Abstract. MicroRNAs have emerged as crucial regulators of tumorigenesis. However, the mechanism by which miR-203 is involved in the pathogenesis of pancreatic cancer (PC) remains elusive. In the present study, PC cell lines were used as an experimental model to investigate the expression and functional role of miR-203 in PC. miR-203 mimic virus, miRNA negative control virus and Survivin shRNA virus were transfected into the PC cell line, CFPAC-1. mRNA and protein levels of Survivin were detected using qPCR and western blot analysis. Proliferation, apoptosis and cell cycle profiles were detected by an MTT assay and flow cytometry. Female BALB/cA-nu nude mice were used to validate the role of miR-203 in vivo. The protein levels of Survivin were found to negatively correlate with miR-203 levels in four PC cell lines. A luciferase assay revealed that Survivin was a direct target of miR-203. Transfection with miR-203 mimic inhibited CFPAC-1 cell proliferation and induced apoptosis and G1 phase cell cycle arrest, similar to knockdown of Survivin. In the in vivo nude mouse model, the downregulation of Survivin by knockdown of Survivin or transfection with miR-203 mimic inhibited tumor growth. Results of the current study indicate that miR-203 regulates the proliferation, apoptosis and cell cycle progression of PC cells by targeting Survivin.

Introduction

Pancreatic cancer (PC) is a highly metastatic malignancy. Despite advanced developments in surgery, radiation therapy and chemotherapy for the treatment of PC, the 5-year survival rate following surgery in PC patients remains extremely poor (15-20%) (1). Therefore, it is urgent to identify new therapeutic targets or early diagnosis markers for PC.

Survivin (BIRC5) is a member of the inhibitors of apoptosis protein (IAP) family, which includes seven other members, as follows: X-linked inhibitor of apoptosis, cIAP1, cIAP2, NAIP, livin, IAP-like protein 2 and BRUCE (2). Survivin functions to regulate cell division, apoptosis, cellular stress responses and surveillance checkpoints, and its expression is abnormally high in a number of human malignancies, including esophageal, stomach, liver, brain, lung, breast, ovary and hematological cancer (3-5). The overexpression of Survivin is associated with advanced disease, resistance to therapy, reduced survival and induced recurrence (6). By contrast, the downregulation of Survivin may reduce cell proliferation and increase sensitivity to radiotherapy and cytotoxic drugs in various cancer cell lines, including head, neck, thyroid, lung, bladder, cervical and renal cancer (7-11). These observations indicate that Survivin may represent a molecular target for human cancer therapy.

MicroRNAs are a novel class of endogenous single-stranded and non-coding RNA molecules. They are 19-24 nucleotides long and function as gene expression regulators by targeting the 3'-untranslated region (UTR) of mRNA for degradation or translational repression (12). microRNAs have been identified to regulate cell proliferation, apoptosis, migration, invasion and the cell cycle in various cancer cell lines (13). In addition, it was previously reported that the elevated expression of miR-203 is associated with poor survival and may be used as a new prognostic marker for PC (14,15). Notably, while miR-203 is downregulated in hematopoietic malignancies and prostate cancer, it is upregulated in ovarian, bladder and colon cancer (16-20). These studies indicate that the role of miR-203 in tumorigenesis is complex. More recent studies have reported that miR-203 inhibits the proliferation of hepatocellular carcinoma (HCC) and laryngeal cancer cells by targeting Survivin (21,22).

In the present study, PC cell lines were used as an experimental model to investigate the expression and role of miR-203 in PC. In addition, we explored the relationship between miR-203 and Survivin expression and function, and aimed to determine whether miR-203 directly targets Survivin in PC cells to inhibit cancer progression.
Materials and methods

Cell culture. The human PC cell lines, SW1990, CFPAC-1, Panc-1 and BxPc-3, were obtained from Shanghai Cell Bank (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; both Wisent Inc., St-Bruno, QC, Canada), 100 µg/ml streptomycin, 100 µg/ml penicillin and 2 mM glutamine in a humidified chamber at 37°C with 5% CO₂.

miRNA and siRNA transfection. miRNA and Survivin shRNA virus (shSurvivin) were designed and synthesized by GenePharma (Shanghai, China). CFPAC-1 cells were seeded in 6-cm tissue culture plates at a density of 50%. After 24 h, the cells were transfected with miRNAs or shSurvivin virus using reduced serum medium (OPTI-MEM-1) according to the manufacturer's instructions. At 48 h post-transfection, the fluorescent index of the cells reached 90%.

qPCR. Total RNA was extracted from the cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), and Primerscript RT reagent (Takara Bio, Inc., Shiga, Japan) was used to synthesize cDNA. qPCR was performed with a 7500 Real-Time-PCR System (Applied Biosystems, Foster City, CA, USA) using the following primers: miR-203 forward, 5'-GTCGTATTACGAGGGTGCGAGG TATTGCACCTCAGCCTCAGT-3' and reverse, 5'-GCCGCGTGAATCCTTGAGAG-3'; U6 forward, 5'-ATTGGAACGATACAGAGAGTT-3' and reverse, 5'-GGACGCCTTCAGCACTTGG-3'; Survivin forward, 5'-AGGACCAGCCGATCTCTACATC-3' and reverse, 5'-CCGGCAGCAACGAAAGACACTTG-3'; and GAPDH forward, 5'-TCCACCATGCTGCCCACATCTACATC-3' and reverse, 5'-CAAGGAAGCAGAACACTTG-3'. qPCR was performed in triplicate.

Statistical analysis. All data are presented as the mean ± SD. A Student's t-test was used to analyze the differences between groups. Statistical analysis was performed with SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

hsa-miR-203 inhibits Survivin protein expression in PC cells. Firstly, hsa-miR-203 and Survivin levels were examined in four PC cell lines. qPCR revealed that miR-203 expression levels were highest in BxPc-3 cells and lowest in CFPAC-1 cells (Fig. 1A). Western blot analysis revealed that Survivin protein levels were highest in CFPAC-1 cells and lowest in BxPc-3 cells (Fig. 1B). These results indicate that Survivin protein and hsa-miR-203 levels are negatively correlated in PC cells.
Since Survivin protein levels were high in CFPAC-1 cells, the 203M miRNA virus (miR-203 mimic) was transfected into CFPAC-1 cells and Survivin mRNA and protein levels were detected. As predicted, miR-203 levels increased significantly in the 203M group (P<0.05 vs. the 203NC group; Fig. 2A). Notably, compared with the control groups, there was no significant change in Survivin mRNA levels in the 203M group (Fig. 2B); however, Survivin protein levels decreased significantly in the 203M group (Fig. 2C). These results indicate that miR-203 inhibits Survivin expression post-transcriptionally.

Survivin is a direct target gene of miR-203 in PC cells. To confirm that Survivin is a direct target gene of miR-203 in PC cells, TargetScan (http://www.targetscan.org) was used to predict the 3'UTR of Survivin and the binding site of miR-203 (Fig. 3A). Based on this prediction, pGL3-Survivin-3'UTR and pGL3-Survivin-3'UTR-mut vectors were constructed as a luciferase reporter and control, respectively, and transfected into CFPAC-1 cells. The luciferase assay revealed that luciferase activity was decreased by ~51% in the 203M group compared with the controls (P<0.05; Fig. 3B). These results indicate that miR-203 directly targets Survivin via the binding site in its 3'UTR region.

hsa-miR-203 inhibits the proliferation and promotes the apoptosis of CFPAC-1 cells. To characterize the role of hsa-miR-203 in PC cells, hsa-miR-203 mimic 203M was transfected into CFPAC-1 cells. Cell proliferation was observed to be significantly inhibited after day 4 when compared with the control (P<0.05; Fig. 4A). Next, flow cytometry was employed to detect cell cycle progression and apoptosis. CFPAC-1 cells transfected with 203M revealed a reduced G2+S phase compared with the control (41.6±5.7 vs. 64.7±5.9%, respectively; P<0.05), but exhibited increased G1 phase cell cycle arrest compared with the control (58.4±5.3 vs. 35.3±4.2%, respectively; P<0.05; Fig. 4B). In addition, the rate of apoptosis was higher in CFPAC-1 cells transfected with 203M than in the controls (12.2±2.1 vs. 5.1±1.3%, respectively; P<0.05; Fig. 4C). Taken together, these results indicate that miR-203 inhibits the proliferation of CFPAC-1 cells via the induction of G1 phase arrest and apoptosis.

Knockdown of Survivin inhibits the proliferation and promotes the apoptosis of CFPAC-1 cells. Next, the functional implication between miR-203 and Survivin was investigated in PC cells. Following knockdown of Survivin in CFPAC-1 cells by shRNA virus, Survivin mRNA and protein levels were decreased significantly compared with the controls (P<0.05; Fig. 5A). The MTT assay revealed that the proliferation of shSurvivin-transfected cells was decreased after 4 days (P<0.05 vs. control; Fig. 5C). Flow cytometric analysis revealed that the G2+S phase was decreased in shSurvivin-transfected cells compared with the controls (37.3±4.6 vs. 67.2±4.8%, respectively; P<0.05; Fig. 5B).
respectively; P<0.05) and the G1 phase was increased in shSurvivin-transfected cells compared with the control (62.7±5.3 vs. 32.8±3.9%, respectively; P<0.05; Fig. 5B). In addition, the rate of apoptosis in shSurvivin-transfected cells was higher than in the control (13.4±4.1 vs. 4.8±1.0%, respectively; P<0.05; Fig. 5D). These observations demonstrated that the loss of Survivin produces a similar phenotype as the gain of miR-203 in PC cells, indicating antagonism between Survivin and miR-203.

**Discussion**

In recent years, the aberrant expression of miRNAs has been reported to be implicated in human malignancies (12,13). While miR-203 is downregulated in hematopoietic malignancies and prostate cancer, it is upregulated in ovarian, bladder and colon cancer (16-20). In the present study, miR-203 levels were found to negatively correlate with Survivin levels in PC cells. In addition, in vitro cell proliferation and apoptosis assays, as well as an in vivo xenograft model, demonstrated that miR-203 mimic inhibited the malignant phenotypes of CFPAC-1 cells. Notably, the knockdown of Survivin similarly inhibited the malignant phenotypes of CFPAC-1 cells. A luciferase assay further confirmed that miR-203 inhibited the expression of Survivin by directly targeting its 3'UTR. Survivin is known to promote cancer cell survival and drug
Figure 5. Knockdown of Survivin inhibits proliferation and induces cell cycle arrest and apoptosis in CFPAC-1 cells. (A) q-PCR and western blot analysis for the determination of Survivin mRNA and protein levels in CFPAC-1 cells. GAPDH mRNA and protein levels were used as internal and loading controls. Survivin mRNA and protein levels were significantly decreased in the shSurvivin group compared with the control group. (B) Cell cycle profiles were measured by flow cytometry. The shSurvivin group exhibited an increased G1 phase and decreased G2 + S phase compared with the control group. (C) Cell proliferation was detected by an MTT assay. The cell proliferation rate in the shSurvivin group was significantly decreased compared with the control group. (D) Apoptosis was measured by flow cytometry. The apoptotic rate in the shSurvivin group was higher than that in the control group. *P<0.05 vs. control. shSurvivin, Survivin shRNA virus; AV, Annexin V fluorescein conjugate; PI, propidium iodide.

Figure 6. Downregulation of Survivin inhibits tumor growth in vivo. (A) Subcutaneous injection of CFPAC-1, CFPAC-1-203NC, CFPAC-1-203M and CFPAC-1-shSurvivin cells into the flanks of mice (n=4) led to tumor growth. (B) Tumors were removed after 30 days and the tumor size was measured. Tumor sizes in the 203M and shSurvivin groups were smaller than those of the other groups. (C) Tumor growth curve. Following injection with tumor cells, the tumor volume was calculated every five days. *P<0.05 vs. the 203NC group. shSurvivin, Survivin shRNA virus.
resistance (6). It is reasonable to hypothesize that miR-203 suppresses the expression of Survivin, leading to its loss of oncogenic function. This prediction is consistent with the downregulation of miR-203 and overexpression of Survivin in PC and indicates that miR-203 is an anti-oncomir, at least in PC. These observations are also consistent with previous results in HCC and laryngeal cancer cells (21,22).

miR-203 was demonstrated to be overexpressed in pancreatic adenocarcinoma samples with advanced disease, and indicated a shorter survival time or poorer prognosis for patients who underwent pancreatectomy (14,15). The same outcome was demonstrated in colon cancer (20). These results indicate that miR-203 functions as an oncomir. By contrast, miR-203 has also been reported to directly target oncogenes, including ABL1, Bcl-w, Scr, AKT2 and DNp63, functioning as a tumor-suppressor in esophageal, gastric, hepatocellular, bladder, prostate and colorectal cancer and hematological malignancies (16,23-27). Therefore, the function of miR-203 in different tissues is complex and further studies are required to understand its underlying mechanisms.

In conclusion, in the present study, miR-203 was demonstrated to inhibit the proliferation and induce the apoptosis of PC cells. In addition, Survivin was identified as a direct target of miR-203. These results indicate that miR-203 functions as an anti-oncomir in PC and represents a potential molecular target for PC therapy.

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