Abstract. MicroRNAs (miRNAs or miRs) are believed to have great potential for use as molecular targets in the diagnosis and treatment of cancer. In this study, we demonstrate that miR-375 is downregulated in pancreatic carcinoma (PC) tissues and PC cell lines. We found that miR-375 negatively regulates the expression of 3-phosphoinositide-dependent protein kinase 1 (PDK1) by directly targeting the 3'UTR of the PDK1 transcript. To investigate the biological roles and the potential mechanisms of action of miR-375, we induced either the up- or downregulation of miR-375 expression by transfecting various PC cells with miR-375 mimics or an inhibitor. Our results revealed that the upregulation of miR-375 inhibited cell growth and induced cell apoptosis, while the downregulation of miR-375 with the inhibitor had the opposite effect. In addition, our data demonstrate that miR-375 suppresses the malignant behavior of PC cells through the Akt signaling pathway rather than mitogen-activated protein kinase (MAPK) signaling pathways. Taken together, our findings indicate that targeting miR-375 by a genetic approach may provide a novel strategy for the treatment of PC.

Introduction

Pancreatic carcinoma (PC) is one of the most lethal and aggressive of all malignancies. It is the sixth leading cause of mortality from malignant disease in China and the fourth leading cause of cancer-related mortality in the United States (1,2). The median overall survival is >6 months and the 5-year survival rate is dismal, ranging between 2 and 6% (3). Despite advances in chemotherapy and radiotherapy over the past few decades, the overall prognosis of PC has remained essentially unchanged (4). Recently, the classical categories of oncogenes and tumor suppressor genes have been expanded to include a novel class of small non-coding RNAs, termed microRNAs (miRNAs or miRs), which can regulate a number of protein-coding genes, including tumor-related genes (5).

miRNAs regulate gene expression by base pairing with partially complementary messenger RNAs (mRNAs), resulting in the degradation of target mRNAs or the inhibition of their translation. The expression patterns of miRNAs appear to be tissue-specific and more accurate than mRNA-based profiling in tumor diagnostics. To date, a number of deregulated miRNAs have been found in human malignancies, including breast cancer, lung cancer, hepatocellular carcinoma (HCC) and colon cancer (6-8). Among these miRNAs, miR-375 was originally reported to be an islet cell-specific miRNA and to regulate insulin secretion (9). Recent studies have demonstrated the downregulation of miR-375 in gastric cancer (10), HCC (11) and colorectal cancer (12). However, knowledge of the mechanisms that regulate PDK1 expression during tumor progression is limited.

In this study, we investigated the biological roles and the potential mechanisms of action of miR-375 in PC. Furthermore, we identified PDK1 as a putative target of miR-375 by compu-
tational prediction and luciferase reporter assays. Our findings demonstrated that miR-375 may have great potential for use as molecular targets in the diagnosis and treatment of PC.

Materials and methods

Clinical samples. Tissue samples from 50 patients with PC were collected during surgical resections performed at the First Affiliated Hospital of Soochow University, Suzhou, China from January 2010 to December 2012. Tumor tissues and adjacent non-tumor tissues were frozen immediately after surgical removal in liquid nitrogen and stored at -80°C. The patients had not received any pre-operative chemotherapy, radiotherapy or immunotherapy. All samples were obtained after receiving patient consent from and approval from the Ethics Committee of Soochow University.

Cell culture and transfection. The human PC cell lines, PANC-1, SW1990, Capan-1, Patu8988, AsPC-1 and BxPC-3 obtained from the Shanghai Institute of Cell Biology (Shanghai, China) were maintained in DMEM or RPMI-1640 supplemented with 10% fetal bovine serum (all from Gibco, Grand Island, NY, USA) and 100 µg/ml each of penicillin and streptomycin (Invirtrogen, Carlsbad, CA, USA) in 5% CO₂ at 37°C.

Cells (1x10⁶) per well were cultured in 6-well plates overnight until they reached 60-70% confluence. Due to their different expression levels of miR-375, the PANC-1 cells express low levels of miR-375 and BxPC-3 cells express high levels. The PANC-1 and BxPC-3 cells were transfected with non-transfected cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as the transfection reagent. Non-transfected cells were used as the blank control (mock group).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR). Total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen). The expression levels of miR-375 were measured using a TaqMan microRNA assay (Applied Biosystems, Foster City, CA, USA) specific for hsa-miR-375 in the PCR reactions of 40 cycles (95°C, 15 sec; 60°C, 60 sec; 70°C, 30 sec), with U6 RNA as an internal control. PDK1 expression was detected with the following primers: forward, 5'-ACCCAACCACAAAGACACAAA-3' and reverse, 5'-TTTTGTTCTTTGTGGTGGTTGGGT-3'. As a negative control, the mutated binding site of miR-375 mimics or miR-negative control (NC) as described above. The cells were harvested 48 h after transfection and luciferase activity was measured using the Dual-Luciferase assay system (Promega, Madison, WI, USA).

Cell proliferation assay. The CCK8 assay was performed to determine cell growth. Following transfection, the cells were seeded in 96-well plates at 5x10⁴ cells per well, followed by the addition of 20 µl CCK8 (Dojindo Laboratories, Kumamoto, Japan) and incubation at 37°C for an additional 2 h. An ultraviolet spectrophotometer was used to measure the absorbance of each well at 450 nm, and each experiment was performed in triplicate and repeated 3 times.

Analysis of cell apoptosis. After 72 h of transfection, the cells were seeded and a volume of 100 µl of cell suspension (1x10⁶ cells/ml) was labeled with 10 µl of propidium iodided (PI) and 5 µl of Annexin V/FITC (both from BD Biosciences, Franklin Lakes, NJ, USA). The cells were incubated in the dark for 15 min at room temperature and the number of early apoptotic cells was determined using a FACSCalibur flow cytometer (BD Biosciences).

Terminal dUTP nick end-labeling (TUNEL) assays. The cells were plated on glass coverslips in 24-well plates and fixed in 4% paraformaldehyde for 60 min. After washing with phosphate-buffered saline, 50 µl/well Cy3-labeling TUNEL (Beyotime, Shanghai, China) reaction mixture were added to the cells followed by incubation for 60 min at 37°C in the dark. The TUNEL-positive cells emitted red fluorescence under a fluorescence microscope. The number of apoptotic cells was calculated by counting the number of positive cells in at least 5 randomly selected microscopic fields (magnification, x200).

Statistical analysis. SPSS software version 17.0 was used for statistical analysis. Data re expressed as the means ± standard deviation (SD). One-way analysis of variance (one-way Anova) or the t-test was performed for inter-group comparisons. Pearson's correlation analysis was used to compare
miR-375 expression with PDK1 expression. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

miR-375 is downregulated and inversely correlates with PDK1 in PC. To determine the expression levels of miR-375 in PC, we examined miR-375 expression in 50 pairs of PC tissues and matched adjacent non-tumor tissues by qRT-PCR. As shown in Fig. 1A, the miR-375 expression levels were significantly suppressed in the PC tissues (P<0.01). Of note, the expression of miR-375 inversely correlated with that of PDK1, a potential target of miR-375 (Fig. 1B and C).

miR-375 is a post-transcriptional regulator of PDK1. We examined the relative expression of miR-375 in 6 PC cell lines (PANC-1, SW1990, Capan-1, Patu8988, AsPC-1 and BxPC-3), from which the PANC-1 and BxPC-3 cells were selected, as they were found to have a low and high expression of miR-375, respectively (Fig. 2A). As shown in Fig. 2B and C, the levels of miR-375 were significantly upregulated in the PANC-1 cells following transfection with miR-375 mimics (P<0.05), while the levels of miR-375 were significantly downregulated in the BxPC-3 cells following transfection with the miR-375 inhibitor (P<0.05).

Furthermore, the expression of PDK1 was examined by qRT-PCR and western blot analysis in these 2 cell lines. Our data revealed no apparent change in PDK1 expression at the transcript level (Fig. 3A), while the PDK1 protein level was significantly downregulated in the cells transfected with miR-375 mimics and upregulated in the cells transfected with the miR-375 inhibitor compared with the NC-transfected cells (Fig. 3B; both P<0.05).

PDK1 is a direct target of miR-375. To confirm the direct targeting of PDK1 by miR-375, we integrated a firefly luciferase reporter plasmid containing a region of PDK1 3’UTR harboring the miR-375 target site, or a fragment whose target site was mutated (Fig. 4A), and used these to co-transfect the PANC-1 cells with miR-375 mimics or miR-NC. As shown in Fig. 4B, our results demonstrated that relative luciferase activity was decreased in the WT 3’UTR-transfected PANC-1 cells that were co-transfected with miR-375 mimics as compared with those cells co-transfected with miR-NC (P<0.05), while the mutation of the miR-375 binding site blocked this suppressive effect. These results strongly suggest that miR-375 negatively regulates the expression of PDK1 by directly targeting the 3’UTR of the PDK1 transcript.

Effect of miR-375 on proliferation and apoptosis of PC cells. Using the transfected cells, CCK8 assays were performed
to examine the effects of miR-375 on PC cell proliferation. As shown in Fig. 5A, the PANC-1 cells transfected with the miR-375 mimics displayed significant growth inhibition compared with the cells transfected with miR-NC at 48 and 72 h (both P<0.05). On the contrary, the BxPC-3 cells transfected with the miR-375 inhibitor showed an enhanced proliferation at 48 and 72 h (P<0.05).

We further assessed the effects of miR-375 on cell apoptosis by flow cytometry and TUNEL assays. The apoptotic rate in the PANC-1 cells transfected with the miR-375 mimics was significantly higher than that of the cells transfected with miR-NC (Fig. 5B; P<0.05). In the BxPC-3 cells, the cells transfected with the miR-375 inhibitor showed a decreased rate of apoptosis compared with the miR-NC-transfected group (P<0.05). As shown in Fig. 5C, TUNEL assays demonstrated that the number of apoptotic cells in the miR-375 mimic-transfected group was greater than that in the miR-NC-transfected group (miR-mimic-transfected cells, 120.16±10.04 cells per field; miR-NC-transfected cells, 31.06±4.57 cells per field; P<0.05). The downregulation of miR-375 induced by the inhibitor led to a decrease in the number of apoptotic cells (miR-375 inhibitor-transfected cells, 42.56±7.11 cells per field; miR-NC-transfected cells, 131.01±12.32 cells per field; P<0.05).

Effect of miR‑375 on mitogen‑activated protein kinase (MAPK) and Akt signaling pathways in PC cells. PDK1 is an oncogene involved in the regulation of cell cycle progression, apoptosis and invasion through the MAPK or Akt signaling pathways. Thus, we investigated the effects of miR-375 on the activation status of MAPK and Akt pathways by western blot analysis (Fig. 6). Our results revealed the decreased expression of phosphorylated (p)-Akt in the miR-375 mimic-transfected cells and an increased expression of p-Akt in the miR-375 inhibitor-transfected group as compared with the controls (NC). There was no change observed in the expression of ERK1/2, p-ERK1/2, p38, p-p38 and Akt. These results suggest that miR-375 suppresses the malignant behavior of PC cells through the Akt signaling pathway.

Discussion

In the present study, we investigated the role of miR-375 in PC and observed a tendency towards the downregulation of miR-375 expression in PC tissues compared to normal (non-tumor) ones. We induced either the up- or downregulation of miR-375 expression by transfecting PC cells with miR-375 mimics or miR-375 inhibitor and observed that miR-375 suppressed tumor cell proliferation and induced apoptosis. Furthermore, our results demonstrated that the 3'UTR of human PDK1 contained a putative target of miR-375 and that miR-375 regulated PDK1 expression at the protein level in PC cells.

miRNAs are small non-coding RNA molecules that play key roles in cell proliferation, apoptosis and differentiation in cancer initiation and progression (15). A great deal of evidence
Figure 5. Effects of microRNA-375 (miR-375) on cell growth and apoptosis. (A) Cell viability following transfection with miR-375 mimics or inhibitor was analyzed by CCK8 assay (*P<0.05). (B) Apoptotic cell rate following transfection with miR-375 mimics or inhibitor was detected by Annexin V/propidium iodide (PI) combined labeling flow cytometry (*P<0.05). (C) The number of apoptotic cells following transfection with miR-375 mimics or inhibitor in PANC-1 or BxPC-3 cells was counted under a fluorescence microscope by Cy3-labeling TUNEL assay (magnification, x200).
miR-375 and PDK1 expression in the PC clinical specimens. PDK1 is a key component of the PI3K/Akt signaling pathway and its overexpression has been associated with cell growth and proliferation in PC. Thus, the identification of miR-375 as a regulator of PDK1 may have potential therapeutic implications in PC and other malignancies.

ERK1/2 and p38, members of MAPKs, are important cellular protein kinases. Akt, also known as protein kinase B, is a serine/threonine protein kinase. The activation of Akt, ERK1/2 and p38 through phosphorylated forms plays a crucial role in the modulation of cell survival and apoptosis (25,26). Previous studies have demonstrated that miR-375 is involved in regulating target genes in the MAPK signal pathway (27,28). In this study, our data demonstrated that miR-375 modulated phosphorylation of Akt in PC cells, while no change was observed in p-ERK1/2 and p-p38 expression. To the best of our knowledge, our observations that miR-375 effectively influenced PC cell proliferation and apoptosis through the Akt signaling pathway have not been previously reported. Therefore, our findings provide a more comprehensive understanding of miR-375 in the development and progression of PC.

In conclusion, this study demonstrates that miR-375 negatively regulates the oncogene, PDK1, and modulates PC cell proliferation and apoptosis through the Akt signaling pathway. Our findings indicate that targeting miR-375 through a genetic approach may provide a novel strategy for the treatment of PC.

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