Downregulation of lncRNA PVT1 expression inhibits proliferation and migration by regulating p38 expression in prostate cancer

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Abstract. Long non-coding RNA (lncRNA) plasmacytoma variant translocation 1 (PVT1) has been reported to be overexpressed in prostate cancer cells and associated with tumorigenesis in various types of cancer. However, the biological function of lncRNA PVT1 remains largely unknown. The aim of the present study was to investigate the effect of lncRNA PVT1 expression on the proliferation and migration of prostate cancer cells. Stably transfected prostate cancer cells with downregulated expression of lncRNA PVT1 were constructed by an efficient siRNA fragment, followed by confirmation by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Proliferation was assessed using CCK-8, colony formation and xenograft assays, and cell migration was evaluated using a wound healing assay. The PathScan® Intracellular Signaling Array kit was utilized to explore the underlying molecular mechanisms of lncRNA PVT1 expression in prostate cancer cells. RT-qPCR results confirmed that the lncRNA PVT1 expression level was successfully knocked down in prostate cancer cells. When lncRNA PVT1 expression was downregulated in prostate cancer cells, proliferation and migration were significantly inhibited, compared with the control lncRNA PVT1 group. Furthermore, PVT1 knockdown decreased the phosphorylation of p38 in DU145 cells. Therefore, the present study demonstrated that lncRNA PVT1 downregulation inhibits the proliferation and migration of prostate cancer cells, and is associated with p38 phosphorylation.

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Introduction

Prostate cancer has been reported as the second leading cause of cancer-associated male mortality in the United States (1,2). The majority of patients with early stage prostate cancer can be treated by radical prostatectomy and androgen deprivation therapy (3). It has been indicated that after 1-3 years of treatment, the majority of prostate cancer cases ultimately progress to hormone refractory prostate cancer, which is classified as an incurable disease (4). However, the underlying mechanism associated with the carcinogenesis and progression of prostate cancer requires further investigation. Therefore, it the development of a novel, target-specific effective treatment strategy for patients with prostate cancer exhibiting androgen-resistance is urgently required.

Long non-coding RNAs (lncRNAs) are 200 nucleotides in length (5) and serve crucial roles in the progression of malignant tumors (6-9). Plasmacytoma variant translocation 1 (PVT1), an oncogenic lncRNA, is located in the chromosomal region 8q24 adjacent to MYC (10). Overexpression of lncRNA PVT1 has been reported in various types of tumor (11-16), including prostate cancer (17). However, the biological function and underlying mechanism of lncRNA PVT1 in prostate cancer requires further investigation. In the present study, the effect of downregulation of lncRNA PVT1 expression on proliferation and migration was investigated, in addition to its potential underlying mechanism in prostate cancer.

Materials and methods

Cell culture. Human prostate cancer cell lines, PC-3 and DU145, were obtained from American Type Culture Collection (Manassas, VA, USA) and cultivated in Roswell Park Memorial Institute 1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Bio-Sciences, Logan, UT, USA). The cells were maintained at 37°C in 5% CO_2.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was

extracted from PC-3 and DU145 cells, using TRIzol™ reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed into cDNA using the PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan) from 1 µg of total RNA in a 20 µl reaction volume, according to the manufacturers' protocols. The qPCR reaction mixture was assessed using the ABI 7500 Fast Real Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) utilizing SYBR Premix Ex Taq™ (Takara Bio, Inc.). The GAPDH fragment was used as an internal control gene for standardizing the expression of target genes. The primers were as follows: lncRNA PVT1 forward, 5'-TGAGAA CTGTCCTTACGTGACC-3' and reverse, 5'-AGAGCACCA AGACTGGCTCT-3'; GAPDH forward, 5-AGCCACATC GCTCAGACAC-3' amd reverse, 5'-GCCCAATACGACCAA ATCC-3'. Each experiment was repeated three times. The reaction conditions were 95°C for 10 min, followed by 95°C for 10 sec for 40 cycles and 60°C for 60 sec. The relative mRNA expression level of each sample compared with GAPDH expression was derived using the $2^{-\Delta\Delta Cq}$ method (18,19).

RNA interference. PC-3 and DU-145 cells ($1x10^5$ cells) were cultured to 60% confluency in 35-mm culture dishes. Subsequently, the cells were transfected with small interfering (si)-lncRNA PVT1-1# (10 nM) sense, 5'-GCUUGGAGGCUG AGGAGUUTT-3' and antisense, 5'-AACUCCUCAGCCUCC AAGCTT3'; si-lncRNA PVT1-2# (10 nM) sense, 5'-CCC AACAGGAGGACAGCUUTT-3' and antisense, 5'-AAGCUG UCCUCCUGUUGGGTT-3'; si-lncRNA PVT1-3# (10 nM) sense, 5'-CCUGUUACACCUGGGGAUUUTT-3' and antisense, 5'-AAAUCCCAGGUGUAACAGGTT-3'; and si-negative control (NC; 10 nM) sense, 5'-UUCUCCGAACGUGUCACG UTT-3' and antisense, 5'-ACGUGACACGUUCGGAGA ATT-3', using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. After 24 h, cells were harvested for RNA isolation. The knockdown efficiency was assessed using RT-qPCR. All siRNA senses were supplied from Shanghai Jima Company (Shanghai, China). The most efficient siRNA fragment was subsequently packaged into recombinant lentivirus vectors to construct stably transfected cell lines exhibiting lncRNA PVT1 downregulation, which was performed and produced by the Shanghai Jima Company. ShPVT1 was the same sequence as siPVT1 and packaged into recombinant lentivirus vectors by the Shanghai Jima Company.

Cell proliferation assay. A CCK-8 cell proliferation assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to assess proliferation, according to the manufacturer's protocol. PC3 and DU145 cells were seeded in 96-well plates at 100 cells per well. Cell were cultured in Dulbecco's modified Eagle's medium, supplemented with 12% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO_2 for 5 days. Absorbance of each well was subsequently measured at 450 nm using a spectrophotometer (xMark; Bio-Rad Laboratories, Inc., Hercules, CA, USA). All assays were performed at least in triplicate.

Wound healing assay. PC-3 and DU145 cells ($1x10^4$) were seeded with Dulbecco's modified Eagle's medium supplemented with 12% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc.) into 6-well plates at 80% confluency

and incubated overnight at 4°C. Wounds were created with a sterile 10-µl pipette tip. Subsequently, detached cells were washed twice and removed with phosphate-buffered saline (PBS). Migration and movement of cells was assessed by measuring the same section of the wound at 0 and 24 h. This test was repeated three times under the same conditions.

Colony formation assay. The PC-3 and DU145 cells stably transfected with shRNA lncRNA PVT1 and NC were seeded in 6-well plates at a density of 500 cells per well. The medium was replaced with fresh culture medium every 4 days. After 14 days, plates were washed twice with PBS, fixed with 4% paraformaldehyde for 30 min at 4°C, and stained with Wright-Giemsa (Ruigen Co., Beijing, China) for 30 min at room temperature. Subsequent to washing with PBS, colonies with >50 cells were counted under an Olympus confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan).

In vivo xenograft studies. A total of 8 male nude mice (4-6 weeks old; weight, 10-12 g) were purchased from the Model Animal Research Center of Southern Medical University (Guangzhou, China). All animal procedures and experiments were approved by the Southern Medical University Animal Research Ethics Committee (Guangzhou, China). To establish a prostate cancer xenograft model, $1x10^7$ PC3 cells in 200 µl PBS were injected subcutaneously into the left and right flanks of mice. The mice were maintained in a pathogen-free environment (Pa 0.65 cm H_2O at 26-28°C, with light for 10 h), with access to sterile food, and tumor length and width were measured every 3 and 4 days after the 13th day. After 33 days, tumor weight and volume were calculated with the formula: Tumor volume=(tumor width)^2 x (tumor length) x0.5.

Intracellular signaling array. The lysates of DU145 cells ($1x10^7$ cells) were prepared for detecting modifications of cellular proteins using the PathScan® Intracellular Signaling Array kit (cat. no. 7323; Cell Signaling Technology, Inc., Danvers, MA, USA), according to the manufacturer's instructions. Each sample was repeated in triplicate.

Western blot analysis. Total protein was obtained from DU145 and PC3 cells using protease buffer and inhibitors containing phenylmethanesufonyl fluoride (100 nM; Biyuntian Co. Shanghai, China). Protein concentration was measured by bicinchoninic acid assay. Protein lysates (50 µg) were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were blocked using 5% skimmed milk in PBST (0.1% triton in PBS) for 2 h at room temperature. Next, membranes were cultured with polyclonal anti-p-P38 (dilution, 1:1,000; cat. no. BS6381; Bioworld Technology, Inc., St. Louis Park, MN, USA) and monoclonal anti-GAPDH (dilution, 1:2,000; cat. no. 5174; Cell Signaling Technology, Inc.) primary antibodies overnight at4°C. Subsequent to being incubated with a horseradish peroxidase-conjugated secondary antibody (cat. no. PV-6000; OriGene Technologies, Inc., Beijing, China) at room temperature for 30 min, signals were developed using enhanced chemiluminescence detection kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.
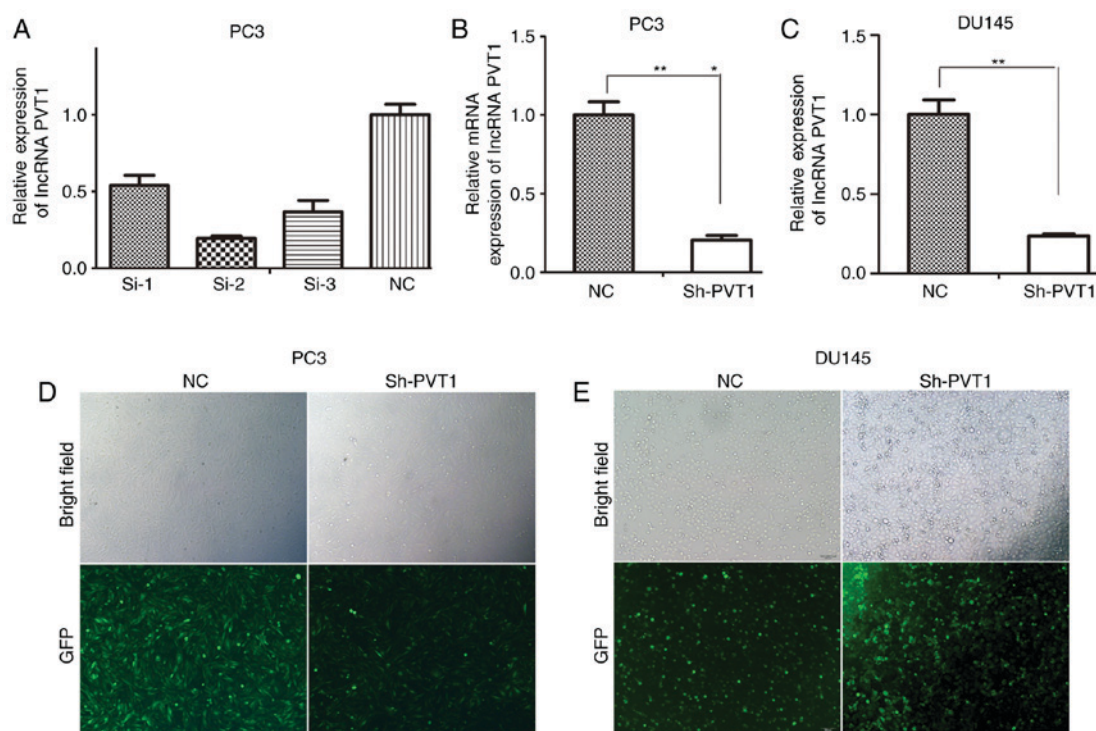
Figure 1. Identification of the most effective siRNA for lncRNA PVT1-knockdown. (A) si-RNA-2# was the most effective for knockdown of lncRNA PVT1 expression. (B) lncRNA PVT1 expression was significantly knocked down in PC3 cells. (C) lncRNA PVT1 expression was significantly knocked down in DU145 cells. (D) Efficiency of lentivirus-mediated RNAi was tested in PC3 cells (magnification, x100). (E) Efficiency of lentivirus-mediated RNAi was tested in DU145 cells (magnification, x100). *P<0.05 vs. NC group, **P<0.01 vs. NC group. siRNA, small interfering RNA; lncRNA, long non-coding RNA; NC, negative control; RNAi, RNA interference; GFP, green fluorescent protein.

Statistical analysis. All analyses were performed using SPSS 22.0 software (IBM Corp., Armonk, NY, USA). All data are expressed as mean ± standard deviation. Differences between two groups were determined using two-tailed Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of the most effective siRNA. To identify the most effective siRNA for lncRNA PVT1-knockdown, PC3 cells were transfected with three lncRNA PVT1 siRNAs and one NC si-RNA. si-RNA-2# was confirmed to be the most effective siRNA for lncRNA PVT1-knockdown compared with other siRNAs (Fig. 1A). si-RNA-2# was subsequently recombined into a lentivirus vector. To obtain stable cell lines exhibiting lncRNA PVT1-knockdown, the lentivirus vectors were transfected into PC3 cells (Fig. 1D) and DU145 cells (Fig. 1E). Subsequently, RT-qPCR results indicated significant knockdown of lncRNA PVT1 expression levels in PC3 and DU145 cells (Fig. 1B and C).

Downregulation of lncRNA PVT1 expression inhibits the proliferation, mobility and colony formation abilities of prostate cancer cells. To investigate the effect of lncRNA PVT1 on the proliferation of prostate cancer cells, a CCK-8 assay was performed in PC3 and DU145 cells. The results indicated that lncRNA PVT1 downregulation significantly inhibited the proliferation of PC3 (Fig. 2A) and DU145 (Fig. 2B) cells, compared with the NC group.

The effect of lncRNA PVT1 was also examined on the colony formation ability of prostate cancer cells. The colony number was significantly decreased in shPVT1 PC3 and DU145 cells compared with NC cells (Fig. 2C and D). These results indicated that downregulation of lncRNA PVT1 expression inhibited the colony formation ability of prostate cancer cells.

A wound-healing assay was conducted to assess the effect of lncRNA PVT1 expression on the mobility of prostate cancer cells. Downregulation of lncRNA PVT1 expression significantly inhibited the migration of PC3 cells (Fig. 3A) and DU145 (Fig. 3B) cells compared with the NC group.

LncRNA PVT1 downregulation inhibited tumorigenesis in a tumor xenograft model. To confirm the effect of lncRNA PVT1 on tumorigenesis in prostate cancer cells, a xenograft tumor formation assay was performed by injecting PC3 cells into mice. After 33 days, all mice were sacrificed, and the size and weight of subcutaneous tumors were calculated (Fig. 4A). Compared with the NC group, the growth rate of xenograft tumors was decreased in the shPVT1 group (Fig. 4B). The mean tumor weight of the shPVT1 group was significantly reduced compared with that of the NC group (Fig. 4C). The results of the present study indicated that lncRNA PVT1 downregulation inhibited tumorigenesis in prostate cancer cells.

Downregulation of lncRNA PVT1 expression inhibits p38 activation. To explore the underlying molecular mechanisms of lncRNA PVT1 downregulation in prostate cancer cells, the PathScan® Intracellular Signaling Array kit (Chemiluminescent
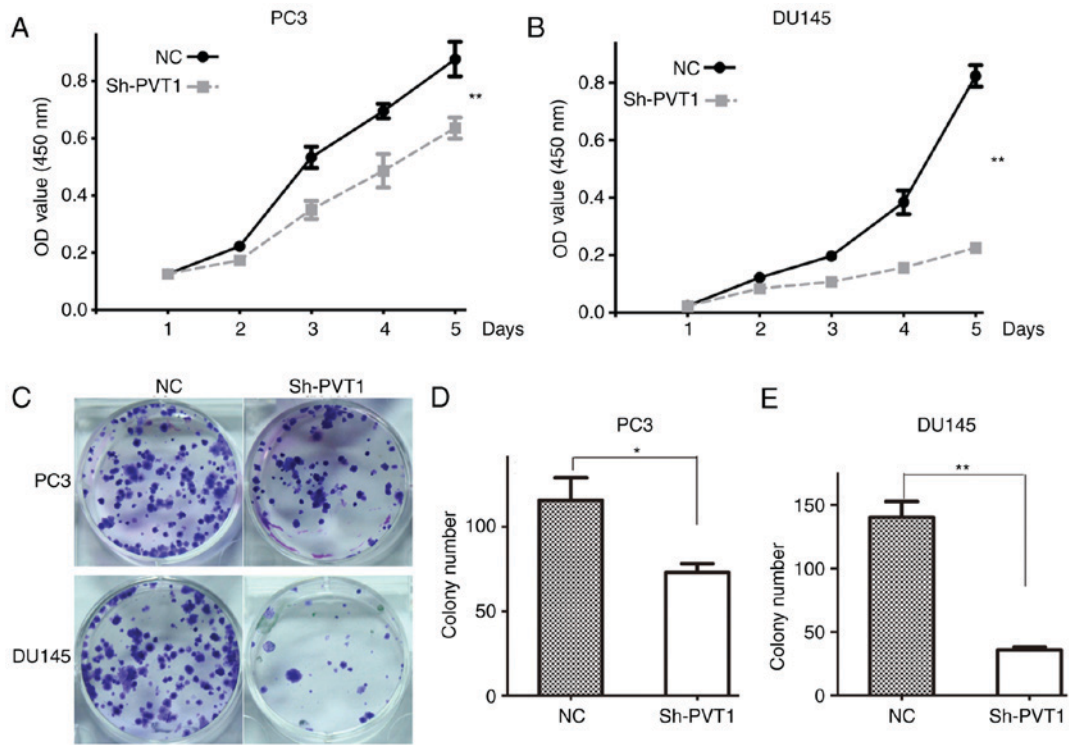
Figure 2. LncRNA PVT1-knockdown inhibits proliferation of prostate cancer cells. (A) Proliferation curves of PC3 cells subsequent to shPVT1 infection. (B) Proliferation curves of DU145 cells subsequent to shPVT1 infection. (C) LncRNA PVT1-knockdown suppressed the colony-formation ability of prostate cancer cells compared with the NC group. (D) Quantification analysis of colony number in PC3 cells. (E) Quantification analysis of colony number in DU145 cells. *P<0.05 vs. NC group, **P<0.01 vs. NC group. lncRNA, long non-coding RNA; NC, negative control; OD, optical density.
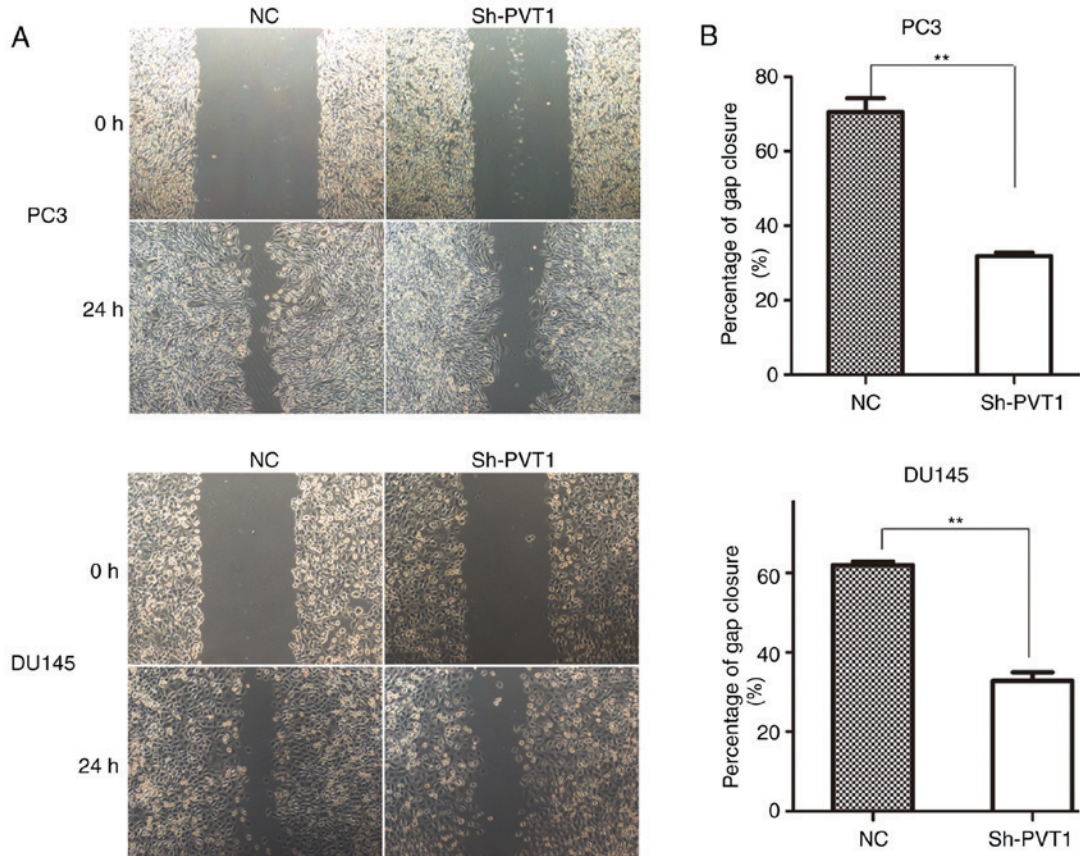

Figure 3. lncRNA PVT1-knockdown inhibits migration of prostate cancer cells. (A) Migration capacity of PC3 and DU145 cells was significantly reduced in lncRNA PVT1-knockdown groups at 24 h, compared with the negative control groups. (B) Migrated distance in PC3 and DU145 cells. **P<0.01 vs. control groups. lncRNA, long non-coding; NC, negative control; shRNA, short hairpin RNA.
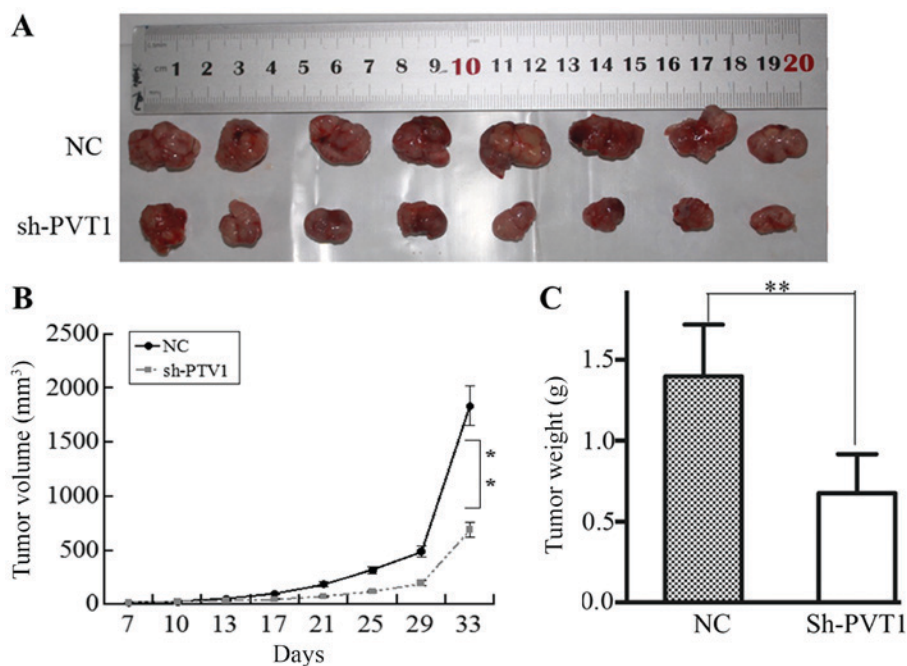
Figure 4. lncRNA PVT1-knockdown inhibits proliferation of xenograft tumors *in vivo*. (A) Photographs of the solid tumors. (B) Tumor growth. (C) Mean weight of solid tumors. lncRNA, long non-coding; NC, negative control. Tumors in (A) are presented to show their best aspect. **P<0.01.
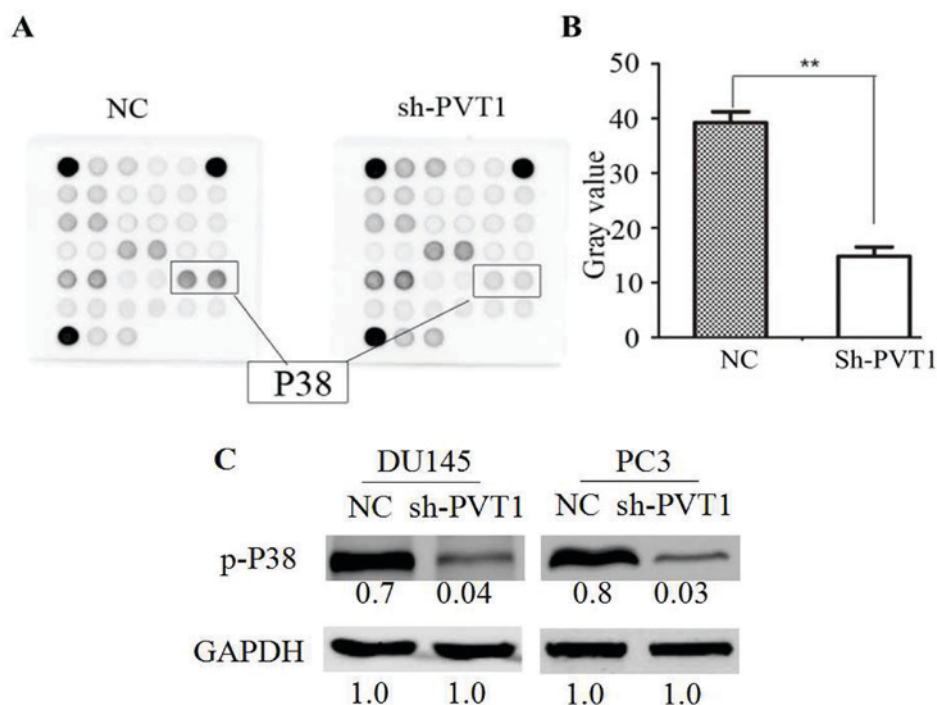

Figure 5. lncRNA PVT1-knockdown suppresses p38 phosphorylation in prostate cancer cells. (A) Decreased phosphorylation of p38 was observed in DU145 cells by intracellular signaling array following shPVT1 infection. (B) The gray value of p38 phosphorylation was statistically analyzed. (C) Decreased phosphorylation of p38 was validated in DU145 and PC3 cells by western blotting. **P<0.01 vs. control. lncRNA, long non-coding; NC, negative control; shRNA, short hairpin RNA; p-phosphorylated.

Readout) was utilized in DU145 cells. The phosphorylation level of p38 (Thr180/Tyr182) was demonstrated to be significantly reduced in the shPVT1 group compared with the NC group (Fig. 5A and B). Western blot analysis indicated that the phosphorylation levels of p38 cells were significantly reduced in the DU145 and PC3 cells with downregulated PVT1 expression compared with the NC group (Fig. 5C). Therefore, the data of the present study indicated that lncRNA PVT1 downregulation suppressed p38 phosphorylation.

Discussion

In previous studies it has been reported that lncRNA PVT1 expression is upregulated in various types of cancer,

including non-small cell lung cancer (NSCLC) (20,21), gastric cancer (22,23), renal cell carcinoma (24), cervical cancer (25), bladder cancer (26), hepatocellular carcinoma (HCC) (27), ovarian cancer (28), acute promyelocytic leukemia (29) and colorectal carcinoma (30). It has been indicated that lncRNA PVT1 is overexpressed in prostate cancer cells and may be a potential biomarker for prostate cancer (31). In previous studies, lncRNA PVT1 expression has been identified as a potential oncogene in multifarious types of cancer. For example, in HCC, high lncRNA PVT1 expression levels have been reported to be associated with a poor clinical prognosis (32). In addition, lncRNA PVT1 has been reported to promote HCC proliferation, the cell cycle, and the acquisition of stem cell-like properties (33). In another study, ectopic lncRNA PVT1 expression was indicated to significantly accelerate proliferation in NSCLC by downregulating p15 and p21 expression (34). In cervical cancer, it has been demonstrated that lncRNA PVT1 expression promoted cervical cell proliferation and migration depending on miR-200b silencing (25). Guan *et al* (35) reported that lncRNA PVT1-knockdown inhibited proliferation and enhanced apoptosis in breast and ovarian cancer cell lines. Downregulation of lncRNA PVT1 expression has been demonstrated to decrease proliferation and colony formation abilities *in vitro* and to suppress xenograft tumor proliferation *in vivo* by regulating p15 and p16 expression (36). The aforementioned findings indicate that lncRNA PVT1 could be a potential therapeutic target for these types of cancer (37).

However, the functional role of lncRNA PVT1 in prostate cancer cells requires further investigation. To examine the detailed underlying mechanism of lncRNA PVT1, lncRNA PVT1 expression was knocked down in PC3 and DU145 cell lines by lentivirus-mediated shRNA. The effect of lncRNA PVT1 on proliferation and migration of prostate cancer cells was evaluated by CCK-8, colony formation, and wound healing assays. The results of the present study indicated that lncRNA PVT1-knockdown inhibited prostate cancer cell proliferation and mobility *in vitro*. Furthermore, to determine the impact of lncRNA PVT1 on *in vivo* proliferation, a PC3 cell line stably transfected with lncRNA PVT1 was used to establish tumorigenicity in a tumor xenograft model. The results of the present study indicated that the tumorigenic capacity and weight in the shPVT1 group were decreased compared with the control group. Furthermore, knockdown of lncRNA PVT1 expression decreased the phosphorylation of p38, which has been identified as a mitosis-associated molecule, involved in the modulation of proliferation and migration of cancer cells (38,39). Therefore, lncRNA PVT1 expression may regulate prostate cancer proliferation through phosphorylation of p38. The results of the present study demonstrated that lncRNA PVT1 downregulation inhibited proliferation and migration of prostate cancer cells, which are associated with p38 expression. Therefore, lncRNA PVT1 may represent a novel diagnostic marker and a therapeutic target for prostate cancer. However, further investigation is required.

Acknowledgements

Funding

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

XMM conceived and designed the experiments. BW, HYW, DJL and XMZ performed the experiments. LRZ, BL and SBZ acquired and analyzed the data. XMM and BW contributed to drafting and revising the manuscript.

Ethics approval and consent to participate

All animal procedures and experiments were approved by the Southern Medical University Animal Research Ethics Committee (Guangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflicts of interest.

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