

MiR-431 inhibits cell proliferation and induces cell apoptosis by targeting CDK14 in pancreatic cancer

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Abstract. – OBJECTIVE: To investigate the role of miR-431 in the proliferation and apoptosis of pancreatic cancer cells.

MATERIALS AND METHODS: The pancreatic cancer cell was used for in vitro experiments. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) method was used to confirm the level of miR-431. Cell Counting Kit-8 (CCK-8) was used to detect the effect of miR-431 on cell proliferation. Flow cytometry was used to evaluate cell apoptosis rate and the changes of cell cycle arrest. The luciferase reporter assay was used to confirm the regulatory mechanism.

RESULTS: MiR-431 expression was reduced both in cancer tissues and cell lines. Cell proliferative ability was effectively lessened after up-regulating miR-431. Elevated miR-431 significantly induced cell apoptosis and modulated cell cycle arrest. Meanwhile, CDK14 (cyclin-dependent kinase 14) was a target gene of miR-431, and over-expression of miR-431 decreased the level of CDK14.

CONCLUSIONS: MiR-431 inhibits cell proliferation and induces cell apoptosis by targeting CDK14 in pancreatic cancer.

Key Words:

MiRNA-431, Pancreatic cancer, Proliferation, Apoptosis.

Introduction

Pancreatic cancer is one of the most malignant tumors. In recent years, surgical resection, radiotherapy, chemotherapy and other treatment have improved the survival time and quality of pancreatic cancer patients to a certain extent. However, the survival rate of pancreatic cancer is still less than 5% in the past 25 years¹. Among them, the prognosis of the patients with pancreatic ductal adenocarcinoma is the poorest. Due to the lack of the diagnostic markers, the majority of patients used to lose the opportunity to undergo

surgery. Hence, the early diagnosis and the appropriate surgical approach are the most effective treatments for pancreatic cancer^{2,3}. Therefore, exploring new and effective markers of diagnosis and treatment for pancreatic cancer is particularly important⁴.

MicroRNAs are derived from endogenous non-coding RNA, which can play a biological effect at the post-transcriptional level by interacting with the target mRNA of the gene⁵. MicroRNAs play a complicated and important role in the development of tumors⁶. MiR-19a functioned as an oncogenic microRNA in cervical cancer⁷. MiR-301a was increased in breast cancer and enhanced tumor metastasis through regulating PTEN and Wnt/ β -catenin signaling⁸. MicroRNA-431 suppressed hepatocellular carcinoma cells migration and invasion via epithelial-mesenchymal transition⁹. MiR-431 was markedly down-regulated in the HCC samples and was correlated with multiple malignant characteristics, including lymph node metastasis, clinical TNM stage¹⁰. Downregulation of microRNA-431 by human interferon- β inhibited viability of medulloblastoma and glioblastoma cells via upregulating SOCS6¹¹. Schultz et al¹² had identified that miR-431 could predict overall survival (OS) of the patients with pancreatic cancer. However, the impact of miR-431 on pancreatic cancer proliferation and apoptosis has not been reported.

In this study, we aimed to detect miR-431 expression in pancreatic cancer, and investigate the relationship between miR-431 dysregulation and cell biological processes.

Materials and Methods

Cell Lines and Main Reagents

Pancreatic cancer cell lines were purchased from ATCC (American Type Culture Collection,

Manassas, VA, USA). The fetal bovine serum, DMEM (Dulbecco's Modified Eagle Medium), RPMI-1640 (Roswell Park Memorial Institute-1640) medium, and 0.05% Ethylene Diamine Tetraacetic Acid (EDTA)-containing trypsin were purchased from Gibco (Rockville, MD, USA). Cell Counting Kit-8 (CCK-8) detection kit was purchased from Beyotime Biotechnology (Shanghai, China). RNA extraction reagent (TRIzol) was purchased from Invitrogen (Carlsbad, CA, USA). SYBR Premix ExTaq kits were purchased from TaKaRa Company (Otsu, Shiga, Japan). The miR-431 mimics was purchased from Guangzhou Ruibo Company (Guangzhou, China).

Cell Culture and Transfection

Pancreatic cancer cells were cultured in RPMI-1640 or DMEM containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), respectively, and incubated in a 37°C, 5% CO₂ incubator. Cells at logarithmic growth phase were seeded in 6-well plates at a density of 2×10^5 /ml. According to the manufacturer's protocol, miR-431 mimic and NC were transfected into cells by lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cell lines were divided into miR-431 over-expression group (miR-431 mimics) and empty vector group (NC).

Cell Proliferation Assay

The proliferative ability of cells was tested using Cell Counting Kit-8 (CCK-8) assay (Beyotime Institute of Biotechnology, Shanghai, China). After transfection for 24 h, cells were counted and seeded in 96-well plates (3×10^3 per well). Before testing, 10 μ L CCK-8 reagents were added into cell lines for incubation for 2 h at 37°C in dark according to the protocol. OD (optical density) value at 490 nm was measured by using the microplate reader (Bio-Rad, Hercules, CA, USA).

Apoptosis Detection

The apoptosis rate of cells was tested using a flow cytometry assay. The transfected cells were collected and washed twice with cold phosphate-buffered saline (PBS). Next, cells were suspended by using 200 μ L Binding Buffer (Invitrogen, Carlsbad, CA, USA), and incubated with 3 μ L Annexin V-fluorescein isothiocyanate (FITC) and 5 μ L PI (propidium iodide) (Invitrogen, Carlsbad, CA, USA) at room temperature for 20 min according to the manufacturer's instructions. Finally, cell apoptosis rate was measured using flow cytometry (EPICS XI-4; Beckman Coulter, Inc., Brea, CA, USA). Each assay was repeated

three times. The analysis of results was used by FlowJo 7.6.1 (FlowJo LLC, Ashland, OR, USA).

Cell Cycle Detection

The transfected cells were washed twice with 1 \times phosphate-buffered saline (PBS), and fixed with 100% ethanol (700 μ L) at 4°C. Subsequently, all the cells were incubated with RNase A (TaKaRa Bio, Inc., Otsu, Shiga, Japan) at 50 μ g/mL for 30 min at room temperature. Next, cell lines were stained with 100 μ g/mL PI (BD Biosciences, San Jose, CA, USA) at room temperature. The cell number in each phase was detected by using Calibur Flow Cytometers. Each assay was repeated for three times.

Luciferase Reporter Assay

The downstream target gene (CDK14) (cyclin dependent kinase 14) of miR-431 was predicted by online software (TargetScanandmicroRNA.org). With the TaKaRa PCR Amplification Kit (TaKaRa, Otsu, Shiga, Japan), 3'UTR of CDK14 mRNA containing the binding site was amplified. The products were cloned into the psiCHECK-2 reporter vector (Promega, Madison, WI, USA). Using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), 200 ng psiCHECK-2-CDK14 or psiCHECK-2-CDK14-Mutant and 100 nmol/L miR-431 mimics were co-transfected into cells. The reporter activity was tested by a Dual Luciferase Reporter Kit (Promega, Madison, WI, USA).

Statistical Analysis

Data were analysis by using Statistical Product and Service Solutions (SPSS22.0, Armonk, NY, USA) statistical software and present as mean \pm standard deviation ($\bar{x} \pm s$); t-test was used to compare the two groups. $p < 0.05$ was acted as statistically significant.

Results

MiR-431 was Lowly Expressed Both in Cancer Tissues and Cell Lines

QRT-PCR was used to identify the level of miR-431 in the pancreatic cancer samples and the matched normal specimen. The result revealed that the expression level of miR-431 in the pancreatic cancer samples was markedly lower than that of matched normal specimen ($p < 0.05$, Figure 1A).

Meanwhile, qRT-PCR was also used to detect the expression level of miR-431 in the pancreatic cancer cell lines and human pancreas ductal epithelial cell line (HPDE6-C7). The expression level of miR-431 was also markedly lower in

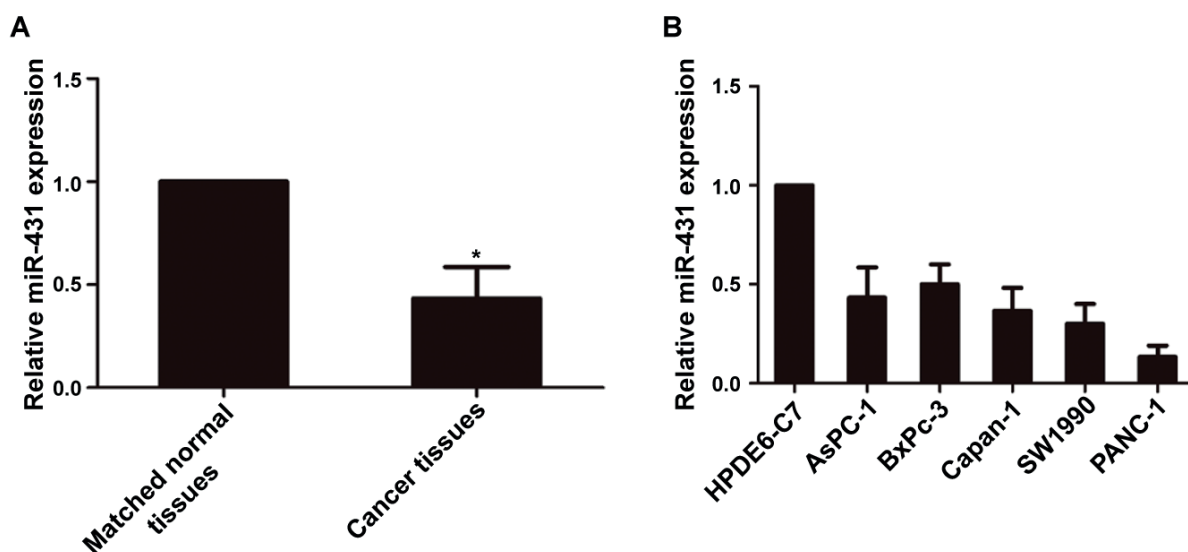


Figure 1. MiR-431 was lowly expressed both in cancer tissues and cell lines. **A**, The expression level of miR-431 was detected between the cancer tissues and the matched normal tissues by qRT-PCR. * $p < 0.05$. **B**, The expression level of miR-431 was detected among cancer cell lines by qRT-PCR.

all the pancreatic cancer cell lines than that of HPDE6-C7 cells, consistently with the *in vivo* expression ($p < 0.05$, Figure 1B).

In summary, all the above data suggested that miR-431 might function as a tumor suppressor in pancreatic cancer, which prompted us to explore the underlying molecular functions of miR-431.

Up-Regulation of miRNA-431 Expression Could Inhibit Pancreatic Cancer Cell Proliferation

First, to gain miR-431 in cells was conducted by transfecting miR-431 mimics to explore the

potential molecular functions of miR-431. The transfection effect was identified by qRT-PCR. The expression level of miR-431 was markedly up-regulated by transfecting miR-431 mimics as compared with NC group (Figure 2A).

Next, we used CCK-8 method to detect cell proliferative capacity. The result of CCK-8 revealed that OD490 at 48 h and 72 h showed a significantly decrease in miR-431 mimics group (over-expression of miR-431) relative to the NC group ($p < 0.05$, Figure 2B). This data indicated that up-regulation of miRNA-431 could inhibit pancreatic cancer cell proliferation.

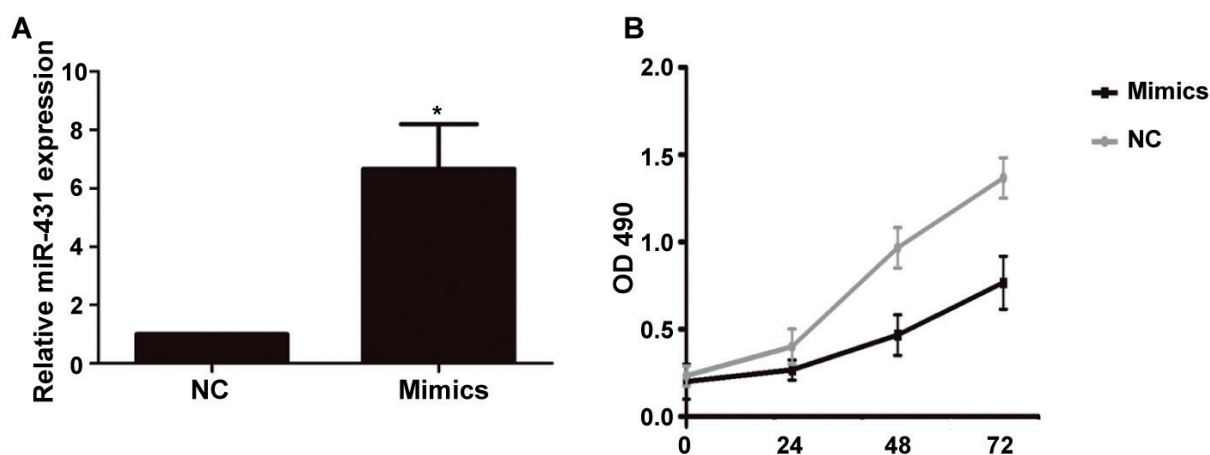


Figure 2. Up-regulation of miR-431 expression could inhibit pancreatic cancer cell proliferation. **A**, MiR-431 was increased by transfection of mimics. * $p < 0.05$. **B**, OD 490 nm was detected at 0, 24, 48 and 72 h.

Over-Expression of miR-431 Might Induce Pancreatic Cancer Cell Apoptosis

Subsequently, we used flow cytometry to detect the changes of cell apoptosis responding to over-expression of miR-431. Significantly, the result showed that cells apoptosis was increased in miR-431 mimics group compared with that of the NC group ($p < 0.05$, Figure 3A), indicating that over-expression of miR-431 might induce pancreatic cancer cell apoptosis.

MiR-431 Dysregulation Could Modulate Pancreatic Cancer Cell Cycle

In additional, we also used flow cytometry to detect the changes of cell cycle. The result showed that G1 phase cells increased in miR-431 mimics group, while S phase and G2/M phase cells decreased. The main cell block point was G1 phase when compared with that of the NC group, and the difference was statistically significant ($p < 0.05$, Figure 3B).

CDK14 Was a Candidate Target Gene of miR-431

CDK14 was one of the target downstream genes of miR-431 via using the software (TargetScan and microRNA.org). 3'UTR of CDK14 mRNA contain-

ing the predicted binding site was shown in Figure 4A. Furthermore, we used the luciferase reporter assay to identify that CDK14 was a target of miR-431. The activity of the wild-type CDK14-3'UTR with miR-431 mimics was decreased. Meanwhile, the effect was not inhibited with the mutant group (Figure 4B). These findings revealed that miR-431 could be binding to the 3'UTR of CDK14 mRNA. Moreover, to further explore the regulatory mechanism of miR-431 on CDK14, we detected the expression of CDK14 responding to miR-431 over-expression by qRT-PCR. We observed that decreased expression of CDK14 was found in cells transfected with miR-431 mimics (Figure 4C). This data suggested that miR-431 could down-regulate CDK14 expression level.

In conclusion, all the data demonstrated that CDK14 was the target downstream gene of miR-431, which was directly inhibited by miR-431.

Discussion

Pancreatic cancer is a malignant tumor with high mortality¹³. The remarkable features of pancreatic cancer are difficult in early diagnosis. Therefore,

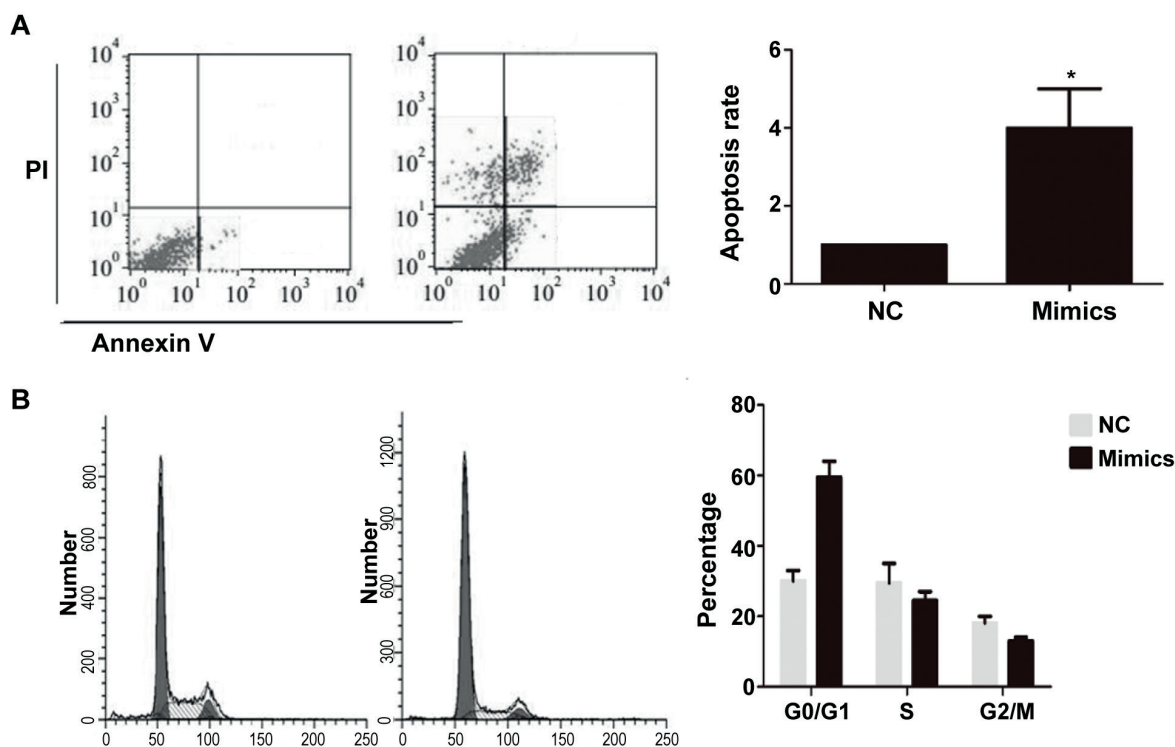


Figure 3. Over-expression of miR-431 might induce pancreatic cancer cell apoptosis and modulate cell cycle. **A**, Cell apoptosis rate was detected by flow cytometer. * $p < 0.05$. **B**, Cell cycle was also detected by flow cytometer.

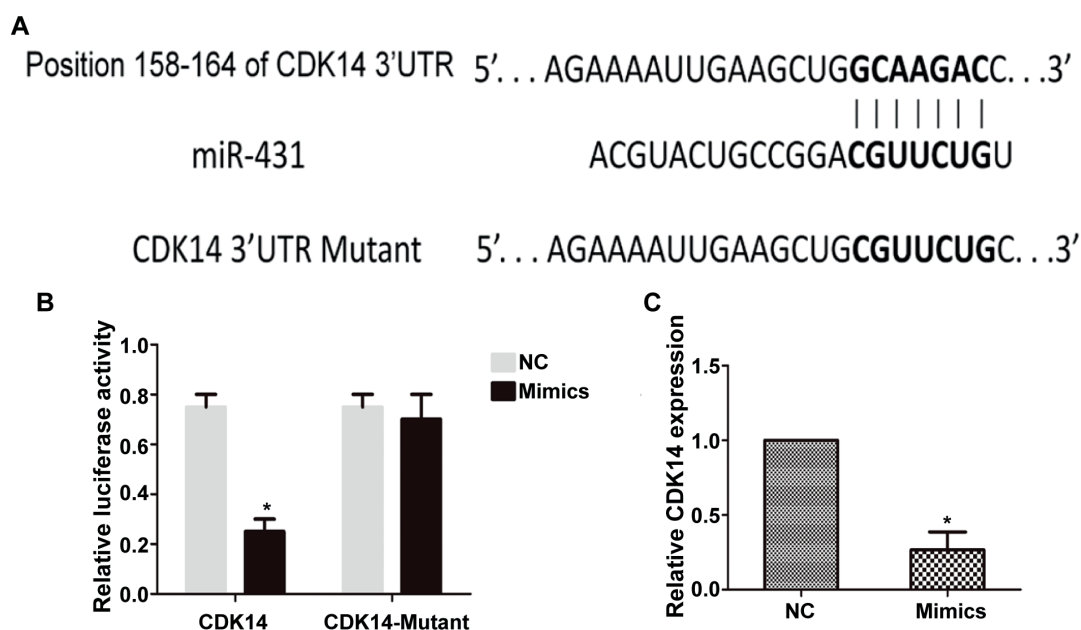


Figure 4. MiR-431 was likely targeted directly to CDK14 and could inhibit its expression. A, The potential binding site was predicted by the software (TargetScan and microRNA.org). B, The relative luciferase activity was detected between mimics group and NC group. * $p < 0.05$. C, The expression level of CDK14 was detected by qRT-PCR. * $p < 0.05$.

early diagnosis and effective treatment are particularly required¹⁴. Researches¹⁵ have shown that miRNAs have significant impact on the progression of pancreatic cancer. MicroRNAs can combine with the target gene by interacting with non-coding region, then degrade mRNA or inhibit the translation so as to control cell differentiation, proliferation and apoptosis and other processes^{16,17}. In recent years, lots of studies have found that miRNAs play important roles in the proliferation and invasion of liver cancer¹⁸, breast cancer¹⁹, and pancreatic cancer^{20,21}. In our study, we found that miR-431 was lowly expressed both in pancreatic cancer tissues and cell lines. The expression level of miR-431 was markedly up-regulated by transfecting miR-431 mimics as compared with that of NC group. Up-regulation of miRNA-431 expression could inhibit proliferation and induce apoptosis of pancreatic cancer cells. MiR-431 dysregulation could modulate pancreatic cancer cell cycle; the main cell block point was G1 phase.

MiRNAs function as oncogenes or suppressors on the protein expression by binding to the target gene²². Therefore, finding the downstream target genes is the key to reveal its functions. Through online software (TargetScan and microRNA.org), the analysis found that CDK14 was one of the target genes of miR-431.

CDK14 (cyclin dependent kinase 14), also known as PFTK1, participated in proliferation, invasion and metastasis of many tumor cells, including breast cancer^{23,24}, osteosarcoma²⁵, glioma²⁶, and hepatocellular carcinoma²⁷. CDK14 regulates cell proliferation, migration and invasion in epithelial ovarian cancer²⁸. Knockdown of CDK14 expression by RNAi inhibits the proliferation and invasion of human non-small lung adenocarcinoma cells²⁹. CDK14 promotes gastric cancer progression by regulating proliferation, migration and invasion³⁰. CDK14 promotes cell proliferation, migration and invasion in ovarian cancer by inhibiting Wnt signaling pathway³¹. In this study, by the luciferase reporter experiment, we found that miR-431 could be binding to the 3'UTR of CDK14 mRNA. The analysis of qRT-PCR observed that decreased expression of CDK14 was found in cells transfected with miR-431 mimics.

Conclusions

We showed that miR-431 was lowly expressed both in cancer tissues and cell lines, could inhibit cell proliferation, and induces cell apoptosis by targeting CDK14 in pancreatic cancer. miR-431 would provide a new vision for understanding the

molecular mechanism of pancreatic cancer development.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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