RecQ helicase BLM regulates prostate cancer cell proliferation and apoptosis

XIAOSONG QIAN, SUJUAN FENG, DAWEI XIE, DALIN FENG, YIHANG JIANG and XIAODONG ZHANG

Institute of Uro-Nephrology, Beijing Chao-Yang Hospital, Capital Medical University, Beijing 100020, P.R. China

Abstract. Prostate cancer (PCa) is a common malignant tumor and the second leading cause of morbidity and mortality in men worldwide. Considering the prevalence and effects of PCa in males, an understanding of the molecular mechanisms underlying PCa tumorigenesis are essential and may provide novel therapeutic strategies for treating PCa. Bloom syndrome protein (BLM) is a member of the RecQ helicase family. The major function of BLM is to uncoil the double-stranded DNA structure. It has previously been demonstrated that BLM acts as a 'genome caretaker', and dysregulation of BLM function has been implicated in the development of multiple tumor types; however, its potential for inducing PCa tumorigenesis remains undetermined. The present study aimed to explore the function of BLM in PCa progression. Reverse transcription-polymerase chain reaction, immunohistochemistry and western blot analyses were performed to detect the BLM expression pattern in PCa patients and cell lines. The proliferation, and migration and invasion capacities of prostate cells were determined by EdU and Transwell assays following transfection with BLM-targeting short hairpin RNA (shRNA). The expression of BLM was significantly increased in PCa tissues and PC3 cells compared with non-PCa tissues and benign prostatic hyperplasia cells. Knockdown of BLM via shRNA inhibited PCa cell proliferation, and promoted PCa cell apoptosis. Notably, reducing the expression of BLM had no effect on the migration or invasive abilities of PCa cells. These results suggest that downregulation of BLM may alleviate PCa development, providing a novel perspective for PCa tumorigenesis and a potential therapeutic target for PCa.

Introduction

Prostate cancer (PCa) is a common malignant tumor and the second leading cause of male morbidity and mortality worldwide. In 2015, PCa alone accounted for ~26% (220,800)

of newly diagnosed cancer cases in men and led to >27,000 deaths (1). Although numerous methods, such as biochemical assays (2), biopsies (3), digital rectal examination (DRE) (4), and transrectal ultrasonography (5), have been widely used in PCa diagnosis, lack of early efficient diagnoses is the major problem affecting precise therapy of PCa. Therefore, research on the mechanisms of PCa tumorigenesis and progression will help to improve prevention strategies and develop effective interventions to treat PCa.

Bloom syndrome protein (BLM), which is mutated in patients with Bloom syndrome, is a member of the RecQ helicase family. The core structure is highly conserved between yeast and humans (6). Usually, BLM utilizes energy from ATP-hydrolysis to unwind double-stranded DNA (dsDNA) with 3' to 5' polarity, and to disassemble complex DNA structures, such as G-quadruplexes (G4s) and Holliday junctions (HJs) that are formed during the S phase of the cell cycle (7). BLM is a crucial helicase required for DNA metabolic processes, including DNA recombination, replication and repair. Such helicases are referred to as 'the caretakers of the genome'. BLM is expressed at high levels in tumor cells, and its level expression is known to be differentially regulated through the cell cycle stages. Considering that abnormalities in BLM are linked to genome instability, accumulating evidence has recently indicated that changes in BLM protein expression are associated with the development of a variety of tumors (8-10). Using exome sequencing and gene analysis, Johnson *et al* (11) identified that BLM missense variants and PCa status were completely co-segregated. However, the role of BLM in PCa, and whether it can influence the cell cycle and apoptosis, still remains unclear. The results of the present study demonstrate that BLM protein expression is upregulated in PCa patient tissues and PC3 cells. Furthermore, the downregulation of BLM can inhibit PC3 cell ischemic proliferation and induce apoptosis *in vitro*.

Materials and methods

Tissue collection. A total of 15 cancerous prostate tissues (PCa) and 10 noncancerous tissues (non-PCa) were collected from patients who underwent surgery at Beijing Chao-Yang Hospital, Capital Medical University (Beijing, China). The present study was approved by the ethics committee of Beijing Chao-Yang Hospital, Capital Medical University, and written informed consent was obtained from each patient.

Correspondence to: Dr Xiaodong Zhang, Institute of Uro-Nephrology, Beijing Chao-Yang Hospital, Capital Medical University, 8 Gong Ti Nan Lu, Beijing 100020, P.R. China
E-mail: drcyzxd@163.com

Immunohistochemistry. All tissues from PCa patients were paraffin-embedded and cut to 4 mm thickness. Endogenous peroxidase activity in deparaffinized sections was blocked by incubation with 3% hydrogen peroxide. Subsequently, sections were boiled in 1 mM EDTA pH 8.0 for 10 min, followed by cooling at room temperature. Fetal bovine serum was applied to block non-specific antigen sites. Sections were incubated with anti-BLM antibody for 3 h at a dilution of 1:200, and then the secondary antibody was applied for 30 min. Finally, the sections were stained with liquid diaminobenzydine and counterstained with Mayer's hematoxylin blue.

Cells and culture. The BPH1 benign prostatic hyperplasia cell line and PC3 PCa cell line were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were routinely cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (Gibco) in a standard culture conditions (5% CO_2 at 37°C).

BLM short hairpin RNA (shRNA) plasmid transfection. PC3 cells were transfected with BLM-targeting shRNA (GeneChem Co. Ltd, Shanghai, China) (target sequence: AAGGAAGTT GTATGCACTA) or non-targeting shRNA (GeneChem Co. Ltd, Shanghai, China) using Lipofectamine™ 2000. Transfection with a negative control (NC) scramble sequence was also conducted. Transfection efficiency was confirmed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot.

Cell proliferation assay. PC3 cells (1x103) were seeded in 6-well plates in RPMI-1640 containing 10% FBS. Cells were harvested and counted using the EdU assay after 2, 4 and 7 days, as follows. Cell proliferation was detected by using the Cell-LightTM 5-ethynyl-2'-deoxyuridine (EdU) imaging detection kit according to the manufacturer's instructions (RiboBio, Guangzhou, China). EdU was added to cells seeded in 6-well plates and the cells were cultured for an additional 2 h. The cells were then collected and washed with PBS twice. Apollo reaction buffer was then incubated with the cells for 10 min in the dark. Subsequently, cells were washed once with PBS, stained with Hoechst33342 for 30 min at room temperature and washed with PBS again. EdU-positive cells were counted using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Springs, MD, USA) in five randomly selected fields.

For cell cycle analysis, cells were seeded on 6-well plates and stained with Vybrant® DyeCycle™ Ruby stain kit at 37°C for 30 min. The DNA content was measured by flow cytometry according to the manufacturer's instructions (Thermo Fisher Scientific, Inc.).

Cell invasion and migration assays. A cell invasion assay was performed using a 24-well Transwell (Corning, New York, NY, USA) with a polycarbonate filter pre-coated with Matrigel at a 1:20 dilution (BD Biosciences, Franklin Lakes, NJ, USA). Cells ($2.5x10^4$) suspended in 0.2 ml serum-free medium were added to the upper well of the chamber, and 500 ml RPMI-1640 supplemented with 10% FBS was added to the lower well. After 24 h incubation, cells remaining in the upper well were removed by scraping. Invaded cells on the bottom

Table I. Clinical and pathological parameters of PCa and Non-PCa patients.

Parameter	PCa (n=15)	Non-PCa (n=10)
Age		
Range	58-76	49-76
Mean	68.40	64.60
Total PSA		
Range	0.005-75.488	1.550-10.383
Mean	17.400	5.126
Clinical stage		
T1c	2	N/A
T2c	5	N/A
T3a	2	N/A
T3b	4	N/A
T4b	2	N/A
Gleason score		
3+3	1	N/A
3+4	2	N/A
4+3	4	N/A
4+4	5	N/A
4+5	2	N/A
5+4	1	N/A

of the membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet solution. Cells were imaged under a microscope, and the cell numbers were counted using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Springs, MD, USA) in five randomly selected fields. A cell migration assay was conducted in a similar manner but without the use of Matrigel.

Apoptosis assay. Cell apoptosis was detected using an Annexin V-PE apoptosis detection kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's protocol. Briefly, cells at density of $1x10^6$ cells/ml were harvested and washed twice with PBS, then 500 μl of cell suspension in binding buffer was transferred to a 5-ml falcon tube, and 5 μl Annexin V-PE conjugate and 5 μl propidium iodide were added. The cells were incubated in the dark for 15 min at room temperature and the fluorescence of the cells was subsequently determined by flow cytometry.

RNA extraction and RT-qPCR. Total RNA was extracted from tissues or cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). A FastQuant RT Kit (With gDNase) and SuperReal PreMix Plus (SYBR Green; TIANGEN BIOTECH, Beijing, China) were used. The PCR primers used were as follows: BLM, forward 5'-GGATCCTGGTTCCGTCCGC-3', reverse 5'-CCTCAGTCAAATCTATTTGCTCG-3'; β-actin, forward 5'-TGACGTGGACATCCGCAAAG-3', reverse 5'-TCTTCATTGTGCTGGGTGCC-3'. PCR cycling conditions were as follows: Initial denaturation period at 95°C for 15 min, followed by a two-step PCR program consisting of denaturation at 95°C for 10 sec and annealing/extension at 60°C for 32 sec
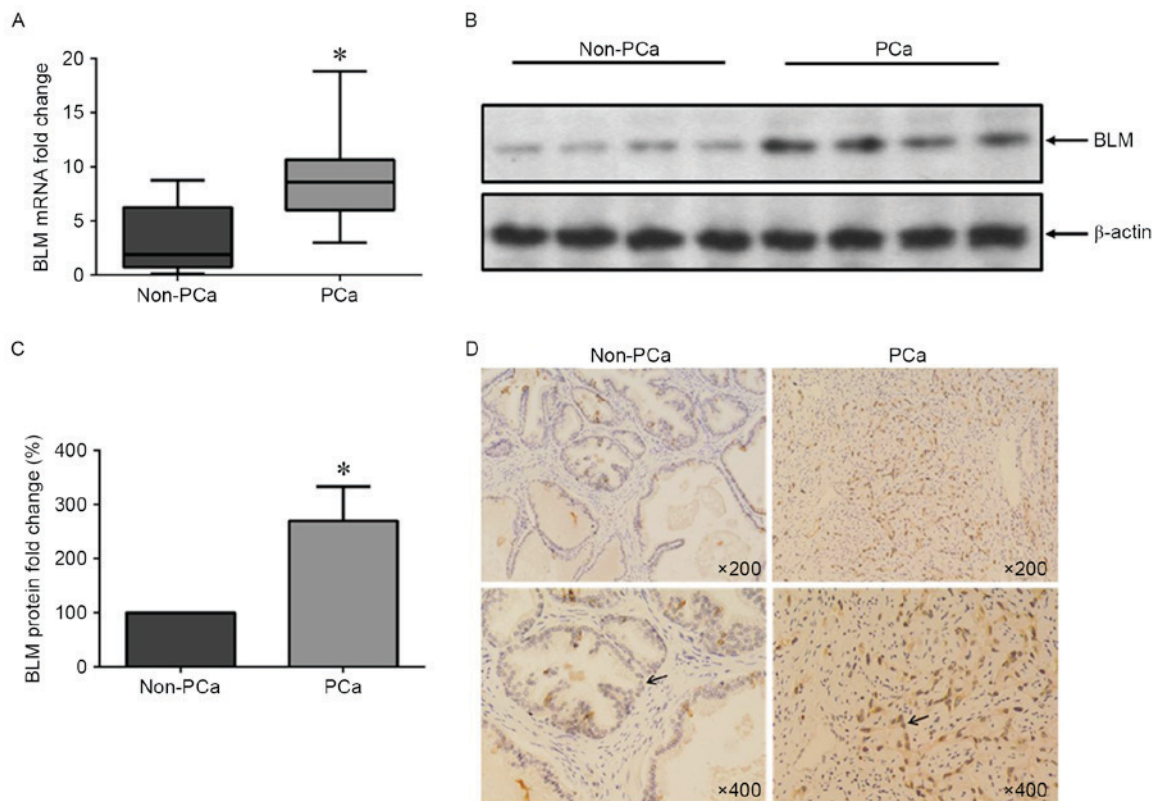
Figure 1. Higher expression of BLM in PCa patients. (A) The relative expression of BLM mRNA in PCa tissues was measured by qRT-PCR. β-actin acted as an internal loading control. Each sample was detected by triplicates, *P<0.05; (B) Typical results of western blot analysis shows BLM protein level in PCa and non-PCa tissues. (C) The quantitative analysis demonstrates that the BLM protein level increases significantly in PCa tissues. *P<0.05; (D) Immunohistochemical staining shows that the BLM is strong positive in the nuclear of prostate tissues (arrows) and significantly increases in PCa tissues compared to the non-PCa tissues. BLM, bloom syndrome protein; PCa, prostate cancer.

for 40 cycles. All samples were normalized against the internal control (β-actin) and analyzed using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis. Total protein was extracted from the cells or tissues using lysis buffer containing protease inhibitors. Proteins (30 μg) from each sample were loaded on 10% SDS-polyacrylamide gels for electrophoresis. Following electrophoresis, the proteins were transferred onto polyvinylidene difluoride membranes (GE Healthcare, Chalfont, UK), which were then blocked with 10% non-fat milk in Tween/Tris-buffered salt solution (TTBS; 20 mm Tris-Cl, pH 7.5, 0.15 M NaCl and 0.05% Tween-20) for 1 h. Following incubation with the anti-BLM antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C overnight, the membrane was washed and incubated with IRDye® 800CW donkey anti-goat IgG secondary antibody at room temperature for 2 h followed by LI-COR Odyssey gel imaging scanner detection (LI-COR Biosciences, Lincoln, NE, USA). To verify equal loading of protein, the blots were reprobed with primary monoclonal antibody against β-actin (ProteinTech Group, Inc., Chicago, IL, USA).

Statistical analysis. Quantitative analysis of immunoblotting was performed using Quantity-One software (Gel Doc 2000 imaging system, Bio-Rad Laboratories, Inc., Hercules, CA, USA). For protein level, the protein ratio of BLM (band density of protein/band density of β-actin) was set as 100% in the BPH or NC group. The data from other groups were expressed as a percentage of the BPH or NC group. The values

are presented as the mean ± standard error. Statistical analysis was conducted by one-way analysis of variance followed by all pairwise multiple comparison procedures using the Bonferroni test and Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Differential expression of BLM in PCa and non-PCa patients. To determine BLM expression status in PCa tissues, the mRNA and protein levels of BLM in PCa and non-PCa tissue samples were detected. The patient profiles are summarized in Table I. We found that the BLM mRNA and protein expression levels were upregulated in the PCa tissues compared the non-PCa group (Fig. 1A-C). Similarly, immunohistochemical staining showed that the expression of BLM protein was increased in the nuclei of tumor cells, but it almost was undetectable in non-PCa tissue (Fig. 1D).

Changes of BLM expression level in PCa and BPH1 cells. To verify the results from tissue samples, the BLM expression level was also analyzed in BPH1 and PC3 cells *in vitro*. As shown in Fig. 2, the mRNA and protein levels of BLM in PC3 cells were significantly higher than that of the BPH1 cells.

Inhibition of BLM reduced PC3 cell proliferation in vitro. To explore the role of BLM in PCa cell proliferation, PC3 cells were transfected with a BLM-targeting shRNA plasmid. As shown in
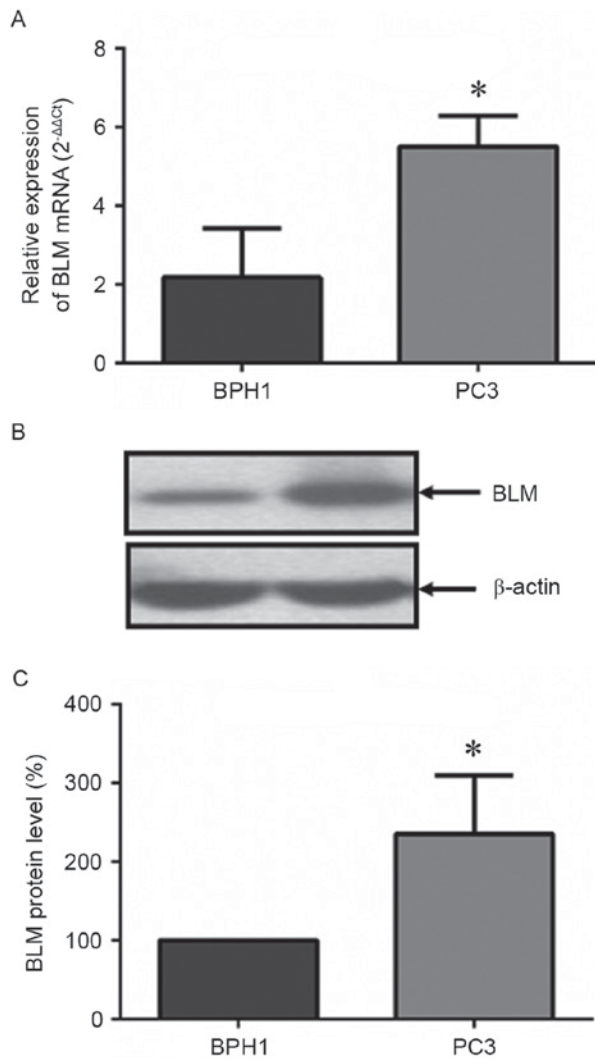
Figure 2. Increase expression of BLM in PC3 cells. (A) Relative expression of BLM mRNA is higher in PC3 cells than in BPH1 cells, n=3 per group, *P<0.05; (B) Western blot analysis shows that BLM protein level higher in PC3 cells than BPH1 cells. (C) The quantitative analysis also demonstrates that the BLM protein level increases significantly in PC3 cells, n=3 per group, *P<0.05. BLM, bloom syndrome protein.

Fig. 3, both of the BLM mRNA and protein expression levels were significantly downregulated in PC3 cells after transfection with the BLM-targeting shRNA. An EdU proliferation assay was conducted to determine the effect of BLM inhibition on PC3 cell proliferation. As shown in Fig. 4A and B, shRNA-mediated inhibition of BLM significantly reduced PC3 cell proliferation. Additionally, cell cycle analysis was performed to examine if BLM inhibition influences the cell cycle. The results in Fig. 5A and B revealed that there was significant decrease in the number of cells in S-phase after transfection with BLM shRNA. Similarly, the PC3 cells exhibited a significantly reduced proliferative capacity when BLM was knocked down (Fig. 5D).

Downregulation of BLM promoted PCa cell apoptosis. To further elucidate the role of BLM in PCa apoptosis, PC3 cells transfected with BLM shRNA plasmid were subjected to Annexin-V and PI staining, and flow cytometry analysis was performed. The results shown in Fig. 6A and B demonstrated that knockdown of BLM led to an increase in apoptosis.
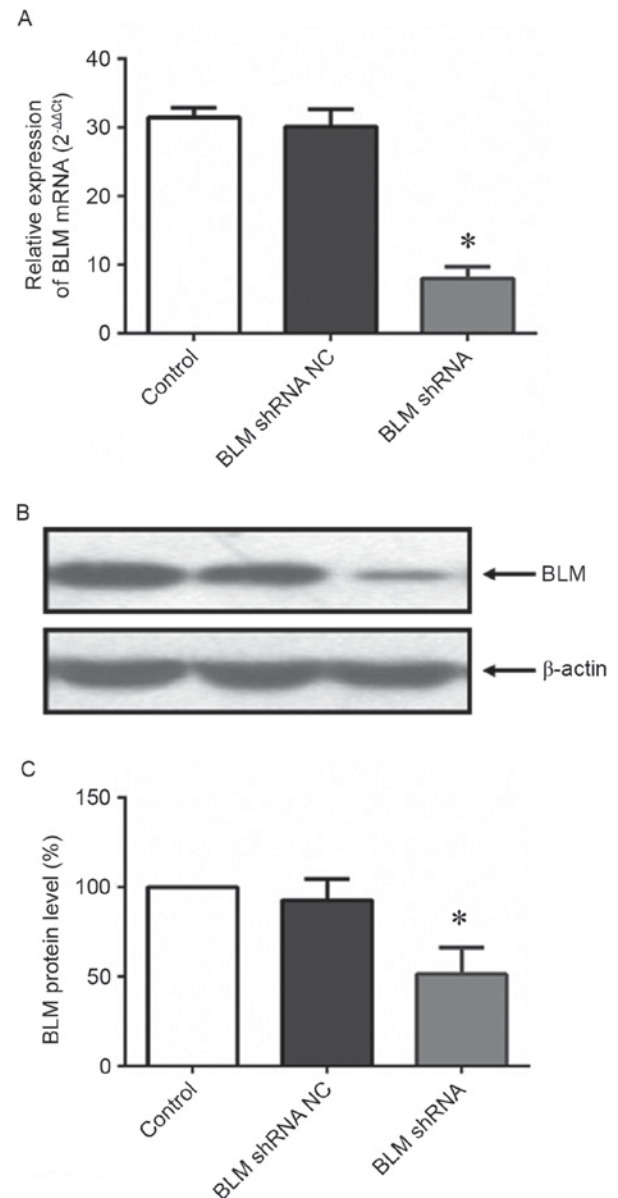

Figure 3. Effect of BLM shRNA on the BLM expression in PC3 cells. (A) BLM mRNA level could be downregulated in BLM shRNA transfected PC3 cells, n=3 per group, *P<0.05. (B) Western blot analysis shows that BLM protein level significantly decreases in the BLM shRNA transfected PC3 cells than shRNA NC transfected PC3 cells. (C) The quantitative analysis shows that the BLM protein level decreases significantly in PC3 cells, n=3 per group, *P<0.05. BLM, bloom syndrome protein.

Inhibition of BLM had no effect on PCa cell migration and invasion. The effect of BLM on PCa cell migration and invasion was examined using Transwell assays. As presented in Fig. 7A and B, PC3 cells transfected with the BLM shRNA plasmid did not exhibit any increase or decrease in migration and invasion compared with the NC cells.

Discussion

It is well-established that PCa development is affected by genetic inheritance, with numerous genes recognized to influence PCa susceptibility, including MED12 (12), HOXB13 (13), BRCA1 and BRCA2 (14,15). As a RecQ helicase, BLM is part
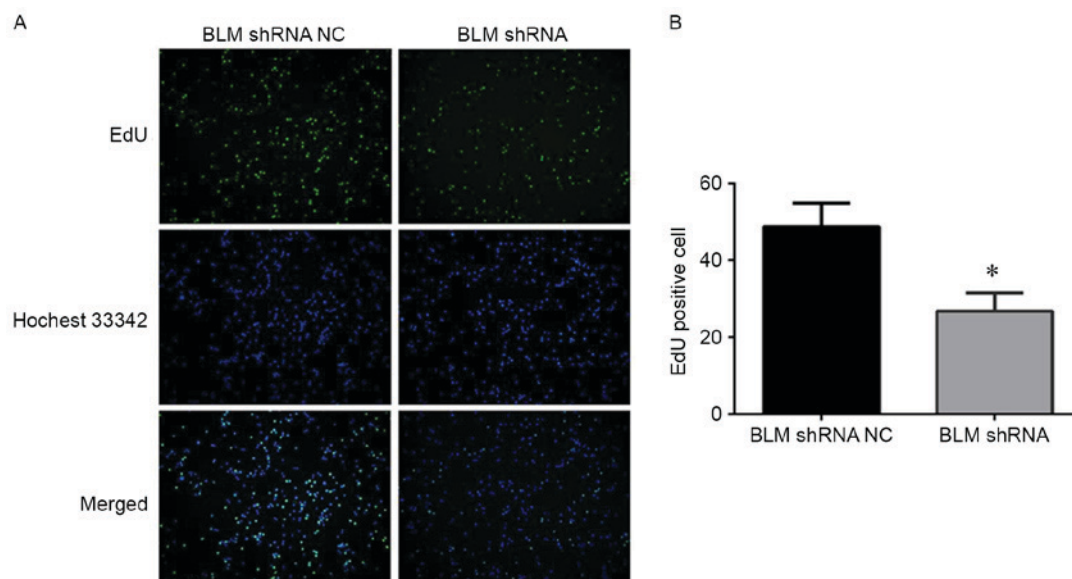
Figure 4. Knockdown of BLM decreases cell proliferation. (A) Representative results of images show the effect of BLM shRNA on PC3 cells proliferation. (B) The quantitative analysis of EdU positive cell number is decreased in PC3 cells transfected with BLM shRNA compared with the NC group, n=3 per group, *P<0.05. BLM, bloom syndrome protein; NC, negative control.
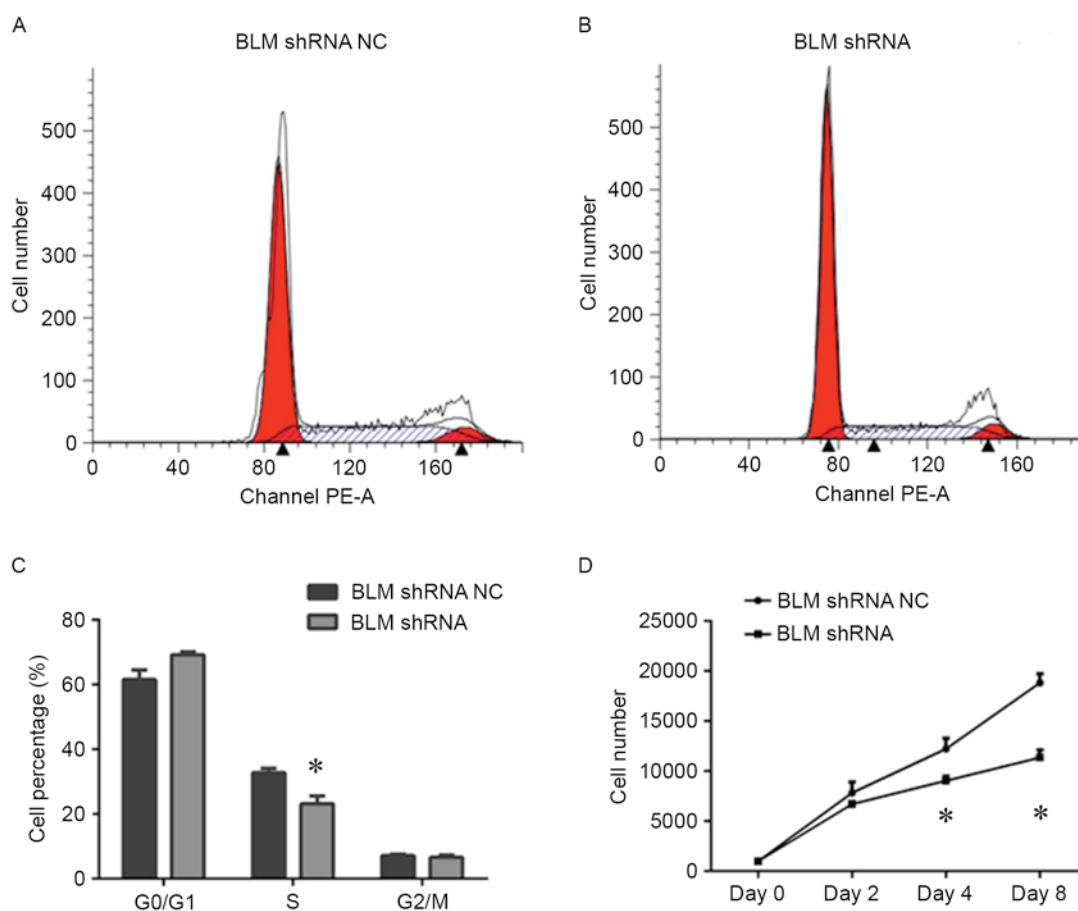

Figure 5. Knockdown of BLM induces cell cycle arrest. (A and B) Typical images of cell cycle progression were measured by flow cytometry. (C) The quantitative analysis demonstrated that knockdown of BLM decreases the number of cells in the S-phase, n=3 per group, *P<0.05. (D) Cell counting decreases in PC3 cells transfected with BLM shRNA after 4 and 8 days seeded compared with the NC group, n=3 per group, *P<0.05. BLM, bloom syndrome protein.

of a family of DNA unwinding enzymes, and has crucial roles at multiple steps in the DNA recombination, replication and repair processes (16). The human genome contains five RecQ genes

that encode Werner syndrome protein (WRN), BLM, RECQ1, RECQ4, and RECQ5. It is worth noting that BLM is mutated in Bloom syndrome, a rare autosomal recessive disorder that
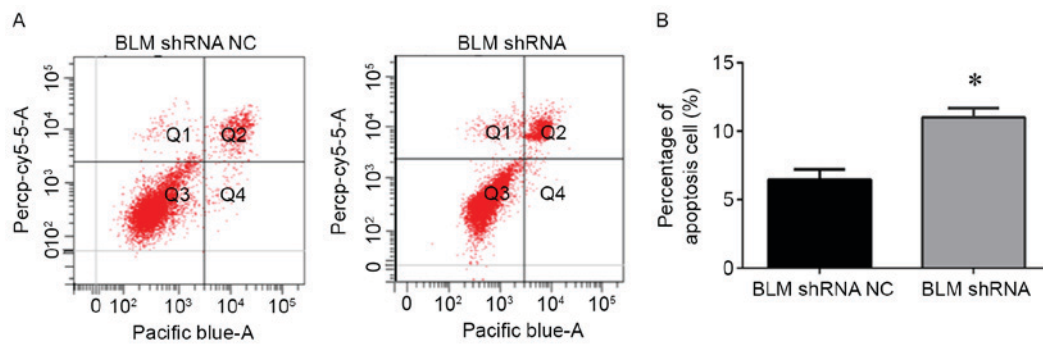
Figure 6. Downregulation of BLM promotes cell apoptosis. (A) Representative images of cell apoptosis were measured by flow cytometry. (B) Quantification of apoptotic cell number indicated that cells transfected with BLM shRNA enhanced PC3 cell apoptosis compared with the NC group, n=3 per group, *P<0.05. BLM, bloom syndrome protein; NC, negative control.
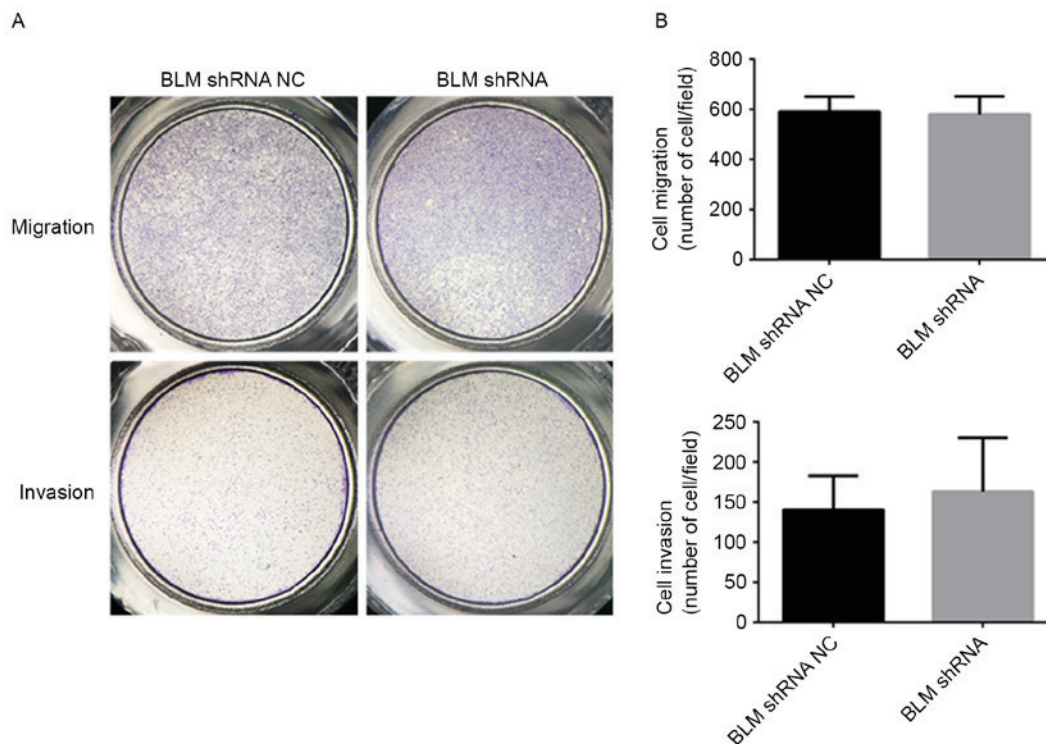

Figure 7. Knockdown of BLM have no effect on PC3 cell migration and invasion. (A) Representative images of cell migration and invasion were detected in PC3 cells by transwell; (B) Quantification of cell number indicates that cells transfected with BLM shRNA could not up- or down-regulate the cell migration and invasion compared with the NC group, n=3 per group. BLM, bloom syndrome protein; NC, negative control.

is characterized by proportional dwarfism, sun-sensitive facial erythema, skin pigmentation abnormalities, immunodeficiency and infertility (17). In addition, mutations in BLM are pathogenic due to an increased predisposition for development of many tumor types, including lymphoid and epithelial-derived tumors (18,19). Balci *et al* (20) reported that colon carcinoma was diagnosed in ~12% of patients with Bloom syndrome, and Thompson *et al* (8) reported that heterozygous truncating mutations in BLM increase the risk of breast cancer.

Cell proliferation and cell cycle control are the major regulatory mechanisms of cell growth. A large body of evidence has established the mechanisms of how BLM mutations affect tumor development, including changes to the regulation of many cellular DNA metabolic processes, including DNA replication, DNA repair, telomere maintenance and RNA

transcription. BLM expression is high in the S-phase of the cell cycle (21), but BLM depletion has been reported to suppress cell proliferation in human fibroblasts, and BLM-deficient cells have defective S-phase progression (22). Other studies also report that BLM plays a role in recovery from S-phase arrest in response to hydroxyurea. This may due to interaction of BLM and p53-binding protein 1 in a Chk1-mediated pathway (23). Our study demonstrated that knockdown of BLM in PC3 cells suppressed cell proliferation and increased cell S-phase arrest. Apoptosis has an important role in tumor progression, Chester *et al* (24) reported that increased apoptosis occurred in BLM mutant embryos, Kaneko *et al* (25) suggested that BLM-deficient cells have abnormal regulation of p53 protein expression and are highly susceptible to apoptosis. This previous study also suggested that p53 mediates cell death by

inducing mitochondria-related apoptosis (25). Consistently, the current study also revealed that BLM deficiency enhanced cell apoptosis. Taken together, these results provide strong evidence that BLM may play an important role in the genomic stability and DNA repair of PCa cells. Therefore, it is tempting to speculate that BLM could be a useful therapeutic target for treating PCa cases that are susceptible to specific DNA-damaging chemotherapeutic agents.

However, in the present study, silencing of BLM expression had no impact on invasion and migration of PC3 cells. This effect of BLM during tumorigenesis predominantly involves changes in DNA replication and repair through increased fragility of hotspots after replication fork stalling. Without functional BLM, inefficient resolution of DNA linkages at fragile sites gives rise to increased numbers of anaphase ultra-fine DNA bridges and fragile site loci that contribute to chromosomal instability and tumorigenesis (26). However, as indicated by the results of our study, loss of BLM function may not influence the invasion and migration potential of PCa cells.

In summary, our results provide a new perspective for PCa tumorigenesis and development. These findings highlight a novel phenomenon in the regulation of PCa progression, which is useful for understanding the effect of BLM on proliferation and apoptosis of PCa cells. Thus, BLM could be a promising biomarker and/or a therapeutic target for PCa treatment. However, further work is necessary to explore the regulatory mechanisms of BLM in PCa.

Acknowledgements

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