Upregulation of MicroRNA-935 Promotes the Malignant Behaviors of Pancreatic Carcinoma PANC-1 Cells via Targeting Inositol Polyphosphate 4-Phosphatase Type I Gene (INPP4A)

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Our goal was to determine the roles and regulatory mechanism of microRNA-935 (miR-935) in the progression of pancreatic cancer. The results showed that, compared with normal pancreatic tissues and cells, the expression of miR-935 was markedly upregulated, while INPP4A expression was obviously downregulated in pancreatic cancer tissues and PANC-1 cells. After transfection with the miR-935 inhibitor, miR-935 was significantly suppressed, and suppression of miR-935 significantly inhibited cell proliferation, suppressed cell migration, and induced cell apoptosis of pancreatic cancer cells. Moreover, suppression of miR-935 resulted in a significant increase in the expression of p27. Also, suppression of miR-935 resulted in significant expression changes of EMT markers; E-cadherin was significantly upregulated, while N-cadherin, Snail, and vimentin were markedly downregulated. In addition, after suppression of miR-935, the expression of apoptosis-related proteins was also changed; Bax was significantly upregulated while Bcl-2, procaspase 3, and active caspase 3 were obviously downregulated. Importantly, opposite effects were obtained when miR-935 was overexpressed by transfection with the miR-935 mimic. In addition, INPP4A was a direct target of miR-935. Silencing of INPP4A significantly counteracted the effects of miR-935 suppression on cell migration and apoptosis, as well as the expression changes of the above EMT- and apoptosis-related molecules. Our findings indicate that upregulation of miR-935 may promote pancreatic cancer cell proliferation and migration and inhibit cell apoptosis by targeting INPP4A. miR-935 and INPP4A may serve as potential targets in the therapy of pancreatic cancer.

Key words: Pancreatic cancer; MicroRNA-935 (miR-935); INPP4A; Cell proliferation; Cell apoptosis; Cell migration

INTRODUCTION

Pancreatic cancer is a common digestive system malignancy with a poor prognosis. It is always diagnosed at an advanced state, and consequently there are few effective therapies. The 5-year survival rate is reported to be only 2% among patients with metastatic pancreatic cancer. Moreover, the incidence and mortality of pancreatic cancer are on the rise in recent years. Therefore, a better understanding of the molecular mechanisms underlying pancreatic cancer will facilitate discovery of novel targeted therapies for this disease.

MicroRNAs (miRNAs) are functional noncoding RNAs, which have been widely identified as being associated with tumor progression in pancreatic cancer. For instance, the miR Let-7c is found to be upregulated after quercetin treatment, which can inhibit the progression of pancreatic cancer via activation of the Notch inhibitor Numb. miR-1291 can suppress cell proliferation and tumorigenesis in pancreatic cancer via targeting the FOXA2–AGR2 pathway. miR-891b can also inhibit the growth of pancreatic cancer cells via targeting Cbl-b and thus is considered to be an independent prognostic factor for pancreatic cancer. In addition, the aberrantly expressed miRNAs are demonstrated to be critically correlated with drug resistance, disease stage, and survival of pancreatic cancer patients, suggesting that targeting the specific miRNAs may provide an efficient strategy for the therapy of pancreatic cancer. Several serum miRNAs are shown to have potential diagnostic value in patients with pancreatic cancer. Therefore, identification of the specific miRNAs associated with pancreatic cancer is of significance. In recent studies, miR-935 is observed to be upregulated in pancreatic cancer. However, the potential roles and possible regulatory mechanism of miR-935 in pancreatic cancer progression are largely unknown.
In this study, the miR-935 expression in pancreatic cancer tissues and human pancreatic cancer PANC-1 cells was investigated. In addition, miR-935 was overexpressed and suppressed in pancreatic cancer cells. The effects of miR-935 overexpression and suppression on cell proliferation, migration, and apoptosis were then investigated. In addition, the association between miR-935 and inositol polyphosphate 4-phosphatase type I gene (INPP4A) was explored. The objective of our study was to investigate the potential roles and regulatory mechanism of miR-935 in the progression of pancreatic cancer. Our findings are expected to provide new insight in the treatment of pancreatic cancer.

MATERIALS AND METHODS

Patients
From January 2014 to April 2016, a total of 37 patients who were diagnosed with pancreatic cancer and received surgical removal in our hospital were enrolled in this study. The diagnosis of pancreatic cancer was pathologically confirmed. Cancer tissues and their adjacent normal tissues were collected, snap frozen with liquid nitrogen, and stored at −80°C. The patients provided their informed consent, and the procedures in this study were approved by the human ethics committee of Qilu Hospital of Shandong University.

Cell Culture
The normal pancreatic ductal epithelial cell line HPDE6-C7 and human pancreatic cancer cell line PANC-1 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). These cells were then cultured in the Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA), mixed with 10% fetal bovine serum (FBS; Sigma-Aldrich), and incubated in a humidified 37°C incubator with 5% CO2.

Cell Transfection
Vectors including the miR-935 mimic, miR-935 inhibitor, and miR-935 scramble were purchased from Sangon Biotech (Shanghai, P.R. China) and then transfected into cells with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) based on the instructions of the manufacturer. Cells transfected with the miR-935 scramble and no vector transfection were considered as the negative and blank controls.

Cell Proliferation Assay
Cell proliferation ability was assessed by the MTT assay and clonogenic assay. For the MTT assay, cells (adjusted to 5×104 cells/ml) at the logarithmic phase were seeded onto 96-well plates. After transfection at different times, 20 μl of MTT solution (Roche) was added to each well, and the plates were then cultured for 4 h. After terminating the reaction, the supernatant was removed by centrifugation and cells were collected. Then 150 μl of dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals by shaking at low speed for 10 min. Absorbance of cells in each well at 570-nm wavelength (A570) was then measured under a microplate reader (Biotek, Winooski, VT, USA). For the clonogenic assay, after 48 h of transfection, cells were plated into the 60-mm culture dishes with a density of 100 cells/dish. DMEM containing 10% FBS was then added to culture cells for 14 days. Afterward, cells were fixed, stained with Diff-Quik, and air dried. Colonies in each culture dish were then counted under a microscope (IX83; Olympus, Tokyo, Japan), and the cell number in each colony was more than 30 cells. All determinations were conducted in triplicate.

Cell Migration Assay
Cell migration was evaluated using Transwell migration chambers (8-μm pore size; Corning, Corning, NY, USA). In brief, after 48 h of transfection, cells (5×104 cells/well) were seeded in the upper portion of the chambers, which were filled with serum-free medium. The lower portion of the chambers was filled with the medium containing 10% FBS as a chemoattractant. After 24 h of incubation at 37°C, the migrated cells were fixed and stained with Diff-Quik staining. The number of migrated cells in each field was then counted using light microscopy. Each experiment was carried out three times with duplicate wells.

Cell Apoptosis Assay
Cell apoptosis was quantified with an annexin V–FITC cell apoptosis kit (Invitrogen) by means of flow cytometry. Briefly, after 48 h of transfection, cells were harvested, washed with ice-cold PBS, and then resuspended in the staining buffer. Cells were then mixed with 5 μl of annexin V–FITC and 5 μl of propidium iodide (PI; 10 mg/L) for 10 min. The mixtures were then analyzed using the FACScalibur flow cytometer (Beckton-Dickinson, San Jose, CA, USA). The apoptotic cells (annexin V+ and PI−) were then analyzed with CellQuest 3.0 software (BD Biosciences, San Jose, CA, USA).

qRT-PCR Analysis
Total RNA was extracted from the tissue samples or cultured cells with TRizol reagent (Takara, Dalian, P.R. China). After detecting the quality of isolated RNA using SMA 400 UV0VIS (Merinton, Shanghai, P.R. China), cDNA synthesis was performed with the PrimerScript 1st Strand cDNA Synthesis Kit (Invitrogen). With a standard protocol provided by the manufacturer, qRT-PCR
was carried out to measure the expressions of targets in tissues or cells using the SYBR Ex Script qRT-PCR Kit (Takara). Each reaction was performed in triplicate. The expressions of targets were normalized to phosphoglyceraldehyde dehydrogenase (GAPDH) and calculated using the comparative threshold (Ct) cycle ($2^{-\Delta\Delta Ct}$) method. Primers used for the amplification of targets are shown in Table 1.

**Western Blotting**

Cells were first lysed with adioimmunoprecipitation (RIPA; Sangon Biotech) lysate mixed with phenylmethanesulfonyl fluoride (PMSF; Sigma-Aldrich). Then the supernatant was collected by means of centrifugation, and protein concentrations were measured with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Afterward, 25 μg of protein was subjected to a 12% SDS-PAGE and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were blocked with TBST (0.1% Triton in PBS) containing 5% nonfat milk for 1 h. Then the membrane was incubated with primary antibodies to INPP4A, P27, P21, and GAPDH (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bax, Bcl-2, caspase 3, E-cadherin, N-cadherin, Snail, and vimentin (1:100 dilution; Invitrogen) overnight at 4°C. The membrane was then probed with horseradish peroxidase-labeled appropriate secondary antibody (1:2,000 dilution; Santa Cruz Biotechnology) at room temperature for 1 h. After incubation with a chromogenic substrate, the protein bands were detected using the enhanced chemiluminescence (ECL) method. GAPDH served as the internal control to normalize the expression of target proteins.

**Bioinformatic Method**

The target of miR-935 was predicted using the predictive software programs TargetScan (http://www.targetscan.org/).

**Luciferase Reporter Assay**

To validate whether INPP4A was a target of an miR-935, the pMIR-REPORT™ vectors including INPP4A containing the wild-type miR-935 binding site (LUX-INPP4A 3'-WT) or with a mutated miR-935 binding sequence (LUX-INPP4A 3'-MUT) were used. Cells were plated onto 24-well plates at a cell density of 100 cells/well and were incubated for another 24 h. Then cells were cotransfected with luciferase vectors (LUX-INPP4A 3'-WT or LUX-INPP4A 3'-MUT) together with the miR-935 inhibitor (or control miRNA) and the Renilla control. After 36 h of transfection, luciferase activity was then measured with the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA) and normalized to Renilla luciferase activity.

**Statistical Analysis**

All data were expressed as mean±standard error of the mean (SEM), and statistical analyses were performed using the GraphPad Prism 5.0 software (GraphPad Prism, San Diego, CA, USA). The differences between the two groups were measured using the independent sample t-test, and the differences among groups were carried out using the post hoc Tukey test. A statistically significant difference was defined with a value of $p<0.05$.

**RESULTS**

**Inverse Expression of miR-935 and INPP4A in Pancreatic Cancer Tissues and Cells**

miR-935 expression in pancreatic cancer tissues was significantly increased in comparison with their adjacent normal tissues ($p<0.05$) (Fig. 1A). However, the expression of INPP4A mRNA in pancreatic cancer tissues was significantly decreased compared with their adjacent normal tissues ($p<0.05$) (Fig. 1B and C). In addition, miR-935 expression was also markedly increased in human pancreatic cancer PANC-1 cells compared with

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**Table 1. Primers Used for Target Amplification**

| Gene      | Forward Primer (5'→3') | Reverse Primer (5'→3') |
|-----------|------------------------|------------------------|
| miR-935   | GATCACAGCAGGATGACATCA  | CTGCCCTGTGGTCTAAAAAC   |
| INPP4A    | GGAGCAGGTGATGCTTAGAAA  | ATGATGAAGCGCAGATGAG    |
| E-cadherin| AAAGCATTGCCACATACAC    | AAGCATTGCCACATACAC     |
| N-cadherin| AACTCCAGGGGACCTTTTC    | CAAATGAAACCCGGCTATC    |
| Vimentin  | TCCAAGTTTGTGACCTTC     | TCAACGGCAAATGCTCTTC    |
| Snail     | TCTAACGTCAAATACTGCAAACAAG | CGTGTTGGCTTCGGATGTG    |
| Bax       | CTGAGCTGACCTTTGGAGC    | GACGCTGCCACAAAGATG    |
| Bcl-2     | CTGGTTGACACATCCTTCT    | GGTCTGCTGACTTCTTCTG    |
| Procaspase 3 | TGTGACTCCTGCTCTGAACAG | AAATGACCCCTTCATACCA    |
| Active caspase 3 | GGTTAATTCTGACAGACATGG | CATGGAATCCTTCTTTTCTG    |
| GAPDH     | TGACTTCAACAGCGACACCCA  | CACCCTGTTGCTGACACAACAAA |
the normal pancreatic ductal epithelial HPDE6-C7 cells ($p < 0.05$) (Fig. 1D). Inverse expression of INPP4A with miR-935 was also found in human pancreatic cancer PANC-1 cells ($p < 0.05$) (Fig. 1E and F).

The effects of miR-935 overexpression and suppression on cell proliferation were then assessed. The results of the MTT assay showed that cell proliferation was markedly promoted after miR-935 overexpression and obviously inhibited after miR-935 suppression ($p < 0.05$) (Fig. 2B), which was in line with the results of the clonogenic assay (Fig. 2C and D). Furthermore, the expression of p27 in different transfected cells was further detected to explore the possible regulatory mechanism of miR-935 in cell proliferation. The results of the Western blot showed that p27 was significantly downregulated in the miR-935 overexpression group, while it was obviously upregulated in the miR-935 suppression group ($p < 0.05$) (Fig. 2E), indicating that overexpression of miR-935 promoted cell proliferation, possibly via regulating the expression of p27.
Overexpression of miR-935 Promoted Pancreatic Cancer Cell Migration

The effects of miR-935 overexpression and suppression on pancreatic cancer cell migration were also evaluated. In comparison with the control group, the number of migrated cells in the miR-935 overexpression group was significantly increased and markedly decreased in the miR-935 suppression group ($p<0.05$) (Fig. 3A and B). Furthermore, the expressions of epithelial–mesenchymal transition (EMT)-related molecules, including N-cadherin, E-cadherin, Snail, and vimentin, were measured to explore the possible regulatory mechanism of miR-935 in cell migration. The expressions of E-cadherin in the miR-935 overexpression group were significantly downregulated, and the expressions of N-cadherin, Snail, and vimentin were markedly upregulated, whereas the opposite effects on the expressions of these EMT-related molecules were obtained in the miR-935 suppression group ($p<0.05$) (Fig. 3C and D). These findings suggest that miR-935 overexpression significantly promoted cell migration, possibly via inhibiting EMT.

Overexpression of miR-935 Inhibited Pancreatic Cancer Cell Apoptosis

The effects of miR-935 on pancreatic cancer cell apoptosis were investigated using flow cytometry. The results showed that the percentage of apoptotic cells in the miR-935 overexpression group was significantly decreased compared with the control group, while it was markedly increased in the miR-935 suppression group ($p<0.05$) (Fig. 4A and B). In addition, in the miR-935 overexpression group, the expression of Bax was significantly decreased, and the expressions of Bcl-2, procaspase 3, and active caspase 3 were obviously increased

Figure 2. The effects of miR-935 on cell proliferation. (A) The expression of miR-935 in different transfection groups. (B) The MTT assay showed the cell viability of different transfection groups. (C, D) The colony assay showed the number of colonies of different transfection groups. (E) The expression level of p27 in different groups determined by Western blot. Error bars indicate means±SD. *$p<0.05$ and **$p<0.01$ (compared with the control group).
Opposite changes in the expressions of these apoptosis-related molecules were observed in the miR-935 suppression group (Fig. 4C and D). These results implied that miR-935 overexpression significantly inhibited cell apoptosis, possibly via regulating these apoptosis-related molecules.

**INPP4A was the Direct Target of miR-935**

In this study, based on the information of TargetScan, INPP4A was predicted as a target for miR-935 (Fig. 5A). To further verify the predicted results, we performed a luciferase reporter assay. Expected results were obtained, showing that cotransfection of the miR-935 inhibitor
miR-935 suppression group ($p<0.05$) (Fig. 5C and D), displaying a negative regulatory relationship between miR-935 and INPP4A.

**miR-935 Regulated Cell Migration and Apoptosis by Targeting INPP4A**

To further explore whether miR-935 played a key role in pancreatic cancer via targeting INPP4A, cells were transfected with si-INPP4A and the miR-935 inhibitor

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**Figure 4.** The effects of miR-935 on cell apoptosis. (A, B) Flow cytometry showed the percentage of apoptotic cells in different transfection groups. (C) The expression levels of Bax, Bcl-2, procaspase 3, and active caspase 3 in different groups determined by qRT-PCR. (D) The expression levels of Bax, Bcl-2, procaspase 3, and active caspase 3 in different groups determined by Western blot. Error bars indicate means±SD. *$p<0.05$ and **$p<0.01$ (compared with the control group).
INPP4A expression was significantly suppressed by si-INPP4A (p < 0.05) (Fig. 6A and B). In addition, the inhibitory effects of the miR-935 inhibitor on cell migration and the expression of EMT-related molecules were counteracted after cells were transfected with si-INPP4A and the miR-935 inhibitor simultaneously (p < 0.05) (Fig. 6C–F). In addition, the promoted effects of the miR-935 inhibitor on cell apoptosis, as well as the expression of apoptosis-related molecules, were neutralized after cells were transfected with si-INPP4A and the miR-935 inhibitor simultaneously (p < 0.05) (Fig. 6G–J). These results indicated that miR-935 inhibited cell migration and induced cell apoptosis by targeting INPP4A.

DISCUSSION

Recently, miR-935 has been shown to be implicated in the tumorigenesis of several human cancers, such as gastric cancer and gastric signet ring cell carcinoma. However, the potential role of miR-935 in the pathogenesis of pancreatic cancer has not been fully investigated. In the present study, miR-935 was significantly upregulated in pancreatic cancer tissues and PANC-1 cells, while inverse expression of INPP4A was observed. Moreover, suppression of miR-935 significantly inhibited pancreatic cancer cell proliferation, suppressed cell migration, and induced cell apoptosis. In addition, INPP4A was confirmed as a direct target of miR-935. Silencing of INPP4A significantly counteracted the effects of miR-935 suppression on cell migration and apoptosis.

Accumulating evidence has demonstrated that p27 is a well-known tumor suppressor in various types of cancer. For instance, miR-25 promotes cell proliferation in osteosarcoma by targeting p27. miR-148a can promote gastric cancer proliferation by inhibiting the expression of p27. Downregulation of miR-429 inhibits cell proliferation in human prostate cancer via targeting p27. In addition, the reduced p27 expression has been shown to be correlated with poor prognosis in some human cancers, including pancreatic adenocarcinoma. In this study, we found that miR-935 was significantly upregulated in pancreatic cancer tissues and PANC-1 cells. Additionally, suppression of miR-935 significantly inhibited pancreatic cancer cell proliferation and resulted in a significant increase in the expression of p27. Therefore, our results prompted us to speculate that miR-935 may promote pancreatic cancer proliferation, possibly via inhibiting p27 expression.

Furthermore, EMT is a key mechanism contributing to the progression of primary tumors toward metastasis. EMT has been shown to play a crucial role in the malignant metastasis in pancreatic cancer. E-cadherin, one of the EMT markers, has been found to have a lower...
Figure 6. The effects of INPP4A on the migration and apoptosis of pancreatic cancer cells. (A, B) The expression of INPP4A after si-INPP4A treatment. (C, D) The Transwell assay showed the number of migrated cells in different groups. (E, F) The expression levels of N-cadherin, E-cadherin, Snail, and vimentin in different groups. (G, H) The flow cytometry showed the percentage of apoptotic cells in different groups. (I, J) The expression levels of Bax, Bcl-2, procaspase 3, and active caspase 3 in different groups. Error bars indicate means ±SD. *p<0.05 and **p<0.01 compared with the control group. #p<0.05 compared with the inhibitor group.
expression in pancreatic cancer tissues compared with nontumor tissues. N-cadherin and vimentin, two other EMT markers, were observed to be upregulated in pancreatic cancer. In our study, overexpression of miR-935 significantly promoted cell migration. Moreover, the expressions of E-cadherin in the miR-935 overexpression group were significantly downregulated and the expressions of N-cadherin and vimentin were markedly upregulated. It can therefore be speculated that miR-935 promotes pancreatic cancer cell migration, possibly via inhibiting EMT.

Another aspect of the present analysis showed that miR-935 overexpression significantly inhibited cell apoptosis. In order to investigate miR-935’s effect on apoptosis, the relationships between miR-935 and the expression of apoptotic molecules were conducted. The results showed that, in the miR-935 overexpression group, the expression of Bax was significantly decreased, and the expressions of Bcl-2, procaspase 3, and active caspase 3 were obviously increased. Retinoids can induce cell apoptosis in pancreatic cancer via altered expression of Bcl-2/Bax. Overexpression of Bax promotes chemotherapeutic agent-induced cell apoptosis in human pancreatic cancer. The expression of Bcl-2 and Bax proteins is also confirmed to have clinicopathological significance in human pancreatic cancer. In addition, previous studies have suggested that caspase 3 is a key mediator of mitochondrial events of apoptosis. Caspase 3 drives pancreatic cancer cell apoptosis after treatment with gemcitabine. Caspase 3 expression is also shown to be correlated with clinical pathologic features and survival time of patients with pancreatic cancer because it can influence the apoptosis of pancreatic cancer tissues and cells. In sum, these results imply that miR-935 may inhibit pancreatic cancer cell apoptosis, possibly by altering the expression of Bcl-2/Bax and caspase 3.

Intriguingly, one of the important findings in this study was that inverse expression of miR-935 and INPP4A was observed in pancreatic cancer tissues and cells. INPP4A, a PtdIns(3,4)P2 phosphatase, is identified as a suppressor of glutamate excitotoxicity in the central nervous system and thus can protect neurons from excitotoxic cell death. However, the roles of INPP4A in cancer progression have not been fully investigated until now. In our study, we found that INPP4A was confirmed to be a direct target of miR-935. Silencing of INPP4A significantly counteracted the effects of miR-935 suppression on cell migration and apoptosis via regulating EMT and apoptosis-related molecules. Therefore, we speculate that miR-935 may promote pancreatic cancer progression via targeting INPP4A.

In conclusion, our findings indicate that upregulation of miR-935 may promote pancreatic cancer cell proliferation and migration and inhibit cell apoptosis through targeting INPP4A. miR-935 and INPP4A may serve as potential targets in the therapy of pancreatic cancer.

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