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Circ PSMC3 inhibits prostate cancer cell proliferation by downregulating DGCR8

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Abstract. – OBJECTIVE: The importance of circular RNAs in malignant tumors has attracted a lot of attention. Circular PSMC3 (CircPSMC3) is identified as a tumor suppressor in gastric cancer. The role of circPSMC3 in prostate cancer (PCa) remains unclear. Our study aims to uncover whether and how circPSMC3 functions in PCa development.

PATIENTS AND METHODS: Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was utilized to determine the level of circPSMC3 in PCa tissues and cell lines. The relation between circPSMC3 expression and patients' prognosis was analyzed as well. CircPSMC3 lentivirus was constructed and transfected into PCa cells. Cell migration and invasion abilities were detected through wound healing assay, transwell assay, and Matrigel assay, respectively. Western blot assay was performed to detect the protein level of DGCR8.

RESULTS: CircPSMC3 was lowly expressed in PCa tissues compared with adjacent normal tissues. Low expression of circPSMC3 was significantly downregulated in PCa cell lines as well. The migration and invasion abilities of PCa cells were significantly inhibited after circPSMC3 was overexpressed *in vitro*. Furthermore, DGCR8 expression increased remarkably *via* the overexpression of circPSMC3.

CONCLUSIONS: CircPSMC3 could suppress PCa cell migration and invasion by upregulating DGCR8.

Key Words:
Circular RNA, CircPSMC3, Prostate cancer, DGCR8.

Introduction

Prostate cancer (PCa) remains one of the most frequent cancers in men, accounting for 27% of all cancer cases[1,2]. Due to increased awareness, aging population, and the use of prostate-specific antigen (PSA) for screening, the morbidity of PCa has been increasing for the past years[13,14]. Approximately 233,000 new cases are diagnosed with prostate cancer annually[5]. It has been reported[6] that more than 29,000 deaths caused by PCa occurred in US in 2018. In the past decade, numerous studies[7] have explored the molecular and biological mechanism underlying the progression of PCa. However, few improvements have been made for advanced cases. Therefore, there is an urgent need to explore the underlying mechanism of PCa and to find out potential biomarkers and therapeutic targets.

Circular RNAs (circRNAs), formed by a covalently closed loop, have been emerging as a new hot topic in the noncoding RNAs network. CircRNAs are differentially expressed in various cancerous tissues or cells. Meanwhile, they are closely related to tumorigenesis and prognosis. It has been detected that circ-ITCH suppresses the proliferation and induces the apoptosis of epithelial ovarian cancer cells. Moreover, it is associated with a prolonged overall survival[8]. Has_circ_0001946 enhances tumorigenesis in lung adenocarcinoma by the activation of the Wnt/β-catenin signaling pathway[9]. The knockdown of circRNACER restrains breast cancer cell proliferation and migration *via* modulating the activity of miR136/MMP13 signaling[10]. By regulating the expression of LATS1 and sponging miR-424-5p, circ-RNA_LARP4 suppresses the proliferation and invasion of gastric cancer cells[11]. Currently, circPSMC3 has been identified as a new tumor suppressor in multiple cancers. However, the exact function of circular RNA in PCa remains unclear.

Also, DGCR8 has been reported to participate in the progression of cancers. Dysregulated DGCR8 played an important role in various malignancies, including PCa. DGCR8 could be regulated by long noncoding RNAs, microRNAs, and circular RNAs.

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In our work, circPSMC3 was significantly downregulated in PCa samples and cell lines. Low circPSMC3 expression was associated with poor disease-free survival of PCa patients. Moreover, circPSMC3 markedly inhibited the proliferation and regulated the cell cycle of PCa cells *in vitro*. Furthermore, circPSMC3 overexpression decreased tumor formation and downregulated DGCR8 in nude mice.

Patients and Methods

Tissue Samples

A total of 55 PCa tissues and para-cancer tissues were collected from patients in the Affiliated Jiangyin Hospital of Southeast University Medical College. The relation between circPSMC3 expression and patients' prognosis was analyzed. No radiotherapy or chemotherapy was performed before the surgery. This research was approved by the Ethics Committee of the Affiliated Jiangyin Hospital of Southeast University Medical College. Signed written informed consents were obtained from all participants before the study.

Cell Culture

Human PCa cell lines (PC3, DU145, and LNCap) and one normal human prostate epithelial cell line (P69) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD, USA) in an incubator with 5% CO_2 at 37°C.

Cell Transfection

PCa cells were first cultured for 24 h on 6-well plates. Next, the cells were transfected with lentivirus targeting specifically targeting circPSMC3 (shRNA) and control (GenePharma; Shanghai, China) according to the instructions of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). GFP-positive cells were chosen for the following experiments.

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

TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) was utilized to extract total messenger ribonucleic acid (mRNA) from tissues and cells. Then, extracted RNA was reverse transcribed into complementary deoxyribonucleic acids (cDNAs) through the Reverse Transcription Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). Primer sequences used for RT-qPCR were as follows: circPSMC3, forward: 3'-GTTTAGGGTCCCTGCCCTTTG-5'; circPSMC3, reverse: 3'-GTGTTGGGCTGGAAGCCATC-5'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers forward: 5′-CCAAAATCAGATGGGGCAATGCTGG-3′ and reverse 5′-TGATGGCATGGACTGTGGTCATTCA-3′. The mRNA expression level of mRNAs was normalized to GAPDH.

Cell Proliferation Assay

2×10^3 transfected cells were first seeded into 96-well plates. Cell proliferation was assessed at 0, 24, 48, 72 h in accordance with Cell Proliferation Reagent Kit I (MTT; Roche, Basel, Switzerland), respectively. Absorbance at 490 nm was assessed using an enzyme-linked immunosorbent assay (ELISA) reader system (Multiskan Ascent, LabSystems, Helsinki, Finland).

Colony Formation Assay

PCa cells were seeded into 6-well plates and cultured for 10 days. Then, formed colonies were fixed with 10% formaldehyde for 30 min and stained with 0.5% crystal violet for 5 min. Image-Pro Plus 6.0 (Silver Springs, MD, USA) was used for data analysis.

Cell Cycle Assay

RNase A solution (250 μg/mL) was used to treat PCa cells (2×10^5) in 90% methanol solution for 30 min at 37°C. After that, the cells were incubated with propidium iodide (PI) for another 15 min. FlowJo software (Version X; TreeStar, Ashland, OR, USA) was used to detect the cell cycle.

Western Blot Analysis

Protein in cells was extracted by reagent radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China). Total protein samples were separated by dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were incubated with primary antibodies of rabbit anti-GAPDH and rabbit anti-DGCR8 (Cell Signaling Technology, CST, Danvers, MA, USA) overnight. Image J software (Silver Springs, MD, USA) was applied for the assessment of protein expression.

Xenograft Model

After circPSMC3 overexpression LNCap cells, the cells were replanted into NOD/SCID mice (6 weeks old). Tumor volume was calculated every 5 days by the following formula (volume = length × width^2 × 1/2). Tumors were extracted after 4 weeks. The research was approved by the Animal Ethics Committee of the Southeast University Medical College.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Graph PAD 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) was applied for image editing. Student's *t*-test was used to compare the difference between the two groups. The Kaplan-Meier method was used to predict patients' prognosis. $p<0.05$ was considered statistically significant.

Results

The Disease-Free Survival Rate of PCa Patients

PCa patients were divided into two groups, including low circPSMC3 expression group and high circPSMC3 expression group. The Kaplan-Meier method was used to analyze the disease-free survival rate of patients after surgery. Compared with patients in high circPSMC3 expression group, the prognosis of those in low circPSMC3 expression group was significantly worse (Figure 1).

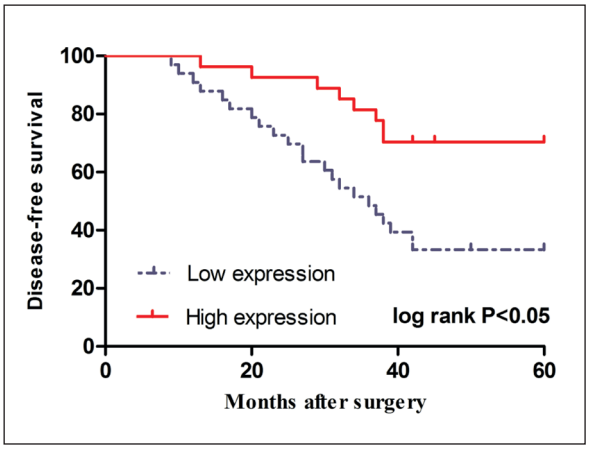

Figure 1. Low expression level of circPSMC3 was associated with worse prognosis of PCa patients. Low expression of circPSMC3 was associated with worse disease-free survival of PCa patients. Data were presented as mean ± standard error of the mean. *$p<0.05$.

The Expression of CircPSMC3 in PCa Tissues and Cells

RT-qPCR was used to detect circPSMC3 expression in 55 PCa patients' tissues samples corresponding to normal tissues. As shown in Figure 2A, circPSMC3 was significantly lower in tumor tissues samples than that of the corresponding samples. Moreover, the circPSMC3 expression level was markedly lower in PCa cells (PC3, DU145, and LNCap) than that in a normal human prostate epithelial cell line (P69; Figure 2B). These results indicated that dysregulated circPSMC3 might participate in the PCa progression.

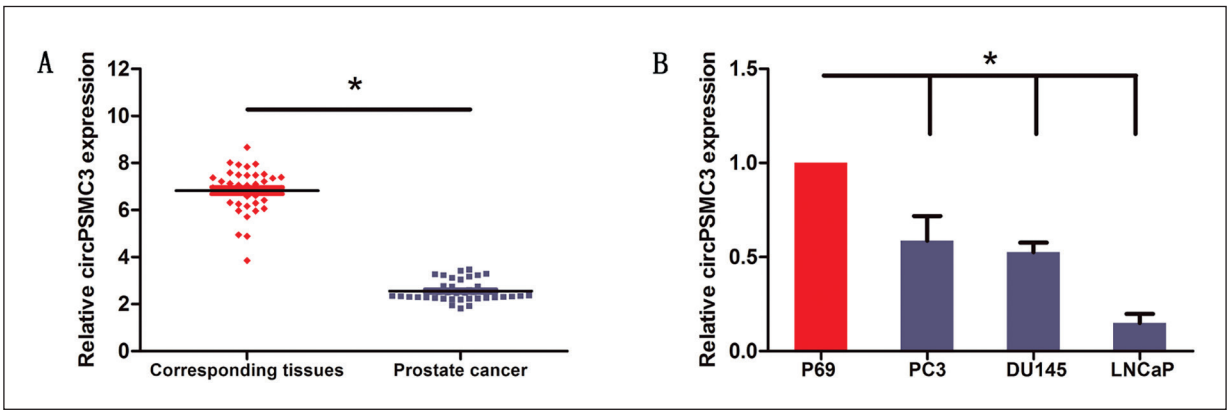

Figure 2. Expression level of circPSMC3 increased significantly in PCa tissues and cell lines. **A,** CircPSMC3 expression was significantly down-regulated in PCA tissues compared with adjacent normal tissues. **B,** Expression levels of circPSMC3 relative to GAPDH were determined in human PCa cell lines and normal human prostate epithelial cell line (P69) by RT-qPCR. GAPDH was used as an internal control. Data were presented as mean ± standard error of the mean. *$p<0.05$.

CircPSMC3 Overexpression Inhibited Cell Proliferation and Regulated Cell Cycle in LNCap Cells

To explore the effect of circ_0067934 on PCa proliferation, MTT assay, colony formation assay, and cell cycle assay were conducted. LNCap were first transfected with circPSMC3 lentivirus. RT-qPCR was used to verify the transection efficiency (Figure 3A). As shown in Figure 3B, the MTT assay showed that the growth ability of LNCap cells was significantly repressed after circPSMC3 overexpression. As shown in Figure 3C, colony formation assay showed that the number of colonies was significantly reduced after circPSMC3 was overexpressed. As shown in Figure 3D, the percentage of cells in G0/G1stage increased remarkably after circPSMC3 was overexpressed in LNCap cells.

However, the percentage of cells in the S stage was significantly reduced after circPSMC3 overexpression.

CircPSMC3 Overexpression Up-regulated DGCR8 Expression in PCa

DGCR8 has been confirmed to suppress the proliferation of numerous cancers, including PCa. In our study, we first explored the interaction between DGCR8 and circPSMC3. RT-qPCR was used to detect DGCR8 expression in LNCap cells transfected with circPSMC3 lentivirus or empty

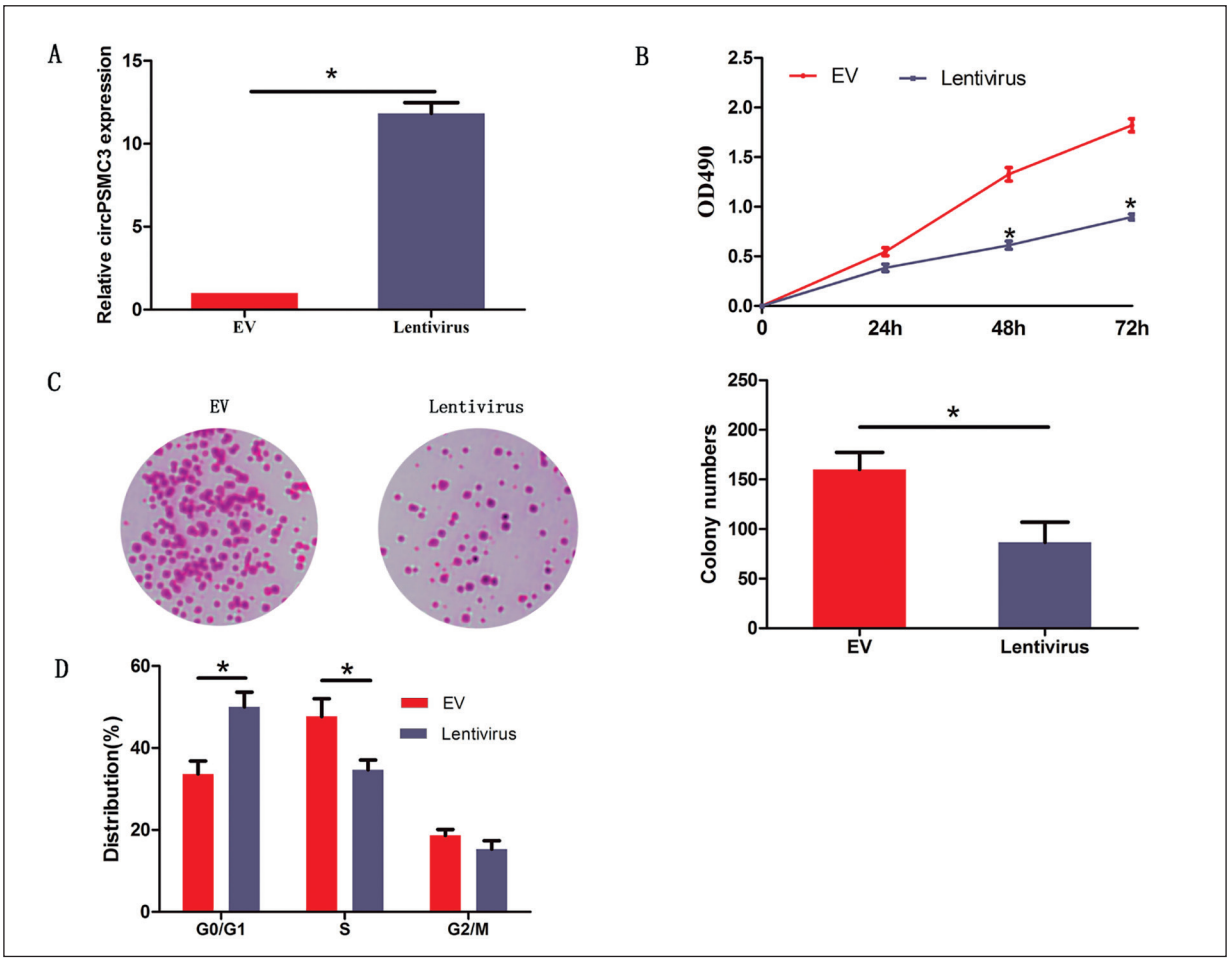

Figure 3. Overexpression of circPSMC3 inhibited PCa cell proliferation and regulated cell cycle. **A,** CircPSMC3 expression in PCa cells transfected with circPSMC3 lentivirus and EV were detected by RT-qPCR, respectively. **B,** MTT assay showed that overexpression of circPSMC3 significantly repressed the viability of PCa cells. **C,** Colony formation assay showed that the number of colonies was significantly reduced *via* overexpression of circPSMC3 in PCa cells (magnification 40 X). **D,** Cell cycle assay showed that the percentage of cells in G0/G1 cells increased remarkably after circPSMC3 was overexpressed in PCa cells. The results represented the average of three independent experiments (mean ± standard error of the mean). *p<0.05, as compared with control cells.

vector (EV), respectively. The results showed that circPSMC3 overexpression significantly decreased the mRNA expression of DGCR8 (Figure 4A). The protein level of DGCR8 was measured through Western blot assay. CircPSMC3 overexpression markedly reduced the protein level of DGCR8 *in vitro* (Figure 4B). Meanwhile, RT-qPCR was used to detect DGCR8 expression in PCa tissues as well. The results demonstrated that DGCR8 was highly-expressed in PCa tissues whn compared with corresponding normal tissues (Figure 4C). Furthermore, correlation analysis indicated that DGCR8 expression was negatively correlated with circPSMC3 expression in PCa tissues (Figure 4D).

To detect the function of circPSMC3 *in vivo*, tumor formation assay was conducted in NOD/SCID mice. Tumor size in circPSMC3 lentivirus group was less than that in EV group (Figure 4E). Four weeks later, tumor tissues were extracted

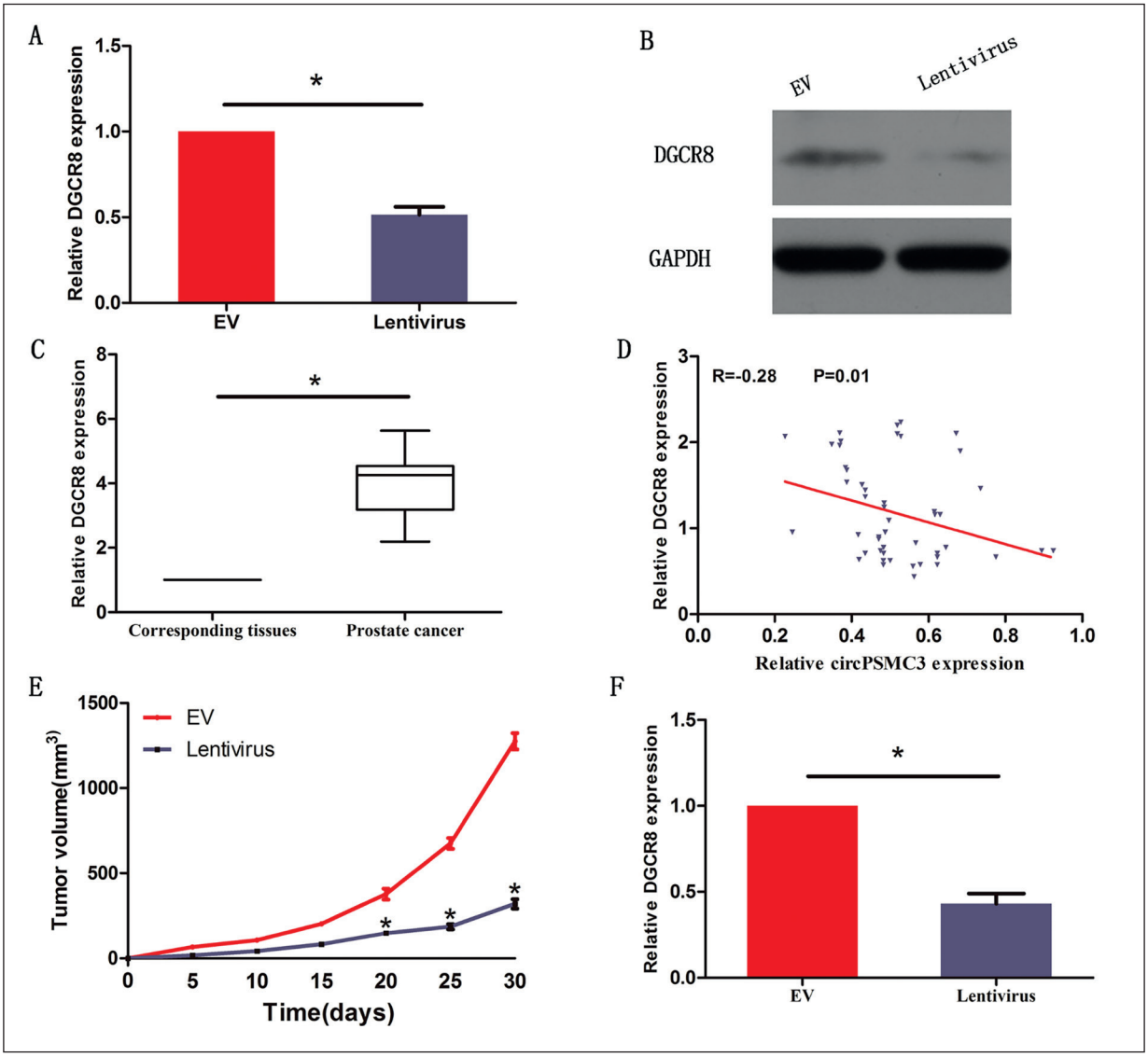

Figure 4. CircPSMC3 overexpression inhibited DGCR8 expression in PCa. **A,** RT-qPCR results showed that DGCR8 expression was significantly up-regulated in circPSMC3 lentivirus group compared with EV group. **B,** Western blot results showed that protein expression of DGCR8 was down-regulated in circPSMC3 lentivirus group compared with EV group. **C,** DGCR8 expression was significantly elevated in PCa tissues compared with adjacent normal tissues. **D,** The linear correlation between the expression level of DGCR8 and circPSMC3 in PCa tissues. **E,** Tumor size in circPSMC3 lentivirus group and EV group. F, DGCR8 expression in dissected tumors in circPSMC3 lentivirus group and EV group. The results represented the average of three independent experiments. Data were presented as mean ± standard error of the mean. *p<0.05.

from treated mice, and DGCR8 expression in tumor tissues was detected by RT-qPCR. As a result, DGCR8 was lowly-expressed in circPSMC3 lentivirus group when compared with EV group (Figure 4F).

Discussion

Several reports have identified that circular RNAs are dysregulated in PCa and participate in its process of development. Circular RNAs regulate the carcinogenesis of PCa by acting as sponges for microRNAs. Responding to androgen, circSMARCA5 is overexpressed in PCa and facilitates the proliferation of PCa cells[12]. By regulating the expression of miR-29a, circMYLK functions as an oncogene and promotes the progression of PCa[13]. The upregulation of circ-102004 enhances the proliferation of prostate cancer cells. Meanwhile, it may be a potential biomarker of PCa as well[14]. As a novel circRNA, circPSMC3 has recently been reported to function as a tumor suppressor in gastric cancer by serving as a competitive endogenous RNA of miR-296-5p[15]. Our results showed that circPSMC3 was downregulated in PCa tissues and cell lines. Low circPSMC3 expression was associated with poor disease-free survival of PCa patients, indicating that circPSMC3 acted as a tumor suppressor in PCa.

To determine the function of circPSMC3 in PCa proliferation, circPSMC3 lentivirus was used for transfection in PCa cells. Functional assays showed that circPSMC3 overexpression significantly repressed the proliferation of PCa cells. The effect of circPSMC3 on cell cycle of PCa cells was investigated as well. The results showed that circPSMC3 overexpression contributed to the arrest at the G0/G1 stage in PCa cells. All the above findings indicated that circPSMC3 inhibited the proliferation and regulated cell cycle of PCa. Next, the related proteins of circPSMC3 were further explored. Encoded by the *DiGeorge syndrome critical region gene 8,* RNA binding protein DGCR8 is a critical protein for microRNA (miRNA) biogenesis[16]. Knockdown of DGCR8 inhibits proliferation, cell migration, and invasion of ovarian cancer cells[17]. In addition, DGCR8 functions as a tumor suppressor in PCa and inhibits its progression[18]. In our work, the potential interaction between DGCR8 and circPSMC3 was first researched in our study. The results showed that circPSMC3 overexpression markedly decreased DGCR8 expression *in vitro*. Meanwhile, circPSMC3 expression was negatively correlated with dgcr8 expression in PCa tissues. Through *in vivo* experiments, we found that circPSMC3 overexpression decreased tumor formation and downregulated DGCR8 in nude mice. The above results indicated that circPSMC3 might inhibit the growth of PCa by downregulating DGCR8.

Conclusions

These findings revealed that circPSMC3 was remarkably downregulated in PCa tissues and indicated poor prognosis of PCa patients. Meanwhile, circPSMC3 inhibited cell proliferation and regulated the cell cycle of PCa by down-regulating DGCR8. All our results suggested that circPSMC3 might contribute to PCa therapy as a prospective target.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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