Original Paper

MicroRNA-195 Suppresses the Progression of Pancreatic Cancer by Targeting DCLK1

Bin Zhou^a Chuandong Sun^a Xiao Hu^a Hanxiang Zhan^b Hao Zou^a Yujie Feng^a
Fabo Qiu^a Shun Zhang^a Liqun Wu^a Bingyuan Zhang^a^aDepartment of Hepatopancreatobiliary Surgery, the Affiliated Hospital of Qingdao University, Qingdao, Shandong Province; ^bDepartment of General Surgery, Qilu Hospital, Shandong University, Jinan, Shandong Province, China

Key Words

Pancreatic cancer • DCLK1 • MiR-195 • Proliferation • Metastasis

Abstract

Background/Aims: Doublecortin-like kinase 1 (*DCLK1*) is emerging as a tumor-specific stem cell marker in pancreatic cancer (PC). *MicroRNA-195* (*miR-195*) plays an important role in many types of tumors. However, the roles of *DCLK1* in cancer and miRNAs that directly regulate *DCLK1* have not been elucidated. The goal of this study is to assess the effects of *miR-195* on inhibiting *DCLK1* and to clarify the regulating mechanism of *miR-195-DCLK1* in PC cells. **Methods:** The expression of *DCLK1* protein and *miR-195* in PC tissues and adjacent healthy pancreatic tissues was detected by Western blot and quantitative reverse transcription polymerase chain reaction (qRT-PCR), respectively and the correlation between overall survival of PC patients and expression of *DCLK1* was measured by Kaplan-Meier analysis. Bioinformatics tools were used to identify the target gene of *miR-195*. Effects of *miR-195* and *DCLK1* on proliferation and cell cycle of PC cells were analyzed by MTT, colony formation assays and flow cytometry. Transwell and wound-healing experiments were employed to examine the cellular migration and invasion. A xenograft mouse model was also used to test the effects of *miR-195* on tumor growth and metastasis *in vivo*. **Results:** The expression level of *DCLK1* and *miR-195* shows an inverse correlation in PC tissues and cell lines. A higher *DCLK1* level is associated with higher TNM (tumor, node, and metastasis) stage, higher rate of lymph node metastasis, and poor survival. Luciferase reporter assay shows that *miR-195* directly targets *DCLK1*. Overexpression of *miR-195* inhibits proliferation, migration and invasion of PC cells, whereas downregulation of *miR-195* has an opposite role. These actions were similar to the effects of knockdown and overexpression of *DCLK1*, respectively. **Conclusions:** These data suggest that *miR-195* has tumor suppressor roles in PC by targeting *DCLK1*. *MiR-195-DCLK1* pathway may provide insight into PC progression and represent a novel, promising diagnostic and therapeutic target for PC.

© 2017 The Author(s)
Published by S. Karger AG, Basel

B. Zhou and C. Sun contributed equally to this work.

Bin Zhou
and Bingyuan ZhangDepartment of Hepatopancreatobiliary Surgery, the Affiliated Hospital of Qingdao University, Qingdao, Shandong Province (China)
E-Mail binalin@163.com, bingyuanzhang@126.com

Introduction

Pancreatic cancer (PC) is a disease associated with high mortality, with a 5-year overall survival rate of less than 5% [1] because of its late presentation, early metastasis, and unresponsiveness to most treatment options [2]. Only 15% to 20% of PC tumors are resectable at the time of diagnosis. In a large proportion of patients with PC, locally advanced or metastatic disease is diagnosed at the time of presentation because PC usually causes no symptoms in its early stage [3, 4]. Nevertheless, PC responds poorly to both chemotherapy and radiation therapy [5]. Therefore, a better understanding of the molecular mechanisms underlying PC development will be useful to identify novel detection biomarkers and to improve current therapeutic strategies.

In our preliminary studies, through exome capture sequencing and bioinformatics analysis, we found that doublecortin-like kinase 1 (*DCLK1*) is a specific molecular candidate associated with PC metastasis [6]. *DCLK1* is a microtubule-binding member of the calmodulin-dependent kinase family and is overexpressed in pancreatic intraepithelial lesions as well as surgical resection specimens of PC [7-9]. Previous studies have demonstrated the therapeutic effects of *DCLK1* in cancer. Inhibiting *DCLK1* with small interfering RNA (siRNA) suppresses epithelial-to-mesenchymal transition of PC cells through regulating important microRNAs (miRNAs) [8]. However, the functional role of miRNAs on targeting *DCLK1* in PC has not been clarified.

A recent study has shown that deregulated expression of series miRNAs correlates with PC development [10]. Through the online tools and luciferase assay, we found that *microRNA-195* (*miR-195*) directly targets *DCLK1*. *MiR-195* is an important member of the *miR-15a*, *-15b*, *-16*, *-195*, and *-497* families, which play an important role in the occurrence and developmental progress of many tumors and in regulation of malignant biological behaviors [11-14]. Nevertheless, the role of *miR-195* in PC development and especially the potential targets of this miRNA in PC cells remain unknown.

In this study, we found that *DCLK1* exhibited a higher level, whereas *miR-195* was significantly downregulated in human PC tissues and cell lines, in which *DCLK1* and *miR-195* have an inverse relationship. Both high level of *DCLK1* and low level of *miR-195* in PC tissues are associated with poor survival of patients with PC. We also clarified the effects of *miR-195* on proliferation and metastasis of PC cells by using *in vitro* assays and animal models. Furthermore, we confirmed that *miR-195* exerts its tumor suppressor effects by inhibiting *DCLK1* in PC cells. Our findings highlight that *miR-195* functions as a tumor suppressor in PC and implicate *miR-195-DCLK1* as a potential diagnostic and therapeutic target for PC.

Materials and Methods

Patient tissue samples and PC cell lines

The human PC tissues and adjacent healthy pancreatic tissues were obtained from the Affiliated Hospital of Qingdao University and Qilu Hospital between January 2011 and May 2016 with patients' informed consent. The category of PC samples was confirmed by pathological analysis. The use of these samples was approved by the Clinical Research Ethics Committee of Qingdao University (Qingdao, People's Republic of China [PRC]). All samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until used. None of the patients received chemotherapy or radiotherapy before the surgical excision. Five PC cell lines (BxPC-3, PANC-1, SW1990, MIAPaCa-2, and AsPC-1) and human normal pancreatic duct epithelial cells (HPDE6-C7) were purchased from the Tianjin Sai'er Biotechnology Co. Ltd (Tianjin, PRC).

Immunohistochemistry staining

Immunohistochemistry assay was performed by BenchMark XT automated slide-staining instrument (Ventana, Arizona, USA) with Ultraview Universal DAB Detection Kit (Ventana, Arizona, USA) after deparaffinization, rehydration, and antigen retrieval. After primary antibody incubation, the sections were incubated with anti-mouse horseradish peroxidase labeled polymer (Roche, Arizona, USA) for 30 min

at room temperature, and then developed with diaminobenzidine. All slides were counterstained with hematoxylin. For *DCLK1* staining, the reactivity assessed was cytoplasmic. All micrographs were taken with a Nikon microscope equipped with a digital color camera and software. The immunostaining was evaluated by a pathologist who was blinded to patient prognosis and clinicopathological outcomes.

Cell culture and transfection

All cells were grown in RPMI 1640 (GIBCO BRL, USA) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL of penicillin, and 100 µg/mL of streptomycin, and incubated at 37°C in a humidified chamber supplemented with 5% CO₂. Transfection was performed with Lipofectamine 2000 Reagent (Invitrogen) following the manufacturer's protocol. Spiked red fluorescent protein (RFP)-expressing vector was used to monitor transfection efficiency.

The prediction of miRNA targeting gene

The *miR-195* targets were predicted on the calculations of mirSVR scores by the computer-aided algorithms of PicTar and TargetScan Release 5.1.

Luciferase-reporter assay

PANC-1 cells were cotransfected using 0.2 µg of luciferase reporter vector with a wild-type or mutant *DCLK1* 3'UTR in 48-well plates and *miR-195* mimics or mimics control. The assay was normalized with 0.05 µg of the RFP expression vector pDsRed2-N1 (Clontech, USA). The cells were lysed with RIPA lysis buffer (0.15 M NaCl, 0.05 M Tris/HCl pH 7.2, 1% Triton X-100, 0.1% SDS) 48 hours later. The luciferase activity and RFP intensity were detected with an F-4500 Fluorescence Spectrophotometer (Hitachi, Japan).

RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA from cultured cells or surgical tissues was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. qRT-PCR was performed using the All-in-One™ miRNA qRT-PCR detection kit (GeneCopoeia, Rockville, MD, USA) for *miR-195* and small nuclear RNA U6 as an endogenous control. The relative expression level of mRNA was detected by SYBR Green qRT-PCR assay (Bio-Rad Laboratories Inc, Hercules, CA, USA), and β-actin was used as an endogenous control. All qRT-PCR procedure was performed on an ABI 7500 thermocycler (Thermo Fisher Scientific, Waltham, MA, USA). Primers used were as follows: β-actin, 5'-AGTGTGACGTGGACATCCGCAAAG-3' (forward), 5'-ATCCACATCTGCTGGAAGGTGGAC-3' (reverse); *DCLK1*, 5'-CCAGCTCAGTTAATGGAAC-3' (forward), 5'-GGACGCAAGTGACGTAGA-3' (reverse). The specific primers for *miR-195* (HmiRQP0376) and U6 (HmiRQP9001) were purchased from RiboBio (Guangzhou, PRC). The relative expression levels were calculated using the 2^{-ΔΔCt} method.

Western blot analysis

Cells were lysed using the protein-extraction reagent radioimmunoprecipitation assay (GIBCO) supplemented with protease inhibitors. Then, approximately 50 µg protein extractions were subject to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, to the transfer onto nitrocellulose membranes (Millipore, Bedford, MA, USA), and incubated with specific primary antibodies against *DCLK1* and GAPDH (Proteintech Group, Inc, PRC). Horseradish peroxidase-conjugated goat anti-rabbit IgG (Proteintech Group, Inc, PRC) and ECL detection systems (PerkinElmer, NEL100001EA, USA) were utilized to visualize the specific bands.

Construction of expression vectors

MiR-195 mimics, mimics control, *miR-195* antisense oligonucleotides (*miR-195* ASO), and ASO control were purchased from RiboBio. *DCLK1*-specific small interfering RNAs (siRNA-*DCLK1*) and the control small interfering RNAs (siRNA-control) were purchased from GeneChem (Shanghai, PRC). The coding sequence of *DCLK1* was amplified and cloned into a pcDNA3.0 vector to generate *DCLK1*-expression vectors, and the empty pcDNA3.0 vector was used as control. Primers used for amplifying the *DCLK1* coding sequence were 5'-GGAATTCCATGTCTTCGGCAGAGAC-3' (forward) and 5'-CGGATCCGTCACATCCTGGTTGCGTC-3' (reverse).

Cell proliferation assay

MTT assay was performed to evaluate cell proliferation. Briefly, the PC cells were plated in 96-well plates with 5,000 per well and were allowed to grow for 12, 24, and 48 hours. Then, the cells were assessed by a colorimetric assay using MTT solution (10 mg/mL) at 570 nm. All the experiments were performed three times.

Colony formation assay

The transfected PC cells were harvested and seeded at a density of 200 cells per well in 12-well plates and incubated at 37°C and 5% CO₂ in a humidified incubator for 2 weeks. During colony growth, the culture medium was replaced every 3 days. The colony number in each well was counted and calculated.

Cell-cycle analysis by flow cytometry

The cells were harvested by trypsinization, washed twice with cold phosphate buffered saline (PBS), and fixed in 70% ethanol overnight at 4°C. The fixed cells were then washed with cold PBS, resuspended in staining solution (50 µg/mL of propidium iodide, 1 mg/mL of RNase A, 0.1% Triton X-100 in PBS), and incubated for 30 minutes at 4°C. The stained cells were analyzed with a flow cytometer (Beckman Coulter Inc, Brea, CA, USA).

Cell migration and invasion assay

Cell migration was assessed by wound-healing assay. In brief, cells were seeded in 6-well plates and cultured to 100% confluence. Using a sterile pipette tip, wounds were generated and the cells were cultured for 48 hours. Then, wound closure was assessed by Scion Image software (Scion Corporation, USA).

Matrigel invasion chambers (Millipore, USA) were used to assess cell invasion ability. Briefly, 1×10^5 cells were seeded in the upper chamber with media containing 0.1% FBS, while the lower chamber was filled with media with 10% FBS. After incubation for 48 hours, noninvading cells were removed with cotton swabs, and cells that migrated through the membrane were fixed with 70% alcohol, stained with crystal violet and imaged, and counted under a microscope (Olympus, Japan).

Tumor xenograft study in vivo

Six-week-old male BALB/c nude mice were purchased from the Shanghai Institute for Biological Sciences (Shanghai, China). For subcutaneous xenograft, the stable *miR-195*-overexpression PANC-1 cells or control cells were inoculated subcutaneously in the right dorsal flank in the 7 pairs of mice with 4×10^6 cells per mouse. The length (L) and width (W) of the tumors were examined every 5 days with calipers and tumor volume was calculated using the formula: $(L \times W^2)/2$. On day 25, all mice were killed and the tumors were excised.

For detecting liver metastatic capacity, the stable *miR-195*-overexpression PANC-1 cells or control cells were injected into the spleen or the tail of the pancreas similarly as previously described [15, 16]. Briefly, mice were anesthetized by continuous inhalation of isoflurane, and the abdominal wall was opened. The pancreatic tail with the spleen was gently exteriorized. 3×10^6 stable *miR-195*-overexpression PANC-1 cells or control cells in 20 µL of 33% Matrigel (Becton Dickinson, NJ, USA) were injected into the spleen or the tail of the pancreas with a 25-gauge needle. After the pancreas was relocated into the abdominal cavity, the abdominal wall was closed with 5.0 absorbable sutures. Two months after PANC-1 cells inoculation, the experiment was terminated. The metastatic nodules in each liver were counted. The mice used in this experiment were maintained under specific pathogen-free conditions and handled in accordance with NIH Animal Care and Use Committee regulations.

Statistical analysis

Data are presented as the median ± standard deviation. Student *t*-test was performed to compare differences in two groups. Comparisons among multiple samples were made by the Statistical Analysis System software (v.9.1.3; SAS Institute, Cary, NC, USA). Correlation between *DCLK1* / *miR-195* expression and clinicopathological features was estimated by the chi-square test and Pearson correlation test. Kaplan-Meier plots were performed with SPSS version 23.0 (SPSS Inc., Chicago, IL). All statistical tests were two-sided; *P* < 0.05 was considered statistically significant.

Results

DCLK1 is upregulated in PC and high DCLK1 indicates a poor clinical outcome

In this study, we evaluated the expression of *DCLK1* in 63 human PC tissues by immunohistochemistry staining. High cytoplasmic immunoreactivity of *DCLK1* was observed in tumor cells but not in the surrounding stroma. Representative staining is shown in Fig. 1A. Meanwhile, we also examined the expression levels of *DCLK1* protein in differently differentiated PC tissues and adjacent healthy pancreatic tissues using western blot analysis. *DCLK1* protein level was significantly increased in differently differentiated tumor tissues compared with those in the non-tumor adjacent tissues. However, its level showed no difference among the differently differentiated PC tissues (Fig. 1B). The association between *DCLK1* level and clinical parameters was shown in Table 1. Patients with higher *DCLK1* level had a higher TNM stage and a higher rate of lymph node metastasis compared with those with lower *DCLK1* level. In addition, the high *DCLK1* expression level was correlated with poorer prognosis (Fig. 1C). These results indicate that *DCLK1* may play a tumor-promoting effect in PC.

Table 1. Clinical characteristics of patients with PC (n=63) and correlations with DCLK1 expression. * Significant difference (P<0.05)

Variable	Total	DCLK1 expression level		P-value
		Low	High	
Total, n	63			
Median age, years (n ± SD)	57.35±7.51			
Age range, years	45-74			
Gender, n (%)				0.986
Male	40(63)	14	26	
Female	23(37)	8	15	
Histological grade, n (%)				0.926
Poorly differentiated	32(51)	11	20	
Well/moderately differentiated	31(49)	11	21	
Tumour stage, n (%)				0.224
T1-T2	56(89)	21	35	
T3-T4	7(11)	1	6	
TNM stage, n (%)				0.006*
I + IIA	40(63)	18	19	
IIB + III + IV	23(37)	4	22	
Lymph node metastasis, n (%)				0.002*
Yes	21(33)	2	20	
No	42(67)	20	21	

Table 2. Clinical characteristics of patients with PC (n=42) and correlations with miR-195 expression. * Significant difference (P<0.05)

Variable	Total	miR-195 expression level		P-value
		High	Low	
Total, n	42			
Median age, years (n ± SD)	57.26±7.76			
Age range, years	45-72			
Gender, n (%)				0.372
Male	26	10	14	
Female	16	10	8	
Histological grade, n (%)				0.516
Poorly differentiated	20	8	11	
Well/moderately differentiated	22	12	11	
Tumour stage, n (%)				0.188
T1-T2	39	19	18	
T3-T4	3	1	4	
TNM stage, n (%)				0.012*
I + IIA	30	15	8	
IIB + III + IV	12	5	14	
Lymph node metastasis, n (%)				0.021*
Yes	12	4	12	
No	30	16	10	

MiR-195 is downregulated in PC and negatively correlated with DCLK1 level, and low miR-195 indicates a poor clinical outcome

By qRT-PCR assay, we found that *miR-195* level was significantly reduced in differently differentiated tumor tissues compared with that in the non-tumor adjacent tissues, and its level in the poorly differentiated PC tissues was lower than that in the well and moderately differentiated tissues (Fig. 2A). Moreover, we found that *miR-195* exhibited a lower level in all the PC cell lines except AsPC-1 compared with the HPDE6-C7 normal PC cells (Fig. 2B). *MiR-195* also showed a negative correlation with *DCLK1* level (Fig. 2C) and lower *miR-195* level is associated with higher TNM stage, higher rate of lymph node metastasis, and poorer prognosis (Table 2 and Fig. 2D). These data suggest that decreased *miR-195* may be associated with PC.

Fig. 1. The expression of DCLK1 in PC tissues and adjacent normal tissues. Immunohistochemical staining (A) and western blot analysis (B) were performed to detect the DCLK1 protein level in well-differentiated human PC tissues (WD tumors, n = 11), moderately differentiated PC tissues (MD tumors, n = 14), poorly differentiated PC tissues (PD tumors, n = 6) and adjacent healthy pancreatic tissues (n = 9) (Left: $\times 100$, Right: $\times 400$). (C) Kaplan-Meier analysis was performed to analyze the overall survival of PC patients with high and low expression levels of DCLK1 (n = 63). *P<0.05.

MiR-195 directly inhibits DCLK1 expression

To expound the molecular mechanism underlying *DCLK1* exerting its effects on PC cells, we predicted potential miRNA of *DCLK1* using the TargetScan, miRanda, and PicTar online tools, and identified two conserved binding sites of *miR-195* in the 3' untranslated region (UTR) of the *DCLK1* mRNA. We speculated that *DCLK1* was a potential target for *miR-195*. To confirm this, we first conducted a luciferase reporter assay. A fragment of wild-type or mutant 3' UTR of *DCLK1* was cloned into the psiCheck-2 reporter vector (Fig. 3A). Our results showed that *miR-195* mimics significantly decreased the firefly luciferase activity of the vector with the wild-type 3' UTR of *DCLK1*, but had no significant effect on the vector with the mutated 3' UTR of *DCLK1* (Fig. 3B). Further, we detected the basic levels of *DCLK1* mRNA and protein in the four PC cell lines including PANC-1, BxPC-3, SW1990, and AsPC-1. The results showed that *DCLK1* mRNA and protein levels were significantly lower in AsPC-1

Fig. 2. MiR-195 expression in PC tissues and cell lines and its correlation with DCLK1 and prognosis. (A) miR-195 level was evaluated by qPCR in well-differentiated human PC tissues (WD tumors, $n = 11$), moderately differentiated PC tissues (MD tumors, $n = 14$), poorly differentiated PC tissues (PD tumors, $n = 6$) and adjacent normal tissues ($n = 9$). (B) miR-195 level in PC cell lines (BxPC-3, PANC-1, SW1990, MIA PaCa-2, and AsPC-1) and normal human pancreatic duct epithelial cells (HPDE6-C7) was detected by qPCR. (C) The correlation between miR-195 and DCLK1 in differently differentiated PC tissues and adjacent normal tissues was analyzed. (D) Kaplan-Meier analysis was performed to analyze the overall survival of PC patients with high and low expression levels of miR-195 ($n = 63$). * $P < 0.05$.

cell line than those in the other PC cell lines. Furthermore, we found that *DCLK1* mRNA and protein levels in the *miR-195* mimics treated PANC-1, BxPC-3, and SW1990 cells were lower than the control cells. Because the relative level of *miR-195* in AsPC-1 cells was high (Fig. 2B), we transfected *miR-195* ASO into the AsPC-1 cells to suppress endogenous *miR-195*. We observed increased *DCLK1* mRNA and protein levels in the *miR-195* ASO treated AsPC-1 cells (Fig. 3C and 3D). These results demonstrated that *miR-195* can directly inhibit *DCLK1* expression by binding to its 3' UTR in PC cells.

MiR-195 inhibits the proliferation, migration and invasion of PC cells in vitro and in vivo

To identify the effects of *miR-195* on PC cells, *miR-195* mimics or *miR-195* ASO was transfected into PC cells to alter *miR-195* level, and qRT-PCR confirmed miR-195's elevation in PANC-1 cells and its reduction in AsPC-1 cells (Fig. 4A). As shown in Fig. 4B and 4C, overexpression of *miR-195* was inhibited, whereas downregulation of *miR-195* promoted the proliferation of AsPC-1 cells by MTT assay. Furthermore, the colony number of the *in vitro* cultured PC cells was negatively correlated with the expression level of *miR-195* (Fig. 4D). To reveal the mechanisms of *miR-195* on suppressing the tumor, we analyzed the PC cell cycle

Fig. 3. MiR-195 inhibits DCLK1 expression in PC cells. (A) Predicted binding sequences of miR-195 in DCLK1 3' UTR is shown. The fragment of wild-type (wt) or mutant (mut) 3' UTR of DCLK1 was cloned into the luciferase reporter vector. (B) PANC-1 cells were co-transfected with miR-195 mimics and luciferase reporters containing wild type (wt) or mutated (mut) DCLK1 mRNA 3' UTR. Luciferase activities measured at 48 hours posttransfection. The DCLK1 mRNA (C) and protein (D) level in 4 PC cell lines (PANC-1, BxPC-3, SW1990, and AsPC-1), miR-195 mimics transfected PANC-1, BxPC-3, SW1990, and miR-195 ASO transfected AsPC-1 cells was measured by qRT-PCR and Western blot, respectively. * $P < 0.05$.

using flow cytometry. As shown in Fig. 4E, overexpression of *miR-195* significantly increased the proportion of G1 phases and reduced the proportion of G2 and S phases in PANC-1 cells, and the *miR-195* ASO resulted in an opposite phenomenon in the AsPC-1 cells. In addition, the mean volumes of xenograft tumors derived from the PANC-1 cells stably overexpressing *miR-195* were smaller than those originated from the control cells ($P = 4.68 \times 10^{-3}$) (Fig. 4F and 4G). Collectively, these data suggest that *miR-195* inhibits PC cell growth *in vitro* and *in vivo*.

The effects of *miR-195* on PC cell invasion and migration were investigated by transwell and wound-healing assays. We found that overexpression of *miR-195* significantly inhibited cell invasion and migration ability in PANC-1 cells. In contrast, after endogenous *miR-195* was inhibited by *miR-195* ASO, there was an obvious increase in invasion and migration ability of AsPC-1 cells (Fig. 4H and 4I). More importantly, animal experiments showed that overexpression of *miR-195* dramatically alleviated PANC-1 cells metastasis *in vivo* (Fig. 4J). These results revealed that *miR-195* suppresses PC cell migration, invasion, and metastasis.

Fig. 4. MiR-195 inhibits the proliferation, migration and invasion of PC cells in vitro and in vivo. (A) miR-195 mimics or ASO was transfected into PANC-1 or AsPC-1 cells, respectively, and miR-195 level measured using qRT-PCR. (B, C) The cell viability of PANC-1 cells transfected with miR-195 mimics (B) and AsPC-1 cells transfected with miR-195 ASO (C) was detected using MTT assay. (D) The colony formation of the transfected PANC-1 and AsPC-1 cells was detected. (E) The effect of miR-195 on PC cell cycle was determined using flow cytometry. (F) The growth of xenograft tumors derived by miR-195-treated PC cells was determined in vivo. (G) The representative image of the xenograft tumors is shown (n = 7 per group). The effects of miR-195 on cell invasion and migration were determined using transwell assays (H) and wound-healing assays (I), respectively (× 100). (J) The schematic representation of the experimental and representative liver metastatic tumors derived by miR-195-treated PC cells in mice. The histogram shows the average number of tumors in the liver (n = five per group; Column: mean; bars: ± standard deviation. Scale bars, 5 mm). *P<0.05.

Fig. 5. MiR-195 performs its roles in PC cells by downregulating DCLK1 expression. (A) The DCLK1 protein level in the AsPC-1 cells cotransfected with miR-195 ASO and si-DCLK1, and in the PANC-1 cells cotransfected with miR-195 mimics and DCLK1 overexpression vector was measured by western blot analysis. (B) The colony formation of the transfected PANC-1 and AsPC-1 cells was detected. The effects of miR-195 on cell invasion and migration were determined using transwell assays (C) and wound-healing assays (D), respectively ($\times 100$). * $P < 0.05$.

MiR-195 performs its tumor suppressor role in PC cells by downregulating DCLK1 expression

DCLK1 protein is involved in the growth, invasion, and metastasis of the PC cells [17, 18]. In this research, short hairpin RNA targeting *DCLK1* mRNA (si-*DCLK1*) was exploited to specifically inhibit the expression of *DCLK1* in miR-195 ASO treated AsPC-1 cells. The *DCLK1*/pcDNA3.0 vector was used to overexpress *DCLK1* in miR-195 mimics treated PANC-1 cells. As shown in Fig. 5A, si-*DCLK1* reduced the high *DCLK1* protein level caused by miR-195

Fig. 6. MiR-195 regulates the expression of p-ERK1/2, p-p38, p21, vimentin, E-cadherin, RhoA, and MMP-9 by targeting DCLK1. (A) Effects of miR-195 on p-ERK1/2, t-ERK1/2, p-p38, p38, vimentin, E-cadherin, MMP-9, RhoA, p53, and p21 were analyzed by western blot analysis in the transfected PC cells. (B) miR-195 inhibits DCLK1 via upregulation of p21 and E-cadherin, and downregulation of p-ERK1/2, p-p38, vimentin, RhoA, and MMP-9, which results in inhibition of proliferation and metastasis in PC cells. * $P < 0.05$.

ASO in AsPC-1 cells. Further, *DCLK1* overexpression vector prevented the reduction of *DCLK1* protein level caused by *miR-195* mimics. These results support the point that *miR-195* plays the same role as si-*DCLK1* in PC cells and serves as a negative regulator of *DCLK1* expression. Inhibition or overexpression of *DCLK1* also reversed the changes of colony formation, migration, and invasion of PC cells induced by *miR-195* ASO or mimics, respectively (Fig. 5B, 5C, and 5D). These results suggest that *miR-195* inhibits PC cell malignancy through targeting *DCLK1*.

MiR-195 regulates p-ERK1/2, p-p38, p21, vimentin, E-cadherin, RhoA, and MMP-9 expression in PC cells by targeting DCLK1

To investigate the mechanisms of *miR-195* in regulation of PC cells, we analyzed the effects of *miR-195* on the proliferation-, invasion- and migration-related genes, such as p-ERK1/2, p-p38, p21, vimentin, E-cadherin, RhoA, and MMP-9. As shown in Fig. 6A, *miR-195* mimics transfected PANC-1 cells had low levels of p-ERK1/2, p-p38, vimentin, RhoA, and MMP-

9, and increased levels of *p21* and *E-cadherin*. Meanwhile, cotransfection of *miR-195* mimics and *DCLK1*/pcDNA3.0 vector into PANC-1 cells resulted in increased levels of *p-ERK1/2*, *p-p38*, *vimentin*, *RhoA*, and *MMP-9*, and decreased levels of *p21* and *E-cadherin*. Furthermore, downregulation of *miR-195* and knockdown of *DCLK1* resulted in opposite changes of these genes in AsPC-1 cells. However, *miR-195* had no significant effect on expression of *p53*, *p38*, and *t-ERK1/2*.

Discussion

DCLK1 (formerly known as *DCAMKL1*) is a putative intestinal and pancreatic stem cell marker and is upregulated in the stroma and epithelium of PC. It has also recently been described as a marker of the relatively undefined tuft/brush cell in the intestine and pancreas [19, 20]. Some studies have shown that *DCLK1* expression was detected at a high level in gastric cancer [21], esophageal cancer [22], and colorectal cancer [23]. We previously found a mutation of *DCLK1* in PC tissues, especially in liver metastatic lesions, and a high *DCLK1* expression level in PC tissues. *In vitro* studies indicated that *DCLK1* might have promoting functional roles in PC cells [6]. This finding was confirmed by other studies. Ito et al. found that *DCLK1* was overexpressed in the metastatic tumors in PC patients, and overexpression of *DCLK1* promoted the migration of PC cells. Knockdown of *DCLK1* profoundly suppressed *in vivo* liver metastasis of PC cells [17]. In this study, we discovered that *DCLK1* is not only upregulated in PC tissues but also positively correlated with TNM stage, lymph node metastasis, and poorer prognosis of patients with PC. This means that the high expression of *DCLK1* might be closely related to the progression and metastasis of PC.

The dysregulation of miRNA is implicated in the development and progression of practically all tumor types [24]. However, the function and mechanism of these deregulated miRNAs in various cancers including PC remain undefined. To date, various miRNAs (including *miR-21*, *miR-34a*, *miR-155*, and *miR-29a*) has been shown to be associated with tumor progression and overall survival in patients with PC [10, 25]. Most miRNAs are known to regulate target mRNAs by binding to the 3' UTR of target genes in a posttranscriptional manner. Thus, establishing the interrelationship of miRNA and its target genes may help improve understanding of the molecular mechanism underlying cancer progression and provide potential therapeutic targets for the clinical treatment of cancers. Our study showed that the levels of *DCLK1* mRNA and protein are both negatively regulated by *miR-195* in PC cells. The direct targeting was further supported through luciferase reporter assay. These results indicate that *miR-195* might influence the function of PC cells by targeting *DCLK1*.

Recently, compelling evidence has demonstrated that *miR-195* is associated with the biological features of cancers, such as oncogenesis, development, and metastasis. *miR-195* is frequently silenced or downregulated in many types of cancers, including prostate cancer [11], non-small cell lung cancer [12], hepatocellular cancer [14], gastric cancer [26], glioma [27], osteosarcoma [28], and colorectal cancer [29]. In these cancers, *miR-195* usually has tumor-suppressive effects. In this study, we found that the expression level of *miR-195* was low in PANC-1, BxPC-3, SW1990, and MIA PaCa-2 cells; however, it was high in AsPC-1 cells. This result suggested that the different expression level of *miR-195* is related to the heterogeneity and complexity of PC cells.

It has been previously demonstrated that antigrowth signals prevent cell proliferation through two different mechanisms. Cells may enter the quiescent state (G0) and not participate in the active proliferative cycle; or cells may be induced to enter postmitotic states and lose the potential to differentiate [30]. In the current study, overexpression of *miR-195* in PANC-1 cells may result in the activation of antigrowth signals, inducing cell cycle arrest at the G0/G1 phase and disturbance to the G1 to S phase transition. Furthermore, we found that overexpression of *miR-195* suppressed the invasion and migration ability of PANC-1 cells, and downregulation of *miR-195* resulted in the opposite phenomenon in AsPC-1 cells using transwell and wound-healing assays. In addition, we demonstrated that tumor growth

and metastasis in the nude mice were well inhibited by overexpressed *miR-195* in PANC-1 cells. Lower expression of *miR-195* in human PC tissues is also associated with a higher TNM stage, higher rate of lymph node metastasis, and poorer prognosis of PC patients. On the basis of these results, we considered that *miR-195* may serve as a negative regulator in the tumorigenesis and development of PC.

However, some studies found that *miR-195* increased in the circulation of breast cancer and chronic lymphocytic leukemia patients compared with those corresponding control subjects [31, 32]. In this research, we also observed that *miR-195* expression significantly decreased in differently differentiated PC tissues and cell lines. But it is not downregulated in AsPC-1 cells, suggesting that the role of *miR-195* in different PC cell lines is distinguishing. As a result, these data suggest that the roles of *miR-195* might vary in different types of cancers.

In our analysis of clinical human PC tissues, the *DCLK1* mRNA level was negatively correlated with *miR-195* level. Our functional studies also revealed that the *DCLK1* was positively correlated with cell proliferation, migration, and invasion of PC. Therefore, these results confirmed *DCLK1* as a functional target of *miR-195* in PC cells and validated the function of *miR-195-DCLK1* interaction in the progression of PC.

To investigate the molecular mechanisms of *miR-195* in inhibiting *DCLK1* in PC cells, we analyzed the known proliferation-, invasion- and migration-related genes. It has been recently reported that activated *p-ERK* and *p-p38 MAPK* signaling pathways and *E-cadherin* take part in regulating proliferation, migration, and invasion of PANC-1 cells [33]. In addition, downregulation of *vimentin* suppresses the proliferation and invasion of PC cells *in vitro* and *in vivo* [34]. Oncogenic *K-Ras* enhances the malignant phenotype and the mitogen-activated protein kinase *p38* was identified as a target to inhibit oncogenic *K-Ras*-induced pancreatic cancer cell migration [35]. Moreover, the expression of *MMP-9*, which was correlated with vascular invasion, lymph node invasion, liver metastases, and TNM stage, was increased in PC [36]. Thus, our discovery demonstrated that abnormal alteration of *miR-195-DCLK1* interaction might occur through regulating *p-ERK1/2*, *p-p38*, *p21*, *vimentin*, *E-cadherin*, *RhoA*, and *MMP-9*, which contribute to the progression and metastasis of PC cells (Fig. 6B).

Conclusion

In summary, our results revealed that *miR-195* is significantly downregulated in PC and plays tumor suppressor roles in inhibiting the proliferation, migration, and invasion of PC cells by negatively regulating *DCLK1*. These data suggest that the *miR-195* targeting *DCLK1* pathway in tumor growth and metastasis may provide a novel possibility to diagnose and treat PC in the future.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (81302077). We thank all the patients and clinical investigators who were involved in this study.

Disclosure Statement

The authors declare no conflicts of interest.

References

- 1 Siegel RL, Miller KD, Jemal A: Cancer statistics, 2016. *CA Cancer J Clin* 2016;66:7-30.
- 2 Maitra A, Hruban RH: Pancreatic cancer. *Annu Rev Pathol* 2008;3:157-188.
- 3 Bussom S, Saif MW: Methods and rationale for the early detection of pancreatic cancer. Highlights from the "2010 asco gastrointestinal cancers symposium". Orlando, fl, USA. January 22-24, 2010. *JOP* 2010;11:128-130.
- 4 Shahrokni A, Saif MW: Metastatic pancreatic cancer: The dilemma of quality vs. Quantity of life. *JOP* 2013;14:391-394.
- 5 Garridolaguna I, Hidalgo M: Pancreatic cancer: From state-of-the-art treatments to promising novel therapies. *Nat Rev Clin Oncol* 2015;12:319-334.
- 6 Zhou B, Irwanto A, Guo YM, Bei JX, Wu Q, Chen G, Zhang TP, Lei JJ, Feng QS, Chen LZ: Exome sequencing and digital PCR analyses reveal novel mutated genes related to the metastasis of pancreatic ductal adenocarcinoma. *Cancer Biol Ther* 2012;13:871-879.
- 7 Bailey JM, Alsina J, Rasheed ZA, Mcallister FM, Fu YY, Plentz R, Zhang H, Pasricha PJ, Bardeesy N, Matsui W: DCLK1 marks a morphologically distinct subpopulation of cells with stem cell properties in preinvasive pancreatic cancer. *Gastroenterology* 2014;146:245-256.
- 8 Sureban SM, May R, Lightfoot SA, Hoskins AB, Lerner M, Brackett DJ, Postier RG, Ramanujam R, Mohammed A, Rao CV: DCAMKL-1 regulates epithelial-mesenchymal transition in human pancreatic cells through a mir-200a-dependent mechanism. *Cancer Res* 2011;71:2328-2338.
- 9 Sureban SM, May R, Ramalingam S, Subramaniam D, Natarajan G, Anant S, Houchen CW: Selective blockade of DCAMKL-1 results in tumor growth arrest by a let-7a microRNA-dependent mechanism. *Gastroenterology* 2009;137:649-659.
- 10 Sun XJ, Liu BY, Yan S, Jiang TH, Cheng HQ, Jiang HS, Cao Y, Mao AW: MicroRNA-29a promotes pancreatic cancer growth by inhibiting tristetraprolin. *Cell Physiol Biochem* 2015;37:707-718.
- 11 Cai C, Chen QB, Han ZD, Zhang YQ, He HC, Chen JH, Chen YR, Yang SB, Wu YD, Zeng YR: Mir-195 inhibits tumor progression by targeting RPS6KB1 in human prostate cancer. *Clin Cancer Res* 2015;21:4922-4934.
- 12 Liu B, Qu J, Xu F, Guo Y, Wang Y, Yu H, Qian B: MiR-195 suppresses non-small cell lung cancer by targeting CHEK1. *Oncotarget* 2015;6:9445-9456.
- 13 Jain M, Zhang L, Boufraquech M, Liu-Chittenden Y, Bussey K, Demeure MJ, Wu X, Su L, Pacak K, Stratakis CA, Kebebew E: ZNF367 inhibits cancer progression and is targeted by miR-195. *PLoS One* 2014;9:e101423.
- 14 Wang R, Zhao N, Li S, Fang JH, Chen MX, Yang J, Jia WH, Yuan Y, Zhuang SM: MicroRNA-195 suppresses angiogenesis and metastasis of hepatocellular carcinoma by inhibiting the expression of VEGF, VAV2, and CDC42. *Hepatology* 2013;58:642-653.
- 15 Zhou B, Zhan H, Tin L, Liu S, Xu J, Dong Y, Li X, Wu L, Guo W: TUFT1 regulates metastasis of pancreatic cancer through HIF1-Snail pathway induced epithelial-mesenchymal transition. *Cancer Lett* 2016;382:11-20.
- 16 Zhou B, Wu Q, Chen G, Zhang TP, Zhao YP: NOP14 promotes proliferation and metastasis of pancreatic cancer cells. *Cancer Lett* 2012;322:195-203.
- 17 Ito H, Tanaka S, Akiyama Y, Shimada S, Adikrisna R, Matsumura S, Aihara A, Mitsunori Y, Ban D, Ochiai T, Kudo A, Arii S, Yamaoka S: Dominant Expression of DCLK1 in Human Pancreatic Cancer Stem Cells Accelerates Tumor Invasion and Metastasis. *PLoS One* 2016;11:e0146564.
- 18 Sureban SM, May R, Qu D, Weygant N, Chandrasekaran P, Ali N, Lightfoot SA, Pantazis P, Rao CV, Postier RG, Houchen CW: DCLK1 regulates pluripotency and angiogenic factors via microRNA-dependent mechanisms in pancreatic cancer. *PLoS One* 2013;8:e73940.
- 19 Gerbe F, van Es JH, Makrini L, Brulin B, Mellitzer G, Robine S, Romagnolo B, Shroyer NF, Bourgaux JF, Pignodel C, Clevers H, Jay P: Distinct ATOH1 and Neurog3 requirements define tuft cells as a new secretory cell type in the intestinal epithelium. *J Cell Biol* 2011;192:767-780.
- 20 Bjerknes M, Khandanpour C, Moroy T, Fujiyama T, Hoshino M, Klisch TJ, Ding Q, Gan L, Wang J, Martin MG, Cheng H: Origin of the brush cell lineage in the mouse intestinal epithelium. *Dev Biol* 2012;362:194-218.
- 21 Nam KT, O'Neal R, Lee YS, Lee YC, Coffey RJ, Goldenring JR: Gastric tumor development in Smad3-deficient mice initiates from forestomach/glandular transition zone along the lesser curvature. *Lab Invest* 2012;92:883-895.

- 22 Vega KJ, May R, Sureban SM, Lightfoot SA, Qu D, Reed A, Weygant N, Ramanujam R, Souza R, Madhoun M, Whorton J, Anant S, Meltzer SJ: Identification of the putative intestinal stem cell marker doublecortin and CaM kinase-like-1 in Barrett's esophagus and esophageal adenocarcinoma. *J Gastroenterol Hepatol* 2012;27:773-780.
- 23 Gagliardi G, Goswami M, Passera R, Bellows CF: DCLK1 immunoreactivity in colorectal neoplasia. *Clin Exp Gastroenterol* 2012;5:35-42.
- 24 Hammond SM: MicroRNAs as oncogenes. *Curr Opin Genet Dev* 2006;16:4-9.
- 25 Song W, Li Q, Wang L, Wang L: Modulation of FoxO1 expression by miR-21 to promote growth of pancreatic ductal adenocarcinoma. *Cell Physiol Biochem* 2015;35:184-190.
- 26 Deng H, Guo Y, Song H, Xiao B, Sun W, Liu Z, Yu X, Xia T, Cui L, Guo J: MicroRNA-195 and microRNA-378 mediate tumor growth suppression by epigenetical regulation in gastric cancer. *Gene* 2013;518:351-359.
- 27 Wang H, Lu Y, Luo L, Li WS, Liang CF, He HY, Ba Y: MicroRNA-195 inhibits the proliferation of human glioma cells by directly targeting cyclin D1 and cyclin E1. *PLoS One* 2013;8:e54932.
- 28 Mao JH, Zhou RP, Peng AF, Liu ZL, Huang SH, Long XH, Shu Y: microRNA-195 suppresses osteosarcoma cell invasion and migration *in vitro* by targeting FASN. *Oncol Lett* 2012;4:1125-1129.
- 29 Yang B, Tan Z, Song Y: Study on the molecular regulatory mechanism of MicroRNA-195 in the invasion and metastasis of colorectal carcinoma. *Int J Clin Exp Med* 2015;8:3793-3800.
- 30 Hanahan D, Weinberg RA: The hallmarks of cancer. *Cell* 2000;100:57-70.
- 31 Heneghan HM, Miller N, Lowery AJ, Sweeney KJ, Newell J, Kerin MJ: Circulating micrornas as novel minimally invasive biomarkers for breast cancer. *Ann Surg* 2010;251:499-505.
- 32 Zanette DL, Rivadavia F, Molfetta GA, Barbuzano FG, Protosiqueira R, Silvajr WA, Falcão RP, Zago MA: Mirna expression profiles in chronic lymphocytic and acute lymphocytic leukemia. *Braz J Med Biol Res* 2007;40:1435-1440.
- 33 Cao L, Chen X, Xiao X, Ma Q, Li W: Resveratrol inhibits hyperglycemia-driven ROS-induced invasion and migration of pancreatic cancer cells via suppression of the ERK and p38 MAPK signaling pathways. *Int J Oncol* 2016;49:735-743.
- 34 Zhu S, He C, Deng S, Li X, Cui S, Zeng Z, Liu M, Zhao S, Chen J, Jin Y: Mir-548an, transcriptionally downregulated by HIF-1 α /HDAC1, suppresses tumorigenesis of pancreatic cancer by targeting vimentin expression. *Mol Cancer Ther* 2016;15:2209-2219.
- 35 Dreissigacker U, Mueller MS, Unger M, Siegert P, Genze F, Gierschik P, Giehl K: Oncogenic K-Ras down-regulates Rac1 and RhoA activity and enhances migration and invasion of pancreatic carcinoma cells through activation of p38. *Cell Signal* 2006;18:1156-1168.
- 36 Xu Y, Li Z, Jiang P, Wu G, Chen K, Zhang X, Li X: The co-expression of MMP-9 and Tenascin-C is significantly associated with the progression and prognosis of pancreatic cancer. *Diagn Pathol* 2015;10:211.