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ORIGINAL ARTICLE

Long non-coding RNA VIM-AS1 promotes prostate cancer growth and invasion by regulating epithelial–mesenchymal transition

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Summary

Purpose: *Long non-coding RNAs (lncRNAs) have been elucidated to participate in the development and progression of prostate cancer (PCa). Here, we aimed to detect the expression, function and further underlying the mechanisms of lncRNA VIM-AS1 in PCa.*

Methods: *A total of 88 PCa and 31 normal prostate tissue samples were collected after surgical resection. Expression of VIM-AS1 in the samples was detected using quantitative real-time polymerase chain reaction (qRT-PCR). Similarly, the relative level of VIM-AS1 in PCa cell lines to normal prostate cell line was also measured. Lentivirus for up- or down-regulating VIM-AS1 was used to establish the experimental cells. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay and colony formation assay were utilized to study the proliferation, and wound-healing, and transwell assay was utilized to study the migration and invasion abilities of established cells. Furthermore, western blot was employed to detect the expression of the related proteins.*

Results: *VIM-AS1 was expressed significantly higher in PCa tissues comparing with normal prostate tissues. Higher VIM-AS1 expression predicted larger tumor size, metastasis and advanced TNM stage. Inhibition of VIM-AS1 reduced cell proliferation, migration and invasion of PC3 cells but overexpression of VIM-AS1 promoted cell growth, migration and invasion. We also found VIM-AS1 promoted the expression of vimentin, which further promoted epithelial-mesenchymal transition (EMT) of PCa cells.*

Conclusions: *lncRNA VIM-AS1 was overexpressed in PCa tissues and cell lines and promoted PCa proliferation and metastasis via EMT through regulating vimentin, which might provide a novel target for the diagnosis and therapy for PCa.*

Key words: *lncRNA, VIM-AS1, prostate cancer, growth, invasion, EMT*

Introduction

Prostate cancer (PCa) ranks second in incidence of male malignancies worldwide. In the United States, prostate cancer is the number one cancer that endangers men's health. PSA screening combined with prostate biopsy is important for the early diagnosis of PCa [1]. However, PSA detection still has many limitations in specificity and sensitivity, and when it is in the gray area (4 to 10 ng/mL), the sensitivity of prostate biopsy for PCa is low [2,3]. Therefore, there is an urgent need for more effective diagnosis and treatment methods that could be applied clinically.

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Long non-coding RNA (lncRNA) is an endogenous transcribed RNA molecule containing 200 to 100 000 nucleotides, located in the nucleus or cytoplasm [4]. LncRNA lacks an obvious open reading frame and has no protein-coding function. It regulates the expression level of genes in the form of epigenetics, transcription and post-transcription, causing development of diseases and tumors [5]. In the gene regulatory network of tumorigenesis and development, the abnormal regulation of lncRNA is already an indispensable component. With the in-depth study of lncRNA, high evidence indicates that the abnormal expression of lncRNA is closely related to the occurrence, development, metastasis and prognosis of PCa [6,7]. For example, in colon cancer, LINC01234 promotes cell proliferation by binding miR-642a-5p competitively [8]. In PCa, lncRNA TINCR is associated with clinical progression and has tumor suppressive role [9]. In hepatocellular carcinoma, lncRNA Linc-GALH accelerates cell invasion and migration via regulating Gankyrin [10]. LncRNA MALAT1 expression inhibition suppresses tongue squamous cell carcinoma proliferation, migration and invasion by inactivating PI3K/Akt pathway and downregulating MMP-9 expression [11].

LncRNA VIM-AS1, located at 10p13, was reported to play a crucial role in colorectal cancer and type 2 diabetes [12-14]. However, the expression of VIM-AS1 in PCa and its function has not been reported before.

In this study, we first detected VIM-AS1 expression in 88 PCa and 31 normal prostate tissue samples. The relationship between VIM-AS1 and clinicopathologic characteristics was confirmed. Expression of VIM-AS1 in PC3 or LNCaP cells was over-expressed or low-expressed using lentivirus. Then, cell proliferation and metastasis were measured through functional experiments. Vimentin and EMT were verified as underlying molecular and pathways of VIM-AS1 in PCa. Therefore, our study might provide new potential targets for cancer diagnosis and treatment.

Methods

Clinical samples

All 88 PCa specimens were obtained from PCa surgically removed between August 2016 and March 2018 in Hongkou Branch of Changhai Hospital, Navy Medical University (Second Military Medical University). Each specimen was confirmed by two pathologists. The 31 normal prostate tissues of the control were obtained from specimens of surgically resected patients with enlarged prostate. Specimens were removed and stored in liquid nitrogen. This study was approved by the Ethics Committee of Hongkou Branch of Changhai Hospital, Navy Medical University (Second Military Medical University). Signed informed consents were obtained from all participants before the study entry.

Cells and culture

Four PCa-derived cell lines (LNCaP, DU145, 22RV1, PC3) and human normal prostate epithelial cell line RWPE-1 were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in the Dulbecco's Modified Eagle Medium (DMEM, Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin (HyClone, South Logan, UT, USA). Cells were maintained in the incubator at 37°C containing 5% CO_2.

Overexpression and interference of VIM-AS1

The lentivirus for overexpressing and shRNA for interfering VIM-AS1 were constructed by GeneWiz (Suzhou, China). LNCaP cells were transfected with the lentivirus while PC3 cells with shRNA according to the manufactures' instructions. The efficiency of the transfection was confirmed by using qRT-PCR.

RNA isolation and qRT-PCR

Total RNAs of PCa tissues and cells were isolated using TRIzol reagent (Beyotime, Shanghai, China). The RNAs were reverse-transcribed into cDNAs using random primers with TaKaRa Reverse Transcription Kit (Tokyo, Japan). QRT-PCR was performed using ABI-7900 (ABI, Foster City, CA, USA) with SYBR Green Kit (TaKaRa, Tokyo, Japan). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control for VIM-AS1 or Vimentin mRNA. The relative expression of lncRNA was calculated using the $2^{-\Delta\Delta Ct}$ method. All the primers were synthesized by GeneWiz. The primers used were as follows (Sequence (5'-3')): VIM-AS1: Forward, TAGTTGGCGAAGCGGTCATT, Reverse, CCAAAGGGAGCTTTGCTGTC; Vimentin: Forward, AGTCCACTGAGTACCGGAGAC, Reverse, CATTTCACGCATCTGGCGTTC; GAPDH: Forward, CTGGGCTACACTGAGCACC, Reverse, AAGTGGTCGTTGAGGGCAATG.

Cell counting kit-8 (CCK-8) assay

Cells treated with shRNA-VIM-AS1 or LV-VIM-AS1 were processed into single cell suspension and seeded in 96-well plates. Then, cells were cultured in 100 µL of 10% FBS. At experimental time points (0 h, 24 h, 48 h, 72 h), the medium was added with 10 µL of CCK-8 reagent (Donjindo, Tokyo, Japan). After 2-h culture, the absorbance at 490 nm was measured using a spectrophotometer. The experiments were repeated at least three times.

Colony formation assay

The experimental cells were prepared as a single cell suspension and planted in 6-well plates at a density of 500 cells per well. After 15 days of culture in 10% FBS, cells colony formation was observed. The cells

were fixed with methanol, stained with crystal violet, and photographed.

Wound healing assay

The treated PC3 or LNCaP cells were placed in 6-well plates and cultured in 10% FBS until cells congested the entire surface of the 6-well plates. Using a 200 μL tip, three wounds were created vertically on the surface of cells at the bottom of the 6-well plates. After washing with phosphate buffered saline (PBS), the cells were cultured in serum-free medium for 48 h, and then the wound healing condition was recorded under a microscope.

Transwell assay

For the migration assay, 8 μm pore size inserts (Millipore, Billerica, MA, USA) were obtained. Established PC3 or LNCaP cells were suspended in serum-free medium and placed into the top chamber of the insert at a density of 3×10^4 cells per well. A total 500 μL medium containing 10% FBS was added in the lower chamber. After incubation for 36 h, cells on the upper chambers that did not pass through the membrane were wiped off with using cotton swabs. Cells on the lower surface were fixed with methanol and stained with crystal violet and recorded under a microscope (Zeiss, Oberkochen, Germany) for 5 random visions at 400× magnification. For the invasion assay, the insert was pre-coated with matrigel (BD, Franklin Lakes, NJ, USA). The other steps were the same with the migration assay.

Protein extraction and western blot

Radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) was employed to extract proteins from experimental cells containing Cocktail inhibitors (Beyotime, Shanghai, China). The extracted protein concentration was detected by a bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China). A total of 20 μg protein was placed on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel for electrophoresis. After electrophoresis was completed, the protein was transferred to the polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After closing non-specific binding sites with 5% skim milk, the stripe was immersed in specific primary antibody at 4°C overnight. Then, washing stripes three times with tris buffered saline-tween (TBST), the horseradish peroxidase-labeled secondary antibody was used to bind the primary antibody. Then, an electrochemiluminescence (ECL) kit (Millipore, Billerica, MA, USA) was used to quantify the protein level. The antibodies used in the experiment was as follows: antibody for Vimentin (1:1000, Abcam, Cambridge, MA, USA), antibody for N-cadherin (1:1000, Abcam, Cambridge, MA, USA), antibody for E-cadherin (1:1000, Abcam, Cambridge, MA, USA), antibody for GAPDH (1:2000, Abcam, Cambridge, MA, USA).

Statistics

SPSS 19.0 software (IBM, Armonk, NY, USA) was utilized to analyze the data. Quantitative data were displayed as mean ± standard deviation (SD). The difference between groups was evaluated using Student's t-test and one-way ANOVA. P value <0.05 was considered to show statistical significance.

Results

VIM-AS1 was overexpressed in PCa tissues and cells

For detecting the expression of VIM-AS1 in PCa tissues, we collected 88 PCa and 31 normal prostate tissue samples and measured the VIM-AS1 level using qRT-PCR. Figure 1A showed that PCa tissue samples group expressed significantly higher VIM-AS1 level than the normal control group. Also, we detected VIM-AS1 level in 4 PCa-derived cell lines (LNCaP, DU145, 22RV1, PC3) comparing to human normal prostate epithelial cells RWPE-1 and found increased VIM-AS1 levels in the 4 PCa cell lines (Figure 1B). These results indicated that VIM-AS1 was upregulated in PCa tissues and cell lines. Furthermore, we divided the 88 samples into

Table 1. Relationship between VIM-AS1 level and the clinicopathological characteristics of 88 patients with prostate cancer

		VIM-AS1 level			
	Group	*Total*	*High*	*Low*	*p value*
Age (years)	<70	51	25	26	0.829
	>70	37	19	18	
Metastasis	Yes	26	21	5	0.0002
	No	62	23	39	
PSA level	<4	18	2	16	0.0002
	4-10	26	12	14	
T stage	>10	44	30	14	0.0000
	T1	54	17	37	
	T2/T3	34	27	7	

The median expression level of VIM-AS1 was used as the cutoff.

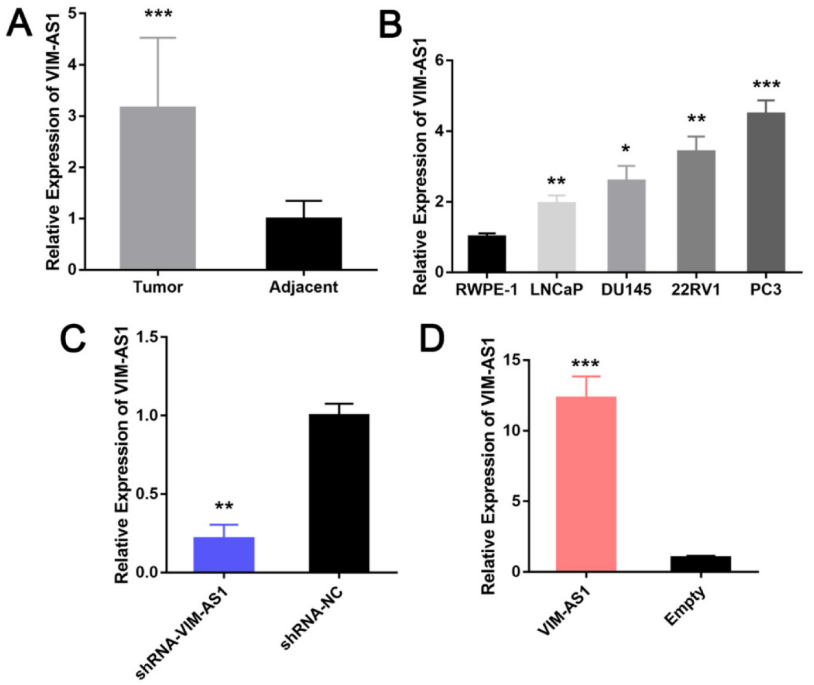

Figure 1. LncRNA VIM-AS1 was highly expressed in prostate cancer (PCa) tissues and cells. **A:** QRT-PCR showed the lncRNA VIM-AS1 expression level in 88 PCa tissues and 31 adjacent tissues. **B:** VIM-AS1 expression level in prostate cancer cell lines (LNCaP, DU145, 22RV1, PC3) and human normal prostate epithelial cell line (RWPE-1). **C:** ShRNA targeting VIM-AS1 (shRNA-VIM-AS1) and negative controls (shRNA-NC) were transfected into PC3 cells. **D:** LV-VIM-AS1 and LV-Empty were transfected into LNCaP cells. ***p<0.001, **p<0.01 *p<0.05 compared to control group.

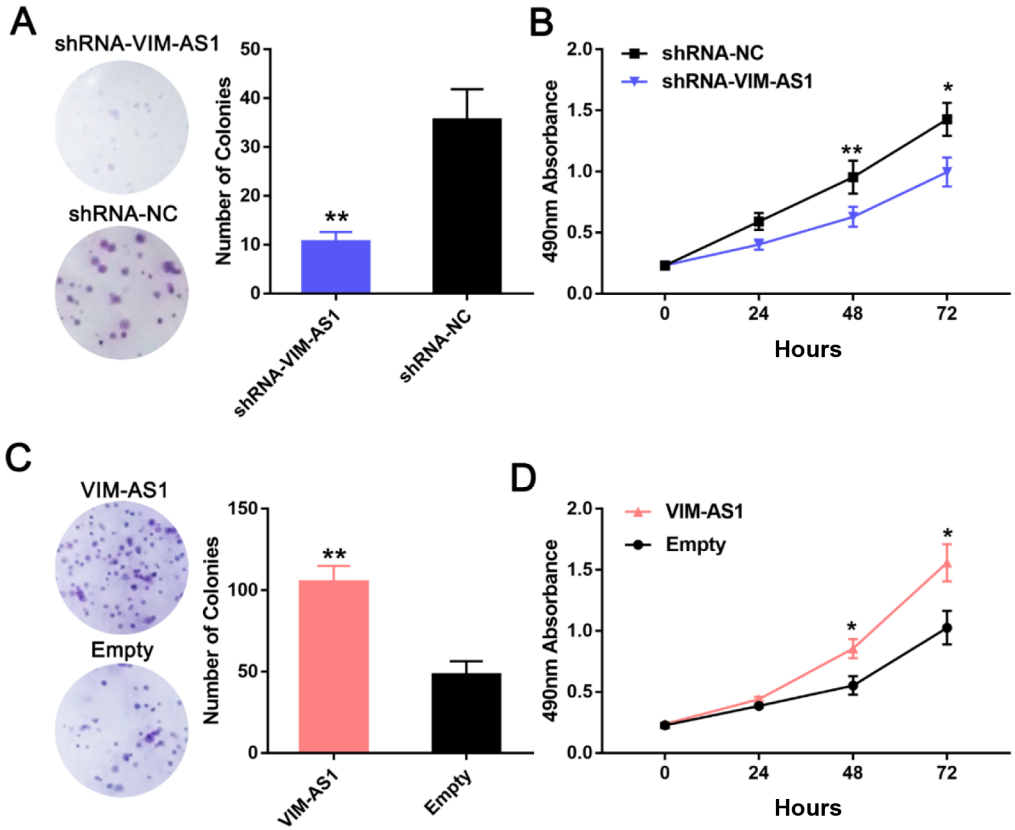

Figure 2. VIM-AS1 affected the proliferation of PCa cells *in vitro*. **A,B:** Clone formation assay showed the proliferation ability of PC3 cells transfected with shRNA-VIM-AS1 and shRNA-NC or LNCaP cells transfected with LV-VIM-AS1 and Empty. **C,D:** CCK-8 assay showed the proliferation ability of PC3 cells transfected with shRNA-VIM-AS1 and shRNA-NC or LNCaP cells transfected with LV-VIM-AS1 and Empty. **p<0.01, *p<0.05 compared to control group.

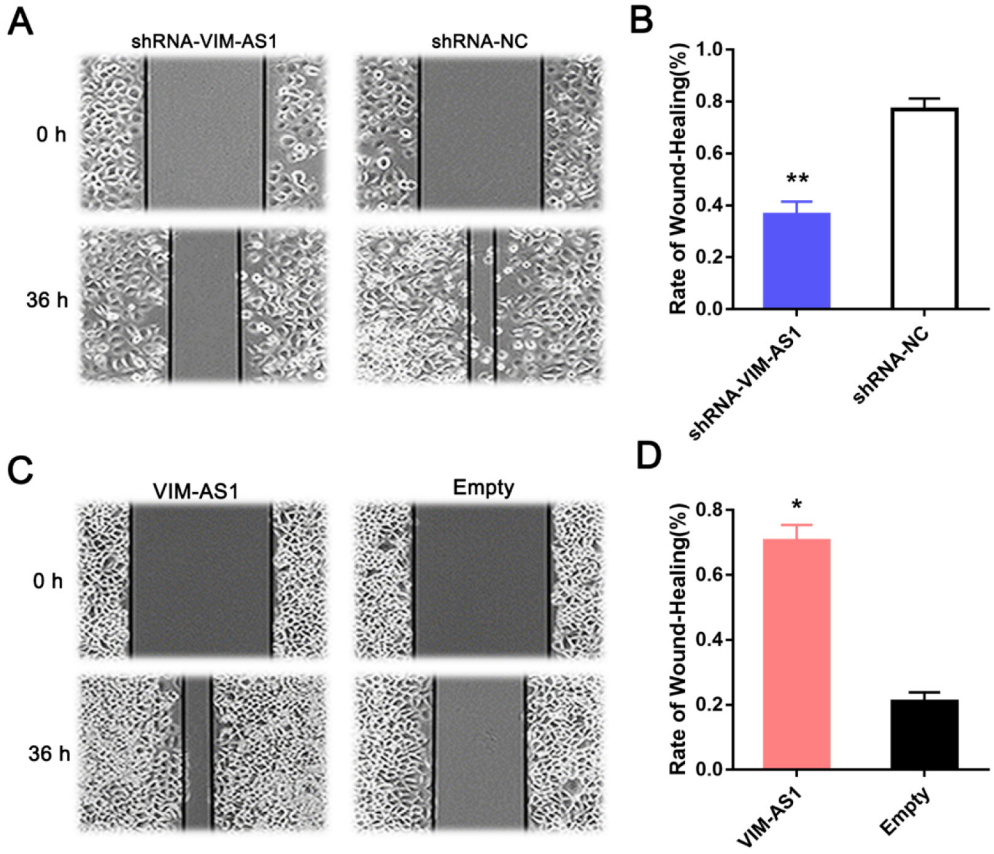

Figure 3. VIM-AS1 affected wound-healing ability of PCa cells. **A,B:** Wound-healing assay was used to detect the migration ability of shRNA-VIM-AS1 treated PC3 cells compared with the shRNA-NC group. **C,D:** Wound-healing assay was used to detect the migration ability of LV-VIM-AS1 treated LNCap cells compared with the Empty group. **p<0.01, *p<0.05 compared to control group.

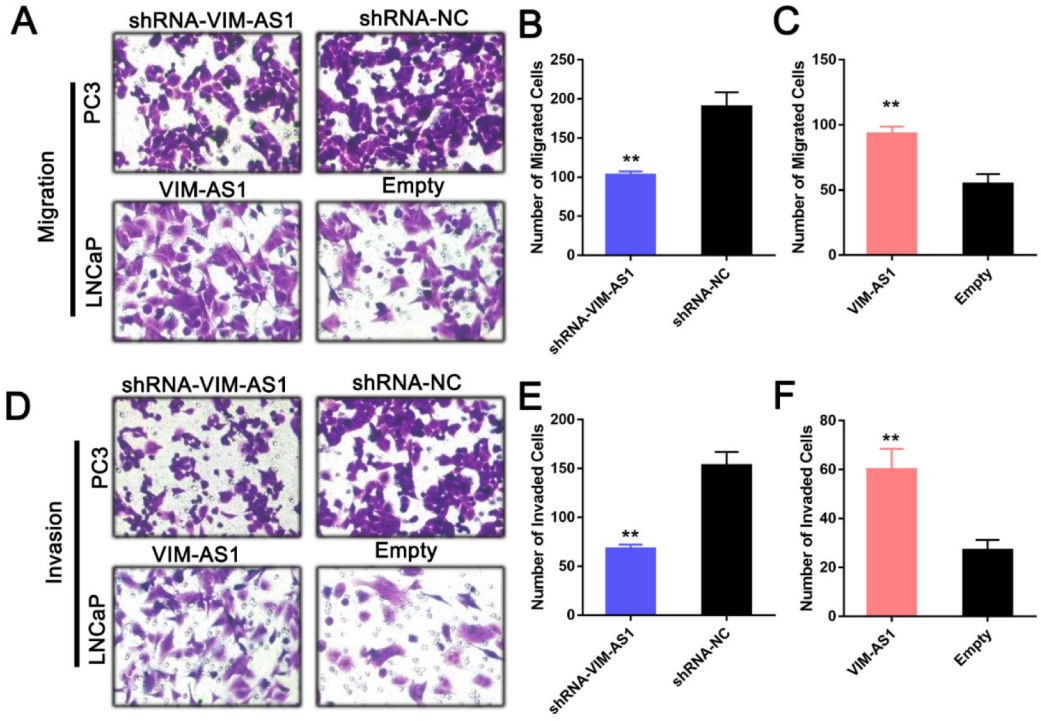

Figure 4. VIM-AS1 affected invasion and migration of PCa cells. **A,B,C:** Transwell migration assay indicated the invaded cell number in established PC3 cells and LNCaP cells. **D,E,F:** Transwell invasion assay showed the migrated cell number in established PC3 cells and LNCaP cells. **p<0.01, and p<0.05 compared to control group.

high VIM-AS1 expression group and low VIM-AS1 expression group according to the median VIM-AS1 expression level and explored the relationship between the VIM-AS1 levels with clinicopathological features. As shown in Table 1, higher VIM-AS1 expression showed higher PSA level, poor T-stage and metastasis. Next, to study the function of VIM-AS1 in PCa cells, we inhibited VIM-AS1 expression using shRNA in PC3 cells and overexpressed using VIM-AS1 lentivirus for LNCAP cells. The shRNA-VIM-AS1 group showed lower VIM-AS1 expression but the VIM-AS1 group showed higher when compared to their relative control group (Figure 1C,1D).

Ectopic VIM-AS1 level affected cell proliferation of PCa

Next, we employed colony formation and MTT assays to elucidate the function of VIM-AS1 on cell proliferation. Clearly, inhibition of VIM-AS1 reduced the colony formation ability of PC3 cells (Figure 2A). However, LNCaP cells formed more colonies after VIM-AS1 upregulation comparing with the control group (Figure 2C). Similarly, MTT assay emphasized that knockdown of VIM-AS1 inhibited the growth of PC3 cells while overexpression of VIM-AS1 promoted the proliferation of LNCaP cells (Figure 2B, 2D). These results indicated that VIM-AS1 could accelerate cell proliferation of PCa cells.

Abnormal VIM-AS1 expression influenced cell migration and invasion of PCa

As we have verified VIM-AS1 could promote PCa cell growth, we then studied the effects of VIM-AS1 on cell metastasis. Using wound healing assay, we found the healing rate of PC3 cells after shRNA-VIM-AS1 treatment was significantly

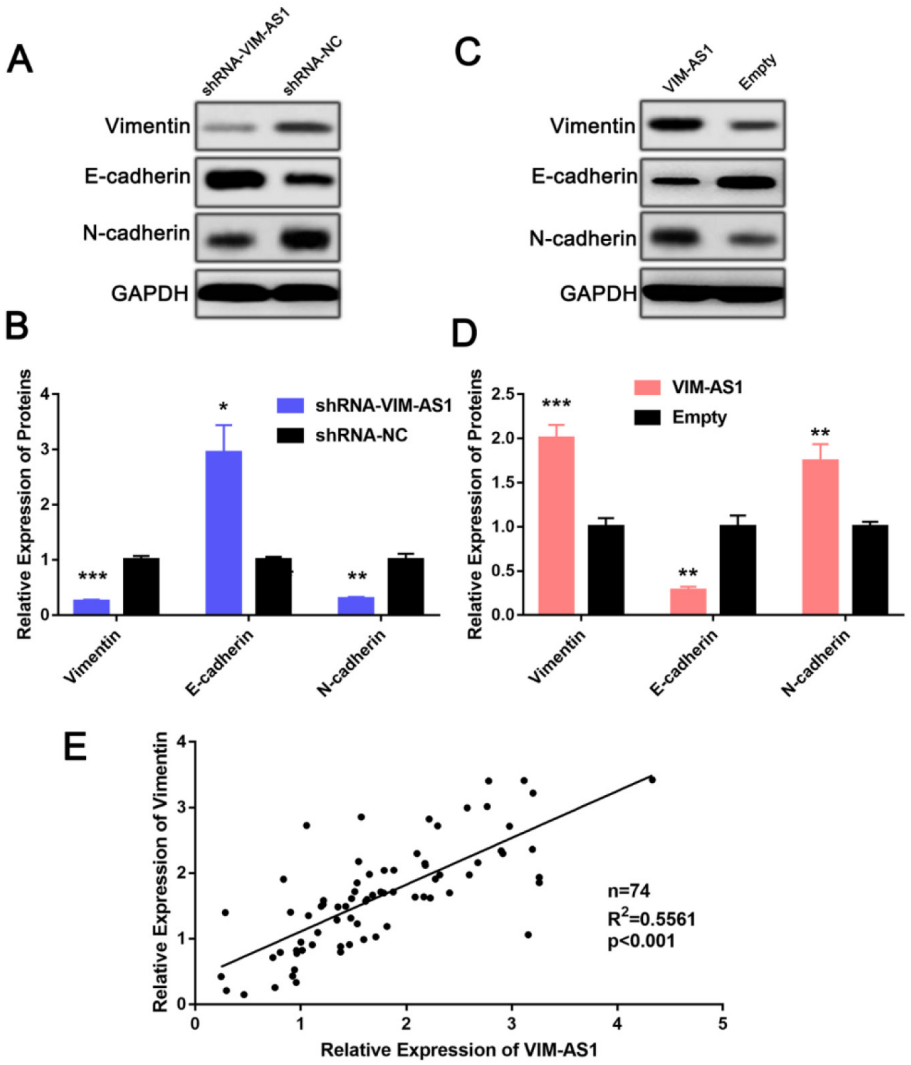

Figure 5. VIM-AS1 promoted EMT of PCa via Vimentin. **A,C:** Expression of EMT markers in experimental cells. **B,D**: Relative level of protein. **E:** relationship between expression of VIM-AS1 and Vimentin mRNA in 88 NSCLC tissues. Data are presented as the mean ± SD of three independent experiments. ***p<0.001, **p<0.01 *p<0.05 compared to control group..

lower than the shRNA-NC group (Figure 3A, 3B). In the contrast, the wound of VIM-AS1 overexpressed LNCaP cells healed faster that the control group (Figure 3C,3D). Furthermore, transwell migration assay demonstrated that downregulation of VIM-AS1 inhibited PC3 cell migration while upregulation of VIM-AS1 increased LNCaP cell migration compared to each control group (Figure 4A-4C). Likewise, transwell invasion assay elucidated that inhibition of VIM-AS1 decreased the invasion ability of PC3 cells but overexpression of VIM-AS1 promoted the invasion ability of LNCaP cells when comparing with their relative control group (Figure 4D-4F). These data suggested VIM-AS1 improved cell migration and invasion of PCa.

VIM-AS1 promoted EMT via promoting Vimentin expression

Next, we further explored the underlying mechanism of VIM-AS1 in PCa to explain the function of VIM-AS1 as a tumor-promoting gene. Several studies have proved "Flank 10" rules which mean that lncRNAs could affect the gene function on the upstream 10 kb. According to this, we found Vimentin as a potential target for VIM-AS1. Using western blot, we revealed that inhibition of VIM-AS1 reduced Vimentin protein level in PC3 cells, while upregulation of VIM-AS1 increased Vimentin expression (Figure 5A-5D). This indicated VIM-AS1 might affect the expression of Vimentin. Next, we measured the Vimentin RNA level in 88 PCa tissue samples comparing to the VIM-AS1 RNA level and found a positive correlation between VIM-AS1 and Vimentin (Figure 5E). This verified Vimentin as a downstream molecule for VIM-AS1 in PCa. In addition, we also tested EMT markers expression including E-cadherin, N-cadherin and Vimentin, which function as a core member in EMT. Expression of epithelial marker E-cadherin was increased after VIM-AS1 knockdown but decreased after VIM-AS1 overexpression; besides, the mesenchymal marker N-cadherin was reduced after VIM-AS1 inhibition but improved after VIM-AS1 upregulation (Figure 5A-5D). All these indicated VIM-AS1 promoted Vimentin expression and accelerated EMT of PCa.

Discussion

In recent years, lncRNAs are subjects of extensive attention. More and more lncRNAs, which are closely related to the development of PCa, have been discovered [15-17]. The effects of lncRNA on the growth, proliferation, invasion and apoptosis of PCa cells have been elucidated. For example, lncRNA PCGEM1, induced by hypoxia, promoted gastric cancer cell invasion and migration via regulating SNAI1 expression [18]. LncRNA ARLNC1 could promote PCa tumorigenesis and development while lncRNA PCA3 could inhibit PCa cell growth and metastasis by regulating PRUNE2 [19,20]. Also, lncRNA HOXD-AS1 regulated PCa cell proliferation and chemoresistance by recruiting WDR5 [21]. In addition, lncRNA ZEB1-AS1 changed the expression of ZEB1 epigenetically to regulate PCa development and progression [22]. LncRNA VIM-AS1 was identified as an oncogene in colorectal cancer. However, the gene regulation mechanism in PCa is not well understood and further research is needed.

Herein, we first detected the VIM-AS1 expression in PCa tissues and cells. The VIM-AS1 was significantly overexpressed in PCa tissues compared with the normal control while PCa-derived cells had higher VIM-AS1 level as well. This is in line with previous studies that VIM-AS1 played a promoting role in tumors. Also, higher VIM-AS1 indicated poor T stage, metastasis, and higher PSA level, suggesting poor prognosis of patients with PCa. Next, we up- or downregulated VIM-AS1 expression in PCa cells to demonstrate the influence of VIM-AS1 in PCa cells. Clearly, inhibition of VIM-AS1 inhibited cell proliferation, migration and invasion of PC3 cells but its overexpression promoted cell growth, migration and invasion of LNCaP cells. These confirmed VIM-AS1 could promote PCa progression.

Furthermore, we found Vimentin as a potential target for VIM-AS1 according to "Flank 10kb" theory, which reveals that almost >65% lncRNAs located within 10 kb of known protein-coding genes may function as cis-acting or trans-acting regulators via regulating these genes [18,22]. Using qRT-PCR, we confirmed that VIM-AS1 level positively correlated to the mRNA level of VIM-AS1 in PCa tissues, which showed that VIM-AS1 affected the progression of PCa through targeting Vimentin. Also, we measured the protein level of Vimentin in established cells and found Vimentin was reduced in VIM-AS1 knocked-down PC3 cells but increased in VIM-AS1 overexpressed LNCaP cells. These verified Vimentin as a target for VIM-AS1 in PCa.

EMT refers to the biological process by which epithelial cells are transformed into mesenchymal phenotype cells by a specific procedure. Through EMT, PCa cells lose their cell polarity, lose their connection to the basement membrane, and gain higher migration and invasion abilities [23-25]. EMT is an important biological process for PCa cells to acquire migration and invasion abilities [26-28]. Next, as Vimentin is an important member

of EMT, we detected several other EMT markers to explore the function of VIM-AS1 on EMT. The epithelial marker E-cadherin level was increased in VIM-AS1 downregulated PC3 cells but decreased in VIM-AS1 upregulated LNCaP cells, while the mesenchymal marker N-cadherin showed opposite trends. These results confirmed VIM-AS1 could promote cell proliferation, invasion, migration and EMT via regulating Vimentin expression.

Conclusions

Taken all together, we demonstrated for the first time VIM-AS1 was highly expressed in PCa tissues and cells, and promoted cell proliferation, migration, invasion and EMT via promoting Vimentin expression. This might provide a novel target for PCa biological diagnosis and therapy.

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Conflict of interests

The authors declare no conflict of interests.

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