Int J Clin Exp Med 2015;8(10):18819-18824
www.ijcem.com /ISSN:1940-5901/IJCEM0012388

Original Article

Urinary BLCA1 is specific for urothelial cancer detection in Chinese ethnicity

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Received July 4, 2015; Accepted September 30, 2015; Epub October 15, 2015; Published October 30, 2015

Abstract: Aim: To study the potential of BLCA1 for detection of urothelial cancer including urinary bladder cancer (UBC) and upper tract urothelial cancer (UTUC). Method: An antibody for BLCA1 was generated and indirect ELISA was used to detect urinary BLCA1 level. Clinicopathological parameters were studied for the association with BLCA1 level. Urine samples from UBC and UTUC patients, together with cases with other urological disorders were collected and tested. Results: Urinary BLCA1 level in UBC and UTUC patients were significantly higher than that in normal controls. This was also proven when urothelial cancers were grouped as a whole, where as the level did not differ between UBC and UTUC samples. BLCA1 was also significantly higher in urothelial cancer samples compared to other urological disorders such as patients with long-dwelling catheter, glandular cystitis, BPH and other benign conditions. Cut-off value at 0.0009 OD/μg yielded a sensitivity of 92.7% and a specificity of 92.9% for UBC. Conclusion: Urinary BLCA1 is a promising marker for urothelial cancers including UBC and UTUC with high sensitivity and specificity.

Keywords: Bladder cancer, BLCA1, tumor marker

Introduction

BLCA1/4 are nuclear proteins specifically expressed in urinary bladder cancer (UBC) and were first reported in 1996 by Getzenberg et al. [1]. The proteins were extracted and identified via proteomics of UBC and were named BLCA1-6.

The Getzenberg group prepared the antibody using single peptide acquired form proteomic extraction and applied an indirect ELISA test for BLCA4 level in urine samples from normal and UBC individuals. By determining suitable cut-off value, they obtained 97.7% of sensitivity and 100% of specificity [2]. BLCA4 was not detected in urological disorders other than UBC such as cystitis, benign prostatic hyperplasia (BPH), and other urological malignancies. Later, a sandwich ELISA test was proposed using antibodies generated by two peptides and yielded a sensitivity of 89% and specificity of 95% for UBC in a modest amount of patients [3]. Urinary BLCA4 was also elevated in patients with spinal injury who were known to have higher risk for UBC, and was not associated with urinary tract infection (UTI), smoking, and catheter-related cystitis.

In our previous studies, we also found that urinary BLCA4 had high sensitivity and specificity in UBC patients of Chines Han nationality and we suggested that the marker worked better for muscle-invasive bladder cancer (MIBC) [4]. Also, we showed that BLCA4 was highly sensitive and specific for upper tract urothelial cancer (UTUC). The result contributed to differential diagnosis for ureteral mass, before biopsy [5]. In combination with the UBC studies, we assume that BLCAs could be a sensitive and specific marker for urothelial cancers regardless of upper or lower urinary tract.

In the current study, we have used BLCA1/4 antibody to study its predictive role in differentiating urothelial cancers from other benign and malignant urological disorders.

Materials and methods

Urine sample collection

Seventy-nine urine samples from patients with pathologically confirmed urinary bladder cancer (UBC), 12 urine samples from patients with pathologically confirmed upper tract urothelial

Table 1. Patient characteristics and urinary BLCA1 level grouped by different clinical parameters

			N	%	Mean (OD/μg)	Std. Deviation
Sex						
	F		12	12.40%	0.0028	0.0013
	M		70	87.60%	0.0034	0.0024
Category						
	UBC		69	86.30%	0.0034	0.0024
	UBC+UTUC		1	1.90%	0.0050	/
	UTUC		12	11.80%	0.0026	0.0014
Stage						
	UBC	Ta	47	58.40%	0.0033	0.0016
		Tis	1	2.60%	0.0070	/
		T1	12	10.30%	0.0023	0.0025
		T2	6	12.00%	0.0054	0.0051
		T3	2	2.80%	0.0038	0.0006
		T4	2	2.10%	0.0028	0.0037
	UTUC	T1	3	1.00%	0.0009	0.0004
		T2	3	4.30%	0.0038	0.0006
		T3	1	1.60%	0.0044	/
		T4	2	1.70%	0.0023	0.0018
		Ta	3	3.20%	0.0029	0.0006
Grade						
	UBC	PUNLMP	4	3.40%	0.0023	0.0001
		LG	32	37.40%	0.0031	0.0009
		HG	34	47.40%	0.0037	0.0033
	UTUC	LG	1	0.80%	0.0022	/
		HG	11	11.00%	0.0027	0.0015

UBC = Urinary bladder cancer; UTUC = Upper tract urothelial cancer; LG = Low grade; HG = High grade; PUNLMP = papillary urothelial neoplasm of low malignant potential.

cancer (UTUC), and 1 urine sample from a patient with both UBC and UTUC were collected. For benign controls, 42 normal urine samples, 12 infected urine samples from catheterized patients, 1 from inverted papilloma patient, and 1 from ureterocele patient, 4 from glandular cystitis patients, and 4 from benign prostatic hyperplasia (BPH) patients were collected. All samples were collected from Huashan Hospital, Fudan University. All samples were morning voiding urines, bar coded, and stored in the -20°C freezer. All patients were of Chinese ethnicity signed the informed consent and ethical approval was obtained from the local ethical committee (HIRB).

Antibody preparation

We have previously applied a modified approach to produce polyclonal antibody of BLCA4 [6-8]. A peptide sequenced as CEIS-QLNAG-NH2 was synthesized and emulsified by intervals with Freund's adjuvant (Sigma-Aldrich, MO, USA) and was subsequently immunized to 4 New Zealand white rabbits aged 3 to 9 months. After 4 times of immunization into the 3 to 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 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, Cölbe, Germany). The BLCA1 antibody was generated using the similar method [9].

ELISA test

An established ELISA protocol was reported in our previous study [6]. Briefly, an indirect immunoblotting test was applied using serial dilutions of BSA-conjugated anti-BLCA1/4 antiserum in all cohorts. 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. Samples were then blocked with 1% BSA blocking buffer. Gradient-diluted antibody was then added at 100 μl/well with blank rabbit serum as negative control. The secondary antibody of goat anti-rabbit IgG was added to each well and color development was performed with TMB solution. Values were read at 450 nm and 630 nm of wavelength.

Statistical analysis

The Prism GraphPad ver. 6 software was used for the statistical analysis. The Student's t-test

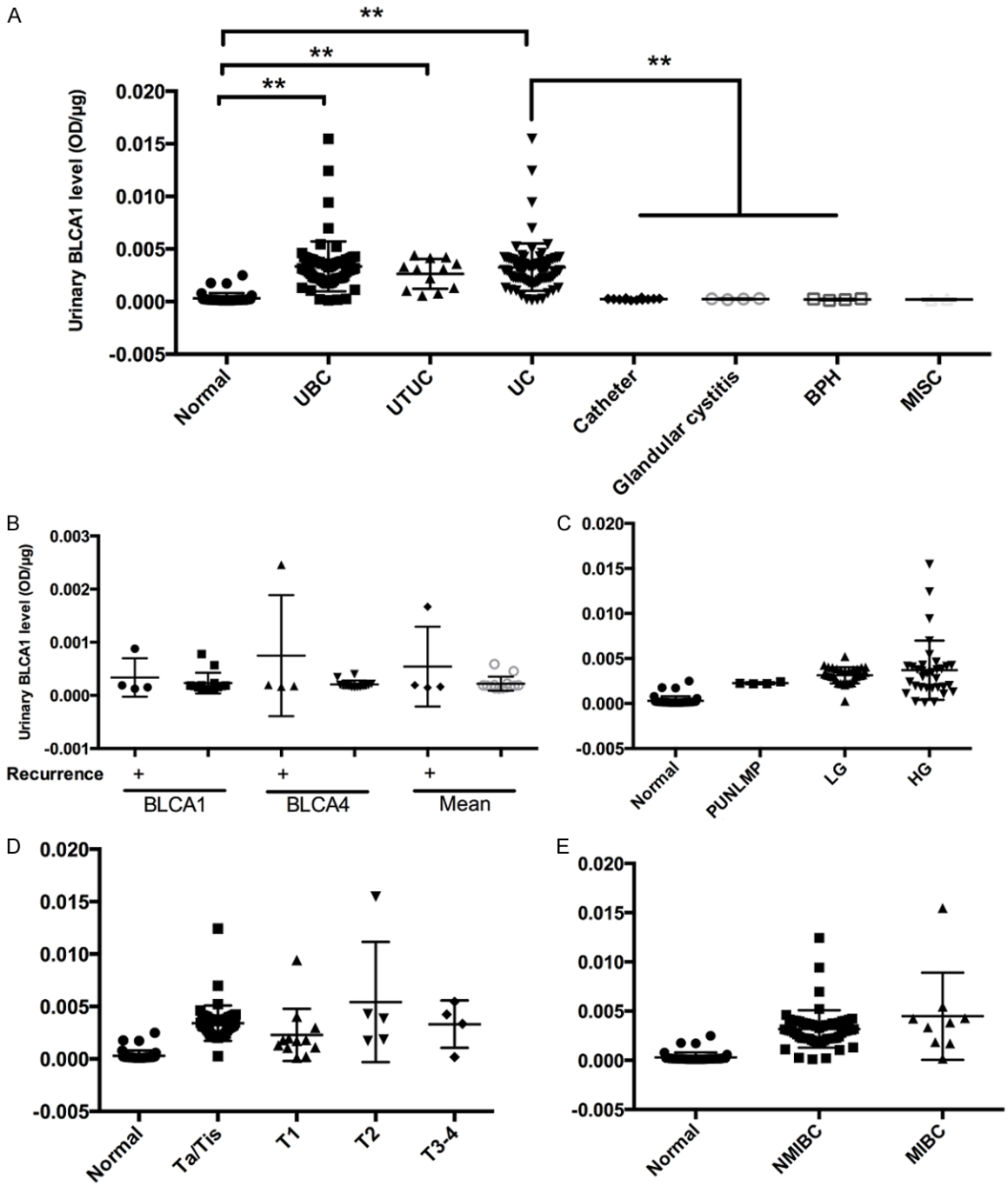

Figure 1. Urinary BLCA1/4 as an indicator of clinicopathological parameters of bladder cancer. A. Urinary BLCA1 level was significantly higher in urothelial bladder cancer (UBC) and upper tract urothelial cancer (UTUC) or as a whole (urothelial cancer, UC), distinguishing other urological diseases like catheter dwelling, glandular cystitis, benign prostatic hyperplasia (BPH), etc; B. Neither urinary BLCA1 or BLCA4 was significantly changed in patients with recurrent bladder mass (cancer, atypical hyperplasia, and inflammatory tissue on biopsy), nor was the mean value of BLCA/1/4; C. Urinary BLCA1 level did not differ in different tumor grades of UBC; D. Urinary BLCA1 level did not differ in different tumor stages of UBC; E. Urinary BLCA1 level did not differ in different muscle involvement of UBC.

was wielded for data comparisons between two groups. For comparisons within more than two groups the Kruskal-Wallis test was applied. The ROC curve was generated to study the sensitivity and specificity. The cut-off value was determined by Youden's index. All data were pro-

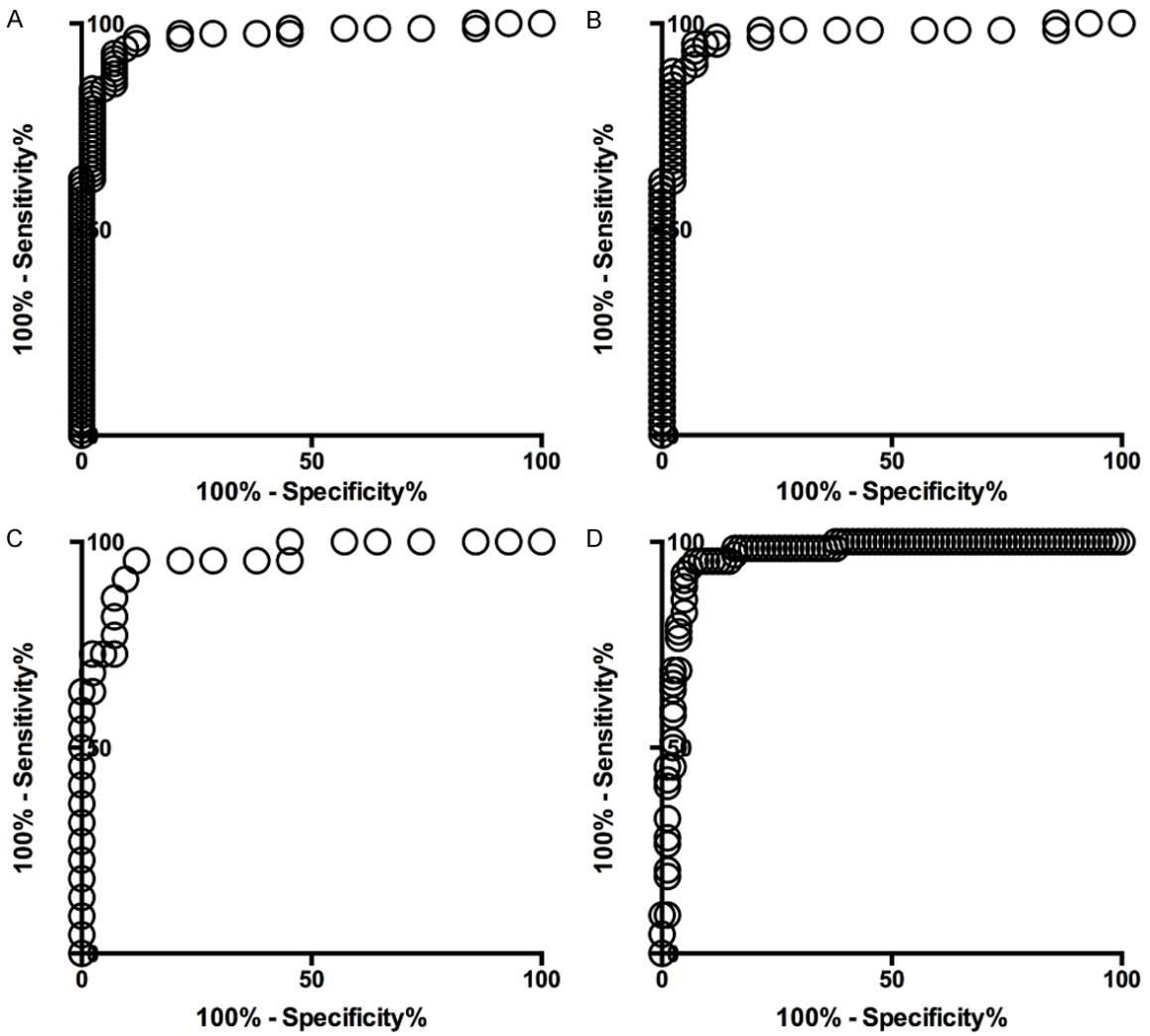

Figure 2. ROC curve demonstrating sensitivity-specificity distribution of when urinary BLCA1 levels are grouped as (A) Normal vs. urothelial cancer; (B) Normal urine vs. urothelial bladder cancer; (C) Normal vs. upper tract urothelial cancer; and (D) benign urological disorders vs. urothelial cancer.

cessed with 2-tailed and the *P* value of < 0.05 was accepted as statistically significant.

Results

The general demographic and clinicopathological data of the patients were summarized in **Table 1**. The reading of urinary BLCA1 level was presented in OD/μg. Urinary BLCA1 level in UBC and UTUC patients were significantly higher than that in normal controls (**Figure 1A**). This was also proven when urothelial cancers were grouped as a whole (**Figure 1A**), where as the level did not differ between UBC and UTUC samples. BLCA1 was also significantly higher in urothelial cancer samples compared to other urological disorders such as patients with long-dwelling catheter, glandular cystitis, BPH and other benign conditions (**Figure 1A**). When we studied the value of BLCA1/4 in the indication of recurrence of UBC, we first investigated the emergence of bulky mass in the cystoscopic follow-ups. Although there was only one case confirmed by pathology to be a cancer recurrence, the rest 3 cases all showed atypical cells microscopically. We found that the level of both BLCA1/4 was apparently higher in the case with cancer recurrence and was insignificantly changed in the rest 3 cases (**Figure 1B**). When grouped together, neither BLCA1/4 nor the mean of BLCA1 and BLCA4 showed the significant predictive power (**Figure 1B**).

We than studied whether BLCA1 level differed in different tumor grade and stage of UBC. We found that although an increasing trend of BLCA1 level was noticed in progressed grade, the difference was not significant (**Figure 1C**). The urinary BLCA1 level was not significantly different between different tumor stages (**Figure 1D**). It was also noted that BLCA1 was not significantly different between bladder cancer with different muscle involvement (**Figure 1E**).

We then studied the optimal urinary BLCA1 level cut-off to detect UBC. The ROC curve showed a satisfactory AUC to detect urothelial cancers (UC = UBC + UTUC) out of normal urine (**Figure 2A**). When divided in to UBC and UTUC respectively, we found that the Youden's index was maximal when the cut-off value was at 0.0009 OD/μg, yielding a sensitivity of 92.7% and a specificity of 92.9% for UBC (**Figure 2B**). Nonetheless, this cut-off value could not distinguish UBC from UTUC (**Figure 2C**). We then compared urinary BLCA1 level between UC and benign urological disorders and the cut-off value still yielded a high sensitivity and specificity (**Figure 2D**).

Discussion

In the current study, we have showed that urinary BLCA1 could be used to detect urothelial cancer with high sensitivity and specificity. This study, together with our previous reports, confirms the role of BLCA1/4 in UC, especially in UBC, which is a common malignancy and entails lifelong surveillance. It has been reported that BLCA4 was also present in adjacent pathologically "normal" bladder tissue, as confirmed in UBC animal models induced by MNU [2, 10]. It has therefore been proposed that BLCA4 participates in the early tumorigenic process of UBC, though not confirmed. As BLCAs are NMPs, which are the skeleton of nuclei, it is possible that BLCAs are not solely a tumor marker but exert certain functions in UBC. The Getzenberg group reported in 2004 cloning the BLCA4 sequence and matched a possible gene to the protein. They speculate that the BLCA4 gene could be a member of ETS transcriptional factor family and could be homologous to the ELK-3 gene. Using the cDNA they once believed it to be, they showed that BLCA4 could modulate the transcription of a series of oncogenes and could substantially promote the proliferation of UBC cells. Unfortunately, relevant reports were retracted in 2014 as the authors now think the cDNA could possibly be attributed to a wrong gene, leaving the BLCAs' origin still an enigma [11, 12].

Our group previously used IHC to study the correlation between BLCA4 and a series of angiogenic factors an found that BLCA4 expression was positively correlated with expression of IL-1α, IL-8, VEGF, and MMP-9, but not correlated with that of PEDF, TNF-α or microvessel density (MVD). TNF-α is considered to be associated with chronic inflammation, which incurs angiogenesis. Lack of association between BLCA4 and TNF-α indicates that BLCA4 is not an inflammation-related factor. PEDF is the most potent natural anti-angiogenic factor. Lack of association between BLCA4 and PEDF also indicates BLCA4 does not directly participate in the angiogenic process. Meanwhile, IL-1α, IL-8, VEGF, and MMP-9 are currently believed to promote tumor aggressiveness in multiple ways other than solely angiogenesis. Taken together, BLCA4 could play a pro-tumorigenic role via signaling other than angiogenesis.

BLCA1 is also one of the 6 NMPs the Getzenberg group extracted. In 2005, Myers-Irvin et al. [9] studied BLCA1 level in UBC tissue and urine and found that unlike BLCA4 that is expressed in both tumor and adjacent tissue, BLCA1 was solely present in morphologically tumor tissue, indicating a different role of BLCAs in UBC tumorigenesis. Notwithstanding that, urinary BLCA1 is significantly higher in UBC patients compared with normal candidates. The level of urinary BLCA1 is not associated with tumor grade. BLCA1 also exhibit high specificity distinguishing prostate cancer and kidney cancer, and yielding a sensitivity of 80% and specificity of 87%, making it a promising detecting tool for UBC.

In this study, we showed that BLCA1 is actually highly specific and sensitive for urothelial cancer. Given the common origin of the urothelium, it warrants further study on other inherent differences between UBC and UTUC both at genetic and molecular level, as the two entities exhibit similar but not the same biological behavior.

Acknowledgements

The authors declare that no conflict of interest exists. This study was supported in part by the Science and Technology Commission Shanghai Municipality. (Grant No. 124119a2000).

Disclosure of conflict of interest

None.

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