microRNA-218 suppresses the proliferation, invasion and promotes apoptosis of pancreatic cancer cells by targeting HMGB1

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Objective: To detect the expression profiles of microRNA-218 (miR-218) in human pancreatic cancer tissue (PCT) and cells and their effects on the biological features of human pancreatic cancer cell line PANC-1 and observe the effect of miR-218 on the expression of the target gene high mobility group box 1 (HMGB1), with an attempt to provide new treatment methods and strategies for pancreatic cancer.

Methods: The expressions of miR-218 in PCT and normal pancreas tissue as well as in various pancreatic cancer cell lines including AsPC-1, BxPC-3, and PANC-1 were determined with quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). The change of miR-218 expression in PANC-1 cells was detected using qRT-PCT after the transfection of miR-218 mimic for 48 h. Cell Counting Kit-8 (CCK-8) was applied for detecting the effect of miR-218 on the activity of PANC-1 cells. The effects of miR-218 on the proliferation and apoptosis of PANC-1 cells were analyzed using the flow cytometry. The effect of miR-218 on the migration of PANC-1 cells was detected using the Trans-well migration assay. The HMGB1 was found to be a target gene of miR-218 by luciferase reporter assay, and the effect of miR-218 on the expression of HMGB1 protein in cells were determined using Western blotting.

Results: As shown by qRT-PCR, the expressions of miR-218 in PCT and in pancreatic cancer cell line significantly decreased when compared with the normal pancreatic tissue (NPT) (P<0.01). Compared with the control group, the miR-218 expression significantly increased in the PANC-1 group after the transfection of miR-218 mimic for 48 h (P<0.01). Growth curve showed that the cell viability significantly dropped after the overexpression of miR-218 in the PANC-1 cells for two days (P<0.05). Flow cytometry showed that the S-phase fraction significantly dropped after the overexpression of miR-218 (P<0.01) and the percentage of apoptotic cells significantly increased (P<0.01). As shown by the Trans-well migration assay, the enhanced miR-218 expression was associated with a significantly lower number of cells that passed through a Transwell chamber (P<0.01). Luciferase reporter assay showed that, compared with the control group, the relative luciferase activity significantly decreased in the miR-218 mimic group (P<0.01). As shown by the Western blotting, compared with the control group, the HMGB1 protein expression significantly decreased in the PANC-1 group after the transfection of miR-218 mimic for 48 h (P<0.01).

Conclusions: The miR-218 expression decreases in human PCT and cell lines. miR-218 can negatively regulate the HMGB1 protein expression and inhibit the proliferation and invasion of pancreatic cancer cells. A treatment strategy by enhancing the miR-218 expression may benefit the patients with pancreatic cancer.

Keywords: Pancreatic cancer; microRNA-218 (miR-218); proliferation; apoptosis; high mobility group box 1 (HMGB1)

Submitted Jan 15, 2015. Accepted for publication Mar 24, 2015.
doi: 10.3978/j.issn.1000-9604.2015.04.07
View this article at: http://dx.doi.org/10.3978/j.issn.1000-9604.2015.04.07
Introduction

Pancreatic cancer is a malignant tumor of the digestive system featured by insidious clinical manifestation, difficult early diagnosis, rapid progression, and extremely poor prognosis (1-3). With worldwide increase in prevalence, pancreatic cancer has become the fourth leading cause of cancer-related death. Most pancreatic cancer patients have already in the advanced stages when the disease is confirmed, and their 5-year survival rate is less than 5% (4,5). Epidemiological data have shown that a higher incidence of pancreatic cancer is associated with factors including smoking, high-fat high-protein diets, alcoholism, coffee drinking, exposure to certain chemical carcinogens, diabetes mellitus, and chronic pancreatitis (6-8). Therefore, it is critically important to carry out basic research on the pancreatic cancer, so as to identify are highly sensitive and specific novel tumor markers (especially feasible for the early diagnosis) and develop new diagnosis and treatment techniques (9-11). Along with the development of the theories and techniques of molecular biology, new advances have been continuously made in the pancreatic cancer-related genes and new hot research topics have emerged (12,13).

According to the basic theories of molecular biology, oncogenesis is a multi-step progress involving the activation of multiple cancer genes and the inactivation of tumor suppressor genes, which cause the abnormal growth of cells as well as the changes in phenotypes and functions; as a result, the normal cells may be transformed into tumor cells (14-16). After many years of efforts, many achievements have been made in terms of oncogenes, tumor suppressor genes, and signal transduction pathways; however, the exact mechanism governing the development of pancreatic cancer remains unclear (17,18).

MicroRNAs (miRNAs) are endogenous, single-stranded, small, ~22-nucleotide noncoding RNAs. miRNAs generally regulate the mRNA expression at the post-transcriptional level by base pairing to complementary sequences within the 3’ untranslated region (3’UTR) of protein-coding mRNA transcripts, and thus are involved in various physiological processes including the proliferation, apoptosis, differentiation, metabolism, and development of cells and pathological conditions such as cardiovascular diseases, neurological diseases, and tumors (19-21). It is estimated that more than one third of human genes are regulated by miRNAs. The roles of miRNA in human diseases, in particular in tumors, are increasingly concerned (22,23).

Recent studies have demonstrated that miRNA is involved in the occurrence, development, and invasion of tumors by regulating the expressions of the genes that are related with the cell cycle, apoptosis, cell migration, and angiogenesis (24-26). miRNAs are found to be closely associated with the development of tumors. Similar studies have also been carried out in pancreatic cancer (27,28). However, relatively few studies have been carried out on the role of miRNA in pancreatic cancer (29). In their study on the brain gliomas, Tu et al. found that miR-218 could suppress the proliferation and migration of glioma cells by regulating the target gene Bmi1 (30). A study on the osteogenic sarcoma showed that miR-218 could also suppress the invasion and migration of osteosarcoma cells by regulating the targeting genes including TIAM1, MMP2, and MMP9 (31). Zhu et al. demonstrated that miR-218 was expressed in the pancreatic ductal adenocarcinima (PDAC) and was associated with the development of pancreatic cancer (32). While miR-218 may be a new marker of pancreatic cancer, the effects of miR-218 on the biological characteristics of pancreatic cancer cells as well as the underlying mechanisms remain unclear.

In our current study, we tried to determine the expression profiles of miR-218 in human pancreatic cancer tissue (PCT) and cells and their effects on the biological features of human pancreatic cancer cell line PANC-1 and observe the impact of miR-218 on the expression of the target gene HMGB1, with an attempt to provide new treatment methods and strategies for pancreatic cancer.

Materials and methods

Specimen

Fresh tumor specimens were surgically isolated from ten patients with pancreatic carcinoma who were treated in our hospital. Also, normal pancreas tissues were harvested from five subjects. All these specimens were immediately preserved in liquid nitrogen until subsequent experiments. All patients were naive to any radiotherapy, chemotherapy, or other therapies and had no other inflammatory disease.

Main reagents

Fetal calf serum, Dulbecco’s modified Eagle’s medium (DMEM), RPMI 1640 medium, L-glutamine, HEPES, and Lipofectamine 2000 were purchased from Invitrogen, USA. TaqMan miRNA isolation kit, TaqMan microRNA assay
kit, TaqMan microRNA assay, and TaqMan Universal PCR Master Mix were purchased from Applied Biosystems. The miR-218 RT primers, miR-218 mimic, and non-specific control (mimic ctrl) were synthesized by Shanghai Genepharm Corporation.

Cell Counting Kit-8 (CCK-8) was purchased from Beyotime Institute of Biotechnology (Shanghai, China). The trypsin, phosphate buffered saline (PBS), and Hoechst 33342 were purchased from Sigma-Aldrich (USA). Cell culture plates or dishes and Transwell invasion chamber were purchased from Corning, USA. Matrigel gum was purchased from BD Bioscience. Annexin V and propidium iodide (PI) were purchased from Roche, Germany. The primary antibodies including rabbit anti-human HMGB1 monoclonal antibody and mouse anti-human β-actin monoclonal antibody were purchased from Abcam, UK. The secondary antibodies including HRP-conjugated affinity purified goat anti-mouse IgG and HRP-conjugated affinity purified goat anti-rabbit IgG were purchased from Sigma-Aldrich. Protein extraction and quantification kit was purchased from Bio-Rad company.

Methods

Cell culture and treatment
The AsPC-1 and BxPC-3 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 10 mM HEPES, 1.5 g/L NaHCO₃, and 2 mM L-glutamine. The PANC-1 cells were cultured in DMEM containing 10% FBS, 1.5 g/L NaHCO₃, and 4 mM L-glutamine. All the AsPC-1, BxPC-3, and PANC-1 cells were inoculated at 37 °C, 5% CO₂ and saturated humidity. The growth of cells was observed under an inverted microscope. When cells were grown to 70-80% confluence, they were detached with 0.25% trypsin. Medium was changed every other day, and the cells were passaged every 4 to 6 days. Cells in the exponential phase were selected for further experiment.

The normally cultured PANC-1 cells were uniformly seeded in 6-well culture plates at a density of 3x10⁵/mL (1,000 μL in each well). After the cells reached complete adherence, the transfections of miR-218 mimic and non-specific control (mimic ctrl) were performed using Lipofectamine 2000 in accordance with the manufacturer’s instruction. Meanwhile, normal control (normal ctrl) group was set. Serum-free minimum essential medium (MEM) was used to dilute the miR-218 mimic and mimic ctrl. Then, the liposome Lipofectamine 2000 was diluted in the MEM, gently mixed, and then inoculated at room temperature for 5 min. The diluted Lipofectamine 2000 was separately mixed with the diluted miR-218 mimic and mimic ctrl and then inoculated at room temperature for 20 min to form complexes. The complexes were then added into the culture plate containing the PANC-1 cells, which was put inside an incubator at 37 °C in the humidified incubator with 5% CO₂. Five hours later, MEM containing 10% FBS was used instead, and the cells were cultured for another 48 h.

Detection of the miR-218 expression in human PCT and cell lines
The expressions of miR-218 in pancreatic cancers including AsPC-1, BxPC-3, and PANC-1 and in PCT and normal pancreatic tissue (NPT) were determined using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). After the in vitro cultured pancreatic cancer cells and tissues were collected, the RNA was extracted using the TaqMan miRNA isolation kit, the expression of the mature miR-218 was determined using the TaqMan microRNA assay and TaqMan Universal PCR Master Mix, with U6 being an internal control. Three duplicate wells were set in each group for the assay. The CT value of the specimen in each reaction tube was recorded. The relative quantification method in the qRT-PCR was employed for analyzing the experimental results.

Detection of the effect of the transfection of miR-218 mimic in PANC-1 cells on the expression of miR-218
Forty-eight hours after the transfection of miR-218 mimic or mimic ctrl in the PANC-1 cells, the RNA of cells in each group was extracted using the TaqMan miRNA isolation kit, and the changes in the miR-218 expression in the PANC-1 cells in each group were determined using the qRT-PCR.

Detection of the effect of miR-218 on cell viability using the CCK-8 method
Based on the manufacturer’s instructions, the CCK-8 solution was added in each well 1, 2, 3, and 4 days after the transfection of miR-218 mimic or mimic ctrl in the PANC-1 cells, the RNA of cells in each group was extracted using the TaqMan miRNA isolation kit and the changes in the miR-218 expression in the PANC-1 cells in each group were determined using the qRT-PCR.
Determination of the effect of miR-218 on cell proliferation using flow cytometry
The PANC-1 cells were transfected with miR-218 mimic or mimic ctrl. Meanwhile, normal control (normal ctrl) group was set. Wash the cells 1-2 times with PBS 48 h after the transfection. Wash the cells twice with PBS after the cells were detached with trypsin. Cells were collected by centrifugation, and then added with PI staining solution and incubated 30 min in the dark at 4 °C. After the mesh filter, the apoptosis was detected using flow cytometry (BD Company, USA). The cells were counted using the CellQuest software (BD, USA), and the data were analyzed using the Macquity software.

Determination of the effect of miR-218 on cell apoptosis using flow cytometry
The PANC-1 cells were transfected with miR-218 mimic or mimic ctrl. Meanwhile, normal control (normal ctrl) group was set. Wash the cells 1-2 times with PBS 48 h after the transfection. Adding Annexin V-FITC and PI dye, and then let incubate at room temperature in dark for 15 min. After the mesh filter, the apoptosis was detected using flow cytometry (BD Company, USA). The cells were counted using the CellQuest software (BD, USA), and the data were analyzed using the Macquity software.

Determination of the effect of miR-218 on cell migration using the Trans-well migration assay
The transfections of miR-218 mimic and non-specific control (mimic ctrl) were performed using Lipofectamine 2000 in accordance with the manufacturer’s instructions. Meanwhile, normal control (normal ctrl) group was set. Twenty-four hours after the transfection, the PANC-1 cells were inoculated in Transwell invasion chamber. The cells were cultured in the normal conditions for 24 hours, then washed with PBS 1-2 times and stained with Hoechst 33342. Count the number of cells that had passed through the Transwell polycarbonate membrane using Leica microscope (Leica company, Wetzlar, Germany). These cells were regarded as invasive cells, and eight random fields were observed.

Determining whether HMGB1 was the target gene of miR-218 by using the luciferase reporter assay
Construction of the luciferase reporter plasmid: After the sequence fragments of the target gene HMGB1 3'-UTR complementary to miR-218 and the mutated HMGB1 3'-UTR sequence fragments were synthesized using chemical approaches, the HMGB1 3′-UTR (wild type, WT) that was used for predicting miR-218 binding sites and the mutated HMGB1 3′-UTR (mutant) were cloned into the Xba I site of the pGL3-Luciferase vector.

The normally cultured HEK293 cells were uniformly seeded in 6-well culture plates at a density of 3×10⁵/mL (1,000 μL in each well). The luciferase plasmid containing the HMGB1 3′-UTR(WT) and the mutated HMGB1 3′-UTR (mutant) as well as miR-218 mimic or mimic ctrl were transfected. Meanwhile, the normal control (normal ctrl) group was set. Cells were collected 48 hours after the transfection and detected using the luciferase kit, and the results were read using an ELISA reader.

The effect of miR-218 on the HMGB1 protein expression was determined using Western blotting. The cellular proteins in each group were collected 48 h after the transfection of miR-218 mimic or mimic ctrl in PANC-1 cells. These cells placed in 6-well cell culture plates. Add 1 mL of RIPA lysis buffer [150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris (pH 7.9), 10 mM NaF, 10 mM PMSF and 1× protease inhibitors (Complete cocktail tablets, Roche)] in each well. Then, the cell lysates were transferred to a 1.5 mL centrifuge tube and centrifuged at 16,000 rpm for 30 min. The protein concentration of the supernatant was determined using bicinchoninic acid (BCA) method. Electrophoretic separation was conducted using 5% stacking gel and 15% separating gel, with 50 μg total protein added in each lane. After the protein had been well transferred onto a PVDF membrane (Bio-Rad, USA), they were blocked in TBST solution (10 mM Tris-HCl, pH7.5, 150 mM NaCl, and 0.1% Tween-20) containing 5% skimmed milk powder at room temperature for 1 h, then added with rabbit anti-human HMGB1 antibody (1:500 dilution) and rabbit anti-human β-actin monoclonal antibody (1:1,000 dilution) and incubated overnight at 4 ℃. Wash three times with fresh TBST for 5 min. Add HRP-labeled goat anti-rabbit IgG or HRP-labeled goat anti-mouse IgG secondary antibody and incubate it at 37 ℃ for 1 h. Wash three times with fresh TBST for 5 min. Autoradiography was conducted using ECL chemiluminescent substrate. The relative concentration of HMGB1 was presented as RKIP/β-actin ratio, and the change of its relative expression was analyzed using PDQuest software (Bio-Rad, Richmond, CA, USA).

Statistical analysis
The experimental data were analyzed using SPSS
17.0 software. Inter-group differences were analyzed by t-test and multiple comparisons among several groups were conducted by analysis of variance. P<0.05 was considered significantly different.

**Results**

*The miR-218 expressions in human PCT and cell lines*

As shown by qRT-PCR, the expression of miR-218 in PCT was significantly lower than in NPT (P<0.01), and its expression in pancreatic cancer cell lines (AsPC-1, BxPC-3, and PANC-1) were significantly lower than those in the NPT (P<0.01) (Figure 1).

*The effect of the transfection of miR-218 mimic in PANC-1 cells on the expression of miR-218*

The miR-218 was over-expressed in PANC-1 cells by the transfection of miR-218 mimic in these cells. As shown by qRT-PCR, the MiR-218 expression in the miR-218 mimic transfection group was significantly higher than that in the normal control (normal ctrl) group (P<0.01) and negative control (mimic ctrl) group (P<0.01) (Figure 2).

*Detection of the effect of miR-218 on cell viability using the CCK-8 method*

As shown by CCK-8 analysis, 2 days after the miR-218 mimic transfection, the cell viability in the miR-218 mimic transfection group was significantly lower than that in the normal control (normal ctrl) group (P<0.05) and negative control (mimic ctrl) group (P<0.05) (Figure 3).

*The effect of miR-218 on cell proliferation using flow cytometry*

The cell proliferation in each group was detected using flow cytometry 48 h after the transfection of miR-218 mimic or mimic ctrl in PANC-1 cells. S-phase fraction = (S)/(G0G1+S+G2M). As shown by flow cytometry, the S-phase fraction in the miR-218 mimic transfection group was significantly lower than that in the normal control (normal ctrl) group (P<0.01) and negative control (mimic ctrl) group (P<0.01) (Figure 4).
Determination of the effect of miR-218 on cell apoptosis using flow cytometry

The S-phase fraction in the miR-218 mimic transfection group was significantly lower than that in the normal control (normal ctrl) group (P<0.01) and negative control (mimic ctrl) group (P<0.01) (Figure 5).

The effect of miR-218 on cell migration using the Transwell migration assay

The cell migration capacity in each group was detected using Transwell migration assay 48 h after the transfection of miR-218 mimic or mimic ctrl in PANC-1 cells. As shown by Hoechst 33342 staining, the transfection with miR-218 mimic was associated with a significantly lower number of cells that passed through a Transwell chamber (P<0.01) (Figure 6).

Determining whether HMGB1 was the target gene of miR-218 by using the luciferase reporter assay

To validate whether the putative target site of miR-218
located at \textit{HMGB1} 3'-UTR, we constructed a luciferase plasmid containing the \textit{HMGB1} 3'-UTR (WT) and the mutated \textit{HMGB1} 3'-UTR (mutant) and used it to co-transfect the HEK293 cells with miR-218 mimic or mimic ctrl. Compared with the mimic ctrl group, the luciferase activity in the plasmid significantly decreased in the miR-218 mimic transfection group (P<0.01) (Figure 7).

The regulatory effect of miR-218 on \textit{HMGB1} expression was detected by using Western blotting, which showed that the \textit{HMGB1} protein expression in the miR-218 mimic transfection group was significantly lower than those in the normal control (normal ctrl) group (P<0.01) and negative control (mimic ctrl) group (P<0.01) (Figure 8).

**Discussion**

Pancreatic cancer is one of the most common forms of gastrointestinal malignancies. As a highly malignant tumor, its prognosis is extremely poor due to its deep anatomic position, insidious onset, difficulty in early diagnosis, poor response to radiochemotherapy, and inoperability (33,34). Therefore, in-depth research on the occurrence and development of pancreatic cancer is urgently required to improve its early diagnosis and optimize the treatment (35,36).

Similar to many other malignancies, the pathogenesis of pancreatic cancer is also a progressive process involving multiple genes, steps, and stages. Therefore, gene therapy has been recognized as one of the most promising strategies (9,37,38). Numerous studies have shown that mRNA disturbances appear during the tumorigenesis (39,40). When over-expressed in tumor cells, some mRNAs (e.g., miR-21 and miR-10b) can induce tumor invasion/metastasis and thus exert the oncogene-like functions. These mRNAs are known as carcinogenic mRNAs (41). On the other hand, the down-regulation of some other mRNAs (e.g., miR-335, miR146a, and miR-29c) is associated with the occurrence, development, and invasion/metastasis of tumors. They are called as tumor suppressor mRNAs (42). As shown by a previous study, miR-218 is regarded as a tumor suppressor mRNA can is down-regulated in many tumors (43). Shi et al. found that the expression of miR-218 was down-regulated in childhood medulloblastoma (44). Tu et al.
**Figure 6** Determination of the effect of miR-218 on cell migration using the Trans-well migration assay. **P<0.01 vs. normal ctrl group or mimic ctrl group.

**Figure 7** (A) Schematic representation of HMGB1 3’-UTR showing putative miR-218 target site; (B) relative viability of luciferase in each group. **P<0.01 vs. mimic ctrl group.

**Figure 8** Effect of the transfection of miR-218 mimic in PANC-1 cells on the HMGB1 protein expression. **P<0.01 vs. normal ctrl group or mimic ctrl group.
reported that miR-218 was down-regulated in glioma and could suppress the proliferation, migration, and invasion of glioma cells (30). In our current study, by using qRT-PCR, we detected the expressions of miRNA-218 in PCT, NPT, and pancreatic cancer cell lines. As shown by qRT-PCR, the expression of miR-218 in PCT was significantly lower than in NPT, and its expressions in pancreatic cancer cell lines (AsPC-1, BxPC-1, and PANC-1) were significantly lower than that in the NPT. Therefore, miR-218 may also act as a tumor suppressor miRNA in pancreatic cancer.

According to Guan et al., the expression of miR-218 was down-regulated in thyroid cancer, and the down-regulated miR-218 was associated with the invasion of thyroid cancer cells and the progression of thyroid cancer (45). In our current study, we used CCK-8, flow cytometry, and Transwell assay to explore the effects of miR-218 on the biological characteristics of pancreatic cancer cell line PANC-1. As shown in our study, after the over-expression of miR-218 in PANC-1 cells, the viability of PANC-1 cells decreased, the proliferation and invasion of PANC-1 cells were suppressed, and the apoptosis was promoted. Therefore, our study demonstrated that miR-218 also served as a tumor suppressor miRNA in PANC-1 cells.

HMGB1 is a member of the high mobility group box family of non-histone chromosomal proteins (46-48). Studies have shown that, after binding with DNA, HMGB1 is involved in the construction and maintenance of the nucleosome and thus plays different roles in the transcription, replication, recombination, DNA repair, and maintenance of genome stability (49,50). In addition, HMGB1 can act as an inflammatory factor after having been actively secreted by inflammatory cells and (or) tumor cells or passively secreted from necrotic cells into the extracellular environment, in which it binds to multiple receptors [mainly the receptor for advanced glycation end product (RAGE)] on cell surface and activates a series of downstream signaling pathways; thus, it plays vital roles in inflammation, cancer, sepsis, autoimmune disease, and vascular diseases (51,52). In our current study, the online software TARGETSCAN was employed to analyze and predict that HMGB1 was a target gene of miR-218. We also validated that the putative target site of miR-218 is located at HMGB1 3’-UTR, as shown by the luciferase reporter assay. Also, the regulatory effect of miR-218 on HMGB1 expression was detected by using Western blotting, which showed that the HMGB1 protein expression in the miR-218 mimic transfection group was significantly lower than those in the normal control (normal ctrl) group and negative control (mimic ctrl) group. Thus, the miR-218 overexpression inhibited the HMGB1 protein expression.

Stimulated by cytokines such as growth factors, tumor cells can excessively express and secret HMGB1 (46,53,54). By binding to its receptor RAGE, the secretory HMGB1 can stimulate the proliferation, migration, and invasion of cells and the angiogenesis, and thus promote tumorigenesis (55,56). Meng et al. demonstrated that the overexpression of HMGB1 in osteogenic sarcoma cell line MG-63 could promote the proliferation and invasion of MG-3 cells and inhibit apoptosis (56). Wang et al. used the lentivirus-mediated RNA to intervene and inhibit the expression of HMGB1 in prostate cancer cells and found that the suppression of HMGB1 gene expression inhibited the proliferation of prostate cancer cells and promoted their apoptosis (57). In our current study, we firstly confirmed that HMGB1 was the target gene of miR-218 and then demonstrated that miR-218 inhibited the HMGB1 protein expression. Thus, miR-218 may inhibit the proliferation and invasion of pancreatic cancer cell line PANC-1 and promote its apoptosis by suppressing the expression of its target gene HMGB1.

In conclusion, the miR-218 expression decreases in human PCT and cell lines. miR-218 can negatively regulate the HMGB1 protein expression and inhibit the proliferation and invasion of pancreatic cancer cells. A treatment strategy by enhancing the miR-218 expression may benefit the patients with pancreatic cancer.

Acknowledgements

Funding: This study was supported by grants: (I) Liaoning Provincial Department of education science research project (L2014299); (II) Liaoning province science and technology plan project (2011404013-4); (III) The Shenyang Municipal Science and technology project (F12-277-1-73).

Disclosure: The authors declare no conflict of interest.

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