Int J Clin Exp Pathol 2012;5(5):422-427
www.ijcep.com /ISSN: 1936-2625/IJCEP1204006

Original Article

High expression level of BLCA-4 correlates with poor prognosis in human bladder cancer

Qian Zhao[1]*, Wen-Hao Shen[2]*, Zhi-Wen Chen[2], Zhan-Song Zhou[2], Hui-Xiang Ji[2]

*[1]Urology Department, Kunming General Hospital of Chengdu Military Region. No. 212, Daguan RD, Kunming, 650032; [2]Urology Department, Southwest Hospital Affiliated to Third Military Medical University. *Contributed to this paper equally.*

Received April 6, 2012; accepted May 11, 2012; Epub May 23, 2012; Published June 30, 2012

Abstract: Objectives: To evaluate the association between BLCA-4 tissue expression and patients' prognosis in bladder cancer (BC). Methods: BLCA-4 expression was analyzed using immunohistochemical staining methods on tissue samples from a consecutive series of 325 BC patients who underwent resections between 2000 and 2006. The correlation of BLCA-4 expression and patients' clinicopathological parameters was evaluated. Survival analysis was performed using the Kaplan-Meier method and Cox's proportional hazards model. Results: BLCA-4 was highly expressed in 54.8% of the BC patients. BLCA-4 overexpression was significantly associated with tumor grade ($P<0.001$), and stage ($P<0.001$). Kaplan-Meier survival analysis showed that high expression level of BLCA-4 resulted in a significantly poor prognosis of BC patients. Multivariate analysis revealed that the BLCA-4 expression level was an independent prognostic parameter for the overall survival rate of BC patients. Conclusions: These findings provide evidence that high expression level of BLCA-4 serves as a poor prognostic biomarker for BC. BLCA-4 may be a potential target of antiangiogenic therapy for BC.

Keywords: BLCA-4, bladder cancer, biomarker, prognosis, immunohistochemical analysis

Introduction

It has been estimated that nearly 70, 530 new cases of bladder cancer (BC) were diagnosed in the United States in 2010, with approximately 14, 680 people dying of the disease [1]. BC is the most common genitourinary malignancy in China, and the incidence of this disease is gradually increasing [2]. Despite improvement in surgical techniques, 5-year disease-free survival (DFS) and cancer specific survival (CSS) after radical cystectomy (RC) remains between 50% and 70% [3, 4]. Conventional clinicopathological parameters, such as tumor-node-metastasis (TNM), stage and grade of the tumor, as prognostic tools for patient counseling and treatment decisions. While these parameters have provided useful estimates of survival outcome, the heterogeneity of tumor biology leads to large variation in outcomes within each stage and grade. Therefore, specific molecular markers which could serve as standard prognostic factors are needed.

Previous studies [5, 6] describe the isolation of six proteins (BLCA-1 to -6) found to be uniquely expressed in the tumor tissue of patients with bladder cancer. BLCA-4, one of the most abundant of these proteins, was isolated by excising gel spots from negatively stained two-dimensional gels. The gel spots were then concentrated to obtain protein sequences and synthesized for antibody production. The first resulting peptide sequence was EISQLNAG, with a 75% homology with a number of nonvertebrate proteins including the epidermin biosynthesis protein, whereas the second peptide of BLCA-4 was VYEDIMQK, with a 75% homology with the ERECTA receptor protein kinase according to the BLAST database [7]. BCLA-4 is a nuclear transcription factor present in bladder tumors and adjacent benign areas of the bladder, but not in benign urothelium. BLCA-4 is one of six such factors which are promising tumor markers in bladder cancer detection. This protein is tested by an ELISA on voided urine. Preliminary

studies indicate a sensitivity of 89%-96% with a specificity of 100% for bladder cancer [8-12]. However, there have been no reports on the actual expression level of BLCA-4 and the correlation between clinicopathologic features and prognosis of BC patients. In this study, we explored the expression of BLCA-4 and investigated its clinicopathological and prognostic significance in human BC.

Materials and methods

Tissue specimens

BC specimens were obtained from 325 patients who underwent surgery between January 2000 and December 2006. Their diagnosis was made by a pathological examination, and all patients did not receive any preoperative treatment before admission. The histomorphology of all specimens was assessed by the Department of Pathology at Southwest Hospital Affiliated to Third Military Medical University. Histological cell types were assigned following the WHO classification criteria. Specimens were fixed in 10% formaldehyde and embedded in paraffin for histological sectioning. Clinical information was collected and stored in a database. Follow-up information for all participants was obtained every 3 months by telephone, at a visit or via a posted questionnaire. During the follow-up period, overall survival was measured from diagnosis to death or the last follow-up (5 years). The death of a patient was ascertained by reporting from the family and verified by a review of public records.

Immunohistochemical analysis

All 325 tissue specimens were subjected to immunohistochemical analysis using the avidin-biotin-peroxidase method. Sections were deparaffinized in xylene and dehydrated using a graded alcohol series before endogenous peroxidase activity was blocked with 0.5% H_2O_2 in methanol for 10 minutes. Nonspecific binding was blocked by incubating sections with 10% normal goat serum in phosphate-buffered saline (PBS) for 1 hour at room temperature. Without washing, sections were incubated with anti-BLCA-4(1:100; Abnova, Taipei, Taiwan) in PBS at 4ºC overnight in a moist box. Biotinylated goat anti-rabbit immunoglobulin G (IgG) (1:400; Sigma, St. Louis, MO, USA) was incubated with the sections for 1 hour at room temperature and detected with a streptavidin-peroxidase complex. The brown color indicative of peroxidase activity was developed by incubating sections with 0.1% 3, 3-diaminobenzidine (Sigma) in PBS with 0.05% H_2O_2 for 5 minutes at room temperature. The tissue specimens were viewed separately by two pathologists under double-blinded conditions, where they had no prior knowledge of the clinical or clinicopathological status of the specimens. Expression of BLCA-4 in the BC specimens was evaluated by scanning the entire tissue specimen under low magnification (×40), and then confirmed under high magnification (×200 and×400). An immunoreactivity score (IRS) system was applied as described elsewhere [14]. The percent of positive cells was scored as '0'(<5%, negative), '1'(5%-25%, sporadic), '2'(25%-50%, focal), and'3'(>50%, diffuse), respectively. The staining intensity was scored as '0' (no staining), '1'(weakly stained), '2'(moderately stained) and '3' (strongly stained), respectively. Both the percent of positive cells and cell staining intensity were decided in a double-blinded manner. The final BLCA-4 immunostaining score was calculated using the percent of positive cell score × staining intensity score ranging 0-9. High BLCA-4 expression level was defined as a total score ^{3} 4, and low BLCA-4 expression level as a total score < 4.

Statistical analysis

All statistical analyses were performed using SPSS software (version 17. 0; SPSS Inc., Chicago, IL, USA). Correlation of BLCA-4 expression with immunohistochemistry and clinicopathologic parameters was evaluated by chi-square test or Fisher's exact probability test. Survival curves were plotted using the Kaplan-Meier product-limit method, and differences between survival curves were tested using the log-rank test. Cox's proportional hazards model was used to identify the factors that had a significant influence on survival. Statistical significance was set at p<0. 05.

Results

Expression of BLCA-4 in BC tissues

The expression level of BLCA-4 protein in 105 BC tissue samples was measured with immunohistochemical staining. BLCA-4 localized at the nuclei of tumor cells. Overall, BLCA-4 was posi-

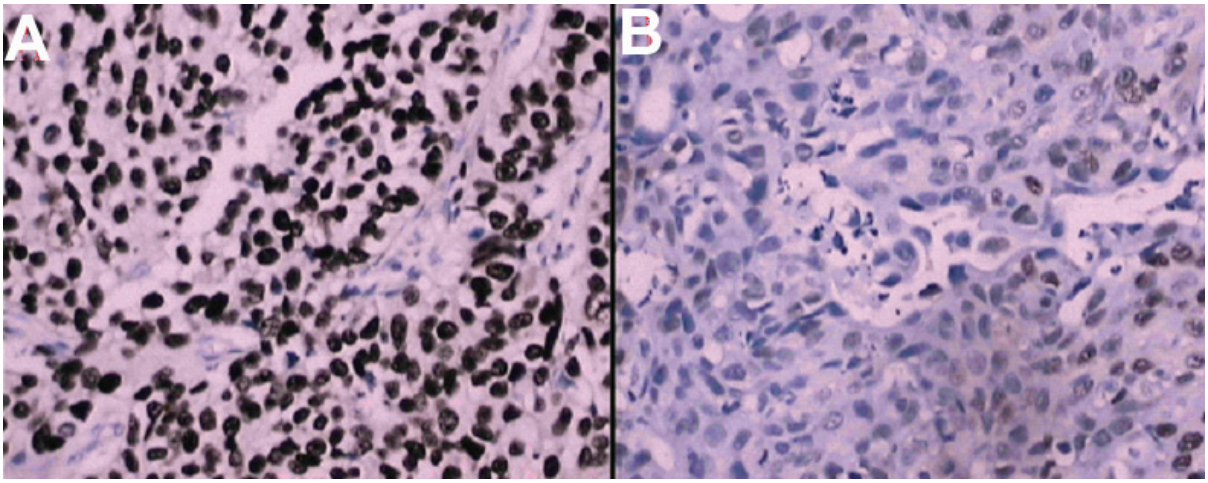

Figure 1. Immunohistochemical analysis of BLCA-4 in bladder cancer patients. A: High expression level of BLCA-4; B:Low expression level of BLCA-4; A, B: Original magnification×200.

Table 1. Correlation Between BLCA-4 Protein Expression and Clinicopathologic Features of the Patients With BC.

		BLCA-4 expression		
Parameter	Total(n=325)	Low expression(n=147)	High expression(n=178)	P value
Gender				
Female	85	45	40	0. 525
Male	240	102	138	
Age(years)				
≤65	131	61	70	0. 734
>65	194	86	108	
Tumor Size(cm)				
≤3	176	85	91	0. 264
>3	149	62	87	
Tumor number				
Unifocal	140	55	85	0. 072
Mutlical	185	92	93	
Grade				
G1	91	66	25	<0. 001
G2-G3	234	81	153	
T stage				
Ta-T1	201	116	85	<0. 001
T2-T4	124	31	93	

tively and negatively expressed in 254 (78. 2%) and 71 (21. 8%) of the 325 BC patients, respectively. BLCA-4 was highly and lowly expressed in 178 (54. 8%) and 147 (45. 2%) of the 325 BC patients, respectively (**Figure 1**). BLCA-4 overexpression was significantly associated with tumor grade (P<0. 001), and stage (P<0. 001), but not with age, gender, tumor size, and tumor number (**Table 1**).

Univariate and multivariate analyses of prognostic variables in BC patients

The prognostic effect of BLCA-4 on the overall survival rate of BC patients with a high or low BLCA-4 protein expression level was compared using Kaplan-Meier survival curves and the log-rank test respectively, showing that high expression level of BLCA-4 protein was a significant

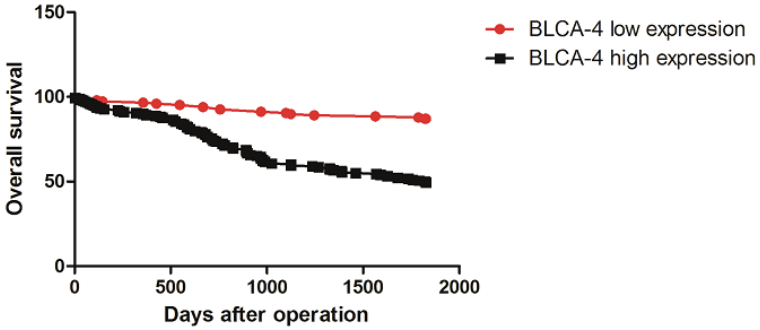

Figure 2. Overall survival rate of bladder cancer patients estimated according to BLCA-4 expression level in bladder cancer tissue samples (Kaplan-Meier method) with immunohistochemical staining (P<0. 001).

Table 2. Univariate analysis and multivariate analysis showing the overall survival rate for bladder cancer patients

Variables	Univariate analysis			Multivariate analysis		
	HR	95%CI	P value	HR	95%CI	P value
BLCA-4	1. 987	1. 023-3. 674	<0. 001	1. 876	1. 082-3. 543	<0. 001
Age	0. 867	0. 435-1. 435	0. 354	0. 846	0. 482-1. 324	0. 126
Gender	0. 679	0. 367-1. 231	0. 442	0. 985	0. 623-1. 584	0. 231
Tumor size	1. 342	0. 897-1. 964	0. 078	1. 231	0. 987-2. 856	0. 063
Histologic grade	1. 213	0. 768-2. 324	0. 014	1. 356	0. 823-1. 978	0. 013
Tumor number	1. 432	1. 121-2. 465	0. 453	1. 543	1. 112-1. 968	0. 078
Tumor stage	1. 465	0. 967-1. 956	0. 003	1. 213	0. 684-1. 563	<0. 001

prognostic factor for poor overall survival rate of BC patients. The 5-year survival rate of BC patients with a high or a low BLCA-4 protein expression level was 50% and 89.8%, respectively. A significant difference was observed on the Kaplan-Meier survival curves for BC patients with a high or a low expression level of BLCA-4 (P<0. 001, log-rank test, **Figure 2**). Univariate Cox regression analysis also identified that clinical variables including age, sex, tumor size, tumor number, tumor grade, and tumor stage and BLCA-4 expression were significantly associated with overall survival (**Table 2**). Furthermore, to evaluate the potential of BLCA-4 expression as an independent predictor for overall survival of BC, multivariate Cox regression analyses were performed. While the others failed to demonstrate independence, tumor grade, tumor stage and BLCA-4 expression may play a role in predicting the overall survival in BC (**Table 2**).

Discussion

Despite many advances in diagnostic imaging of tumors, combination chemotherapy, and radiation therapy, little improvement has been achieved within the last decade in terms of prognosis and quality of life for patients with BC. Given the frequent failure of conventional treatment strategies, many cancer-related molecules have been characterized toward the goal of developing novel anticancer therapies such as molecular-targeted drugs and antibodies or cancer vaccines [13, 14]. Molecular-targeted therapies are expected to be highly specific to malignant cells, with minimal adverse effects due to their well-defined mechanisms of action. Equally desirable in prospect are minimally invasive, highly sensitive, and specific new diagnostic methods that would adapt readily to clinical settings. From these points of view, tumor-specific transmembrane/secretory proteins should have significant advantages because they are presented either on the cell surface or within the extracellular space and/or in serum, making them easily accessible as molecular markers and therapeutic targets. Some tumors specific markers already available, such as CYFRA or Pro-GRP, are transmembrane/secretory proteins [15, 16]; the example of rituximab (Rituxan), a humanized monoclonal antibody against CD20-positive lymphomas, provides

proof that targeting specific cell surface proteins can result in significant clinical benefits [17]. As an approach to identifying novel cancer-specific cell surface or secretory proteins, we have been exploiting the power of genome-wide expression analysis to select genes that are overexpressed in cancer cells. Analysis of candidate molecules revealed BLCA-4 as a potential target for development of novel tools for diagnosis and treatment of bladder cancer.

BCLA-4 is a nuclear transcription factor present in bladder tumors and adjacent benign areas of the bladder, but not in benign urothelium. BLCA-4 is one of six such factors which are promising tumor markers in bladder cancer detection. This protein is tested by an ELISA on voided urine. Preliminary studies indicate a sensitivity of 89%-96% with a specificity of 100% for bladder cancer [8-12]. BLCA-4 seemed likely to have a potential role in tumor development or progression. In this study, we evaluated the association between BLCA-4 tissue expression and patients' prognosis in bladder cancer. In our tumour material, BLCA-4 was positively expressed in 78.2% of BC patients and highly expressed in 54.8% of BC patients. BLCA-4 overexpression was significantly associated with tumor grade ($P<0.001$), and stage ($P<0.001$), but not with age, gender, tumor size, and tumor number (**Table 1**). In this study, the prognosis of BC patients with a high expression level of BLCA-4 was poor, and Cox regression analysis indicated that high expression level of BLCA-4 was a significant prognostic factor for a poor overall survival rate of BC patients, suggesting that BLCA-4 may become a novel prognostic marker for BC.

In conclusion, our results provide a basis for the concept that high expression level of BLCA-4 in bladder cancer may be important in the tumor progression and serves as an independent biomarker for poor survival. Thus, high expression level of BLCA-4 identifies patients at high risk and is a potential novel therapeutic target for bladder cancer.

Funding

This study was supported by a grant from Chongqing Municipal Major Scientific and Technological Project (CSTC, 2011AC5027).

Address correspondence to: Dr. Hui-Xiang Ji, Vice professor, Urology Department, Southwest Hospital Affiliated to Third Military Medical University. No. 33, Gaotanyanzheng RD, Chongqing, 400038, China. E-mail: huixiangji@yahoo.com.cn

References

[1] Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA Cancer J Clin 2010; 60: 277-300.

[2] Zhang W, Xiang YB, Liu ZW, Fang RR, Ruan ZX, Sun L, Gao LF, Jin F, Gao YT. Trends analysis of common urologic neoplasm incidence of elderly people in Shanghai, 1973-1999. Ai Zheng 2004; 23: 555-558.

[3] Ghoneim MA, Abdel-Latif M, el-Mekresh M, Abol -Enein H, Mosbah A, Ashamallah A, el-Baz MA. Radical cystectomy for carcinoma of the bladder: 2,720 consecutive cases 5 years later. J Urol 2008; 180: 121-127.

[4] Shariat SF, Karakiewicz PI, Palapattu GS, Lotan Y, Rogers CG, Amiel GE, Vazina A, Gupta A, Bastian PJ, Sagalowsky AI, Schoenberg MP, Lerner SP. Outcomes of radical cystectomy for transitional cell carcinoma of the bladder: A contemporary series from the Bladder Cancer Research Consortium. J Urol 2006; 176: 2414-2422.

[5] Getzenberg RH, Konety BR, Oeler TA, Quigley MM, Hakam A, Becich MJ and Bahnson RR. Bladder cancer-associated nuclear matrix proteins. Cancer Res 1996; 56: 1690-1694.

[6] Konety BR, Nguyen T-ST, Lavelle JP, Zeidel ML and Getzenberg RH. Expression of BLCA-4, a nuclear matrix protein (NMP) marker in a carcinogen induced model of bladder cancer. J Urol 2000; 163: abstract 597.

[7] Murphy WM and Soloway MS. Urothelial dysplasia. J Urol 1982; 127: 849-854.

[8] Konety BR, Nguyen TS, Brenes G, Sholder A, Lewis N, Bastacky S, Potter DM, Getzenberg RH. Clinical usefulness of the novel marker BLCA-4 for the detection of bladder cancer. J Urol 2000; 164: 634-639.

[9] Van Le TS, Miller R, Barder T, Babjuk M, Potter DM, Getzenberg RH. Highly specific urine-based marker of bladder cancer. Urology 2005; 66: 1256-1260.

[10] Feng CC, Wang PH, Guan M, Jiang HW, Wen H, Ding Q, Wu Z. Urinary BLCA-4 is Highly Specific for Detection of Bladder Cancer in Chinese Han Population and Is Related to Tumour Invasiveness. Folia Biologica 2011; 57: 242-247.

[11] Konety BR, Nguyen TS, Dhir R, Day RS, Becich MJ, Stadler WM, Getzenberg RH. Detection of Bladder Cancer Using a Novel Nuclear Matrix Protein, BLCA-4. Clinical Cancer Research 2000; 6: 2618-2625.

[12] Myers-Irvin JM, Van Le TS, Getzenberg RH. Mechanistic Analysis of the Role of BLCA-4 in Bladder Cancer Pathobiology. Cancer Res 2005; 65: 7145-7150.

[13] Kelly K, Crowley J, Bunn PA Jr, Presant CA,

Grevstad PK, Moinpour CM, Ramsey SD, Wozniak AJ, Weiss GR, Moore DF, Israel VK, Livingston RB, Gandara DR. Randomized phase III trial of paclitaxel plus carboplatin versus vinorelbine plus cisplatin in the treatment of patients with advanced non-small-cell lung cancer: a Southwest Oncology Group trial. J Clin Oncol 2001; 19: 3210-3218.

[14] Hennessy BT, Hanrahan EO, Daly PA. Non-Hodgkin lymphoma: an update. Lancet Oncol 2004; 5: 341-353.

[15] Pujol JL, Grenier J, Daures JP, Daver A, Pujol H, Michel FB. Serum fragment of cytokeratin subunit 19 measured by CYFRA 21-1 immunoradiometric assay as a marker of lung cancer. Cancer Res 1993; 53: 61-66.

[16] Miyake Y, Kodama T, Yamaguchi K. Pro-gastrin-releasing peptide (31-98) is a specific tumor marker in patients with small cell lung carcinoma. Cancer Res 1994; 54: 2136-2140.

[17] Tuck AB, O'Malley FP, Singhal H, Harris JF, Tonkin KS, Kerkvliet N, Saad Z, Doig GS, Chambers AF. Osteopontin expression in a group of lymph node negative breast cancer patients. Int J Cancer 1998; 79: 502-508.