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ORIGINAL ARTICLE

Role of miR-489 in the proliferation and apoptosis of pancreatic carcinoma

Maolan Zeng[1], Yanhua Zhou[2], Yinxia Zhang[3], Tiancheng Wang[4], Jing Wang[5]

[1]Department of Nursing, Northwest Minzu University, Lanzhou 730030, China; [2]Department of Hemodialysis, Lanzhou Second People's Hospital, Lanzhou 730046, China; [3]Department of Health, Northwest Minzu University, Lanzhou 730030, China. [4]Department of Epilepsy, Lanzhou University Second Hospital, Lanzhou 730030, China; [5]Department of Cardiology, Gansu Baoshihua Hospital, Lanzhou 730060, China.

Summary

Purpose: *The purpose of the present study was to detect the expression of miR-489 in pancreatic cancer (PC) tissues and cells, and to explore the effects of miR-489 on cell proliferation and apoptosis of human PC cells and to also uncover the underlying mechanism.*

Methods: *miR-489 expression was assessed by quantitative real time-polymerase chain reaction (qRT-PCR) in PC tissues and PANC-1 and HPDE6-C7 cell lines. The binding-site predictions by bioinformatics showed that AKT Serine/Threonine Kinase 3 (AKT3) might be a potential target of miR-489. AKT3 expression in PC tissues and cells was detected by qRT-PCR, luciferase report assay and Western blotting assay were used to verify the rationality of the target gene. The biological role of miR-489 on cell proliferation, cell cycle and apoptosis were determined in PANC-1 cells by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay and flow cytometry after transfection with miR-NC, miR-489 mimics and si-AKT3.*

Results: *Compared with normal adjacent tissues and normal pancreatic cells, the expression of miR-489 was markedly down-regulated in PC tissues and cells. AKT3 was considered as a downstream gene of miR-489 and it was found that the expression levels of miR-489 and AKT3 were inversely proportional to each other, which was further confirmed by luciferase and Western blot assays. In subsequent experiments, up-regulation of miR-489 by transfection with miR-489 mimics significantly inhibited cell proliferation, blocked the G1/S transition and induced cell apoptosis of PANC-1 cells. However, overexpression of AKT3 significantly counteracted the biological effects of miR-489.*

Conclusions: *Our findings indicate that up-regulation of miR-489 could suppress PC cell proliferation and facilitate cell apoptosis through targeting AKT3. miR-489 and AKT3 might serve as potential targets in the therapy of PC.*

Key words: *pancreatic cancer, mir-489, AKT serine/threonine kinase 3, proliferation, apoptosis*

Introduction

Pancreatic cancer (PC) is one of the most common solid tumors of the digestive system. It is usually a high-grade malignancy with vague clinical features in the early stage. However, the progress of the disease is rapid, the treatment is not effective and the prognosis remains particularly poor. The incidence and mortality of PC have been increasing each year, and the 5-year overall survival rate of PC is extremely low, only less than 5% [1,2]. The onset of PC is hidden, and there is no specific diagnostic marker for PC in the early stage. In addition, PC is prone to local infiltration and distant metastasis in the early stage. Therefore, it is particularly important to study the molecular mechanism of the occurrence and development of PC.

MiRNAs are a kind of small non-coding RNA with approximately 22 nucleotides in length, that can specifically bind to the 3'-untranslated region

Corresponding author: Jing Wang, MM. Department of Cardiology, Gansu Baoshihua Hospital, No.733 Fuli West Rd, Xi Gu District, Lanzhou, Gansu, 730060, China.
Tel: +86 013669380068, Email: 2820692415@qq.com
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(3'UTR) sequence of mRNA and regulate the transcription of downstream target gene, eventually cancelling the biological functions of target gene [3,4]. There are thousands of miRNAs in the human genome, and about 33% of protein-coding genes in humans are regulated by miRNAs. Each miRNA could directly regulate about 200 target genes, and play an important role in a variety of pathophysiological processes, such as cell proliferation, differentiation, migration, invasion and apoptosis [3, 5, 6]. Therefore, miRNAs hold an extremely important position in human gene expression.

With the deepening of studies related to miRNAs in recent years, their roles in the occurrence and development of PC has been increasingly prominent. A number of studies focused on the abnormal expression (up-regulation or down-regulation) of miRNAs in PC, and many miRNAs were found to play regulatory roles as tumor suppressors or oncogenes in the development of PC, such as miR-34 [7], miR-146a [8], miR-216a [9] and miR-137 [10].

MiR-489 is a miRNA attracting much attention, and studies have proved that the miR-489 level in tumor tissues significantly declined when compared with that in corresponding normal tissues, including hypopharyngeal squamous cell carcinoma (HSCC) [11], non-small cell lung cancer (NSCLC) [12], gastric cancer [13] and bladder cancer [14]. However, miR-489 is less reported in the occurrence and development of PC. The present study focused on the role of miR-489 in PC through detecting its expression level in PC tissues and cell lines combined with *in vitro* experiments, so as to provide new thoughts for the research on PC at the molecular biological level.

Methods

Clinical samples and cell lines

Carcinoma and para-carcinoma tissue specimens (more than 5 cm away from the edge of carcinoma tissue) were collected from 40 patients pathologically diagnosed with PC after operation in our hospital from March 2014 to September 2017. All specimens were cryopreserved in liquid nitrogen, and no patient had undergone preoperative radiotherapy and chemotherapy. Signed informed consent was obtained from patients, and the study was approved by the Ethics Committee.

PC cell line (PANC-1) together with normal pancreatic cell line (HPDE6-C7) were purchased from the Institute of Biochemistry and Cell Biology (Beijing, China). All cells were cultured in the RPMI-1640 medium containing 10% fetal bovine serum (FBS) and placed in incubatot with 5% CO_2 at 37°C. The medium was replaced once every 1-2 days, and cells in the logarithmic growth phase were taken for subsequent experiments.

Target gene prediction

The target gene of miR-489 was predicted using the bioinformatics prediction software Targetscan and miRanda, and appropriate target genes were selected combined with differentially-expressed proteins.

Dual luciferase activity assay

PANC-1 cells were inoculated into a 24-well plate and cultured for 24 h. Cells were co-transfected with wild-type AKT3 mRNA 3'UTR (pGL3-AKT3-3' UTR-WT) and mutant-type AKT3 mRNA 3'UTR (pGL3-AKT3-3'UTR-MUT) recombinant luciferase reporter plasmids and miR-489 mimics and scrambled control using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), respectively. After 48 h, the luciferase activity was detected via dual-luciferase reporter system.

Transfection

PANC-1 cells in the logarithmic growth phase were obtained and inoculated into a 6-well plate (3×10^5/well). When the confluence reached 70-80%, transfection was performed according to instructions of the Lipofectamine 2000 transfection reagent. Cells were divided into 3 groups in the experiment: NC group (negative control), miR-489 mimics group (cells transfected with miR-489 mimics) and mimics + AKT3 group (cells transfected with miR-489 mimics and si-AKT3).

Quantitative real time-polymerase chain reaction (qRT-PCR)

The total RNA was extracted from cell lines and PC tissue specimens using the TRIzol kit (Invitrogen, Carlsbad, CA, USA), and reversely transcribed into complementary DNA (cDNA). The miR-489 expression level was detected using the TaqMan microRNA kit (TaKaRa, Tokyo, Japan), with U6 snRNA as internal reference. The AKT3 mRNA expression level was detected using the Power SYBR Green PCR Master Mix kit (TaKaRa, Tokyo, Japan), with glyceraldheyde 3-phosphate dehydrogenase (GAPDH) as internal reference.

Western blot

Cells were lysed using the radioimmunoprecipitation assay (RIPA) lysis solution (Beyotime, Shanghai, China), the total protein was extracted from cells in each group, and the protein concentration was determined by bicinchoninic acid (BCA) (Pierce, Rockford, IL, USA) at 48 h after transfection. Protein was taken (50 μg/well) for 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein separated via electrophoresis was transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA), sealed with sealing solution containing 5% skim milk at room temperature for 1 h, and added with the primary antibody according to instructions for reaction at 4°C overnight. After the protein was washed with tris buffered saline-tween (TBST), the corresponding secondary antibody (diluted at 1:1000) was added for reaction in the dark at room temperature for 1 h. Finally, the protein was washed again with TBST, followed by image development via electrochemiluminescence (ECL) and image acquisition (Thermo Fisher Scientific, Waltham, MA, USA).

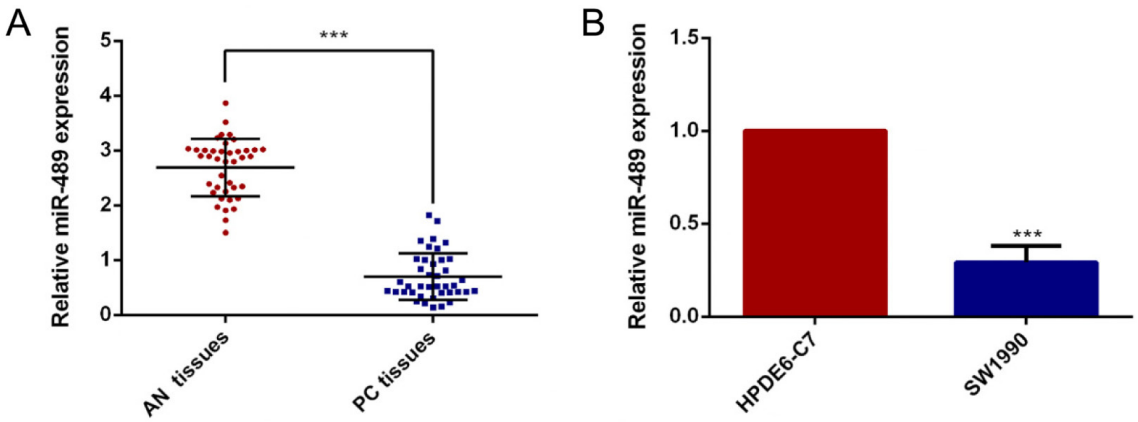

Figure 1. The expressions of miR-489 in PC tissue samples and cells. **A:** Difference in the expression of miR-489 between PC tissues and adjacent normal tissues (***p<0.001 compared with adjacent normal tissue). **B:** The expression of miR-489 in PC cell lines (PANC-1) and normal pancreatic cell lines (HPDE6-C7). (***p<0.001 compared with HPDE6-C7).

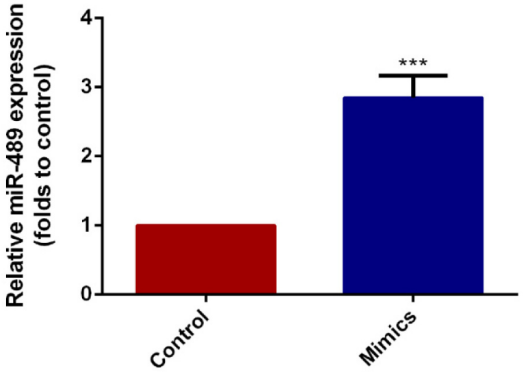

Figure 2. Transfection efficiency detection by qRT-PCR. This Figure shows that the expression of miR-489 in PANC-1 cells was clearly upregulated after transfection with miR-489 mimics (***p<0.001).

Cell proliferation

At 6 h after transfection, cells in each group were collected and inoculated into a 96-well plate (3×10^3/well). Five repeated wells were set in each group. After culture for 24, 48, 72 and 96 h, 20 μL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (5 mg/mL) (Beyotime, Shanghai, China) were added into each well for culture for another 4 h. The supernatant was discarded, and 150 μL dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) were added into each well and vibrated at room temperature for 10 min. The optical density (OD) was measured and averaged at a wavelength of 492 nm using a full-automatic quantitative microplate reader.

Cell cycle

For cell cycle analysis, PC cells were obtained 48 h after transfection. The number of PC cells in different cell phase was measured with the cell cycle staining kit (Multi Sciences Biotech Co., Ltd., Hangzhou, China) by flow cytometry. The rates of cells in G0/G1 or S phases are presented in the Results.

Cell apoptosis

According to instructions of the flow cytometry kit, PANC-1 cells in each group were collected, washed with phosphate buffered saline (PBS), digested and prepared into cell suspension with a concentration of 1×10^6/mL, followed by centrifugation. After the supernatant was discarded, cells were washed with PBS again, added with 5 μL Annexin V-FITC and 10 μL propidium iodide (PI), and gently mixed evenly, followed by incubation in the dark at room temperature for 5-15 min. Finally, the apoptosis ratio was detected using Influx Flow Cytometer & Cell Sorter System (BD, Franklin Lakes, NJ, USA).

Statistics

Statistical analysis was performed by Prism 6.02 software (La Jolla, CA, USA) with Student's t-test or F-test. All p values were two-sided and p<0.05 was considered as statistically significant. All experiments were repeated for three times.

Results

Abnormal expression of miR-489 in PC tissues and cells

The expression level of miR-489 and AKT3 in 40 PC tissues and the corresponding para-carcinoma tissues was detected via qRT-PCR. The results revealed that the expression level of miR-489 in PC tissues was significantly lower than that in the corresponding para-carcinoma tissues while AKT3 expression level was obviously higher (Figure 1A).

Consistent with tissue results, the same results were obtained from cells (Figure 1B).

Detection of miR-489 transfection efficiency

The transfection efficiency was detected by qRT-PCR assay. As shown in Figure 2, the expression of miR-489 in PANC-1 cells was clearly up-regulated

after transfection with miR-489 mimics. This result confirmed that the transfection of mimics could significantly increase the expression of miR-489.

Target gene prediction results

AKT3 was found as a target gene of miR-489, and the complementary base sequence of miR-489 and AKT3 mRNA 3'UTR was predicted by the software.

The AKT3 expression was detected by qRT-PCR in tissues and cells, and it was found that the expression levels of miR-489 and AKT3 were inversely proportional to each other. Importantly, the AKT3 expression in human PC tissues and cells was markedly up-regulated (Figure 3).

The results of dual-luciferase assay proved that miR-489 mimics could increase the luciferase activity of PANC-1 cells transfected with pGL3-AKT3-3'UTR-WT compared with scrambled control, and the luciferase activity had no significant changes in cells transfected with pGL3-AKT3-3'UTR-MUT, confirming that miR-489 exerted an inhibitory effect on AKT3 expression (Figure 4A).

Western blot results revealed that AKT3 protein expression in PANC-1 cells which were transfected with miR-489 mimics was declined compared with that in the control group, suggesting that miR-489 could inhibit the AKT3 expression (Figure 4B).

MiR-489 suppressed the cell proliferation

Results from MTT assay indicated that the absorbance of PANC-1 cells was significantly decreased and the proliferation speed was slowed

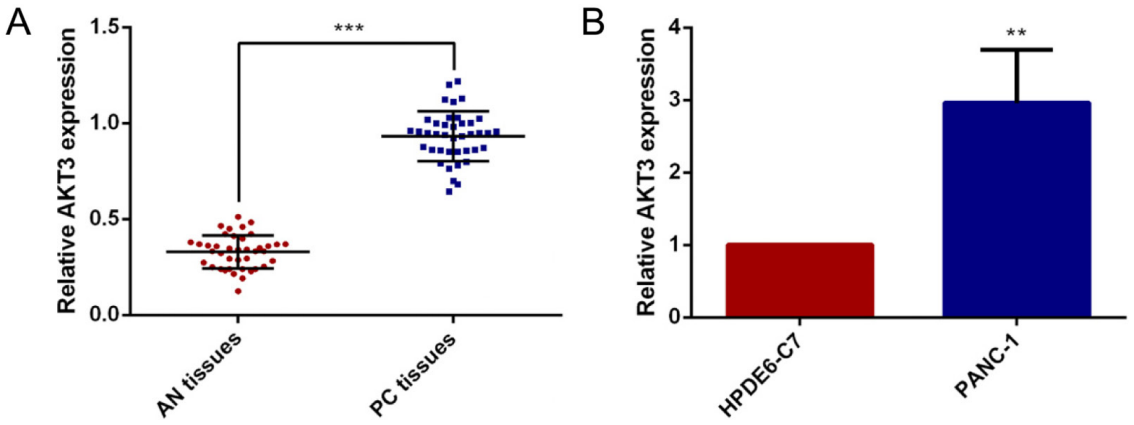

Figure 3. The expressions of AKT3 in PC tissue samples and cells. **A:** Significant difference in the expression of AKT3 between PC tissues and adjacent normal tissues was detected (***p<0.001 compared with adjacent normal tissue). **B:** The expression of AKT3 in PANC-1 cells and HPDE6-C7 cells (**p<0.01 compared with HPDE6-C7).

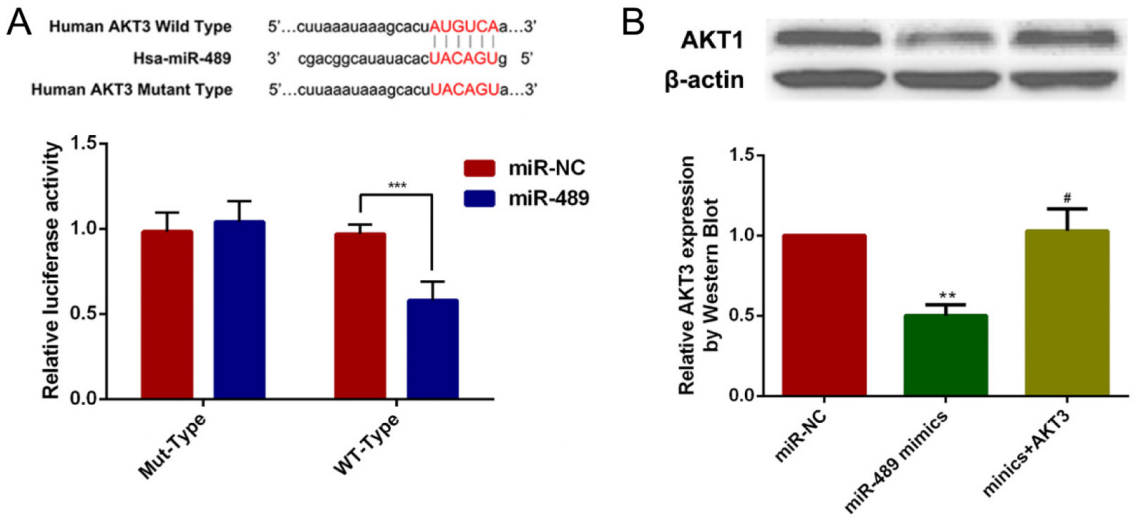

Figure 4. AKT3 is a direct and functional target of miR-489. **A:** Diagram of putative miR-489 binding sites of AKT3 and relative activities of luciferase reporters (***p<0.001). **B:** MiR-489 decreased the expression level of AKT3 detected by Western blot analysis (**p<0.01 vs. NC group; #p<0.05 vs. mimics group).

down after transfection with miR-489 mimics. Interestingly, addition of AKT3 reversed the above effects of miR-489 (Figure 5A).

For cell cycle, flow cytometry showed that after transfection with miR-489 mimics, the proportion of PC cells in G0/G1 phase was significantly increased, while that in S phase was decreased. However, after overexpression of AKT3, the number of cells in S phase was increased, and the conversion from G0/G1 phase to S phase was advanced (Figure 5B).

MiR-489 increased the apoptosis rate of PANC-1 cells

Flow cytometry analysis showed that in the miR-489 mimics group, the apoptosis rate of PANC-1 cells was significantly higher than that of the control group, as shown in Figure 6. However, the apoptosis rate of cells which co-transfection of miR-489 and AKT3 rose to the level of the control group.

Discussion

The role of miRNAs in tumors is mainly realized through the regulation of different target genes, and the major regulatory way is to promote the degradation or inhibit the translation through binding to the 3'UTR of target gene. It was found in previous studies that miR-489 could bind to the 3'UTR of HER2 oncogene in a targeted manner in breast cancer, thus inhibiting its expression and proliferation capacity of breast cancer cells [15]. In NSCLC, miR-489 could downregulate suppressor of zest 12 (SUZ12) and inhibit the proliferation and migration of tumor cells [12]. However, there have been no reports on the role of miR-489 in PC cells and its corresponding target genes. In our study, after detection of 40 cases of PC and the corresponding normal tissue specimens it was found that miR-489 was significantly downregulated in PC tissues. It was speculated, based on the above findings, that miR-489 might play a role as tumor suppressor in PC. Moreover, in our study it was also found in PC cell lines that the miR-489 expression was significantly reduced compared with that in normal pancreatic cells, which further aroused the interest in the interaction between miR-489 and PC. We believe that

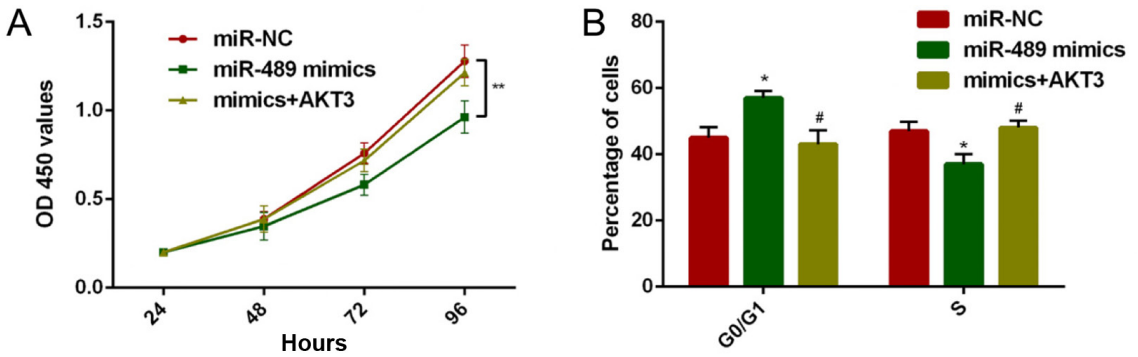

Figure 5. MiR-489 suppressed the cell proliferation and inhibited the G1/S transition of PC cells. **A:** Cell proliferation detected by MTT assay (**p<0.01). **B:** The cell cycle phases of PC cell analyzed using flow cytometry (*p<0.05 vs. NC group; #p<0.05 vs. mimics group).

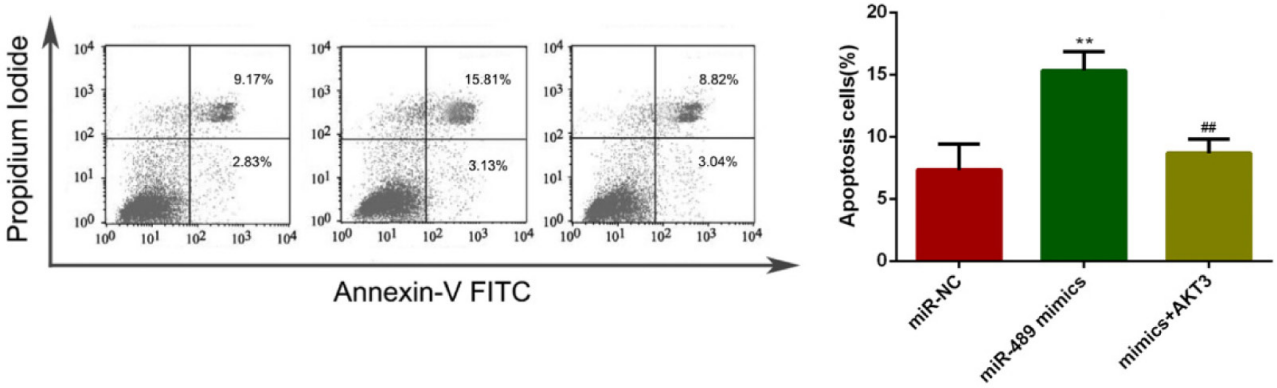

Figure 6. MiR-489/AKT3 axis promoted the apoptosis of PC cells. Apoptosis level of PANC-1 cells tested by flow cytometry. All data are presented as mean±standard deviation (**p<0.01 vs. NC group; ##p<0.01 vs. mimics group).

searching the target gene of miR-489 in playing a role in PC would help clarify its molecular mechanism. Therefore, the possible downstream target gene of miR-489 was predicted and analyzed using bioinformatics prediction software, followed by verification via the reporter gene assay, so as to deeply understand its relevant regulatory pathway and possible molecular mechanism in inhibiting PC.

As it is well known, one miRNA can regulate several target genes, and one target gene can be regulated by several miRNAs, thus forming a complex regulatory network in the occurrence and development of tumors. The role and expression level of the same miRNA may vary from tumor to tumor, and they may even be different in the same tumor in different types or stages [16-18]. The in-depth research on the molecular mechanism of one miRNA in the occurrence and development of specific tumors can provide new targets for the early diagnosis and treatment of tumors. To study the abnormally low expression of miR-489 in PC and its molecular mechanism in regulating the biological behaviors of PC, a possible target gene for miR-489, AKT3, was screened using bioinformatics combined with gene chip technique.

AKT3 is a subtype of serine/threonine protein kinase/protein kinase B (AKT/PKB) and a key point connecting multiple signaling pathways [19], which mainly acts on cells through adjusting transcription factors, regulating apoptotic proteins and controlling the metabolic mode. A large number of studies had proved that the abnormality in AKT/PKB signaling pathway could lead to excessive cell proliferation and inhibition of apoptosis, which was closely related to the occurrence and development of a variety of malignant tumors. Currently, most studies have focused on AKT1 and AKT2. AKT1 was often overexpressed in malignant tumors such as gastric cancer and lung cancer, which is related to continuous proliferation of tumor cells [20]. AKT2 mainly existed in ovarian cancer and breast cancer, which was associated with sustained survival of tumor cells [21,22]. There were few studies on AKT3. According to some studies, the AKT3 expression was increased in glioma, melanoma and ovarian cancer, and it was involved in the proliferation, apoptosis, invasion and metastasis of these three kinds of malignant tumors. Moreover, it was found that AKT3 overexpression often indicated poor prognosis of patients [23,24]. However, the role of AKT3 in PC and its mechanism is less studied, so there is a lot to be explored. Therefore, a series of experiments were designed to verify the correlation between AKT3 and miR-489 in PC and its mechanism. It was found that miR-489 could directly bind to AKT3 to inhibit its expression. To further confirm such a view, the expressions of miR-489 and AKT3 in human PC tissues were evaluated, and it was found that the expression levels of miR-489 and AKT3 were inversely proportional to each other, and the AKT3 expression in human PC tissues was up-regulated. Therefore, what effects could be obtained after down-regulation of AKT3 expression in PC cells? To verify the above hypothesis, miR-489 mimics were transfected into PANC-1 cells to reduce the AKT3 expression artificially and it was found that down-regulation of AKT3 expression in PANC-1 cells could inhibit cell proliferation and induce cell apoptosis. Then, we had another idea: what impact would be produced if the AKT3 expression was up-regulated together with transfection of miR-489? In order to realize this idea, AKT3 overexpression plasmid and miR-489 mimics were co-transfected into PANC-1 cells. The results showed that the AKT3 expression in PANC-1 cells co-transfected with AKT3 overexpression plasmid and miR-489 mimics was significantly increased compared with that in cells only transfected with miR-489 mimics. Then results of the *in vitro* experiments confirmed that the proliferation and apoptotic capacity of PANC-1 cells which were co-transfected with AKT3 overexpression plasmid and miR-489 mimics were returned to the level of the control group. The above experimental results prove that increase of AKT3 expression can reverse the effect of miR-489.

Conclusions

In conclusion, this study found that AKT3 was the direct target gene of miR-489, and it was verified that miR-489 could regulate the AKT3 expression in a targeted manner, thus getting involved in the inhibition of PC cell growth, and partially revealing the mechanism of miR-489 in exerting an anti-tumor effect and providing new possible ways for the treatment of PC. However, the mechanism of miR-489 found in current study was just one regulatory network, so further in-depth studies are needed to improve the regulatory network of PC.

Conflict of interests

The authors declare no conflict of interests.

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