miR-148b Functions as a Tumor Suppressor in Pancreatic Cancer by Targeting AMPKα1

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Abstract

miRNAs are small noncoding RNAs that participate in a variety of biologic 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 biologic 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. Furthermore, the deregulated miR-148b was correlated with increased tumor size, late tumor–node–metastasis 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 tumorigenicity 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 RNA interference 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 of pancreatic cancer. Mol Cancer Ther; 12(1); 1–11. ©2012 AACR.

Introduction

miRNAs are a class of small noncoding RNAs, which contain of about 22 nucleotides. miRNAs bind to partially complementary sequences in the 3′-untranslated region (UTR) of specific target mRNA, resulting in either mRNA degradation or translation inhibition. miRNAs are small noncoding RNAs that participate in a variety of biologic 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 biologic 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. Furthermore, the deregulated miR-148b was correlated with increased tumor size, late tumor–node–metastasis 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 tumorigenicity 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 RNA interference 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 of pancreatic cancer. Mol Cancer Ther; 12(1); 1–11. ©2012 AACR.
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, 19). AMPK is a heterotrimeric complex consisting of a catalytic (α) and 2 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 shown that AMPK plays critical regulatory roles in cancer cell growth and tumorigenesis of cancer cell (21–23). For example, some research showed that activation of AMPKα1 suppressed the growth of human colon cancer cell (24), whereas 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 and colleagues displayed that AMPKα1-RNA interference (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 pancreatic cancer.

Materials and Methods

Patients and tumor tissues

Human pancreatic cancer tissues and corresponding adjacent normal pancreas tissues 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. Immediately after surgical removal, tissue specimens 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 histologic 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 (Wuhan, China).

Immunohistochemistry

AMPKα1 expression was determined by immunohistochemistry as previously described (27). Sections of paraffin-embedded tissue were incubation with the primary antibody to AMPKα1 (dilution 1:100, Santa Cruz) and a rabbit anti-goat horseradish peroxidase–labeled secondary antibody (dilution 1:200, Cell Signaling). All slides were independently analyzed by 2 experienced pathologists blinded to patient status. Cases with 30% or more positive tumor cells in a section were considered as positive expression (28).

RNA isolation and quantitative real-time reverse transcription 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) according to the manufacturer’s protocol. To quantify AMPKα1 expression, total RNA was converted to cDNA using M-MLV reverse transcriptase (Invitrogen). Quantitative real-time reverse transcription PCR (qRT-PCR) was conducted 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 miRNA-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^−ΔΔCt method. All qRT-PCR reactions were carried out in triplicate. The primers used in qRT-PCR are shown in Supplementary 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; they were tested and authenticated for genotypes by DNA fingerprinting. These cell lines were passaged for less than 6 months after resuscitation, and no reauthorization was done. Cells were cultured in RPMI-1640 mediums supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

miRNA 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.). siRNA duplexes with nonspecific sequences were used as siRNA-negative control. RNA oligonucleotides were transfected by using Lipofectamine 2000 (Invitrogen) and medium was replaced 6 hours after transfection. A final concentration of 50 nmol/L miR-148b, 100 nmol/L Inh-148b, and 50 nmol/L siAMPKα1 was used, and the expression levels of miR-148b and mRNA were quantified 48 hours 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, 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 Supplementary Table S2, and sequences of LV-shAMPKα1 were consistent with the sequences of siAMPKα1-2 that were 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 minutes 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 x 10^4 cells per well, treated with 50 nmol/L miR-148b and/or different concentrations of 5-fluorouracil (5-FU; Sigma-Aldrich), gemcitabine (Eli Lilly and Co.) and cisplatin (Sigma-Aldrich) for 48 hours 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 3 independent experiments, each of which used n = 6 replicate wells per assay condition.

Evaluation of cell apoptosis
To quantify cell apoptosis, Annexin V/propidium iodide (PI) staining was conducted. Briefly, cells were collected, washed in cold PBS for twice, and resuspended in binding buffer at a cell density of 1 x 10^6/mL. Cells were then stained with Annexin V-fluorescein isothiocyanate (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 mmol/L Tris-Cl (pH 7.4), PI, 0.1% Triton X-100 buffer, and 100 µg/mL RNase A). Cell-cycle analysis was accomplished by fluorescence-activated cell sorting (FACS) flow-cytometry.

Matrigel invasion assay
Cell invasion experiment was assessed using the Matrigel Invasion Chamber of pore size 8 mm (Corning, Fisher Scientific). A total of 5 x 10^4 cells was seeded into the upper compartment of the chamber precoated with Matrigel (Sigma). Medium containing 30% FBS was in the lower compartment of the chamber precoated with Matrigel Invasion Chamber. After the cells were incubated for 48 hours and fixed and stained with 0.5% crystal violet for 30 minutes, and the noninvasive 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 5 random fields.

Western blot analysis
Western blot analysis was conducted 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).

Luciferase activity assay
Luciferase activity assay was conducted using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. PANC-1 cells of 85% to 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 cotransfected into PANC-1 cells in a 96-well plate with 50 nmol/L miR-148b or 50 nmol/L miR-NC by using Lipofectamine 2000. Luciferase activity assay was conducted 48 hours after transfection using the Dual-Luciferase Assay System. Firefly luciferase activity was normalized to the corresponding Renilla luciferase activity. All experiments were carried out 3 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 x 10^6) in 100 µL PBS were injected subcutaneously into the right flank of 3-week-old female BALB/c nu/nu mice (n = 10 mice/group). The tumor size was measured every 4 days using a digital caliper and calculated by the formula: tumor volume = length x width^2 x 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 mean ± SD. Comparisons between groups were conducted with the unpaired t test. The relationships between miR-148b expression and clinic characteristics were analyzed by χ^2 tests. Survival of patients with pancreatic cancer was analyzed by log-rank test. The relationship between miR-148b and AMPKα1 expression was explored by Spearman correlation. Values were considered to be significantly different at P < 0.05. All statistical analysis was conducted using SPSS 13.0 software.

Results
miR-148b is deregulated in pancreatic cancer and correlates with clinical characteristics
Compared with noncancerous tissues, 41 pancreatic cancer 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 downregulated 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 Mia-paca-2 cells (0.07 ± 0.03-fold; Fig. 1B), respectively.
Furthermore, the results showed that deregulated expression of miR-148b significantly correlated with increased tumor size, late tumor–node–metastasis (TNM) stage, lymphatic invasion, and distant metastasis. There were no significant correlations between miR-148b with other clinical pathologic characteristics including sex, age, tumor location, histologic 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

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

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<td>Positive</td>
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<td>5</td>
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<td>0.003(^a)</td>
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<tr>
<td>Negative</td>
<td>28</td>
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\(^a\)\(P < 0.05\).
proliferation of PANC-1 and BXPC-3 cells was significantly inhibited by 32.14% ± 2.58% and 26.72% ± 2.95%, respectively. Although 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 as 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 with miR-NC, miR-148b mimics transfection that significantly increased the apoptosis rate in PANC-1 (5.63% ± 1.76% vs. 16.00% ± 0.96%) and BXPC-3 cells (2.67% ± 2.76% vs. 9.93% ± 0.80%; Fig. 3A). Moreover, the results showed that the S-phase was significantly increased in both PANC-1 (4.02% ± 3.52% vs. 44.10% ± 3.49%) and BXPC-3 cells (21.12% ± 3.00% vs.

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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 negative controls (50 nmol/L) for 48 hours. B, the effect of transfection of miR-148b or Inh-148b and their respective negative controls (50 nmol/L) 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 3 independent experiments. *, P < 0.05; **, P < 0.01 compared with control.
28.99% ± 2.32%) following transfection with miR-148b after 48 hours (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 48 hours, the IC₅₀ of PANC-1 cells was significantly decreased in 5-FU (8.33 ± 1.48 vs. 40.36 ± 3.39 µg/mL), gemcitabine (21.39 ± 1.89 vs. 83.66 ± 1.73 µg/mL), and cisplatin (5.77 ± 1.97 vs. 118.65 ± 1.50 µmol/L; Supplementary 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 µg/mL), gemcitabine (23.94 ± 1.28 vs. 75.20 ± 2.23 µg/mL), and cisplatin (5.72 ± 0.89 vs. 19.63 ± 1.59 µmol/L; Supplementary 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 (Supplementary Fig. S2A and S2B). qRT-PCR results showed that miR-148b expression levels were obviously increased in LV-miR-148–transfected tumors compared with control tumors (Supplementary Fig. S2C).

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

To show 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 cotransfection 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
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 analysis (right) were used to monitor AMPKα1 expression in PANC-1 cells 48 hours after transfection with miR-148b or Inh-148b. *, P < 0.05; **, P < 0.01 compared with control.

The results showed that the AMPKα1 expression levels in pancreatic cancer tissues were significantly higher than in adjacent normal pancreatic tissues (Supplementary 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 (Supplementary Fig. S3B). Although AMPKα1 expression levels in pancreatic cancer tissues always exceeded those of matched normal pancreas, some patient-to-patient variability was also obvious. We also found that AMPKα1 was upregulated in miR-148b–downregulated pancreatic cancer lines (Supplementary Fig. S3C). Furthermore, as shown in Supplementary Fig. S3D, a significant inverse correlation was observed between AMPKα1 and miR-148b expression in pancreatic cancer tissues (Spearman 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.

The expression of AMPKα1 mRNA and protein expression in PANC-1 cells. On the contrary, transfection of miR-148b inhibitor was able to upregulate 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 with control tumors (Supplementary Fig. S2D).

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

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

Western blot analysis results showed 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 analysis 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 conduct colony formation assays. The data showed that knockdown of AMPKα1 induced much fewer and smaller colonies, whereas 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. Furthermore, 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; Supplementary Fig. S4A and S4B). 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 with negative control group, AMPKα1-RNAi increased the cell apoptosis (Supplementary Fig. S5A) and caused a significant S-phase arresting of PANC-1 cells (Supplementary Fig. S5B). We also observed that PANC-1 cells invasion ability was significantly inhibited following AMPKα1-RNAi (Supplementary Fig. S5B). 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 (Supplementary Fig. S6A–S6C). 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 miRNAs has been frequently observed in various types of cancer (4, 5, 33), the current knowledge about the effects of miRNAs on pancreatic cancer and corresponding molecular mechanisms
miR-148b Targets AMPKα1 in Pancreatic Cancer

is still preliminary. Therefore, identifying the valuable miRNAs 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 downregulating 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 patients with pancreatic cancer. Hence, determination of miR-148b expression could be a novel biomarker to predict the prognosis of patients with pancreatic cancer.

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 know, chemotherapy resistance is still a major challenge for the therapy for pancreatic cancer (34, 35). 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, gemcitabine, and cisplatin. 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 (36, 37). 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 analysis 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 been 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 (29, 38), ultimately resulting in changes of cellular energy levels detected by AMPK (18, 39), 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, 40), which suggested that AMPKα1 might be an inhibitor for cancer. On the other hand, other research showed that AMPKα1 could increase cell migration through upregulation of integrin (25, 41) 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 in vivo, consistent with Kato and colleagues' 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 showed 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: G. Zhao, C.-Y. Wang
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