MicroRNA-21 modulates biological functions of pancreatic cancer cells including their proliferation, invasion, and chemoresistance

Taiki Moriyama,1 Kenoki Ohuchida,1,2 Kazuhiro Mizumoto,1,4 Jun Yu,1 Norihiro Sato,1,3 Toshinaga Nabe,1 Shunichi Takahata,1 Hiroki Toma,1 Eishi Nagai,1 and Masao Tanaka 1

Departments of 1Surgery and Oncology, 2Advanced Medical Initiatives, and 3Cancer Therapy and Research, Graduate School of Medical Sciences, Kyushu University, and 4Kyushu University Hospital Cancer Center, Fukuoka, Japan

Abstract

Due to the poor prognosis of pancreatic cancer, novel diagnostic modalities for early diagnosis and new therapeutic strategy are urgently needed. Recently, microRNA-21 (miR-21) was reported to be strongly overexpressed in pancreatic cancer as well as in other solid cancers. We investigated the functional roles of miR-21, which have not been fully elucidated in pancreatic cancer. miR-21 expression was assessed in pancreatic cancer cell lines (14 cancer cell lines, primary cultures of normal pancreatic epithelial cells and fibroblasts, and a human normal pancreatic ductal epithelial cell line) and pancreatic tissue samples (25 cancer tissues and 25 normal tissues) by quantitative realtime reverse transcription-PCR amplification. Moreover, we investigated the proliferation, invasion, and chemoresistance of pancreatic cancer cells transfected with miR-21 precursor or inhibitor. miR-21 was markedly overexpressed in pancreatic cancer cells compared with nonmalignant cells, and miR-21 in cancer tissues was much higher than in nonmalignant tissues. The cancer cells transfected with the miR-21 precursor showed significantly increased proliferation, Matrigel invasion, and chemoresistance for gemcitabine compared with the control cells. In contrast, inhibition of miR-21 decreased proliferation, Matrigel invasion, and chemoresistance for gemcitabine. Moreover, miR-21 positively correlated with the mRNA expression of invasion-related genes, matrix metalloproteinase-2 and -9, and vascular endothelial growth factor. These data suggest that miR-21 expression is increased in pancreatic cancer cells and that miR-21 contributes to the cell proliferation, invasion, and chemoresistance of pancreatic cancer. [Mol Cancer Ther 2009;8(5):1067–74]

Introduction

Pancreatic cancer is the fifth leading cause of cancer deaths in Japan and the fourth leading cause of cancer-related deaths in the United States. Annually, >17,000 patients in Japan and ~30,000 in the United States die from the disease. The prognosis for pancreatic cancer is the worst among all cancers due to a lack of improvement in its detection, diagnosis, and treatment strategies (1, 2). Therefore, novel diagnostic modalities for early diagnosis and new therapeutic strategies are urgently needed.

Over the past few years, microRNAs (miRNA) have emerged as a prominent class of gene regulators. miRNAs comprise a class of small noncoding RNAs that modulate gene expression by targeting mRNAs for translational repression or cleavage (3). Although 530 human miRNAs have been reported to date (ref. 4; miRBase at the Sanger Institute),5 the functional roles of these miRNAs in many cellular processes have not been fully elucidated. Recently, miRNAs were reported to have diverse functions, including the regulation of cellular differentiation, proliferation, and apoptosis (5). Moreover, a growing number of direct and indirect lines of evidence suggest relationships between altered miRNA expression levels and cancer, including alterations of miR-15a and miR-16-1 in chronic lymphocytic leukemia (6, 7), miR-143 and miR-145 in colorectal cancer (8), let-7 in lung cancer (9, 10), and miR-155 in diffuse large B-cell lymphoma (11). Expression profiling has identified several miRNAs that are differentially expressed in other cancers, such as breast cancer (12), glioblastoma (13), and papillary thyroid cancer (14).

Previous microarray or Northern blot analyses have shown that several miRNAs are aberrantly expressed in pancreatic cancer (15, 16). It has been reported that microRNA-21 (miR-21) is strongly overexpressed in pancreatic cancer as well as glioblastoma (13), breast cancer (17), cholangiocarcinoma (18), and hepatocellular cancer (19). Most of these studies indicated that miR-21 could play roles in carcinogenesis or cancer progression (20). Moreover, several researchers have reported that in vitro inhibition of miR-21

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Requests for reprints: Kenoki Ohuchida, Department of Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Fukuoka 812-8582, Japan. Phone: 81-92-642-5440; Fax: 81-92-642-5458. E-mail: kenoki@med.kyushu-u.ac.jp

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5 http://microrna.sanger.ac.uk/sequences/
activity significantly reduces cell viability accompanied by elevated levels of intracellular caspase activity, although the mechanism that underlies these effects is largely unknown (13, 17, 18).

Despite several reports that miR-21 is one of the representative miRNAs with functions as an oncogene, there are no reports regarding its biological functions in pancreatic cancer. In the present study, we investigated the biological functions of miR-21 in pancreatic cancer cells using a precursor or an inhibitor of miR-21 in vitro. Our data suggest that miR-21 plays roles in the tumor growth, invasion, and chemoresistance of pancreatic cancer.

Materials and Methods

Cell Lines and Cultures
The following 14 pancreatic cancer cell lines were used: AsPC-1, KP-1N, KP-2, KP-3, PANC-1, and SUIT-2 (Dr. H. Iguchi, National Shikoku Cancer Center); MIA PaCa-2 (Japanese Cancer Resource Bank); CAPAN-1, CAPAN-2, CFPAc-1, H48N, HS766T, and SW1990 (American Type Culture Collection); and NOR-P1 (established by Dr. N. Sato in our laboratory; ref. 21). Three primary cultures of pancreatic fibroblasts were used and maintained as described previously (22). A human pancreatic ductal epithelial (HPDE) cell line (HPDE6-E6E7 clone 6) immortalized by transduction with the E6/E7 genes of human papillomavirus 16 was kindly provided by Dr. Ming-Sound Tsao (University of Toronto) and maintained as described previously (22). Primary cultures of human normal pancreatic epithelial cells were obtained from Cell Systems and maintained in CS-C medium containing 10% fetal bovine serum according to the supplier's instructions. Total cell numbers were quantified using a particle distribution counter (CDA500; Sysmex).

Tissue Samples
The tissue samples analyzed in this study were obtained from patients who underwent a surgical procedure to resect a portion of the pancreas in Kyushu University Hospital from February 2000 to March 2008. Normal pancreatic tissues were also taken from areas of peripheral tissues away from the tumor or from nonneoplastic pancreas resected due to bile duct disease. Tissues were removed as soon as possible after resection. A part of each sample was embedded in OCT compound (Sakura) as described previously (23). The PCR primer sequences used in this study were derived in our laboratory; ref. 21). Three primary cultures of pancreatic cancer cell lines were used and maintained as described previously (22). A human pancreatic ductal epithelial (HPDE) cell line (HPDE6-E6E7 clone 6) immortalized by transduction with the E6/E7 genes of human papillomavirus 16 was kindly provided by Dr. Ming-Sound Tsao (University of Toronto) and maintained as described previously (22). Primary cultures of human normal pancreatic epithelial cells were obtained from Cell Systems and maintained in CS-C medium containing 10% fetal bovine serum according to the supplier's instructions. Total cell numbers were quantified using a particle distribution counter (CDA500; Sysmex).

Transfections
Transfections were done by electroporation using a Nucleofector system (Amaxa Biosystems). All studies were done in triplicate. Cells (1 × 10^6-2 × 10^6) were centrifuged into a pellet at 1,200 rpm for 5 min, and the medium was removed. The cells were resuspended in 98 μL Nucleofector solution (Amaxa Biosystems) at room temperature followed by the addition of 2 μL of 50 μmol/L miR-21 precursor (pre-miR-21) or miR-21 inhibitor (anti-miR-21, specific antisense oligonucleotide) and a negative control precursor or control inhibitor (all obtained from Ambion). According to the manufacturer’s information,6 pre-miR negative controls and anti-miR negative control are random sequence molecules that have been extensively tested in human cell lines and tissues and validated to not produce identifiable effects on known miRNA function. The transfected cells were resuspended and cultured in regular culture medium containing 10% serum for 24 h before analysis.

Quantitative Real-time Reverse Transcription-PCR Assay for miR-21 Expression
Total RNA and miRNA fractions were isolated from tissues and cell lines using a mirVana microRNA isolation kit (Ambion), and their RNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) at 260 and 280 nm (A260/A280). RNA integrity was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies). Quantitative real-time reverse transcription-PCR (qRT-PCR) assays for frozen tissue samples were done using the TaqMan miRNA Assay (Applied Biosystems) in accordance with the manufacturer's instructions. Normalization was done with the small nuclear RNA U6 (RNU6B; Applied Biosystems). qRT-PCR assays for cell lines were done using SuperTaq polymerase, a mirVana qRT-PCR miRNA Detection Kit, and mirVana Primer Sets (all from Ambion) according to the manufacturer’s instructions. The expression levels of miR-21 in all cell lines were also normalized by the expression level of small nuclear RNA U6. RT-PCR was done in a Chromo4 Real-time PCR Detection System (Bio-Rad Laboratories) for 40 cycles of 15 s at 95°C and 1 min at 60°C.

qRT-PCR Assay for mRNA Expression
We assessed the mRNA expression of various genes, which have been suggested as direct or indirect targets for miR-21. One-step qRT-PCR was done using a Quantitect SYBR Green RT-PCR Kit (Qiagen) as described previously (23). The PCR primer sequences used in this study were described in the Supplementary Table S1.7

Cell Proliferation Assay
Cell proliferation was evaluated by measuring the fluorescence intensity of propidium iodide (PI) as described previously (23, 24). Pancreatic cancer cells were transfected with either the miR-21 precursor or inhibitor (antisense oligonucleotide), and negative control precursor or control inhibitor, and were seeded in 90 mm dishes at 1 × 10^6. At 24 h after the transfection, only viable cells were plated at 2 × 10^4 per well in 24-well tissue culture plates (Becton Dickinson Labware) and cultured for 24 h. Cell proliferation was

6 http://www.ambion.com/index.html
7 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
evaluated after culture for a further 96 h after the initial cell number determination by the PI assay. To evaluate chemoresistance, the PI assay was done as for cell survival. At 24 h after the transfection, the viable cells were plated at $3 \times 10^4$ per well in 24-well tissue culture plates and cultured for 24 h. The transfected pancreatic cancer cells were further cultured in the presence of 1 to 1,000 nmol/L gemcitabine for 72 h. PI (30 μmol/L) and digitonin (600 μmol/L) were added to each well to label all nuclei with PI. The fluorescence intensity, corresponding to the total cell number, was measured using a CytoFluor II multiwell plate reader (PerSeptive Biosystems) with 530 nm excitation and 645 nm emission filters. A separate well, which possessed the same medium but no cells, was used for a baseline PI signal as a control. We evaluated the difference between each sample well and the control well. Cell proliferation was defined relative to the cell number measured at the beginning of the experiment. All experiments were done in triplicate wells and repeated at least three times.

Invasion Assay
Using PANC-1, SUIT-2, and CFPAC-1 cells, the invasion of pancreatic cancer cells was measured by the number of cells invading Matrigel-coated Transwell chambers (Becton Dickinson). Transwell inserts with 8 μm pores were coated with Matrigel (40 μg/well for PANC-1 cells and 20 μg/well for SUIT-2 and CFPAC-1 cells; Becton Dickinson) and reconstituted with 10% fetal bovine serum-containing medium for 2 h before the experiment. Cells ($2 \times 10^5$/mL) were seeded into the upper chambers in 250 μL DMEM supplemented with 10% fetal bovine serum. The same medium (750 μL) was placed in the lower wells. Thereafter, the PANC-1 and CFPAC-1 cells were incubated for 72 h. Cells that had degraded the Matrigel and invaded to the lower surface of the Matrigel-coated membranes were fixed with 70% ethanol, stained with H&E, and counted in five random fields at ×200 magnification under a light microscope. The results were expressed as the average number of invasive cells per field.

Flow Cytometric Analysis and Apoptosis Measurement
Cells were seeded into six-well plates at $1 \times 10^5$ per well in triplicate. The next day, nonadhered cells were removed by gentle washing, and the medium was replaced with fresh medium containing 1 μmol/L gemcitabine. After 24 h, the cells were retrieved and analyzed by an EPICS ALTRA flow cytometer (Beckman Coulter) using an Annexin V-FITC/PI kit (BD Biosciences) as described previously (25). Positive Annexin V cells were considered apoptotic regardless of PI staining. Cells staining positive for PI uptake were considered dead regardless of Annexin V staining (25).

Statistical Analysis
Tissue sample data were analyzed using the Mann-Whitney U test for comparisons involving two groups because a normal distribution was not obtained. Other data were expressed as the mean ± SD and evaluated with a double-sided Student’s t test. Values of $P < 0.05$ were accepted as statistically significant in any analysis. All experiments were repeated at least three times.

Results
miR-21 Is Markedly Overexpressed in Pancreatic Cancer Cell Lines Compared with Nonmalignant Cells
We measured the expression of miR-21 in 14 pancreatic cancer cell lines, HPDE cells, and primary cultures of normal pancreatic epithelial cells and pancreatic fibroblasts by qRT-PCR (Fig. 1A). There was a wide range of relative miR-21 expression levels among the pancreatic cancer cell lines. However, all of the pancreatic cancer cells and HPDE cells expressed remarkably higher levels of miR-21 than the primary normal pancreatic epithelial cells, which did not express detectable levels of miR-21. All the pancreatic cancer cells expressed 1.1- to 5.0-fold (median, 1.8) higher levels of miR-21 than the HPDE cell line, which is an immortalized normal pancreatic ductal epithelial cell line. The levels of miR-21 in pancreatic cancer-related fibroblasts were lower than those in all of the pancreatic cancer cell lines, except AsPC-1 and NOR-P1.

miR-21 Is Markedly Overexpressed in Pancreatic Cancer Tissues Compared with Normal Pancreatic Tissues
We measured the expression of miR-21 in 25 pancreatic cancer tissues and 25 normal pancreatic tissues by
qRT-PCR (Fig. 1B) and found that the expression of miR-21 in cancer tissues was much higher than in nonmalignant tissues \((P < 0.001\), Mann-Whitney). These results are consistent with the previous studies in pancreatic cancer \((15, 26)\). Although we investigated the correlation between miR-21 expression levels and prognosis or other clinicopathologic findings in pancreatic cancer, we did not find any correlation between them (Supplementary Table S2; Supplementary Fig. S2).7

Overexpression of miR-21 Promotes Proliferation of Pancreatic Cancer Cells

To investigate the transfection efficiency of the miR-21 precursor or inhibitor, we first measured the levels of mature tmiR-21 in pancreatic cancer cells transfected with the miR-21 precursor, inhibitor, and each negative control by qRT-PCR. For these experiments, we used a miR-21 precursor to PANC-1 and CAPAN-1 cells that expressed relatively lower level of miR-21, whereas we used a miR-21 inhibitor to CFPAC-1 and SUIT-2 cells that expressed the highest level and the third highest level of miR-21 among the pancreatic cancer cell lines (Fig. 1A). At 48 h after transfection with 100 pmol of the miR-21 precursor or miR-21 inhibitor, CAPAN-1 and PACAN-1 cells transfected with the miR-21 precursor exhibited 3.0- to 3.5-fold higher level of mature miR-21 than the cells transfected with the negative control precursor (Supplementary Fig. S1A).7 In contrast, the CFPAC-1 and SUIT-2 cells transfected with the miR-21 inhibitor showed 0.6-fold lower levels of mature miR-21.
than the cells transfected with the negative control inhibitor (Supplementary Fig. S1B). There were no morphologic differences between the transfected cells.

Next, we performed PI assays on PANC-1 cells, which expressed relatively lower levels of miR-21, after transfection with the miR-21 precursor or negative control precursor to examine the effects of miR-21 on cell proliferation. PANC-1 cells transfected with the miR-21 precursor showed significantly increased cell proliferation (3.0-fold) compared with the control cells at 120 h after transfection (Fig. 2A; \( P < 0.001 \)). We also investigated the effect of the miR-21 inhibitor on the proliferation of CFPAC-1 cells, which expressed the highest levels of miR-21. We found that the CFPAC-1 cells transfected with the miR-21 inhibitor showed significantly decreased cell proliferation (0.7-fold) compared with the control cells (Fig. 2B; \( P < 0.001 \)).

**Invasion of Pancreatic Cancer Cells Is Affected by miR-21**

Next, we investigated the effects of miR-21 on invasion, which is an important function for malignant progression and metastases, using a Matrigel invasion assay. We used PANC-1, AsPC-1, and CFPAC-1 cells for these experiments. PANC-1 cells, which expressed relatively lower levels of miR-21, exhibited significantly increased cell invasion (2.2-fold) compared with the control cells at 72 h after seeding the cells transfected with the miR-21 precursor (Fig. 3A; \( P = 0.002 \)), although there were no changes in the proliferation of PANC-1 cells at this time point (Fig. 2A). CFPAC-1 cells, which expressed the highest levels of miR-21, also exhibited markedly decreased cell invasion (0.3-fold) at 72 h after transfection of the miR-21 inhibitors compared with the control cells (Fig. 3B; \( P < 0.001 \)). We found no change in invasion of AsPC-1 and PANC-1 cells with low levels of miR-21 expression after transfection of miR-21 inhibitors (data not shown).

**miR-21 Affects Matrix Metalloproteinase-2 and -9 and Vascular Endothelial Growth Factor Expression**

There is a previous report that miR-21 mediates matrix metalloproteinase (MMP)-2 and MMP-9 mRNA expression in hepatocellular cancer (19). Therefore, we assessed the expression of both genes by RT-PCR and found that miR-21 positively modulates the mRNA expression of both MMP-2 and MMP-9 (Fig. 4A). These results suggest that miR-21 promotes cell invasion of pancreatic cancer by indirectly mediating the expression of MMP-2 and MMP-9. Additionally, we also evaluated the expression of vascular...
endothelial growth factor (VEGF), which promotes migration and invasion of pancreatic cancer cells (27, 28), and found that overexpression of miR-21 significantly up-regulates the mRNA expression of VEGF (Fig. 4B).

miR-21 Decreases the Gemcitabine Sensitivity of Pancreatic Cancer Cells

To further assess the effect of the miR-21 precursor and miR-21 inhibitor on the sensitivity to chemotherapy, we investigated the cell survival rates of PANC-1 and SUIT-2 cells after treatment with an anticancer agent because its treatment is accepted as the first-line chemotherapy for advanced pancreatic cancer (29). The cell survival rates were evaluated at 72 h after treatment with 1 to 1,000 nmol/L gemcitabine. The survival rate of PANC-1 cells, which expressed relatively lower levels of miR-21, transfected with the miR-21 precursor was higher than the survival rate of PANC-1 cells transfected with the negative control precursor after treatment with 10 to 100 nmol/L gemcitabine (Fig. 5A; P < 0.01). We also evaluated apoptosis of miR-21 in PANC-1 cells at 24 h after the treatment with 1 μmol/L gemcitabine and found that the increase in miR-21 expression reduced the number of apoptotic cells (Fig. 5B; P = 0.008). Moreover, the survival rate of SUIT-2 cells, which expressed relatively higher levels of miR-21, transfected with the miR-21 inhibitors was lower than that of SUIT-2 cells transfected with the negative control inhibitor after treatment with 5 to 20 nmol/L gemcitabine (Fig. 5C; P < 0.05).

In earlier studies, several tumor suppressor genes have been reported as targets of miR-21, including tropomyosin 1 (30), programmed cell death 4 (31, 32), and phosphatase and tensin homologue (18, 19). To investigate the more detailed mechanism of miR-21-induced chemoresistance and antiapoptotic effects, we evaluated the expression levels of these direct target genes of miR-21 in pancreatic cancer cells. However, qRT-PCR analyses revealed that the basic levels of tropomyosin 1 and programmed cell death 4 genes were undetectable or extremely low in CAPAN-1, CFPAC-1, PANC-1, and SUIT-2 cells and that inhibition of miR-21 did not up-regulate the levels of these genes. The results suggest that these genes may not be functional in pancreatic cancer cells (data not shown). We also found no significant change in the level of phosphatase and tensin homologue expression after transfection with miR-21 precursors and inhibitors (Supplementary Fig. S3A and B).7

Discussion

In the present study, we have obtained the first data regarding the biological functions of miR-21 in pancreatic cancer. We found that miR-21 expression was increased in pancreatic cancer cells and that miR-21 contributed to cell proliferation, invasion, and chemoresistance. Our present data are consistent with previous reports on various types of tumors, which revealed that miR-21 is strongly overexpressed in malignant cells and that inhibition of miR-21 activity leads to a significant reduction in cell viability (13, 17–19).

The present study showed that the level of miR-21 expression in HPDE is similar to that of several pancreatic cancer cell lines and much higher than that of normal pancreatic epithelial cells. Although HPDE cells have a near normal genotype and phenotype as a previous study demonstrated (33), the cells are immortalized unlike the primary normal epithelial cells. Therefore, miR-21 expression may be related to cell immortalization. We also found that inhibition of miR-21 decrease proliferation of AsPC-1 and PANC-1 cells even at low levels of miR-21. However, we found no change in invasion of these cells after transfection with miR-21 inhibitors. These data suggest that miR-21 have a functional role only in proliferation but not in invasion of pancreatic cancer cells with low levels of miR-21. However, these differences are cell context dependent and are not surprising. Motility and proliferation may use the same redundant signaling pathways regulated by miR-21.

Figure 4. Effects of miR-21 on MMP-2, MMP-9, and VEGF mRNA expression. A, MMP-2 and MMP-9 mRNA were assessed by qRT-PCR. miR-21 modulates positively the mRNA expression of MMP-2 and MMP-9 (*, P = 0.002; **, P = 0.010, Student’s t test). B, VEGF mRNA was also assessed by qRT-PCR. miR-21 positively modulates VEGF mRNA expression (*, P = 0.042; **, P = 0.005, Student’s t test). Mean ± SD of triplicate measurements.

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Slaby et al. (34) showed that the expression of miR-21 is positively correlated with clinical stage, lymph node metastasis, and distant metastasis in patients with colorectal cancer. Although the present study revealed that the expression of miR-21 in pancreatic cancer tissues was much higher than in nonmalignant tissues, we did not find any correlation between levels of miR-21 expression and clinicopathologic findings. This may be due to the small number of cancer tissue samples and/or the use of bulk tissues. Further studies based on large-scale analyses of microdissected cells are needed.

It has been reported that miR-21 mediates MMP-2 and MMP-9 mRNA expression in hepatocellular cancer (19). The present data also revealed that cell invasion of pancreatic cancer was significantly promoted by overexpression of miR-21. Therefore, we investigated the effect of miR-21 on invasion-related genes (27, 28) and found that the mRNA expression of MMP-2, MMP-9, and VEGF were positively correlated with miR-21 expression. Although MMP-2, MMP-9, and VEGF are “indirect” targets of miR-21, identifying such indirect genes is important to understand the function of miR-21 on cell invasion of pancreatic cancer. However, further studies are needed to identify the direct targets of miR-21.

The present study revealed that miR-21 affected sensitivity to gemcitabine in pancreatic cancer cells and reduced the number of gemcitabine-induced apoptotic cells. These findings are consistent with a previous report regarding cholangiocarcinoma (18). To date, there is no report to clarify the detailed mechanism of the miR-21-induced chemoresistance, although several studies have revealed significant correlations between miR-21 expression and resistance to anticancer agents (18, 35) and anti-apoptotic effect in other tumor cells (13, 17). Meng et al. reported that gemcitabine-induced apoptosis is inhibited by miR-21 via phosphatase and tensin homologue and the phosphatidylinositol 3-kinase pathway (18). Also, tropomyosin 1 (30) in breast cancer and programmed cell death 4 in breast and colon cancer (31, 36) have been reported as target tumor suppressor genes of miR-21. However, the present study suggests that the pancreatic cancer cells did not express significant levels of tropomyosin 1 and programmed cell death 4 genes and that miR-21 did not affect the level of phosphatase and tensin homologue expression in pancreatic cancer cells. Therefore, further studies are needed to identify the target chemoresistance-related genes of miR-21 in pancreatic cancer cells.

In conclusion, we found that miR-21 is overexpressed in pancreatic cancer and that miR-21 promotes pancreatic cancer cell proliferation and invasion in vitro. Moreover, miR-21 contributed to the increased chemoresistance in pancreatic cancer cells. These data suggest that miR-21 may serve as a potential target for pancreatic cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
miR-21 Elicits Malignant Progression in Pancreatic Cancer

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