MiR-148b functions as a tumor suppressor in pancreatic cancer by targeting AMPKα1

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No potential conflicts of interest were disclosed.

Running title: miR-148b targets AMPKα1 in pancreatic cancer
Abstract

MicroRNAs are small non-coding RNAs that participate in a variety of biological processes, and dysregulation of miRNA is always associated with cancer development and progression. Aberrant expression of miR-148b has been found in some types of cancer, but its expression and potential biological role in pancreatic cancer are still largely unknown. In this study, our data showed that miR-148b was significantly downregulated in 48 pairs of human pancreatic cancer tissues and five cell lines. Further, the deregulated miR-148b was correlated with increased tumor size, late TNM stage, lymphatic invasion, distant metastasis and worse prognosis in pancreatic cancer. Functional studies indicated overexpression of miR-148b dramatically suppressed the growth of cancer cells, attributable to induction of apoptosis and cell cycle arrest at S phase. Meanwhile, miR-148b remarkably inhibited invasion and enhanced chemosensitivity of pancreatic cancer cells. Moreover, ectopic expression of miR-148b was able to inhibit tumourigenicity in nude mice. Further studies revealed that AMPKα1 might be the direct target gene of miR-148b, and overexpressed AMPKα1 inversely correlated with miR-148b in pancreatic cancer. Silencing of AMPKα1 with RNAi inhibited the growth of pancreatic cancer cells in vitro and in vivo, and also induced apoptosis, cell cycle arrest and inhibited invasion of cancer cells, which is consistent with the effects of miR-148b overexpression. In conclusion, miR-148b can inhibit cell proliferation, invasion and enhance chemosensitivity of pancreatic cancer by targeting AMPKα1. Our present results implicate the potential effects of miR-148b on prognosis and treatment for pancreatic cancer.

Keywords

miR-148b; pancreatic cancer; proliferation; invasion; AMPKα1
**Introduction**

MicroRNAs are a class of small non-coding RNAs, which contain of about 22 nucleotides. MicroRNAs bind to partially complementary sequences in the 3'-untranslated region (UTR) of specific target mRNA, resulting in either mRNA degradation or translation inhibition (1). Growing evidence suggests that microRNAs play an important role in various biological processes, including cell proliferation, development and differentiation (2, 3). Furthermore, increasing numbers of microRNAs have been observed in various types of cancer and may be involved in modulating cancer cell behaviors (4-7). These data emphasize the importance of microRNAs in cancer development and provide new insights into understanding the molecular mechanism of tumorigenesis.

Pancreatic cancer is one of the most common cancers worldwide and the fourth leading cause of cancer related deaths in the United States (8). The poor prognosis is due to its tendency for late presentation, early metastasis and poor response to chemotherapy (9). The development of pancreatic cancer is a multistep process with accumulation of genetic and epigenetic alterations (10, 11). Microarray studies have identified a number of microRNAs that are up- or down-regulated in pancreatic cancer. Recently, miR-148b has been shown to be deregulated in some other types of cancers, such as overexpressed in ovarian cancer (12) and lung cancer (13), while downregulated in pancreatic cancer (14, 15), gastric cancer (16) and colorectal cancer (17). However, the exact role of miR-148b in carcinogenesis of pancreatic cancer has not been revealed yet.

Hence, the present study evaluated the miR-148b expression in human pancreatic cancer tissues and cell lines. Furthermore, the correlations between dysregulation of miR-148b and clinical pathological characteristics were analyzed. Also, the effects of miR-148b on proliferation, invasive ability and chemosensitivity of pancreatic cancer cells were identified.

Moreover, the microRNA software indicated that AMP-activated protein kinase α1 (AMPKα1) might be the downstream target of miR-148b. AMPK is a highly conserved energy-sensing serine/threonine kinase, which can maintain energy stores and enhance oxidative metabolism (18,
AMPK is a heterotrimeric complex consisting of a catalytic (α) and two regulatory (β and γ) subunits. Two isoforms are known for catalytic subunit (α1, α2) and they are encoded by different genes (20). Recently, some studies have demonstrated that AMPK plays critical regulatory roles in cancer cell growth and tumorigenesis of cancer cell (21-23). For example, some research demonstrated that activation of AMPKα1 suppressed the growth of human colon cancer cell (24), while some other research showed that AMPKα1 could increases chondrosarcoma cancer cell migration (25), which indicated that AMPKα1 was controversial in different cancer cells. Moreover, Kato K et al displayed that AMPKα1-RNAi could inhibit the growth of pancreatic cancer (26). Therefore, we selected the AMPKα1 as the target for miR-148b to further explore the effects of miR-148b/AMPKα1 signal on pancreatic cancer.

Materials and methods

Patients and Tumor Tissues

Human pancreatic cancer tissues (PC) and corresponding adjacent normal pancreas tissues (NP) were acquired by surgical removal from 48 patients at Pancreatic Disease Institute, Union Hospital (Wuhan, China) between 2007 and 2009 (30 males and 18 females; median age, 56 years; age range, 31–77 years). None of the patients had received chemotherapy or radiotherapy before surgery excision. The original histopathological reports were obtained from each case and the diagnosis of pancreatic cancer was confirmed. Immediately after surgical removal, tissue samples were either snap-frozen in liquid nitrogen (for miR-148b and AMPKα1 extraction) or fixed in 10% buffered formalin solution and then embedded in paraffin (for histological analysis). Informed consent was obtained from all patients before surgery and the study protocol was approved by the ethics committee of Huazhong University of Science and Technology.

Immunohistochemistry

AMPKα1 expression was determined by immunohistochemistry as our previously described (27). Sections of paraffin-embedded tissue were incubation with the primary antibody to AMPKα1 (dilution 1:100, Santa Cruz) and a rabbit antigoat horseradish peroxidase-labeled secondary antibody (dilution 1:200, Cell Signaling). All slides were independently analyzed by two experienced pathologists blinded to patient status. Cases with ≥30% positive tumor cells in a
section were considered as positive expression (28).

**RNA Isolation and Quantitative Real-time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)**

Total RNA from tissue specimens and cell lines was prepared using Trizol reagent (Invitrogen). To quantify mature miR-148b expression, total RNA was polyadenylated using poly-A polymerase based First-Strand Synthesis kit (TaKaRa Bio, Japan) according to the manufacturer’s protocol. To quantify AMPKα1 expression, total RNA was converted to cDNA using M-MLV reverse transcriptase (Invitrogen). QRT-PCR was performed using the quantitative SYBR Green PCR kit (TaKaRa) after reverse transcription. The expression of miR-148b was normalized to that of U6 small nuclear RNA. One primer is microRNA specific and the other is a universal primer. AMPKα1 expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative amounts of miR-148b and AMPKα1 were measured with the $2^{-\Delta\Delta CT}$ method. All qRT-PCR reactions were performed in triplicate. The primers used in qRT-PCR are shown in Table S1.

**Cell Culture**

The pancreatic cancer PANC-1, ASPC-1, BXPC-3, SW1990 and Miapaca-2 cell lines were obtained from American Type Culture Collection (ATCC, Manassas, USA); they were tested and authenticated for genotypes by DNA fingerprinting. These cell lines were passaged for less than 6 months after resuscitation, and no re-authorization was done. Cells were cultured in RPMI-1640 mediums supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin.

**MicroRNA and RNA interference**

MiR-148b mimics (miR-148b), miR-148b negative control (miR-NC), miR-148b inhibitor (Inh-148b), miR-148b inhibitor negative control (Inh-NC), siRNA duplexes targeting human AMPKα1 (siAMPKα1-1, siAMPKα1-2 and siAMPKα1-3) were synthesized and purified by RiboBio (RiboBio Co., Guangzhou, China). SiRNA duplexes with non-specific sequences were
used as siRNA negative control (NC). RNA oligonucleotides were transfected by using Lipofectamine 2000 (Invitrogen) and medium was replaced 6 hours after transfection. A final concentration of 50 nM miR-148b, 100 nM Inh-148b and 50 nM siAMPKα1 was used, and the expression levels of miR-148b and mRNA were quantified 48h after transfection. Lentiviral miR-148b (LV-miR-148b), lentiviral AMPKα1 (LV-AMPKα1), lentiviral shAMPKα1 (LV-shAMPKα1) or empty lentiviral vector (LV-NC) was constructed by genechem Company (Shanghai, China), and transfected to the PANC-1 cells according to the manufacturer’s instructions, in the presence of virus at a multiplicity of infection of 10. All oligonucleotide sequences are listed in Table S2, and sequences of LV-shAMPKα1 was consistent with the sequences of siAMPKα1-2 that was the most effective.

**Colony formation assay**

Twenty-four hours after transfection, 300 transfected cells were placed in a fresh 12-well plate and maintained in RPMI-1640 containing 10% FBS for 12 days. Colonies were fixed with methanol and stained with 0.1% crystal violet solution for 15 min and photographed. Colonies of more than 50 cells were counted (29).

**Cell viability assay**

Cell survival was determined by MTT assays as previously described (30). Cells were seeded in 96-well plates at densities of $5 \times 10^3$ cells per well, treated with 50nM miR-148b and/or different concentrations of 5-Fluorouracil (5-FU) (Sigma-Aldrich), Gemcitabine (GEM) (Eli Lilly and Co., Suresnes Cedex, France) and Cisplatin (DDP) (Sigma-Aldrich) for 48h and then estimated by a colorimetric assay using MTT dye (5 mg/mL). The absorbance was determined in an ELISA plate reader using an activation wavelength of 570 nm. Cell viability values were determined by comparison with untreated control cells for three independent experiments, each of which used $n = 6$ replicate wells per assay condition.

**Evaluation of cell apoptosis**

To quantify cell apoptosis, Annexin V/PI staining was performed. Briefly, cells were collected, washed in cold phosphate buffered saline (PBS) for twice and resuspended in binding buffer at a
cell density of $1 \times 10^6$/mL. Cells were then stained with Annexin V-FITC and PI according to the manufacturer’s protocol and acquired by a FACS Calibur flow cytometer (BD Biosciences) and analyzed with Cellquest software.

**Cell cycle distribution analysis**

Cells were harvested by trypsinization, washed with cold PBS, and fixed in 70% ethanol overnight at -20°C. After fixation, cells were treated with DNA staining solution (3.4 mM Tris-Cl (pH 7.4), propodium iodide, 0.1% triton X-100 buffer and 100 μg/ml RNase A). Cell cycle analysis was accomplished by FACS flow-cytometry.

**Matrigel invasion assay**

Cell invasion experiment was assessed using the Matrigel Invasion Chamber of pore size 8 mm (Corning, Fisher Scientific, UK). A total of $5 \times 10^4$ cells were seeded into the upper compartment of the chamber pre-coated with matrigel (Sigma). Medium containing 30% fetal bovine serum was in the lower chamber. After the cells were incubated for 48 h and fixed and stained with 0.5% crystal violet for 30 min, and the non-invading cells were removed with cotton swabs. The number of invasive cells on the lower surface of the membrane was then counted under a microscope at a magnification of 400 × in five random fields.

**Western blot analysis**

Western blot was performed as described recently (31), with some modifications. Total cell lysates (40 μg) were denatured and resolved on SDS–polyacrylamide gels, and transferred onto polyvinylidene difluoride membranes. After blocking in 5% skim milk, membranes were probed with primary antibodies followed by horseradish peroxidase-linked secondary antibodies. The membrane was visualized using ECL (Pierce) and exposed to a Kodak X-OMAT film (Sigma-Aldrich). Band intensities were quantified using the Alpha DigiDoc 1201 (Alpha Innotech, San Leandro, USA).

**Luciferase activity assay**
Luciferase activity assay was performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions. PANC-1 cells of 85%-90% confluence were seeded in 96-well plates. For AMPKα1 3'UTR luciferase reporter assay, 100 ng wild type or mutant luciferase reporter constructs (termed WT or MUT) were co-transfected into PANC-1 cells in 96-well plate with 50 nM miR-148b or 50 nM miR-NC by using lipofectamine 2000. Luciferase activity assay was performed 48 hours after transfection using the Dual-Luciferase Assay System. Firefly luciferase activity was normalized to the corresponding Renilla luciferase activity. All experiments were performed three times.

Pancreatic cancer mouse models

LV-NC or LV-miR-148b, and LV-NC or LV-shAMPKα1 transfected PANC-1 cells were harvested by trypsinization and resuspended in RPMI 1640. The cells were washed once in serum-free medium and resuspended in PBS. The cells (1.5×10^7) in 100μL PBS were injected subcutaneously (SC) into the right flank of 3-week-old female BALB/c nu/nu mice (n = 10 mice per group). The tumor size was measured every 4 days using a digital caliper and calculated by the formula: tumor volume = length×width^2×0.5 (32). All nude mice were used following protocols approved by the Animal Care and Use Committee of Tongji Medical College of Huazhong University of Science and Technology.

Statistical analysis

All results are expressed as means ±standard deviation (SD). Comparisons between groups were performed with the unpaired t test. The relationships between miR-148b expression and clinic characteristics were analyzed by χ^2 tests. Pancreatic cancer patient survival was analyzed by log-rank test. The relationship between miR-148b and AMPKα1 expression was explored by Spearman's correlation. Values were considered to be significantly different at P<0.05. All statistical analysis was performed using SPSS 13.0 software.

Results

MiR-148b is deregulated in pancreatic cancer and correlates with clinical characteristics

Compared with noncancerous tissues, 41 PC tissues showed low expression of miR-148b, and the
median fold change was 1.89 (P<0.01; Fig. 1A). Compared with adjacent normal pancreas, miR-148b was down-regulated with different expression levels in PANC-1 (0.06±0.02-fold), ASPC-1 (0.07±0.03-fold), BXPC-3 (0.24±0.04-fold), SW1990 (0.42±0.01-fold) and Miapaca-2 cells (0.07±0.03-fold; Fig. 1B), respectively.

Further, the results showed that deregulated expression of miR-148b significantly correlated with increased tumor size, late TNM stage, lymphatic invasion and distant metastasis. There were no significant correlations between miR-148b with other clinical pathological characteristics including sex, age, tumor location, histological grade or vessel infiltration in pancreatic cancer (Table 1).

**Downregulation of miR-148b correlates with worse prognosis of patients with pancreatic cancer**

As shown in Fig. 1C, the survival analysis revealed that lower miR-148b expression levels significantly correlated with reduced survival (P<0.05). This result suggested that deregulation of miR-148b might promote the progress in pancreatic cancer.

**MiR-148b inhibits cell proliferation in vitro**

As shown in Fig. 2A, miR-148b mimics caused a 24.03 and 9.04-fold increase in the expression of miR-148b in PANC-1 and BXPC-3 cells relative to miR-NC. Meanwhile, miR-148b inhibitor decreased the expression of miR-148b by 2.19 and 1.54-fold in PANC-1 and BXPC-3 cells. After transfection with miR-148b mimics, the proliferation of PANC-1 and BXPC-3 cells was significantly inhibited by 32.14% ±2.58% and 26.72%±2.95%, respectively. Whereas miR-148b inhibitor increased cell growth in PANC-1 and BXPC-3 cells by 21.44% ±3.48% and 26.94%±5.35% (Fig. 2B). Compared with LV-NC transfected cells, the Proliferation of LV-miR-148b transfected cells was significantly inhibited and displayed much fewer and smaller colonies compared with LV-NC transfected cells (Fig. 2C and D).

**MiR-148b induces apoptosis and cell cycle arrest and inhibits invasion of pancreatic cancer cells**

Compared to miR-NC, miR-148b mimics transfection significantly increased the apoptosis rate in
PANC-1 (5.63%±0.76% vs 16.00%±0.96%) and BXPC-3 cells (2.67%±0.76% vs 9.93%±0.80%) (Fig. 3A). Moreover, the results demonstrated that the S phase was significantly increased in both PANC-1 (24.02%±3.52% vs 44.10%±3.49%) and BXPC-3 cells (21.12%±3.00% vs 28.99%±2.32%) following transfection with miR-148b after 48 h (Fig. 3B).

Moreover, we observed that the invasion ability of PANC-1 cells was significantly inhibited following transfection with miR-148b (miR-148b group, 63±8 cells / HP; miR-NC group, 117±10 cells / HP; HP means high power magnification field) (Fig. 3C). The similar results were also observed in BXPC-3 cells.

**MiR-148b enhances chemosensitivity of pancreatic cancer cells**

After transfected with miR-148b mimics for 48h, the IC50 of PANC-1 cells was significantly decreased in 5-FU (8.33±1.48 vs 40.36±3.39 ug/mL), GEM (21.39±1.89 vs 83.66±1.73 ug/mL) and DDP (5.77±1.97 vs 118.65±1.50 uM) (Supplemental Fig.S1A). Similarly, the IC50 of BXPC-3 cells transfected with miR-148b mimics was reduced in 5-FU (6.33±1.7 vs 33.12±2.32 ug/mL), GEM (23.94±1.28 vs 75.20±2.23 ug/mL) and DDP (5.72±0.89 vs 19.63±1.59 uM) (Supplemental Fig.S1B).

**MiR-148b suppresses tumorigenicity of pancreatic cancer cells in vivo**

After 32 days, the tumor volume of mice injected with LV-NC transfected cells was 623.97±25.05 mm³, whereas the tumor size of mice injected with LV-miR-148 cells was 148.89±28.46 mm³ (Supplemental Fig. S2A and B). QRT-PCR results demonstrated that miR-148b expression levels were obviously increased in LV-miR-148 transfected tumors compared with control tumors (Supplemental Fig. S2C).

**AMPKα1 is a direct target of miR-148b**

To demonstrate the direct interaction between miR-148b and AMPKα1, we cloned 3’UTR sequences that contain the predicted target site (wild type, WT) or mutated sequences (mutant type, MUT) of miR-148b into the pGL3 control vector, respectively (Fig. 4A). The results showed that co-transfection of miR-148b mimics significantly decreased the firefly luciferase activity of the reporter with wild type 3’UTR but not that of the mutant reporter (Fig. 4B), which indicates that
miR-148b can directly target the 3'UTR of AMPKα1. The effect of miR-148b on endogenous expression of AMPKα1 was subsequently observed by qRT-PCR and western blot. Transfection of miR-148b mimics led to a decrease of AMPKα1 mRNA and protein expression in PANC-1 cells. On the contrary, transfection of miR-148b inhibitor was able to up-regulate AMPKα1 expression (Fig. 4C). Our results also showed that the expression levels of AMPKα1 were dramatically decreased in miR-148b-overexpressed tumors from mice, compared to control tumors (Supplemental Fig. S2D).

AMPKα1 is overexpressed in human pancreatic cancer tissues and cells and inversely correlates with miR-148b levels

The results demonstrated that the AMPKα1 expression levels in pancreatic cancer tissues were significantly higher than in adjacent normal pancreatic tissues (Supplemental Fig. S3A). Simultaneously, the immunohistochemical analysis also showed that AMPKα1 was overexpressed and located primarily to cytoplasm in pancreatic cancer cells. In contrast, normal pancreatic tissue samples exhibited weak AMPKα1 immunoreactivity (Supplemental Fig. S3B). Although AMPKα1 expression levels in pancreatic cancer tissues always exceeded those of matched normal pancreas, some patient-to-patient variability was also obviously. We also found that AMPKα1 was upregulated in miR-148b-downregulated pancreatic cancer lines (Supplemental Fig. S3C). Further, as shown in supplemental Fig. S3D, a significant inverse correlation was observed between AMPKα1 and miR-148b expression in pancreatic cancer tissues (Spearman's correlation, r = -0.5998; P < 0.001) and adjacent noncancerous tissues (r = -0.5093; P < 0.001). This result further confirms that endogenous AMPKα1 was regulated by miR-148b.

Downregulation of AMPKα1 inhibits growth of pancreatic cancer cell in vitro and in vivo

Western blot results demonstrated that all of 3 siRNA targeting AMPKα1 were able to effectively knockdown the expression of AMPKα1 in PANC-1 and BXPC-3 cells. The MTT assays showed that the silencing of AMPKα1 significantly reduced growth of pancreatic cancer cells (Fig. 5A). The qRT-PCR and western blot results showed that LV-shAMPKα1 significantly silenced AMPKα1 expression and LV-AMPKα1 effectively rescued the above silencing of AMPKα1 in mRNA and protein levels (Fig. 5B). Then, we chose PANC-1 and BXPC-3 cells transfected with
LV-shAMPKα1 and LV-AMPKα1 to perform colony formation assays. The data showed that knockdown of AMPKα1 induced much fewer and smaller colonies, while it is could be rescued by overexpression of AMPKα1 by LV-AMPKα1 transfection (Fig. 5C). Meanwhile, MTT assays showed that the enforced expression of miR-148b significantly inhibited cells proliferation, but overexpression of AMPKα1 moderately promoted cells proliferation. Further, the overexpression of AMPKα1 was able to rescue miR-148b-inhibited proliferation of pancreatic cancer cells (Fig. 5D). To further validate the effect of miR-148b targeted AMPKα1 on growth of pancreatic cancer cells in vivo, the PANC-1 cells transfected with LV-shAMPKα1 were subcutaneously injected into 10 BALB/c athymic nude mice of 3-week-old. Compared with LV-NC group, the growth rate and size of LV-shAMPKα1 tumors were significantly reduced (621.00±28.48 vs 168.09±25.61 mm³; Supplemental Fig. S4A and B). Hence, our findings suggest that miR-148b targeted AMPKα1 might contribute to the tumorigenicity of pancreatic cancer cells in vitro and in vivo.

**Downregulation of AMPKα1 induced apoptosis, cell cycle arrest and inhibits invasion of pancreatic cancer cells**

Compared to NC group, AMPKα1-RNAi increased the cell apoptosis (Supplemental Fig. S5A) and caused a significant S phase arresting of PANC-1 cells (Supplemental Fig. S5B). We also observed that PANC-1 cells invasion ability was significantly inhibited following AMPKα1-RNAi (Supplemental Fig. S5C). On the other hand, overexpression of AMPKα1 rescued miR-148b-induced apoptosis and cell cycle arrest and invasion inhibition of pancreatic cancer PANC-1 cells (Supplemental Fig. S6A, B and C). Similar results were obtained in BXPC-3 cells (date not shown). These results indicate that miR-148b regulates apoptosis, cell cycle arrest and invasion by the direct target of AMPKα1.

**Discussion**

Although ectopic expression of microRNAs has been frequently observed in various types of cancer (33-35), the current knowledge about the effects of microRNAs on pancreatic cancer and corresponding molecular mechanisms is still preliminary. Therefore, identifying the valuable microRNAs and their targets that are essential for pancreatic cancer development may provide...
promising therapeutic significance. In the present research, we revealed that miR-148b was significantly downregulated in pancreatic cancer and its overexpression could inhibit growth and invasion of pancreatic cancer cells, as well as enhance chemosensitivity through down-regulating AMPKα1. These results strongly suggested that miR-148b might be an inhibitor for pancreatic cancer.

Our clinical data showed that miR-148b deregulation was significantly associated with large tumor size, late TNM stage, lymphatic invasion and distant metastasis, which further indicated that deregulated miR-148b might facilitate the development of pancreatic cancer. Furthermore, the survival analysis revealed that downregulation of miR-148b correlated with shorter survival time of pancreatic cancer patients. Hence, determination of miR-148b expression could be a novel biomarker to predict the prognosis of pancreatic cancer patients.

Furthermore, overexpression of miR-148b could significantly inhibit proliferation by inducing apoptosis and cell cycle arresting at S phase, whereas, deregulation of miR148b promoted cell growth. On the other hand, overexpression of miR-148b inhibited invasion of pancreatic cancer cells. This study first showed that miR-148b functioned as a tumor suppressor in pancreatic cancer cells. As we known, chemotherapy resistance is still a major challenge for the therapy of pancreatic cancer (36, 37). Therefore, the identification of biomarkers beneficial for chemoresistance and understanding the related mechanisms will display advisable strategies to overcome this problem. Interestingly, our study showed that miR-148b mimics transfection promoted the sensitivity of PANC-1 and BXPC-3 cells to 5-FU, GEM and DDP. Therefore, therapeutic approaches to introduce miR-148b into cancer cells might be potentially feasible not only in prohibiting the development of tumorigenesis but also in sensitizing cancer cells to chemotherapeutic drugs.

To explore the mechanisms underlying the inhibition of pancreatic cancer cell growth mediated by miR-148b, we next set out to identify the potential target genes of miR-148b. The bioinformatics analysis indicates that AMPKα1 may be the potential target for miR-148b. Moreover, AMPKα1
plays important roles in linking metabolic syndrome and cancer (38, 39). Thus, we applied luciferase activity assay to identify the effect of miR-148b on AMPKα1 expression. The luciferase activity data showed that miR-148b was able to directly target the 3’UTR of AMPKα1. QRT-PCR and western blot results showed that the overexpression of miR-148b downregulated AMPKα1 expression in PANC-1 and BXPC-3 cells by both interfering and degrading mRNA. Therefore, the present results showed that AMPKα1 was upregulated in pancreatic cancer tissues and cell lines and inversely correlated with miR-148b expression. These data confirmed that miR-148b could downregulate the expression of AMPKα1, which also implied that AMPKα1 might be a promoter in pancreatic cancer.

There have accumulating evidences in recent years for an intrinsic link between cancer and metabolism. Highly anabolic malignant cells need to constantly increase protein translation and DNA synthesis to support their ongoing growth (40, 41), ultimately resulting in changes of cellular energy levels-detected by AMPK (18, 42), which suggests that AMPK is required for malignant progression. Previously, some research confirmed that activation of AMPKα1 inhibited the growth of human cancer cell by effecting mTOR signaling (24, 43), which suggested that AMPKα1 might be an inhibitor for cancer. On the other hand, other research showed that AMPKα1 could increase cell migration through up-regulation of integrin (25, 44) levels in cancer cells, which also indicated that AMPKα1 might be a promoter in cancer. In the present study, we further reveal that knockdown of AMPKα1 with RNAi inhibited pancreatic cancer cell growth both in vitro and vivo, consistent with Kato K results in vivo (26). These contradictory results suggest that the role of AMPKα1 is heterogeneous in different cancers.

Moreover, silencing of AMPKα1 caused apoptosis, cell cycle S-phase arrest, and inhibited invasion of pancreatic cancer cells, which was similar to the phenotypes induced by miR-148b overexpression. Furthermore, AMPKα1 overexpression could rescue the growth suppressive effect of miR-148b, and further rescue miR-148b-induced apoptosis and cell cycle arrest and invasion inhibition of pancreatic cancer. These findings strongly indicated that AMPKα1 might be a promoter in development of pancreatic cancer.
In conclusion, our present study demonstrated that miR-148b was downregulated in pancreatic cancer tissues and cell lines. And the low expression pattern was observed to be significantly correlated with increased tumor size, late TNM stage, lymphatic invasion, distant metastasis and worse prognosis. We also found that miR-148b can inhibit cell proliferation, invasion and enhance chemosensitivity of pancreatic cancer by targeting AMPKα1. Our data implicated the potential application of miR-148b as a tumor suppressor in pancreatic cancer therapy and also as a tumor marker for predicting prognosis.

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References


Table 1. Relationship between miR-148b and clinical pathological characteristics in 48 patients with pancreatic cancer

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**Figure Legends**

**Figure 1. Analysis of miR-148b expression in human pancreatic cancer tissues and cell lines by qRT-PCR**

(A) The relative expression levels of miR-148b in human pancreatic cancer specimens (n=48) and matched adjacent noncancerous pancreatic tissues (n=48). PC, pancreatic cancer tissue; NP, adjacent noncancerous pancreatic tissues. (B) Expression of miR-148b in five pancreatic cancer cell lines (PANC-1, ASPC-1, BXPC-3, SW1990 and Miapaca-2). Data are presented in pancreatic cancer cell lines relative to control. There is no normal pancreatic cell line, so we randomly chose three normal pancreatic tissues as control. U6 was used as a control for RNA loading, and miRNA abundance was normalized to U6 RNA. **P<0.01 compared with control. (C) Kaplan-Meier curves of overall survivals of 48 pancreatic cancer patients, according to miR-148b expression scored as low expression (below the median value, n=24) and high expression level (above the median value, n=24).

**Figure 2. MiR-148b inhibits growth of pancreatic cancer cells in vitro**

(A) The expression levels of miR-148b were tested by qRT-PCR in pancreatic cancer cells transfected with miR-148b mimics (miR-148b), miR-148b inhibitor (Inh-148b) and their respective NCs (50nM) for 48h. (B) The effect of transfection of miR-148b or Inh-148b and their respective NCs (50nM) on the growth of PANC-1 and BXPC-3 cells was examined by MTT assays. (C) The effect of miR-148b on cell proliferation was measured by MTT assays. 300 cells per well were incubated in 96-well plates for 1 to 7 days. Absorption values were read at 570 nm using an ELISA plate reader. (D) Proliferation was quantified using the colony formation assay. The colonies were evaluated and values were analyzed according to the ratio between LV-miR-148b-infected cells and LV-NC-infected cells at a multiplicity of infection of 10. All data are representative of three independent experiments. *P<0.05; **P<0.01 compared with control.

**Figure 3. MiR-148b induces apoptosis and cell cycle arrest and inhibits invasion of pancreatic cancer cells**
(A) PANC-1 and BXPC-3 cells were transfected with miR-148b or miR-NC (50nM) for 48h, and the apoptosis was measured by Annexin V staining and flow cytometry. (B) PANC-1 and BXPC-3 cells were treated as above, and cell cycle distribution was measured by PI staining and flow cytometry. (C) PANC-1 and BXPC-3 cells were treated as above, and the cell invasion ability was measured by matrigel invasion assays. Quantification was performed by counting the stained cells invading to the lower chamber under a light microscopy. The results were reproducible in three independent experiments. **P<0.01 compared with control.

**Figure 4. AMPKα1 is a direct target of miR-148b in PANC-1 cells**

(A. Top) Human AMPKα1 3’UTR fragment containing wild-type or mutated miR-148b–binding sequence. (Bottom) MiR-148b and the miR-148b-binding site in the 3’UTR of AMPKα1. (B) Luciferase reporter assays in PANC-1 cells, with cotransfection of wild-type or mutant 3’UTR and miR-148b or miR-NC as indicated. Firefly luciferase activity was normalized by Renilla luciferase activity. (C) The effects of miR-148b mimics or inhibitor on the expression of endogenous AMPKα1. QRT-PCR (left) and western blot (right) were used to monitor AMPKα1 expression in PANC-1 cells 48h after transfection with miR-148b or Inh-148b. *P<0.05; **P<0.01 compared with control.

**Figure 5. MiR-148b targeted AMPKα1 contributes to pancreatic cancer cell growth in vitro**

(A) The effect of siRNA targeting AMPKα1 on AMPKα1 expression and growth of PANC-1 and BXPC-3 cells was examined by western blot and MTT assays. PANC-1 and BXPC-3 cells transfected with 3 different siRNA targeting AMPKα1 (siAMPKα1-1, siAMPKα1-2 and siAMPKα1-3) or NC for 48h. (B) The effect of transfection of LV-shAMPKα1 and LV-AMPKα1 targeting AMPKα1 in PANC-1 and BXPC-3 cells was examined by qRT-PCR and western blot at 48 h following transfection. (C) The effect of transfection of LV-shAMPKα1 and LV-AMPKα1 on the growth of PANC-1 and BXPC-3 cells was examined by colony formation assays at 48 h following transfection. (D) The effect of miR-148b, LV-AMPKα1 and co-transfection of miR-148b and LV-AMPKα1 on AMPKα1 expression and pancreatic cancer cells viability were examined by western blot and MTT assays, respectively. All results were reproducible in three independent experiments. *P<0.05, **P<0.01.
Figure 1

A

miR-148b relative expression

B

miR-148b relative expression

C

Percent survival (%) vs. Follow up (months)

High miR-148b (n=24)
Low miR-148b (n=24)
Figure 2

Panel A: Relative expression of miR-146b in PANC-1 and BXPC-3 cells.

Panel B: Cell viability of PANC-1 and BXPC-3 cells.

Panel C: Growth curve of PANC-1 and BXPC-3 cells.

Panel D: Colony formation assay of PANC-1 and BXPC-3 cells.
Figure 3

A

PANC-1  

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B

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C

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Figure 4

A

B

C

Position 2009-2016 of
AMPKα2 3' UTR

WT MUT

WT MUT

WT MUT

WT MUT

Relative luciferase activity

AMPKα1 relative expression

AMPKα1

GAPDH

**

**

**

**
Figure 5

A

B

C

D

AMPKα1

GAPDH

Cell Viability (% of control)

Cell Viability (% of control)

Cell Viability (% of control)

Cell Viability (% of control)

NC  si-1  si-2  si-3

NC  si-1  si-2  si-3

NC  si-1  si-2  si-3

NC  si-1  si-2  si-3

PANC-1

AMPKα1

GAPDH

BXPC-3

AMPKα1

GAPDH

BXPC-3

AMPKα1

GAPDH

BXPC-3

AMPKα1

GAPDH

PANC-1

BXPC-3

PANC-1

BXPC-3

LUCNC  L1-C-AMPKα1  L1-A-AMPKα1  L1-L-AMPKα1

LUCNC  L1-C-AMPKα1  L1-A-AMPKα1  L1-L-AMPKα1

LUCNC  L1-C-AMPKα1  L1-A-AMPKα1  L1-L-AMPKα1

LUCNC  L1-C-AMPKα1  L1-A-AMPKα1  L1-L-AMPKα1

Colony Formation(%)
Molecular Cancer Therapeutics

MiR-148b functions as a tumor suppressor in pancreatic cancer by targeting AMPK α1

Gang Zhao, Jungang Zhang, Yang Liu, et al.

Mol Cancer Ther Published OnlineFirst November 20, 2012.

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