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Circular RNA_LARP4 inhibits cell migration and invasion of prostate cancer by targeting FOXO3A

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Abstract. – OBJECTIVE: The importance of circular RNAs in malignant tumors causes more attention in researchers. Circular RNA_LARP4 is identified as a tumor suppressor in gastric cancer, but the role of circular RNA_LARP4 in prostate cancer (PCa) remains unclear. Our work aims to uncover whether and how circular RNA_LARP4 functions in the PCa development.

PATIENTS AND METHODS: Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was utilized to determine the level of circular RNA_LARP4 in PCa tissues and cell lines. The patients' prognosis was analyzed. Circular RNA_LARP4 lentivirus was constructed for transfection of PCa cells. Cell migrated and invaded ability was detected through wound healing assay and transwell assay. Western blot assay was performed to analyze the protein level of FOXO3A.

RESULTS: The low circular RNA_LARP4 expression was associated with poor prognosis of PCa patients. The circular RNA_LARP4 was lowly expressed in PCa tissues compared with adjacent samples. The expression of circular RNA_LARP4 was downregulated in PCa cell lines. The cell migrated and invaded ability of PCa cells was inhibited after circular RNA_LARP4 was overexpressed. Furthermore, FOXO3A expression was increased via the overexpression of circular RNA_LARP4.

CONCLUSIONS: Circular RNA_LARP4 could suppress cell migration and invasion of PCa by upregulating FOXO3A.

Key Words:
Circular RNA, Circular RNA_LARP4, Prostate cancer, FOXO3A.

Introduction

Prostate cancer (PCa) remains one of the leading malignancies in men globally, accounting for 27% of all cancer cases[1,2]. Moreover, the incidence of PCa has kept increasing for the past years. There are approximately 233,000 new cases diagnosed with prostate cancer annually. Although numerous studies have been carried out to help to understand the molecular and biological mechanism underlying the progression of PCa, few improvements have been made for the advanced cases[3]. Drug resistance and metastasis, which are a significant medical problem for the patients, remain two major causes of cancer-related mortalities in advanced prostate cancer. Thus, new biomarkers are urgently needed to explore the underlying mechanism of PCa to find out the therapeutic targets.

Circular RNAs (circRNAs) are one subtype of noncoding RNAs (ncRNAs) which are tissue-specific, ubiquitously expressed. Circular RNAs are naturally resistant to degradation by exonucleases and thus are more stable than linear RNA[4]. Serving as microRNA (miRNA) sponges was one of the functions of circRNAs firstly described. Recently, it has been revealed that circRNAs have a variety of biological functions, including tumorigenesis. So, hsa_circ_0071589 promotes carcinogenesis *via* the miR-600/EZH2 axis in colorectal cancer[5]. Circ_0067934 facilitates tumor growth and cell migration in hepatocellular carcinoma by modulating miR-1324/FZD5/Wnt/β-catenin signaling[6]. Knockdown of circRNACER restrains cell proliferation and cell migration in breast cancer *via* modulating the activity of miR136/MMP13 signaling[7]. CircPSMC3 functions as a tumor suppressor in gastric cancer by serving as a competitive endogenous RNA of miR-296-5p[8].

Recently, circular RNA_LARP4 is reported to be a new tumor suppressor in cancers. However, the function of circular RNA in PCa remains unclear. In our study, circular

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RNA_LARP4 expression was detected in PCa tissues, and its function in PCa was further explored.

Patients and Methods

Tissue Samples

Fifty-five cases of PCa tissues and para-cancer tissues were obtained at the Yinzhou People's Hospital. The prognosis of patients was analyzed. These patients have received no radiotherapy or chemotherapy prior to surgery. This work was approved by the Ethics Committee of the Yinzhou People's Hospital. Signed written informed consents were obtained from all participants before the study.

Cell Culture and Transfection

Human PCa cell lines (LNCaP, DU145, and 22Rv1) and normal human prostate epithelial cell line (P69) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured with Dulbecco's Modified Eagle's Medium (DMEM; Promega, Madison, WI, USA) and 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA) in an incubator containing 5% CO_2 at 37°C. After PCa cells were cultured for 24 h on 6-well plates, cells were transfected with lentivirus targeting specifically targeting circular RNA_LARP4 (Lentivirus) and negative control (GenePharma; Shanghai, China) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Those GFP-positive cells were chosen for following investigations.

RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

TRIzol RNA isolation kit (Invitrogen, Carlsbad, CA, USA) was utilized for separating the total mRNA from tissues and cells. The synthesis of complementary deoxyribose nucleic acids (cDNAs) was conducted through reverse Transcription Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). The primer sequences used for RT-qPCR were as follows: circular RNA_LARP4 forward: 5′-GGGCATCAGGAGCAAACTTA-3′ and reverse: 5′-CTGGCGAATTAAAGCCATTC-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers forward: 5′-CCAAAATCAGATGGGGCAATGCTGG-3′ and reverse: 5′-TGATGGCATGGACTGTGGTCATTCA-3′. The mRNA expression level was normalized to GAPDH.

Scratch Wound Assay

Scratch wound assay was used to measure the area of the wounded surface using a light microscope (Olympus, Tokyo, Japan) at 48 h. 1×10^5 PCa cells were seeded in each well of 6-well plates. After 48 h transfection, a scratch was performed by a pipette tip. Images of the same fields were taken at 48 h after the scratch.

Transwell Assay

Migration and invasion assays were conducted through a 24-well transwell plate (8 μm pore size; Corning, Corning, NY, USA). 1×10^5 cells in 200 μL serum-free DMEM were replanted in the upper chamber for migration assays, while 1×10^5 cells in 200 μL serum-free DMEM were replanted in the upper chamber with 50 μg Matrigel (BD Biosciences; Bedford, MA, USA) for invasion assays. DMEM and FBS were added to the lower chamber. Then, they were cultured overnight in an incubator supplemented with 5% CO_2 at 37°C. The top surface of chambers was treated by methanol for 30 min after wiped by a cotton swab. Next, they were stained in crystal violet for 20 min. Five fields were randomly chosen using a digital microscope (Nikon, Tokyo, Japan).

Western Blot Analysis

The protein was extracted from cells using a lysis buffer. Bicinchoninic acid (BCA) method was used for the quantification of the protein concentration. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was utilized to extract the target proteins which were then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were immersed into Phosphate Buffered Saline and Tween (PBST) solution for 2 h with 5% non-fat milk and incubated with the antibodies, rabbit anti-GAPDH, and rabbit anti-FOXO3A (Cell Signaling Technology; Danvers, MA, USA) used in this study, as well as goat anti-rabbit secondary antibody (Cell Signaling Technology; Danvers, MA, USA). Image J software was applied for the assessment of protein expression.

Statistical Analysis

Data analysis was performed using Statistical Product and Service Solutions (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA). GraphPad 4.0 (GraphPad Software, Inc., La Jolla, CA, USA) helped to present these consequences. Kaplan-Meier method

and Student's *t*-test were used when appropriate. Quantitative data was presented as mean ± SD. The statistical significance was defined as $p<0.05$.

Results

The Association Between Circular RNA_LARP4 Expression and Prognosis of PCa Patients

Before the analysis PCa patients' prognosis, they were divided into two groups, low and high circular RNA_LARP4 expression groups. The patients' survival after surgery was analyzed through the Kaplan-Meier method. As is shown in Figure 1, the PCa patients in the low circular RNA_LARP4 expression group had poorer overall survival compared with those in high circular RNA_LARP4 expression group.

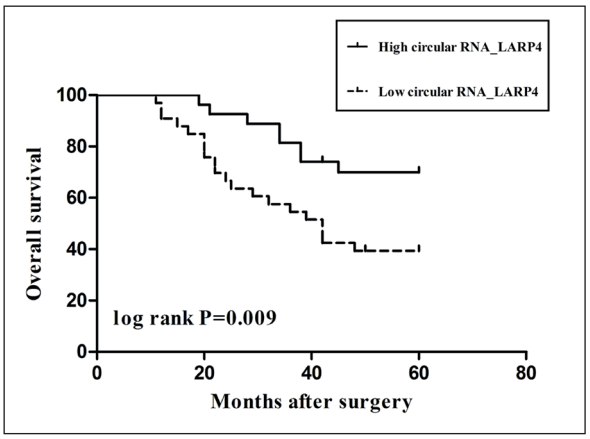

Figure 1. Low expression level of circular RNA_LARP4 was associated with poorer prognosis of PCa patients. Low level of circular RNA_LARP4 was associated with poorer overall survival of PCA patients. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

Circular RNA_LARP4 Expression Level Was Lower in PCa Tissues and Cells

The circular RNA_LARP4 expression was detected *via* RT-qPCR in PCa patients' tissue samples and matched adjacent samples. Results showed that circular RNA_LARP4 was lower in tumor tissue samples than that in adjacent tissues (Figure 2A). Moreover, the circular RNA_LARP4 expression level was lower in PCa cells than in P69 cells (Figure 2B). The results suggested that downregulated circular RNA_LARP4 might be associated with PCa development. Then, we chose DU145 PCa cell line for

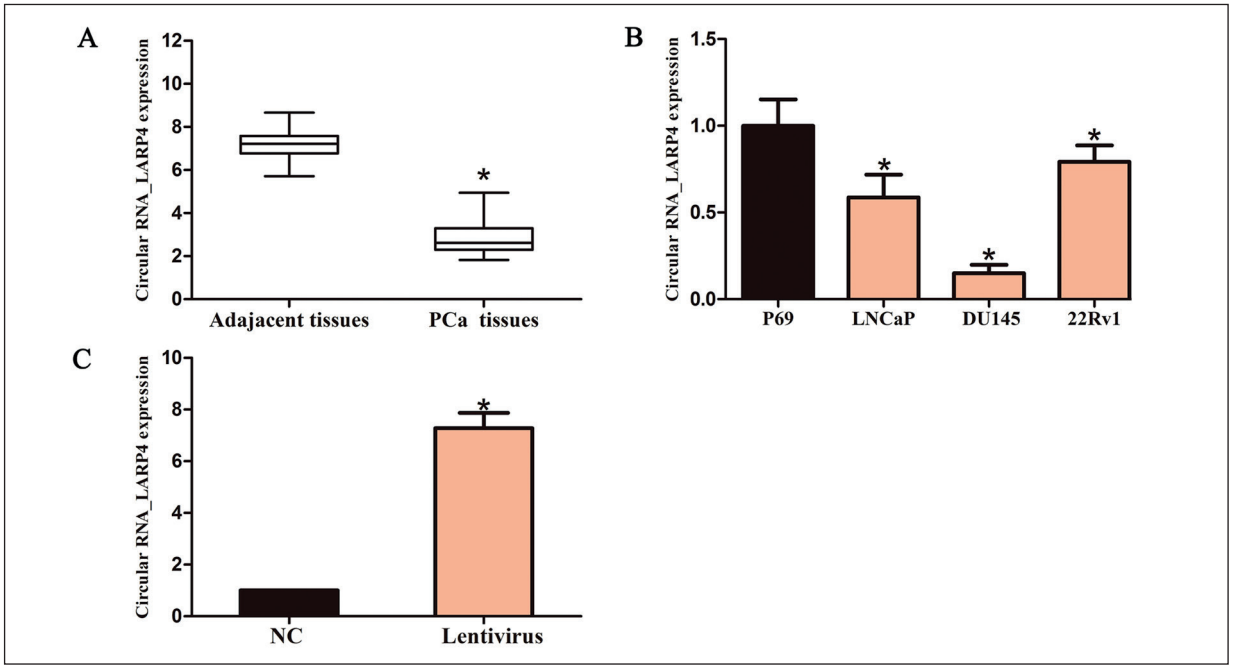

Figure 2. Expression levels of circular RNA_LARP4 were increased in PCa tissues and cell lines. **A,** Circular RNA_LARP4 expression was significantly increased in the PCa tissues compared with adjacent tissues. **B,** Expression levels of circular RNA_LARP4 relative to GAPDH were determined in the human PCa cell lines and normal human prostate epithelial cell line (P69) by RT-qPCR. **C,** Circular RNA_LARP4 expression in DU145 cells transfected with circular RNA_LARP4 lentivirus and control were detected by RT-qPCR. GAPDH was used as an internal control. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

the transfection of circular RNA_LARP4 lentivirus. The transfection efficiency was detected by RT-qPCR (Figure 2C).

Circular RNA_LARP4 Overexpression Inhibited Cell Migration and Invasion in DU145 Cells

To explore how circular RNA_LARP4 affected PCa metastasis, scratch wound assay and transwell assay were performed. As shown in Figure 3A, circular RNA_LARP4 overexpression reduced cell migrated length of DU145 cells. As shown in Figure 3B, the number of migrated cells was markedly decreased after circular RNA_LARP4 was overexpressed in DU145 cells. As shown in Figure 3C, the number of invaded cells was remarkably decreased after circular RNA_LARP4 was overexpressed in DU145 cells.

Circular RNA_LARP4 Overexpression Promoted FOXO3A in PCa

Previous researches have reported that FOXO3A, a tumor suppressor, participates in regulating tumor metastasis. In our work, the interaction between FOXO3A and circular RNA_LARP4 was studied. We found that circular RNA_LARP4 overexpression increased the mRNA expression of

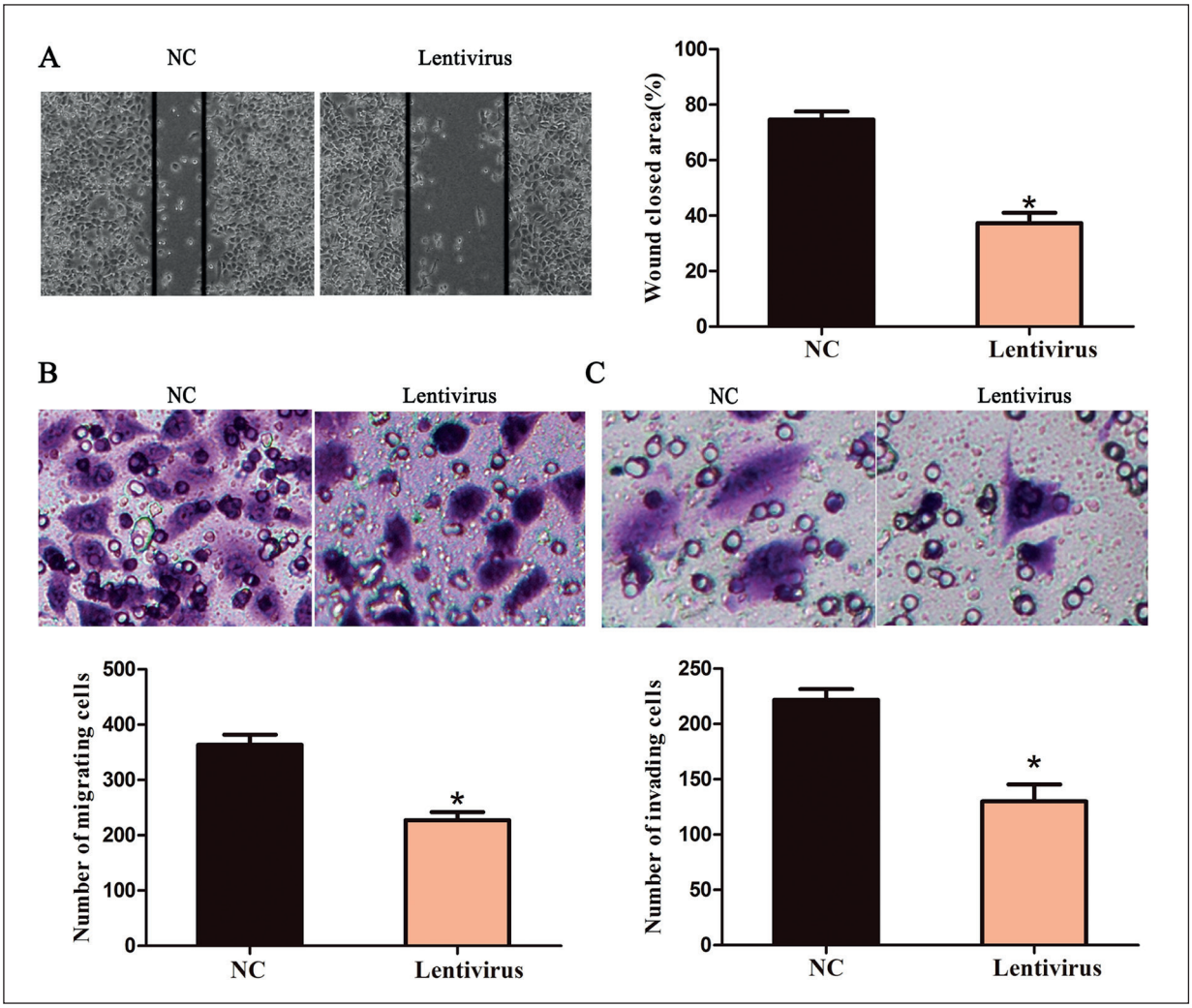

Figure 3. Overexpression of circular RNA_LARP4 inhibited DU145 cell migration and invasion. ***A,*** Scratch wound assay showed that overexpression of circular RNA_LARP4 significantly repressed cell migration and invasion in DU145 cells (magnification:10×). ***B,*** Transwell assay showed that the number of migrated cells was significantly decreased *via* overexpression of circular RNA_LARP4 in DU145 cells (magnification: 40×). ***C,*** Matrigel assay showed that the number of invaded cells was significantly decreased *via* overexpression of circular RNA_LARP4 in DU145 cells (magnification: 40×). The results represent the average of three independent experiments (mean ± standard error of the mean). *$p<0.05$, as compared with the control cells.

FOXO3A (Figure 4A). Moreover, circular RNA_LARP4 overexpression increased the protein level of FOXO3A (Figure 4B). Furthermore, correlation analysis demonstrated that FOXO3A expression level positively correlated to circular RNA_LARP4 expression in PCa tissues (Figure 4C).

Discussion

CircRNAs are formed during reverse splicing of RNA. Depending on its origination, it can be divided into exonic circular RNA (ecircRNA), intronic circular RNA (ciRNA), and ElciRNA. CircRNAs are dysregulated in PCa and may serve as promising biomarkers of PCa. For example, by sponging miR-193a-3p and modulating MCL1, circHIPK3 facilitates cell proliferation and tumor invasion in PCa[9]. Upregulation of circ-102004 enhances the proliferation of prostate cancer cell which may be a potential biomarker of PCa[10]. Responding to androgen, circSMARCA5 is overexpressed in PCa and facilitates cell proliferation in PCa[11]. By regulating the expression of mir-29a, circ MYLK functions as an oncogene and promoting progression of PCa[12].

Derived from LARP4 gene locus, circular RNA_LARP4 has been revealed recently to inhibit cancer cell migration and invasion by serving as a La-related RNA-binding protein. Recently, circular RNA_LARP4 has been reported to be significantly down-regulated in ovarian cancer which may serve as a potential biomarker for prognosis of ovarian cancer patients[13]. In addition, circular RNA_LARP4 suppresses cell proliferation and cell invasion in gastric cancer by sponging miR-424-5p and modulating the expression of LATS1[14]. We showed that circular RNA_LARP4 was downregulated in PCa samples and cell lines, and low circular RNA_LARP4 expression was associated with poor prognosis of PCa patients, which indicated that circular RNA_LARP4 could be a tumor suppressor in PCa.

Then, the related proteins of circular RNA_LARP4 were further explored. Forkhead box (FOX) proteins are a conserved transcription factor family of proteins which regulate a variety of biological processes, including normal homeostasis and development[15]. The forkhead box class O (FOXO) family, one of FOX subfamilies, has been reported to modulate developmental processes and tumorigenesis in many tissues.

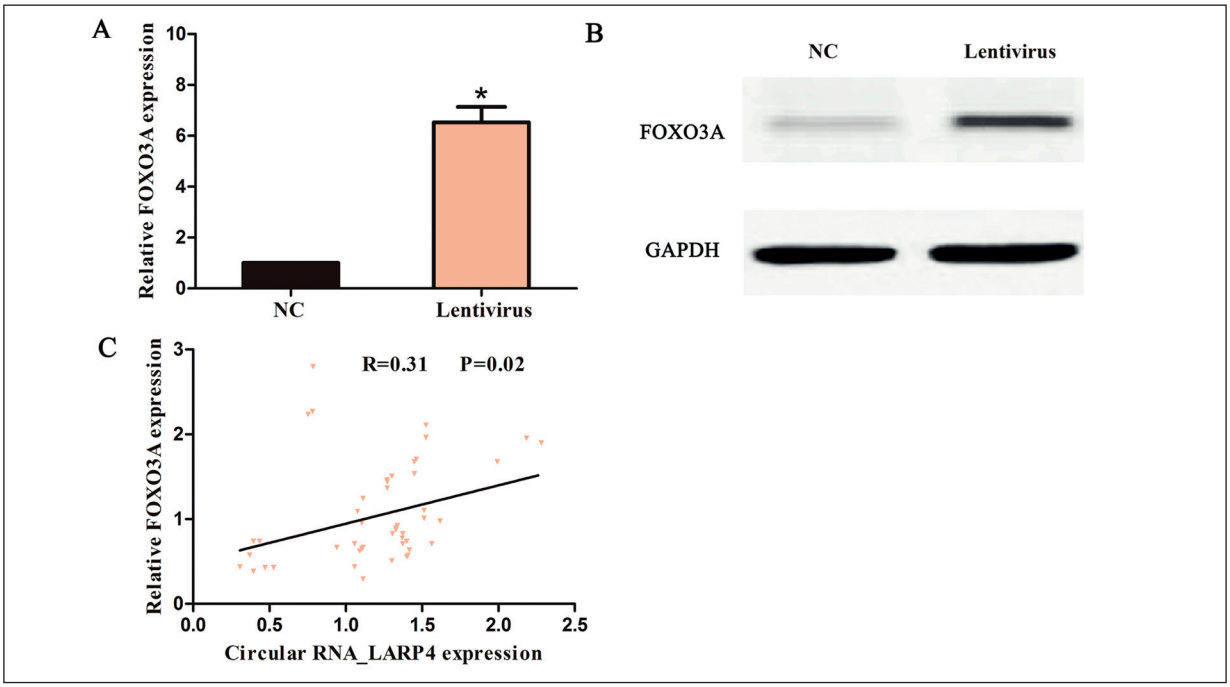

Figure 4. Circular RNA_LARP4 overexpression inhibited FOXO3A in PCa. ***A,*** RT-qPCR results showed that FOXO3A expression was increased in circular RNA_LARP4 lentivirus group compared with control group in DU145 cells. ***B,*** Western blot results showed that FOXO3A expression was increased in circular RNA_LARP4 lentivirus group compared with control group. ***C,*** Linear correlation between the expression level of FOXO3A and circular RNA_LARP4 in PCa tissues. The results represent the average of three independent experiments. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

As a member of FOXO subfamily, FOXO3a gene is located on chromosome 6q21 which has been found to play a vital role in regulating a variety of cellular processes involved in the progression of cancers. So, PAX3 functions as a tumor suppressor in thyroid cancer by modulating the activities of transcription factor FOXO3a[16]. Through FOXO3a-mediated pathways, the silencing of SIRT1 depresses cell proliferation and cell migration, and induces cell cycle arrest in bladder cancer[17]. Crosstalk of FOXO3a and miRNA155-5p contributes to the induction of IGFBP1 expression and inhibits cell growth in human lung cancer[18]. Moreover, by modulating the WNT/β-catenin signaling, FOXO3a inhibits epithelial-to-mesenchymal transition in prostate cancer[19].

The potential interaction between FOXO3A and circular RNA_LARP4 was first researched in our study. Results showed that circular RNA_LARP4 overexpression increased FOXO3A expression *in vitro* and was positively correlated with FOXO3A expression in PCa tissues. Above findings indicated that circular RNA_LARP4 might inhibit metastasis of PCa by upregulating FOXO3A.

Conclusions

We showed that circular RNA_LARP4 was remarkably downregulated in PCa tissues and could indicate the prognosis of PCa patients. Circ_0000885 inhibits cell migration and invasion of PCa by upregulating FOXO3A, which suggests that circular RNA_LARP4 may contribute to therapy for PCa as a prospective target.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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