BLCAP arrests G_1/S checkpoint and induces apoptosis through downregulation of pRb1 in HeLa cells

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Abstract. *BLCAP* (bladder cancer-associated protein) gene exhibited tumor suppressor function in different tumors and is regarded as a candidate tumor suppressor gene; however, the mechanism by which BLCAP exerts its function remains elusive. This study investigated the functional association between BLCAP and proliferation or apoptosis in cervical cancer cells, to identify the functional motifs of BLCAP. The *BLCAP*-shRNA expression vector based on pRNA-U6.1/Hygro plasmid was used to specifically inhibit BLCAP activity in HeLa cells. The optimal shRNA plasmid was selected to knock down *BLCAP* expression and the biological effects were investigated. The effects on cell cycle and apoptosis were detected by flow cytometric or Annexin V-FITC staining analysis. The gene expression profiles of HeLa cells transfected with blcap-wt and *BLCAP*-shRNA were analyzed using human signal pathway gene Oligochips. The levels of protein expression and interaction of BLCAP with Rb1 proteins were determined by western blotting and Co-IP assays. The site-specific mutagenesis assay was used to identify amino acid residues important for BLCAP. Significantly differentially expressed genes were found by gene Oligo chips analysis. These genes were all correlated with proliferation, cell cycle and apoptosis. The results of western blotting and Co-IP assays confirmed that overexpression of BLCAP could interact with Rb1 and inhibit Rb1 phosphorylation. Further investigation revealed that SAXX mutation in the key regions of BLCAP suppressed the function of BLCAP and significantly increased the level of phosphorylated Rb1 protein. Here our findings suggested that the functional association of BLCAP and Rb1 might play important roles in proliferation and apoptosis of HeLa cells. It suggested that BLCAP could be a novel therapeutic target for cervical cancer.

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Introduction

Cervical cancer is the second most common gynecological malignancy among women worldwide, and there are an estimated 530,000 cases of cervical cancer and 275,000 deaths from the disease per year (1,2). The mechanisms of cervical carcinoma formation remain unclear. Cervical carcinoma emerges from a defined series of preneoplastic lesions with increasing cellular dysplasia referred to as cervical intraepithelial neoplasia (CIN) grade I, II and III. The development and progression of cervical carcinoma have been demonstrated associated with various genetic and epigenetic events, especially alterations in the cell cycle checkpoint machinery. However, the steps of the progression from low-grade CIN to carcinoma remain elusive. Epidemiological studies have established a causal relationship between cervical carcinogenesis and infection of high risk HPV types (HR-HPV, such as HPV 16 and 18) (3). It is explicit that integration of HR-HPV DNA into the host cell genome resulting in persistent overexpression HPV E6 and E7 oncoproteins, subsequently induce immortalization of cells and allow virus to replicate through their inhibitory effects on the tumor suppressor proteins p53 and pRb, respectively (4-6). However the E6-p53 and E7-Rb model is not sufficient to inevitably produce cervical carcinoma (7). Other factors must have contributed to the initiation of the cervical cancer (8,9). Recent studies suggested a strong association between HR-HPV types and cell cycle regulators (10). It is well-known that the cell cycle is regulated by a family of cyclins (cyclins A, B, D, E), cyclin dependent kinases (CDKs, CDK1, CDK2, CDK4, CDK6) and their inhibitors (CDKIs) through activating and inactivating phosphorylation events. Attention has been focused on altered expression of G1

cyclins and Cdks because the major regulatory events leading to cell proliferation and differentiation occur within the G1 phase of the cell cycle (11-13). However, it is unclear how and when cell cycle factors that are innate to the HPV-infected cells, including genetic aberrations launch the host cell into an irreversible progression to cancer.

BLCAP is a small 87-amino acid, evolutionary conserved protein with no homology to any known protein in mammalians (14). Studies have shown *BLCAP* gene exerts tumor suppressor function in bladder cancer, osteosarcoma, tongue carcinoma, renal cancer, breast cancer and other malignant tumors (15,16). Our previous studies found that BLCAP protein putatively includes two trans-membrane domains, cytoplasmic domains at the N and C terminals, a phosphorylation site that might bind DNA. We found that *BLCAP* mRNA could be detected in normal cervical tissue, but it was absent or reduced in cervical cancer tissue. Overexpression of BLCAP could play its function as a tumor suppressor gene to inhibit the growth of cervical cancer cell line *in vitro* (17). However, little was known about the regulation and function of BLCAP protein. It seems likely that BLCAP might play a role not only in regulating cellular proliferation but also coordinating the cell cycle and apoptosis via a novel way independent of p53 and NF-κB as previously reported by us (18).

By analyzing the signal peptides of BLCAP protein, we found a PXXP (proline-X-X-proline) and an SPXX (Ser-Pro-X-X) motif located within it (17). In addition, using NetPhos and KinasePhos program analysis we identified several putative phosphorylation sites in the BLCAP protein: Ser66, Ser71 (Ataxia Telangiectasia Mutated phosphorylation site), Ser73 (cdc2 phosphorylation site) and Ser78 (casein kinase II phosphorylation site) (19-22). Through a computer-based search *BLCAP* was identified as a target of adenosine to inosine (A-to-I) by RNA editing.

In this study, we determined the molecular targets of BLCAP by protein interaction technology, combined with the cell cycle signal path analysis and apoptosis induction by regulating expression of key molecules to explore the molecular mechanism of *BLCAP* gene.

Materials and methods

Cell culture and reagents. Human cervical cancer cell line (HeLa) was used in this study. HeLa cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS, Invitrogen) at 37˚C in a humidified 5% CO_2 atmosphere. The sources of antibodies were as follow: antibodies against pRB1, E2F were from Cell Signaling Technology. Antibodies against cyclin D1, cyclin B1, CDK4, CDK6, caspase-3 and p53 were from Abcam, Inc. Antibodies against β-actin were obtained from Santa Cruz Biotechnology. Goat peroxidase (HRP), conjugated secondary antibodies, and protease inhibitor were from Roche. Chemiluminescence substrate was obtained from Pierce.

Plasmid construction. The pRNA-U6.1/Hygro vector was purchased from GenScript (Piscataway, NJ, USA) to use to construct shRNA plasmids of *BLCAP*. DNA template corresponding to *BLCAP* gene (GenBank accession no. NM006698) was used for design of shRNA. shRNAs of *BLCAP* gene were designed and synthesized through database of https://www.genscript.com. The sequences of shRNA are shown in Table I.

*Bam*HI/*Hin*dIII fragments from the sequences were subcloned into the same sites of pRNA-U6.1/Hygro to generate the pshRNA-B1, pshRNA-B2, pshRNA-B3 and pRNA-NS (a non-specific shRNA) plasmid, respectively. The recombinant vectors were confirmed by digestion analysis of restriction endonuclease and DNA sequencing. The full length cDNA of *BLCAP* was acquired from recombinant pCD-3.1(-)-*BLCAP* plasmid (it was stored by our group) and subcloned into pEGFP-N1 and pEF-CARD-3x Flag plasmids to generate recombinant pEGFP-*BLCAP* and pEF-*BLCAP*-3xFlag expression plasmids. The pEGFP-*BLCAP* plasmid is co-transfected with shRNA plasmids of *BLCAP* to detect the effects of RNAi with the help of alteration of EGFP in cells. The Flag protein of pEF-*BLCAP*-3xFlag was used to detect the BLCAP protein in immunoprecipitation reactions.

Plasmid transfection and stable selection. HeLa cells ($1x10^5$) were seeded in 6-well plates and subsequently transfected with recombinant plasmid containing selection marker using Lipofectamine 2000 (Grand Island, NY, USA) according to the manufacturer's protocols. Transfectant cells were selected with 50 μg/ml of G418 or 8 μg/ml of hygromycin (Sigma, USA) for two weeks, respectively. Single clones were isolated and expanded for an additional one months in media containing selection antibiotics. The stable transfectants were named HeLa-wt (transfected with wild-type of *BLCAP* gene), HeLa-M1 (transfected with *BLCAP* AXXA type), HeLa-M2 (transfected with *BLCAP* SAXX type) and HeLa-M3 (transfected with *BLCAP* Ala type). The stable transfectants of shRNA in HeLa were HeLa-B1 (transfected with pshRNA-B1), HeLa-B2 (transfected with pshRNA-B2), HeLa-B3 (transfected with pshRNA-B3) and HeLa-NS (transfected with pshRNA-NS), respectively.

Detection of apoptosis. Apoptosis analysis was performed by Annexin V-FITC Apoptosis Detection kit (BD Biosciences, San Jose, CA, USA) following the manufacturer's instructions. Briefly, the kit includes Annexin V conjugated to FITC and propidium iodide. For each sample, $1x10^5$ cells were harvested and washed twice with cold PBS buffer. All cells were gently suspended in 100 μl of binding buffer, and 5 μl Annexin V-FTIC and 5 μl propidium iodide was added. Then, cells were gently vortex and incubated for 15 min at room temperature (RT) (25˚C) in the dark. Finally, cells were analyzed with a flow cytometer. At least 10,000 cells were counted per analysis.

RNA extraction and cellular signal pathway chip analysis. To determine the role that BLCAP play in the signal transduction of cell cycle and to determine the target molecules for interaction, signal pathway chip experiment was performed using human signal pathway gene Oligo chips (CapitalBio Corp., Beijing, China). This chip contains 897 genes related to signal transduction. All cells were divided into two cell groups, one group includes HeLa cells and HeLa *BLCAP*wt (transfected with wild-type *BLCAP* plasmid, pCD-3.1(-)-*BLCAP*) cells. The other includes HeLa-*BLCAP*wt and HeLa-*BLCAP*wt-siRNA(HeLa- *BLCAP*wt cells transfected with siRNA against

Table I. The sequences of shRNA targeting *BLCAP*.

Name	Sequence of shRNA
pRNA-B1	5'-GGATCCCGTTGTGCAAGGCTTCCGTTCCATTGATATCCGTGGAACGGAAGCCTTGCACAA-3'
pRNA-B2	5'-GGATCCCGTGCAAGGCTTCCGTTCCAGGATTGATATCCGTCCTGGAAGCCTTGCA-3'
pRNA-B3	5'-GGATCCCGAATCGGAGCAGTGGTACAGGTTGATATCCGCCTGTACCACTGCTCCGATTC-3'
NS	5'-GCGAGATCTGTGCCGCTCCTCATCATCCATGTTCAAGAGACATGGATGATGAGGAGCGGCA-3'

BLCAP gene) cells. Total RNA was isolated with NucleoSpin RNA II kit according to the manufacturer's instructions. Procedures for cDNA synthesis, labeling and hybridization were carried out according to the manufacturer's protocol. Sequences of oligo genes were obtained from database of human genome oligo (Operon). The arrays were hybridized, washed and scanned according to the standard protocol. The gene chips were scanned with a luxScan 10KA (CapitalBio Corp.). Data analysis was performed using GenePix Pro 4.0 software (Axon Instruments). After background correction, we performed normalization for each array and gene. Gene activity was considered to differ between HeLa cells and wild-type *BLCAP* or siRNA plasmid-transfected HeLa cells ($P<0.01$) when compared by the unpaired Student's t-test using multiple testing correction. Classification of differentially expressed genes was also analyzed. All assays were performed in 2-4 independent experiments run in triplicates.

Western blotting. Approximately $1x10^5$ cells were harvested and washed with PBS buffer, and lysed using RIPA buffer in the presence of protease inhibitor according to the manufacturer's protocols. BCA protein assay was used to measure the protein concentration of the lysates. Equivalent amounts of protein were resolved and boiled in loading buffer. Then, total proteins were fractionated by SDS-PAGE and electrophoretically transferred onto PVDF (polyvinylidene difluoride) membrane for western blotting. Subsequently, the membranes were blocked with 5% non-fat milk and then incubated with the primary antibodies at 4˚C overnight, following washed with TBST buffer, and incubated again with an appropriate HRP-conjugated secondary antibody at room temperature for 1 h. Quantification of blotting was done using chemiluminescence detection. The detected bands were quantitated with laser densitometry.

Co-immunoprecipitation. Immunoprecipitations from extracts of HeLa cells were performed according to Mehta and Ticku (23). First, recombinant pEF-3xFlag/*BLCAP* plasmid containing full length *BLCAP* gene and three copies of Flag tag was constructed to express BLCAP-Flag fusion protein. The Flag protein in fusion protein is a tag to detect the BLCAP protein in immunoprecipitation reaction due to the lack of special antibody of BLCAP protein. After transient transfection with Lipofectamine 2000 reagent (Invitrogen), HeLa cells were incubated with ice-cold lysis buffer for 30 min and homogenized with a Pyrex glass homogenizer. The cell lysate were centrifuged at 10,000 x g for 10 min at 4˚C. The supernatants were incubated with 50% protein A/G plus agarose (Santa Cruz Biotechnology) at 4˚C for 2 h. Anti-Flag antibody (Santa Cruz Biotechnology) was added to the reaction mixture and incubated at 4˚C for 4 h. Then the mixture was incubated with 50% protein A/G plus agarose on a rocking platform at 4˚C overnight. Subsequently, protein A/G agarose complexes were collected by centrifugation at 1000 x g for 3 min, washed three times with ice-cold phosphate-buffered saline, eluted with 2X SDS sample buffer, and finally separated by SDS-polyacrylamide gel electrophoresis and subjected to immunoblot analysis.

Site-specific mutagenesis. Site-specific mutagenesis has been extensively used to study gene function. Three highly conserved amino acid positions which may potential interact with other proteins or genes were singled out to identify the importance of amino acid residues. Wild-type and three mutation types of *BLCAP* gene were subcloned into PCI-neo eukaryotic expression vector, respectively. Each recombination plasmid was confirmed by PCR, digestion analysis of restriction endonuclease and DNA sequencing.

According to the results of bioinformatics analysis of BLCAP protein, the mutation of proline of PXXP and SPXX motifs into Alanine, and the mutation of Serine78 into Alanine78 were performed with the PCR-based DpnI-treatment. The primer sequences of the *BLCAP* gene were designed with Primer 5.0 software, and the coding region of wild-type *BLCAP* gene was amplified by PCR using pfu high fidelity polymerase. The forward primer: 5'-CTAGTCTA GATTAGGTGCCCACAACGC-3', and reverse primer: 5'-GCAGAATTCATGTATTGCCTCCAGTG-3' were generated to amplify the wild-type *BLCAP* gene. Three mutation type *BLCAP* genes were amplified using site-specific mutagenesis PCR, the primers were as follows. For AXXA gene type, forward primer: 5'-ACAGGGCGGCGTTGAGGGCC TTGGGGATG-3', reverse primer: 5'-CATCCCCAAGGC CCTCAACGCCGCCCTGTG-3'; For SAXX gene type, forward primer: 5'-GCTCCGATTCCGCGCTTCCAGAA-3', reverse primer: 5'-GATTCTGGAAGCGCGGAATCGG AGC-3'; For Ala gene type, forward primer: 5'-CGCTT CCAGAAGCGGCGCATGATCC-3', reverse primer: 5'-GATCATGCGCCGGTTCTGGAAGC-3'. Finally, recombination plasmids were confirmed by PCR, digestion analysis of restriction endonuclease and DNA sequencing.

Statistical analysis. Statistical analyses were performed using SPSS 16.0 software. Student t-test was used for the comparison between two samples. The results were considered statistically significant at $P<0.05$.

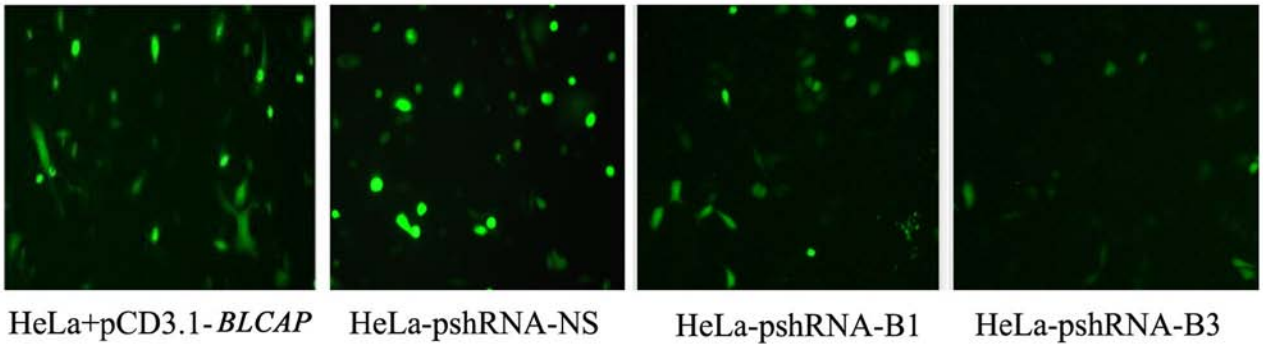

Figure 1. Effects of siRNA on wild-type BLCAP protein expression in HeLa cells. The efficiency of silencing and expression level of BLCAP protein was measured with expression of green fluorescent proteins. The representative fields were photographed at x200 magnification. HeLa cells that were co-transfected with pCD(3.1) EGFP/*BLCAP*, pshRNA-B1, pshRNA-B3 and pshRNA-NS (a negative control shRNA). The EGFP expression was significantly decreased when cells were transfected with pshRNA-B3 ($P<0.05$).

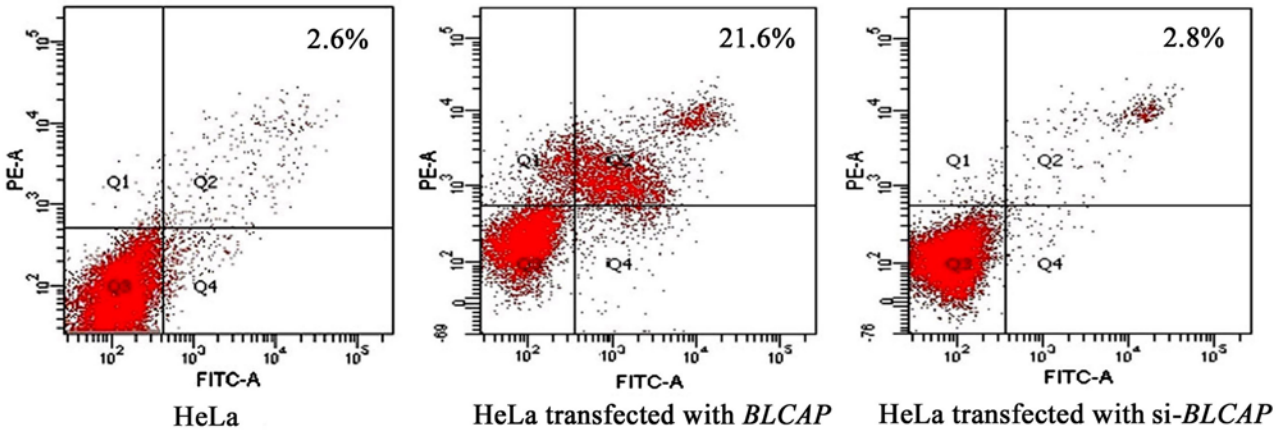

Figure 2. Apoptosis analysis of HeLa cells by flow cytometry. All cells were pre-treated with Annexin V-FITC/PI staining. Upper right quadrant shows the apoptosis rate. The apoptosis rate of HeLa cells with expression of *BLCAP* gene was 21.6%, significantly higher than HeLa cells or HeLa transfected with siRNAs of *BLCAP*.

Results

BLCAP might increase apoptosis in HeLa cells. To investigate the potential role of *BLCAP* gene as an inhibitor of cell proliferation, we assessed effects of wild-type *BLCAP* gene and its siRNA in HeLa cells by use of a fluorescence microscope and flow cytometry. The shRNAs of *BLCAP* gene (pshRNA-B1, pshRNA-B2, and pshRNA-B3) and pshRNA-NS (a negative control shRNA) was transfected into the HeLa cells. The efficiency of silencing and expression level of BLCAP protein was measured with expression of green fluorescent proteins. We found shRNAs of *BLCAP* did decrease the levels of BLCAP protein, and pshRNA-B3 had the best effect in silencing (Fig. 1).

To elucidate the mechanism of induced apoptosis by BLCAP, we assessed the effect of BLCAP knockdown on the cellular apoptosis with flow cytometry. The results showed that the apoptosis rate was increased to 21.6% after transfection with the wild-type *BLCAP* gene while in control HeLa cells it was only 2.6%. When the expression of *BLCAP* was silenced by pshRNA-B3, the apoptosis rate of cells returned to 2.8%. This result suggested that BLCAP can increase apoptosis in HeLa cells (Fig. 2).

BLCAP regulates the expression of genes. In order to detect whether BLCAP protein may modulate signaling pathways in HeLa cells, we subjected the differently treated HeLa cells (HeLa-*BLCAP*wt, HeLa-*BLCAP*wt-siRNA-B3) to gene expression analyses using human signal pathway gene Oligo chips. A comparison of the expression profiles in the *BLCAP*wt treatment vs. control (HeLa without treatment) revealed 46 genes which were significantly up- or down-regulated with a mean change ≥2-fold. A total of 11 genes were significantly downregulated in HeLa-*BLCAP*wt cell lines, while remaining 35 genes were upregulated. Furthermore, the blcapwt treatment vs. HeLa-*BLCAP*-siRNA revealed that there were 61 genes which were significantly up- or down-regulated. Ten genes were significantly downregulated while 51 genes were upregulated in HeLa-*BLCAP*-siRNA cells.

BLCAP regulates G1 to S phase of the cell cycle. From gene expression analyses using human signal pathway gene Oligo chips, we found 30 differential expression genes in HeLa cells (Table II), and at least half of them played important roles in the cell cycle, growth or proliferation. Among them at least 7 genes were related with G1 to S phase of the cell cycle,

Table II. The characteristics of 30 differential expression genes by GO analysis.

Acession code	Unigene. ID	Gene symbol	Gene name	FC	Dir	CC	CG	CS	CF
NM_006609	Hs.28827	*MEKK2*	Mitogen-activated protein kinase kinase 2	4.7291	↑	1	0	1	0
NM_001786	Hs.334562	*cdk1 (cdc2)*	Cell division cycle 2, G1 to S and G2 to M	4.3154	↑	1	0	0	0
NM_005721	Hs.380096	*ARP3*	ARP3 actin-related protein 3 homolog (yeast)	3.9601	↑	0	0	1	1
NM_002734	Hs.183037	*PRKAR1A*	Protein kinase, cAMP-dependent, regulatory, type I	3.7921	↑	1	0	1	0
NM_005722	Hs.393201	*ARP2*	ARP2 actin-related protein 2 homolog (yeast)	3.4129	↑	0	0	1	1
NM_003670	Hs.171825	*l-Dec*	Basic helix-loop-helix domain containing, class B, 2	3.3637	↑	0	0	0	1
NM_000321	Hs.75770	*Rb1*	Retinoblastoma 1 (including osteosarcoma)	3.0727	↑	1	0	0	0
NM_003816	Hs.2442	*ADAM9*	A disintegrin and metalloproteinase domain 9	3.0339	↑	0	0	1	0
NM_001892	Hs.283738	*CSNK1A1*	Casein kinase 1, α1	2.6738	↑	0	1	1	0
NM_004156	Hs.80350	*PPP2CB*	Protein phosphatase 2, β isoform	2.6287	↑	0	1	1	0
NM_001752	Hs.76359	*Catalase*	Catalase	2.6148	↑	0	0	0	1
NM_031966	Hs.23960	*cyclin B1*	Cyclin B1	2.5594	↑	1	1	0	0
NM_005655	Hs.82173	*TIEG*	TGF-β inducible early growth response	2.4786	↑	0	1	0	1
NM_016026	Hs.179817	*ARSDR1*	CGI-82 protein	2.4469	↑	0	1	0	0
NM_002592	Hs.78996	*PCNA*	Proliferating cell nuclear antigen	2.3775	↑	1	1	0	0
AF059531	Hs.152337	*PRMT3*	Protein arginine N-methyltransferase 3-like 3	2.3432	↑	0	0	0	1
AF073310	Hs.143648	*IRS2*	Insulin receptor substrate 2	2.3229	↑	0	1	1	0
NM_005359	Hs.75862	*DPC4*	MAD, mothers against decapentaplegic homolog 4	2.2822	↑	1	0	1	0
NM_005171	Hs.36908	*ATF-1/TREB*	Activating transcription factor 1	2.2552	↑	0	0	0	1
AB036063	Hs.94262	*p53R2*	Ribonucleotide reductase M2 B (TP53 inducible)	2.1887	↑	0	0	0	1
NM_005917	Hs.75375	*MDH1*	Malate dehydrogenase 1, NAD (soluble)	2.1807	↑	0	0	0	1
NM_002884	Hs.865	*RAP1A*	RAP1A, member of RAS oncogene family	2.1792	↑	1	0	0	0
NM_002227	Hs.50651	*JAK1*	Janus kinase 1 (a protein tyrosine kinase)	2.0847	↑	0	0	1	0
NM_004329	Hs.2534	*ALK-3*	Bone morphogenetic protein receptor, type IA	2.0835	↑	0	0	1	0
X68560	Hs.154295	*Sp3*	Sp3 transcription factor	2.0498	↑	0	0	0	1
NM_001239	Hs.514	*cyclin H*	Cyclin H	2.0299	↑	1	0	0	0
NM_002467	Hs.79070	*c-myc*	V-myc myelocytomatosis viral oncogene homolog	2.015	↑	1	1	0	0
NM_022740	Hs.236131	*HIPK2*	Homeodomain interacting protein kinase 2	0.4943	↓	0	0	1	1
S76638	Hs.73090	*NFKB2*	Nuclear factor of kappa light polypeptide gene	0.4135	↓	0	0	0	1
NM_001955	Hs.2271	*EDN1*	Endothelin 1	0.2809	↓	0	0	1	1

Microarray results with characteristics of 30 genes regulated by BLCAP ≥2-fold or ≤0.5-fold compared to control which were identified by GO pathway analysis. Pathways analysis identified 30 BLCAP associated genes in HeLa cell line to be involved in 'cell cycle', 'cell growth and proliferation' and 'cell signal pathway' functions. The data illustrate the distribution and overlapping of BLCAP regulated genes among the different functions. FC, fold change; Dir, direction of changed expression; (↑= increased or ↓= decreased expression compared to control cells); CC, cell cycle; CG, cell growth and proliferation; CS, cell signal pathway; CF, cellular molecular function as analyzed by pathway analysis (1, yes; 0, no).

followed by cell signal pathway, protein synthesis or transcription factors (Table III).

Interaction of BLCAP and Rb1 proteins. We performed RT-PCR, western blotting and Co-IP assays to confirm the exact association between BLCAP and Rb1 proteins. Firstly, the candidate protein Rb was identified to participate in BLCAP signal pathway with chip analysis (Table III). We prepared HeLa cell line expressing flag-tagged BLCAP and examined the phosphorylation status of Rb1. As shown in Fig. 3A and B, upregulation of BLCAP proteins could specifically increase RB1 protein expression level, but did not phosphorylate Rb1 proteins. The pRb1/Rb1 was significantly decreased (Fig. 3C, D and E). These data implied that almost all of the Rb1 protein was phosphorylated in HeLa cells, but only half of Rb1 protein was phosphorylated in HeLa cells transfected with BLCAP expression plasmid. The interaction between *BLCAP* and Rb1 or pRb1 was clearly observed with co-immunoprecipitation analysis (Fig. 4A and B). These above results further suggested that overexpression of BLCAP protein might interact and inhibit the phosphorylation of Rb1 in HeLa cells.

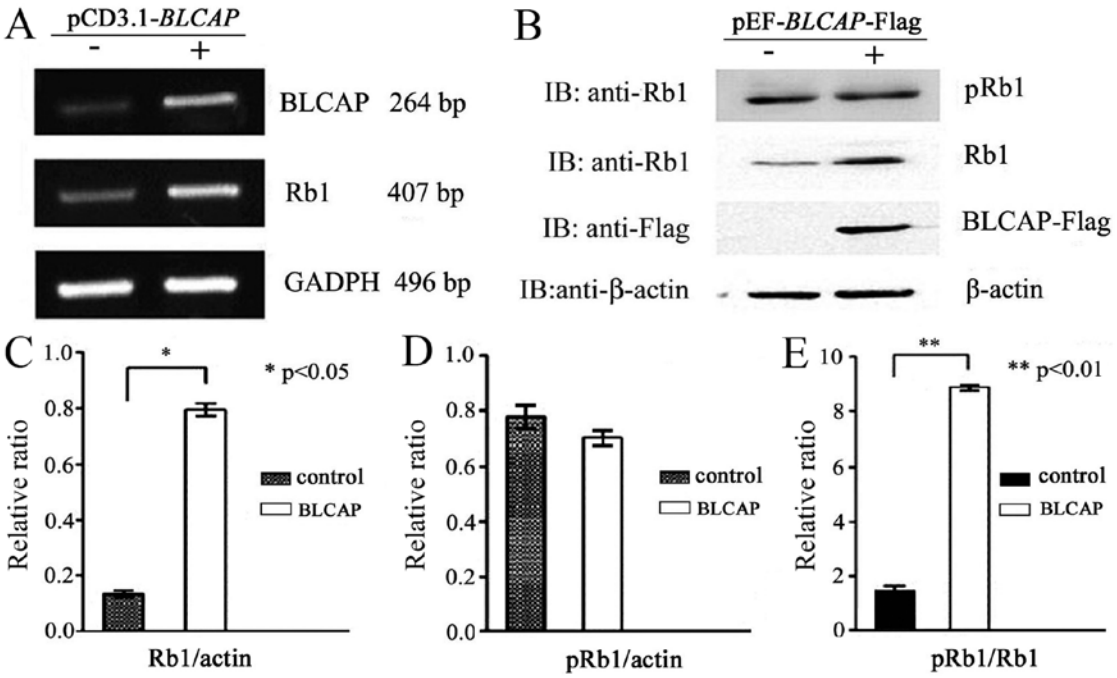

Figure 3. Differential expression of BLCAP, Rb1 and pRb by RT-PCR and western blot analysis in HeLa cells. Interaction of BLCAP and Rb1 proteins in HeLa cells was analyzed with or without pCD3.1-*BLCAP* expression plasmids. Synchronous upregulation of expression of BLCAP and Rb1 proteins was observed. (A) RT-PCR analysis of BLCAP and Rb1 expression. (B) Western blot analysis of BLCAP and Rb1 expression. (C, D and E) Graphs showing relative ratio between the expression of Rb1, pRb1 and β-actin. The significant differences are reported in each graph as evaluated by t-tests. Differences were considered significant when P<0.05.

SAXX mutation of BLCAP promotes the expression of pRb1 protein. In this study, we targeted three highly conserved amino acid positions within the BLCAP protein that potentially correspond to amino acids predicted to directly interact with other proteins or genes, and combined site-specific mutagenesis to identify amino acid residues important for BLCAP (Fig. 5). The effects of the mutants in cells were tested by protein expression analyses for a potential BLCAP target gene. We found that both AXXA and Ala78 mutation of BLCAP could inhibit the expression of cell cycle G1/S regulators such as cyclin D1 and CDK4 proteins similarly to wild-type BLCAP in cells. Thus, AXXA and Ala78 motifs of BLCAP protein were not the key regions in terms of BLCAP structure-function relationships. However, SAXX mutation of BLCAP significantly suppresses the BLCAP inhibition of expression of cyclin D1 and promotes the expression of pRb1 proteins in HeLa cells. (Fig. 6). As mentioned above, SPXX (Ser-Pro-X-X) motif located in many regulatory proteins could attend the regulation of gene expression by the phospholated site in BLCAP protein. These results provided novel information regarding the role of these residues in BLCAP function, and how BLCAP regulates expression of genes involved in the cell cycle and apoptosis.

Discussion

It is widely recognized that cervical carcinogenesis is related to various genetic and epigenetic events, especially the alterations in cell cycle checkpoint. *BLCAP* gene located on chromosome 20 is regarded as a tumor-suppression gene and was identified in human bladder carcinoma (19,24-26). Until now, the exact function and mechanisms have been obscure.

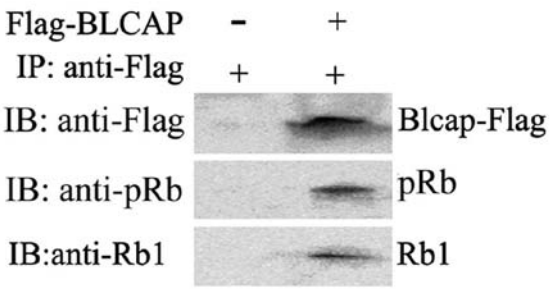

Figure 4. BLCAP proteins co-immunoprecipitated with Rb1 and pRb1. To detect the interaction of BLCAP and Rb1 protein, anti-Flag, anti-Rb and anti-pRb1 antibody were used to detect Rb in immunoprecipitated complex by western blotting. Results show that the positive bands appeared on the sample position due to co-immunoprecipitated targeted proteins in HeLa cells transfected with *BLCAP* expression plasmid, while there were no positive bands in HeLa cells.

By bioinformatics analysis BLCAP was found to contain SPXX, PXXP sequences and the phospholation site was located at 78th amino acid. A number of studies have shown that these domains of protein usually play a role in cell signaling pathways. PXXP is often involved in the multi-protein interactions, such as the activation of transcription and signal transduction. SPXX participated in regulating BLCAP function of gene expression by the phospholated site in BLCAP protein (21,22). Therefore, we hypothesized that BLCAP may play an important role in growth, reproduction, or malignant transformation of cervical cells through signal transduction pathway. In order to confirm this hypothesis, we used the cell signal transduction chip to analyze the alteration of gene profiles in HeLa cells which were transfected with wild-type *BLCAP* gene and

Table III. Significant signal pathway analysis through human signal pathway gene oligo chips.

Pathway name	P-value	Number of genes found in the pathway	Gene name and accession number
Hs_Cell_cycle_KEGG	0.00316	7	*CCNB1* NM_031966 *CDC2* NM_001786 *SMAD4* NM_005359 *RB1* NM_000321 *PRKAR1A* NM_002734 *CCNH* NM_001239 *PCNA* NM_002592
Hs_G1_to_S_cell_cycle_Reactome	0.02107	5	*CCNB1* NM_031966 *RB1* NM_000321 *CCNH* NM_001239 *MYC* NM_002467 *PCNA* NM_002592

Among the 30 differentially expressed genes, 7 genes belong to Hs_Cell_cycle, 5 genes belong to Hs_G1_to_S_cell_cycle. P-value <0.05, indicates that this pathway is very important.

Figure 5. The sequences of site-specific mutations of BLCAP protein. The wild-type coding sequence of *BLCAP* is shown in upper case letters and the mutant sequences in lower case letters. The three mutation domains are underlined in the nuclear sequence and marked.

siRNA targeted *BLCAP* gene. BLCAP was found to exert anti-tumor activity in cervical cancer cells. We identified at least 30 up- or down-regulated genes that might represent potential target genes for BLCAP in HeLa cells using microarray assay combined with GO pathways analysis. Among the potential targets, seven genes belong to the regulation of cell cycle, including *RB1, cyclin D1, CDC2*.

Rb protein has profound effect on multiple cellular processes and has been reported to regulate the expression of genes involved in cell cycle progression, differentiation, development, proliferation and apoptosis (27,28). Rb protein molecular weight is about 110 kDa, with localization in the nucleus. The most important structure domains of Rb protein is the A/B pocket. A variety of protein such as viral oncogene proteins, SV40 large T antigen, adenovirus E1A, HPV E7 protein and cellular E2F protein was able to combine with A/B pocket of Rb protein (29). Rb protein function was regulated by the phosphorylation state through a cascade of cell cycle dependent kinases, and the binding transcription factor E2F, to determine cell entry into S-phase of the cell cycle (30,31).

Diverse essential molecular processes within a cell are carried out by a large number of protein components organized by protein-protein interactions. Co-immunoprecipitation technology is a classic method for the study of protein-protein interactions. In this study, co-immunoprecipitation and immunoblotting were applied to detect the interactions between BLCAP and other biological macromolecules. We constructed the recombinant eukaryotic expression plasmid pEF *BLCAP*-3xFlag as a commercial BLCAP monoclonal antibody is not available. The Flag protein in fusion protein is a tag to detect the BLCAP protein in immunoprecipitation reaction and Western Blot detection after HeLa transient transfection with plasmids. We found that BLCAP could co-immunoprecipitate with Rb1 and pRb1 in physiological conditions. The results were further confirmed in HeLa cells transfected with wild-type BLCAP expression plasmid showing that the expression levels of Rb1 were significantly upregulated, and pRb1 level was significantly downregulated. It suggested that BLCAP can inhibit phosphorylation of Rb1, which blocks the cells from going through the G1/S checkpoint of the cell cycle. The cell proliferation is inhibited and apoptosis induction follows the overexpression of BLCAP. Our results indicate the important role that BLCAP plays in its biological function through Rb1 pathway, and provide a novel way for clinical treatment of malignant tumors such as cervical cancer.

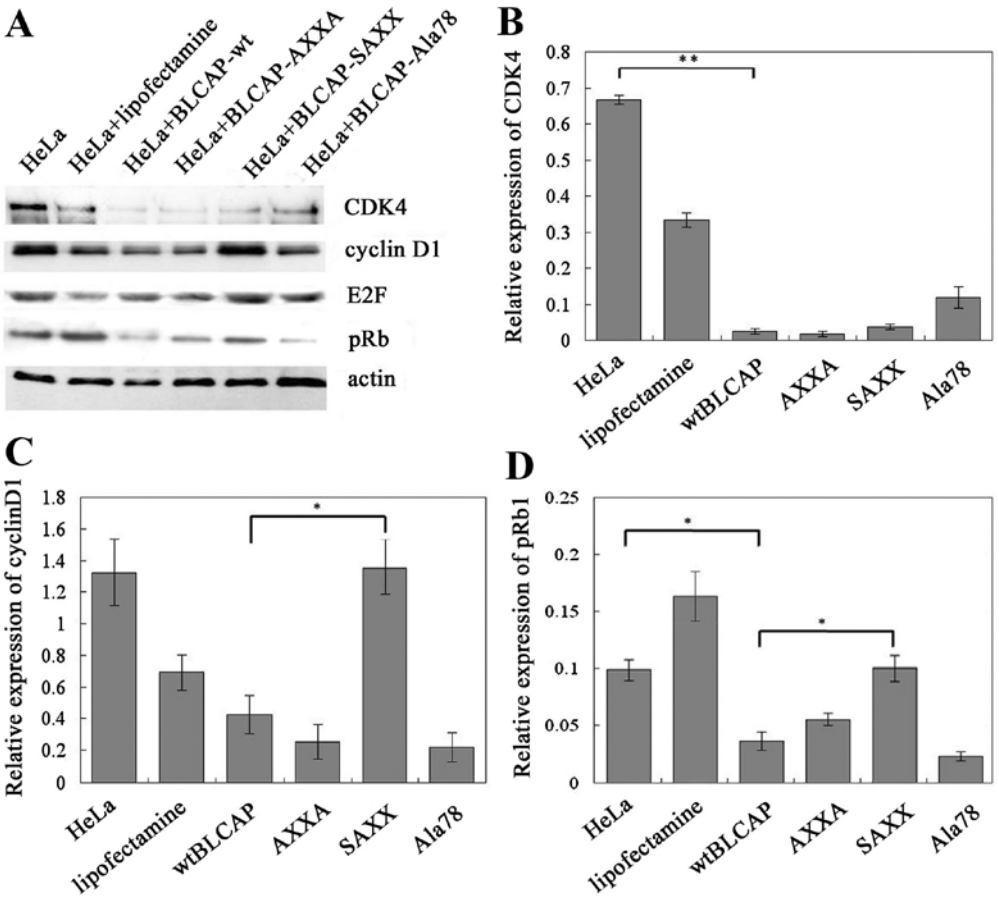

Figure 6. Detection of the key regulatory proteins in G1/S phase of the cell cycle. The expression of cell cycle factors in HeLa cell line transfected with wide-type BLCAP and mutant BLCAP expressing constructs. (A) Western blot analysis of CDK4, cyclin D1, E2F and pRb1 expression in HeLa cell line transfected with *BLCAP*-wt and three mutant *BLCAP* expressing plasmids. Graphs showing the ratio of the relative density of CDK4 (B), cyclin D1 (C), and pRb1 (D), protein expression and normalized with β-actin. Bars represent the mean of triplicate measurements with error bars corresponding to standard error of the mean. Asterisks indicate significant differences between groups ($^{*}p<0.05$; $^{**}p<0.01$ as determined by Student's t-test).

Next we designed a working model of BLCAP to mutate the potential functional motif of BLCAP including AXXA, SPXX and Ser78 site. The recombination plasmids were transfected into HeLa cells to investigate the function model of BLCAP protein *in vitro*. We identified that motif SPXX was a key region for the function of BLCAP, and SPXX motif showed a significant effect on inhibition of BLCAP function. As mentioned above, SPXX (Ser-Pro-X-X) was the phosphorylation site in BLCAP protein. When the SPXX (Ser-Pro-X-X) motif was mutated to SAXX (Ser-Ala-X-X) by site-specific mutagenesis in our study, the expression of cyclin D1 and pRb1 proteins were significantly upregulated. In contrast, AXXA mutation and Ala78 mutation of BLCAP did not show these effects in the same assay.

Transcription factor *E2F* family members played a major role in regulating cell cycle process by promoting the timely expression of genes required for DNA synthesis at the G1/S phase transition and their altered expression contribute to a number of human diseases, including cancer (32). During cancer cells growth and progression, E2F promotes tumor cell proliferation, but inhibits apoptosis. It was clear that E2F activity was controlled by the Rb1. Commonly Rb1 binds to E2F which represses E2F activity, affecting the G1/S phase of the cell cycle, while Rb phosphorylated by CDK4/cyclin D complexes, then E2F was released to activate its target genes. Cyclin D1, CDK4 and Rb1 played important roles in the cell cycle G1/S regulation in several human tumors (33,34).

Overexpression of cyclin D1 and CDK4 is a commonly observed alteration in tumors. Some reports suggest that the overexpression of cyclin D1 may serve as a driving force through its cell cycle regulating function (35). Our results indicated that BLCAP could induce overexpression of pRb1 and promote CDK4/cyclin D activity, resulting in increased Rb phosphorylation and thus E2F accumulation, eliciting its potential tumor-suppression effects. Based on our studies, we concluded that wild-type BLCAP protein plays its biological function through regulating the expression of cyclin D1, CDK4 and pRb1 proteins. In cervical cancer, the role of cyclin D1 and CDK4 in cervical carcinogenesis was not clearly understood and controversial results have been described. The inactivation of the E2F repressor resulting in increased E2F activity was a key step for cervical carcinogenesis (9). Our work provided detailed information regarding the role of BLCAP, and novel insight into how BLCAP regulates gene expression involving the cell cycle and apoptosis in Hela cells. Further, our studies suggested that BLCAP might be a new Rb1 activator or a potential E2F repressor. *BLCAP* is suggested as a prospective biomarker and a possible new therapeutic target of cervical cancer (36).

Our results showed that knock down of *BLCAP* by the use of siRNA or mutagenesis was useful to inhibit the cell growth and induce apoptosis of HeLa cells *in vitro*. This study strongly suggested that interaction between BLCAP and Rb1 was a frequent event in HeLa cells leading to cell growth inhibition and apoptosis induction. Therefore, BLCAP played an important role in the pathogenesis of cervical cancer, which might be due to the regulation of Rb expression.

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