ORIGINAL ARTICLE

Urinary BLCA-4 level is useful to detect upper urinary tract urothelial cell carcinoma

C.-C. Fenga,*, Z. Wua, H.-W. Jianga, H. Wena, M. Guana, Q. Dinga

a Servicio de Urología, Hospital Huashan, Universidad Fudan, Shanghai, China
b Centro de Medicina de Laboratorio, Hospital Huashan, Universidad Fudan, Shanghai, China

Received 1 March 2012; accepted 5 March 2012
Available online 1 March 2013

KEYWORDS
Upper urinary tract; Urothelial carcinoma; BLCA-4

PALABRAS CLAVE
Vías urinarias superiores; Carcinoma urotelial; BLCA-4

Abstract
Objective: Upper urinary tract urothelial cell carcinomas (UUT-UCCs) are rare but usually invasive at diagnosis. Early diagnosis of UUT-UCCs is thus warranted. UUT has the same embryological origin with bladder and BLCA-4 is a highly sensitive and specific marker for bladder cancer. We intend to investigate the viability of BLCA-4 in detecting UUT-UCCs.

Material and methods: Urines from 30 UUT-UCC patients, 10 ureteral polyp patients, 20 infected patients with incarcerated ureteral stones, and 30 normal controls were included. BLCA-4 antibody was produced and applied in an indirect ELISA assay.

Results: Urinary BLCA-4 is significantly higher in UUT-UCC group than ‘Polyp’ group (p = 0.0017), ‘Infection’ group (p < 0.0001), or ‘Normal’ group (p < 0.0001). The ‘Polyp’ group is also higher than ‘Infection’ group (p = 0.015), or ‘Normal’ group (p = 0.0009). ROC curve revealed at cutoff of 5.5 × 10−4 A, sensitivity was 93.3% and specificity was 100%. When grouped as ureteral mass vs. normal, same cut-off value yielded 93.3% sensitivity and 83.3% specificity. At 2.4 × 10−4 A, sensitivity was 56.7% and specificity was 97.2%.

Conclusions: Urinary BLCA-4 is also highly specific in UUT-UCCs detection. For incidentally identified ureteral mass, BLCA-4 can be considered an auxiliary indicator besides biopsy.

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El nivel de BLCA-4 urinario es útil para la detección del carcinoma de células uroteliales de vía urinaria superior

Resumen
Objetivo: Los carcinomas de células uroteliales de las vías urinarias superiores (CCU-VUS) son poco comunes, pero normalmente invasivos en el diagnóstico. Se garantiza por lo tanto el diagnóstico temprano de CCU-VUS. El CCU tiene el mismo origen embriológico que el de vejiga y el BLCA-4 es un marcador específico altamente sensible para el cáncer de vejiga. Tenemos la intención de investigar la viabilidad del BLCA-4 para detectar CCU-VUS.


† Corresponding author.
E-mail address: dingqiangd@hotmail.com (C.-C. Feng).

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Material y métodos: Se incluyó la orina de 30 pacientes con CCU-VUS, 10 pacientes con pólipo ureteral, 20 pacientes infectados con piedras ureterales incarceradas y 30 controles normales. El anticuerpo BLCA-4 se produjo y aplicó en un ensayo indirecto ELISA.

Resultados: El BLCA-4 urinario es significativamente mayor en el grupo de CCU-VUS que en el grupo «pólipo» (p = 0,0017), grupo «infección» (p < 0,0001) o grupo «normal» (p < 0,0001). El grupo «pólipo» también es mayor que el de «infección» (p = 0,015) o «normal» (p = 0,0009). La curva ROC revelada en el punto de corte fue de 5,5 × 10⁻⁴ A, la sensibilidad fue del 93,3% y la especificidad del 100%. Cuando se agruparon como masa ureteral vs. normal el mismo valor del punto de corte dio un 93,3% de sensibilidad y un 83,3% de especificidad. A 2,4 × 10⁻⁴ A, la sensibilidad fue del 56,7% y la especificidad del 97,2%.

Conclusiones: El BLCA-4 urinario también es muy específico en la detección del CCU-VUS. Para la masa ureteral identificada incidentalmente el BLCA-4 se puede considerar un indicador auxiliar además de la biopsia.

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Introduction

Urothelial cell carcinomas (UCCs) are the fourth most common malignancy worldwide.1,2 Bladder tumors account for most of the UCCs while upper urinary tract UCCs (UUT-UCCs) only take up 5–10% of all.1,3,5 The pyelocaliceal tumors are twice as common as ureteral tumors. Due to common embryologic origin, the concomitant bladder tumors are found in 8–31% of UUT-UCC cases. Nonetheless, some biological behavior of UUT-UCCs differs from that of bladder cancer: 60% of UUT-UCCs are invasive at diagnosis compared to merely 15% of bladder cancer3,5,6; there are almost no tumors of low malignant potential.3,5,6 Such characteristic, together with lack of early symptoms, has made early detection of UUT-UCCs fairly difficult. Although the multidetector computed tomographic urography (MDCTU) together with the urine cytology can now make a highly accurate diagnosis,9–11 those approaches are usually done after the suspicious symptoms and can thus hardly be used for screening.

However, there is a dearth of studies focusing on urinary markers for UUT-UCC detection. Studies on ImmunoCyto,12 BTA stat,13 and microsatellite instability14 have shown indicative value of those urinary markers, but the sensitivity and specificity are not satisfied. To date, none of the reported markers has been externally validated and can thus be introduced to clinical application.15 The scenario of bladder cancer is the opposite. Many urinary markers with high specificity and sensitivity have been studied for bladder cancer screening. Some of them have already been involved in commercial use.16 The BLCA-4 belongs to the nuclear matrix protein (NMP) and is specifically found in bladder cancer.17 Amid all markers reported, this proteomic marker has thus far yielded the highest specificity and sensitivity,16 which are further confirmed by our institute.18 It has also been reported that BLCA-4 is expressed in the very early stage of tumorigenesis of bladder urothelial cancer and the sensitivity does not decline for early staged disease.19 The aforementioned traits of BLCA-4 as well as the embryologic ties between UUT-UCCs and bladder cancer have triggered our interest of testing urinary BLCA-4 of UUT-UCC patients. Furthermore, previous researches did not include UUT-UCCs in the examination of the specificity of BLCA-4.20,21 In the current study, we have introduced the urinary BLCA-4 detection in a cohort of 30 UUT-TCC patients and 60 controls in order to evaluate the clinical contribution of this marker for UUT-UCCs detection.

Materials and methods

Urine sample collection

From June 2010 to December 2011, 30 urine samples from patients with pathologically confirmed unilateral UUT-UCCs were collected. All patients underwent open or laparoscopic radical nephroureterectomy (RNU). Cases with concurrent bladder cancer at the time of surgery were excluded. Three groups were set as controls: The ‘pulp’ groups consisted of urine samples of 10 patients who were diagnosed with ureteral mass on admission and were pathologically diagnosed with ureteral inflammatory or fibroepithelial polyps via ureteroscopic cup-biopsy. Those patients rejected re-biopsy or other surgical approaches and chose intact surveillance of the mass; the ‘infection’ group consisted of urine samples of 20 urinary tract infection (UTI) patients with incarcerated ureteral calculi who were later treated by ureteroscopic laser lithotripsy; the ‘normal’ group consisted of urine samples of 30 candidates without urological disorders. All the samples were morning voided urine, bar coded, and stored in the –20 °C freezer. All the patients signed the informed consent and ethical approval was obtained from the local ethical committee.

Antibody preparation and Western blotting

We have previously applied a modified approach to produce polyclonal antibody of BLCA-4.18,22 A peptide sequenced as CEISQLHAG-NH2 was synthesized and emulsified by intervals with Freund’s adjuvant (Sigma–Aldrich, MO, USA), and it was subsequently immunized to 4 New Zealand white rabbits (Youke Biotechnology Co. Ltd, Shanghai, China) aged 3–9 months. After 4 times of immunization into the 3–4 s.c. dorsal sites, the animals were bled from the auricular artery and the serum was collected. Binding pillars were used for serum purification and the resulting antibody was therefore acquired. Standard western blotting procedure was carried out for specificity examination. Four samples were used to conduct the blotting: one from a high-grade bladder urothelial cancer; one from pathologically confirmed
normal bladder mucosa proximate to the bladder cancer in a cystectomy specimen; one from normal bladder tissue acquired via cup-biopsy; and one was (phosphate buffered saline) PBS (PAA Laboratories GmbH, Colbe, Germany). Protein extractions together with PBS control were loaded with marker. The samples were then transferred to a polyvinylidene difluoride membrane and were incubated overnight. All the samples were rinsed with Tris-buffered saline (TBS) (Dako Corp., CA, USA), the membrane was treated with anti-BLCA-4 antiserum at 1:400 dilution with non-fat dry milk. Goat anti-rabbit IgG conjugated with horseradish peroxidase was then added at 1:1000 of dilution. Color development was finally conducted with enhanced chemiluminescence (ECL) (Amersham International, Buckingham, UK).

ELISA test

Using a BSA-conjugated anti-BLCA-4 antiserum, we conducted the indirect ELISA test in all urine samples, as previously described in bladder cancer samples.\(^{18}\) The total protein of all urine samples was extracted and the concentration was determined by BCA kit (Amresco, OH, USA) and read at absorbance at 450 nm. Coating was conducted by adding 1 \(\mu\)g/ml of diluted antigen at 100 \(\mu\)l/well which was kept overnight at 4 °C. The samples were then blocked with 300 \(\mu\)l/well of 1% BSA blocking buffer (Amresco). Antibody was added at 100 \(\mu\)l/well with gradient dilution and unimmunized rabbit serum was used to generate control. Goat anti-rabbit IgG (100 \(\mu\)l/well) was then applied with final color development using TMB solution (Biopanda, Zhejiang, China). Values were read, respectively, at 450 nm and 630 nm. Detectable range of BLCA-4 was tested preliminarily by serial dilution in samples selected from each group. The concentration of protein extracted ranged from 3.29 to 7.66 \(\mu\)g/\(\mu\)l.

Statistical analysis

The SPSS ver. 17 software was used for statistical analysis. The Student t-test was used for data comparisons between 2 groups. The ROC curve was applied for sensitivity-specificity analysis. All analyses were 2-tailed and \(p\) value of <0.05 was accepted as statistically significant.

Results

General information of the 30 patients with UUT-UCC was summarized in Table 1. All cases were primary. The median age was 57.4 years (48–78 years). Tumor occurred on the left side in 16 cases and on the right in 14 cases. Regional lymph node invasion was found in 4 cases, yet metastasis was not noticed in any case. Although concomitant CIS (cancer in situ) was not reported in any case, the imbalanced distribution of tumor stage and grade, namely lack of pTa-1 and pT4 tumors and very few papillary urothelial neoplasias of low malignant potential (PUNLMP) and low grade tumors, made it impossible to conduct statistical analysis between BLCA-4 expression and these parameters (Table 1). Urinary BLCA-4 level was expressed in OD units/\(\mu\)g protein (A). Specificity of BLCA-4 was demonstrated by western blotting (Fig. 1A). The faint band indicated that paracancerous cells also express BLCA-4, a trait conforming to previous reports (Fig. 1A).\(^{18,23}\) Urine from UUT-UCC patients yielded significantly higher level of BLCA-4 ((17.4±1.9) × 10\(^{-4}\) A) compared to 'Polyp' group (6.16 ± 1.3) × 10\(^{-4}\) A, \(p=0.0017\), 'Infection' group (3.42 ± 0.4) × 10\(^{-4}\) A, \(p<0.0001\), or 'Normal' group ((2.8 ± 0.3) × 10\(^{-4}\) A, \(p<0.0001\)) (Fig. 1B). It is, however, noteworthy that the 'Polyp' group also yielded a significantly higher level of BLCA-4 compared to 'Infection' (\(p=0.0152\)) or 'Normal' (\(p=0.0009\)) group (Fig. 1B). The latter 2 groups had no significant difference in BLCA-4 level (\(p=0.2080\)) (Fig. 1B). In order to obtain clinical rather than biological significance of the data, we compared the urinary BLCA-4 level in patients with ureteral mass (cancer + polyp) to the other 2 control groups. The BLCA-4 level was still higher in the ureteral mass group (\(p<0.0001\), respectively).

The ROC curve was generated to determine the ideal cut-off value (Fig. 1C and D). When urine samples from UUT-UCC patients and normal candidates were studied, the cut-off value at 5.5 × 10\(^{-4}\) A, the sensitivity was 93.3% (95%CI: 77.93–99.18%) and the specificity was 100% (95% CI: 86.77–100.0%). When grouping, they were assigned as ureteral vs. normal mass, the aforementioned cut-off value merely yielded a sensitivity of 93.33% (95% CI: 77.93–99.18%) and specificity of 83.33% (95% CI: 67.19–93.63%). We thus chose the value where the likelihood ratio was largest. When the ratio reached 20.40, the cut-off value of 2.4 × 10\(^{-4}\) A yielded a sensitivity of 56.67% (95% CI: 37.43–74.54%) and specificity of 97.22% (95% CI: 85.47–99.93%).

Discussion

Early detection of UUT-UCCs has always been a predicament for clinicians. Without prominent clinical presentations, it is difficult to detect the disease in its early stage. The MDCTU has 96% sensitivity and 99% specificity for polypoid

<table>
<thead>
<tr>
<th>Parameters</th>
<th>N (%)</th>
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<tbody>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>24 (80.0%)</td>
</tr>
<tr>
<td>F</td>
<td>6 (20.0%)</td>
</tr>
<tr>
<td>Incidence</td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>28 (93.3%)</td>
</tr>
<tr>
<td>Multiple</td>
<td>2 (6.7%)</td>
</tr>
<tr>
<td>Tumor size</td>
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</tr>
<tr>
<td>0.5–2 cm</td>
<td>(mean 0.83 cm)</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
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<tr>
<td>PUNLMP</td>
<td>1 (3.3%)</td>
</tr>
<tr>
<td>Low</td>
<td>4 (13.3%)</td>
</tr>
<tr>
<td>High</td>
<td>25 (83.3%)</td>
</tr>
<tr>
<td>Stage</td>
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</tr>
<tr>
<td>pTa-1</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>pT2</td>
<td>26 (86.7%)</td>
</tr>
<tr>
<td>pT3</td>
<td>4 (13.3%)</td>
</tr>
<tr>
<td>pT4</td>
<td>0 (0.0%)</td>
</tr>
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Table 1: Clinicopathological parameters of 30 patients with urothelial cell carcinomas of the upper urinary tract (UUT-UCC).
lesion between 5 and 10 mm. However, both indexes drop for smaller lesions and the difficulty that remained for this type of image is the detection of flat lesions.  

Urine cytology, though with a decisive specificity, has a low sensitivity and is recommended to be tested in situ. A recent report has further questioned the viability of the urine cytology due to its poor performance. To sum up, both methods lack the ability for early detection and are usually carried out based on heralding clinical presentation. In the search of the ‘PSA’ for UUT-UC patients, potential markers or approaches should meet certain criteria: non-invasive; high specificity and sensitivity, especially for early staged disease; and methodological feasibility for clinical application. It is thus natural to take reference to the numerous potential urinary markers of bladder cancer in light of the same embryologic origin of the urothelium. The fact that the proportion of invasive and high-grade UUT-UCs is predominantly high at diagnosis is possibly due to failure of early detection of the disease. Albeit examination for molecular abnormalities by fluorescence in situ hybridization (FISH) is now prevailing for bladder cancer screening, the results are still preliminary and distant from clinical application.

BLCA-4 belongs to one of the 6 NMPs specifically found in bladder cancer. An antibody of BLCA-4 is later raised and is used to detect urinary BLCA-4 level, which shows high sensitivity and specificity for bladder cancer. Furthermore, the BLCA-4 expression is also detected in the pathologically confirmed paracancerous tissue of the bladder cancer specimen, indicating that BLCA-4 is expressed in a very early stage of tumorigenesis. This highly sensitive and specific maker is then verified by our institute and is found to be related to tumor invasiveness. However, the urinary BLCA-4 level is also elevated in 19% of the patients with spinal cord injury (CSI) since patients with CSI are at much higher risk for bladder cancer than the healthy population. The mechanistic analysis revealed that BLCA-4 belongs to the ETS transcription factor family and in vivo studies confirmed the expression of BLCA-4 at the early stage of tumorigenesis of bladder cancer in a mouse model. Microarray analysis shows that BLCA-4 overexpression induces up-regulations of interleukin-1α (IL-1α), IL-8, and thrombomodulin expression, which are subsequently confirmed in our study. Additionally, we have discovered the interaction between BLCA-4, matrix metalloproteinase-9 (MMP-9), and vascular endothelial growth factor (VEGF), and we have found that BLCA-4 is not related to angiogenesis.

In the current study, we have included 30 patients with UUT-UCs and have compared their urinary BLCA-4 level
with controls. The major outcome, that urinary BLCA-4 is significantly higher in UUT-UCC patients, indicates that BLCA-4 can be considered a candidate for UUT-UCCs screening with a sensitivity of 93.3% and specificity of 100% at the cut-off value of 5.5 × 10⁻⁴ A. Nonetheless, further interpretation of the data raises noteworthy issues. First, the 10 candidates with ureteral polyps also have elevated urinary BLCA-4 level while the patients with polyps due to stone incarceration have not. Though the current data show that semirigid especially flexible ureteroscope is capable to determine tumor grade in 90% of cases with a low false-negative rate, there is thus a possibility that there are concurrent early staged lesions that cannot yet be identified optically other than the polyp. The drop of both sensitivity and specificity when BLCA-4 is used to differentiate the ‘ureteral mass’ group from the normal one indicates that for incidentally identified ureteral mass, BLCA-4 can be considered an important auxiliary indicator aside from biopsy. Patients diagnosed with ureteral polyps with high urinary BLCA-4 level should keep close follow-ups even if pathologically proven to be fibroepithelial polyp [30]. Secondly, under the 2004 WHO classification, there is almost no lesion in UUT-UCCs diagnosed as PUNLMP. In our series, however, one female patient was diagnosed as ureteral PUNLMP both in the biopsy and the final pathology. The patient was diagnosed on a fortuitous finding because of the ipsilateral incidentally discovered renal mass. The final pathology confirmed synchronous renal clear cell carcinoma and ureteral PUNLMP. Even in this very early-staged case, the urine BLCA-4 level is above the cut-off value. Nonetheless, further study to rule out influence of renal cell carcinoma on urinary BLCA-4 level is warranted. Thirdly, this study has certain limitations that need further investigation: the subject number recruited herein is relatively small. Confirmation in larger population should be conducted; since the marker is capable of detecting both UCCs in the upper and lower urinary tracts, studies on how the cut-off range should be defined to differentiation should be conducted; recurrence of low-grade bladder tumors after RNU is common and there is thus far no study on the surveillance merits of BLCA-4, which is highly warranted.

Conclusion

Early detection of UUT-UCCs is critical for better oncologic outcome. We currently used a highly specific and sensitive urinary marker for bladder cancer, BLCA-4 to detect UUT-UCC on a urine-based ELISA test. Results indicate that BLCA-4 is still highly sensitive and specific for UUT-UCC detection. Further study on its merits in surveillance is warranted.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgement

This project was sponsored in part by the Science and Technology Commission of Baoshan District (08-E-14), Shanghai Municipality, China.

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