Original Article

Urinary BLCA-4 is Highly Specific for Detection of Bladder Cancer in Chinese Han Population and Is Related to Tumour Invasiveness

(bladder cancer / BLCA-4 / Chinese / Han nationality)

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Abstract. BLCA-4 is currently the most sensitive and specific urinary marker for bladder cancer. As the incidence of bladder cancer varies by ethnic and territory, we tended to evaluate the feasibility of bladder cancer detection using urinary BLCA-4 in Chinese Han nationality. Urines from 79 bladder cancer patients, 31 urinary tract infection patients and 29 normal controls were included. Tissue specimens of 53 bladder cancers, 24 pathologically normal tumour-adjacent urothelium and 15 healthy controls were involved. BLCA-4 antibody was produced and applied in an indirect ELISA assay for urine samples and immunohistochemistry study in tissue samples. Urinary BLCA-4 was significantly higher in the bladder cancer group (P = 0.0001). The level was in no relation to age, gender, growth pattern, grade or stage. Discrepant to reported data, a cut-off value of 1.7×10^{-4} *A* was acquired here, which yields a sensitivity of 97.37 % and specificity of 100 %. Muscle invasiveness was related to a higher BLCA-4 level (P = 0.0175). Tumour tissues were also scored higher in staining (P = 0.0001), yet this was not related to invasiveness. In 41.67 % of adjacent normal tissue we found positive BLCA-4 expression. Urinary BLCA-4 was also highly specific in bladder cancer detection in the Chinese, with additional indicative value in muscle invasiveness detection. A cut-off value of 1.7×10^{-4} *A* may be more adaptive to Chinese Han population.

Introduction

The necessity of lifelong surveillance has entitled bladder cancer one of the most expensive malignancies of the patient from diagnosis to decease (Botteman et al., 2003). Complete assessments include cytology, imaging and routine cystoscopy, which elevates the economic burden on the one hand, while bringing a certain extent of trauma on the other. The currently available non-invasive tools such as cytology, FISH, or imaging yield either unsatisfactory sensitivity or specificity, which confines their single utilization to bladder cancer detection (Gaston et al., 2010). In the search of a potential "PSA" for bladder cancer indication, various markers have been investigated, among which some have presented promising applicability. Generally, the markers can be categorized into genomic, proteomic and epigenetic markers. BLCA-4, identified in the proteomic surveys as one of the six nuclear matrix proteins (NMPs) that are bladder cancer specific (Getzenberg et al., 1996), has yielded the highest sensitivity and specificity thus far in a series of studies (Shirodkar et al., 2009).

Nonetheless, the incidences of bladder cancer vary between different regions and ethnics, revealing a relatively lower rate in Chinese population compared to occidental countries (Surveillance Epidemiology and End Results Database, 2010). Therefore, it is critical to investigate the urinary BLCA-4 level in the population of Han nationality, which is predominant in China, before defining a universal cut-off value of the marker. In the present study, we used the self-produced BLCA-4 antibody to detect the levels of urinary BLCA-4 in patients with bladder cancer, urinary tract infection (UTI) and cancer-free urine controls. The tissue expression of BLCA-4 was also investigated by immunohistochemis-

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Abbreviations: DAB – diaminobenzidine tetrahydrochloride, IHC – immunohistochemistry, NMPs – nuclear matrix proteins, PUNLMP – papillary urothelial neoplasm of low malignant potential, UTI – urinary tract infection.

try (IHC) of bladder cancer samples, tumour-adjacent "normal" urothelium and normal uroepithelium controls.

Material and Methods

Urine and tissue specimen collection

Seventy-six urine samples from patients with pathologically confirmed bladder cancer together with 31 UTI urine samples and 29 normal controls were collected from Huashan Hospital, Fudan University. All samples were morning voiding urines, bar coded, and stored in the -20 °C freezer. Infected urine samples were from patients excluded from urologic tumours. Tissue samples from 53 patients with bladder tumour were involved, among which 38 were collected via transurethral resections and 15 were from radical cystoprostatectomies. Pathologically normal urothelium adjacent to bladder tumour was collected in 24 cases. Fifteen normal urothelium controls were all collected from subjects without urologic tumours, amid whom three were organ donors and the rest were biopsies from histologically healthy bladder mucosa. All patients signed the informed consent and ethical approval was obtained from the local ethical committee (HIRB). Tissue samples were all confirmed by the pathological grading and staging according to WHO/ISUP (Eble et al., 2004) and UICC-TNM (Rosai et al., 2002), respectively. Both urine and tissue samples included in the current study were from Chinese patients of Han nationality.

Antibody preparation

A slightly modified protocol was followed in the production of the BLCA-4 polyclonal antibody (Konety et al., 2000a). The sequence we adopted was CEISQLNAG--NH2 and the peptide was used to immunize two New Zealand White rabbits aged 3 to 9 months (Youke Biotechnology Co. Ltd, Shanghai, China). The resulting antigens were emulsified by intervals with Freund's adjuvant (Sigma-Aldrich, St. Louis, MO). The animals were bled for serum collection after four times of immunization over a 3-month period. Serum was purified through antigen-specific binding pillar and the corresponding polyclonal antibody was collected. Standard western blotting was performed for specificity detection of the product. Protein extractions of adjacent "normal" tissue, bladder cancer tissue, normal bladder tissue, and PBS (PAA Laboratories GmbH, Cölbe, Germany) control together with markers were loaded and further transferred to a polyvinylidene difluoride membrane. After being incubated overnight and washed with TBS (Dako Corp., Carpinteria, CA), anti-BLCA-4 antiserum was added to the membrane at 1 : 400 dilution together with non-fat dry milk. Secondary antibody of goat anti-rabbit IgG conjugated with horseradish peroxidase was applied at 1 : 1,000 dilution, followed by final colour development with ECL (Ammersham International, Buckingham, UK).

ELISA test

An indirect immunoblotting test using serial dilutions of BSA-conjugated anti-BLCA-4 antiserum was carried out in urine samples from patients with bladder cancer, UTI and normal control (Konety et al., 2000a). Urine samples were precipitated and protein concentration was detected using the BCA kit (Amresco, Solon, OH). Data was read at an absorbance of 450 nm. Coating was performed by adding 1 μg/ml of diluted antigen at 100 μl/well, which was kept overnight at 4 °C. After five washes the samples were blocked with 300 μl/well of 1% BSA blocking buffer (Amresco). Gradient-diluted antibody was then added at 100 μl/well with blank rabbit serum as negative control, followed by thorough rinses. The secondary antibody of goat anti-rabbit IgG (100 μl/well) was added to each well and colour development was performed with TMB solution (Biopanda, Zheijang, China). Values were read at 450 nm and 630 nm of wavelength. The detectable range of urinary BLCA-4 was determined by preliminary serial dilution in five samples, of which three were from the cancer group, one was from the infected group and one was normal urine. The protein concentration extracted varied from 3.31 to 7.24 μg/μl.

Immunohistochemistry

A previously established protocol was used for standard immunohistochemical study (Feng et al., 2011a). Endogenous peroxidase of deparaffinized tissue sections was blocked with 3% hydrogen peroxide. Sections were then treated without boiling at pH 6.7. Non-specific antigen sites were blocked with non-immunized rabbit serum. Primary antibody of BLCA-4 was subsequently applied at a dilution of 1 : 100, followed by secondary antibody application and diaminobenzidine tetrahydrochloride (DAB) (KPL, Gaithersburg, MD) colour development. Final counter-stain with Mayer's haematoxylin (Sigma-Aldrich) was performed in all sections. Sections treated with solely PBS instead of primary antibody were chosen as negative control. All slides were assessed with a Nikon 80i microscope (Nikon Instruments, Tokyo, Japan) for extensity and intensity. The extensity was graded pertaining to the percentage of tumour cells stained, namely 0 for 0–5 %; 1 for 6–20 %; 2 for 21–50 %; and 3 for > 50 %. Intensity was graded as: 1 for light yellow, 2 for dark yellow, and 3 for brown. The final score of each section was attained from the sum of the two aspects as: 0 for negative (0~2), 1 for mild (3), 2 for moderate (4), and 3 for strong (5~6) (Feng et al., 2011b).

Statistical analysis

The Student's *t*-test was wielded for data comparisons between two groups. For comparisons within more than two groups the Kruskal-Wallis test was applied. Correlations were analysed with the Spearman test, and the Fisher's exact test was utilized for specificity and sensitivity determination. All data were processed with 2-tailed

tests and a P value of < 0.05 was accepted as statistically significant.

Results

Among the 76 bladder cancer patients from whom the urine specimen was collected, nine were graded as papillary urothelial neoplasm of low malignant potential (PUNLMP), 39 graded as low grade, and 28 as high grade. Stages were categorized as follows: 19 cases for Ta, 34 cases for T1, 15 cases for T2 and eight cases for T3–4. The patients consisted of 14 females and 62 males at the mean age of 57.20 ± 1.4 years (36 years to 83 years). Control with UTI and normal urine samples were collected from subjects excluded from urologic neoplasms. The urinary level of BLCA-4 was expressed in OD units/μg protein (*A*). Urine samples from tumour patients yielded a significantly higher level of BLCA-4 ($(7.262 \pm 0.9) \times 10^{-4}$ *A*) in comparison to UTI samples ($(1.334 \pm 0.03) \times 10^{-4}$, $P < 0.0001$) or normal urine ($(1.299 \pm 0.03) \times 10^{-4}$ *A*, $P < 0.0001$). The latter two did not change significantly in BLCA-4 expression (P = 0.4491), as shown in Fig. 1a. There was neither age (P = 0.710) nor gender (P = 0.7547) preference associated with BLCA-4 expression. The urinary BLCA-4 level did not change significantly along with progressed tumour grading (P = 0.9759, Fig. 1b) or staging (P = 0.0544, Fig. 1c). Notwithstanding, when subjects were grouped as NMIBC (non-muscle invasive bladder cancer, Ta + T1) and MIBC (muscle invasive bladder cancer, T2 + T3–4), the urinary BLCA-4 level was weakly yet significantly higher in the MIBC population ($(5.909 \pm 1.0) \times 10^{-4}$ *A* vs. $(10.380 \pm 1.7) \times 10^{-4}$ *A*, P = 0.0175). At the cut-off value of 1.7×10^{-4} OD units /μg protein (*A*), the test yielded a specificity of 100 % (95 % CI: 88.06 % to 100 %) and a sensitivity of 97.37 % (two samples below the cut-off level, 95 % CI: 90.81 % to 99.68 %), compared to normal urine samples.

The specificity of the manufactured BLCA-4 antibody was examined by western blotting (Fig. 2). The tumour tissues all exhibited specific bands in the corresponding location. Normal urothelium or PBS mock control failed to show the bands. A discrepancy was observed when pathologically normal urothelium samples adjacent to bladder tumour were evaluated. Some (10/24, 41.67 %) revealed BLCA-4 expression whilst the rest did not. The same phenomenon was also noted in the IHC assessment. The IHC demonstrated immunopositive regions chiefly confined within the nucleus of bladder tumour cells (Fig. 3a, b). Consistently, the normal urothelium was not stained in any case (Table 1, Fig 3c) whilst some sections of the tumour-adjacent normal tissue exhibited staining and were thus scored above 0 (Table 1). Generally, BLCA-4 was expressed to a significantly higher extent in tumour sections compared with normal control or adjacent "normal" tissue (Table 1). The adjacent "normal" urothelium also displayed higher scoring than the healthy uroepithelium (Table 1). Expression of BLCA-4 in tumour tissue was not related to age, gender, onset pattern, occurrence, tumour size, grade, stage (Table 1) or, unlike the urinary expression, the muscle invasiveness (P = 0.421).

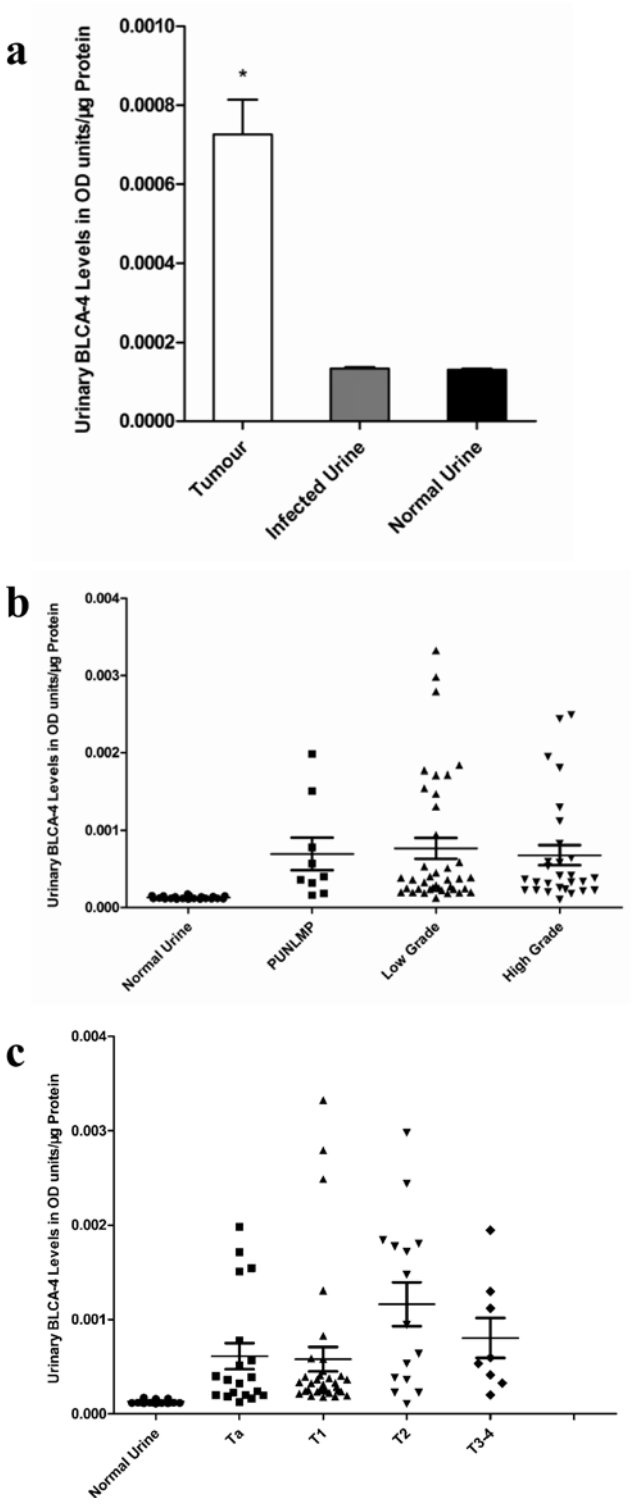

Fig 1. Statistical study of urinary BLCA-4 level demonstrating that (A) the urinary BLCA-4 level is significantly higher in patients with bladder cancer in contrast to UTI or normal patients; the urinary BLCA-4 level cannot distinguish between different (B) grades or (C) stages of bladder cancer. (*P < 0.05, statistically significant)

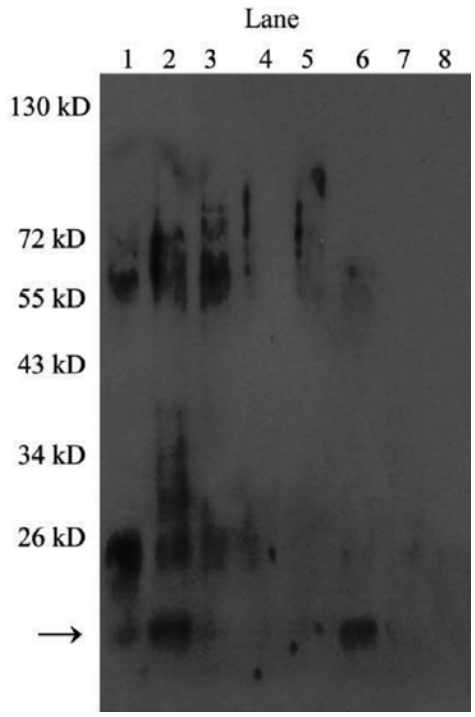

Fig 2. Western blotting showing target bands (arrow indicated) in lanes 1, 2 and 6 verifying the BLCA-4 expression. (lanes 1 and 5: adjacent "normal" urothelium in bladder cancer samples #20 and #34; lanes 2 and 6: bladder cancer samples #20 and #34; lanes 3 and 7: normal urothelium controls #6 and #7; lanes 4 and 8: PBS replaced primary substrate "mock" controls)

Table 1. Immunohistochemical expression of BLCA-4 in comparisons between different clinicopathological parameters (data presented as mean ± standard error, SEM)

Parameters	N	BLCA-4
Gender		
M	39	1.103 ± 0.1
F	14	1.143 ± 0.2
P		0.8695
Occurrence		
Single	37	1.108 ± 0.1
Multiple	16	1.125 ± 0.2
P		0.9428
Tumour size		
< 3 cm	35	1.114 ± 0.1
≥ 3 cm	18	1.111 ± 0.2
P		0.9889
Tumour onset		
Primary	39	1.231 ± 0.1
Recurrent	14	0.786 ± 0.2
P		0.0651
Tissue		
Carcinoma	53	1.113 ± 0.1
Normal	15	0.000
P		0.0001
Carcinoma	53	1.113 ± 0.1
*Adjacent Normal	24	0.417 ± 0.1
P		0.000
Normal	15	0.0001
*Adjacent Normal	24	0.417 ± 0.1
P		0.0035
Grade		
PUNLMP	6	1.500 ± 0.4
Low	25	1.040 ± 0.1
High	22	1.091 ± 0.2
P		0.4891
Stage		
pTa	15	1.000 ± 0.2
pT1	18	1.167 ± 0.1
pT2	13	1.154 ± 0.3
pT3-4	7	1.143 ± 0.3
P		0.8377

*Defined as pathologically normal tissue adjacent to the bladder carcinoma

Discussion

Early detection of bladder cancer has been urged to an unprecedented importance as the NMIBC can be effectively managed in an early stage. However, in the absence of haematuria bladder cancer is more likely to advance in stage. The screening of high-risk population has become critical to diminish the mortality. Nonetheless, current approaches have limitations in predictive value, and have thus led us to intravesical remedies and unnecessary removal of the bladder in a number of cases. Urinary cytology is recommended in several major guidelines for suspicion of bladder tumour (Babjuk et al., 2008; AUA Clinical Guidelines, Bladder Cancer, 2010). Though with a high specificity, the test has a sensitivity leaning much on the stage and grade of the tumour and thus varies within a large range (Habuchi et al,

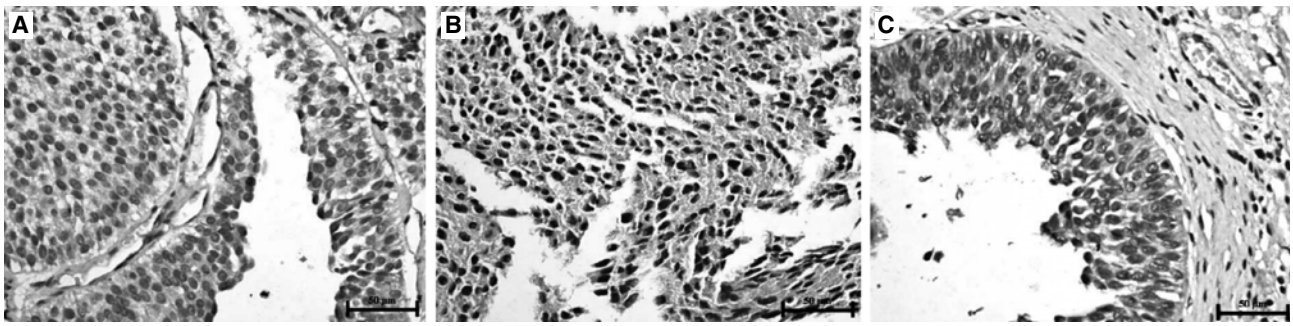

Fig 3. Immunohistochemistry of BLCA-4 revealing chiefly nucleic positive staining in (A) low-grade bladder cancer and (B) PUNLMP and (C) no immunopositive cells in normal bladder urothelium.

2005). Thus far, a series of novel urinary markers have been discovered which can be generally categorized as proteomic, genomic and epigenetic makers (Shirodkar et al., 2009). Some have been reported to be both highly sensitive and specific, exceeding the cytology assay, and with the technical simplicity are more welcome amid patients (Yossepowitch et al., 2007).

A major problem before putting these tests into clinical practice is that the markers are generally studied by individual centres or institutes where the specimen source lacks the diversity to define a universal or ethnic-based cut-off value in light of the difference of disease incidence of bladder cancer in occidental and oriental population. Among almost all the reported urinary markers such as BTA, NMP-22, hyaluronic acid, hyaluronidase, cytokeratin-8, cytokeratin-18, cytokeratin-19, tissue polypeptide antigen, and tissue polypeptide-specific antigen, etc. (Shirodkar et al., 2009), BLCA-4 has been reported to yield the highest sensitivity and specificity so far. Yet likewise, reports on BLCA-4 are all conducted by the University of Pittsburgh associated with several sister institutes (Getzenberg et al., 1996; Konety et al., 2000a, b; Van Le et al., 2004, 2005; Myers-Irvin et al., 2005a, b; Lamm et al., 2000), and we are thus the first institute to verify the effectiveness of urinary BLCA-4 in the detection of bladder cancer in a distinctive centre with subjects of the Chinese ethnic.

BLCA-4 is among the six nuclear matrix proteins (NMPs) derived from bladder cancer tissue using proteomic technology and was found to be expressed exclusively in the tumour specimens (Getzenberg et al., 1996). Later, the protein was also identified within pathologically normal urothelium adjacent to bladder tumours when an anti-BLCA-4 antibody was raised and utilized in immunoblotting assay. In the same study, an indirect ELISA method was developed using this single-peptide-raised antibody to detect urinary BLCA-4 levels in contrast to normal urine, and a cut-off value of 13 absorbance units/μg protein was defined yielding a sensitivity of 97.7 % and specificity of 100 % (Konety et al., 2000a). The study also shows that the urinary BLCA-4 level is irrelevant to tumour grade or stage. With the same cut-off value, BLCA-4 was subsequently revealed to be elevated in patients with spinal cord injury, in no relation to UTI, smoking, catheterization or cystitis (Konety et al., 2000b). Functional study indicated that BLCA-4 is a novel member of the ETS transcription factor family, and is not found in benign urologic diseases. *In vivo* studies also imply that BLCA-4 functions in the early stage of bladder cancer occurrence (Van Le et al., 2004). When a "sandwich" immunoassay was developed later using antibodies raised from an additional peptide, the urinary BLCA-4 still yielded an agreeable sensitivity of 89 % and specificity of 95 % at the cut-off value of 0.04 absorbance units (Van Le et al., 2005). Microarray analysis of interactions of gene expression revealed up-regulation of interleukin-1α (IL-1α), IL-8, and thrombomodulin induced by over-expression of BLCA-4, which were confirmed by immunoblots (Myers-Irvin et al., 2005a).

In the present study, we have adopted the indirect ELISA method for urinary BLCA-4 detection, and the same antibody has also been applied in the IHC evaluation. In accordance to previous literature, the BLCA-4 detected in urine samples of bladder tumour patients was significantly higher than in infected or normal urine, leaving the latter two unchanged in the BLCA-4 level. Also, the urinary BLCA-4 level was accordingly in no correlation with age, gender, grade or stage. At the cut-off value of 1.7×10^{-4} *A*, a high sensitivity and specificity was acquired parallel to previous reports. Notwithstanding the first time of studying BLCA-4 with IHC, we have gained satisfactory staining outcomes of the sections. Nonetheless, there are several points that necessitate addressing. First, the cut-off value is distant from the literature, either in indirect immunoblots or sandwich assays (Van Le et al., 2005). Second, we have acquired a significant elevation in urinary yet not IHC BLCA-4 levels in MIBC patients, which has not been reported previously. Third, only some of the tumour-adjacent "normal" uroepithelium exhibited immunopositive results in our series, whereas 100 % cases in the previous study showed BLCA-4 expression (Konety et al., 2000a).

We assume that these discrepancies may result from distinction of manufactural craft in the production of BLCA-4 antibody or from the intrinsic difference of tumorigenesis between Chinese Han nationality and western population, which may be the explanation for the vast difference of disease incidence within the two groups. Furthermore, we propose that a cut-off value of 1.7×10^{-4} *A* may be more adaptive to Chinese Han nationality, and higher levels of urinary BLCA-4 are more indicative for muscle invasiveness in the Chinese population. Of note, in the current study we have detected positive staining in PUNLMP in all cases, which implies that these papillary lesions are actually cancerous tissue with a benign morphologic feature (Babjuk et al., 2008). Such result, together with the data mentioned above, indicates that BLCA-4 is a marker with higher resolution in distinguishing urothelial malignancy.

Conclusion

Urinary BLCA-4 detection has been proved highly sensitive and specific in bladder cancer patients. As the incidence of bladder cancer varies from western to eastern populations, we analysed urinary BLCA-4 levels in the Chinese Han population. The results show a high sensitivity and specificity of urinary BLCA-4 level at 1.7×10^{-4} *A* to distinguish bladder cancer from normal urine. This cut-off value is more adaptive to Han nationality and a higher value is associated with muscle invasion in Chinese population.

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