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ORIGINAL ARTICLE

LncARSR promotes prostate cancer proliferation by activating PTEN/PI3K/Akt pathway

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Summary

Purpose: *To investigate the expression characteristics of LncARSR in prostate cancer tissues and further exploring the specific mechanism by which LncARSR promotes the proliferation of prostate cancer (PCa) cells.*

Methods: *Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out to examine LncARSR expression in 50 pairs of PCa tumor tissues and adjacent normal ones, and the relationship between LncARSR and clinicopathologic indicators or prognosis of these PCa cases were also analyzed. Meanwhile, qRT-PCR further verified LncARSR expression in PCa cell lines. LncARSR knockdown models were constructed in PCa cell lines 22RV1 and PC-3. The impacts of LncARSR on the function of PCa cells were assessed by cell counting kit-8 (CCK-8), plate cloning experiments, and 5-Ethynyl-2'- deoxyuridine (EdU) assay. Finally, the relationship between LncARSR and PTEN / PI3K / AKT pathway was analyzed by Western blotting.*

Results: *Our data showed that LncARSR expression in PCa tumor specimens was remarkably higher than in adjacent ones. In comparison to patients in group of low LncARSR expression, patients in high expression group showed higher pathologic grade, larger tumor size, and lower overall survival. In vitro cell experiments suggested that knockdown of LncARSR significantly suppressed the proliferative capacity of PCa cells, enhanced the expression of the key protein PTEN in the PTEN / PI3K / AKT signaling, while reduced the other proteins such as p-PI3K, p-AKT, and p-mTOR. In addition, silencing PTEN reversed the inhibitory effect of knockdown of LncARSR on the proliferation of PCa cells.*

Conclusions: *LncARSR, highly expressed in PCa tissues and cell lines, is remarkably associated with pathologic stage, tumor size, and poor prognosis of patients with PCa. In addition, LncARSR may promote the malignant progression of PCa through activating the PTEN / PI3K / AKT signaling pathway.*

Key words: *LncARSR, PTEN / PI3K / AKT, prostate cancer, malignant progression*

Introduction

Prostate cancer (PCa) is one of the most common malignancies, with the second highest incidence and sixth highest death rate worldwide. Although the incidence of PCa in China is far lower than that in western countries, its growth is much faster than in western countries [1,2]. As China gradually enters an aging society, PCa will become the major tumor "killer" that endangers the health of elderly men, and the clinical diagnosis, treatment and basic research of PCa have entered an urgent stage [3-5]. The early symptoms of PCa are insidious, often without any clinical symptoms, and the clinical stage is often advanced when the clinical symptoms such as dysuria and urinary retention occur [5,6]. Currently recognized early diagnosis way include rectal digital rectal examination (DRE), transrectal ultrasound (TRUS) and serum PSA testing [6-8]. However, the positive rate of DRE

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and TRUS is low. Even though PSA sensitivity is better, its specificity is lower, especially in the gray area of PSA diagnosis (PSA 4-10 ng/mL), and the positive rate of prostate puncture is less than 30% [6-8]. This leads to a large number of unnecessary piercings, which increases the economic burden of the society and the psychological pressure of patients [9,10]. In recent years, a large number of studies have focused on exploring molecular diagnostic markers that can make up for the defects of PSA diagnosis, so as to improve the positive rate of punctures, detect clinically significant high-grade PCa and avoid unnecessary punctures [11-13].

LncRNAs are transcripts larger than 200 bases that encode no protein or a very short polypeptide [14-16]. A great number of studies have shown that lncRNA can regulate gene expression through multiple ways, including participation in chromatin imprinting, binding epigenetic modification complexes or transcription factors for transcriptional regulation, and binding miRNA/miR, mRNA or protein for post-transcriptional regulation [16,17]. There is increasing evidence that lncRNA can be involved in regulating multiple biological characteristics of tumors, including proliferation, apoptosis, metastasis and metabolism of tumor cells [18-20]. Studies have revealed that lncRNA plays a significant part in the development of PCa [21,22]. As an emerging lncRNA, the function of LncARSR has been confirmed in other tumors, while the role of LncARSR in PCa has not been reported [23,24]. Hence, this study explored whether LncARSR could be a serological marker for the early diagnosis of PCa and thus provide a possible option for target gene therapy.

Methods

Patients and PCa samples

40 pairs of PCa tissue specimens and adjacent ones were collected from PCa patients diagnosed in our hospital. All cases were diagnosed by two senior deputy chief pathologists. This study was approved by the Ethics Committee of Tianjin Fifth Central Hospital. Signed informed consents were obtained from all participants before the study entry.

Cell lines and reagents

Four human-derived PCa cell lines (PC-3, DU-145, 22RV1, Lncap) and one human normal prostate stromal immortalized cell (WPMY-1) provided by American Type Culture Collection (ATCC) (Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and 1% penicillin/streptomycin in a 37°C cell incubator with 5% CO_2.

Transfection

Lentivirus transfection was performed with sh-NC and sh-LncARSR (GenePharma, Shanghai, China) when cell confluence reached 30-50%. Cells were collected 48 h later for quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting analysis and cell function experiments.

Cell counting kit-8 (CCK-8) assay

Cells were plated in 96-well plates (2×10^3 cells/well) in 100 ul culture medium. CCK-8 assay (Dojindo, Kumamoto, Japan) was performed based on the manufacturer's protocol.

Colony formation assay

After 48 h of transfection, cells were collected, and 200 cells were seeded in each well of 6-well plates and cultured with complete medium for 2 weeks. The medium was changed after one week and then twice a week. After 2 weeks, the cells were cloned and then fixed in 2 mL of methanol for 20 min. After the methanol was aspirated, the cells were stained with 0.1% crystal violet (Solarbio, Beijing, China), photographed and counted under a light-selective environment.

5-Ethynyl-2'-deoxyuridine (EdU) assay

After transfection for 24 h, EDU test (RiboBio, Nanjing, China) was carried out according to the manufacturer's requirements.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the RNA was reverse transcribed into cDNA using Primescript RT Reagent. QRT-PCR reaction was carried out using SYBR® Premix Ex Taq ™ (TaKaRa, Tokyo, Japan) kit. The following primers were used in the qPCR reaction: LncARSR: forward: 5'-GCTGGTTGTGGGTTACTCTC-3', reverse: 5'-GCCCTCTGTGCTACTTACTC-3'; β-actin: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-TGCCGTAGGTGTCCCTTTG-3'.

Western blotting assay

Cells were lysed, shaken on ice for 30 min, and centrifuged at 4°C, 14000 × g for 15 min. Total protein concentration was calculated by bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). The extracted proteins were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Western blot analysis was carried out based on standard procedures.

Statistics

Kaplan-Meier and log-rank test were used for statistical analyses and GraphPad Prism 5 V5.01 software (La Jolla, CA, USA) was used for drawing analysis. Differences between two groups were analyzed by the Student's t-test. Comparison between multiple groups was

done using one-way ANOVA test followed by *post hoc test* (Least Significant Difference). P less than 0.05 was considered statistically significant.

Results

LncARSR showed an increased expression in PCa

qRT-PCR results showed that LncARSR had significantly higher expression in collected PCa tumor tissue samples than in paracancer normal ones (Figure 1A), suggesting that LncARSR may function as an oncogene in PCa. Meanwhile, *in vitro* cell qRT-PCR detection also revealed an increased expression in PCa cell lines, especially 22RV1 and PC-3 cell lines (Figure 1B).

LncARSR expression was correlated with clinical stage and overall survival in PCa patients

According to qRT-PCR results of LncARSR expression, we divided the 40 pairs of tissue samples into high and low expression group. Additionally, high expression of LncARSR was positively correlated with the clinical stage of PCa and tumor size (Figure 1C). Therefore, the above results suggest that LncARSR may act as a new biological index to predict the malignant progression of PCa. In addition, relevant follow-up data were collected and Kaplan-Meier survival curves showed that high expression of LncARSR was closely relevant to the poor prognosis of PCa (the higher the LncARSR expression, the worse the prognosis).

Knockdown of LncARSR inhibited proliferation ability of PCa cells

To specify the impact of LncARSR on the proliferation ability of PCa cells, we constructed a LncARSR knockdown model in PCa cell lines 22RV1 and PC-3 and verified the transfection efficiency by qRT-PCR (Figure 2A). Subsequently, both CCK-8 test, plate cloning experiment and EdU assay demonstrated that the proliferation rate and ability of PCa cells was remarkably reduced after downregulation of LncARSR (Figures 2B, 2C and 2D).

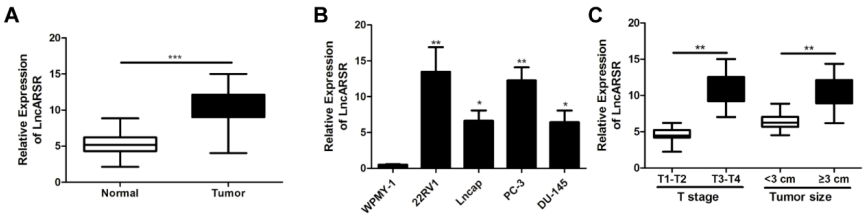

Figure 1. LncARSR is highly expressed in prostate cancer tissues and cell lines. **A:** qRT-PCR detection of LncARSR expression in prostate cancer tumor tissues and non-tumor tissues adjacent to the cancer. **B:** qRT-PCR detection of LncARSR expression levels in prostate cancer cell lines. **C:** qRT-PCR detection of the expression difference of LncARSR in tumor tissues of PCa patients with different pathological stages or tumor size. Data are average ± SD. *p<0.05, **p<0.01, ***p<0.001.

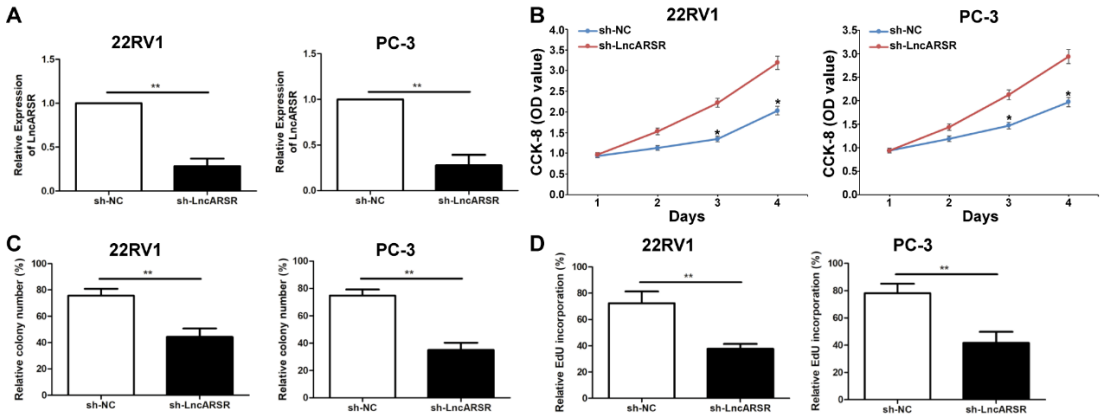

Figure 2. Silencing LncARSR inhibited prostate cancer cell proliferation. **A:** qRT-PCR validated the interference efficiency of LncARSR knockdown vector in prostate cancer 22RV1 and PC-3 cell lines. **B:** CCK-8 test detected the proliferation rate of prostate cancer cells after knockdown of LncARSR in prostate cancer cell lines 22RV1 and PC-3. **C:** Cell cloning experiment detected the number of prostate cancer positive proliferative cells after knockdown of LncARSR in prostate cancer cell lines 22RV1 and PC-3. **D:** EdU detected the proliferation of prostate cancer cells after transfection of LncARSR knockdown vector in prostate cancer 22RV1 and PC-3 cell lines. Data are average ± SD. *p<0.05, **p<0.01.

Knockdown of LncARSR inhibited PI3K/AKT signaling

To further clarify the ways by which LncARSR promotes the malignant progression of PCa, we examined the changes in the expression of key proteins PTEN, p-PI3K, p-AKT, and p-mTOR in the PTEN/PI3K/AKT pathway after knocking down LncARSR by Western Blot. Figure 3 shows that knockdown of LncARSR markedly enhanced the expression of PTEN while reduced that of p-PI3K, p-AKT, and p-mTOR.

LncARSR inhibited PTEN gene expression in PCa

To explore whether LncARSR promotes the malignant progression of PCa through PTEN, we simultaneously transfected LncARSR and PTEN knockdown vectors into PCa cell lines 22RV1 and PC-3. As a result, compared to the sh-LncARSR group, simultaneous knockdown of LncARSR and PTEN reduced PTEN expression more significantly, measured by Western blot assay (Figure 4A), and enhanced cell proliferation capacity, which was observed in the results of CCK-8, clone formation experiments, and EdU assay (Figures 4B, 4C and 4D).

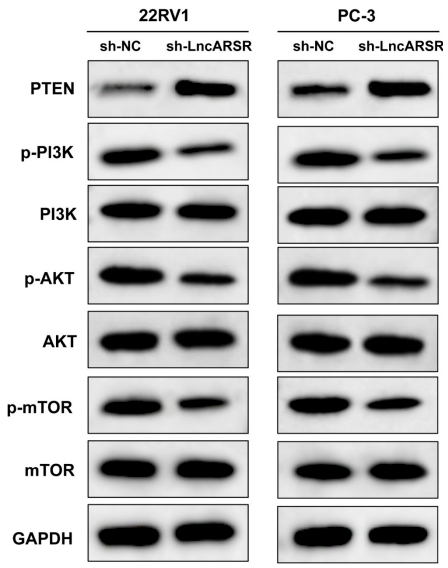

Figure 3. LncARSR works by regulating PTEN/PI3K/AKT in prostate cancer cells. Western blotting was performed to verify the expression levels of PTEN, p-PI3K, p-AKT and p-mTOR after transfection of LncARSR knockdown vector in prostate cancer 22RV1 and PC-3 cell lines.

Discussion

At present, surgical treatment and endocrine therapy are still the main treatment methods for PCa. Endocrine therapy is often initially effective, but almost all patients will gradually enter into hormone-independent PCa after a median time of 14-30 months [4-7]. It turns out that many patients have experienced the process from hormone-dependent to hormone-independent, and eventually died of hormone-independent PCa, which is the final form and inevitable result [8,9]. To date, there is no standard effective treatment for advanced PCa [6-10]. The androgen-independent growth and distant metastasis of cancer cells have become the two major obstacles affecting the clinical treatment and prognosis of PCa [4,5,9]. Therefore, how to reduce the side effects of hormone therapy and how to effectively treat advanced PCa has become a difficult and hot spot for many researchers worldwide [10-13].

LncRNA is a type of ncRNA with a transcript longer than 200 bases which does not encode proteins or only encodes short peptides [14-17] In this study, a large number of clinical specimens of PCa were collected for the first time to explore the role of LncARSR in the development of PCa. We demonstrated that LncARSR expression was increased in PCa tissues and PCa cell lines as compared with normal controls, indicating that the high expression of LncARSR plays an extremely important role in the occurrence and development of PCa. In order to verify the effect of LncARSR on the cell function of PCa cell lines, we downregulated LncARSR expression and examined cell proliferation

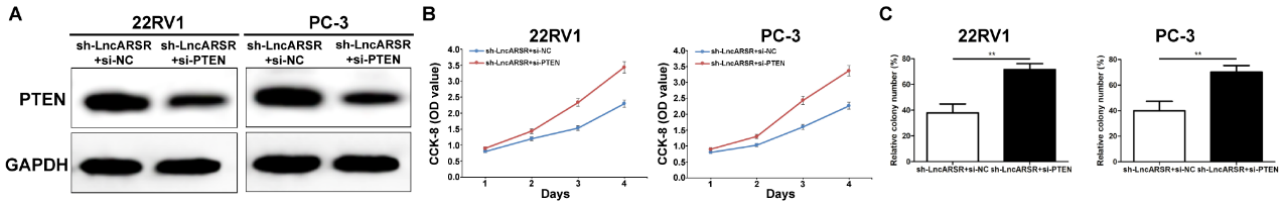

Figure 4. LncARSR promote prostate cancer cell proliferation through regulating PTEN expression. **A:** Western blotting verified the transfection efficiency of LncARSR and PTEN knockdown vectors in prostate cancer 22RV1 and PC-3 cell lines. **B:** CCK-8 experiments detected the proliferation of prostate cancer cells after co-transfection of LncARSR and PTEN knockdown vectors. **C:** Cell cloning experiments detected the number of prostate cancer positive proliferative cells after co-transfection of LncARSR and PTEN knockdown vectors in prostate cancer 22RV1 and PC-3 cell lines. Data are average ± SD. **p<0.01.

by CCK-8, cell cloning and EDU experiments, the results of which showed that knockdown of LncARSR suppressed the proliferation ability of PCa cells. However, the specific molecular mechanism remains unclear.

Cellular signaling pathway is the communication bridge inside and outside the cell. For external stimulus signals, they are transferred into the cell through these specific pathways, and different intracellular signals are produced to regulate specific genes [25,26]. Among the many signaling pathways that promote tumor cell proliferation, the most important is the PTEN/PI3K/AKT signaling pathway, which exerts an essential effect on the progression of PCa [26,27]. Studies have demonstrated that the tumor suppressor gene PTEN is an essential gene for tumor development. Its continuous expression can prevent the occurrence of malignant tumors, while its inactivation often promotes the progression of malignant tumors. Related studies have shown that the progression of prostate cancer from androgen-dependent to androgen-independent is closely relevant to the activation of serine/threonine protein kinase Akt, which is highly expressed and plays a crucial part in the androgen-independent prostate cancer cells [28-30]. In our study, we showed that knocking down LncARSR enhanced the protein expression of PTEN, while reduced p-PI3K, p-AKT, and p-mTOR, indicating that LncARSR can play a pivotal role in promoting proliferation of PCa cells through PTEN/PI3K/AKT pathway. Meanwhile, we demonstrated that silencing PTEN reversed the inhibitory effect of silencing LncARSR on PCa cell proliferation, suggesting that LncARSR may promote the proliferation of PCa through activating PI3K/AKT signaling pathway. As research continues, further understanding of the biological function of LncARSR gene and its role in the development and progression of PCa will further contribute to the diagnosis, treatment and prognostic evaluation of tumors.

Conclusions

In summary, LncARSR, highly expressed in PCa tissues and cell lines, has great relevance to the pathological stage, tumor size, and poor prognosis of PCa patients. In addition, LncARSR may accelerate the malignant progression of PCa by activating the PTEN/PI3K/AKT signaling pathway.

Conflict of interests

The authors declare no conflict of interests.

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