

LncRNA625 modulates prostate cancer cells proliferation and apoptosis through regulating the Wnt/ β -catenin pathway by targeting miR-432

J.-B. LI¹, F. LIU², B.-P. ZHANG³, W.-K. BAI⁴, W. CHENG¹, Y.-H. ZHANG¹, L.-J. YU⁵

¹Department of Ultrasound, Harbin Medical University Cancer Hospital, Harbin, Heilongjiang Province, China

²Department of Medical Oncology, Harbin Medical University Cancer Hospital, Harbin, Heilongjiang Province, China

³Department of Neurology, the Second Affiliated Hospital, Harbin, Heilongjiang Province, China

⁴Department of Ultrasound in Medicine, Shanghai Institute of Ultrasound in Medicine, Shanghai Jiao tong University Affiliated 6th People's Hospital, Shanghai, China

⁵Center of Positron Emission Tomography-Computed Tomography, Harbin Medical University Cancer Hospital, Harbin, Heilongjiang Province, China

Abstract. – OBJECTIVE: Prostate cancer (PCa) is the second leading contributor to male malignancy-associated death in developed countries. The study aimed to evaluate the effects of lncRNA625/miR-432 on the prostate cancer cells and the underlying molecular mechanism.

PATIENTS AND METHODS: The cell proliferation was detected using the MTT and colony formation, and cells apoptosis and cell cycle were analyzed with the flow cytometry. Luciferase reporter assay was carried out to detect the correlation between miR-432 and TRIM29 and PYGO2. Besides, reverse transcription-PCR and Western blot were performed to detect the mRNA and protein levels in prostate tissues and PC3 cells.

RESULTS: lncRNA625 and miR-432 levels were consistently reduced in the PCa tissues compared with the healthy control and lncRNA625 levels significantly affect the miR-432 expression in PC3 cells, indicating that miR-432 is a direct target of lncRNA625. Besides, lncRNA625 overexpression could inhibit the cancer cells growth, arresting cell cycle progression at the G1/S phase, and significantly induce apoptosis of PC3 cells, but reversed by the miR-432 inhibitor. Most importantly, we further found that miR-432 could deactivate Wnt/ β -catenin pathway via suppressing TRIM29 and PYGO2 directly.

CONCLUSIONS: lncRNA625 could functionally inhibit PC3 cells proliferation and promote cells apoptosis through regulating the Wnt/ β -catenin pathway by targeting miR-432.

Key Words:

Prostate cancer, lncRNA625, miR-432, Proliferation, Wnt/ β -catenin.

Introduction

Prostate cancer (PCa) has been reported globally as a type of cancer exclusively diagnosed in males and is the second leading contributor to male malignancy-associated death in developed countries¹. Pathogenesis of prostate cancer is a complex process involving several factors, among which the genetic factor is known as the main determining factor². Cell proliferation and invasion are the most prominent biological features of prostate cancer, but the regulatory mechanism of prostate cancer cell proliferation and invasion is elusive, and the corresponding clinical targeted drugs are also short³. Currently, chemotherapy, radiotherapy, and surgery remain the primary treatment methods for prostate cancer, although there are several drawbacks, such as the side effects during chemotherapy and potential bleeding in surgery^{4,5}. Therefore, providing some new clues for producing more protective drugs for PCa treatment is in urgent need. Long non-coding RNAs (lncRNAs) are an RNA species 4200 bp in

length and expressed in a tissue-specific manner⁶. Mounting researches suggested that lncRNA was involved in the genomic imprinting, chromatin modification, promoting transcription, regulating post-transcription and protein function⁷. Multiple tumors have been found to be characterized by lncRNA disorders and its contribution to tumor progress has been a research focus⁸. Some studies indicated that lncRNAs played important biological and clinical roles in PCa and overexpression of the oncogenic lncRNA might promote tumor cells proliferation and metastasis^{9,10}. LncRNA625 is a novel contributor to the development and progression of PCa discovered in a research¹¹, which elucidated that LncRNA625 could inhibit the migration and invasion in prostate cancer cells. Thus, LncRNA625 is closely related with clinical characteristics and prognosis of PCa. MicroRNAs (miRNAs) are short non-coding RNAs that repress protein synthesis through mRNA degradation or translational inhibition¹². miRNAs have been found to participate in the modulation of cell survival and differentiation, as well as tissue metabolism and remodeling¹³. Growing evidence showed that miRNAs played a central role in the pathogenesis of PCa¹⁴. Previously, studies have demonstrated that miR-432 overexpression could functionally and mechanistically inhibit the cell proliferation and tumor formation¹⁵. Besides, the down-regulation of miR-432 has been reported to activate Wnt/ β -catenin signaling pathway, which has been found participating in the promotion of kinds of carcinoma progression¹⁶. Due to the fact that most miRNAs are highly pleiotropic and act differentially in various cancer cells, the detailed function and regulation of miR-432 in the pathogenesis of PCa remain to be elucidated further. We detected the lncRNA625 and miR-432 levels in the prostate tumor tissues and the healthy control and explored the lncRNA625/miR-432 potential effects on the prostate cells proliferation and apoptosis. Besides, the relationship between lncRNA625 and miR-432 expressions in the PC3 cells was also elucidated. Moreover, the underlying mechanism of lncRNA625/miR-432 effects on the PC3 cells was further investigated.

Patients and Methods

Samples and Cells

Frozen PCa tissue was obtained from patients undergoing radical resections at the Harbin Medical University Cancer Hospital. Informed con-

sent was confirmed according to the Declaration of Helsinki and the agreement of the Ethical Committee of Harbin Medical University. The prostate cancer cell line PC3 was purchased from Peking Medical College (Beijing, China). The cancer cell line was cultivated in Roswell Park Memorial Institute (RPMI) 1640 containing 10% fetal bovine serum (FBS), 50 μ g/mL amphotericin B and 100U/mL penicillin, and maintained at 37°C in 5% CO₂ incubator. The control cells were untreated in medium containing vehicle dimethyl sulfoxide (DMSO).

Cell Transfection

Cells (1×10^5 /well) were treated with the sh-lncRNA625, lncRNA625 overexpression plasmids, and miR-432 overexpression plasmids (GenePharma, Shanghai, China) following the manufacturer's protocol. The miR-432 inhibitor was purified and obtained from RiboBio (Guangzhou, Guangdong, China) as described previously¹⁶. Transfection for 48 h, PC3 cells were carefully collected and conserved for the further analysis.

MTT Assay

Transfection experiments were performed after cells attachment to the plates. Then after overnight culture, baseline values were obtained by a methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay (Life Technologies, Waltham, MA, USA). It is a colorimetric assay to test viable cells by measuring formazan reduced from MTT. After 20 min of incubation with MTT, we extracted formazan by DMSO and tested the optical density at 540 nm. MTT assay was performed at 48 h. Experiments were triplicated (n=3).

Colony Formation Assay

After transfection, the PC3 cells were seeded in a six-well plate at a density of 800 cells per well and cultured for 10-14 days until colonies appeared. Then, the cells were stained with crystal violet. The colonies number was counted only when they contained more than 50 cells.

Cell Apoptosis Analysis

After the transfections of sh-lncRNA625, lncRNA625 and miR-432 inhibitor and incubated for 48 h, the cells for the apoptosis analysis were first re-suspended with the Annexin-V binding buffer, then stained in dark with the Annexin-V and propidium iodide (PI) simultaneously at 37°C for 15 min. Then the apoptotic cells were identi-

fied by the flow cytometry analysis on a FACS Calibur system (Becton-Dickinson FacsScan, Franklin Lakes, NJ, USA).

Cell Cycle Analysis by Flow Cytometry

PC3 cells were collected following the treatment of sh-lncRNA625, lncRNA625 and miR-432 inhibitor and were incubated for 48 h. Then, they were fixed in the ethanol (70%) at -20°C overnight, and after incubated in the 10 μM RNase at 37°C for 30 min. Then, the cells were stained with PI at 4°C for 1 h. Analysis of cell cycle was carried out on a FACS Calibur flow cytometer and the obtained data were measured with the CellQuest™ software (BD Biosciences, Franklin Lakes, NJ, USA).

Luciferase Reporter Assay

Briefly speaking, the miR-432 binding sites in the 3'-UTR of TRIM29 and PYGO2 (mutant or wild type) were cloned downstream of firefly luciferase genes in the pGL3 promoter vector. Luciferase reporter assays were carried out according to the manufacturer's protocols. The luciferase activities were analyzed with the dual luciferase reporter assay system (Promega, Madison, WI, USA).

Western Blot

After treatment, the cells were collected, and the protein was extracted with Radioimmuno-precipitation assay (RIPA) lysis buffer. The protein concentrations were quantified by the bicinchoninic acid (BCA) method. 30 mg protein samples were run on 10% gels, and then transferred to the polyvinylidene fluoride (PVDF) membranes. After 1 hour of blocking in the 5% milk, membranes were incubated respectively with the primary rabbit anti-β-catenin and the rabbit anti-p84 antibody (1:1000, Abcam, Cambridge, MA, USA) at 4°C over night. Washing in Tris buffered saline-Tween 20 (TBST) for 3 times, the membrane was incubated with a peroxidase labeled secondary antibody (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h. The bands were washed again, enhanced with chemiluminescence reagents and visualized with the ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA).

Reverse-Transcription PCR

Tissues from PCa patients and healthy controls or cells after treatment were collected to determine the expression of mRNA levels. Total RNA was

carefully extracted using the Trizol RNA reagent (Invitrogen, Carlsbad, CA, USA) following protocols and quantified by spectrophotometer method. Purified RNA with equal volume was reverse transcribed (RevertAid First Strand cDNA Synthesis Kit, Thermo, K1622, Waltham, MA, USA). The analysis was carried out by the PCR thermal cycler instrument (TaKaRa, Otsu, Shiga, Japan).

Statistical Analysis

All data were presented as mean ± standard error of the mean (SEM), and were analyzed using ANOVA followed by Bonferroni-Dunn correction. Statistical analysis was carried out using the SPSS software, version 20.0 (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

Results

Levels of lncRNA625 and miR-432 Are Down-Regulated in Prostate Cancer

The RT-qPCR analysis was performed to detect the lncRNA625 and miR-432 levels in the prostate tumor tissues and the healthy control. Interestingly, we found a consistent expression between lncRNA625 and miR-432 in the prostate cancer tissues. Our results suggested that the lncRNA625 and miR-432 levels were dramatically reduced in the tumor tissues compared with the healthy control (Figure 1). Data showed that lncRNA625 and miR-432 might play a critical protective role in prostate cancer.

lncRNA625 Regulates miR-432 Expression In Prostate Cancer Cell Line

We searched the binding area between lncRNA625 and miR-432 with the miRanda predictor software as shown in Figure 2A. The RT-qPCR analysis was carried out to further verify the relationship between lncRNA625 and miR-432 expressions in the PC3 cells. After transfection with sh-lncRNA625 plasmid into PC3 cells to silence lncRNA625, miR-432 level was significantly reduced in the treated cells compared with the control group. Adversely, transfection of the lncRNA625 overexpression plasmid could dramatically increase the mRNA level of miR-432. Data showed that lncRNA625 levels significantly affect the miR-432 expressions and miR-432 is a direct target of lncRNA625 (Figure 2B).

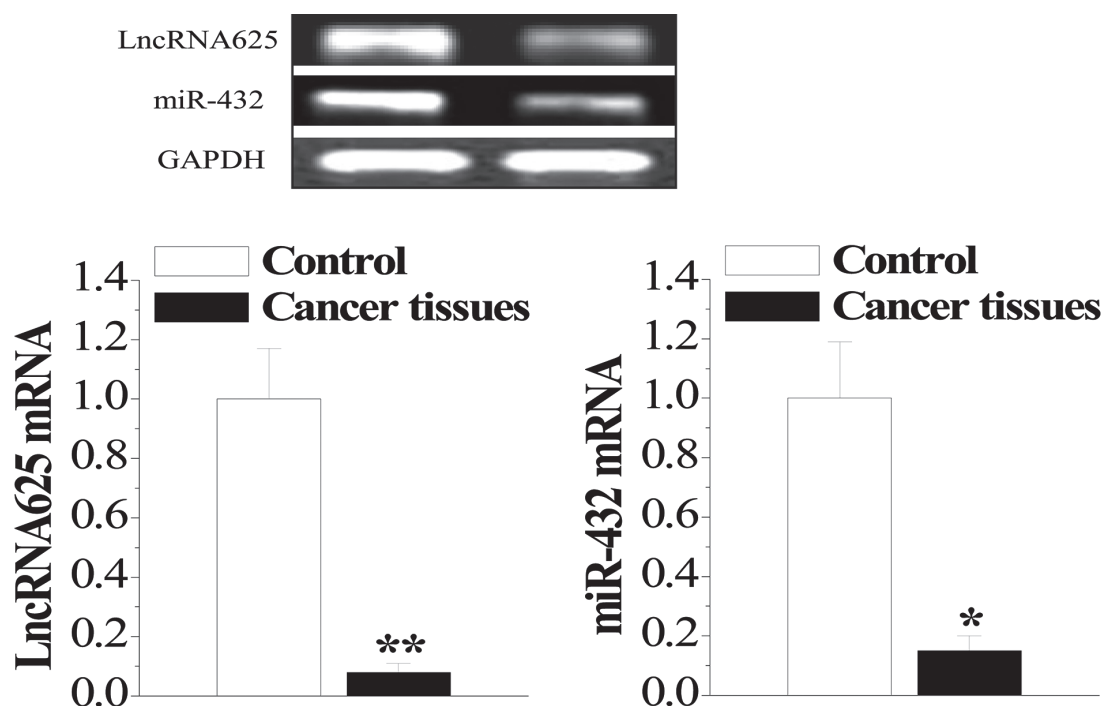


Figure 1. The LncRNA625 and miR-432 levels in PCa tissues. The relative LncRNA625 and miR-432 expressions of cancer and healthy tissues were analyzed by RT-qPCR. Statistical analysis was calculated by one-way ANOVA. * $p < 0.05$, ** $p < 0.01$ vs. healthy control.

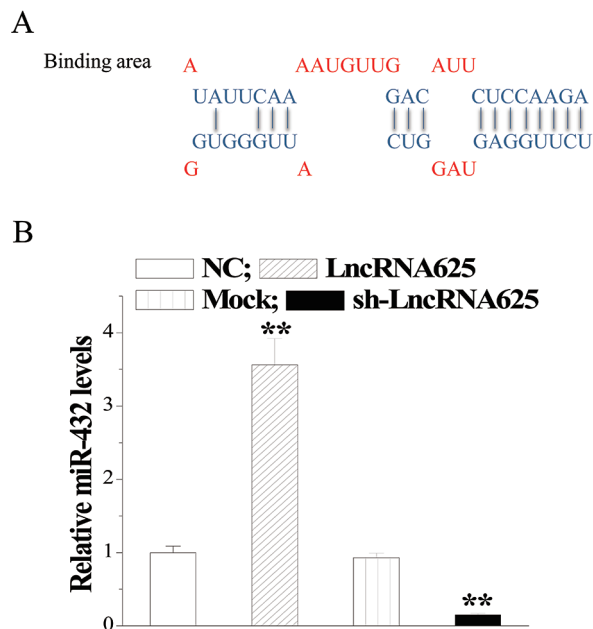


Figure 2. LncRNA625 regulates miR-432 expression in PC3 cells. **A**, The prediction of the binding area between LncRNA625 and miR-432 with the miRanda predictor software. **B**, RT-qPCR analysis of miR-432 expressions after transfections with sh-LncRNA625 and LncRNA625. Data are representative images or expressed as the mean \pm S.D. of each group from three separate experiments. * $p < 0.05$, ** $p < 0.01$ vs. mock control.

LncRNA625 Overexpression Inhibits the Proliferation of PC3 Cells by Targeting miR-432

MTT assay and the colony formation assay were carried out to investigate the cytotoxicity of LncRNA625 overexpression plasmid in the PC3 cell line. Data suggested that the cells growth were significantly inhibited after the transfection with LncRNA625 overexpression plasmid (Figure 3). The inhibition rates were 0.09, 0.13, 0.65 respectively in the NC, mock and LncRNA625 groups after 48 h of incubation. However, the effect of LncRNA625 on cells proliferation was reversed if cells were treated along with the miR-432 inhibitor simultaneously. Results suggested that LncRNA625 overexpression inhibited the proliferation of PC3 cells through targeting miR-432.

LncRNA625 Overexpression Modulates the PC3 Cells Cell Cycle

After transfection with LncRNA625 overexpression plasmid for 48 h, the flow cytometry analysis was performed to investigate the PC3 cells cell cycle according to the cell cycle stage distribution (G0, G1, S, G2, and M). We have found a greatly decreased S phase in the In-

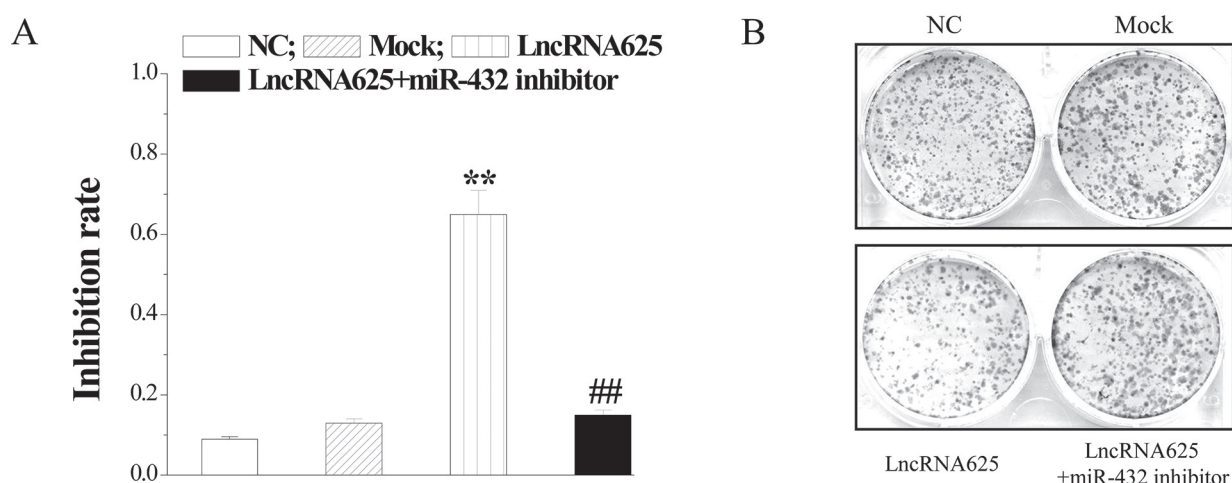


Figure 3. LncRNA625 overexpression inhibits the proliferation of PC3 cells. Inhibition rates were significantly increased in the LncRNA625 group compared with the mock control group, while was reversed if cells were treated along with the miR-432 inhibitor simultaneously. **A**, MTT assay. **B**, Colony formation assay. * $p < 0.05$, ** $p < 0.01$ vs. mock control. # $p < 0.05$, ## $p < 0.01$ vs. LncRNA625 group.

cRNA625 group compared with mock control group elucidating that LncRNA625 overexpression could arrest the progression of cell cycle at G1/S phase. Nevertheless, the LncRNA625 effect on the cell cycle was significantly reversed if treated along with the miR-432 inhibitor simultaneously (Figure 4).

LncRNA625 Overexpression Induces PC3 Cells Apoptosis

With the Annexin V/FITC and PI method, the LncRNA625 ability to induce PC3 cells apoptosis was explored. LncRNA625 overexpression significantly induced the PC3 cells apoptosis compared with mock control group. The number of the apoptotic cells was dramatically increased after the treatment of LncRNA625 overexpression plasmid compared with the mock control ($22.7 \pm 1.8\%$ vs. $7.7 \pm 1.1\%$). However, the LncRNA625 effect on the cell apoptosis was significantly reversed if cells were treated along with the miR-432 inhibitor simultaneously (Figure 5).

MiR-432 deactivates Wnt/ β -catenin Pathway by Suppressing TRIM29 and PYGO2 Expressions

To investigate the underlying mechanism that LncRNA625/miR-432 inhibited PC3 cells growth and induced cells apoptosis, we further assessed the effects of miR-432 on the Wnt/ β -catenin pathway, which has been reported playing a critical role in prostate cancer. As shown in Figure 6A, miR-432 overexpression markedly decreased the

luciferase activity of TOPflash or FOPflash reporter, while miR-432 inhibitor gained the adverse results. Besides, cellular fractionation showed that miR-432 overexpression inhibited the nuclear accumulation of β -catenin (Figure 6B), suggesting that miR-432 deactivated Wnt/ β -catenin pathway by inhibiting β -catenin nuclear accumulation. TRIM29 and PYGO2 are two critical components of Wnt/ β -catenin pathway and are often reported participating in malignant disease. Figure 6C showed that TRIM29 and PYGO2 might be potential targets of miR-432. The luciferase reporter assay further confirmed that miR-432 overexpression could decrease the luciferase activities of the 3'UTRs of TRIM29 and PYGO2 so as to suppress TRIM29 and PYGO2 directly (Figure 6D).

Discussion

In this work, the LncRNA625/miR-432 potential effects on the prostate cells proliferation and apoptosis were evaluated. Our findings suggested that the LncRNA625 and miR-432 levels were greatly reduced in the tumor tissues compared with the healthy control, implying an important protective role in prostate cancer. Besides, the binding area exists between LncRNA625 and miR-432, and the level of miR-432 was significantly regulated by the LncRNA625 expression in PC3 cells. Moreover, LncRNA625 overexpression could inhibit the cancer cells growth, arresting

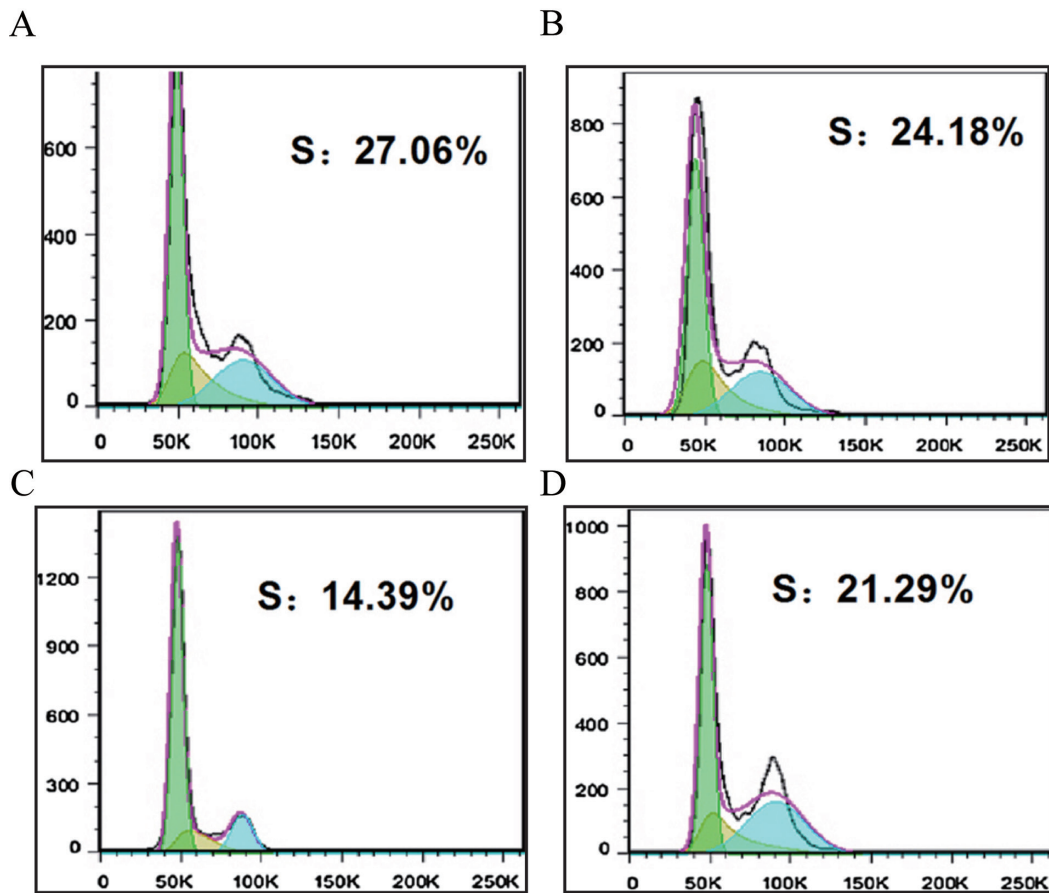


Figure 4. LncRNA625 overexpression modulates the PC3 cells cell cycle. Flow cytometry analysis on cell cycle progression in PC3 cells was carried out after transfection with LncRNA625. **A**, NC; **B**, Mock; **C**, LncRNA625; **D**, LncRNA625+miR-432 inhibitor.

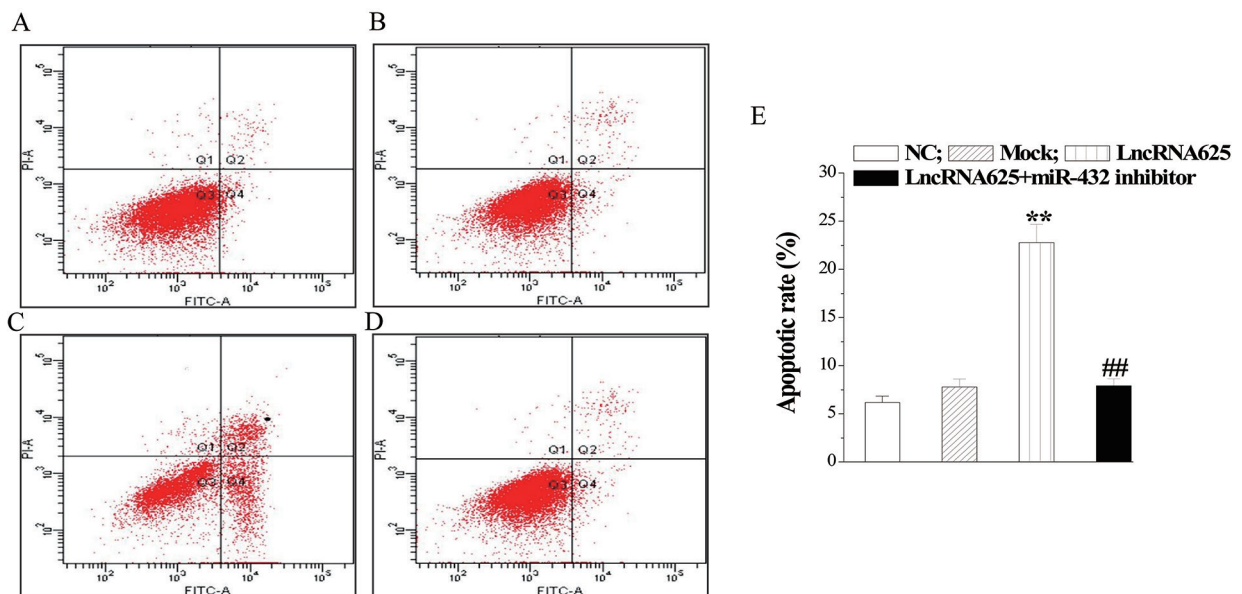


Figure 5. LncRNA625 overexpression induces PC3 cells apoptosis. Flow cytometric analysis of apoptosis in the PC3 cells after transfections with LncRNA625 or treated along with miR-432 inhibitor. **A**, NC; **B**, Mock; **C**, LncRNA625; **D**, LncRNA625+miR-432 inhibitor; **E**, Percentage of apoptotic cells in different groups. * $p < 0.05$, ** $p < 0.01$ vs. mock control. # $p < 0.05$, ## $p < 0.01$ vs. LncRNA625 group.

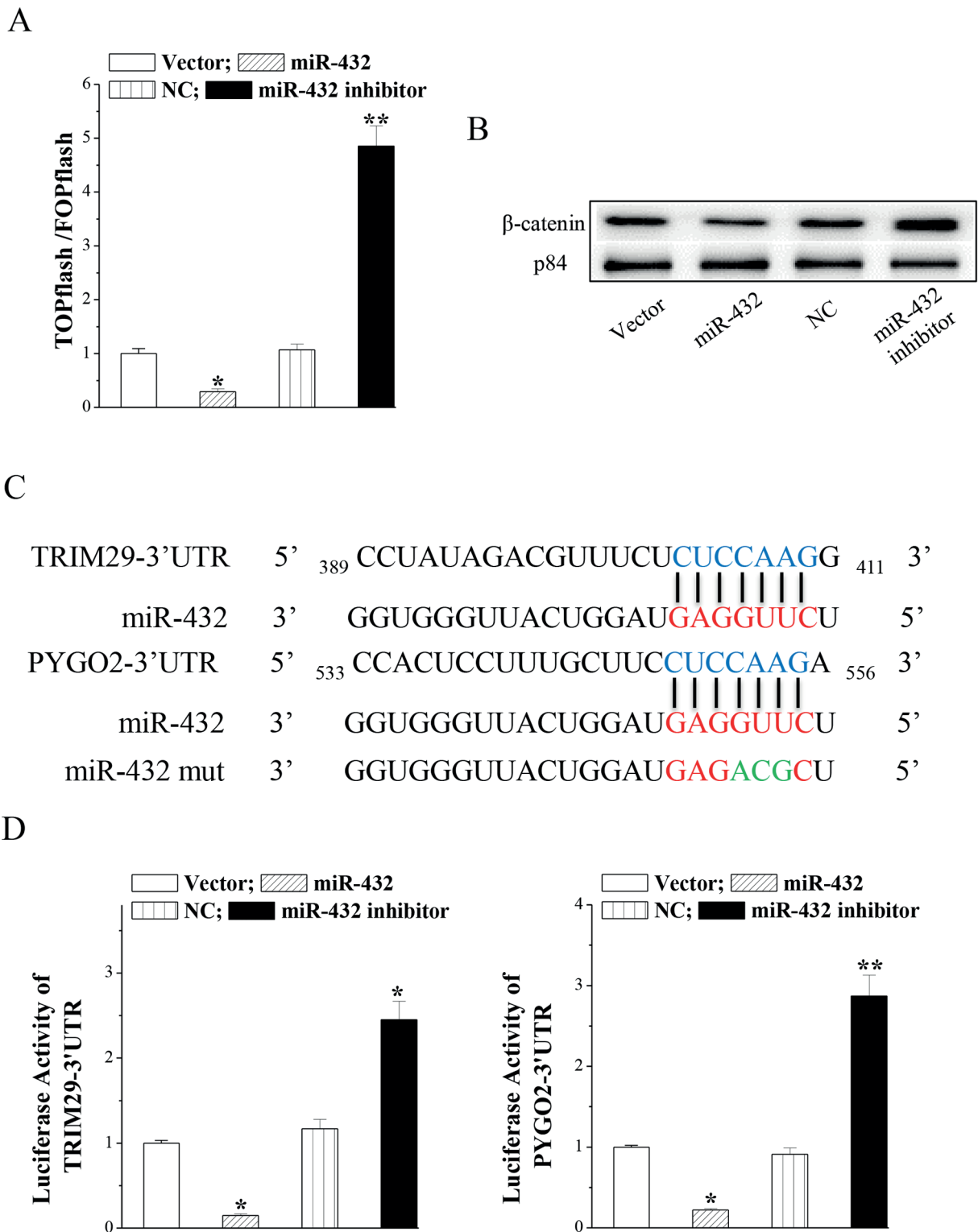


Figure 6. MiR-432 deactivates Wnt/ β -catenin pathway by suppressing TRIM29 and PYGO2 expressions. **A**, Indicated cells transfected with TOPflash or FOPflash and Renilla pRL-TK plasmids were subjected to dual-luciferase assays 48 hours after transfection. **B**, Altered nuclear translocation of β -catenin in response to miR-432 expression. Nuclear fractions of indicated cells were analyzed by Western blotting; p84 was used as the loading control. **C**, Predicted miR-432 target sequences in the 3'UTRs of TRIM29 and PYGO2. **D**, Luciferase assay of pGL3-TRIM29-3'UTR and pGL3-PYGO2-3'UTR reporter cotransfected with miR-432 and miR-432-inhibitor in PC3 cells.

cell cycle progression at the G1/S phase, and significantly induce apoptosis of PC3 cells, while the effect of lncRNA625 on cells was reversed if cells were treated along with the miR-432 inhibitor simultaneously. Notably, we further explored the underlying mechanism of lncRNA625/miR-432 effects on the PC3 cells and found that miR-432 could deactivate Wnt/ β -catenin pathway by suppressing TRIM29 and PYGO2 directly. Prostate cancer is one of the most frequently diagnosed malignancies and endangers public health severely¹⁷. The pathological development of PCa is a multistep complex process, including a series of dysregulated oncogenes and tumor suppressor genes¹⁸. lncRNAs may participate in cancer development, prognosis, metastasis, and recurrence via multiple aspects, involving transcriptional, posttranscriptional and epigenetic regulations¹⁹. A random walk strategy has been used to find out prostate cancer-associated lncRNAs in lncRNA-PCG bipartite network based on the sample correlation, and lncRNA-PCG co-expression network has been constructed to predict lncRNAs functions¹¹. Thus, lncRNAs can be used as diagnostic and prognostic biomarkers for PCa^{20,21}. In our present work, the prostate cancer cells displayed decrease of cell growth and tumor formation after the overexpression of lncRNA625, further validating lncRNA625 as a tumor suppressor gene in PCa. miRNAs act as oncogene or tumor suppressor in various tumors and accumulating evidence suggests that miRNAs contribute greatly to the pathogenesis of PCa^{22,23}. MiR-432 was reported as a cancer suppressor, and its downregulation in multiple tumors was found, for instance, ovarian cancer, cervical cancer, and hepatocellular carcinoma^{16,24}. In this study, we found that miR-432 is significantly suppressed in PCa tissues and the potential involvement of miR-432 in the pathological progression of PCa prompted us to investigate its exact functions. Deregulation of Wnt/ β -catenin pathway is familiar in kinds of cancer, and the activation of this signaling is considered as an early event in tumorigenesis²⁵⁻²⁷. Thus, it may provide some new insights into the molecular mechanisms underlying tumor genesis if we understand more interactions with multiple regulators of β -catenin signaling. Our current study showed that miR-432 simultaneously repressed the expression of two important factors of β -catenin pathway: TRIM29 and PYGO2, which subsequently suppressed β -catenin activation in PC3 cells. These two identified mediation molecules have all been

suggested to be up-regulated in multiple tumors and played critical roles in the modulation of oncogenic β -catenin pathway from plasma membrane to the nucleus level^{28,29}. The new results in our present research were that lncRNA625 and miR-432 levels were consistently reduced in the PCa tissues compared with the healthy control and lncRNA625 levels significantly affect the miR-432 expression in PC3 cells, indicating that miR-432 is a direct target of lncRNA625. Besides, lncRNA625 overexpression could inhibit the cancer cells growth, arresting cell cycle progression at the G1/S phase, and significantly induce apoptosis of PC3 cells, while reversed by the miR-432 inhibitor. Of note, we further explored the underlying mechanism and found that miR-432 could deactivate Wnt/ β -catenin pathway via suppressing TRIM29 and PYGO2 directly. Consequently, we suggested that lncRNA625 could functionally inhibit PC3 cells proliferation and promote cells apoptosis through regulating the Wnt/ β -catenin pathway by targeting miR-432. The close correlation between the prostate cancer cells and levels of lncRNA625 and miR-432 may provide some experimental evidence for the possible effect of the lncRNA625 and miR-432 over-expression against the proliferation of PC3 cells. The clear mechanisms underlying the lncRNA625/miR-432 action and its utility for the treatment of prostate cancer in human beings still need to be investigated further.

Conclusions

The present work demonstrated that lncRNA625/miR-432 overexpression in the human PC3 cells could inhibit the cancer cells proliferation and significantly induce apoptosis. The anti-proliferative effects of lncRNA625/miR-432 on PC3 cells are mainly through the inhibition of Wnt/ β -catenin pathway via suppressing TRIM29 and PYGO2 directly. Our findings suggested that lncRNA625/miR-432 could serve as an innovative and prospective therapeutic target for human prostate cancer.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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