Original Article

microRNA-137 modulates pancreatic cancer cells tumor growth, invasion and sensitivity to chemotherapy

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Abstract: Background: We intended to investigate the role of microRNA 137 (miR-137) in regulating pancreatic cancer cells' growth in vitro and tumor development in vivo. Methods: QTR-PCR was used to examine the expression of miR-137 in pancreatic cancer cell lines and tumor cells from human patients. Lentiviral vector containing miR-137 mimic was used to overexpress miR-137 in PANC-1 and MIA PaCa-2 cells. The effects of overexpressing miR-137 on pancreatic cancer cell invasion and chemo-sensitivity to 5-fluorouracil (5-FU) were examined by cell migration and survival essays in vitro. The molecular target of miR-137, pleiotropic growth factor (PTN), was down-regulated by siRNA to examine its effects on cancer cell invasion. MIA PaCa-2 cells with endogenously overexpressed miR-137 were transplanted into null mice to examine tumor growth in vivo. Results: We found miR-137 was markedly underexpressed in both pancreatic cancer cell lines and tumor cells from human patients. In cancer cells, transfection of lentivirus containing miR-137 mimic was able to markedly upregulate endogenous expression of miR-137, inhibited cancer cell invasion and increased sensitivities to chemotherapy reagent 5-FU. PTN was significantly down-regulated by siRNA to examine its effects on cancer cell invasion. MIA PaCa-2 cells with endogenously overexpressed miR-137 were transplanted into null mice to examine tumor growth in vivo. Results: We found miR-137 was markedly underexpressed in both pancreatic cancer cell lines and tumor cells from patients. In cancer cells, transfection of lentivirus containing miR-137 mimic was able to markedly upregulate endogenous expression of miR-137, inhibited cancer cell invasion and increased sensitivities to chemotherapy reagent 5-FU. PTN was significantly down-regulated by overexpressing miR-137 in pancreatic cancer cells, and knocking down PTN was effective to rescue the reduced cancer cell invasion ability caused by miR-137 overexpression. More importantly, overexpressing miR-137 led to significant inhibition on tumor formation, including reductions in tumor weight and tumor size in vivo. Conclusion: Our study demonstrated that miR-137 played an important role in pancreatic cancer development. It may become a new therapeutic target for gene therapy in patients suffered from pancreatic cancer.

Keywords: miRNA, miR-137, invasion, PTN, pancreatic cancer

Introduction

Pancreatic cancer is the seventh leading cause of cancer deaths in China and the fourth leading cause of cancer deaths in the United States [1, 2]. In the United States alone, more than quarter million patients die from pancreatic cancer each year. The prognosis of patients with pancreatic cancer is one of the worst in all cancer forms, since there has been little progress in cancer diagnosis and treatment strategies during the past decades [3, 4]. Thus, it is of great interest to both research and clinic communities to seeking novel methods for early detections and therapeutic strategies to treat patients suffered from pancreatic cancers.

MicroRNAs (miRNAs) comprise a class of 19–23 nucleotides of noncoding RNAs regulating endogenous gene expression by targeting translational cleavage or repression [5]. In recent years, miRNAs have been shown to modulate various biological processes, including embryogenesis, tissue development, neural differentiation and apoptosis [6-8]. Most importantly, a growing number of evidence had demonstrated that miRNA expression levels were directly associated with cancer cell formation, development and pathology [6, 9-11]. Among them, the family of miR-137s has been shown to be expressed in various carcinoma tissues and its over- or down-expression played essential role in tumor formation or cancer cell apoptosis, including lung cancer [12, 13], breast cancer [14] and melanoma [15, 16]. However, little is known about the expression of miR-137 or its functional role in regulating pancreatic cancer cells either in vitro or in vivo.

In the present study, we first examined the molecular expressions of miR-137 in both pancreatic cancer cell lines and tumors from pan-
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creatic cancer patients. Second, we investigated the biological function of miR-137 in pancreatic cancer cells using a lentiviral vector of miR-137 mimic to overexpress miR-137 in vitro. Third, we explored the possible target of miR-137 regulation in pancreatic cancer cells and used siRNA to investigate its functional role. Finally, we overexpressed miR-137 in transplanted pancreatic cancer cells to examine its effect on tumor growth in vivo. Our data suggested that miR-137 played important roles in regulating cancer cell invasion, chemo-resistance and tumor growth of pancreatic cancer.

Materials and methods

Cell lines and cultures

There were 8 pancreatic cancer cell lines used: AsPC-1, BxPc-3, Capan-1, Capan-2, CFPAC-1, PANC-1, MIA PaCa-2 and SW1990 (American Type Culture Collection). Human pancreas ductal epithelial (HPDE) cells were obtained from M.S. Tsao (Ontario Cancer Institute, Ontario, Canada) and maintained as previously described [17]. Primary human normal pancreatic epithelial cells were obtained from the Cell Systems and cultured in CS-C medium containing 10% fetal bovine serum according to the manufacturer’s instructions.

Tissue samples

The human pancreatic tissue samples were obtained from the patients through a surgical protocol to resect a portion of the pancreas in the Department of Hepatobiliary Surgery, Affiliated Hospital of Guiyang Medical College, China, from January 2013 to February 2014. Normal pancreatic tissues were also taken from areas of peripheral tissues away from the tumor. Tissues were quickly removed and a part of each sample was embedded in OCT compound. Histological examination was then performed of all tissues adjacent to the specimens. Consent forms were obtained from all patients. All procedures of the present study were reviewed and approved by the Ethics Committees of Affiliated Hospital of Guiyang Medical College and Affiliated Tongji Hospital.

Lentivirus construction and transfection

The oligonucleotides of hsa-miR-137 mimic and its non-specific control were synthesized by Ribobio (RiboBio, Shanghai, China). The coding sequences were then amplified and cloned into pCDH-CMV-MCS-EF1-coGFP constructs (System Biosciences, California, USA) to generate miR-137 mimic oligonucleotide vector (miR-137 mimic) and its non-specific control vector (control). Then, according to manufacturer’s instruction, the lentiviral expression constructs and pPACK packaging plasmid mix were co-transfected into 293T cells and viral particles were collected and the titer was determined. Pancreatic cells were then transfected with miR-137 mimic or control by using Lipofectamine 2000 (Invitrogen, Foster city, CA).

Quantitative real-time reverse transcription-PCR (qRT-PCR)

Total RNA and miRNA fractions were isolated from tissues and cell lines with Trizol reagent according to manufacturer’s protocol (Invitrogen, USA). Total RNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) at 260 and 280 nm (A260/280), and examined with an Agilent 2100 Bioanalyzer (Agilent Technologies). Quantitative real-time reverse transcription-PCR (qRT-PCR) assays were done using the TaqMan miRNA Assay according to manufacturer’s protocol (Applied Biosystems). The amplification conditions were 40 cycles of 15 s at 95°C and 1 min at 60°C. The expression levels of miR-137 were also normalized by the expression level of house keeping gene U6.

Matrigel invasion assay

The invasion capabilities of pancreatic cancer cells PANC-1 and MIA PaCa-2 were measured by the number of cells invading Matrigel-coated Transwell chambers (Becton Dickinson). Transwell inserts were coated with Matrigel (40 μg/well, Becton Dickinson) and reconstituted with 10% fetal bovine serum-containing medium for 2 h before the experiment. Pancreatic cancer cells (2 × 10^5/mL) were cultured into the upper chambers in 250 μL DMEM supplemented with 10% fetal bovine serum for 72 hours. Culture medium without cells was placed in the lower wells. Cells that invaded into the lower surface of the Matrigel-coated membranes were fixed with 70% ethanol, stained with H&E, and averaged across five random fields at ×200 magnification under a light microscope.

Cell survival assay and 5-FU treatment

Cell survival or proliferation was evaluated by measuring the fluorescence intensity of prop-
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Figure 1. miR-137 expression levels in human pancreatic cell lines and clinical samples. A. miR-137 expression levels relative to HPDE as examined by qRT-PCR (*, P < 0.05, as compared to HPDE). Data were presented as Mean ± SE of triplicate. B. miR-137 expression levels in 25 cancer tissues and 25 nonmalignant tissues as examined by qRT-PCR (***, P < 0.05). Data were presented as mean ± SD of triplicate.

idium iodide (PI) as previously described [18]. PANC-1 and MIA PaCa-2 cells were transfected with either the miR-137 mimic or control, or without transfection, and were seeded in 60 mm dishes with total cell number of one million. At 24 h after the transfection, the viable cells were plated at in a 24-well tissue culture plate (2 × 10^4 per well) for 24 h, then treated with 1 to 100 mg/mL 5-FU for 72 h. Then, PI (30 μmol/L) and digitonin (600 μmol/L) were added to each well to label all nuclei with PI. The fluorescence intensity was measured using a CytoFluor II multiwell plate reader (PerSeptive Biosystems) with 530 nm excitation and 645 nm emission filters. Surviving cell numbers were normalized to the cell number measured at the beginning of the experiment. All experiments were done in triplicate wells and repeated at least three times.

Western blot analysis

For western blot analysis, lysates of PANC-1 or MIA PaCa-2 cells were extracted with lysis buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 1 mM EDTA, 10% glycerol, and 0.5% NP-40 and protease inhibitor cocktail (Sigma, USA). Protein products were then resolved with 10% SDS-PAGE gel and transferred to the nitro-
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Figure 2. Transfection efficiency with miR-137 control or mimic in pancreatic cancer cells. A. MIA PaCa-2 cells transfected with lentiviral vector expressing GFP were shown under transmitted lights (left) and TRIC fluorescence (right). B. miR-137 expression was assessed in MIA PaCa-2 cells 48 hours after transfection with 100 pmol of miR-137 control or mimics. C. miR-137 expression was assessed in PANC-1 cells 48 hours after transfection with 100 pmol of miR-137 control or mimics. (*, P < 0.05).

cellulose membranes. They were then incubated with primary antibody of PTN (SantaCruz, USA) according to manufacturer’s instruction and then incubated with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA). The western blots were visualized with an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ) according to the manufacturer’s instructions.

Transfection of siRNA

The specific PTN siRNA (PTN-siRNA) and its non-specific scramble siRNA (NC-siRNA) were purchased from Stanta Cruz (Santa Cruz Biotechnology, USA), and the transfection of siRNAs with Lipofectamine 2000 were conducted according to manufacturer’s recommended protocol. Briefly, in both PANC-1 and MIA PaCa-2 cells, after 24 hours transfection of lentivirus of miR-137 mimic, cells were further transfected with either NC-siRNA (100 nM) or PTN-siRNA (100 nM) for additional 48 hours before examination. The efficiency of siRNA on knocking down PTN was examined by western blot analysis 48 hours after transfection.

Statistical analysis

In vivo tumor volumes were analyzed using the one-way ANOVA. Other data were presented as the mean ± SD and evaluated with a Student’s t test. Statistically significance was determined if P < 0.05 in any analysis. All experiments were repeated at least three times.

Results

miR-137 is markedly underexpressed in pancreatic cancer tissues and pancreatic cancer cell lines

We used qRT-PCR to examine the expression levels of miR-137 in 9 pancreatic cancer cell lines, HPDE cells and primary culture of normal pancreatic epithelia cells (Figure 1A). The results showed that, in all of the pancreatic cancer cells the expression levels of miR-137 were markedly lower than the level in HPDE cell or primary normal pancreatic epithelia cells.

We then used qRT-PCR to examine the expression of miR-137 in 12 pancreatic cancer tissues and 12 normal pancreatic tissues (Figure 1B). We found that the expression of miR-137 in cancer tissues was much lower than in non-malignant tissues (***P < 0.05).

Overexpression of miR-137 inhibited pancreatic cancer cell invasion

To investigate the transfection efficiency of lentivirus of miR-137, we first measured the levels of mature tmiR-137 in pancreatic cancer cells transfected with the miR-137 negative control and mimics by qRT-PCR. First, while MIA PaCa-
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Figure 3. miR-137 inhibited invasion of pancreatic cancer cells. PANC-1 and MIA PaCa-2 cells were transfected with the lentivirus of miR-137 control or mimics and the cell invasion activities were assessed after 72 h. A. Decreased Matrigel invasion with PANC-1 cells was seen with miR-137 transfection (***, \( P < 0.05 \), compared to control). B. Decreased Matrigel invasion with CFPAC-1 cells was also seen with miR-137 mimics transfection (***, \( P < 0.05 \), compared to control).

2 was transfected with lentivirus expressing GFP, 95% of the cells showed green fluorescence, confirming that the efficacy of lentiviral transfection was very high (Figure 2A). Then, we transfected two pancreatic cancer cell lines, MIA PaCa-2 and PANC-1, with lentivirus containing either miR-137 negative control or miR-137 mimics (100 pmol) for 48 hours. The expression levels of miR-137, as measured by qRT-PCR, were significantly increased by miR-137 mimics in both cell lines. In MIA PaCa-2 cells, the cells transfected with the mimics exhibited ~170.4 folds higher level of miR-137 than the cells transfected with the negative control (Figure 2B, \( P < 0.05 \)). In PANC-1 cells, the cells transfected with the mimics exhibited ~99.1 folds higher level of miR-137 than the cells transfected with the negative control (Figure 2C, \( P < 0.05 \)).

Then, we examined the effects of miR-137 on pancreatic cancer cell invasion with a Matrigel invasion assay. We used PANC-1 and MIA PaCa-2 cells for these experiments and transfected them with either miR-137 control or miR-137 mimic lentiviruses. In both cell lines, cell invasions were significantly decreased by miR-137 mimics, as compared to the control condition after 72 hours (Figure 3, \( P < 0.05 \)).
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Figure 4. Effects of miR-137 on pancreatic cancer cell 5-fluorouracil chemoresistance. PANC-1 and MIA PaCa-2 cells were transfected with lentiviruses of miR-137 control or mimics, compared with the cells without transfection (non-transfection). Their chemosensitivities were examined by the cell survival assay 72 h after treatment with 0 to 100 μg/mL 5-fluorouracil (5-FU). In both PANC-1 cells (A) and MIA PaCa-2 cells (B), the survival rates of the cells transfected with the miR-137 mimic were significantly lower than those of the cells transfected with control vector, or without transfection, after treatment with 1 - 20 μg/mL 5-FU (*, $P < 0.05$, Student’s t test).

Overexpression of miR-137 increased 5-FU sensitivity of pancreatic cancer cells

To further investigate the role of the miR-137 in regulating the sensitivity to chemotherapy, we investigated the cell survival rates of PANC-1 and MIA PaCa-2 cells after treatment with an anticancer agent 5-FU (Figure 4). The cells were either without transfection, or transfected with lentiviruses expression miR-137 mimic or non-specific control vectors. The cell survival rates were evaluated 72 h after treatment with 0 to 100 mg/mL 5-FU. The result showed that after treatment with 1 to 20 mg/mL 5-FU, in both PANC-1 and MIA PaCa-2 cells, the survival rates were markedly lower in the cells transfected with miR-137 mimic, than the survival rate of cells transfected with the negative control or of cells without transfection (Figure 5A, 5B; $P < 0.05$).

PTN was the target of miR-137 regulation in pancreatic cancer cells

Then, we intended to explore the molecular targets of miR-137 during its regulation in pancreatic cancer. Through the analysis of several miRNA prediction software including TargetScan, miRanda or MicroCosm, we identified that pleiotropic growth factor (PTN) was likely the candidate to be bound by miR-137. We looked into the mRNA expression patterns of PTN in both PANC-1 and MIA PaCa-2 cells under the regulation of miR-137, and found that PTN were significantly down-regulated in both pancreatic cancer cell lines by overexpressing miR-137 (Figure 5A).

We then speculated that PTN had a direct role in miR-137’s regulation on pancreatic cancer cell functions. We applied siRNA to knock down PTN expressions in cancer cells. First, both PANC-1 and MIA PaCa-2 were treated with either non-specific scrambled siRNA (NC-siRNA, 100 nM) or PTN specific siRNA (PTN-siRNA, 100 nM) for 48 hours, and the knockdown efficiency was verified by western blotting analysis. It showed that PTN-siRNA was very effective to down-regulate PTN expression in both PANC-1 and MIA PaCa-2 cells (Figure 5B). Second, we examined whether knocking down PTN could affect the cancer cell invasion capability reduced by miR-137 overexpression. The results of Matrigel invasion essays demonstrated that, in both PANC-1 and MIA PaCa-2 cells, after the initial treatment of lentivirus of miR-137 mimic, cell invasion capabilities were greatly restored by PTN-siRNA, suggesting that PTN was the direct target of miR-137 in pancreatic cancer (Figure 5C).

Overexpression of miR-137 inhibited the MIA PaCa-2 tumor formation in nude mice in vivo

Finally, we examine the effect of miR-137 overexpression on tumor initiation in vivo. MIA PaCa-2 cells were transfected with miR-137 mimic or control for 24 hours. Cells were col-
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Figure 5. PTN is the direct target of miR-137 in pancreatic cancer. A. Both PANC-1 and MIA PaCa-2 cells were either treated without lentivirus (control), or with miR-137 mimic lentivirus (miR-137 mimic), followed by q-PCR to measure the expression of PTN in 24 hours. Data were normalized to the expression level under control condition. (*, P < 0.05). B. Both PANC-1 and MIA PaCa-2 cells were either treated with non-specific scrambled siRNA (NC-siRNA, 100 nM), or PTN siRNA (PTN-siRNA, 100 nM) for 48 hours, followed by western blotting analysis. C. Both PANC-1 and MIA PaCa-2 were initially transfected with the lentivirus of miR-137 mimic for 24 hours, then treated with NC-siRNA (100 nM) or PTN-siRNA (100 nM) for another 48 hours, followed by matrigel invasion essay. (***, P < 0.05).

In the present study, we also demonstrated that PTN was very likely the direct target of miR-137 in pancreatic cancer. The expression levels of PTN were significantly up-regulated by miR-137 overexpression, and down-regulating PTN restored the invasive capability of cancer cell in vitro induced by miR-137 overexpression. It has been shown that PTN was also acting as tumor suppressor in pancreatic cancer [22]. But its directly involved signaling pathways were largely unknown. The results of our study showing that down-regulation of PTN was associated with over-expression of miR-137 confirmed that, miR-137 was at least one of the initiator to

Discussions

In the present study, we presented the first group of data characterizing the biological functions of miR-137 in pancreatic cancer. We demonstrated that miR-137 expression was markedly under-expressed in pancreatic cancer cell lines and tumor cells from patient samples, and that overexpressing miR-137 inhibited cell invasion, increased chemoresistance and suppressed tumor growth in vivo. These results are in line with previous studies showing inhibition of miR-137 activity led to a significant increase in cancer cell growth and carcinogenesis [19-21]. Thus, it is very likely that the molecular mechanism of miR-137 in pancreatic cancer was similar to the mechanism in other cancers, that miR-137 often acted as a tumor suppressor in normal tissues and its down-regulation induced carcinogenesis and tumor growth.

lected and inoculated into female athymic nude mice subcutaneously (s. c.). Our results showed that, miR-137 overexpression significantly inhibited MIA PaCa-2 tumor sizes in vivo (Figure 6). The average tumor weight in mice inoculated with miR-137 mimic was also significantly smaller than the weight with control lentivirus (Figure 6B, 6C).
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Figure 6. miR-137 overexpression inhibited the MiaPaCa2 tumor initiation in nude mice. MIA PaCa-2 cells were transfected with miR-137 mimic or control for 24 hours. Cells were collected and inoculated into female athymic nude mice subcutaneously (s.c.) on both sides of flank with the concentration of 5 million/mL. A. Tumor volume was calculated using the formula: (length × width 2)/2 from day 5 to day 35 (*, P < 0.05, one-way ANOVA). B. On day 35, all tumors were collected to measure the tumor weights (***, P < 0.05, student’s t-test). C. Also on day 35, images of the tumors were shown.

exert anti-cancer effect through PTN in pancreatic cancer. Moreover, our result showing that knocking down PTN was also able decrease pancreatic cancer cell invasive capability. This result suggested miR-137 and PTN can also act independently, activate their own cascade of down-stream molecular pathways to regulate pancreatic cancer development. Thus, further experiments of elucidating the independence or association of miR-137 and PTN -directed pathways would greatly advance our knowledge on the regulation of miRNAs in pancreatic cancer.

In conclusion, our study demonstrated, for the first time, that miR-137 was underexpressed in pancreatic carcinoma or cells. Overexpression of miR-137 could reduce cancer cell invasion, increase chemosensitivity and inhibit tumor growth. Moreover, we identified that PTN was likely the direct target of miR-137 as knocking down PTN also reduced pancreatic cancer cell invasion capability. Our results provided novel insight into how miR-137 worked in pancreatic cancer cells. The method of enhancing tumor suppressor miR-137 may provide a novel therapeutic approach for patients with pancreatic cancer.

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Disclosure of conflict of interest

None.

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References