Abstract. MicroRNAs (miRNA/miRs) are small, non-coding RNA molecules (19-25 nucleotides in length), which function to regulate gene expression. It has been reported that miR-128 serves an important role in regulating cancer cell growth; increasing evidence has indicated that the expression of miR-128 is decreased in pancreatic cancer (PC) cells. However, the specific mechanisms of miR-128 in regulating PC cell growth are unclear. In the present study, it was confirmed that the expression of miR-128 was significantly decreased within PC tissues compared with adjacent normal tissues via reverse transcription-quantitative polymerase chain reaction analysis. In addition, miR-128 mimics inhibited PC MIA-PaCa2 cell growth by enhancing cell apoptosis in a caspase-dependent manner. Furthermore, the results of the present study demonstrated that double minute 4 (MDM4) may be a direct target for miR-128 via a dual luciferase report assay; miR-128 may inhibit MDM4 expression, and increase p53 and cleaved caspase-3 protein expression levels. In summary, the present study indicated that miR-128 is downregulated in PC, and it may be a promising target for future PC diagnosis and treatment.

Introduction

Pancreatic cancer (PC) is one of the most common fatal cancer types (1). The overall 5-year survival rate is <5%; annually, almost half of patients diagnosed with PC will succumb to the disease (2,3). At present, the options for chemotherapy only minimally prolong the life span of patients with PC (4-6). It is urgent to investigate and validate the mechanisms of PC growth (7,8). Extensive efforts have been made to identify biomarkers for this disease. Research has indicated that some miRNAs may exhibit variable expression levels within some cancer tissues (9-11).

MicroRNAs (miRNAs/miRs) are endogenous short non-coding RNAs, 19-25 nucleotides in length (11). miRNAs have been reported to serve an important role in regulating the expression and function of numerous genes and proteins (12). Previous studies have demonstrated that miRNA may facilitate cancer cell growth, invasion and migration via degradation of mRNA, by pairing with bases in its 3'-untranslated region (UTR) (13,14). Thus, miRNAs are considered to be key markers for the diagnosis and investigation of PC (15). In recent decades, a number of studies have been conducted to profile miRNA expression within PC cells, in order to identify differential miRNA expression between PC and adjacent normal tissues (16,17). Numerous miRNAs have been identified, including miR-128, miR-21-5p, miR-31-5p, miR-210, miR-217 and miR-375 (9,12,13,15,18-22); however, the specific mechanisms of these identified miRNAs require further investigation.

Double minute (MDM) family proteins are key regulators for the onco-suppressor p53. Marine and Jochemsen (23) identified that MDM4 was a p53 binding protein and knockdown of MDM4 could induce embryonic lethality within mice. The utero stage death could be rescued by deletion of the Trp53 gene.

In the present study, miR-128 expression levels within PC cells were significantly decreased compared with adjacent normal tissues, as confirmed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Additionally, MDM4 may be associated with miR-128 expression and was demonstrated to be a target of miR-128 via a dual luciferase assay. Furthermore, the expression levels of p53 were upregulated by miR-128 by targeting MDM4 to suppress tumor growth. The results of the present study may contribute to the developments of PC treatment and diagnosis.

Materials and methods

Patient recruitment and tissue sample collection. A total of 30 patients (14 females and 16 males; age, 22-63) diagnosed with PC were recruited from The Third People's Hospital
(Yancheng, China) from September 2015 to June 2017. In-hospital histories of patients were reviewed, including the diagnosis, American journal of critical care (AJCC, version 8) PC stage or metastasis (24). All PC and adjacent noncancerous tissues were obtained intraoperatively and fast frozen in liquid nitrogen, then stored at -80°C until experimental analysis. The present study was approved by the Institutional Review Board for Human Research of The Third People's Hospital. All recruited patients provided written informed consent.

**Cell culture.** Human pancreatic carcinoma cell line MIA PaCa-2 was provided by the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂ in a humidified cell culture incubator (Sanyo Electric Co., Ltd., Osaka, Japan).

**Cell transfection.** MIA PaCa-2 cells (1x10⁴ cells/well) were plated in 6-well plates and transfected with 100 nM miR-128 mimics or scramble sequence control (Shanghai GenePharma Co., Ltd., Shanghai, China) using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol, when MIA PaCa-2 cells reached 50-60% confluence within the plate. The mimic sequence was: 5'-UAU AUG GCU UUA GAU ACU GUG AA-3'. The scramble sequence was: 5'-UAU AUG GCU UUA GAU 3'. Subsequently, cells were washed out following transfection 6 h and 2 ml Dulbecco's modified DMEM with 10% fetal bovine serum were added to each well. cells were harvested with trypsin at 37°C with 5% CO₂ for 5 min for following experiments at certain time points after transfection, including: 24, 48 and 72 h.

**Cell proliferation assay.** Cell proliferation was determined with a Cell Counting kit (CCK)-8 (C0038; Beyotime Institute of Biotechnology, Shanghai, China) along with the normalization to the control group treated with MDM4 wild-type and miR-128 scramble vectors. WI, USA) along with the normalization to the control group treated with MDM4 wild-type and miR-128 scramble vectors. All vectors were purchased from Ambion (Thermo Fisher Scientific, Inc.). Vectors carried the MDM4 sequence, which contained the predicted miR-128 binding sites with wild-type or mutant 3'UTR. MIA PaCa-2 cells were plated into 24-well plates (1x10⁴ cells/well) and transfected with MDM4 vector or mutant vector using Lipofectamine® 2000 transfection reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. After 4 h, 50 nM of miR-128 mimics or scramble control was transfected into the cells respectively. After another 48 h, cells were lysed with 0.5% trypsin at 37°C containing 5% CO₂; and luciferase activities were analyzed with a dual luciferase assay kit (Promega Corporation, Madison, WI, USA) along with the normalization to the control group treated with MDM4 wild-type and miR-128 scramble vectors.

**3'UTR-luciferase reporter gene assay.** All vectors were purchased from Ambion (Thermo Fisher Scientific, Inc.). Vectors carried the MDM4 sequence, which contained the predicted miR-128 binding sites with wild-type or mutant 3'UTR. MIA PaCa-2 cells were plated into 24-well plates (1x10⁴ cells/well) and transfected with MDM4 vector or mutant vector using Lipofectamine® 2000 transfection reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. After 4 h, 50 nM of miR-128 mimics or scramble control was transfected into the cells respectively. After another 48 h, cells were lysed with 0.5% trypsin at 37°C containing 5% CO₂; and luciferase activities were analyzed with a dual luciferase assay kit (Promega Corporation, Madison, WI, USA) along with the normalization to the control group treated with MDM4 wild-type and miR-128 scramble vectors.

**RT-qPCR analysis.** Total RNA was isolated from tumor and adjacent tissues using a PicoPure™ RNA isolation kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Then, 2 µg of RNA was utilized for reverse transcription reactions, qPCR was performed with TaqMan™ gene expression master mix and respective primers (Thermo Fisher Scientific, Inc.). The specific PCR primer for miR-128 was as follows: forward, 5'-AACACTCCACGCTGGTCA CAGTGAACCGTCT-3' and reverse, 5'-CTCAACTGTGGT CGTGGA-3'. The primer for GAPDH was as follows: Forward, 5'-AATGCACTTCGTGACCACCAA-3' and reverse, 5'-GTA GCCATATCTGTCATA-3'. The qPCR procedure was performed as follows: For hold stage: Step 1: Heating from 25 to 95°C at a rate of 1.6°C/sec, holding for 2 min at 50°C. Step 2: Heating from 50 to 95°C at a rate of 1.6°C/sec, holding for
were significantly decreased within PC tissues compared to normal tissues. The results indicated that the levels of miR-128 were significantly decreased within PC tissues compared to normal tissues. Therefore, the following experiments were performed to investigate the biological role of miR-128 in PC cells. miR-128 mimics or mock vectors were transiently transfected into MIA PaCa-2 cells. Subsequently, PC cell viability was determined with a CCK-8 assay at various time points post-transfection. As presented in Fig. 2A and B, miR-128 inhibited the growth of MIA PaCa-2 cells. The cell morphology and density observed under the microscope, reduced cell proliferation was observed within the miR-128 mimics group, particularly at 48 and 72 h post-transfection. In addition, cell morphologies were round instead of epithelial indicating that miR-128 may not only inhibit cell growth, but may also induce cell apoptosis. Furthermore, the result of the colony formation assay indicated that colony formation was significantly inhibited by miR-128 mimics transfection (P<0.01; Fig. 2C and D).

miR-128 induced apoptosis in PC cells. As observed in Fig. 2A, MIA PaCa-2 cells revealed apoptotic characteristics following transfection with miR-128 mimics. Thus, flow cytometry analysis was performed to investigate the proportion of apoptotic cells in the miR-128 mimics-transfected cells using Annexin V-FITC and PI staining. The results of the present study demonstrated that miR-128 mimics markedly increased the early apoptotic cell number (Annexin V-FITC+/PI-) and late apoptotic cell number (Annexin V-FITC+/PI+; Fig. 3A). The apoptotic rate following miR-128 mimics transfection was 32.1%, which was significantly increased compared with in the control (P<0.01; Fig. 3B). These results demonstrated that miR-128 may induce MIA PaCa-2 cell apoptosis.

miR-128 directly targets MDM4. To investigate the underlying mechanisms of miR-128-mediated inhibition of MIA PaCa-2 cell growth and apoptosis induction, bioinformatics analysis was performed to identify the targets of miR-128 in humans. MDM4 was of particular interest among all the potential targets due to the high prediction scores in TargetScan (http://www.targetscan.org/vert_61/) and microRNA.org (http://www.microrna.org/microrna/) online bioinformatics methods. Therefore, the following experiments were performed to confirm whether MDM4 was a direct target of miR-128.

### Results

**Expression of miR-128 in PC tissues and normal adjacent tissues.** The expression of miR-128 within 30 pairs of PC tissues and normal adjacent tissues was determined by RT-qPCR. The results indicated that the levels of miR-128 were significantly decreased within PC tissues compared with normal adjacent tissues (P<0.05; Fig. 1). In order to investigate the biological role of miR-128 in PC cells, miR-128 mimics or mock vectors were transiently transfected into MIA PaCa-2 cells. Subsequently, PC cell viability was determined with a CCK-8 assay at various time points post-transfection. As presented in Fig. 2A and B, miR-128 inhibited the growth of MIA PaCa-2 cells. The cell morphology and density observed under the microscope, reduced cell proliferation was observed within the miR-128 mimics group, particularly at 48 and 72 h post-transfection. In addition, cell morphologies were round instead of epithelial indicating that miR-128 may not only inhibit cell growth, but may also induce cell apoptosis. Furthermore, the result of the colony formation assay indicated that colony formation was significantly inhibited by miR-128 mimics transfection (P<0.01; Fig. 2C and D).

**Western blotting.** MIA PaCa-2 cells were harvested 72 h post-transfection with miR-128 mimics or mock, then total proteins were extracted using 1X NuPAGE™ LDS sample buffer (Thermo Fisher Scientific, Inc.). Protein concentration was quantified using a Bicinchoninic Acid Protein Assay kit (Thermo Fisher Scientific, Inc.). A total of 30 µg of protein was loaded per lane and electrophoresis was performed in 10% Tris-SDS gel. Following electrophoresis, the proteins were transferred to polyvinylidene fluoride membranes (Thermo Fisher Scientific, Inc.). For subsequent blocking and antibody incubation, an iBind™ kit (Thermo Fisher Scientific, Inc.) was used according to the manufacturer's protocol. The prepared diluted primary antibodies, iBind™ Flex/iBind™ Flex FD solution, diluted secondary antibodies and iBind™ Flex/iBind™ Flex FD solution were sequentially added to each lane. Membranes were then incubated with primary antibodies overnight at room temperature. Following this, membranes were incubated with secondary antibodies for 1 h at room temperature, and were rinsed in water prior to immunodetection. The primary antibodies were as follows: Anti-p53 (cat. no. 2527, 1:300; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-MDM4 (cat. no. sc-374147, 1:300; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-cleaved caspase-3 (cat. no. sc-98785, 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-β-actin (cat. no. 7210, 1:1,000 Santa Cruz Biotechnology, Inc.). The secondary antibody (horseradish peroxidase conjugated anti-rabbit; cat. no. 7074) was used at 1:3,000 and was obtained from Cell Signaling Technology, Inc. The signal was detected with SuperSignal west femto maximum sensitivity substrate (Thermo Fisher Scientific, Inc.), and the specific proteins were detected with ChemiDoc™ imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), using Image Lab software version 4.0 (Bio-Rad Laboratories, Inc.).

**Statistical analysis.** Statistical analysis between two groups was performed with two-tailed Student's t-test and statistical differences among multiple groups were analyzed by one-way analysis of variance followed by Dunnett's test using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference. All experiments were performed in triplicate unless otherwise stated and all data are presented as the mean ± standard deviation.

**Figure 1.** miR-128 expression profile of pancreatic cancer tissues and normal adjacent tissues. Total RNA was extracted from tumor tissues and normal tissues of 30 patients with pancreatic cancer. The expression levels of miR-128 were analyzed by reverse transcription-quantitative polymerase chain reaction. For all samples, analysis was conducted in triplicate. P<0.05. miR, microRNA.
the present study, wild-type and mutant MDM4-3’-UTR expression plasmids were constructed, which were fused with a luciferase reporter gene (Fig. 4A). The results indicated that the miR-128 mimics significantly inhibited the luciferase activity of cells transfected with wild-type MDM4 (P<0.01). However, no significant variations between the control and miR-128 mimics treatment were observed with mutant MDM4 (Fig. 4B). Thus, the findings of the present study suggested that miR-128 may directly target MDM4.
miR-128 induces apoptosis in PC cells via upregulation of p53 expression. As MDM4 was considered to be the target of miR-128, MDM4 protein expression was measured in PC cells following transfection with miR-128 mimics. MDM4 levels were significantly decreased post-transfection compared with the control (P<0.01; Fig. 5A and B). Additionally, miR-128 may induce MIA PaCa-2 cell apoptosis; in order to further determine the possible mechanisms involved in this process, molecules were selected that have been reported to be involved in the caspase signaling pathway. The results of the present study indicated that the expression levels of p53 and cleaved caspase-3 protein were significantly upregulated in MIA PaCa-2 cells post-transfection with miR-128 mimics compared with the control (P<0.01; Fig. 5C and D). Collectively, these results indicated that miR-128 promoted MIA PaCa-2 cell apoptosis via the p53 and caspase-3 dependent pathway.

Discussion

The role of miR-128 has been discussed in various types of cancer as its expression may be suppressed at the mRNA level (10,15). It has also been reported that miR-128 is decreased in PC and may inhibit PC cell proliferation (26); however, further investigation is required to understand the mechanisms of miR-128 in the regulation of PC cell growth (10,27,28).

The present study clarified one of the biological roles of miR-128 in regulating PC cell growth. Compared with in adjacent normal tissue, miR-128 expression levels were significantly downregulated in pancreatic cancer tissues. Additionally, miR-128 may significantly inhibit MIA PaCa-2 cell growth in functional experiments. miR-128 was indicated to induce cell apoptosis, as demonstrated by flow cytometry analysis. To the best of our knowledge, the present study is the first to report the induction of cell apoptosis by miR-128 in PC.

In the present study, MDM4 was identified as the direct target of miR-128 using bioinformatics analysis (29). miR-128 may bind to the MDM4 3'UTR via base pairing. Based on this knowledge, the dual luciferase assay demonstrated that restored miR-128 expression levels could decrease luciferase activity, whereas no alterations were observed in the luciferase activity with mutant MDM4 3'UTR. In addition, analysis of protein expression levels indicated that miR-128 decreased MDM4 protein expression. Furthermore, along with decreased MDM4 protein, significant increases in p53 and cleaved caspase-3 protein expression levels were observed. The results of the present study suggested that miR-128 may regulate the expression of MDM4 by directly binding to the 3'UTR of MDM4. Therefore, the increased p53 expression promoted the caspase-3 dependent cell apoptosis program.

In conclusion, the results of the present study demonstrate that miR-128 may inhibit the proliferation of pancreatic cancer cells by targeting MDM4 to induce cell apoptosis. These findings add to the current understanding of miR-128 in pancreatic cancer cells and may have potential applications in the development of treatment strategies for pancreatic cancer. However, the detailed mechanism of MDM4 in the pathogenesis of pancreatic cancer requires further investigation.

Acknowledgements

The authors would like to thank Dr Hongli Wang (The Third People's Hospital, Yancheng, China) for bioinformatics analysis.
References


