictive value (NPV) was 63%.

UCB compared to those without (0 ng/ml vs 207 ng/ml; $p < 0.001$). Among UCB patients, urinary CEACAM1 levels were higher in patients with invasive disease compared with noninvasive disease (median: 400 ng/ml vs 188 ng/ml; $p < 0.001$). The ability of urinary levels of CEACAM1 to predict UCB presence was analyzed using nonparametric ROC analyses. Urinary CEACAM1 was more accurate than guessing for predicting UCB presence (area under the curve: 0.87; 95% CI: 0.81–0.93; $p < 0.001$; Fig. 4B). In multivariable logistic regression analysis that adjusted for the effects

Table 1 – Patient characteristics and carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein; CEACAM1) detection in urine*

Pathologic T stage	No. of patients: CEACAM1 negative	No. of patients: CEACAM1 positive	Total
pTa	19	24	43
pTis	5	12	17
pT1	0	12	12
pT2	0	8	8
pT3	0	7	7
pT4	0	6	6
Grade			
Low grade	17	17	34
High grade	7	52	59
Total	24	69	93
Controls			
No tumor with history of bladder cancer	40	2	42
Healthy volunteers	29	1	30
Severe cystitis	5	0	5
Benign prostatic hyperplasia	5	0	5

* CEACAM1 was considered positive above a cut-off value of 110 ng/ml.

of age (HR: 1.01; 95% CI: 0.97–1.05; $p = 0.638$) and gender (HR: 1.23; 95% CI: 0.51–2.96; $p = 0.644$), higher CEACAM1 levels were associated with cancer presence (HR: 2.89; 95% CI: 2.01–4.15; $p < 0.001$). Using the cut-off level of 110 ng/ml, urinary CEACAM1 had a sensitivity of 74%, a specificity of 95%, a positive predictive value of 97%, and a negative predictive value of 63% (Fig. 4C). Two of the 42 patients with a history of non–muscle-invasive UCB but without current disease showed a positive CEACAM1 test when using this cut-off level. Interestingly, these patients were the same two who were CEACAM1 positive in western blot analyses and showed tumor recurrence in cystoscopy after 3 months.

In multivariable logistic regression analysis that adjusted for the effects of age (HR: 0.97; 95% CI: 0.92–1.02; $p = 0.224$) and gender (HR: 0.34; 95% CI: 0.11–1.03; $p = 0.056$), higher CEACAM1 levels were associated with muscle-invasive cancer (HR: 5.53; 95% CI: 1.68–18.24; $p = 0.005$). Using the cut-off level of 110 ng/ml, urinary CEACAM1 had a sensitivity of 88% (52 of 59 patients) for detecting high-grade UCB (Table 1). CEACAM1 levels were detectable in the urine of 56% (24 of 43) of patients with pTa UCB versus 83% (24 of 29) of patients with pT1 UCB or pTis ($p = 0.017$).

4. Discussion

We confirmed the loss of expression of CEACAM1 in UCB tissue compared with normal urothelium and concomitant gain in CEACAM1 expression in tumor-associated vasculature. This finding led us to hypothesize that CEACAM1 may be detectable in urine of patients with UCB. First, using western blot analyses, we demonstrated that CEACAM1 forms are present in the urine of UCB patients. Then we constructed an ELISA assay for CEACAM1 and measured CEACAM1 levels in voided urine specimens from patients with UCB and control subjects without cancer, with common nonmalignant urologic pathologies, or with past history of UCB without present disease. We found that CEACAM1 forms can be detected in human urine. More important, higher urinary levels of CEACAM1 were associated with UCB presence and advanced stage.

Epithelial CEACAM1 expression is downregulated in several tumors such as colon, breast, and prostate cancers [20–22]. This information led to the postulation that CEACAM1 acts as tumor suppressor [23–25]. Later it was shown that CEACAM1 acts proangiogenically [13,16] and potentiates vascular endothelial growth factor–induced angiogenesis [13]. Epithelial CEACAM1 downregulation, however, increases the expression of proangiogenic factors in bladder and prostate cancers [14,15,26,27]. These data suggest that the tumor-suppressor role previously attributed to CEACAM1 is probably mediated through suppression of proangiogenic factors by its epithelial presence. Furthermore, soluble CEACAM1 forms were reported after its overexpression in endothelial cells [13,16]. Recently, serum levels of CEACAM1 were shown to be elevated in patients with pancreatic cancer [28]. To date, however, no studies have evaluated the presence of CEACAM1 in body fluids of bladder cancer patients.

Using western blot analyses, we demonstrated that CEACAM1 forms can be detected in human urine. While the bands at $\leq$75 kDa probably represent soluble CEACAM1 forms, the 120-kDa band might still represent membrane-bound CEACAM1 in exosomes or membrane vesicles secreted into the urine. Additional studies are needed to clarify the generation and secretion mechanisms of urine CEACAM1 forms. For a test to be clinically useful, it needs to be performed on a high-throughput platform such as an ELISA. Therefore, we set out to develop an ELISA assay for CEACAM1.

We found that higher urinary levels of CEACAM1 were associated with increased risk of UCB presence. Within UCB patients, higher CEACAM1 levels were associated with invasive tumor stage. Using ROC analyses, we attempted to identify a biologic and clinically relevant cut-off point for UCB detection. Using the cut-off point of 110 ng/ml, we found a sensitivity of 74% and a specificity of 95%. These performance characteristics, especially the high specificity, are above those reported for currently used urinary markers for UCB detection such as BTA stat (Polymedco, Cortlandt Manor, NY, USA), BTA TRAK (Polymedco), NMP22 (Inverness Medical Innovations, Waltham, MA, USA), urine fibrin/fibrinogen degradation product (FDP), ImmunoCyt (Scimedx, Denville, NJ, USA), and fluorescence in situ hybridization (FISH; UroVysion, Abbott Molecular, Des Plaines, IL, USA). These markers generally possess higher sensitivity and lower specificity than urinary cytology. Among these, FISH (a complex and expensive test), BTA TRAK, and NMP22 had the highest sensitivity, at 79%, 71%, and 71%, respectively. ImmunoCyt, a test based on the visualization of tumor-associated antigen leading to high interobserver variability, had the highest specificity (75%) of the tests approved by the US Food and Drug Administration. BTA stat and NMP22 showed false-positive results in benign inflammatory

conditions in >25% of cases [7,8]. Nevertheless, final conclusions on superiority of our assay to other tests cannot be drawn until larger studies have been conducted. Our assay remains experimental, requiring further validation of its sensitivity, sensibility, reliability, accuracy, and standardization.

Urine levels of CEACAM1 were measurable in only a limited proportion of noninvasive UCB patients. This limitation may be explained by lack of angiogenic activation in early stage UCB. This theory is further supported by our finding showing that the percentage of CEACAM1-positive urine samples in ELISA analyses is significantly higher in patients with bladder cancer stage pT1 or pTis compared with pTa. One of the critical parameters of tumor transition from stage pTa to pT1 is the activation of angiogenesis with onset of tumor vascularization. Moreover, urinary CEACAM1 levels were higher in patients with high-grade UCB. The expression of CEACAM1 forms in western blot analyses in two patients with severe erosive cystitis is most likely a reflection of destruction of the bladder wall and neovascularization.

Two patients with UCB history and without apparent tumor but with tumor recurrence 3 months later had false-positive tests in both western blot and ELISA analyses. These results might imply that these patients possibly had a tumor at the time of urine analysis that was missed in initial cystoscopy. Nevertheless, this theory cannot be verified retrospectively.

Varying sources of CEACAM1 production can contribute to its level found in urine. The differential contribution from blood levels of CEACAM1 passing the renal filter and from direct release from cells in the urinary epithelium remains to be elucidated. The glomerular capillary wall functions as a filter that allows passage of small molecules but almost completely restricts passage of large molecules such as albumin (66 kDa). The full length CEACAM1 has an estimated weight of 150 kDa, but, to our knowledge, its glomerular permeability has not been evaluated. Indirect evidence, however, suggests that serum CEACAM1 is too large to be filtered by the kidney. In immunohistochemical analyses, the expression of CEACAM1 in tumor cells of the stages pTa, pTis, and pT1 disappeared, but CEACAM1 was expressed on small tumor blood vessels. Thus, we assume that the activated or newly formed tumor vessels or the interaction between tumor vessels and tumor cells serve as a basis for the generation of soluble CEACAM1 forms in the urine samples.

Our study has important limitations. First, despite an acceptable linearity over the concentration ranges expected clinically, the CEACAM1 ELISA assay used in the current analysis did not comply with all validation criteria appropriate for analytic techniques. Prior to use as bladder cancer marker, the sample acquisition protocols and the CEACAM1 assay need to be further refined and standardized. Moreover, we did not directly compare the performance of CEACAM1 to that of currently used urine-based markers for bladder cancer surveillance such as cytology. As part of a phased approach to biomarker discovery and validation, we are in the process of testing the value of CEACAM1 in a prospective study, comparing it with the performance of cytology and FISH [29]. Additionally, prior to introduction of CEACAM1 in patient care, the findings of our study need to be confirmed in large, prospective, multicenter collaborative phase 2 and 3 trials. Toward this end, we have previously proposed clinical guidelines and protocols to ensure a systematic and critical evaluation of new biomarkers by multidisciplinary groups of experts [30].

5. Conclusions

Our data suggest that higher urinary levels of CEACAM1 are associated with UCB presence and invasive stage. Larger studies are needed to determine the potential role (prognostic, predictive, target for therapy) of urinary CEACAM1 in the management of patients who are at risk for UCB.

Author contributions: Derya Tilki had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Tilki, Ergün.
Acquisition of data: Tilki, Singer, Behrend, Irmak, Fernando.
Analysis and interpretation of data: Tilki, Ergün, Reich, Hooper.
Drafting of the manuscript: Tilki, Ergün, Reich, Shariat.
Critical revision of the manuscript for important intellectual content: Stief, Hooper.
Statistical analysis: Buchner, Tilki, Shariat.
Obtaining funding: Tilki, Ergün.
Administrative, technical, or material support: Tilki.
Supervision: Ergün.
Other (specify): None.

Financial disclosures: I certify that all conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject matter or materials discussed in the manuscript (eg, employment/affiliation, grants or funding, consultancies, honoraria, stock ownership or options, expert testimony, royalties, or patents filed, received, or pending), are the following: None.

Funding/Support and role of the sponsor: This work was supported by a scientific grant from Else Kröner-Fresenius-Stiftung.

Acknowledgment statement: The authors are grateful to Mrs. B. Maranca-Hüwel for excellent technical assistance and thank Professor C. Wagener (Department of Clinical Chemistry, University Hospital Hamburg-Eppendorf, Germany) for providing the CEACAM1 antibodies 4D1/C2 and T84.1. Parts of this study were performed within the doctoral thesis of Andreas Behrend at the medical faculty of Ludwig-Maximilians-University Munich, which is in preparation.

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