Bladder cancer-associated protein is suppressed in human cervical tumors

MIN PENG[1,3], TINGBO XIE[2,3], JUAN YU[2,3], BIN XU[1], QIBIN SONG[1] and XINXING WU[3]

[1]Oncology Center, Renmin Hospital of Wuhan University; [2]Wuhan Institute of Biologic Products;
[3]Institute of Virology, School of Medicine, State Key Laboratory of Virology,
Wuhan University, Wuhan, Hubei, P.R. China

Abstract. Bladder cancer-associated protein (BLCAP) is downregulated in bladder cancer and has been identified as a prognostic biomarker for human bladder cancer. We previously reported that BLCAP mRNA is decreased in cervical cancer tissues, and overexpression of BLCAP was found to inhibit cell growth and induce apoptosis in the human cervical cancer HeLa cell line To investigate the BLCAP protein expression in cervical cancer and its potential clinical indications, we developed a polyclonal antibody against human BLCAP to assess the BLCAP protein expression in 30 cervical cancer tissues and 30 non-tumor cervical tissues from patients. Western blotting data showed that a single band of recombinant protein was probed by antiserum of BLCAP and no band was probed by pre-immune serum. BLCAP expression was significantly downregulated in cervical carcinoma tissues compared with its expression in the non-tumor cervical tissues. Moreover, cervical carcinoma tissues from patients with stage III-IV had significantly lower BLCAP expression percentage compared with stage I-II. Similarly, a significantly lower BLCAP expression percentage was observed in moderately/poorly differentiated tumor tissues and in the tumor tissues from patients with lymphatic metastasis (LM) compared with well-differentiated tumor tissues and non-LM patients, respectively. Our results suggest that decreased BLCAP protein expression is associated with poor prognosis and it could be a potential bio-index to predict cervical tumor patient outcome.

Introduction

Cervical carcinoma is the second leading cause of cancer-related death in women worldwide, with over 500,000 new cases reported each year in developing countries. High-risk human papillomavirus (HPV) infection such as HPV16 and/or HPV18 is regarded as the major etiological factor for cervical carcinoma (1,2), however, genetic factors may play an important role in its occurrence. The activation of oncogenes and/or the inactivation of tumor-suppressor genes play an important role in tumor development and progression (3,4). Therefore, it is important to study the genes associated with cervical carcinoma at the molecular level.

The bladder cancer-associated protein (*BLCAP*) gene (GenBank accession no. NM006698) is located on chromosome 20 and is a novel tumor-suppression gene identified from human bladder carcinoma. The mRNA level of *BLCAP* has been found to be markedly downregulated in bladder carcinoma (5,6), as well as human tongue carcinoma (7). In our previous study, BLCAP mRNA expression was decreased in cervical tumor tissues (7) and overexpression of *BLCAP* was found to inhibit cell growth and to induce apoptosis in the human cervical cancer HeLa cell line (8). Since *BLCAP* may be a cervical carcinoma-related suppressor gene, it is important to determine protein expression levels in cervical carcinoma.

Here, we established a pET prokaryotic expression system to express the His-tagged BLCAP fusion protein to immune rabbits for preparing the polyclonal antibody. This purified BLCAP polyclonal antibody was used to detect the BLCAP protein expression level in 30 cervical carcinoma tissues and 30 normal cervical tissues.

Materials and methods

Plasmid construction. The primer sequences of the BLCAP were designed with Primer 5.0 software, and the coding region of the mature BLCAP protein was amplified using polymerase chain reaction (PCR). Primer 1 (5'-GCA **GAA TTC** ATG TAT TGC CTC CAG TG-3') and primer 2 (5'-GC **AAG CTT** TTA GGT GCC CAC AAC G-3') were synthesized by Invitrogen (Shanghai, China). Primer 1 was synthesized with an *Eco*RI site (shown in bold) and primer 2 was synthesized with a *Hin*dIII site (shown in bold). The amplification profile included one initial hot-start denaturing step at 94°C for 5 min, followed by 30 cycles of the following conditions: 94°C for 1 min, 58°C for 30 sec, and 72°C for 1 min, and a final extension at 72°C for 10 min. The expression vector pET-32(a) was digested with *Eco*RI and *Hin*dIII. The digested pET-32(a) was purified by agarose gel and extracted using the QIAquick Gel Extraction kit.

A recombinant plasmid was constructed by inserting the PCR amplified fragment (also digested with *Eco*RI and *Hin*dIII) into the pET-32(a) vector and transformed into the *E. coli* strain Rosetta. The transformants [pET-32(a)-BLCAP] were confirmed by PCR, restriction enzyme digestion and DNA sequencing.

Prokaryotic expression and purification of full length BLCAP. The prokaryotic expression vector pET-32a-BLCAP was introduced into the bacterial host *E. coli* strain Rosetta following standard protocol. Rosetta, the derivational strain of BL21, contains the extra gene copies for coding rare tRNA, which facilitates the prokaryotic expression of eukaryotic protein in *E. coli* Rosetta. The transformants were cultured in LB medium containing ampicillin (100 μg/ml) at 37°C in a shaking incubator until an OD600 of 0.8 to 1.0 was attained. Recombinant BLCAP expression was induced by adding 1 mM isopropyl-1-thio-D-galactopyranoside (IPTG) and further incubation at 37°C for 6 h. The cells were harvested by centrifugation at 5,000 x g for 15 min at 4°C, and the pellets were re-suspended in lysis buffer [50 mM Tris (pH 8.9), 100 mM NaCl, 1 mM EDTA, 100 μg/ml lysozyme, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF) and 1 μg/ml each of pepstatin, leupeptin and aprotinin], and further incubated at 4°C for 1 h. Thereafter, the cells were sonicated and centrifuged at 12,000 x g for 30 min. The clear supernatant was collected and used for protein purification.

The recombinant protein was purified based on its N-terminal His$_6$-tag by affinity chromatography using a Ni^{2+}-NTA HiTrap chelating Sepharose column (Qiagen) that was equilibrated with 20 ml buffer A containing 10 mM imidazole. The cell lysate was applied to the column and allowed to bind using a flow rate of 1.0 ml/min. The bound protein was eluted by applying a gradient of 10-500 mM imidazole in buffer A using a flow rate of 1.5 ml/min. Peak fractions were collected between 200 and 300 mM imidazole. The Bradford method was used to determine the protein concentration, and the protein was confirmed by western blot analysis using an anti-His monoclonal antibody.

Production and purification of polyclonal antibody against BLCAP. New Zealand white rabbits received an intradermal injection of BLCAP protein (500 μg/rabbit) mixed with CFA in a 1:1 ratio. After 4 week, the rabbits were boosted subsequently 3 times at two-week intervals with the BLCAP protein (250 μg/rabbit) mixed with IFA in a 1:1 ratio. Prior to immunization, blood samples were taken from the marginal vein of the rabbit ear, and the sera were obtained to determine the antibody titer by western blotting. The polyclonal antibody was purified by following a standard protocol for the purification of the antibody.

Western blot analysis and agar gel precipitin test. Purified proteins were analyzed by western blot analysis (12% SDS PAGE gel) following a standard procedure. After an overnight blocking in 5% BSA the membrane was incubated with an anti-BLCAP fusion protein polyclonal antibody for 1 h at room temperature (RT) and then incubated in alkaline phosphatase (Ap)-conjugated goat anti-rabbit IgG at a dilution of 1:1500 for 1 h at RT. The membrane was washed and the specific protein bands were visualized using 3,3'-diaminobenzidine

(DAB). To determine antibody titer, the agar gel precipitin test was performed as described previously with modification (9). Briefly, a cluster of six wells surrounding a center well was cut into the solidified agar. Antiserum (25 μl) with a series of dilution (1/2, 1/4, 1/8, 1/16, 1/32 and 1/64) was delivered to the outside well, and purified BLCAP antigen was delivered to the center well. The plates were incubated for 24 h and the precipitin reaction was determined.

Patient tissue specimens. A total of 60 cervical specimens consisting of 30 age-matched carcinoma tissues and 30 normal cervical tissues were collected from the Pathology Department at Zhong Nan Hospital (Wuhan University, China) between 2005 and 2006. The patients were evaluated based on the AJCC TNM classification system (10) as: stage I-II (n=16), stage III-IV (n=14); 13 patients had lymphatic metastasis; well-differentiated tumors (n=16) and moderatedly/poorly differentiated tumors (n=14); squamous cell (SCC) tumors (n=26) and adenosquamous (AC) tumors (n=4). The average age of the patients was 43.3 years ranging from 33 to 63. None of the patients received radiochemotherapy prior to surgery.

Immunohistochemistry. Sections (5-μm thick) from paraffin-embedded blocks were mounted on slides. These sections were deparaffinized and then rehydrated with gradient alcohols. Heat-induced antigen retrieval was performed in citrate buffer (pH 6.0) by heating the slides in a microwave oven (700 W for 15 min) and then processed for immunohistochemistry. In brief, the tissue sections were washed three times with phosphate-buffered saline (PBS) and incubated with normal horse serum for 30 min at RT to block non-specific binding. Endogenous peroxidase activity was quenched by incubating sections in 3% H$_2$O$_2$ in PBS for 20 min. Sections were then incubated with anti-BLCAP rabbit polyclonal antibody for 60 min at RT and washed with PBS-T containing 0.05% Tween-20 (3 x 5 min) before incubating with horseradish peroxidase-labeled secondary antibody for 30 min. Slides were washed again (3 x 5 min) with PBS-T. The reaction color was developed by incubating sections with 3,3'-diaminobenzidine reagent (DAB) as per the manufacturer's instructions. The slides were washed with water, counterstained with hematoxylin, dehydrated, mounted and examined under light microscopy. A normal murine IgG was used for the negative control.

Evaluation of immunohistochemistry (IHC) and statistical analysis. IHC was performed as described previously. Immunostaining was graded as negative (no cells stained), weak (<10% cells stained), moderate (11-50% cells stained) and strong (>51%). For statistical analysis, the staining results were classified into two groups: group I was the 'negative group' (no staining); and group II the 'positive group' (weak intensity, moderate intensity and strong intensity). Statistical analysis for group differences was performed with the χ^2 test. For all statistical tests, a P-value <0.05 was considered statistically significant.

Results

Construction of expression plasmid pET-32a-BLCAP. The 264 bp fragment of the BLCAP gene was amplified by PCR.
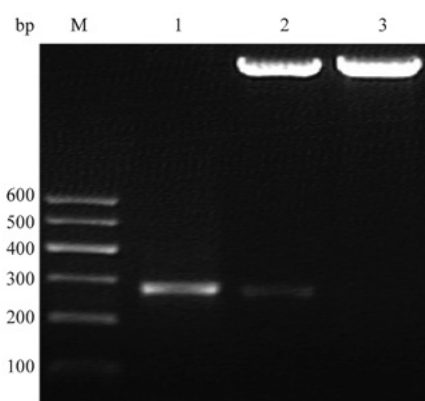
Figure 1. Identification of the recombinant pET-32a-BLCAP by enzymatic digestion. Lane 1, PCR product of the BLCAP gene; lane 2, pET-32a-BLCAP/*Eco*RI + *Hin*dIII; lane 3, pET-32a *Eco*RI + *Hin*dIII.
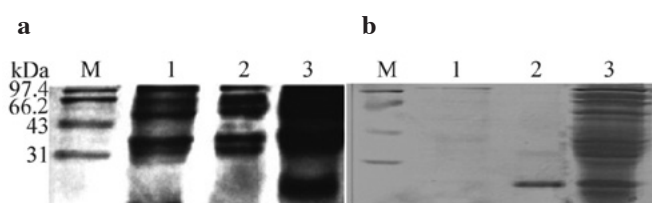

Figure 2. Proteins were separated on 12% acrylamide gel and stained with Coomassie blue. (a) Expression of the recombinant protein. Lane M, molecular mass markers; lane 1, cells with pET-32a after IPTG induction for 6 h; lane 2, cells with pET-32a-BLCAP before IPTG induction; lane 3, cells with pET-32a-BLCAP after IPTG induction for 6 h. (b) Purification of the recombinant protein. Proteins were separated on 12% acrylamide gel and stained with Coomassie blue. Lane M, molecular mass markers; lane 1, purified proteins of cells with pET-32a after IPTG induction (as a positive control); lane 2, purified recombinant proteins; lane 3, cells with pET-32 a-BLCAP after IPTG induction. The square frame indicates the BLCAP bands.

The product was cloned into pET-32a and confirmed by PCR and restriction digestion (Fig. 1). DNA sequencing revealed that DNA was the reported sequence. The recombinant plasmid DNA contained the BLCAP gene in-frame with N-terminal Trx-tag and His-tag encoding the ~28 kDa, 762-amino acid fusion protein.

Expression, purification and analysis of the protein. After induction with IPTG, *E. coli* Rosetta transformed with pET-32a-BLCAP produced a 28-kDa protein shown in Fig. 2a. The size of the protein matched its theoretical molecular weight. To determine the optimal induction period, we used different IPTG concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mM), different temperatures (20, 25, 30 and 37˚C), and varied induction times (1, 2, 3, 4, 5, 6, 8 and 12 h). The results showed that the yield was increased with an incubation time and reach a plateau after 6 h. The final concentration of IPTG used in this experiment was 1 mmol/l, and the bacteria were cultivated at 37˚C for 6 h. Ni-NTA affinity chromatography was applied for purification of the BLCAP fusion protein. The target BLCAP appeared as a single band on SDS-PAGE (Fig. 2b, lane 2), which is in agreement with the molecular weight reported.
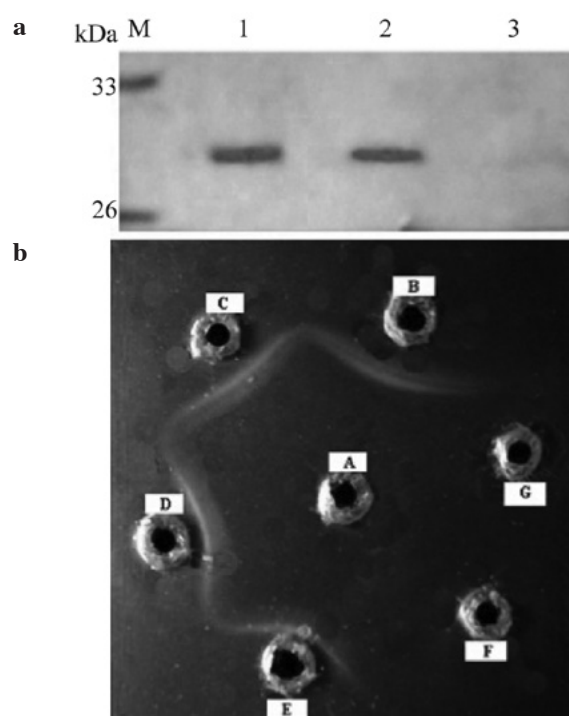

Figure 3. (a) Western blot analysis of recombinant protein using antiserum. Lanes 1 and 2, antiserum; lane 3, pre-immune serum. (b) Determination of antibody titer by agar gel precipitin test, A, antigen, B-G, diluted antiserum, 1/2, 1/4, 1/8, 1/16, 1/32 and 1/64, respectively.

Western blotting and titer determination. A homogenized preparation containing a single protein for BLCAP could be confidently used for immunizing the New Zealand White rabbit. The antiserum was collected 15 weeks after the initial injection. Three addition booster injections were given and the antiserum was again collected after each booster. A 1:8,000 dilution of the 28-kDa His-tagged BLCAP protein was detected in western blot analysis using IPTG-induced *E. coli* Rosetta cell extract. There was no immune-reactivity observed in un-induced cells (Fig. 3a). Agar gel precipitin test demonstrated that distinct bands were observed between the antigen and antiserum wells, as a result of the antigen migrating through the agar matrix toward and interacting with antiserum (Fig. 3b).

Tissue immunohistochemistry. The purified polyclonal antibody was used to detect BLCAP protein levels in cervical tissues by immunohistochemistry. This positive expression of BLCAP protein was detected in 27 of 30 (90%) normal cervical tissues with 15 of the 27 (55%) having moderate to strong cytoplasmic staining. However, in cervical carcinoma tissues 16 of 30 (53.33%) expressed BLCAP protein with 15 of the 16 (93.75%) having weak cytoplasmic staining (Table I). The expression level of BLCAP in cervical carcinoma was significantly lower than that in the corresponding normal tissues (P<0.05)(Fig. 4). Moreover, the expression level of BLCAP in cervical carcinoma was significantly associated with clinical degree and tumor differentiation of cervical carcinoma (P<0.05). No significant difference was observed between SCC and AC (Table II).

Table I. Immunohistochemical analysis of BLCAP expression in cervical tissues.

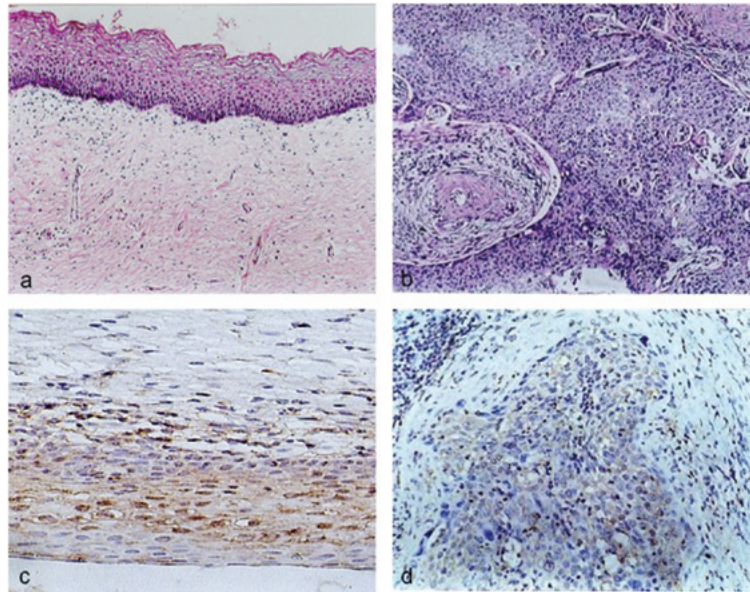| | Total | BLCAP expression | | | | Positive rate (%) |
		-	+	++	+++	
Normal cervical tissues	30	3	8	15[a]	4	90.00[a]
Cervical carcinoma tissues	30	14	15	1	0	53.33

[a]P<0.05.

Figure 4. Immunohistochemical staining of BLCAP in normal cervical and cervical carcinomal tissues. (a) H&E staining in normal cervical tissues (magnification, x100), (b) H&E staining in cervical carcinoma tissues (magnification, x100), (c) positive expression of BLCAP in normal cervical tissues (magnification, x200), (d) negative expression of BLCAP in cervical carcinoma tissues (magnification, x200).

Table II. Relationship between the expression of BLCAP and the clinical and histopathological features of cervical carcinoma.

Clinical pathological characteristic	n	BLCAP expression n (%)	P-value
Histological type			
SCC	26	14 (81.85)	>0.05
AC	4	2 (50)	
Stage			
I-II	16	10 (62.5)	<0.05
III-IV	14	6 (42.86)	
Degree of differentiation			
High	10	7 (70)	<0.05
Moderate/low	20	9 (45)	
Lymphatic metastasis			
Non-LM	17	12 (70.59)	<0.05
LM	13	4 (30.77)	
Total	30	16 (53.55)	

SCC, squamous cell carcinoma; AC, adenocarcinoma.

Discussion

The pET-32(a) vector which contains the thioredoxin (Trx) tag was used in this study to successfully produce a BLCAP fusion protein with a sufficient quantum. The Trx tag increases the molecular weight of the fusion protein. Athough this makes the protein more soluble it does not affect its activity (11,12). Western blot analysis revealed the molecular weight of the protein to be approximately 28 kDa (10 kDa plus the 18 kDa Trx/His protein) as predicted. To screen *E. coli* strains for expression, both Rosetta and BL21 were used as host cells to perform prokaryotic expression in the initial stage. The fusion proteins were expressed in the Rosetta but not in BL21. The reason is that BL21 contains rare tRNA codons in the coding sequence of BLCAP, which impedes the expression of eukaryotic protein (13,14). Using Rosetta as the host cell we optimized the culture and induction parameters to get a more soluble fusion protein.

The expressed His-tagged protein was purified by Ni^{2+} affinity chromatography column and monitored by western blotting with an anti-hexahistidine tag antibody. The purified recombinant proteins were found to be immunogenic in rabbits and produced polyclonal antibodies. Western blot analysis showed that this antibody was highly sensitive and specific.

The expression rate of BLCAP in cervical carcinoma tissues was significantly lower than that in the normal tissues (P<0.05). This suggests that decreased expression of BLCAP may be directly related to the development of cervical carcinoma. Meanwhile, the expression of BLCAP was related to clinical stage and cell differentiation, which indicates that the loss of BLCAP may be involved in tumor invasion and metastasis.

In conclusion, a high titer, highly specific BLCAP polyclonal antibody was produced. The differential expression of BLCAP at the protein level was consistent with that of mRNA levels. BLCAP may be a cervical carcinoma-related suppressor gene, which is the foundation for studying the function of BLCAP in clinical applications as a tumor marker.

Acknowledgements

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