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Downregulated LINC00460 inhibits cell proliferation and promotes cell apoptosis in prostate cancer

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Abstract. – OBJECTIVE: LINC00460 has been confirmed to contribute to cancer development. However, the role and function of LINC00460 in prostate cancer is not identified. The purpose of this study was to evaluate the expression and effect of LINC00460 on prostate cancer cell malignant behaviors.

PATIENTS AND METHODS: The expression of LINC00460 in cancer tissues and cell lines were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) assay. The LINC00460 expression was downregulated by siRNA. The cell counting kit-8 (CCK-8) assay was used to detect cell proliferation. The cell migration and invasion were detected by migration and Matrigel invasion assays. The Western blot assay was used to detect the altered expression levels of Ki67, Cyclin D1, PI3K, p-AKT, T-AKT, Bcl2, and Bax.

RESULTS: LINC00460 was increased in human prostate cancer tissues and cell lines. LINC00460 high expression was related to Tumor Size (T1–T2/T3–T4; *p*=0.004), and high Gleason Score (≤8/>8, *p*=0.000). Downregulation of LINC00460 by siRNA could inhibit cancer cell proliferation and decreased Ki67 and Cyclin D1 expression. Meanwhile, downregulation of LINC00460 promoted apoptosis of cell lines and was related to PI3K/AKT pathway.

CONCLUSIONS: LINC00460 could regulate cell proliferation and cell apoptosis, which might be a novel marker in prostate cancer.

Key Words:
LINC00460, Cell proliferation, Cell apoptosis, PI3K/AKT, Prostate cancer.

Introduction

About 1,111,700 newly cases were diagnosed as prostate cancer, and 307,500 patients died of this disease all over the world in 2012[1]. In China, 60,300 new patients with prostate cancer and more than 26,000 deaths were estimated in 2015[2]. Prostate cancer is the fifth most common cause of cancer-related death. The prognosis of patients with prostate cancer is unfavorable, partly because of a poor understanding of the complicated molecular mechanisms involved in prostate cancer development[3]. Therefore, looking for new diagnostic and therapeutic markers is needed, and exploring the underlying molecular mechanisms is urgent.

Up to data, increasing studies revealed that long non-coding RNAs (lncRNAs) as novel molecules are expressed abnormally in various cancers and function as tumor suppressors and oncogenes, which regulates the development and progression of tumors. In gastric cancer, lncRNA MALAT1 potentiates autophagy-associated cisplatin resistance through modulating the miR-30b/ARG5[4]. LncRNA FEZF1-AS1 can regulate liver cancer cell proliferation and invasion *via* sponging miR-4443[5]. LncRNA LOC441178 inhibits squamous cancer cell invasion and migration through targeting ROCK1[6]. LncRNA XIST controls bladder cancer progression by modulating p53 *via* binding to TET1[7].

Besides, LINC00460 were found to participate in various cancers. For example, LINC00460 can regulate the KDM2A gene to promote gastric cancer cell proliferation and migration through interaction with miR-342-3p[8]. In colorectal cancer, lncRNA LINC00460 suppresses cancer cell proliferation[9] and apoptosis[10] through affecting KLF2 and CUL4A expression, respectively. LncRNA LINC00460 is related to the miR-539/MMP-9 axis to promote metastasis and progression of meningioma[11]. In lung cancer, LncRNA LINC00460 enhances tumor growth through targeting miR-302c/FOXA1[12] and affects epitheli-

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al-mesenchymal transition and tumor cell migration[13,14]. Additionally, LINC00460 facilitates the tumorigenesis of nasopharyngeal cancer through sponging miR-149 to increase IL6[15]. LINC00460 up-regulated by CBP/P300 may enhance the progression of esophageal squamous cancer[16]. However, the role and function of LINC00460 in prostate cancer development are not investigated.

Our study found that LINC00460 was increased in prostate cancer tissues and was involved in tumor size and Gleason score in prostate cancer. The *in vitro* experiments indicated that downregulated LINC00460 could inhibit cell proliferation and induce cell apoptosis. Moreover, downregulated LINC00460 could influence the PI3K/AKT signaling pathway.

Patients and Methods

Patients and Samples

All tumor tissues and the adjacent tissues were obtained from 84 prostate cancer patients with the informed consents of our hospital. All the samples were preserved in liquid nitrogen. This investigation was approved by the Ethics Committee of The Second Hospital of Yulin.

Cell Culture and Transfection

One normal prostate cell (P69) and four cancer cell lines (22Rv1, DU145, LNCaP, and PC3) were purchased from the Chinese Academy of Sciences. Cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS, Gibco, Waltham, MA, USA). All the cells were cultured at 37°C with 5% CO_2.

Small interfering RNA (siRNA) and control siRNA (si-control) were synthesized by GenePharma Co., Ltd. (Shanghai, China). The sequences of si-LINC00460 were as follows: si-RNA1, 5'-AGACCTAATA GCCAATAAG-3'; si-RNA2, 5'-CCATGTGAAGTGTAGAACA-3'. According to the manufacturer's protocol, cells were transfected using Lipofectamine™ 3000 reagent (Invitrogen, Carlsbad, CA, USA). The transfection efficiencies were confirmed by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR).

RNA Extraction and qRT-PCR

Through using TRIzol Reagent (TaKaRa Bio, Dalian, China), RNA was extracted from all the tissues and cell lines. Through using PrimeScript RT-polymerase (TaKaRa Bio, Dalian, China), 1 μg RNA and 20 μL reaction mixture was reversely transcribed to cDNA. Through using SYBR Green PCR Master Mix (TaKaRa Bio, Dalian, China), RNA levels were detected by RT-qPCR, which was conducted by using 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an internal control. The primers were LINC00460-forward: 5'-GGATGAACCAC-CATT GCC-3'. LINC00460-reverse: 5'-CCCAC GCTCA GTCTT TCT-3'.

Cell Counting Kit-8 (CCK-8) Assay

The transfected cells were collected and seeded into 96-well plates (3×10^3 cells/well). According to the protocol, the CCK-8 assay was performed following 0, 24, 48, or 72 h culture. The CCK-8 reagent (10 μL, Beyotime Institute of Biotechnology, Shanghai, China) was added to each well for 2 h. The absorbance at a wavelength of 450 nm was evaluated using an ELx808 absorbance reader (BioTek Instruments, Biotek Winooski, VT, USA). Every experiment was performed in triplicate.

Apoptosis Assay

Through using flow cytometry with an Annexin V-fluorescein isothiocyanate/propidium iodide kit (Nanjing KeyGen Biotech Co., Nanjing, China), the cell apoptosis was measured according to the protocol. A FACSAria flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used.

Cell Migration and Invasion

The cell invasion was detected by using transwell cell culture inserts (BD Biosciences, Franklin Lakes, NJ, USA) in 24-well plates. The cells (2×10^5) in serum-free medium (100 μL) with 1 mg/ml Matrigel (60 μL, BD Biosciences, Franklin Lakes, NJ, USA) were placed into the upper chamber. The RPMI-1640 medium with 10% FBS (600 μL) was placed into the bottom well. After 24 h, the cells that had crossed the filter pores were fixed with methanol (800 μL) and stained with 0.1% crystal violet solution (800 μL). The migration assay was performed in a similar manner without the Matrigel coating.

Western Blotting Assay

Through using bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Waltham,

USA), the protein concentration was assessed. 20 μg proteins were separated on 10% SDS-PAGE gel. Then, the proteins were transferred to the polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Scientific, Waltham, MA, USA) with blocking with 5% milk for 1 h, and incubated with the primary antibody anti-Ki67, anti-Cyclin D1, anti-PI3K, anti-p-AKT, anti-T-AKT, anti-Bcl2 or anti-Bax antibodies (Abcam, Cambridge, MA, USA) at 4°C overnight. After washed three times, they were incubated with secondary antibody for 2 h. The blots were visualized using enhanced chemiluminescence (ECL) kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Statistical Analysis

For the comparison between the two groups, the Student's *t*-test was used. The *t*-test was also used for LINC00460 comparison between tumor tissues and the adjacent tissues. The comparison between groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). The values were analyzed as mean ± standard deviation (SD). Statistical analyses were assessed through GraphPad Prism v6.0 (GraphPad Software, La Jolla, CA, USA). p-values < 0.05 were considered statistically significant.

Results

LINC00460 Was Increased in Human Prostate Cancer

Firstly, as presented in Figure 1A, we detected that LINC00460 expression was observably elevated in prostate cancer tissues ($p<0.05$). Secondly, we measured the expression of LINC00460 in one normal prostate cell (P69) and four cancer cell lines (22Rv1, DU145, LNCaP, and PC3). The results of qRT-PCR revealed that, compared to P69, LINC00460 was increased in the four tumor cell lines ($p<0.05$, Figure 1B). Among those cell lines, 22Rv1 and LNCaP had the highest expression of LINC00460; DU145 and PC3 had a relatively low expression of LINC00460. Thus, 22Rv1 and LNCaP cell lines were chosen for further study in our work.

Increased LINC00460 Was Related to the Malignant Status of the Patients With Prostate Cancer

Based on the above results, we further assessed the relationship between LINC00460 expression and clinicopathological characteristics of the patients with prostate cancer. According to LINC00460 expression, we divided the patients into two groups: LINC00460 high-expression group (n=42) and LINC00460 low-expression group (n=42). As shown in Table I, LINC00460 expression was not related to age ($p=0.072$), smoking ($p=0.334$), and lymph node metastasis ($p=0.362$). However, LINC00460 high expression was related to tumor size (Figure 1C), and high Gleason score (Figure 1D).

Downregulation of LINC00460 by siRNA Could Inhibit Cancer Cell Proliferation

Furthermore, based on the findings from the relationship between LINC00460 expression and clinical factors, we conducted cell experiments to investigate the effect of LINC00460 on cell proliferation *in vitro*. Firstly, LINC00460 expression levels of cell lines were downregulated by siRNA (Figure 2A and 2B).

Next, we tested cell proliferation by CCK-8 assay. The data of CCK-8 revealed that downregulated LINC00460 expression suppressed cell proliferation as relative to the control groups (Figure 2C and 2D). Then, the protein expression levels of two major molecules related with cell proliferation (Ki67 and Cyclin D1) were detected by Western blot assay. The results showed that reduced levels of Ki67 and Cyclin D1 were observed in cells transfection with siRNA- LINC00460 as relative to the control groups (Figure 2E and 2F).

Downregulation of LINC00460 Promoted Apoptosis of Cell Lines, and Could Not Regulate Cell Invasion and Migration

Additionally, we conducted TUNEL assays to explore the effect of LINC00460 on cell apoptosis. The results showed a promotion of cell apoptosis in cells transfected with siRNA- LINC00460 as relative to the control groups (Figure 3A and 3B). In summary, all the above findings suggested that the downregulation of LINC00460 by siRNA could inhibit cancer cell proliferation and promote cell apoptosis.

Moreover, we also assessed cell migration and invasion by migration assays and Matrigel invasion assays. The results showed no influence of downregulated LINC00460 on cell migration and invasion, which indicated that downregulated LINC00460 did not influence cell migration and invasion (Figure 3C and 3D).

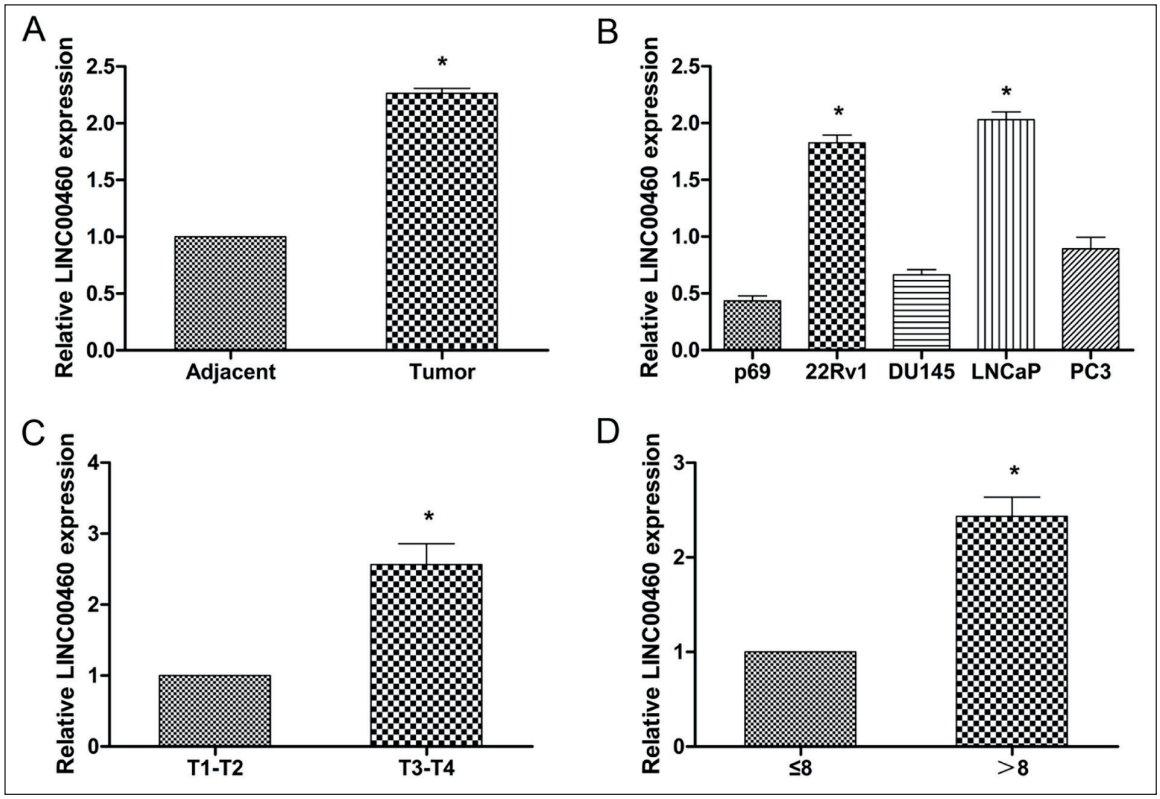

Figure 1. LINC00460 was increased in human prostate cancer. ***A,*** Analysis of LINC00460 expression in 84 pairs of prostate cancer and the adjacent tissues. ***B,*** Analysis of LINC00460 expression in prostate cancer cell lines. ***C,*** qRT-PCR was used to measure the expression of LINC00460 between T1-T2 and T3-T4. ***D,*** qRT-PCR was used to detect the expression of LINC00460 between ≤ 8 and > 8. **p*<0.05.

Dysregulation of LINC00460 Was Related to PI3K/AKT Pathway

PI3K/AKT pathway was involved in different cancer cell behaviors, especially cell proliferation[17]. Thus, we investigated whether LINC00460 control cancer cell proliferation by the PI3K/AKT pathway. The Western blot assay revealed a reduction of PI3K and phosphorylated AKT (p-AKT) expression in cells transfection with siRNA-LINC00460 as relative to the control groups. Meanwhile, a reduction of the downstream gene Bcl-2 and an increased expression of the downstream gene Bax were observed in cells transfection with siRNA-LINC00460 as relative to the control groups (Figure 4A and 4B). These results suggested that dysregulation of LINC00460 could regulate the PI3K/AKT pathway.

Discussion

Prostate cancer is one of the most common malignancies in men. No effective diagnosis biomarker and a poor understanding of the mechanism involving cancer progression have limited the effectiveness of therapy for prostate cancer. Thus, our study found that 1) increased LINC00460 expression was observed both in prostate cancer tissues and cell lines. 2) LINC00460 functioned as an oncogene that regulating prostate cancer progression through a promotion of cell proliferation and a reduction of cell apoptosis. 3) *In vitro*, LINC00460 could modulate the important molecules in PI3K/AKT signal pathway.

The previous reports have demonstrated that non-coding RNAs, especially lncRNAs, participate in different diseases[18]. In cervical cancer, upregulation of lncRNA ZEB1-AS1 enhances cell invasion and epithelial to mesenchymal transition by elevating ZEB1 expression[19]. lncRNA UCA1 is increased in thyroid cancer and represses cell proliferation and cell invasion by interacting with miR-204/IGFBP5[20]. LncRNA IGF2AS is reduced in human prostate cancer and functions as a tumor suppressor by regulating

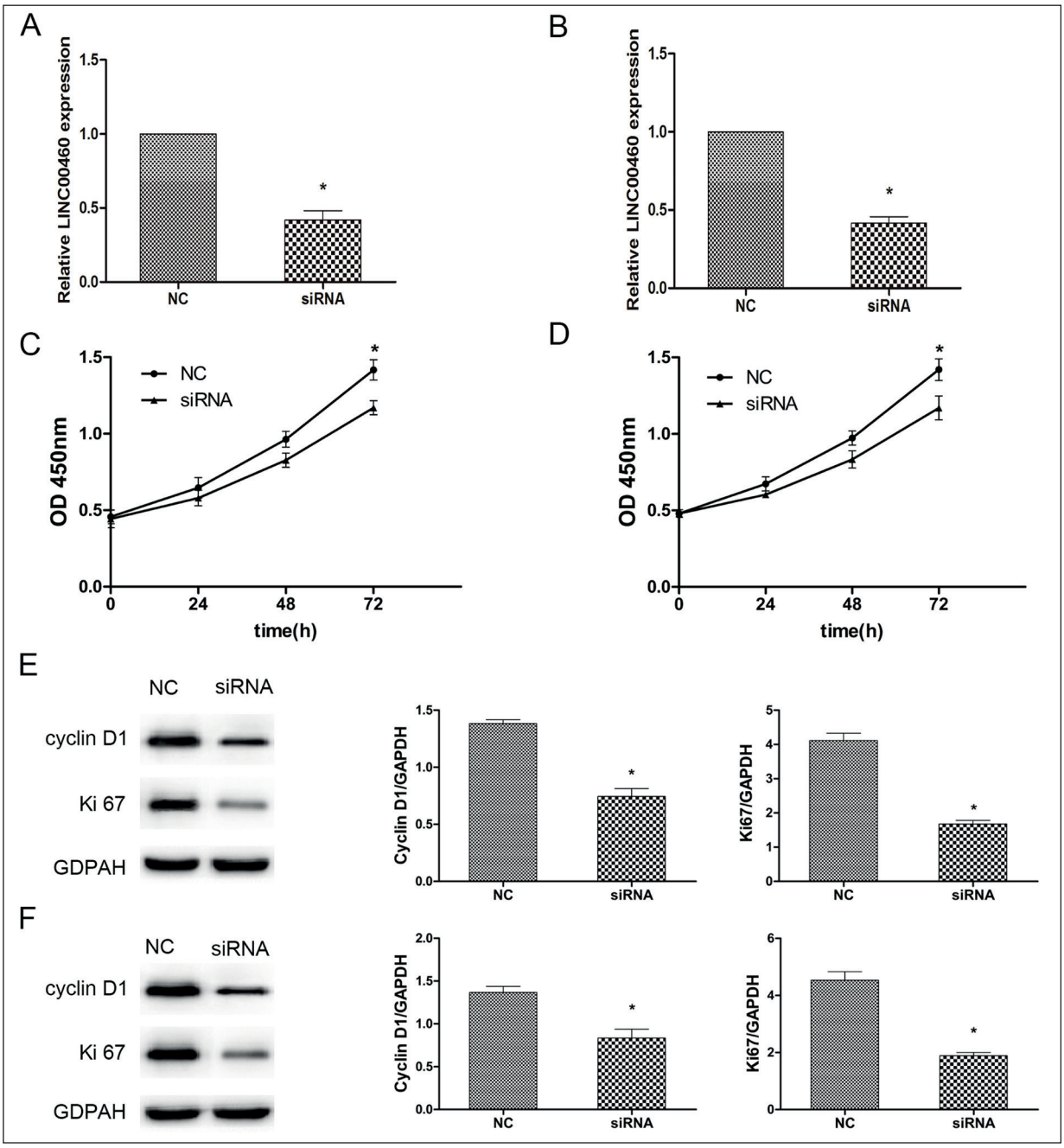

Figure 2. Downregulation of LINC00460 by siRNA could inhibit cancer cell proliferation. ***A,*** and ***B,*** LINC00460 expression levels of cell lines were downregulated by siRNA. ***C,*** and ***D,*** We tested cell proliferation by CCK-8 assay. ***E,*** and ***F,*** The protein expression levels of Ki67 and Cyclin D1 were detected by Western blot assay. *p<0.05. **p<0.01. ***p<0.001.

IGF2 expression[21]. lncRNA CA3-AS1 inhibits colorectal cancer cell proliferation and invasion, and it induces cell apoptosis by miR-93/PTEN axis[22]. The downregulation of LINC00152 suppresses the progression of gastric cancer through controlling miR-193b-3p/ETS1 axis[23]. Our study revealed that LINC00460 acted as an oncogene that contributes to the progression of prostate cancer. LINC00460 was higher expressed in prostate cancer tissues than the adjacent tissues. Increased LINC00460 was related to the malignant status (tumor size and high Gleason score) of the patients with prostate cancer. Furthermore, through CCK-8 assay, we found that

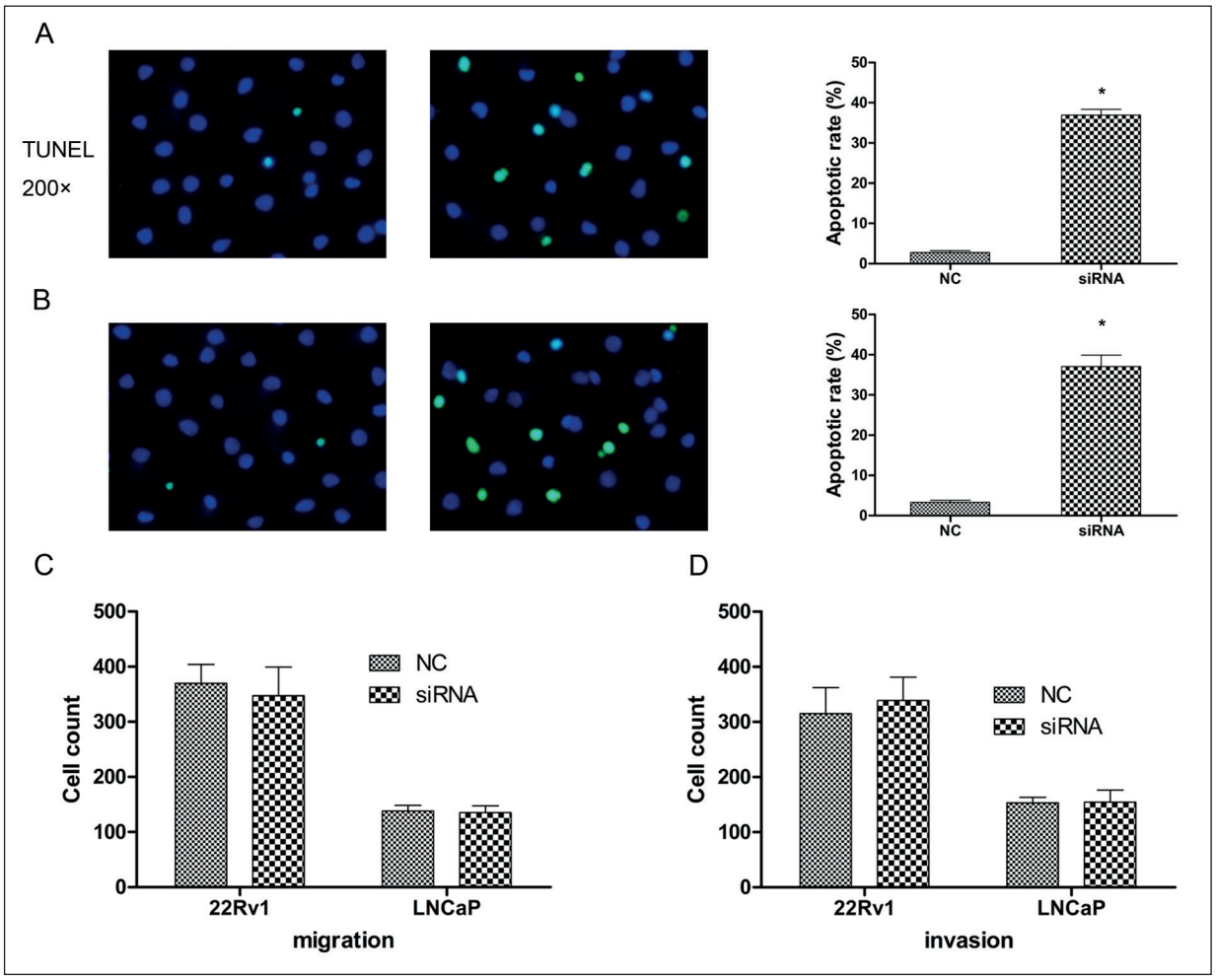

Figure 3. Downregulation of LINC00460 promoted apoptosis of cell lines and could not regulate cell invasion and migration. ***A,*** and ***B,*** We conducted TUNEL assays to explore the effect of LINC00460 on cell apoptosis. ***C,*** and ***D,*** We also assessed cell migration and invasion by migration assays and Matrigel invasion assays. **p*<0.05.

knockdown of LINC00460 could inhibit the cell proliferation. Meanwhile, Western blot assay showed that knockdown of LINC00460 caused a decreased expression of Ki67 and Cyclin D1. TUNEL experiments showed that downregulation of LINC00460 induced the cell apoptosis.

Emerging literature has identified that PI3K/AKT signaling is involved with prostate cancer progression, such as cell apoptosis and cell proliferation. CCR9 and CCL25 interact to elevate the PI3K/AKT pathway and led to a decrease of cell apoptosis[24]. Prosaposin enhances prostate cancer cell survival and inhibits cell apoptosis by PI3K/AKT pathway[25]. PGD2 enhances the accumulation of prostate cancer cell proliferation *via* the FP and PI3K/AKT signaling pathways[26]. The EGFR/PI3K/AKT, ErbB/PI3K/AKT, and HER family/PI3K/AKT pathways are involved in the prostate cancer cell proliferation[27]. Increasing studies have found that lncRNAs are able to regulate PI3K/AKT signaling pathways in tumors[28]. LncRNA MALAT1 enhances ovarian cancer cell proliferation and metastasis through the PI3K-AKT pathway[29]. LINC003121 represses thyroid cancer cell proliferation and invasion *via* modulating the PI3K/Akt signaling pathway[30]. We found that the knockdown of LINC00460 downregulated the protein expression levels of PI3K, p-AKT, and Bcl-2; and it upregulated the protein expression of Bax. Thus, this revealed that LINC00460 influenced the activity of the PI3K/AKT signaling pathway. LINC00460 might be a novel regulator of this pathway and will result in therapeutic benefit.

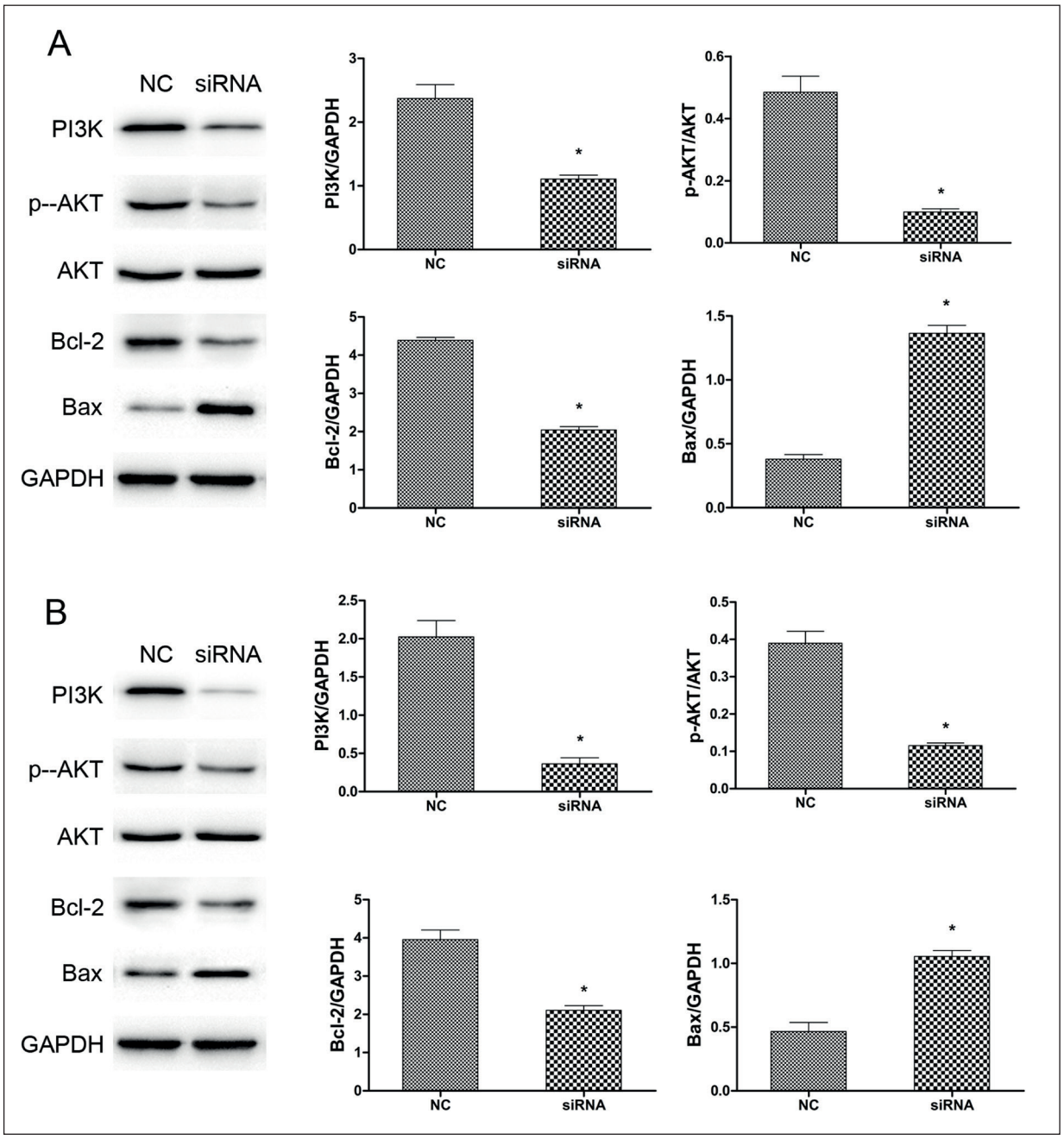

Figure 4. Dysregulation of LINC00460 was related to PI3K/AKT pathway. The Western blot assay was used to detect PI3K, p-AKT, Bcl-2, and Bax expression in cells transfection with siRNA-LINC00460 as relative to the control groups. *$p<0.05$.

Conclusions

In summary, we firstly provide evidence that LINC00460 is significantly upregulated in prostate cancer tissues and cell lines. Increased LINC00460 was related to Tumor Size and high Gleason Score of the patients with prostate cancer. LINC00460 functions as an oncogene and could be a novel diagnosis biomarker through influencing the PI3K/Akt signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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