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Circ_0004417 inhibits the progression of prostate cancer through sponging miR-1228

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Abstract. – OBJECTIVE: Circular RNAs (circRNAs) have been proved to play a vital role in tumorigenesis and progression. Nevertheless, the potential mechanism of circRNAs in prostate cancer (PC) remains unclear. In the present study, we aimed to investigate the exact role of circ_0004417 in the progression of prostate cancer.

PATIENTS AND METHODS: Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the expression of circ_0004417 in primary PC tissues and cell lines. In vitro experiments were conducted to explore the function of circ_0004417 in PC progression, including cell counting kit-8 (CCK-8) assay, colony formation assay and transwell assay. Furthermore, the regulatory function of circ_0004417 on miRNA, p-Akt and E-cadherin was investigated to elucidate the potential mechanisms.

RESULTS: Circ_0004417 was significantly down-regulated in PC tissues and cells ($p<0.05$). Functional experiments proved that circ_0004417 overexpression markedly inhibited the proliferation and invasion of PC cells ($p<0.05$). In addition, the results demonstrated that circ_0004417 served as a sponge for miR-1228 and regulated expressions of p-Akt and E-cadherin.

CONCLUSIONS: Circ_0004417 inhibits the progression of prostate cancer through sponging miR-1228. All our findings suggest that circ_0004417 can be used as a potential therapeutic target for PC.

Key Words:
Circ_0004417, Proliferation, Metastasis, MiR-1228, Prostate cancer (PC).

Introduction

As one of the most malignant tumors in the genitourinary system, the incidence of prostate cancer (PC) ranks first among all male malignant tumors[1]. The increasing annual incidence of PC is strongly linked to the ever-increasing life expectancy, demographic ageing, changes in diet structure and unceasing advancements of diagnostic techniques[2,3]. Mid-advanced PC poses a huge threat to men's health and life worldwide. After endocrine therapy, most patients ultimately become anti-cast cut PC (CRPC) that is insensitive to radiation therapy and chemotherapy. Currently, the worldwide problem is that the efficacy of various therapies is far from satisfactory[4,5]. Therefore, it is of great importance to find out the biological molecular mechanism of PC progression and offer targeted therapy with theoretical basis. Therefore, we hope to reduce the incidence of PC and alleviate the prognosis of PC patients.

CircRNA (Circular RNA), a novel kind of non-coding RNA, is abundant in eukaryotic cells. It has a highly conserved sequence, stable structure, and tissue and cell specificity[6]. The function of circRNAs has been gradually revealed in recent years. For example, circCDR1 regulates EGFR expression by adsorbing microRNA-7 in non-small-cell lung cancer[7]. Circ_SLC19A1 promotes the proliferation and metastasis of PC by modulating the microRNA-497/septin 2 pathway[8]. A growing body of evidence suggests that circRNAs are expected to become new diagnostic markers and even therapeutic targets for malignant tumors due to the reason that they are associated with the initiation and progression of malignancies[9-11]. But to date, we still have trouble fully understanding the expression characteristics and biological mechanisms of circRNAs in PC. Therefore, it is of scientific significance and vital clinical value to investigate the roles of circRNAs in PC.

Circ_0004417 is located on chr1:219366423-219414650 and homologous to the RNA sequence

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of LYPLAL1, one of protein-coding genes[12]. Currently, the biological function of circ_0004417 has not been fully elucidated. Therefore, in this study, we aimed to discover the function of circ_0004417 on the growth and invasion of PC cells by detecting the expression levels of circ_0004417 in PC tissues and cell lines, and to further elucidate the possible underlying mechanism. Explaining the pathogenesis of circ_0004417 on PC may provide effective and precise treatment goal.

Patients and Methods

Tissues

From May 2018 to May 2019, 44 pairs of PC tissues and adjacent normal tissues were collected from patients who received treatment in the Urology Department of Nanjing First Hospital, Nanjing Medical University. The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). This study was approved by the Ethics Committee of Nanjing First Hospital, Nanjing Medical University. Informed consents were obtained from patients before the study. Patients who had received radio-chemotherapy or hormone therapy before surgery were excluded. Paired PC tissues and adjacent normal tissues were taken out promptly after the operation and stored in liquid nitrogen for subsequent use.

Cell Culture

Human normal prostate epithelial cell line RWPE-1 and three PC cell lines (including DU-145, VCaP and PC-3) were purchased from the Typical Culture Collection Center of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA) in an incubator with humid atmosphere and 5% CO_2 at 37°C.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNAs were extracted from PC tissues and cells according to the instructions of RNeasy Protect Mini Kit (Qiagen, Hilden, Germany). For circ_0004419 detection, the linear RNA was digested by using RNase R (Epicentre, Madison, WI, USA), while for miR-1228 detection, the linear RNA was not digested. Subsequently, extracted RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) by the PrimeScript RT reagent (TaKaRa, Otsu, Shiga, Japan). Afterward, PCR was performed on an ABI 7900 fast real-time PCR system (ABI, Foster City, CA, USA). The relative expression levels of RNA were quantified by the $2^{-\Delta\Delta CT}$ method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal reference for circ_0004417, p-Akt and E-cadherin, while U6 was used as the internal reference for miR-1228. All the primers used in this study were as follows: circ_0004417 F: GGAGAAGCCATCCAACCT; R: CTGGGCAGTCATTGGTTA; miR-1228 F: AGTGGGCGGGGGCAGG; R: AGTGCAGGGTCCGAGGTATT; GAPDH F: GGAGCGAGATCCCTCCAAAAT; R: GGCTGTTGTCATACTTCTCATGG; U6 F: CTCGCTTCGGCAGCACATATACTA; R: ACGAATTTGCGTGTCATCCTTGC.

Cell Transfection

Circ_0004417 overexpression and negative control plasmids were synthesized by Ribobio (Guangzhou, China). PC cells transfected with circ_0004417 overexpression plasmid were named as OE-Circ group, while those transfected with negative control plasmid were named as OE-NC group. Cell transfection was performed according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 hours, the expression level of circ_0004417 was detected by qRT-PCR. Similarly, 48 hours after transfection of miR-1228 mimics and mimics NC in PC cells, the expression of miR-1228 was detected by qRT-PCR as well.

Cell Counting Kit-8 (CCK-8) Assay

At 0, 24, 48 and 72 hours after transfection, the growth rate of cells was determined in strict accordance with CCK-8 kit (Dojindo Molecular Technologies, Kumamoto, Japan). Optical density (OD) value was detected by a micro-plate reader.

Colony Formation

Transfected PC cells were first seeded into 6-well plates (Corning, Corning, NY, USA) at a density of 1000 cells per well. After 10 days of culture, the plates were washed twice with phosphate-buffered saline (PBS) (HyClone, South Logan, UT, USA), stained with crystal violet solution (Beyotime, Shanghai, China) and photographed. Colonies with over than 50 cells were observed, and the number of formed colonies was finally counted.

Invasion Assay

Invasion assay was performed according to the manufacturer's instructions of the transwell chamber kit (Millipore, Billerica, MA, USA). Briefly, 2×10^4 cells were seeded in an 8 mm pore size transwell insert coated with extracellular matrix (BD Biosciences, Franklin Lakes, NJ, USA). After incubation at 37°C for 36 hours, a cotton swab was used to remove the attachment to the filter. Cells on the upper surface were then stained with crystal violet (Beyotime, Shanghai, China). Finally, the number of invasive cells was counted.

Luciferase Reporter Gene Assay

Circ_0004417-wt or circ_0004417-mut was first cloned into psiCHECK-2 vector (Promega, Madison, WI, USA). Next, PC-3 and DU-145 cells were co-transfected with recombinant vector (circ_0004417-wt or circ_0004417-mut) and miR-1228 mimics or mimics NC. Luciferase activity was detected through the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) after 48 hours.

Western Blot

Total protein was first extracted from transfected cells. Protein samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Beyotime, Shanghai, China) and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). After blocking with skimmed milk, the membranes were incubated with specific primary and secondary antibodies. Immuno-reactive bands were finally exposed by enhanced chemiluminescence (ECL) kit (Millipore, Billerica, MA, USA). GAPDH was used as an internal reference. Goat anti-p-Akt, E-cadherin (1:1000) were purchased from Cell Signaling Technology (no.4060; no.3195) (Danvers, MA, USA).

Statistical Analysis

GraphPad Prism 8.0 (GraphPad Software, CA, USA) was used for all statistical analysis. Experimental data were presented as means ± standard deviation (SD). Unpaired Student's *t*-test was used to compare the differences between two groups. $p<0.05$ was considered statistically significant.

Results

Circ_0004417 Was Lowly Expressed in PC Tissues and Cells

The expression level of circ_0004417 in PC tissues and adjacent normal tissues was detected by qRT-PCR. The results showed that the expression of circ_0004417 in PC tissues was markedly lower than that in adjacent normal tissues ($p<0.05$, Figure 1A). Meanwhile, circ_0004417 was lowly expressed in PC cells as well ($p<0.05$, Figure 1B). By collecting the clinical characteristics of

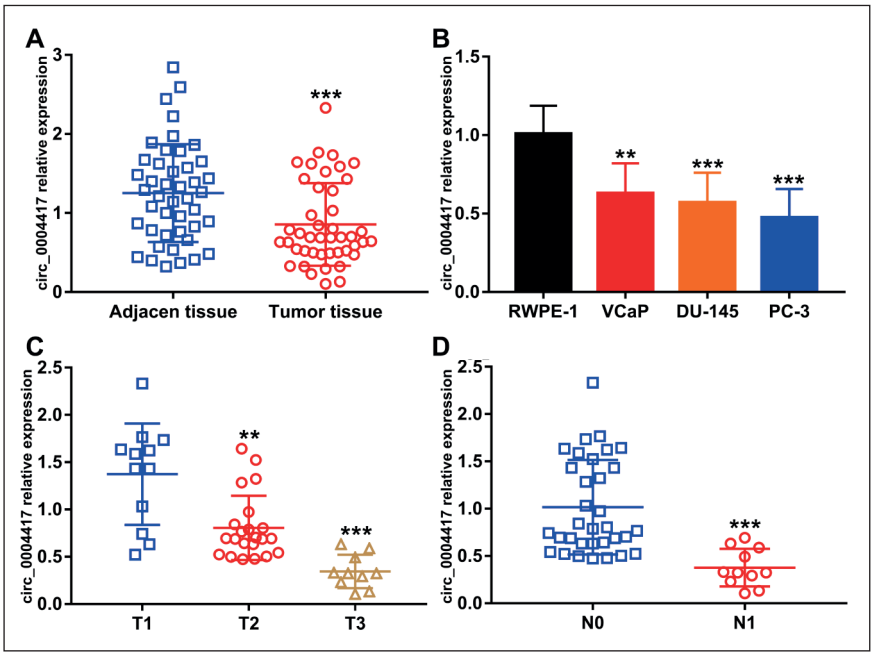

Figure 1. Circ_0004417 was down-regulated in PC tissues and cells. **A,** Circ_0004417 expression in PC tissues was detected by qRT-PCR. **B,** Circ_0004417 expression in PC cell lines and RWPE-1 cells. **C,** The relationship between circ_0004417 expression and T stage. **D,** The relationship between circ_0004417 expression and N stage. Data are represented as mean ± SD. (**$p<0.01$; ***$p<0.001$).

44 patients, we found that the expression level of circ_0004417 decreased significantly with the increase of primary tumor volume (Figure 1C). In addition, the expression level of circ_0004417 in PC patients with lymph node metastasis also decreased significantly (Figure 1D). The above results revealed that circ_0004417 might be closely associated with the proliferation and metastasis of PC.

Circ_0004417 Inhibited PC Cell Growth and Invasion

PC-3 and DU-145 cells were selected for further cellular function experiments. After transfection, circ_0004417 expression in cells of OE-Circ group was markedly higher than that in OE-NC group ($p<0.05$, Figure 2A). CCK-8 results demonstrated that the proliferation rate of cells in OE-Circ group was remarkably lower than that in OE-NC group at 48 and 72 hours after transfection ($p<0.05$, Figure 2B). As shown in Figure 2C, colony formation experiments proved that the colony formation ability of cells in OE-Circ group was remarkably weaker than that of OE-NC group ($p<0.05$). 24 hours after transfection, the number of invasive cells in OE-Circ group was significantly reduced when compared with OE-NC group ($p<0.05$, Figure 2D).

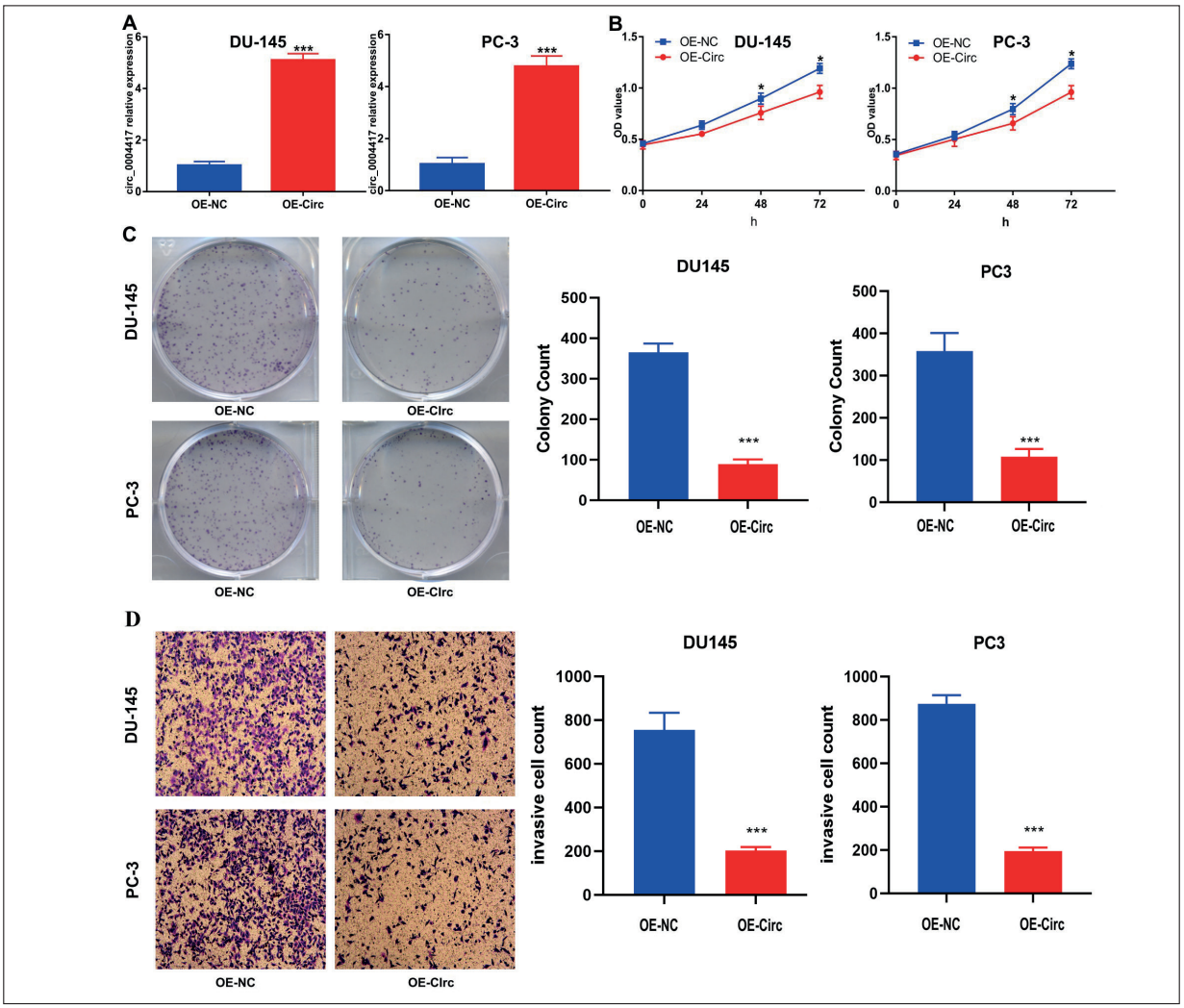

Figure 2. Circ_0004417 inhibited PC cell proliferation. **A,** DU-145 and PC-3 cells were transfected with OE-NC or OE-Circ, and circ_0004417 expression was detected by qRT-PCR. **B,** CCK-8 assay indicated significant differences in the proliferation of PC cells between OE-NC group and OE-Circ group at 48 h and 72 h. **C,** Colony forming assay also revealed significant differences in the proliferation of PC cells between OE-NC group and OE-Circ group. **D,** Transwell assay verified significant differences in the invasion of PC cells between OE-NC group and OE-Circ group. (magnification: 100×) Data are represented as mean ± SD. (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

In summary, overexpression of circ_0004417 inhibited PC cell proliferation and invasion *in vitro*.

Circ_0004417 Might Sponge MiR-1228

The above findings suggested that circ_0004417 could suppress the growth and invasion abilities of PC cells. However, the biological molecular mechanism of such phenomenon remained to be further explored. In this study, the binding sequences between circ_0004417 and miR-1228 were predicted by TargetScan[13] (Figure 3A). To verify whether circ_0004417 could regulate miR-1228, Luciferase reporter gene plasmids were constructed, including wild-type (wt) or mutant-type (mut). Subsequent results indicated that the Luciferase activity of PC cells in wt group was notably weaker than that of mut group ($p<0.05$, Figure 3B). To further explore the biological function of miR-1228, qRT-PCR was performed to detect the expression levels of miR-1228 in PC tissues and cells. Compared with adjacent normal tissues, the expression level of miR-1228 increased significantly in PC tissues ($p<0.05$, Figure 3C). Likewise, miR-1228 was highly expressed in PC cells as well ($p<0.05$, Figure 3D). The expression level of miR-1228 showed a negative correlation with the expression level of circ_0004417 in PC tissues (Figure 3E). With the increase of circ_0004417 expression, the expression levels of miR-1228 decreased notably in PC cells ($p<0.05$, Figure3F). It could be inferred that miR-1228 might be a downstream target of circ_0004417.

Circ_0004417 Sponged MiR-1228 and Regulated p-Akt and E-cadherin Expression

Our results showed that circ_0004417 could suppress the growth and invasion of PC cells by sponging miR-1228. We also found that after

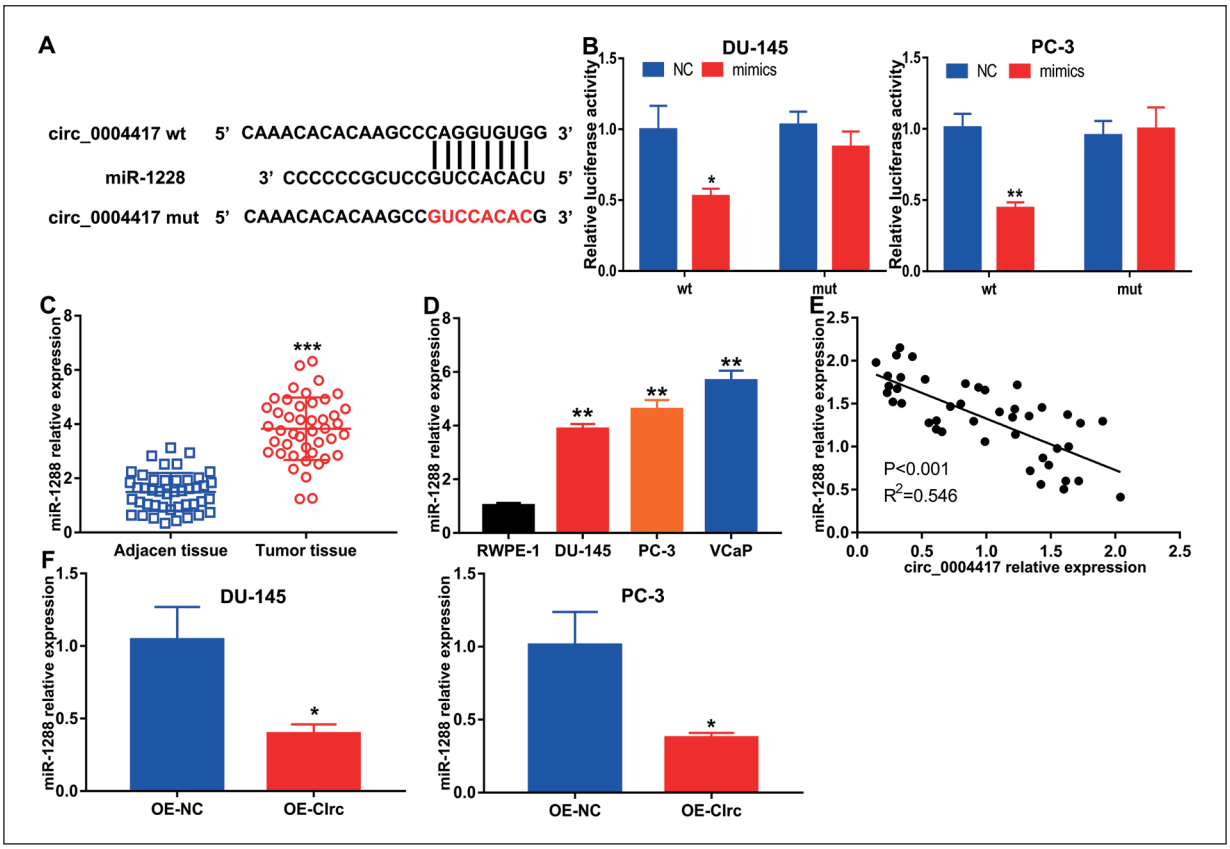

Figure 3. Circ_0004417 might sponge miR-1228. **A,** MiR-1228 might be a potential target of circ_0004417. **B,** Luciferase reporter gene assay was conducted to verify that miR-1228 directly bound to the 3'-UTR sequences of circ_0004417 in PC cells. **C,** MiR-1228 expression in PC tissues and adjacent normal tissues was detected by qRT-PCR. **D,** MiR-1228 expression in PC cells and RWPE-1 cells was detected by qRT-PCR. **E,** A negative correlation was found between the expressions of miR-323-3p and circ_0004417 in PC tissues. **F,** MiR-1228 expression in OE-NC group and OE-Circ group was detected by qRT-PCR. Data are expressed as mean ± SD. (*$p<0.05$; **$p<0.01$).

overexpression of circ00044417 in DU-145 and PC-3 cells, the expression of p-Akt and E-cadherin increased remarkably (p<0.05, Figure 4A and 4B). After overexpression of miR-1228 (Figure 4C), the expression of p-Akt and E-cadherin was significantly reduced (p<0.05, Figure 4D and 4E). The above data emphasized that hsa_circ_0004417 could sponge miR-1228 to modulate the expression of p-Akt and E-cadherin.

Discussion

PC is a common tumor of the genitourinary system, whose incidence rate is increasing in males[14]. In recent years, there have been multiple treatment options for PC, including radical prostatectomy, radiotherapy and endocrine therapy. However, the rates of relapse remain high in PC patients, with poor prognosis[1].

CircRNAs have been found to play a key role in gene regulation, such as the adsorption of miRNA as miRNA sponge, the regulation of related gene and protein expression, etc[15]. As a new regulatory endogenous RNA, circRNA has attracted increasingly more attention in the progression of malignant tumors. Many circRNAs have been reported to be abnormally expressed in different malignancies[16,17]. However, the evidence about the role of circRNAs in the pathogenesis of PC is preliminary but promising. Recent studies have indicated that circ_KATNAL1 regulates the proliferation and metastasis of PC through miR-145-3p/WISP1 pathway[11]. Circ_0005276 promotes the growth and migration of PC cells by interacting with FUS to activate XIAP[18]. These findings mentioned above suggest that some circRNAs may play vital regulatory roles in the development of PC.

The epithelial-mesenchymal transition (EMT) is an important cellular mechanism, which play

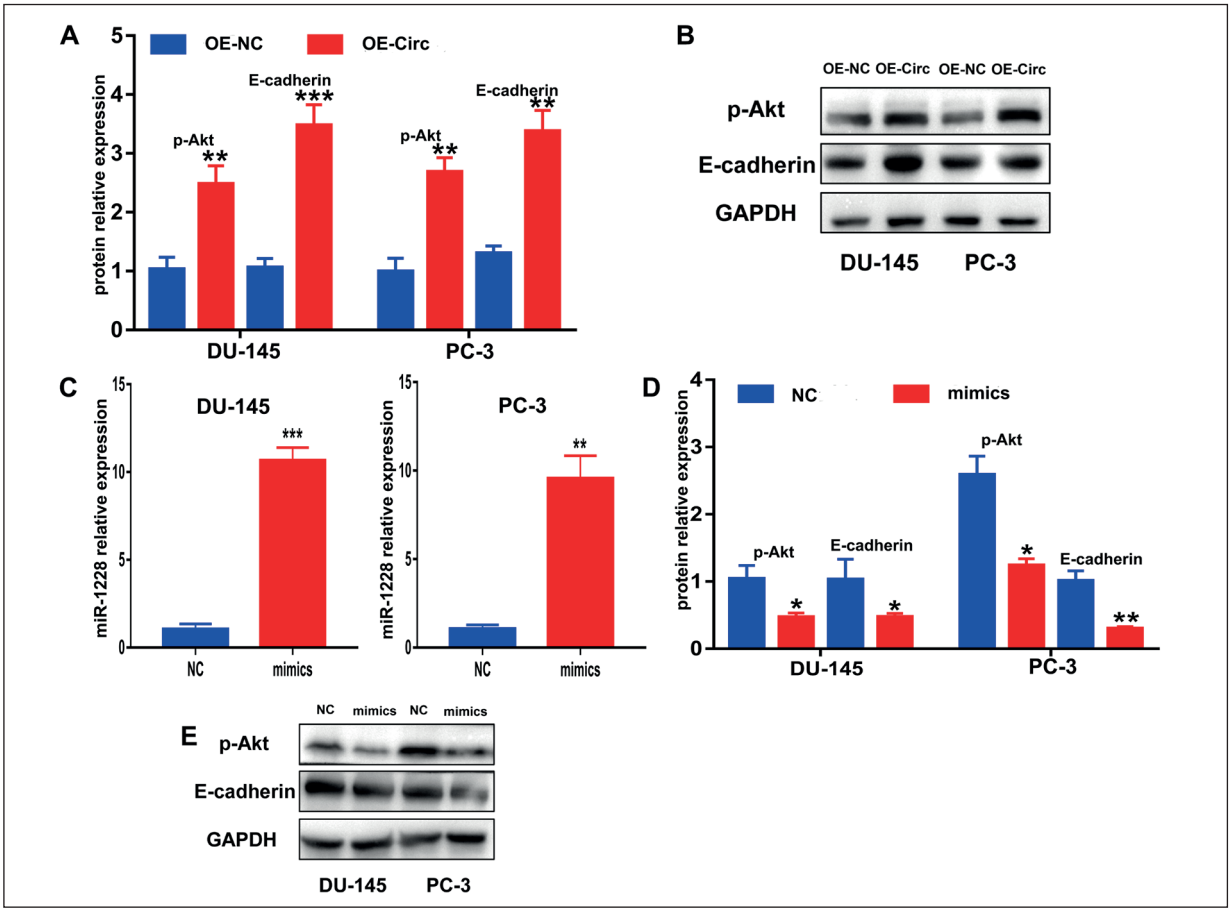

Figure 4. Circ_0004417 regulated PC cell proliferation and invasion by regulating miR-1228/ p-Akt/E-cadherin axis. **A, B,** The expression of p-Akt and E-cadherin was verified by qRT-PCR and Western blot in OE-NC group and OE-Circ group. **C,** MiR-1228 expression in PC cells transfected with NC or mimics was verified by qRT-PCR. **D, E,** The expression of p-Akt and E-cadherin in NC group and mimics group was verified by qRT-PCR and Western blot. Data are expressed as mean ± SD. (*p<0.05; **p<0.01).

a crucial role in embryonic development, tissue repair, and many diseases. Cells could gain a higher migrative and invasive abilities *via* EMT. Meanwhile, circRNAs play an important role in the regulation of the EMT. In this research, circ_0004417 was shown to significantly inhibit PC cell proliferation and invasion. In addition, circ_0004417 antagonized the promotion of miR-1228 on PC cell growth. The inhibitory effect of circ_0004417 vector on p-Akt and E-cadherin expression attenuated the above effects. Existing studies have shown that miR-1228 promotes the growth and invasion of various cancer cells, including hepatocellular carcinoma, breast cancer, gastric cancer, lung cancer and ovarian cancer[19-24]. At the same time, miR-1228 is stable in the peripheral blood of cancer patients and has the potential to become a tumor marker[25-31]. In this study, miR-1228 was highly expressed in PC tissues and cells, which could promote E-cadherin expression and Akt phosphorylation. In summary, these findings support the following view that circ_0004417 plays a vital regulatory role in the occurrence and progression of CRC and may achieve the purpose of regulating PC cell proliferation and invasion through sponging miR-1228.

Of course, there were some limitations in this investigation. First, due to limited laboratory conditions, circ_0004417 treatment was not performed *in vivo*. In addition, besides miR-1228, we failed to elucidate whether circ_0004417 regulated other miRNAs. In the future, we will further study the above two issues to clarify the molecular mechanism of circ_0004417 in regulating PC proliferation and invasion. Furthermore, the number of clinical samples was so small that we could not fully assess the sensitivity and specificity of circRNA_0004417 in predicting the prognosis of PC patients. Hence, in the following studies, more tissue samples should be collected after surgery to identify the biological function of circRNA_0004417.

Conclusions

Circ_0004417 expression was significantly down-regulated in PC. Overexpression of circ_0004417 suppressed the growth and invasion of PC cells through regulating miR-1228/p-Akt/E-cadherin axis. The novelty of this study was that circ_000417 could be used as a promising biomarker for the treatment of PC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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