European Review for Medical and Pharmacological Sciences 2019; 23: 1978-1985

Effects of miR-132 on proliferation and apoptosis of pancreatic cancer cells *via* Hedgehog signaling pathway

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Abstract. – OBJECTIVE: Micro ribonucleic acids (miRNAs) and Hedgehog (Hh) signaling pathway play key roles in the proliferation, migration and invasion of tumor cells. The aim of this study was to investigate the role of miR-132 and Hh signaling pathway in the proliferation and apoptosis of pancreatic cancer cells, and to investigate the possible underlying mechanism.

MATERIALS AND MATERIALS: The expressions of miR-132 and Shh (a ligand of Hh) in clinical pancreatic cancer specimens and pancreatic cancer cell lines were determined by qRT-PCR. Meanwhile, the correlation between the two molecules was analyzed. Pancreatic cancer cell line (MiaPaCe-2a) was transfected with miR-132 mimics and inhibitor. The effects of miR-132 up- and down-regulation on the expressions of miR-132, Shh, Cyclin-D1, cleaved Caspase-3 and cleaved Caspase-9 were detected. In addition, the exact role of miR-132 in the proliferation, apoptosis and distribution of MiaPaCe-2a cells were investigated.

RESULTS: The expression level of miR-132 in pancreatic cancer specimens and pancreatic cancer cell lines was significantly elevated when compared with that of control group. Meanwhile, miR-132 expression was negatively correlated with the expression level of Shh. Moreover, transfection with miR-132 mimics evidently up-regulated miR-132 expression. Moreover, miR-132 up-regulation significantly decreased the mRNA and protein expressions of Shh, facilitated the proliferation of MiaPaCe-2a cells, reduced the protein expressions of Cyclin-D1, cleaved Caspase-3 and cleaved Caspase-9, and suppressed cell apoptosis. On the contrary, miR-132 inhibitor transfection significantly inhibited the proliferative activity of MiaPaCe-2a cells, decreased the proportion of cells in G1 phase, and increased the proportion of cells in G2/M phase.

CONCLUSIONS: MiR-132 promotes proliferation and inhibits apoptosis of pancreatic cancer cells through Hh signaling pathway.

Key Words:
MiR-132, Hedgehog signaling pathway, Pancreatic cancer, Cell proliferation and apoptosis.

Introduction

Pancreatic cancer is a disease with strong lethality all over the world. According to relevant data, there were 36,800 new deaths from pancreatic cancer in the United States in 2010[1]. Since the 21st century, the scientific community has carried out more in-depth research on pancreatic cancer. However, no effective treatment regimens have been developed. Meanwhile, the therapeutic efficacy of traditional chemotherapy and radiotherapy is far from satisfactory. Resection is the only attempt to cure pancreatic cancer. Less than 25% of patients meet the conditions for surgery when first diagnosed. The 5-year survival rate of pancreatic cancer is only about 20%[2]. Therefore, it is of great practical significance to study the molecular pathogenesis of pancreas. Micro ribonucleic acids (miRNAs) are a type of small non-coding RNAs. They can regulate protein expression at post-transcriptional level by binding to the 3'-untranslated re-

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gion (3'-UTR) of target messenger RNAs (mRNAs)[3]. MiRNAs are differentially expressed in many solid tumors, which often create unique features for each type of malignancy[4]. Studies have proved that miRNAs are closely related to pancreatic cancer[5,6]. It is predicted that miR-132 may be closely correlated with carcinogenesis. Previous studies have indicated that the expression of miR-132 is significantly increased in lung cancer, endocrine pancreatic tumor, squamous cell carcinoma of tongue, breast cancer and colorectal cancer, whereas declined in osteosarcoma[7-11]. The different expression levels of miRNA in various cancers highlight its diverse functions in cells. Hedgehog (Hh) protein is a precursor protein automatically catalyzing, cleaving and attaching to cell surface. Hh pathway is a typical signaling pathway associated with embryonic development and differentiation. Meanwhile, it involves Hh ligands (Shh, Dhh and Ihh), membrane proteins, transcription factors (Gli1, Gli2 and Gli3) and target genes[12]. Shh, the best-studied ligand in Hh pathway, plays a vital role in ectoderm development. Abnormal activation of Shh leads to up-regulation of Myc, Ptch and Cyclin-D, thereby promoting the division of tumor cells[13,14]. Furthermore, the activation of Hh signaling pathway and sustained activation of target genes are associated with multiple malignant tumors[15-18]. Excessively activated Hh signaling pathway results in the development and progression of tumors[19]. In this study, the effects of miR-132 on proliferation and apoptosis of pancreatic cancer cells through Hh signaling pathway were explored. Moreover, we investigate the possible intrinsic mechanism of action.

Materials and Methods

Main Experimental Reagents

TaqMan miRNA Assays and SYBR® Green polymerase chain reaction (PCR) Master Mix (purchased from Applied Biosystems, Foster City, CA, USA), CelLytic™ (Sigma-Aldrich St. Louis, MO, USA), bicinchoninic acid (BCA) protein assay kits (Pierce, Rockford, IL, USA), Shh, Cyclin-D1, cleaved Caspase-3 and cleaved Caspase-9 antibodies (Abclone, Wuhan, China), miR-132 mimics and inhibitors (RiboBio, Guangzhou, China), Cell Counting Kit-8 (CCK-8) (NeoBioscience, Shenzhen, China), Annexin-V and propidium iodide (PI) (BD Biosciences, Franklin Lakes, NJ, USA).

Sample Sources and Cell Lines

A total of 76 pancreatic specimens (including 25 normal pancreatic specimens, 18 adjacent benign pancreatic specimens and 23 pancreatic adenocarcinomas) analyzed in this study were obtained from the Affiliated Yantai Yuhuangding Hospital of Qingdao University. This study was approved by the Ethics Committee of the Affiliated Yantai Yuhuangding Hospital of Qingdao University. Signed written informed consents were obtained from all participants before the study. Tissues were fixed with 4% paraformaldehyde, embedded in paraffin and cut into sections for subsequent analysis. Human pancreatic cancer cell line MiaPaCe-2a and normal pancreas cell line HPDE6-C7 were purchased from Shanghai Guandao Biological Engineering Co., Ltd. (Shanghai, China).

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

Total RNA in tissues and cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Next, gene-specific circular primers were used to amplify total RNA of miR-132. Then, TaqMan miRNA assays was utilized to quantify complementary deoxyribonucleic acid (cDNA). The total RNA (1 μg) was synthesized into cDNA using random primers. Gene expression analysis was performed *via* qRT-PCR in accordance with the instructions of SYBR® Green PCR Master Mix. 18S rRNA was used as an internal reference. Data were analyzed using the comparative CT method. Primers used in this study were as follows: Shh: forward: CTCGCTGCTGGTATGCTCG, reverse: ATCGCTCGGAGTTTCTGGAGA, miR-132: forward: GGCAACCGTGGCTTTGGA, reverse: TTTGGCACTAGCACATT.

Western Blotting

Live tissue cells were lysed with CelLytic™ M lysis buffer. Total protein was obtained through addition of protease and phosphatase inhibitors. The concentration of extracted protein was measured using the BCA protein assay kit (Beyotime, Shanghai, China). Protein sample was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was carried out for Shh, Cyclin-D1, cleaved Caspase-3 and cleaved Caspase-9. β-actin was used as a loading control. Enhanced chemiluminescence (ECL) Western blotting analysis system was utilized for detection (Thermo Fisher Scientific, Waltham, MA, USA).

Cell Transfection

MiR-132 mimics and inhibitor were stored in freeze-dried powder at -20°C. Subsequently, they were diluted to solution at a concentration of 20 μm with sterile ribonuclease (RNase)-free H_2O. MIAPaCa-2a cells were transfected with specific RNA oligonucleotides according to the manufacturer's protocol Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). After 6 h, the medium containing miR-132 mimics (50 nm) and miR-132 inhibitor (100 nm) was replaced with new medium, followed by culture for another 48 h.

Cell Proliferation by CCK-8

After cells were confluent in a single layer at the bottom of the well, they were re-suspended and adjusted to the concentration of 1×10^5 cells/mL. Then, cells were inoculated into 96-well plates and cultured for 24 h. Next, the medium was changed and starvation medium was added for 6 h of culture. After discarding the medium, cells in experimental group were added with serum-free starvation medium of miR-132 mimic or inhibitor. Meanwhile, serum-free starvation medium blank controls and mimics or inhibitor controls were set. After incubation for 24 h, 48 h and 72 h, respectively, CCK-8 reagent was added in cells. Optical density (OD) value was measured using a microplate reader.

Cell Cycle and Apoptosis

For cell cycle: cells were suspended in absolute ethanol and stored at -20°C. Subsequently, binding buffer containing PI, NP-40 and RNaseA was added, followed by incubation at room temperature for 15 min. For cell apoptosis: cells were stained with Annexin V and PI at 4°C for 15 min. Cell cycle distribution and apoptosis were determined *via* flow cytometry. Experimental results were analyzed using Kaluza software (Miami, FL, USA).

Statistical Analysis

GraphPad Prism 6 (La Jolla, CA, USA) was utilized for all statistical analysis. The relationship between miR-132 expression and Shh expression was detected by regression analysis. One-way analysis of variance was employed to compare the differences among different groups, followed by Post-Hoc Test LSD (Least Significant Difference). *t*-test was used to compare the differences between two groups. Each experiment was repeated for at least three times. $p < 0.05$ was considered statistically significant.

Results

Expression of miR-132 in Clinical Pancreatic Cancer Tissues, as Well as Its Relationship With Shh

Firstly, qRT-PCR was performed to analyze the expressions of mature miR-132 and Shh in 76 clinical pancreatic specimens (25 normal pancreatic specimens, 18 adjacent benign pancreatic specimens and 23 pancreatic adenocarcinomas). The results (Figure 1A) revealed that compared with normal pancreatic tissues and adjacent benign pancreatic tissues, miR-132 expression was significantly upregulated in pancreatic cancer tissues ($p < 0.001$). Meanwhile, miR-132 expression was negatively correlated with Shh expression (Figure 1B).

Expression of miR-132 and Shh in Pancreatic Cancer Cells

In addition, the expressions of miR-132 and Shh in pancreatic cancer cell lines were detected *in vitro*. QRT-PCR and Western blotting assay were carried out to measure the expression levels of miR-132 and Shh in pancreatic cancer cell line MIAPaCa-2a and normal pancreas cell line HPDE6-C7. It was found that the mRNA expression of miR-132 in pancreatic cancer cell line MIAPaCa-2a was evidently increased. Moreover, both the mRNA and protein expression levels of Shh in pancreatic cancer cell line MIAPaCa-2a were significantly increased when compared with those of normal cell line ($p < 0.05$) (Figure 2A and 2B).

Impacts of miR-132 Over-Expression on Proliferation and Apoptosis of Pancreatic Cancer Cells

MiaPaCe-2a cells were transfected with miR-132 mimics, and the effects of miR-132 on the proliferation and apoptosis of pancreatic cancer cells were verified. Transfection efficiency of miR-132 mimics was first verified. Meanwhile, the effects of miR-132 on the mRNA and protein expressions of Shh were determined. CCK8 assay was used to determine the role of miR-132 mimics in proliferation of MiaPaCe-2a cells, and the changes in apoptosis-related proteins (Cyclin-D1, cleaved Caspase-3 and cleaved Caspase-9). Flow cytometry was employed to analyze the impact of miR-132 mimics on apoptosis of pancreatic cancer MiaPaCe-2a cells. As shown in Figure 3A, miR-132 mimics transfection significantly increased the mRNA expression of miR-132 ($p < 0.01$). This proved that miR-132 mimics promoted

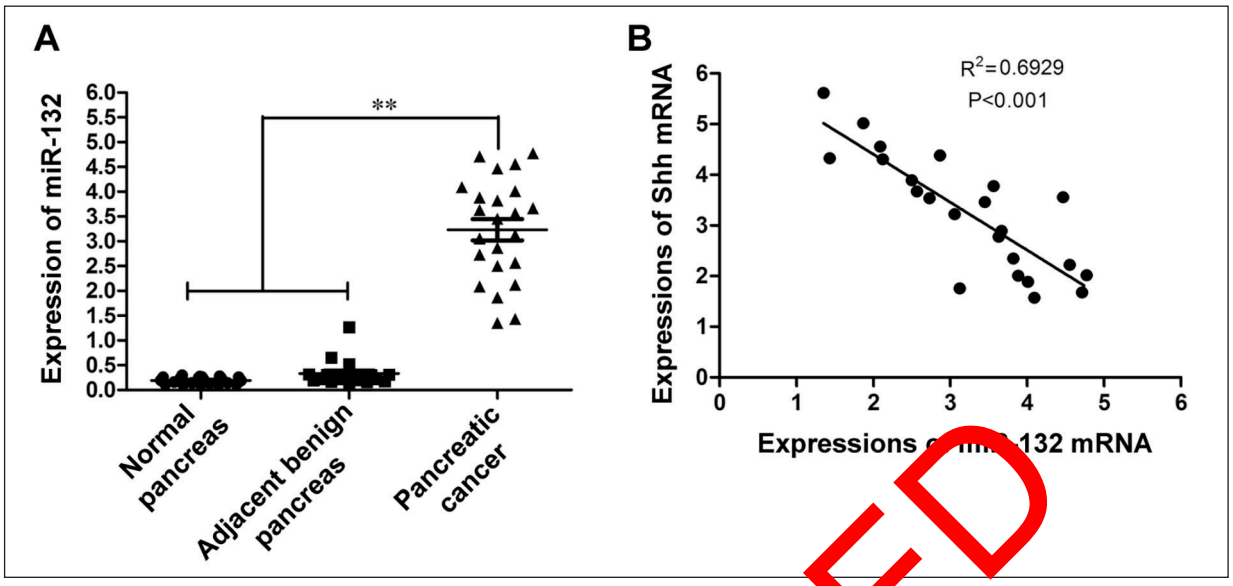

Figure 1. Expression of miR-132 in pancreatic cancer tissues as well as its correlation with Shh. ***A,*** Expression of miR-132 in normal pancreas, adjacent benign pancreas and pancreatic cancer. ***B,*** Linear correlation analysis between miR-132 and Shh. *$p < 0.05$, **$p < 0.01$.

the expression of miR-132. In addition, miR-132 mimics significantly reduced the mRNA and protein expressions of Shh ($p < 0.01$). Results of CCK-8 assay showed that miR-132 over-expression significantly promoted the proliferation of pancreatic cancer MiaPaCe-2a cells when compared with blank controls and mimic control ($p < 0.05$) (Figure 3C). However, miR-132 mimics down-regulated the expressions of Cyclin-D1, cleaved Caspase-3 and cleaved Caspase-9 at the protein level (Figure 3D). The effect of miR-132 mimics on the apoptosis was analyzed through flow cytometry. Results indicated that miR-132 over-expression significantly reduced the per-

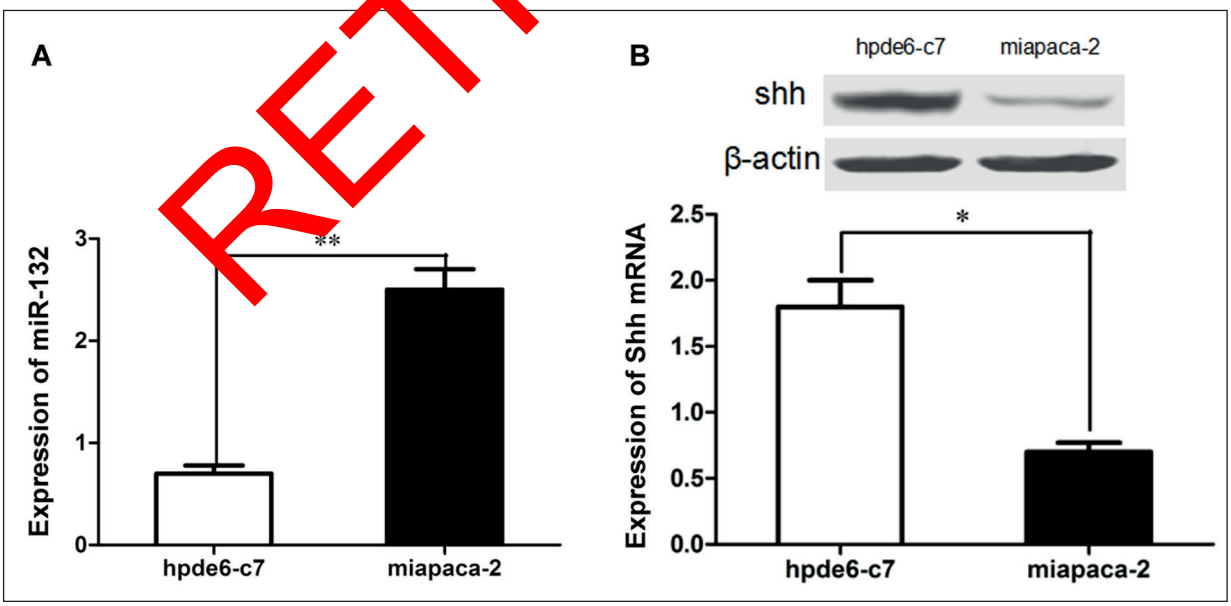

Figure 2. Expressions of miR-132 and Shh in pancreatic cancer cell lines. ***A,*** MRNA expression of miR-132 in MIAPaCa-2a and HPDE6-C7 cell lines detected *via* qRT-PCR. ***B,*** Expression of Shh in MIAPaCa-2a and HPDE6-C7 cell lines determined through qRT-PCR and Western blotting. *$p < 0.05$, **$p < 0.01$.

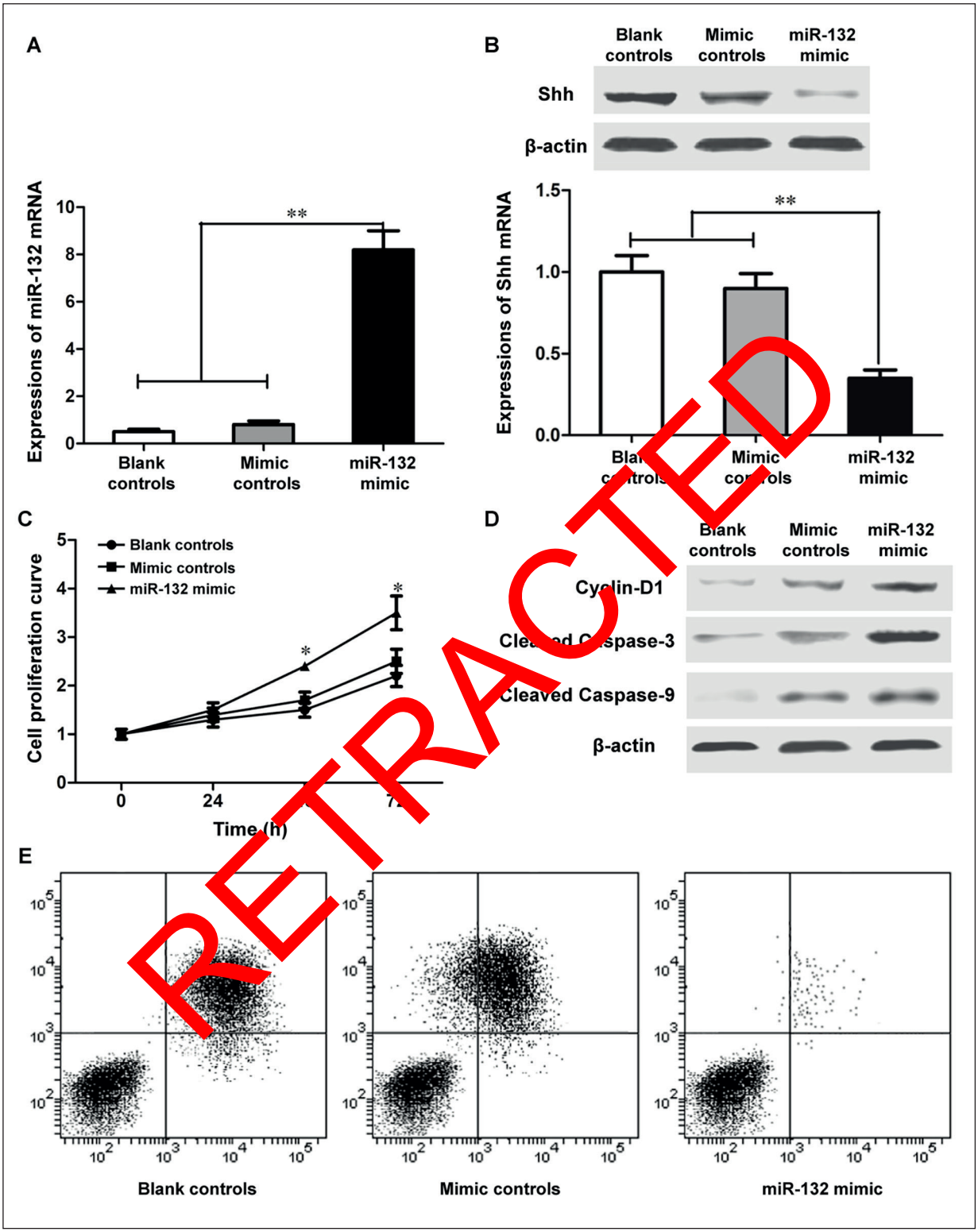

Figure 3. Impacts of miR-132 over-expression on proliferation and apoptosis of MiaPaCe-2a cells. A, Up-regulation of miR-132 on the mRNA expression of miR-132. ***B,*** Effect of miR-132 on Shh mRNA and protein expression. ***C,*** Proliferation of cells detected *via* CCK8. ***D,*** Effect of miR-132 mimics on apoptosis-related proteins determined *via* Western blotting. ***E,*** Apoptosis of MiaPaCe-2a cells determined through flow cytometry. *$p < 0.05$, **$p < 0.01$.

centage of apoptotic cells when compared with that of blank controls and mimics controls. These findings confirmed that miR-132 over-expression facilitated proliferation and repressed apoptosis of pancreatic cancer cells.

Influences of miR-132 Inhibitor on Proliferation and Apoptosis of MiaPaCe-2a Cells

The effects of miR-132 inhibitor on proliferation and apoptosis of pancreatic cancer cells were investigated as well. CCK8 assay and flow cytometry were performed to analyze the influences of miR-132 inhibitor on proliferation and apoptosis of pancreatic cancer cells, respectively. It was discovered that miR-132 inhibitor significantly suppressed the proliferative capacity of pancreatic cancer MiaPaCe-2a cells ($p < 0.05$) (Figure 4A). Results of cell cycle determination *via* flow cytometry indicated that miR-132 inhibition resulted in an obvious increase in MiaPaCe-2a cells to 42.2% from 18.5% in G2/M phase, and a significant decrease in MiaPaCe-2a cells to 17.5% in G1 phase (Figure 4B).

Discussion

In recent years, miRNAs have been extensively studied, which has also become the focus of researches on malignant tumors. Moreover, much progress has been made to improve the knowledge and understanding of miRNAs. In this study, it was found that over-expression of miR-132 evidently promoted the proliferation of pancreatic cancer cells through Hh signaling pathway. The signal transduction mechanism of pancreatic cancer development remains unclear. More and more studies have revealed that Hh signaling pathway plays an important role in the progression and prognosis of pancreatic cancer. Shh is the best-studied ligand in Hh pathway. Therefore, it was used as a marker for Hh signaling pathway in this study. Moreover, the expressions of miR-132 and Shh in clinical pancreatic cancer specimens, and the relationship between the two molecules were firstly analyzed in this study. Results revealed that there was an obvious negative correlation between high expression of miR-132 and low expression of Shh. As a cancer-related miRNA, miR-132 is upregulated in many cell carcinomas. Research by Liu et al[20] has manifested that miR-132 is lowly expressed in hepatocellular carcinoma (HCC) tissues compared with adjacent non-cancerous liver tissues. Meanwhile, miR-132 expression is negatively related to HBV-related HCC. This is contrary to the results of this study. Furthermore, miR-132 mimics were utilized in this study to verify the direct interaction between miR-132 with Shh. It was discovered that up-regulation of miR-132 significantly inhibited the expression of Shh. These results suggested that miR-132 contained a sequence complementary to the 3'-UTR of Shh. Moreover, its binding led to Shh mRNA degradation or translational suppression[21]. These findings fully clarified the relationship between miR-132 and Shh. Previous studies

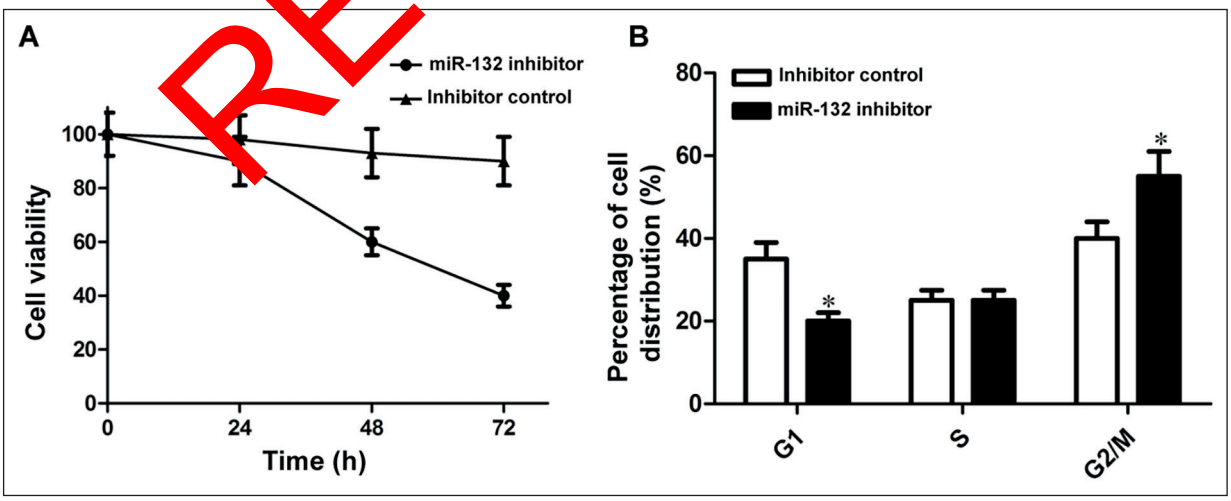

Figure 4. Effects of miR-132 inhibitors on proliferation and apoptosis of MiaPaCe-2a cells. ***A,*** Impact of miR-132 inhibitor on the proliferative activity of pancreatic cancer MiaPaCe-2a cells determined *via* CCK8. ***B,*** Influence of miR-132 inhibitor on cell cycle distribution of MiaPaCe-2a cells examined through flow cytometry. *$p < 0.05$, **$p < 0.01$.

have indicated miR-132 is closely correlated with the development and progression of pancreatic cancer. According to this research, miR-132 mimics transfection significantly promoted the proliferation of pancreatic cancer cells. However, miR-132 over-expression inhibited Shh expression, decreased the expressions of apoptotic proteins, and ultimately suppressed apoptosis of pancreatic cancer cells. These results implied that miR-132 might facilitate tumor cell growth by increasing the expressions of cell cycle regulatory genes. Moreover, it was also interestingly found in this study that inhibition of miR-132 induced G2/M arrest. Shh may affect the transition of G1/S and G2/M of cell cycle[22]. Therefore, G2/M arrest may be due to the targeting of miR-132 to Hh signaling pathway. Moreover, G2/M arrest is aggravated via Hh signaling pathway[23]. It is hypothesized that miR-132 may be involved in DNA damage and cell cycle arrest by targeting ATM and ATR. The mechanism by which miR-132 is up-regulated in pancreatic cancer remains unclear. However, a study by Park et al[24] has demonstrated that beta-2 adrenergic receptor (B2AR) agonists increase the expression of miR-132. B2AR induces transactivation of cyclic adenosine monophosphate (cAMP)-response element binding protein (CREB) *via* cAMP-protein kinase A (PKA) pathway. Transcription is induced by phosphorylation of CREB induced by PKA and recruitment of coactivator CREB binding protein (CBP) or its paralog p300. MiR-132 is activated by CREB transcription in neurons[25]. It has been proved that miR-132 is a part of the feedback loop in the antiviral innate immunologic process. Furthermore, p300 induces miR-132, while miR-132 expression inhibits p300. DNA synthesis of pancreatic cell lines MiaPaCa-2a and BXPC-3 is stimulated by β2 adrenergic signaling pathway. B2AR agonists promote the proliferation of pancreatic cancer cells by activating PKA, arachidonic acid and MAPK pathways. In addition, B2AR antagonists inhibit the activation of CREB[26, 27].

Conclusions

We revealed that miR-132 is over-expressed in pancreatic cancer. Meanwhile, it regulates the proliferation and apoptosis of pancreatic cancer cells *via* Hh signaling pathway. In addition, miR-132 leads to increased proliferative activity of pancreatic cancer cells and arrested apoptosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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