MicroRNA-874 inhibits proliferation and invasion of pancreatic ductal adenocarcinoma cells by directly targeting paired box 6

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Abstract. Studies have demonstrated that a number of microRNAs (miRNAs) are dysregulated in pancreatic ductal adenocarcinoma (PDAC), and alterations in their expression may affect the onset and progression of PDAC. Therefore, the expression patterns, biological functions and associated molecular mechanisms of miRNAs in PDAC should be elucidated for the development of novel therapeutic methods. Previous studies reported significant miRNA‑874 (miR‑874) dysregulation in multiple types of human cancer. However, the expression pattern, possible roles and underlying mechanisms of miR‑874 in PDAC remain to be elucidated. This study evaluated miR‑874 expression in PDAC and examined its biological functions and underlying mechanism of action in PDAC progression. miR‑874 expression was downregulated in multiple types of human cancer. However, the expression pattern, possible roles and underlying mechanisms of miR‑874 in PDAC remain to be elucidated. This study evaluated miR‑874 expression in PDAC and examined its biological functions and underlying mechanism of action in PDAC progression. miR‑874 expression was downregulated in PDAC tissues and cell lines. Functional experiments demonstrated that upregulation of miR‑874 inhibited cell proliferation and invasion in PDAC. Additionally, paired box 6 (PAX6) was predicted as a putative target of miR‑874 using bioinformatics analysis. Further experiments demonstrated that PAX6 may be the direct target gene of miR‑874 in PDAC. PAX6 knockdown exhibited similar inhibitory effects to miR‑874 overexpression in PDAC cells. In addition, restored PAX6 expression may reverse the suppressive roles of miR‑874 overexpression in PDAC cells. The results demonstrated that miR‑874 may serve tumor suppressive roles in PDAC by directly targeting PAX6. Therefore, miR‑874 may exhibit potential applications for treatment of patients with PDAC.

Introduction

Pancreatic cancer ranks the fifth most frequent cancer and the second leading cause of cancer-associated mortality worldwide (1). A total of ~227,000 patients are estimated to succumb to mortality from pancreatic cancer each year worldwide (2). The validated risk factors of pancreatic cancer include smoking, high-fat and high-protein diet, excessive drinking, high coffee consumption, exposure to certain chemical carcinogens, diabetes and chronic pancreatitis (3-5). Pancreatic ductal adenocarcinoma (PDAC) is a major type of primary pancreatic cancer and accounts for 96% of all cases of pancreatic cancer (6). Currently, the primary therapeutic method for early-stage PDAC is surgery. However, only 10-20% of patients with PDAC may be treated with surgery at the time of diagnosis (5). Despite considerable progress in therapy management, the outcome for patients with PDAC remains poor, with a low 5-year survival rate of <5% (7). The unfavourable prognosis of PDAC is primarily due to its aggressive characteristics, including rapid growth, invasion and metastasis (8). Therefore, the underlying mechanisms associated with PDAC occurrence and progression must be elucidated to provide novel insights into the development of new therapeutic methods for patients with this disease.

microRNAs (miRNAs) are a series of endogenous, non-coding and small RNA molecules composed of 18-24 nucleotides. miRNAs have been identified as gene regulators through interaction with the 3'-untranslated regions (3'-UTRs) of their target genes, causing mRNA degradation or inhibition of translation (9,10). Over one half of miRNAs are located at cancer-associated genomic regions or in fragile sites; therefore, miRNAs may serve key roles in tumorigenesis and tumor development (11). Considerable evidence indicates that miRNAs are dysregulated in almost all types of human malignancy (12-14). Deregulated miRNAs are implicated in the regulation of a wide variety of pathological processes, including cell proliferation, cycle, apoptosis, survival, invasion and metastasis (15,16). Furthermore, miRNAs may serve as oncogenes or tumor suppressors in tumor initiation and progression which mainly depends on the characteristics of their target genes (17). Therefore, cancer-associated miRNAs must be further investigated to identify new therapeutic targets for anticancer treatment.
Previous studies reported significant miRNA-874 (miR-874) deregulation in several types of human cancer (18-21). However, the expression pattern, possible roles and associated molecular mechanisms of miR-874 in PDAC remain to be elucidated. The present study evaluated miR-874 expression in PDAC, and its biological function and underlying mechanism of action in PDAC progression.

Materials and methods

Tissue specimens and cell lines. A total of 29 pairs of PDAC tissues and matched adjacent non-tumor tissues were collected from patients (17 males, 12 females; age range, 48-73 years) who were treated with surgery at Jilin Cancer Hospital between May 2014 and January 2016. No patients underwent chemotherapy or radiotherapy prior to surgery. This project was approved by the Ethical Committee of Jilin Cancer Hospital. Written informed consent was also provided by all participants before the study. Tissue specimens were immediately frozen in liquid nitrogen and then stored at -80°C prior to RNA isolation.

Four human PDAC cell lines, Bxpc-3, Panc-1, Sw1990 and Aspc-1, were acquired from Cell Bank Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). A normal human pancreatic cell line HPDE6c7 was obtained from American Type Culture Collection (Manassas, VA, USA). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. Following an incubation for 6 h, the culture medium was discarded and fresh DMEM containing 10% FBS was added into each well.

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Transfection of miRNA mimics, small interfering RNA (siRNA) and plasmid. Cells were plated into six-well plates at a density of 7x10^4 cells per well. Following an incubation overnight, the cells were transfected with miR-874 mimics, miRNA mimic negative control (miR-NC; both 100 pmol; both from Shanghai GenePharma, Co., Ltd., Shanghai, China), small interfering RNA (siRNA) targeting the expression of paired box (PAX) 6, negative control siRNA (NC siRNA; both 100 pmol; both from Guangzhou RiboBio, Co., Ltd., Guangzhou, China), PAX6 overexpression plasmid pcDNA3.1-PAX6 or empty pcDNA3.1 plasmid (both 4 µg; both from Chinese Academy of Sciences; Changchun, China) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells were cultured at 37°C in a humidified incubator with 5% CO₂.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). TRIzol reagent (Thermo Fisher Scientific, Inc.) was used to isolate total RNA from tissue specimens or cells. For the quantification of miR-874, total RNA was converted into complementary DNA using a TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). The temperature protocol for reverse transcription was as follows: 16°C for 30 min, 42°C for 30 min and 85°C for 5 min.

Subsequent RT-qPCR was conducted using a TaqMan MicroRNA PCR kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). The cycling conditions for RT-qPCR were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec; and annealing/extension at 60°C for 60 sec. To analyze PAX6 mRNA expression levels, reverse transcription was performed with a PrimeScript RT Reagent kit (Takara Biotechnology, Co., Ltd., Dalian, China). The temperature protocol for reverse transcription was as follows: 37°C for 15 min and 85°C for 5 sec. Subsequently, a SYBR Premix Ex Taq™ II kit (Takara Biotechnology, Co., Ltd.) was utilized to perform qPCR. The cycling conditions for RT-qPCR were as follows: 5 min at 95°C, followed by 40 cycles of 95°C for 30 sec and 65°C for 45 sec. U6 snRNA and GAPDH were used as control for normalization of miR-874 and PAX6 mRNA, respectively. The primers were designed as follows: miR-874 forward, 5'-GGCCCTGAGGAAGACTG AG-3' and reverse, 5'-TGGAGATCCAACAGGCTTTGAC-3'; U6 forward, 5'-GCTTCGCGAAGCATACTATACAA T-3' and reverse, 5'-CGGTTAATGGGTACCTACCA-3'; PAX6 forward, 5'-GAATCAGAAGAGACGCAAC3' and reverse, 5'-GTGTAAGTATCATACTACCGG-3'; and GAPDH forward, 5'-CAGGATGCAACGGATTTGTCGAT-3' and reverse, 5'-AGCTTTCCTCATGGTGTTGAAC-3'. Relative gene expression was calculated using the 2^(-ΔΔCq) method (22).

Cell Counting kit-8 (CCK-8) assay. At 24 h post-transfection, cells were collected and seeded into 96-well plates in triplicate at a density of 3,000 cells/well. The extent of proliferation was determined with a CCK-8 assay (Beyotime Institute of Biotechnology, Haimen, China) at 0, 24, 48 and 72 h after inoculation. Briefly, a total of 10 µl CCK-8 reagent was added into each well and further incubated at 37°C for 2 h. Subsequently, the absorbance was measured at a wavelength of 450 nm using a plate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All assays were repeated three times.

Transwell invasion assay. A Transwell chamber with 8 µm pores (BD Biosciences, San Jose, CA, USA) was utilized to assess cell invasive ability. Prior to the measurement of invasion ability, the upper chamber was coated with 100 µl diluted Matrigel (BD Biosciences) and incubated at 37°C in 5% CO₂ for 2 h. A total of 5x10⁴ cells in 200 µl of FBS-free DMEM medium were plated into the upper chambers. A total of 500 µl DMEM supplemented with 20% FBS was used as a chemoattractant in the lower chambers. After culturing for 24 h at 37°C with 5% CO₂, the non-invasive cells that remained on the upper surface of the Transwell chamber were removed with a cotton swab. The invasive cells were fixed with 100% methanol at room temperature for 20 min and stained with 0.5% crystal violet (Beyotime Institute of Biotechnology) at room temperature for 20 min. The cells were washed with PBS, and images of the stained cells were captured and counted in ≥5 randomly selected fields under an inverted light microscope (magnification, x200; Olympus Corporation, Tokyo, Japan). All experiments were performed independently in triplicate and repeated three times.
Target prediction. Target gene detection software, TargetScan (version 7.1; www.targetscan.org) and miRanda (August 2010 Release; www.microrna.org) were used to predict the potential target genes of miR-874.

Luciferase reporter assay. The wild-type (Wt) and mutant (Mut) 3′-UTR of PAX6 were designed and produced by Shanghai GenePharma Co., Ltd., and subcloned into the pGL3 reporter plasmid (Promega Corporation, Madison, WI, USA) and named pGL3-PAX6-3′-UTR Wt and pGL3-PAX6-3′-UTR Mut, respectively. For the luciferase reporter assay, cells were seeded into 24-well plates, cultured to ~60% confluence and co-transfected with pGL3-PAX6-3′-UTR Wt or pGL3-PAX6-3′-UTR Mut together with miR-874 mimics or miR-NC using Lipofectamine® 2000 in accordance with the manufacturer’s protocol. Transfected cells were cultured at 37˚C with 5% CO₂ for 48 h and then luciferase activity was determined using a dual-luciferase reporter analysis system (Promega Corporation) in accordance with the manufacturer’s protocol. Renilla luciferase activity was normalized to firefly luciferase activity.

Western blot analysis. Cells or tissue specimens were harvested and homogenized in ice-cold radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology). A bicinechonic acid protein quantitation kit (Beyotime Institute of Biotechnology) was used to detect the concentration of total protein. An equal quantity of protein (30 µg) was separated on 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked at room temperature for 2 h with 5% fat-free milk dissolved in Tris-buffered saline containing 0.1% Tween-20 (TBST) and incubated overnight at 4°C with the following primary antibodies: mouse anti-human PAX6 monoclonal antibody (1:1,000 dilution; cat no. sc-32766) and mouse anti-human GAPDH monoclonal antibody (1:1,000 dilution; cat no. sc-166574; all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA). After washing with TBST three times, the membranes were probed with goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:5,000 dilution; cat no. sc-2005; Santa Cruz Biotechnology, Inc.) at room temperature for 2 h. The protein signals were visualized via an enhanced chemiluminescence kit (GE Healthcare, Chicago, IL, USA) and band intensity was analyzed with Quantity One software (version 4.62; Bio-Rad Laboratories, Inc.). GAPDH was used as loading control.

Statistical analysis. Data are presented as the mean ± standard deviation and analyzed using a Student’s t test and one-way analysis of variance for multiple comparisons followed by a Student-Newman-Keuls test. All statistical analysis was performed with SPSS software (version 18.0; SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

miR-874 is downregulated in PDAC tissues and cell lines. To determine the expression pattern of miR-874 in PDAC, this study first detected miR-874 expression in 29 pairs of PDAC tissues and matched adjacent non-tumor tissues. RT-qPCR analysis revealed that miR-874 expression was significantly downregulated in PDAC tissues compared with the adjacent non-tumor tissues (Fig. 1A; P<0.05). To further characterize miR-874 in PDAC, this study determined miR-874 expression levels in four PDAC cell lines (Bxpc-3, Panc-1, Sw1990 and Aspc-1) and one normal human pancreatic cell line (HPDE6c7) via RT-qPCR analysis. The results indicated that miR-874 expression levels were lower in all PDAC cell lines compared with the adjacent non-tumor tissues (Fig. 1A; P<0.05). To further characterize miR-874 in PDAC, this study determined miR-874 expression levels in four PDAC cell lines (Bxpc-3, Panc-1, Sw1990 and Aspc-1) and one normal human pancreatic cell line (HPDE6c7) via RT-qPCR analysis. The results indicated that miR-874 expression levels were lower in all PDAC cell lines compared with HPDE6c7 (Fig. 1B; P<0.05). Panc-1 and Sw1990 cells, which demonstrated relatively lower expression levels of miR-874 among the four PDAC cell lines, were selected for further experiments. Therefore, decreased miR-874 expression may be associated with PDAC progression.

miR-874 inhibits proliferation and invasion of PDAC cells. To elucidate the biological functions of miR-874 in PDAC, miR-874 mimics were introduced into Panc-1 and Sw1990 cells. RT-qPCR analysis confirmed that miR-874 was overexpressed in Panc-1 and Sw1990 cells transfected with miR-874 mimics (Fig. 2A; P<0.05). CCK-8 assay was performed to investigate the effect of miR-874 overexpression on PDAC...
miR-874 directly targets PAX6 by binding to its 3′-UTR in PDAC. To identify the mechanisms underlying the action of miR-874 in PDAC, bioinformatics analysis was performed to predict the putative targets of miR-874. PAX6, which participates in the regulation of PDAC carcinogenesis and development (23), was predicted as the major target of miR-874 and used in the experiment (Fig. 3A). To confirm their targeting association, luciferase reporter assay was conducted in Panc-1 and Sw1990 cells co-transfected with miR-874 mimics or miR-NC and pGL3-PAX6-3′-UTR Wt or pGL3-PAX6-3′-UTR Mut. The luciferase activity of Wt PAX6 3′-UTR was suppressed in miR-874 mimic-transfected Panc-1 and Sw1990 cells when compared with those of the miR-NC group (Fig. 2C; P<0.05). Therefore, miR-874 may have tumor-suppressive roles in PDAC growth and metastasis.

PAX6 mRNA and protein expression levels, respectively, in Panc-1 and Sw1990 cells following transfection with miR-874 mimics or miR-NC. The results revealed that miR-874 upregulation reduced PAX6 expression in Panc-1 and Sw1990 cells at both mRNA (Fig. 3C; P<0.05) and protein (Fig. 3D; P<0.05) levels. Based on the aforementioned data, miR-874 may negatively regulate PAX6 expression in PDAC by directly binding to its 3′-UTR.

PAX6 inhibition attenuates proliferation and invasion of PDAC cells. PAX6 was identified as the direct target of miR-874 in PDAC. Therefore, the present study hypothesized that the suppressive effects of miR-874 overexpression on PDAC cell proliferation and invasion may be due to PAX6 knockdown. To verify this hypothesis, PAX6 siRNA was introduced into Panc-1 and Sw1990 cells to knockdown PAX6 endogenous levels. Following transfection, western blot analysis demonstrated that PAX6 was downregulated in PAX6 siRNA-transfected Panc-1 and Sw1990 cells compared with the NC siRNA-transfected cells (Fig. 4A; P<0.05). Subsequent functional experiments indicated that downregulation of PAX6 expression reduced Panc-1 and Sw1990 cell proliferation (Fig. 4B; P<0.05) and invasion (Fig. 4C; P<0.05), which was similar to the effect caused by miR-874 overexpression. These results further suggested that PAX6 may be a functional downstream target of miR-874 in PDAC.

PAX6 upregulation counteracts the inhibitory effects of miR-874 overexpression on PDAC cells. To determine whether
the tumor-suppressive roles on PDAC cell proliferation and invasion are mediated by the downregulation of PAX6, this study performed a series of rescue experiments. Panc-1 and Sw1990 cells were transfected with miR-874 mimics in combination with empty pcDNA3.1 plasmid or PAX6 overexpression plasmid pcDNA3.1-PAX6 that lacked the 3'-UTR. The results of western blot analysis revealed that PAX6 protein levels were restored in the Panc-1 and Sw1990 cells co-transfected with miR-874 mimics and empty pcDNA3.1 plasmid (Fig. 5B; P<0.05). Subsequent functional experiments demonstrated that restoring PAX6 expression rescued the suppressive effects on proliferation (Fig. 5B; P<0.05) and invasion (Fig. 5C; P<0.05) of Panc-1 and Sw1990 cells induced by miR-874 overexpression. Accordingly, miR-874 may serve as a tumor suppressor in PDAC at least in part by directly inhibiting PAX6.

Discussion

A large number of studies have shown that many miRNAs are dysregulated in PDAC, and alterations in their expression levels may affect the onset and progression of PDAC (24-26). Therefore, the expression patterns, biological functions and associated molecular mechanisms of miRNAs in PDAC must be elucidated for the development of novel therapeutic methods. The present results indicated that miR-874 was significantly downregulated in PDAC tissues and cell lines. The overexpression of miR-874 inhibited proliferation and invasion of PDAC cells. Through bioinformatics analysis, PAX6 was predicted to be a major target of miR-874. Additionally, luciferase report assay, RT-qPCR and western blot analysis indicated that miR-874 may negatively regulate PAX6 expression in PDAC cells by directly binding to the 3'UTR of PAX6. PAX6 inhibition exhibited similar inhibitory effects to miR-874 overexpression in PDAC cells. Furthermore, restoring PAX6 expression rescued the suppressive effects of miR-874 overexpression on PDAC cells. These data suggested that miR-874 exerted tumor-suppressive effects on PDAC by targeting PAX6 and may be an innovative candidate target for the treatment of patients with this fatal disease.

The dysregulation of miR-874 has been observed in multiple types of human cancer. For example, miR-874 was downregulated in osteosarcoma tissues and cell lines (27). Decreased miR-874 expression was associated with tumor-node-metastasis (TNM) stage, tumor size and lymph node metastasis in osteosarcoma (27). miR-874 expression levels were decreased in colorectal cancer and this downregulation exhibited a strong association with TNM stage and lymph node metastasis (28-30). In hepatocellular carcinoma, miR-874 expression levels were lower in tumor tissues and cell lines. Low miR-874 expression was associated with tumor stage, differentiation and lymph node metastasis (31,32). Additionally, patients with hepatocellular carcinoma with low miR-874 levels exhibited poorer prognosis than those with high miR-874 levels (31,32). In gastric cancer, miR-874 was downregulated in clinical samples and cell lines. miR-874 expression levels were associated with lymphatic invasion and histological type in patients with gastric cancer (33). The downregulation of miR-874 was also observed in breast cancer (18), maxillary sinus squamous cell...
carcinoma (19), head and neck squamous cell carcinoma (20) and non-small cell lung cancer (21). These results suggest that miR-874 deregulation may be developed as a promising prognostic biomarker in these types of human cancer.

miR-874 has been implicated in the regulation of cancer initiation and progression. For instance, resumption expression of miR-874 suppressed osteosarcoma cell growth and metastasis, increased apoptosis in vitro and reduced tumour growth in vivo (27,34). Numerous studies have indicated that miR-874 upregulation inhibited cell proliferation, induced apoptosis and reversed chemoresistance in colorectal cancer (28-30). Que et al (30) and Leong et al (31) demonstrated that enforced expression of miR-874 inhibited cell proliferation, colony formation, metastasis and epithelial-mesenchymal transition, and promoted the apoptosis of hepatocellular carcinoma. Jiang et al (33) and Zhang et al (35) reported that miR-874 overexpression repressed gastric cancer cell growth and metastasis and decreased angiogenesis in vitro and in vivo. Wang et al (18) revealed that miR-874 overexpression reduced cell proliferation and promoted apoptosis in breast cancer. Nohata et al (19) demonstrated that restoring the expression of miR-874 significantly inhibited the cell proliferation and invasion of maxillary sinus squamous cell carcinoma. Kesana And et al (21) found that restoration of expression of miR-874 decreased the cell invasion ability in vitro and tumor growth in vivo in non-small cell lung cancer. These results suggested that miR-874 may be investigated as a novel and effective therapeutic target in the treatment of specific types of cancer.

Scientists have validated several targets of miR-874, including E2F transcription factor 3 in osteosarcoma (27), X-linked inhibitor of apoptosis (28), signal transducer and activator of transcription 3 (29) in colorectal cancer, peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (31) and SRY-box 12 (32) in hepatocellular carcinoma, aquaporin 3 (33) in gastric cancer, cyclin dependent kinase 9 (18) in breast cancer, protein phosphatase 1 catalytic subunit α (19) in maxillary sinus squamous cell carcinoma and histone deacetylase 1 (20) in head and neck squamous cell carcinoma. In the present study, PAX6 was identified as a novel target of miR-874 in PDAC. PAX6, a member of the PAX gene family, serves as a key regulator in the development of eyes, central nervous system and pancreas (36,37). PAX6 was found to be expressed at elevated levels in in several types of human cancer, including colorectal cancer (38), retinoblastoma (39), breast cancer (40) and non-small cell lung cancer (41). Furthermore, deregulated PAX6 is implicated in the regulation of tumor formation and progression through regulating...
cell proliferation, cell cycle, apoptosis, migration and invasion (38,39,42). PAX6 is also overexpressed in PDAC. PAX6 downregulation reduces cell cycle, growth, differentiation, invasion and metastasis (23). Therefore, the miR-874/PAX6 pathway may provide novel and efficient therapeutic targets in treating this aggressive cancer.

In conclusion, miR-874 was downregulated in PDAC tissues and cell lines. miR-874 may serve tumor-suppressive roles in PDAC by directly targeting PAX6. The results of the present study may provide novel evidence for the potential of miR-874/PAX6-based targeted therapy for patients with PDAC.

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Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.
Authors' contributions

YY and YL designed the present study. JD, XS and LC performed the experiments. All authors have read and approved the final draft.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of Jilin Cancer Hospital and was performed in accordance with the Declaration of Helsinki and the guidelines of the Ethics Committee of Jilin Cancer Hospital. Written informed consent was obtained from all patients for the use of their clinical tissues.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

10. Lytle JR, Yario TA and Steitz JA: Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5′ UTR as in the 3′ UTR. Proc Natl Acad Sci USA 104: 9667-9672, 2007.