

Suppression of LASP-1 attenuates the carcinogenesis of prostatic cancer cell lines: Key role of the NF- κ B pathway

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Abstract. Prostate cancer (PCa) is one of the most frequently diagnosed cancers among males worldwide and causes a considerable number of deaths each year. One of the newly explored targets for the development of therapies against PCa is LIM and SH3 protein 1 (LASP-1). In the present study, the function of LASP-1 in the oncogenesis and metastasis of PCa was investigated using a series of *in vitro* experiments. Moreover, the mechanism through which LASP-1 exerted its effect on the carcinogenesis of PCa was also explored. The expression levels of LASP-1 in clinical PCa specimens were determined both at the mRNA and protein levels. Afterwards, the activity of LASP-1 in human PCa cell lines PC3 and DU145 was inhibited using a short hairpin RNA (shRNA) interfering method. The effects of LASP-1 knockdown on the cell growth, apoptosis, cell cycle distribution, migration and invasion were assessed. It was demonstrated that the expression of LASP-1 was significantly higher in the clinical PCa tissues than the level in the corresponding para-carcinoma tissues. Following the knockdown of the LASP-1 gene in human PCa cell lines, the viability, migration and invasion of the cancer cells were decreased. It was also demonstrated that the change in the cell viability and motile ability were associated with an induction of cell apoptosis and G1 phase cell cycle arrest. Based on the results of the detection of the expression of NF- κ B-related factors, it was indicated that LASP-1 may affect the carcinogenesis of PCa through a NF- κ B inhibition-dependent manner. Although the detailed explanation of the mechanism of LASP-1 in the carcinogenesis of PCa requires further elucidation, the present study highlights the potential of LASP-1 as a promising therapeutic target to ameliorate the oncogenesis and metastasis of PCa.

Introduction

Prostate cancer (PCa) continues to be the most frequently diagnosed cancer among males and is the cause of severe health issues in males worldwide (1). According to 2015 statistics (2), PCa ranks second as a leading cause of cancer-related deaths in men in the USA. Although most cases of PCa are curable, there is still 10-20% of PCa patients with poor prognosis who develop castration-resistant prostate cancer (3). Generally, the risk factors for PCa may be divided into an exogenous category, including aging, oxidative stress and family, and an endogenous category, including a rich fat diet and environmental agents (4,5). In addition to the multiple risk factors, the prognosis following traditional treatment modalities also varies greatly. The overall survival time of patients may range from 15 years to 2 years without a clear explanation (6,7). Thus, it is difficult to provide accurate clinical recommendations for PCa.

In recent years, studies regarding the dysregulation of microRNAs in PCa have been extensively performed. Molecular alterations associated with the miR regulation derived from these investigations have provided various potential targets involved in the oncogenesis and development of PCa (8-11). One of these newly explored targets is the LIM and SH3 protein 1 (LASP-1), which encodes a membrane-bound protein of 261 amino acids containing an N-terminal LIM domain (12,13). Although the exact biological function of LASP-1 has yet to be revealed, its association with multiple malignancies, including breast, colorectal, hepatocellular and bladder cancer, is well established (14-18). However, even with extensive interest in the function of LASP-1 in carcinogenesis, little effort has been made to elucidate the role of LASP-1 in PCa, not to mention assessment of its potential as an anti-PCa therapy.

A well establishment pathway involved in the growth of multiple types of tumor cells is nuclear transcription factor NF- κ B (19,20). This factor regulates the expression of immediate-early and stress response genes which are mainly implicated in acute inflammatory responses in various diseases. In addition, regulation of the level of NF- κ B has achieved considerable outcome in the treatment of various cancer types (21,22). Regarding PCa, the critical role of NF- κ B in the proliferation of PCa cells was also verified (23,24), but

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to the best of our knowledge, no studies have given attention to the association between LASP-1 and NF- κ B in PCa. Thus, a comprehensive study attempting to reveal the interaction between the two indicators may promote the development of anti-PCa therapies and the understanding of the mechanism of the carcinogenesis of PCa.

In the present study, for the first time, the expression level of LASP-1 in human PCa clinical samples was investigated. Then, the expression of LASP-1 in human PCa cell lines PC3 and DU145 was regulated by transfection of specific short hairpin RNA (shRNA) and the influence of LASP-1 knockdown on cell growth, cell cycle distribution, apoptosis, migration and invasion was detected. To preliminarily reveal the mechanism through which LASP-1 exerts its function in PCa cells, the expression levels of NF- κ B-related molecules were quantified by RT-qPCR and western blot assay. It was expected that the function of LASP-1 in PCa and the pathway through which LASP-1 contributes to the formation and progression of PCa could be partially elucidated by the findings in the present study.

Materials and methods

Chemicals and cell cultures. Antibodies against LASP-1, NF- κ B subunit p65 (P65), I κ B α and GAPDH were purchased from Promega (Madison, WI, USA). Human PCa cell lines DU145 and PC3 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). DU145 cells were cultured in Eagle's minimum essential medium (EMEM) supplemented with 1.8 mM CaCl₂ and PC3 cells were cultured in F-12 medium. Both media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. For experimental use, cells from three to six passages were employed.

Patients and PCa specimen collection. For detection of LASP-1 expression in clinical samples, 15 pairs of PCa and para-carcinoma tissues were collected from patients at The Second Hospital of Shandong University. All the samples met the following criteria: i) carcinoma tissues collected from nephrectomy were defined as primary prostatic adenocarcinoma while para-carcinoma samples were collected from kidney tissues of benign prostatic hyperplasia via renipuncture; ii) detailed information was available concerning the clinicopathological and prognostic characteristics of all the patients enrolled in the present study (Table I). Patients who had undergone any therapy for PCa before the surgical procedure were excluded. The present study was approved by the Ethics Committee of the Second Hospital of Shandong University. The Ethics Committee approved the related screening, inspection, and data collection of the patients, and all subjects signed a written informed consent form. All studies were undertaken following the provisions of the Declaration of Helsinki.

Knockdown of the LASP-1 gene in PCa cells by shRNA. The target sequence for LASP-1-specific shRNA (5'-GAAUCAGAAGACCCAGGATT-3') and the negative control (NC) shRNA (5'-UUCUCCGAACGUGUCACGUTT-3') were obtained from GeneChem Biotech (Shanghai, China). Two PCa lines were grouped into two treatment groups, respec-

tively: cells treated with NC-shRNA and cells treated with LASP-1-shRNA. Transfections were conducted using transfection agents obtained from Applygen Technologies Inc. (Beijing, China) (no. c1507) according to the manufacturer's instructions. Stable transfected cells for further experiments were screened in medium in the presence of G418 (0.5 μ g/ μ l).

Real-time quantitative PCR (RT-qPCR). Whole RNA in the different samples was extracted using RNAsimple Total RNA kit according to the manufacturer's instructions (no. DP419; Tiangen, Beijing, China). GAPDH was selected as the reference gene. Then, the RNA was reversely transcribing to cDNA templates using Super M-MLV Reverse Transcriptase (no. RP6502; BioTeke, Beijing, China). The final RT-qPCR reaction mixture of volume 20 μ l consisted of 10 μ l of SYBR-Green Master Mix, 0.5 μ l of each primer (LASP-1 forward, 5'-TTCCATTGCGAGACCTGC-3' and reverse, 5'-TGCCACTACGCTGAAACCT-3'; P65 forward, 5'-CTTACACTTAGCAATCATCCACCTT-3' and reverse, 5'-GCAAATCCTCCACCACATCTT-3'; I κ B α forward, 5'-CTGATGGAGTACCCTGAGGCTAT-3' and reverse, 5'-TGTCCGCAATGGAGGAGAAGT-3'; GAPDH forward, 5'-TATGATGATATCAAGAGGGTAGT-3' and reverse, 5'-TGTATCCAACTCATTGTCATAC-3'), 1 μ l of the cDNA template, and 8 μ l of RNase-free H₂O. Thermal cycling parameters for the amplification were set-up as follows: a denaturation step at 94°C for 2 min, followed by 40 cycles at 94°C for 20 sec, 58°C for 20 sec and 72°C for 20 sec. Relative expression level of the targeted gene was calculated with Exicycler™ 96 (Bioneer, Daejeon, Korea) according to the method 2^{- $\Delta\Delta$ Ct}.

Western blot analysis. Protein product of different samples was extracted using Whole Protein Extraction kit according to the manufacturer's instructions (WLA019; Wanleibio, Shenyang, China) and GAPDH was used as a reference protein. The concentration of the extracted protein samples was determined according to the BCA method. For western blot assay, 40 μ g of protein in 20 μ l solution was subject to a 13% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were subsequently transferred onto polyvinylidene difluoride (PVDF) membranes. Then, the membranes were washed in TTBS for 5 min and incubated with 5% skim milk powder solution for 1 h. The primary antibody against LASP-1 (1:1,500), P65 (1:2,000), I κ B α (1:2,000) or GAPDH (1:1,000) was added into the solution and incubated with the membranes at 4°C overnight. The membranes were then washed with TTBS four times and incubated with secondary IgG-HRP antibodies (1:20,000) for 45 min at 37°C. After the final six washes with TTBS, the blots were developed using Beyo ECL Plus reagent and the results were observed using a gel imaging system. The relative expression levels of LASP-1 in the different groups were calculated with Gel-Pro Analyzer (Media Cybernetics, Inc., Rockville, MD, USA).

CCK-8 assay. The cell viability of the PCa cells in the different groups was measured by Cell Counting Kit-8 (CCK-8) assay. In brief, 50 μ l of exponentially growing cells (3 \times 10³ cells/ml) were seeded into one well of a 96-well plate and cultured for 24 h. Each group was represented by 20 replicates and the cell viability of five randomly selected wells at 24, 48, 72 and 96 h

Table I. Clinicopathological information of the patients employed for clinical tissue collection.

No.	PCa or non-PCa	Age (years)	PSA (ng/ml)	Free/total PSA ratio (f/t)	Gleason score
1	PCa	68	133.2	0.14	5+4
2	PCa	55	1.25	0.26	4+5
3	PCa	72	43.0	0.11	4+4
4	PCa	83	14.0	0.10	4+3
5	PCa	70	89.6	0.05	4+4
6	PCa	65	28.6	0.14	3+4
7	PCa	75	209.6	0.04	4+4
8	PCa	83	31.3	0.07	4+4
9	PCa	62	6.29	0.12	3+3
10	PCa	67	7.5	0.22	3+3
11	PCa	74	10.7	0.15	3+3
12	PCa	75	100	0.56	4+5
13	PCa	83	575.6	0.06	4+4
14	PCa	78	152.1	0.06	4+4
15	PCa	76	9.47	0.06	2+3
1	Non-PCa				
2	Non-PCa				
3	Non-PCa				
4	Non-PCa				
5	Non-PCa				
6	Non-PCa				
7	Non-PCa				
8	Non-PCa				
9	Non-PCa				
10	Non-PCa				
11	Non-PCa				
12	Non-PCa				
13	Non-PCa				
14	Non-PCa				
15	Non-PCa				

PCa, prostate cancer; PSA, prostate-specific antigen.

was determined, respectively. For each well, CCK-8 solution (10 μ l) was added and the cultures were incubated at 37°C for 1 h. The optical density (OD) values at 450 nm in different wells were recorded using a microplate reader.

Flow cytometry. Cell cycle distribution and apoptotic rates in the different groups were determined using flow cytometry. Cells in the different groups were collected using centrifugation at 2,000 rpm for 5 min. Cell cycle distribution was detected according to standard procedure. In brief, the cells were fixed with 70% alcohol at 4°C for 2 h. Then, 500 μ l propidium iodide (PI)-FITC was added to the different samples to stain DNA in the dark at 4°C for 30 min. After a 20-min incubation at room temperature, the DNA contents of the cells were analyzed using a flow cytometer (BD Accuri C6; BD Biosciences, San Jose, CA, USA). Then, the cell apoptotic rates were also measured using an Annexin V-FITC apoptosis detection kit (WLA001c;

Wanleibio) according to the manufacturer's instructions. In brief, 5 μ l of Annexin V was added to the different wells. After incubation with Annexin V for 10 min at room temperature, the cells were resuspended with 1X binding buffer and 5 μ l PI was added. Then, the apoptotic rates were analyzed using a FACScan flow cytometry (Accuri C6). The apoptotic cells (UR + LR quadrants; the percentage of early and late apoptotic cells) was equal to the sum of the late apoptotic cells (UR, upper right quadrant; percentage of advanced stage apoptotic cells) and the early apoptotic cells (LR, lower right quadrant; percentage of prophase apoptotic cells).

Transwell experiment. The Transwell experiment which evaluated the migration ability of PCa cells in the different groups was performed. An amount of 200 μ l incubation (with 1 mM MgCl₂) medium containing 1x10⁴ cells were seeded into the upper chamber of Transwell chambers (Corning Star, Cambridge, MA, USA). Then, the cells were incubated at 37°C for 24 h to allow migration through the porous membrane. Upon completion of the culture, the cells remaining on the upper surface of the chamber were completely removed. The lower surfaces of the membranes were fixed with 4% paraformaldehyde for 20 min and stained in a solution containing 0.5% (w/v) crystal violet for 5 min. After being washed using ddH₂O, the numbers of cells in the different groups were determined using Image-Pro Plus 6.0 software (Nikon, Tokyo, Japan). Then, the invasion ability of the PCa cells was measured as described above with polycarbonate membranes being previously coated with 40 μ l Matrigel (1.5 mg/ml; BD Biosciences) at 37°C for 2 h to form a reconstituted basement membrane.

Statistical analysis. All the data are expressed in the form of mean \pm SD. Student's t-test was performed and a significant level of 0.05 was assigned. Statistical analysis was carried out using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA).

Results

LASP-1 expression in clinical samples is upregulated. To investigate the status of expression of LASP-1 in clinical samples, the mRNA and protein levels in 15 pairs of PCa specimens and corresponding para-carcinoma tissues were detected. All the PCa samples showed upregulation of LASP-1 expression when compared with the levels in their corresponding para-carcinoma tissues, and the average difference between carcinoma and para-carcinoma samples was statistically significant ($P < 0.05$) (Fig. 1).

LASP-1 knockdown attenuates the viability of the PC3 and DU145 cells. The cell viability of the cells following the different treatments was quantified using the CCK-8 assay. As shown in Fig. 2A, compared with control and NC groups, transfection of LASP-1-specific shRNA decreased the proliferation of the PC3 cells in the shRNA group at 48 h of the assay, and the differences between NC and shRNA groups were statistically significant for the last three time points ($P < 0.05$) (Fig. 2A). Similar results were also detected for the DU145 cells. Inhibition of LASP-1 in the PCa cells significantly decreased the cell viability (Fig. 2B).

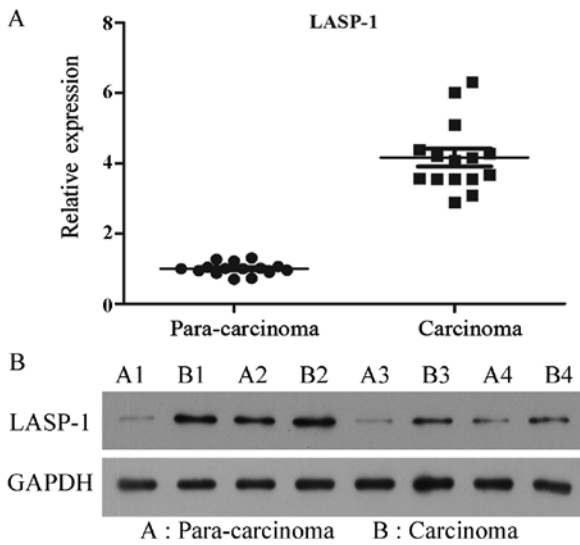


Figure 1. Expression of LASP-1 is upregulated in clinical prostate cancer (PCa) samples. (A) Quantitative analysis of the RT-qPCR results in the clinical samples. The expression levels of LASP-1 in all PCa patient samples were higher than those in their corresponding controls. (B) Representative images of western blot assay of four pairs of randomly selected clinical samples.

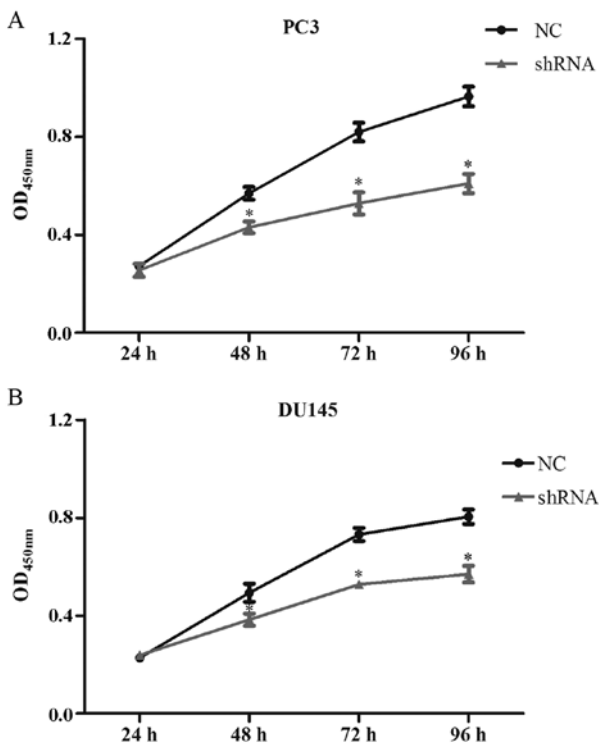


Figure 2. Knockdown of LASP-1 attenuates the cell viability of both prostate cancer cell lines. (A) Quantitative analysis results of cell viability of PC3 cells. (B) Quantitative analysis results of cell viability of DU145 cells. * $P < 0.05$, significantly different from the NC group.

LASP-1 knockdown increases cell apoptosis and induces cell cycle arrest in the PC3 and DU145 cells. Apoptosis and cell cycle distribution of cells in the different groups were analyzed by flow cytometry. After knockdown of LASP-1, late apoptotic (13.8±1.3%) and early apoptotic rates (20.4±3.1%) of the PC3 cells transfected with LASP-1-specific shRNA were

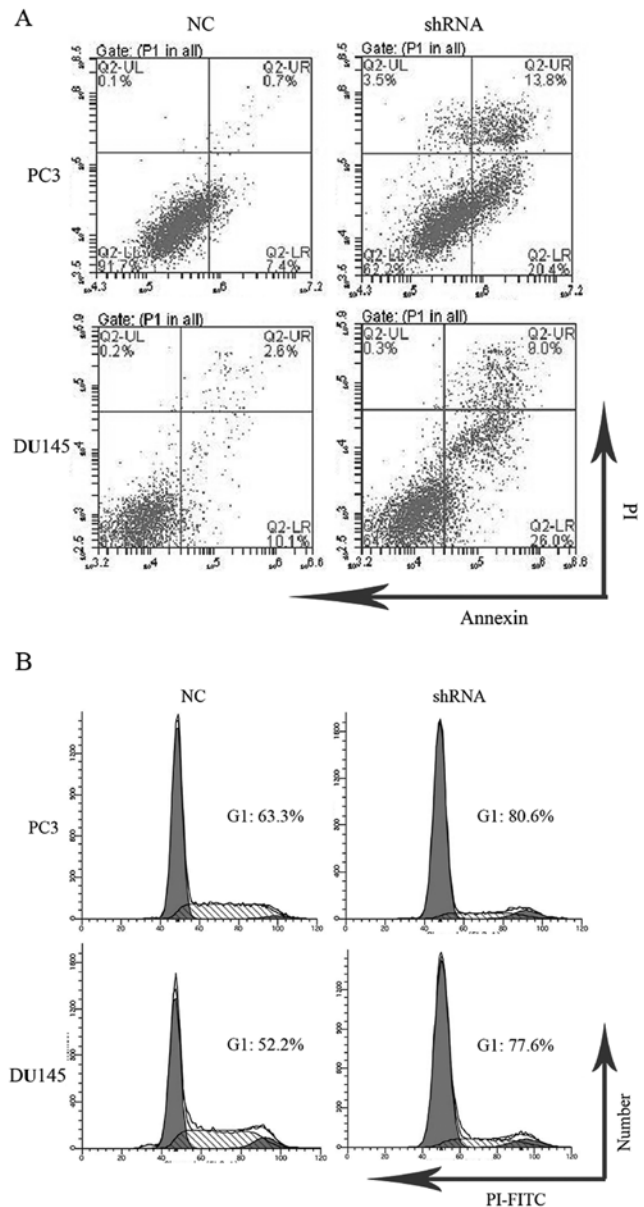


Figure 3. Knockdown of LASP-1 induces the apoptosis and G1 phase cell cycle arrest in both prostate cancer cell lines. (A) Representative images of the apoptotic rates. (B) Representative images of cell cycle distribution.

increased compared with those in the NC groups (0.7±0.02% for late apoptotic rate and 7.4±1.7% for early apoptotic rate) (Fig. 3A). Similar changes were observed for DU145 cells as well (Fig. 3A). As illustrated in Fig. 3B, PC3 cells following LASP-1 knockdown treatment showed G1 arrest by increasing the percentage of cells in the G1 phase (80.6±2.3%), which was markedly different from the results of the NC group (63.4±1.9%). The downregulation of LASP-1 in the PC3 cells also resulted in a concomitant decrease in the fraction of cells in the S phase, revealing that inhibition of LASP-1 could halt the cell proliferation of PC3 cells via cell cycle arrest at the G1 phase. A similar change in the pattern of cell cycle distribution was also recorded for the DU145 cells (Fig. 3B).

LASP-1 knockdown decreases cell migration and invasion ability of the PC3 and DU145 cells. The effect of LASP-1 on

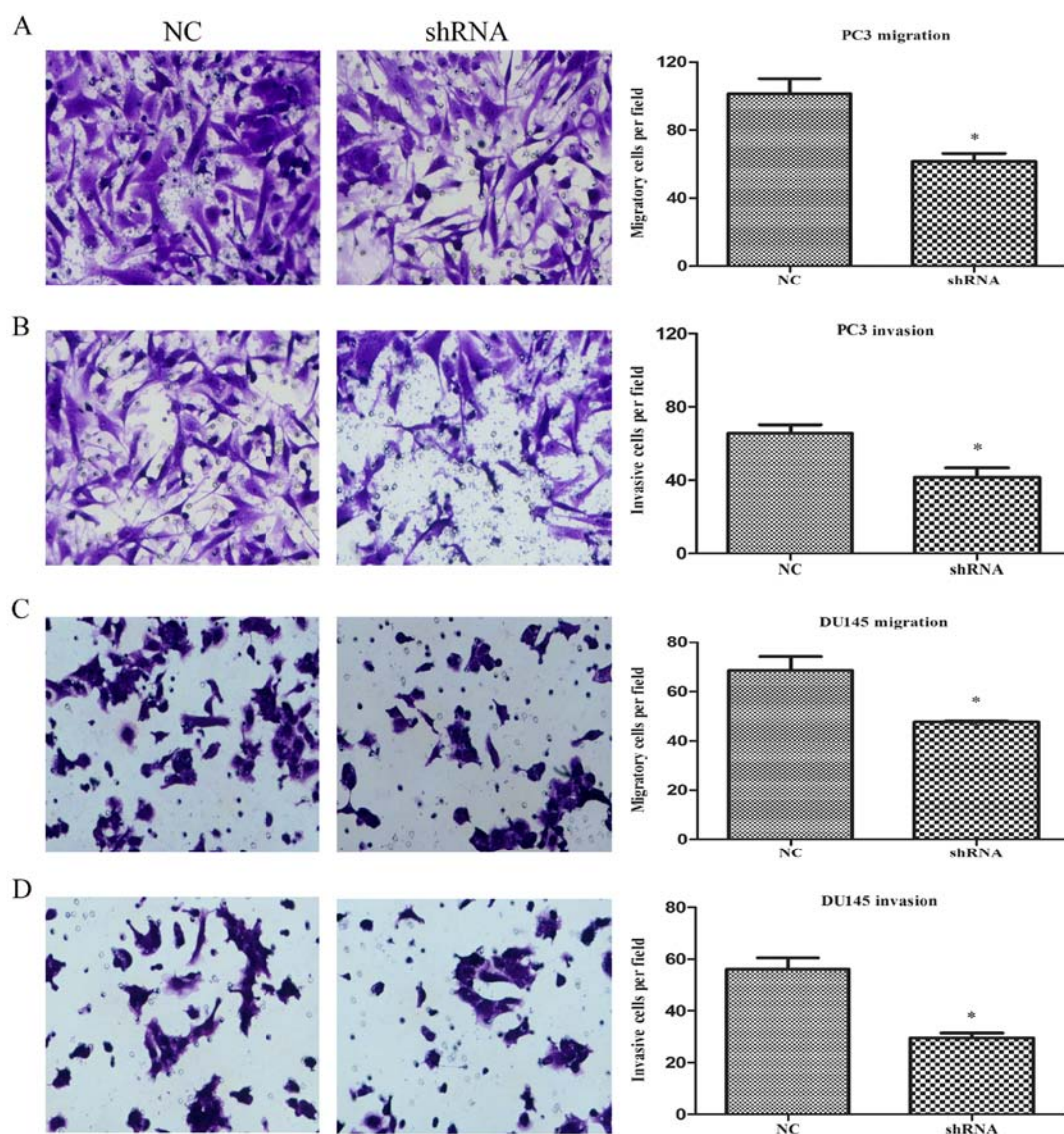


Figure 4. Knockdown of LASP-1 decreases the cell migration and invasion ability in both prostate cancer (PCa) cell lines. (A) Representative images and quantitative analysis of the effect of LASP-1 knockdown on the migration ability of PC3 cells. (B) Representative images and quantitative analysis of the effect of LASP-1 knockdown on the invasion ability of PC3 cells. (C) Representative images and quantitative analysis of the effect of LASP-1 knockdown on the migration ability of DU145 cells. (D) Representative images and quantitative analysis of the effect of LASP-1 knockdown on the invasion ability of DU145 cells. * $P < 0.05$, significantly different from the NC group; .

the migration of PCa cells was determined using Transwell assay. For measurement of the cell invasion ability, the polycarbonate membranes were previously coated with Matrigel to form a reconstituted basement membrane. As shown in Fig. 4A and B, the numbers of cells migrating through the porous membrane in the shRNA group (62 ± 4 for migration and 42 ± 2 for invasion assays) were significantly lower than those in the NC group (102 ± 4 for migration assay and 62 ± 2 for invasion assay) ($P < 0.05$), indicating an inhibitory effect on the cell motile ability due to the knockdown of LASP-1. Similar to the migration and invasion abilities of the PC3 cells, knockdown of LASP-1 in DU145 cells also resulted in a decrease in migration and invasion abilities (Fig. 4C and D).

LASP-1 knockdown downregulates the expression of NF- κ B-related molecules. To explore the mechanism which drives the effect of LASP-1 knockdown on the biological alterations in PCa cells, the expression of LASP-1, P65 and

I κ B α was quantified with RT-qPCR and western blot assay. It was found that inhibition of LASP-1 suppressed the expression of all three indicators at both the mRNA and protein levels (Figs. 5 and 6). Based on the quantitative analysis, the difference between shRNA and NC groups was statistically significant ($P < 0.05$). The result of RT-qPCR and western blot assay demonstrated that LASP-1 may play a role in the carcinogenesis of PCa through a NF- κ B-dependent manner.

Discussion

Despite the progress in the treatment of prostate cancer in the past few decades, PCa remains a major cause of health issues worldwide among males. Traditional treatments including surgery and radiation therapies seem to be less effective against PCa cases once metastasis occurs. Thus, identification of the biological markers related to oncogenesis and mobility of PCa cells is crucial for the development of therapeutic strategies to

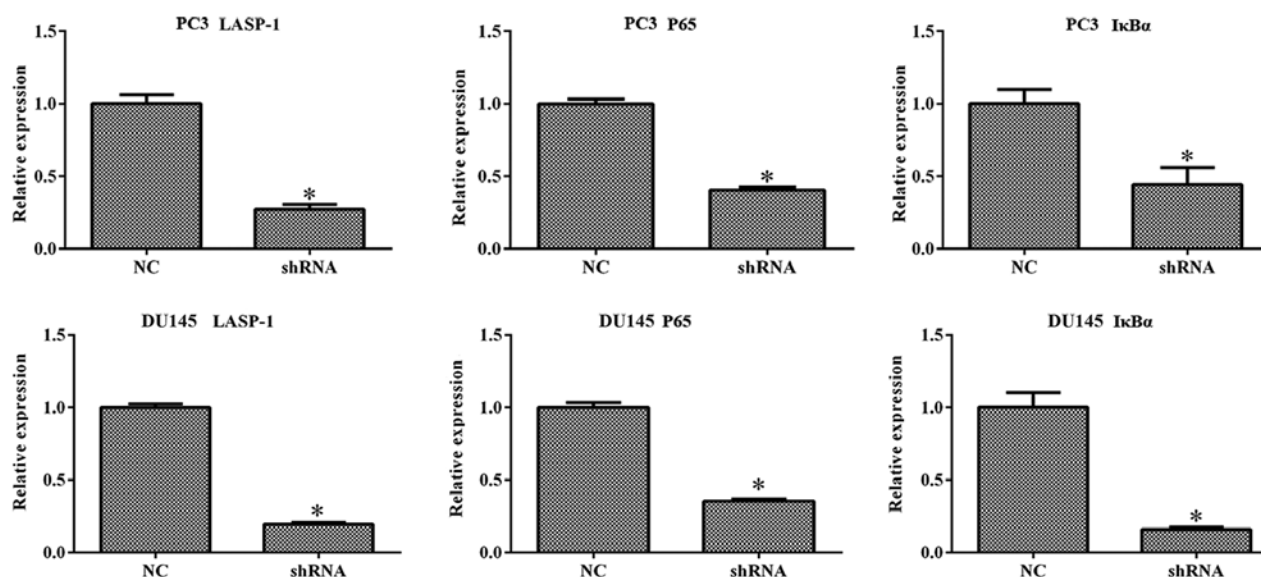


Figure 5. Knockdown of LASP-1 inhibits the expression of LASP-1, NF- κ B subunit p65 and I κ B α in both prostate cancer cell lines at the mRNA level. * $P < 0.05$, significantly different from the NC group.

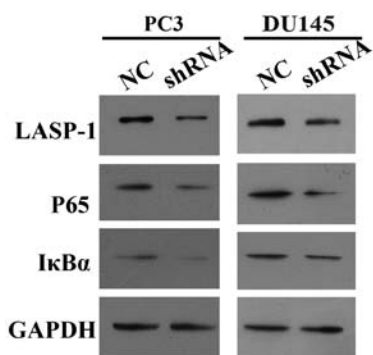


Figure 6. Knockdown of LASP-1 inhibits the expression of LASP-1, NF- κ B subunit p65 and I κ B α in both prostate cancer cell lines at the protein level.

improve the outcome and survival of this malignant cancer. In the present study, the expression of an important tumor-associated factor, LASP-1 in clinical PCa tissues and its function in the carcinogenesis of PCa were investigated for the first time. It was demonstrated that both LASP-1 mRNA and protein levels were significantly upregulated in clinical PCa tissues. Experimental observation based on the knockdown of the LASP-1 gene in human PCa cell lines PC3 and DU145 also clearly inferred that while growth and movement of PCa cells were attenuated, the apoptotic process was induced. All of these results suggest a key role of LASP-1 in the proliferation and motility of PCa cells.

The central role of LASP-1 in the progression and metastasis of various types of cancers was previously investigated (14-18). Results of the present study further support the conclusions of these studies by verifying the important function of LASP-1 in PCa. In clinical PCa samples, the production of LASP-1 at the mRNA and proteins levels was enhanced. Moreover, knockdown of LASP-1 in human PCa cell lines resulted in a deteriorating effect on cell growth. Furthermore, flow cytometric assay also showed that the decrease in cell

proliferation in response to LASP-1-specific shRNA treatment was accompanied by the induction of cell apoptosis and G1 phase cell cycle arrest. However, contradiction regarding the function of LASP-1 in cancer cell viability still exists. According to the study of Grunewald *et al* (14), no significant influence of LASP-1 knockdown on the apoptotic process in BT-20 and MCF-7 cell lines was observed. Although the two studies focused on distinct cancer types, this information is indicative of the complicated mechanism of LASP-1 in regulating cancer cell apoptosis.

LASP-1 is a well-known focal adhesion adaptor protein and is closely associated with the modulation of cytoskeleton and cancer cell migration and invasion (12,25-27). The factor is capable of interacting and co-localizing with a series of focal adhesion proteins, such as F-actin, zyxin and LPP. Thus, silencing of LASP-1 causes cellular localization change of its binding partners at the focal adhesion site and influences the mobility of cells. In the present study, it was found that cells treated with LASP-1-specific shRNA exhibited a significant decrease in cell migration and invasion ability, which proved the critical function of LASP-1 in the metastasis of cancer cells as well. However, as previously reported, cells depleted of LASP-1 may still attach to the extracellular matrix (ECM) and form focal adhesions. It is hypothesized that the molecule may play a supportive role in focal adhesion dynamics rather than actual formation of the related structures (25).

To further elucidate the mechanism through which LASP-1 exerts its function in the onset and development of PCa, the interaction between LASP-1 and NF- κ B was investigated. NF- κ B is a transcriptional factor which regulates the apoptosis and inflammation in various diseases (28). The inactive form of NF- κ B is sequestered in the cytoplasm by binding with I κ B α . Once I κ B α is phosphorylated into p-I κ B α , P65 may be translocated into the nucleus and activates transcription of the targeted genes. In the present study, the results showed that LASP-1-specific shRNA reduced NF- κ B production and P65 translocation to the nucleus, which would lead to the inhibition

of NF- κ B activity. In fact, activation of NF- κ B has been implicated in both carcinogenesis and drug resistance in cancer cells (29). Thus, inhibition of NF- κ B activation may represent a promising opportunity for expanding therapeutic windows in translational cancer research (30,31). Considering the high efficiency of LASP-1 knockdown in attenuating NF- κ B activity in the present study, it was reasonable to demonstrate that therapies based on LASP-1 interference may antagonize PCa through a NF- κ B inhibition-dependent manner.

Conclusively, the major findings outlined in the present study elucidated that the expression of LASP-1 was abnormally high in clinical PCa tissues, and following knockdown of LASP-1 in human PCa cell lines, the proliferation and mobility of cancer cells were markedly inhibited. Preliminarily, it was hypothesized that LASP-1 may activate the carcinogenesis of PCa through an NF- κ B-dependent manner. Although a detailed explanation of the effect of LASP-1 on PCa was not fully elucidated, the present study highlights that LASP-1 has the potential to be a promising target for alleviation of PCa in the clinic. To elaborate the role of LASP-1 in PCa, more comprehensive studies need to be conducted in the future.

Acknowledgements

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