Insight into the effects of microRNA-23a-3p on pancreatic cancer and its underlying molecular mechanism

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Abstract. Previous studies have demonstrated that microRNA (miR)-23a-3p plays a role as an oncogene that is involved in several different types of carcinoma. However, few studies investigated the association between miR-23a-3p and pancreatic cancer (PC). The aim of the present study was to elucidate the biological functions of miR-23a-3p in PC and to investigate its underlying molecular mechanisms. The expression of miR-23a-3p in PC and adjacent normal tissues was investigated using microarrays. In order to validate the outcomes of the microarray results, reverse transcription-quantitative (RT-q)PCR was used to determine the expression levels of miR-23a-3p in PC tissues and cell lines. Furthermore, functional analyses were conducted following miR-23a-3p inhibition and overexpression, in order to assess the proliferation, invasion and migration of PC cells. Bioinformatics analysis indicated transforming growth factor-β receptor type II (TGFBR2) as a potential direct target of miR-23a-3p. Western blotting was performed in order to determine the protein expression of TGFBR2 in PC cell lines. The findings from the microarray demonstrated upregulation of miR-23a-3p in PC compared with normal tissues. RT-qPCR revealed significantly higher levels of miR-23a-3p expression in PC compared with normal control tissues or cells. Furthermore, miR-23a-3p was demonstrated to promote the proliferation, invasion and migration of PC cells, which was suppressed by the inhibition of miR-23a-3p. In addition, the miR-23a-3p expression level was negatively associated with TGFBR2 expression. Overall, the present study demonstrated the tumor-promoting effects of miR-23a-3p in PC cells. Furthermore, miR-23a-3p is a potential oncogenic regulator of PC, by targeting TGFBR2, and a biomarker or target for molecular therapy.

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Introduction

Pancreatic cancer (PC) is considered as a fatal disease and is the third leading cause of cancer-associated mortality in United States in 2019 (1). The overall 5-year survival rate was 9.3% between 2009 and 2015 in United States (1). Compared with other types of cancer, the early diagnostic rate of PC is low, which remains to be one of the critical factors contributing to its dismal prognosis (2). However, there has been no feasible biomarker for the prediction or treatment of PC. Thus, the investigation of the underlying molecular mechanism of PC progression and development, and the identification of novel biomarkers, is likely to be of benefit.

The microRNAs (miRNAs/miRs) are a group of small non-coding class of RNAs containing >1,500 shortened non-coding RNA molecules. miRNAs are single-stranded and ~22 nucleotides in length (3-5). By controlling and targeting downstream genes, miRNAs are likely to facilitate tumorigenesis or suppress the growth of tumors (3,6).

A receptor for transforming growth factor-β is the transmembrane serine/threonine kinase, TGF-β receptor type II (TGFBR2). There are seven exons that encode 567 amino acids to constitute a gene, which can be detected at chromosome 3p22, forming a transmembrane region (referred to as a NH_2-terminal of ligand binding domain) and an active COOH-terminal serine/threonine kinase domain (7). As a member of the TGF-β signal pathway, TGFBR2 is vital for several biological processes, such as cell proliferation, migration, apoptosis and differentiation (8,9). The expression levels of TGFBR2 have been demonstrated as commonly altered in various types of cancer (10).

The present study reported that the TGFBR2 may be targeted by miR-23a-3p. Several different methods were adopted to demonstrate the upregulation of miR-23a-3p expression in PC tissues and cells. Furthermore, the promotion of proliferation, invasion and migration of PC cells was likely to be associated with the inhibition of TGFBR2 expression, mediated by the overexpression of miR-23a-3p.

Materials and methods

Patients and microRNA arrays. PC and adjacent tissues obtained from regions outside the tumor margin (>1 cm) were collected simultaneously from 32 patients who underwent surgical resection in the Department of General Surgery, The Affiliated Changzhou No. 2 People's Hospital of Nanjing Medical University (Changzhou, China). Tumors and adjacent tissues were stored in liquid nitrogen at -80˚C. The tissues included in the present study were collected between February 2017 and May 2018. Of the samples, three were used for microarrays, and the remainder were used for reverse transcription-quantitative (RT-q)PCR. The demographics, clinical information and procedural data were collected from patients' medical records. The staging system and version used for Tumor-Node-Metastasis staging of PC was based on the American Joint Committee on Cancer and the Union for International Cancer Control (11,12).

The high-throughput genome analysis of miRNAs was conducted by Guangzhou RiboBio Co., Ltd. The results were analyzed using the Partek Genomics suite software (version 6.6; Partek, Inc.) for multi-dimensional scaling, clustering and heatmap drawing. Upregulated miRNAs were screened based on the fold change >2.

RT-qPCR analysis. Total RNA was extracted from tissues and cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA concentration was evaluated at an absorbance of 260 nm using the Thermo NanoDrop2000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). The Prime-Script™ RT Reagent kit (Takara Biotechnology Co., Ltd.) was used to synthesize cDNA in the following conditions: 37˚C for 15 min, 85˚C for 5 sec, and finally maintained at 4˚C. qPCR was conducted using the SYBR® Premix Ex Taq® kit (Takara Biotechnology Co., Ltd.) using a Real Time PCR system (Bio-Rad Laboratories, Inc.). The housekeeping gene U6 was used as a control to normalize the expression levels of the miRs. The primers for qPCR were designed by Guangzhou RiboBio Co., Ltd., and the sequences were as follows: miR-23a-3p: Forward, 5'-GCGATCACATTG CCAGGG-3'; and reverse, 5'-CAGTGCGTGTCGTGGAGT-3'; U6: Forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3'; and reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'. The thermocycling conditions were as follows: 95˚C for 20 sec, followed by 40 cycles of denaturation at 60˚C for 30 sec and dissociation at 95˚C for 1 min and 55˚C for 1 min. The miR-23a-3p level was calculated using the $2^{-\Delta\Delta Cq}$ method (13).

Cell culture. Human PC cell lines (AsPC-1, MIA-Paca-2, BxPC-3, Sw1990 and PANC-1) and human pancreatic ductal cell line (HPDE6-C7) were purchased from the The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. All cell lines were cultured in DMEM or RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin and streptomycin (Sigma-Aldrich; Merck KGaA) at 37˚C in a 5% CO_2 atmosphere.

Transfection. miR-23a-3p mimics, inhibitors and relative controls were purchased from Guangzhou RiboBio Co., Ltd. The sequences of the negative controls for the mimic and inhibitor are non-targeting. The sequences were as follows: Mimics, 5'-AUCACAUUGCCAGGGAUUUCC-3'; 3'-UAG UGUAACGGUCCCUAAAGG-5'; inhibitors, 5'-GGAAAU CCCUGGCAAUGUGAU-3'; mimics negative control, 5'-UUUGUACUACACAAAAGUACUG-3'; 3'-AAACAU GAUGUGUUUUCAUGAC-5'; inhibitor negative control, 5'-CAGUACUUUUGUGUAGUACAAA-3'. The mimics were transfected at a concentration of 100 nM/well and the inhibitors at 150 nM/well into PANC-1 and SW-1990 cells using the riboFECT™CP reagent (Guangzhou RiboBio Co., Ltd.) at 37˚C, according to the manufacturer's protocol. The cells were cultured for 72 h following transfection prior to further experiments.

Cell proliferation assay. Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) was used to measure cell proliferation. PANC-1 and SW-1990 cells were seeded at a density of 3,000 cells/well on 96-well plates. Cell viability was assayed at 0, 24, 48 and 72 h post-transfection using CCK-8 reagent. The absorbance values at 450 nm were measured using the Quant Micro-plate spectrophotometer (BioTek Instruments, Inc.).

Migration and invasion assays. For the migration assay, PANC-1 or SW-1990 cells ($3x10^4$) were transfected for 72 h prior to seeding onto the upper chamber of Transwell inserts (24-well insert, 8-μm pore size; BD Biosciences). For the invasion assay, the membranes were coated with Matrigel for 4 h at 37˚C to form a matrix barrier prior to seeding the PC cells. The upper chamber was filled with 200 μl serum-free DMEM, and 500 μl DMEM supplemented with 10% fetal bovine serum was used in the lower chamber. The cells were incubated at 37˚C for 36 h for both migration and invasion assays. The membranes of the Transwell inserts were fixed with absolute methanol for 20 min and stained with 0.1% crystal violet for 30 min at room temperature. Cells were photographed at x10 magnification using an inverted microscope and counted.

Target genes prediction. The target genes of miR-23a-3p were predicted using TargetScan (http://www.targetscan.org/), miRDB (http://www.mirdb.org/miRDB/), starBase (http://starbase.sysu.edu.cn/) and miRtarBase (http://mirtarbase.mbc.nctu.edu.tw/php/index.php) online analysis tools. The overlapping genes between above target genes were screened for further study. The seed match sites of miR-23a-3p on TGFBR2 were obtained from TargetScan.

Western blot analysis. Following the transfection of PANC-1 and SW-1990 cells with miR-23a-3p mimics, inhibitors and mimic controls, total protein was extracted from these cells using radioimmunoprecipitation assay lysis buffer containing protease inhibitor (Beyotime Institute of Biotechnology). Protein concentration was determined using a Nanodrop 2000 spectrophotometer (NanoDrop; Thermo Fisher Scientific, Inc.) by measuring the optical density at 280 nm. Protein (20 μg/lane) was loaded and separated via SDS-PAGE (12.5% gel). The proteins were transferred to 0.22 μg polyvinylidene fluoride membranes. Following blocking with 5% non-fat milk at room temperature

for 1 h, the membranes were incubated with antibodies targeting TGFBR2 (1:1,000; cat. no. 79424s) and β-actin (1:1,000; cat. no. 8457s) at 4˚C overnight. Subsequently, the membranes were incubated with anti-rabbit IgG (H+L) biotinylated antibody (1:3,000; cat. no. 14708s) diluted in TBS-Tween-20 (0.1% Tween-20) for 15 h at room temperature. All antibodies were obtained from Cell Signaling Technology, Inc. The signals were exposed to film (FujiFilm), using the enhanced chemiluminescence reagent (ECL plus; Beyotime Institute of Biotechnology), in accordance with the manufacturer's protocol. The ChemDoc imaging system (Abcam) (Pierce; Thermo Fisher Scientific, Inc.) was used for visualizing the bound antibodies. The ImageJ software (version 1.46r; National Institutes of Health) was used for the semi-quantification of densitometry, which was normalized to β-actin.

Statistical analysis. All data were obtained from at least three independent experiments, and are presented as the mean ± standard deviation. Paired student's t-test was used to compare differences between two groups and one-way analysis of variance followed by the least significant difference post hoc test was used to compare differences among three or more groups. Clinical features of the patients, alongside miR-23a-3p expression, were assessed using Fisher's exact probability test. P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS software (version 22.0; IBM Corp.).

Results

Association between miR-23a-3p and clinical pathology of PC. The age, sex and lymph node metastasis of patients were not associated with the expression of miR-23a-3p (Table I). However, high miR-23a-3p expression was associated with low Tumor-Node-Metastasis (TNM) stage and large tumor size.

miRNA expression profile of PC. In order to identify the dysregulated miRNAs that may participate in the tumorigenesis of PC, miRNA microarrays were performed on three individual pairs of tissues from PC and adjacent non-cancerous pancreatic tissues (Fig. 1). Paired student's t-test was used for the analysis and the dysregulated miRNAs were demonstrated to have ≥2-fold higher number of changes in their expression levels. The P-value threshold was set at 0.01 to screen for a total of 100 differential miRNAs compared with the paired tissues (Fig. 2A).

miR-23a-3p was highly expressed in PC tumors compared with the adjacent normal tissues (Fig. 2B).

miR-23a-3p expression in PC cell lines and tissues. In order to detect the expression of miR-23a-3p in PC cell lines and tissues, RT-qPCR was performed. The expression of miR-23a-3p was upregulated in PC tissues compared with adjacent tissues (Fig. 2C). Subsequently, the expression level of miR-23a-3p was determined in five PC cell lines (AsPC-1, PANC-1, MIA Paca-2, BxPC-3 and SW1990) and a pancreatic cell line (HPDE6-C7). The expression of miR-23a-3p was increased in PC cell lines, notably in PANC-1 and the SW1990 cells, compared with HPDE6-C7 cells (Fig. 2D).

Table I. Association between miR-23a-3p expression and clinical features.

		miR-23a-3p expression, n		
Variables	Total, n	Low	High	P-value
Age, years				>0.500
<60	8	0	8	
≥60	21	3	18	
Sex				>0.500
Male	17	2	15	
Female	12	1	11	
Tumor size[a], cm				0.003
<3	5	3	2	
≥3	24	0	24	
TNM stage[a]				0.005
I-II	23	0	23	
III-IV	6	3	3	
Lymph node metastasis				>0.500
Positive	13	1	12	
Negative	16	2	14	

[a]Based on The 7th American Joint Committee on Cancer for the Future of TNM (12). miR, microRNA; TNM, Tumor-Node-Metastasis.

Cell proliferation of PC cells is promoted by miR-23a-3p. The effect of miR-23a-3p on the proliferation of PC was investigated in the present study. The expression of miR-23a-3p was affected by mimics and inhibitors in PANC-1 and SW1990 (Fig. 3A). PANC-1 cells were transfected with the miR-23a-3p inhibitor at different concentrations. The CCK-8 assay was performed at 24, 48 and 72 h after transfection (Fig. 3B), which revealed that the amount of proliferation decreased as the dose of inhibitor increased. Subsequently, the effects of miR-23a-3p inhibitor and mimic on the proliferation of PANC-1 and SW1990 cells were investigated using the CCK-8 assay. The proliferation was decreased at 48 and 72 h in PANC-1 and SW1990 cells transfected with the miR-23a-3p inhibitor 150 nM, whereas those transfected with the miR-23a-3p mimic 100 nM demonstrated enhanced proliferation (Fig. 3C and D).

miR-23a-3p expression enhances cell invasion and migration. The invasion and migration capabilities of PANC-1 and SW1990 cells transfected with miR-23a-3p mimics and inhibitors were assessed via Transwell migration and matrigel invasion assays. Following a 24-h incubation period the number of migrated/invaded cells were counted. The invasion and migration capability of PANC-1 and SW1990 were significantly increased following the overexpression of the miR-23a-3p compared with the control cells; the opposing effect was observed in cells transfected with the miR-23a-3p inhibitor (Fig. 4).

miR-23a-3p decreases the expression of the target TGFBR2. Several candidate targets of miR-23a-3p were predicted

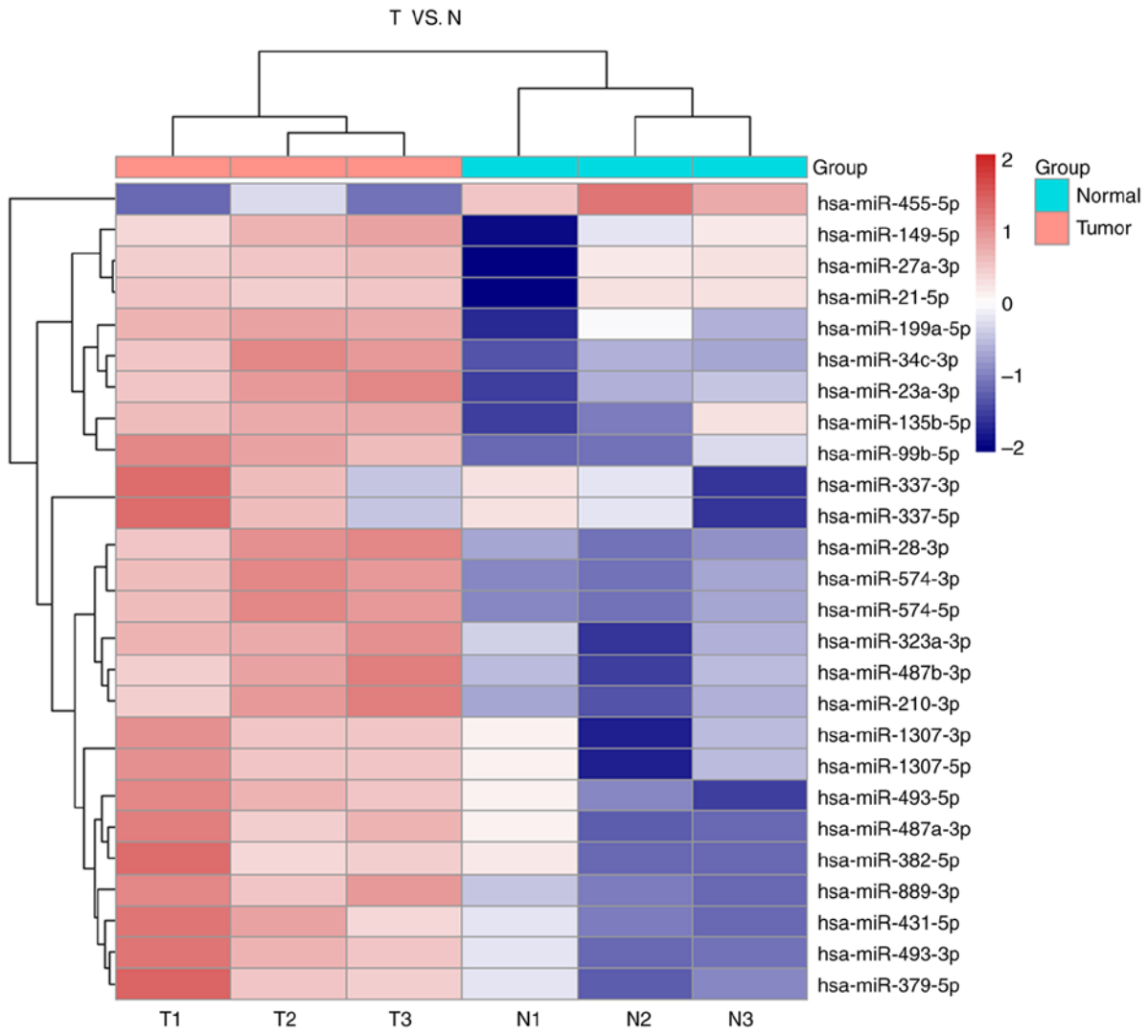

Figure 1. Heatmap presenting the expression levels of various miRNAs from microarray analysis according to tissue type. The higher the expression level, the darker the color (red, upregulated; blue, downregulated). T, tumor tissue; N, normal tissue; miRNA/miR, microRNA.

by online analysis tools, of which TGFBR2 was further investigated in the present study (Fig. 5A). Western blotting revealed decreased ratio of TGFBR2 to β-actin in PANC-1 and SW1990 cells transfected with miR-23a-3p mimic compared with those transfected with mimic control (Fig. 5B and C). Thus, the expression of miR-23a-3p was negatively associated with TGFBR2 protein expression.

Discussion

miRNAs act as both oncogenes and tumor suppressor genes in various types of cancer (14,15) and are critical to the pathological and the physiological processes (such as proliferation, metabolism, differentiation and apoptosis) (16).

Several recent studies demonstrated that miR-23a-3p was associated with the development of several cancer types, such as melanoma cancer, liver cancer and renal cell carcinoma (17-20). Thus, miRNAs are potential biomarkers and therapeutic targets (17,21). It has been reported that there are higher levels of miR-23a-3p expression in the serum of patients with colon cancer compared with that of healthy donors (22,23). The higher expression level of miR-23a-3p could be vital for the early processes of carcinogenesis. The metastasis suppressor 1 (MTSS1) was demonstrated to be a direct target of miR-23a-3p, which potentially participates in the invasion of cancer (24). Zhu *et al* (25) demonstrated higher expression levels of miR-23a-3p in esophageal squamous cell cancer due to its close association with tumor differentiation, and could play a significant role in the microenvironment of esophageal carcinoma. Furthermore, high expression levels of miR-23a-3p were detected in lung adenocarcinoma, as well as in cervical cancer (26,27).

Since the finding by Calatayud *et al* (28) that miR-23a-3p was upregulated in PC, few studies have investigated the detailed roles and other molecular mechanisms of miR-23a-3p in PC; thus far the conclusions remain unclear and contradictory.

In the present study, the ≥2-fold change in expression level was defined as differentially expressed, and the P-value threshold was set as 0.01. Subsequently, miR-23a-3p expression in PC was detected using three pairs of PC samples via high-throughput genome analysis. The microarray results revealed 19 differentially expressed genes, of which miR-23a-3p expression was upregulated in PC. In order to verify the feasibility of the microarray, the remaining tissue specimens and

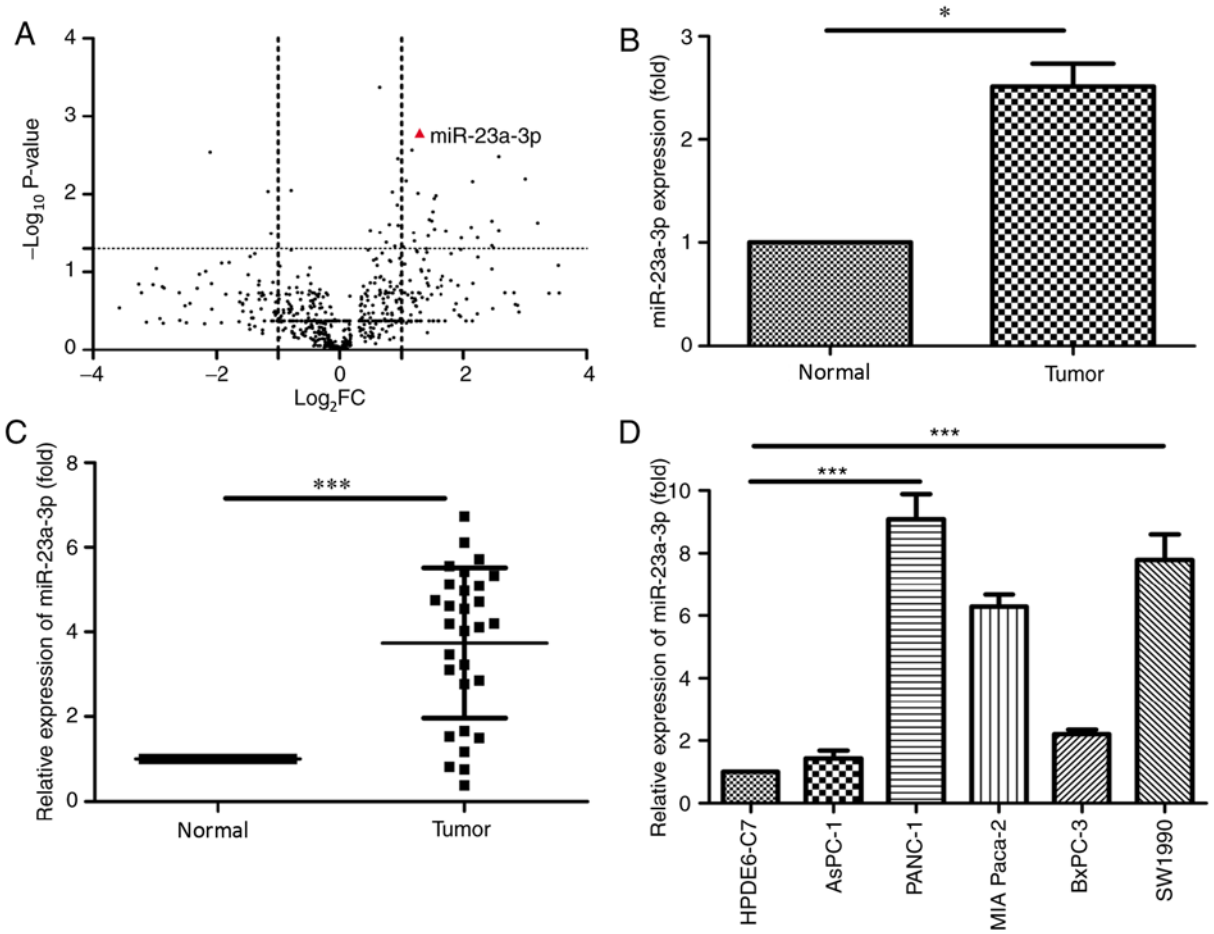

Figure 2. Expression levels of miR-23a-3p in PC tissues and cell lines. (A) The expression levels of miRNAs from microarray analysis visualized in a Volcano plot, constructed using the FC and P-values. The association between fold change and statistical significance, which considers both the magnitude of change and variability, is visualized. The vertical lines mark the 2.0-FC (decrease and increase) and the horizontal line represents P=0.05. The red point in the plot represents miR-23a-3p. (B) miRNA arrays analysis reveals overexpression of miR-23a-3p in 3 samples of PC compared with normal tissues. (C) Analysis of miR-23a-3p expression levels in 29 pairs of PC tissues and normal tissues. (D) Expression level of miR-23a-3p in pancreatic cell line (HPDE6-C7) and pancreatic cancer cell lines (AsPC-1, MIA-Paca-2, BxPC-3, SW1990 and PANC-1). *P<0.05 vs. normal. ***P<0.001 vs. normal or HPDE6-C7. PC, pancreatic cancer; miRNA/miR, microRNA; FC, fold change.

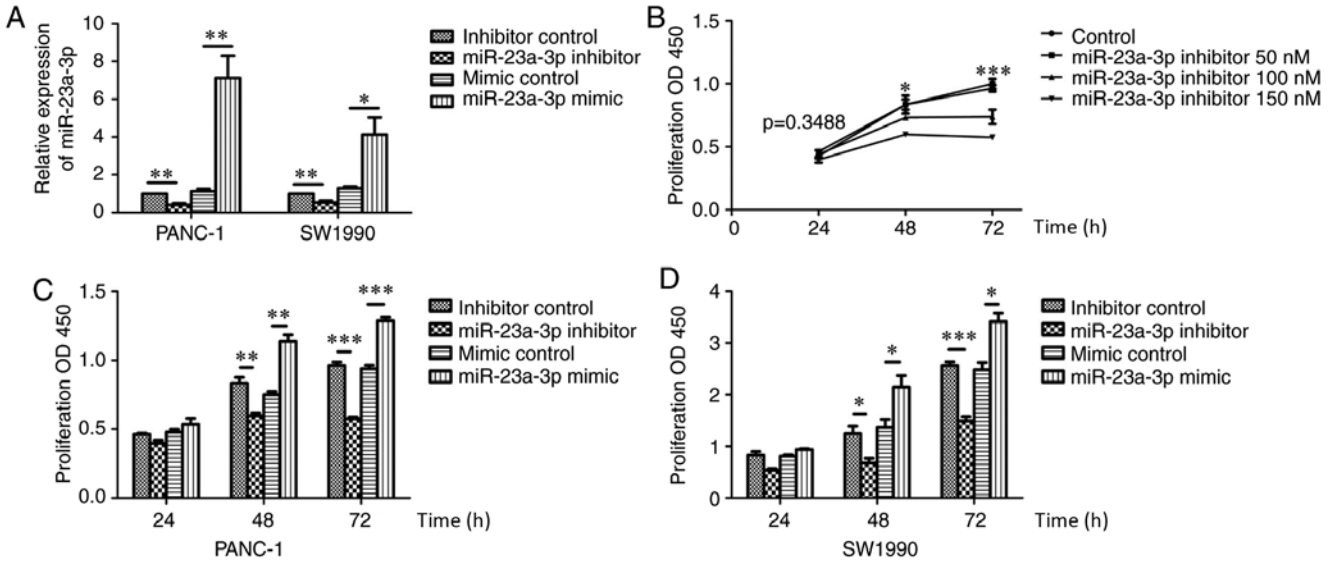

Figure 3. miR-23a-3p promotes the proliferation of PC cells. (A) Expression of miR-23a-3p in PANC-1 and SW1990 cells transfected with miR-23a-3p mimics or inhibitors for 48 h was detected to confirm transfection efficiency. (B) Concentration-dependent inhibition of PANC-1 cell proliferation by miR-23a-3p inhibitors. The effect of miR-23a-3p inhibitor and mimic on the proliferation of (C) PANC-1 and (D) SW1990 cells at 24, 48 or 72 h. *P<0.05 vs. control or inhibitor control or mimic control. **P<0.01 vs. inhibitor control or mimic control. ***P<0.001 vs. control or inhibitor control or mimic control. PC, pancreatic cancer; miR, microRNA; OD, optical density.

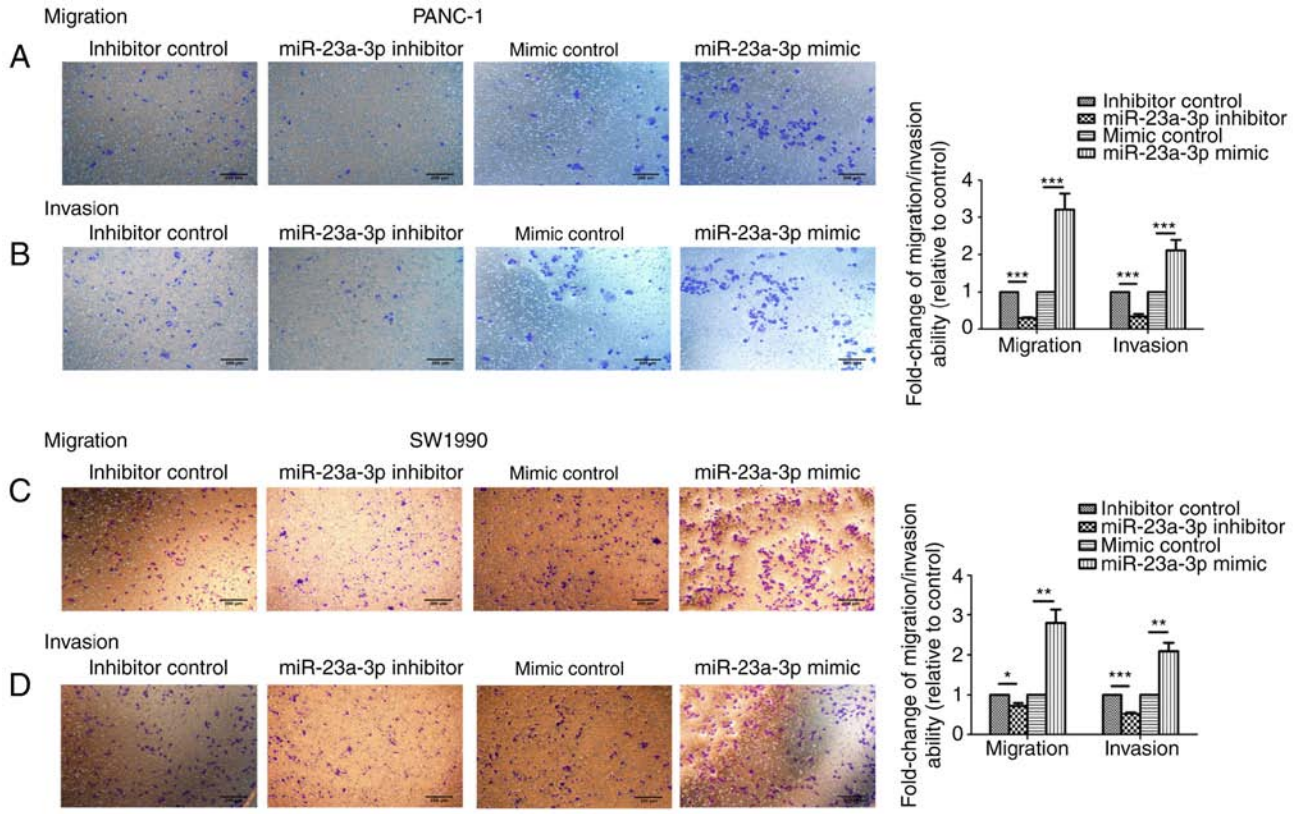

Figure 4. Promotion of cell migration and invasion by miR-23a-3p in pancreatic cancer cells. (A and B) Cell migration was evaluated using Transwell inserts and (C and D) invasion was evaluated using Matrigel-coated Transwell inserts in PANC-1 and SW1990 cells transfected with miR-23a-3p mimic, inhibitor or respective controls. Representative images of the migratory/invasive cells are shown. Scale bar, 200 μm. *P<0.05 vs. inhibitor control. **P<0.01 vs. mimic control. ***P<0.001 vs. inhibitor control or mimic control. miR, microRNA.

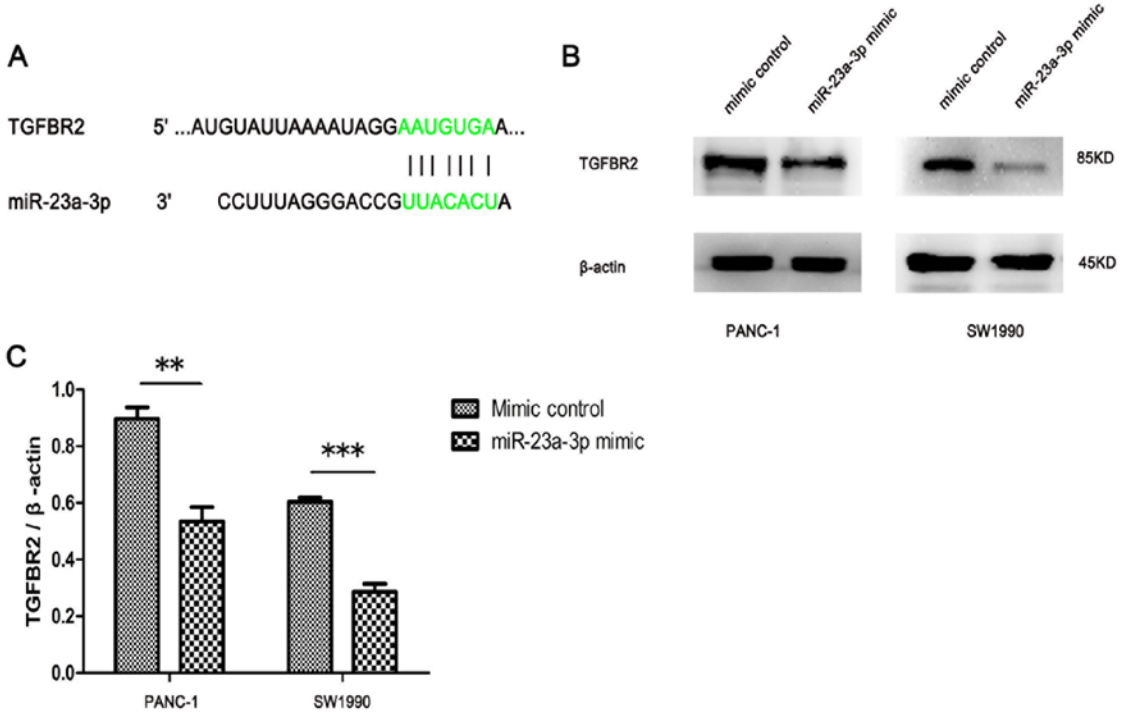

Figure 5. Inhibition of TGFBR2 by miR-23a-3p in PANC-1 and SW1990 cells. (A) Diagram demonstrating the predicted miR-23a3p binding site in the 3'-untranslated region of TGFBR2 mRNA. The seed match sites of miR-23a-3p on TGFBR2 were predicted by TargetScan. (B and C) Western blot analysis of PANC-1 and SW1990 cells treated with miR-23a-3p mimics and mimic control for 72 h. The histogram demonstrates decreased expression levels of TGFBR2 in PANC-1 and SW1990 cells transfected with miR-23a-3p mimic compared with mimic control. **P<0.01 vs. mimic control. ***P<0.001 vs. mimic control. miR, microRNA; TGFBR2, TGF-β receptor type II.

five PC cell lines were employed to perform RT-qPCR, which demonstrated increased expression levels of miR-23a-3p in PC tissues and cells compared with non-neoplastic controls. Furthermore, clinical information indicated the association of high miR-23a-3p expression with larger tumor size. Thus, it was speculated that miR-23a-3p may exhibit oncogenic activities in PC. Furthermore, miR-23a-3p expression promoted cell proliferation and facilitated cell invasion and migration. However, lymph node metastasis was not associated with miR-23a-3p expression, whereas miR-23a-3p expression was negatively associated with TNM stage. There were two explanations considered regarding the findings of the present study: i) miR-23a-3p primarily affected PC by enhancing the ability of invasion, rather than lymph node metastasis; and ii) insufficient organized specimens limited the study, and more samples are required in order to enhance feasibility.

In addition, bioinformatics analysis predicted TGFBR2 as a potential target gene of miR-23a-3p. Despite little emphasis on TGFBR2 in the literature, its mutation and downregulation was detected in various types of cancer such as colorectal cancer, lung cancer and breast cancer (20,29-32). Furthermore, Shima *et al* (33) reported that mutations in TGFBR2 were associated with 5-year survival rates in colorectal cancer. Zhou *et al* (34) reported that the linc00462/miR-665/TGFBR1-TGFBR2/smad2/3 axis was vital for cell migration, invasion, proliferation and tumor metastasis in PC. Furthermore, Yang *et al* (35) demonstrated that lower TGFBR2 expression levels in patients were associated with poor prognosis in cervical cancer. In the present study, western blotting revealed a negative association between the expression levels of miR-23a-3p and TGFBR2 protein levels. Thus, the dysregulation of miR-23a-3-p by targeting TGFBR2 could impact the pathological process of PC.

The limitations of the present study included insufficient number of specimens, lack of rescue experiments and *in vivo* experiments.

Overall, the present study indicated that the expression of miR-23a-3p may be associated with the expression of TGFBR2, and partially facilitate the progression of PC. Furthermore, the findings of the present study could provide novel approaches for PC diagnosis and treatment.

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Availability of data and materials

The datasets used and analyzed during the present study are available from the corresponding author on reasonable request. The datasets are available from TargetScan (http://www.targetscan.org/), miRDB (http://www.mirdb.org/miRDB/), starBase (http://starbase.sysu.edu.cn/) and miRtarBase (http://mirtarbase.mbc.nctu.edu.tw/php/index.php).

Authors' contributions

CZ, JC, LJ, XZ, DD, SW and XQ contributed to the conception and design of the study. CZ, JC, LJ, LM, BZ, SS, XY, PG and JL provided the study materials and patients. JC, LM, BZ, SS, XY, PG, JL, CZ and XQ contributed to the collection and assembly of data. All authors contributed to data analysis and interpretation. JC wrote the manuscript. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Study Committee of Changzhou No. 2 People's Hospital [approval no. (2018) KY024-01]. Written informed consent was obtained from each participating patient (or guardian) prior to entry into the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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