miR-193b directly targets *STMN1* and *uPA* genes and suppresses tumor growth and metastasis in pancreatic cancer

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Abstract. Pancreatic cancer has the poorest prognosis among all cancer types, due to its late diagnosis and the lack of effective therapies. Therefore, identification of novel gene targets, which are differentially expressed in pancreatic cancer and functionally involved in the malignant phenotype, is critical to achieve early diagnosis and develop effective therapeutic strategies. microRNAs (miRNAs) are small non-coding RNAs, which negatively regulate the expression of their targets. Due to their various targets, miRNAs play a key role in a number of physiological processes and in oncogenesis. Therefore, investigating the role of miRNAs in tumor may contribute to the development of new diagnostic and therapeutic tools for various types of cancer, including pancreatic cancer. Here, we investigated the role of miR-193b in pancreatic cancer. Our data showed that the expression of miR-193b is markedly decreased in pancreatic cancer tissues compared to adjacent healthy tissues. The Panc-1 cell line transfected with the miR-193b exhibited significantly decreased proliferative, migratory, and invasive ability compared to untransfected cells. Moreover, miR-193b inhibited the expression of stathmin 1 (STMN1) and urokinase-type plasminogen activator (uPA) in Panc-1 cells. These data suggest that miR-193b acts as a tumor suppressor in pancreatic cancer. Therefore, miR-193b may constitute a promising therapeutic agent for the suppression of pancreatic cancer cell growth and metastasis.

Introduction

Pancreatic cancer is a malignant tumor type that shows one of the highest mortality rates; it ranks as the eighth leading cause of cancer-related deaths worldwide (1). The poor prognosis of pancreatic cancer is attributed to its late manifestation, lack of accurate biomarkers for early diagnosis and assessment of curative resection options, its propensity for early metastasis, as well as the limited effects of standard chemotherapeutic agents and radiotherapy (2). Therefore, novel diagnostic modalities for early diagnosis and new therapeutic strategies are urgently needed for the treatment of pancreatic cancer.

microRNAs (miRNAs) are a class of small, endogenously expressed, well-conserved, non-coding RNA molecules, 18-25 nucleotides long. They play important regulatory roles on their gene targets, by degrading their mRNA or inhibiting their translation (3,4). Growing evidence suggests that miRNAs play an important role in various biological processes, including cell proliferation, development, and differentiation (5,6). Furthermore, emerging evidence suggests that miRNAs play essential roles in tumorigenesis, and thus may function as promising targets for the treatment of cancer (7,8). Recent studies have demonstrated that miR-193b is downregulated in a variety of cancers, and that it regulates cancer cell proliferation, migration, invasion, and metastasis (9-13). Although Ikeda *et al* (14) reported that the expression of miR-193b is altered by the mitogen-activated protein kinase (MAPK) in pancreatic cancer cells and that its exogenous overexpression markedly inhibits cell proliferation, the exact role of miR-193b in pancreatic cancer remains unclear.

In the present study, we not only investigated the expression of miR-193b in pancreatic cancer tissue, but also studied the regulatory effects of miR-193b on panceatic cancer cell proliferation, migration and invasion *in vitro*, as well as the underlying molecular mechanisms, which may help develop potential diagnostic and therapeutic tools for pancreatic cancer. Our data indicated that miR-193b may be downregulated in pancreatic cancer and affects the behavior of Panc-1 cells, namely their proliferation, apoptosis, migration and invasion. We also demonstrated that the stathmin 1 (*STMN1*) and urokinase-type plasminogen activator (*uPA*) genes are negatively regulated by miR-193b.

Materials and methods

Patients and tumor samples. Pancreatic cancer and adjacent tissues were obtained from 27 patients (17 males and 10 females, median age 63 years, range 38-79 years) undergoing pancreatic cancer surgery at the Xiangya Hospital at the Central South University, Changsha, China (Table I). Six healthy pancreatic samples were collected at surgery from patients with acute pancreatic injury. The samples were

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Table I. Clinical features and miR-193b expression profiles in the studied patients.

Patient	Age (years)	Gender	Tumor size (cm)	TNM stage	Normalized miR-193b level
1	60	F	3.6x3.9x4.5	II	0.1869
2	38	M	2.8x3.6x4.4	IV	0.0409
3	63	M	2.7x2.8x2.6	I	2.1585
4	58	M	1.9x1.8x2.3	I	0.2517
5	75	F	2.2x2.8x3.6	III	0.0797
6	66	M	2.5x3.5x4.4	II	0.1111
7	63	M	3.0x3.0x3.1	II	0.2432
8	73	F	2.5x3.2x3.3	I	0.1267
9	61	M	4.0x5.0x3.5	II	0.0896
10	70	F	2.0x3.0x2.2	I	1.5369
11	62	M	2.1x2.6x3.1	I	0.1989
12	48	M	3.4x3.8x4.5	III	0.0813
13	57	F	3.8.x4.0x3.6	III	0.4323
14	73	M	2.5x2.9x3.6	II	0.0981
15	71	M	1.7x2.4x2.6	I	0.2717
16	56	F	2.3x3.0x2.2	II	0.1869
17	77	M	3.2x4.2x4.1	II	0.0608
18	59	F	4.0x4.2x4.6	III	0.0836
19	79	M	6.0x5.0x4.2	IV	0.0902
20	77	M	3.0x4.0x4.0	III	0.1387
21	69	F	3.1x4.3x3.7	III	0.4234
22	65	F	2.7x3.5x3.0	III	0.0703
23	56	M	3.8x4.5x3.3	II	0.1285
24	61	M	3.3x2.9x2.7	II	0.3635
25	64	F	4.2x4.5x4.4	III	0.1975
26	39	M	2.6x1.9x3.0	II	0.0915
27	57	F	2.5x3.8x3.6	I	0.1368

Quantification of miRNA expression was performed with reverse transcription-quantitative PCR (RT-qPCR) and the $2^{-\Delta\Delta Ct}$ method, using the expression level of adjacent healthy pancreatic tissue as a calibrator. Data show the means from three independent analyses, performed after the RNA extraction step. ΔCt values obtained from RT-qPCR were compared with a Wilcoxon signed rank test (ΔCt = Ct miR-193b - Ct U6 small nuclear RNA). The expression level of miR-193b is significantly lower in tumor tissuescompared to the adjacent healthy tissues ($P<0.01$, $Z = -4.469$). M, male; F, female; miR, microRNA.

immediately snap-frozen in liquid nitrogen and stored at -80˚C for RNA extraction. Informed consent was obtained from all patients. Both tumor and non-cancerous samples were histologically confirmed. The study was approved by the Ethics Committee of the Central South University.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA was extracted from the 27 pancreatic cancer tissues and their adjacent non-carcinoma tissues, the six healthy pancreatic tissues and the cultured cells. For miR-193b expression analysis, total RNA was poly-adenylated using a Poly (A) Tailing kit, according to the manufacturer's instructions (GeneCopoeia, Guanzhou, China) and 10 ng of poly(A) mRNA were converted to cDNA using miR-193b-specific primers of U6, which were synthesized forward 5'-CTCGCTTCGGCAGCACA-3' and reverse 5'-AACGCTTCACGAATTTGCGT-3' (cat. no HmiRQP0278; GeneCopeia), and an Applied Biosystems® TaqMan® MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA). Following reverse transcription, qPCR was performed on an ABI 7500 thermocycler (Thermo Fisher Scientific) with the following cycling conditions: 95˚C, 5 min, 1 cycle, 95˚C, 10 sec, 65˚C, 20 sec, 72˚C, 10 sec, 40 cycles. The U6 gene was used as a normalization control. Each sample was analyzed in triplicate.

Cell culture. The human pancreatic cancer cell line Panc-1 (Institute of Biochemistry and Cell Biology, Shanghai, China) was maintained in our laboratory and cultured in Hepes-buffered Dulbecco's modified Eagle's medium (H-DMEM) supplemented with 10% Gibco® fetal bovine serum (FBS) (Thermo Fisher Scientific). Cells were cultured at 37˚C in 5% CO_2.

Cell transfection. Panc-1 cells were transiently transfected for 48 h with chemically synthesized miR-193b and the negative

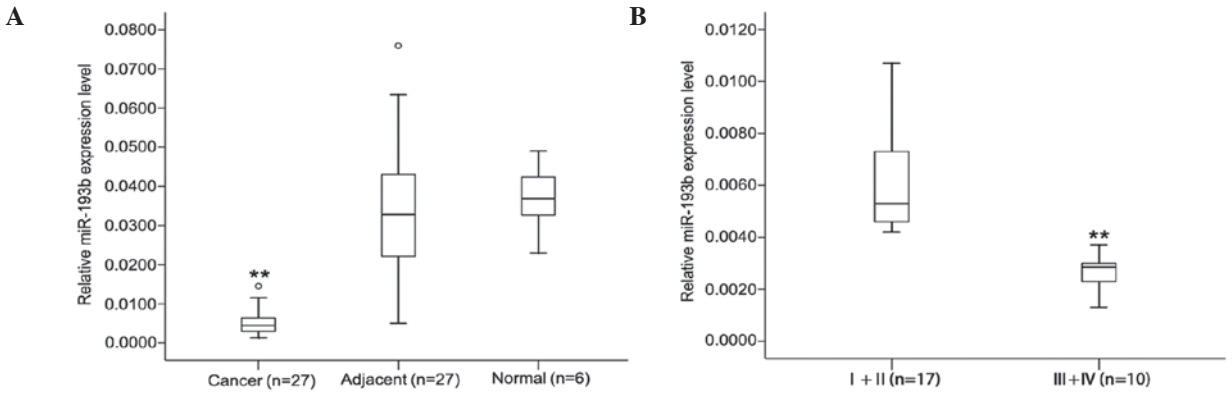

Figure 1. Detection of miR-193b in healthy pancreatic tissue (normal) and pancreatic cancer tissues (cancer and adjacent), as assessed by reverse transcription-quantitative PCR. Relative miR-193b expression levels in (A) 27 pairs of primary pancreatic cancer and adjacent matched non-tumorous tissues, and in 6 healthy pancreatic tissues and (B) pancreatic cancer tissues at different stages, as classified by the TNM system: early stages (I and II), and advanced stages (III and IV). Horizontal lines within each box represent the median and vertical lines the interquartile range of the normalized cycle threshold (Ct) values. miR-193b expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method and normalized to the level of the U6 small nuclear RNA.$^{**}P<0.01$.

control miRNA (miR-NC) (GenePharma, Shanghai, China) (Table II). Transfection was performed with Invitrogen™ Lipofectamine® 2000 (Thermo Fisher Scientific) according to the manufacturer's recommendations.

Cell proliferation assay. The MTT assay was used to measure cell proliferation. At 48 h post-transfection, the transfection medium in each well was replaced by 100 μl of fresh serum-free medium supplemented with 100 μl 0.5 g/l MTT solution. After incubation at 37˚C for 4 h, the MTT medium was removed by aspiration and 50 μl of dimethyl sulfoxide were added to each well. After incubation at 37˚C for an additional 10 min, the absorbance of the sample at 570 nm was measured using a plate reader (cat.no. AD 340C; Beckman Coulter, Miami, FL, USA).

Cell apoptosis assay. Cells were collected and washed with phosphate-buffered saline (PBS) mixed with 2% ethylene diamine tetraacetic acid. Then, each sample, containing 10^5-10^6 cells/ml, was stained with 5 μl Annexin V-fluorescein isothiocyanate (FITC) and 10 μl propidium iodide (PI) (Annexin V-FITC/PI Apoptosis Detection kit; KeyGen Biotech Co., Ltd., Nanjing, China) for 15 min. Afterwards, the cells were diluted using 400 μl Annexin V binding buffer (Haoran Biological Technology Co, Ltd., Shanghai, China) and analyzed on a FACSAria flow cytometer (BD Biosciences, Bedford, MA, USA).

Cell migration and invasion assays. Invasion assays were performed in a 24-well transwell chamber purchased from Corning (Cambridge, MA, USA). The chamber contained an 8 μm-pore size polycarbonate membrane filter and was precoated with 100 μg of Matrigel (BD Biosciences). Panc-1 cells transfected with miR-193b or miR-NC were collected and resuspended in serum-free H-DMEM medium at a concentration of $1x10^5$ cells/ml. Then, the cell suspensions were added into the top chambers (200 ml/well) and the bottom chambers were filled with H-DMEM medium containing 10% FBS (500 ml/well), followed by a 24-h incubation at 37˚C. The cells that did not penetrate the polycarbonate membrane were swabbed using a cotton bud. The cells that had passed through the membrane and adhered to the bottom of the polycarbonate membrane were stained for 20 min with a solution containing 0.1% crystal violet and 20% methanol, and were photographed and manually counted under an inverted fluorescence microscope (Olympus, IX70; Olympus, Tokyo, Japan). The migration assay was performed following similar procedures except for the use of Matrigel coating on the filters. Two independent experiments were performed for each assay. The average of cell counts in five randomly selected fields (x400) was recorded as the value of each chamber. The experiment was repeated twice, with triplicate measurements in each experiment.

Bioinformatics analysis. To examine the potential downstream target genes of miR-193b, the TargetScan (http://www.targetscan.org) (15), miRBase (http://www.mirbase.org) (16) and PicTar (http://www.mirbase.org) (17) were used. The results revealed that a series of 3' UTR of human genes contained potential miR-193b-binding sequences. Amonth these, the oncogenes, STMN1 and uPA were of interest. The 3' UTR of STMN1 (59-65 nt, Genebank accession no. NM_005563) gene and the uPA (777-783 ntl Genebank accesion no. NM_00145031) gene contained the miRb-binding site .

Dual luciferase reporter assay. Construction of the luciferase report vectors were as described by Chen *et al* (18). Briefly, DNA fragments were amplified from human genomic DNA and cloned into the multiple cloning sites (XhoI and NotI) distal to the Renilla luciferase coding region of the psiCHECK2 vector (Promega, Madison, WI, USA). PCR was performed using KOD hot start DNA polymerase (Novagen, Madison, WI, USA). The primer sequences used to construct the psiCHECK-STMN1 vector containing the wild type STMNI 3' UTR were as follows: XhoI-STMN1, forward 5'-ACGCCTCGAGTTGTTCTGAGAACTGACTTTCTC-3' and NotI-STMN1 reverse 5'-ATAAGAATGCGGCCGCATATTCTGATTCTCGTGTCATAGC-3'. Overlapping PCR was used to generate psiCHECK-STMN1-mutant (M), which contained a deletion at the miR-193b seed binding site. The following two primers were also used: STMN1-M, forward 5'-ATATCCAAAGACTGTACTTCATTTTATTTTTTCCCTG-3 and reverse 5'-AGTACAGTCTTTGGATAT-3'. The wild-type and deletion mutant of the uPA 3' UTR were generated using the same technique and were cloned

Table II. Sequences of the chemically synthesized miR-193b, miR-NC, miR-193b inhibitor and NC inbibitors.

Synthesized miRNA	Sequence 5'-3'
miR-193b	AACUGGCCCUCAAAGUCCCGCU
miR-NC	UUCUCCGAACGUGUCACGU
miR-193b inhibitor	AGCGGGACUUUGAGGGCCAGUU
NC inbibitor	CAGUACUUUUGUGUAGUACAA

miR, microRNA; miR-NC, negative control miRNA; NC inhibitor, negative control inhibitor. All chemically synthesized miRNAs were purchased from GenePharma.

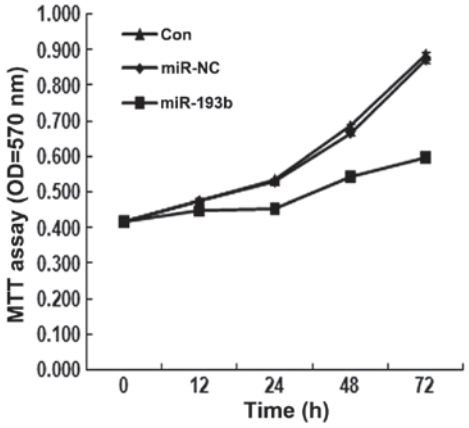

Figure 2. miR-193b inhibits the proliferation of Panc-1 cells, as shown by the MTT assay. Proliferation of Panc-1 cells transfected with miR-193b is significantly decreased as compared to that of the miR-NC-transfected and the untransfected Panc-1 cells. Data represent the mean ± standard deviation of three independent experiments, assayed in triplicate. Con, untransfected Panc-1 cells; miR-NC, Panc-1 cells transfected with the negative control miRNA; miR-193b, Panc-1 cells transfected with miR-193b.

into multiple cloning sites (XhoI and NotI) distal to the Renilla luciferase coding region of the psiCHECK vector (Promega). The primer sequences used to construct the psiCHECK-STMN1 containing wild type and mutant uPA 3' UTR were as follows: XhoI-uPA, forward 5'-CGTCTAG AGGGTCCCCAGGGAGGAAAC-3' and NotI-uPA, reverse 5'-CGCATATGTCATCAGAAAAATCACATT-3', uPA-M forward 5'-CAGTTTCACTTTCACATATCCCTTCCTTTT AGC-3' and reverse 5'-GCTAAAAGGAAGGGATATGTG AAAGTGAAACTG-3'. The following cycling conditions were used: pre-denaturation at 95˚C for 5 min; denaturation at 95˚C for 30 sec, annealing at 60˚C for 30 sec and elongation at 72˚C for 30 sec for a total of 30 cycles, followed by elongation at 72˚C for 10 min. They were then inserted into multiple cloning sites of the psiCHECK™-2 vector (Promega, Madison, WI, USA) to obtain the pMIR-STMN1, -mut STMN1, -uPA, and -mut uPA vectors. For the luciferase assay, Panc-1 cells were transfected in 24-well plates using the Lipofectamine® 2000 transfection reagent. Each well was transfected with 100 ng of either pMIR-STMN1/uPA or pMIR-mut STMN1/mut uPA vector, along with 10 pmol of hsa-miR-193b or miR-NC. The vector pRL-TK (Promega) was also transfected as a control. Forty-eight hours after transfection, the luciferase activity was measured using the Dual Luciferase® Reporter Assay system (Promega).

Western blot analysis. Cells were solubilized in cold RIPA lysis buffer (Santa Cruz Biotechnology, Inc.). Proteins were extracted from the cell lysate using the Total Protein Extraction kit (BestBio, Shanghai, China) and were quantified using the BCA Protein assay kit (Santa Cruz Biotechnology, Inc.). The proteins were separated by 5% SDS-PAGE, and then transferred onto a polyvinylidene difluoride membrane. The membrane was blocked in 5% non-fat dried milk in PBS with Tween-20 (PBST) for 3 h, and then incubated overnight with the primary rabbit anti-STMN1 polyclonal (1:500; Abcam, Cambridge, UK) and anti-uPA polyclonal antibodies (1:1,000; Santa Cruz Biotechnology, Inc.). GAPDH was used as the loading control. After incubation for 1 h at room temperature with the secondary antibodies, goat ant-rabbit immunoglobulin (Ig) G/horseradish peroxidase (HRP) at a dilution of 1:30,000 (Santa Cruz Biotechnology, Inc.) and goat anti-mouse IgG/HRP (1:50,000; Santa Cruz Biotechnology, Inc.). The immune complexes were detected using an enhanced chemiluminescence (ECL) kit (Beyotime Institute of Biotechnology, Shanghai, China).

Statistical analysis. The data were expressed as mean ± standard deviation and were analyzed using the SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Comparisons of the *STMN1* mRNA expression data were performed with the Mann-Whitney U-test. The statistical significance of differences among groups was analyzed by paired-sample t-tests or a one-way analysis of variance (ANOVA), followed by post-hoc least significant difference (LSD) multiple testing correction, when appropriate. $P<0.05$ was considered to indicate statistically significant differences.

Results

miR-193b expression is decreased in pancreatic cancer tissues. To investigate the roles of miR-193b in pancreatic cancer, we examined its expression in 27 primary pancreatic cancer and adjacent matched non-tumorous tissues, and in 6 healthy pancreatic tissues. Table I shows the miR-193b relative expression data in the 27 samples; the expression of miR-193b was decreased (from 2.3- to 24.4-fold) in >90% of the pancreatic cancer samples (25 out of 27 patients). The level of miR-193b in the pancreatic cancer tissues was lower compared to the adjacent matched non-tumorous tissues (0.0084±0.0094 vs. 0.0341±0.0217, $P<0.01$; Fig. 1A); however, no significant differences were found between adjacent matched non-tumorous tissues and healthy tissues (0.0341±0.0217 vs. 0.0368±0.0089, $P>0.05$). Moreover, analysis of expression data in the pancreatic cancer samples of different TNM stages showed that there is a difference between early- and advanced-stage miR-193b expression levels. As shown in Fig. 1B, in early pancreatic cancer at stages I and II (n=17), miR-193b expression was significantly higher than that observed in the advanced stages III and IV (n=10). These results suggested that downregulation of miR-193b may be related to the development of human pancreatic cancer.

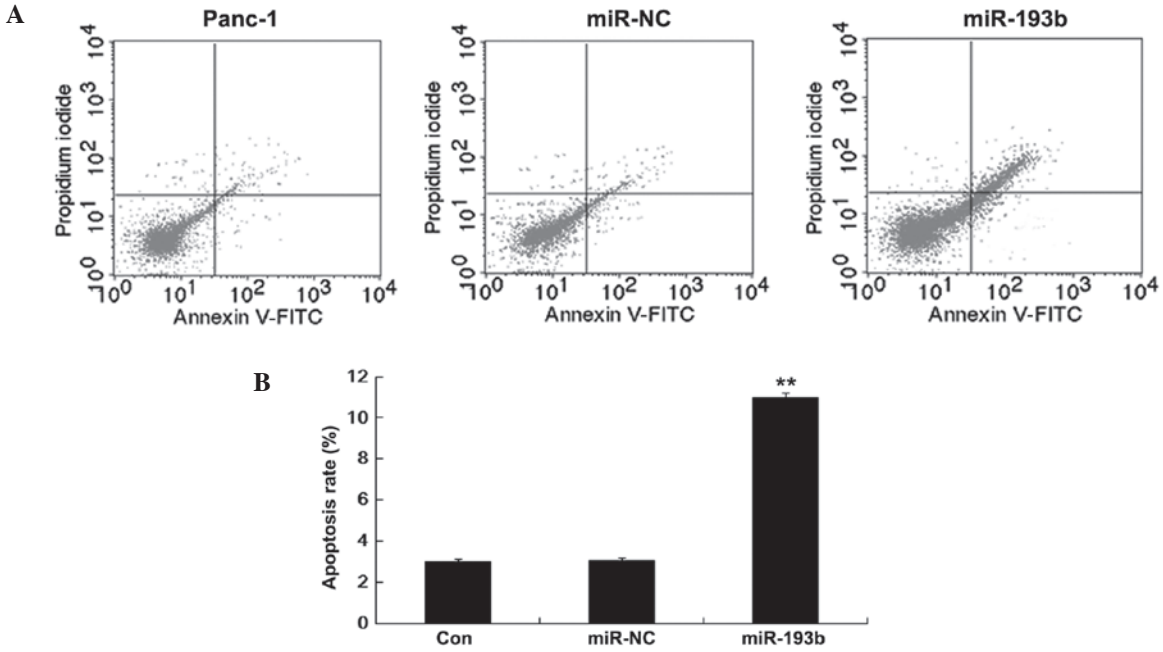

Figure 3. Flow cytometry reveals that the increased expression of miR-193b enhances apoptosis in Panc-1 cells. (A) The cells were stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI). The right upper quadrant represents late apoptotic and necrotic cells; the right lower quadrant represents early apoptotic cells; the left upper quadrant represents cells that were mechanically injured; and the left lower quadrant represents healthy proliferating cells. (B) Apoptotic rate in the different groups of Panc-1 cells after transfection. Bars denote standard deviation of the mean. **P<0.01, compared to the miR-NC and the Con group. Con, untransfected Panc-1 cells; miR-NC, Panc-1 cells transfected with the negative control miRNA; and miR-193b, Panc-1 cells transfected with miR-193b.

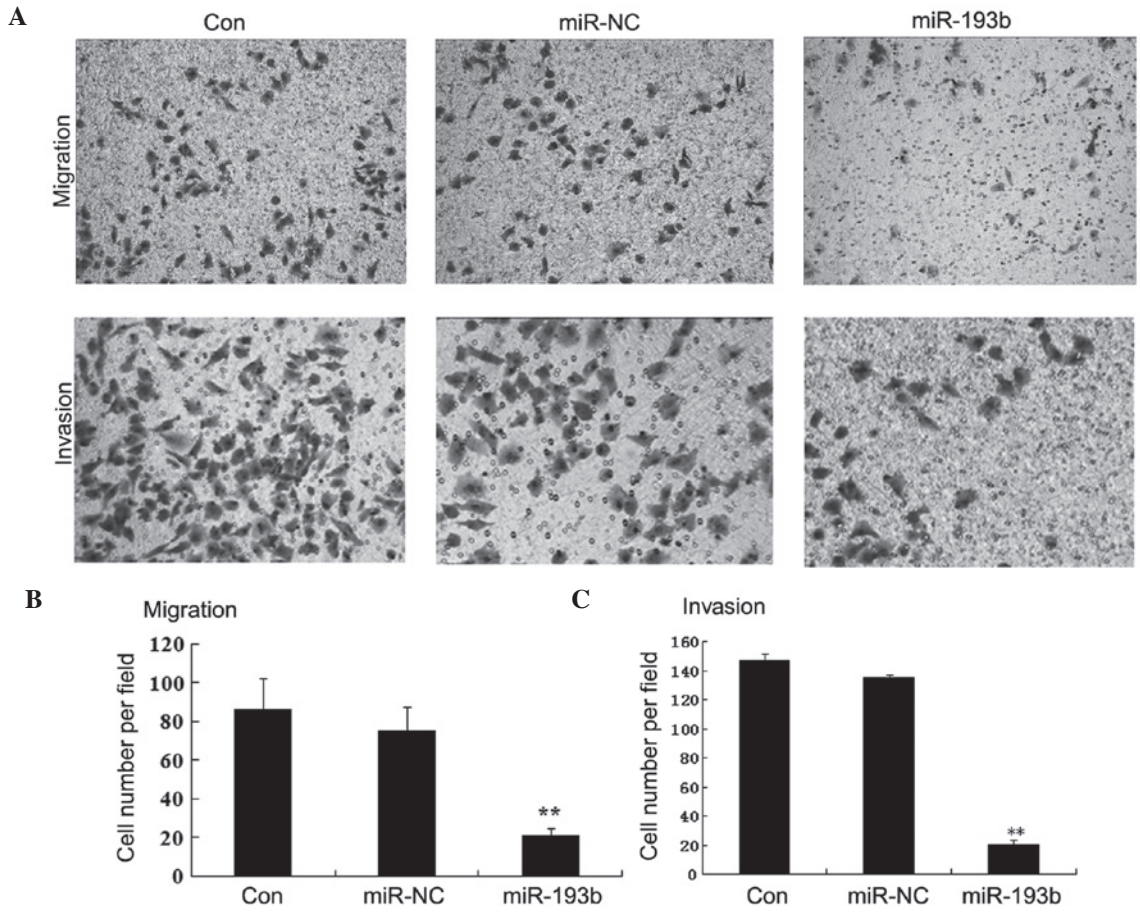

Figure 4. miR-193b reduces the migratory and invasive ability of Panc-1 cells, as shown by the Transwell assay. (A) Migrating and invading cells were stained with crystal violet and visualized using an Olympus IX70 inverted fluorescence microscope (original magnification, x200). Average number of (B) migrating and (C) invading cells per field. Bars denote standard deviation of the mean. **P<0.01, compared to the miR-NC and the Con group. Con, untransfected Panc-1 cells; miR-NC, Panc-1 cells transfected with the negative control miRNA; miR-193b, Panc-1 cells transfected with the miR-193b.

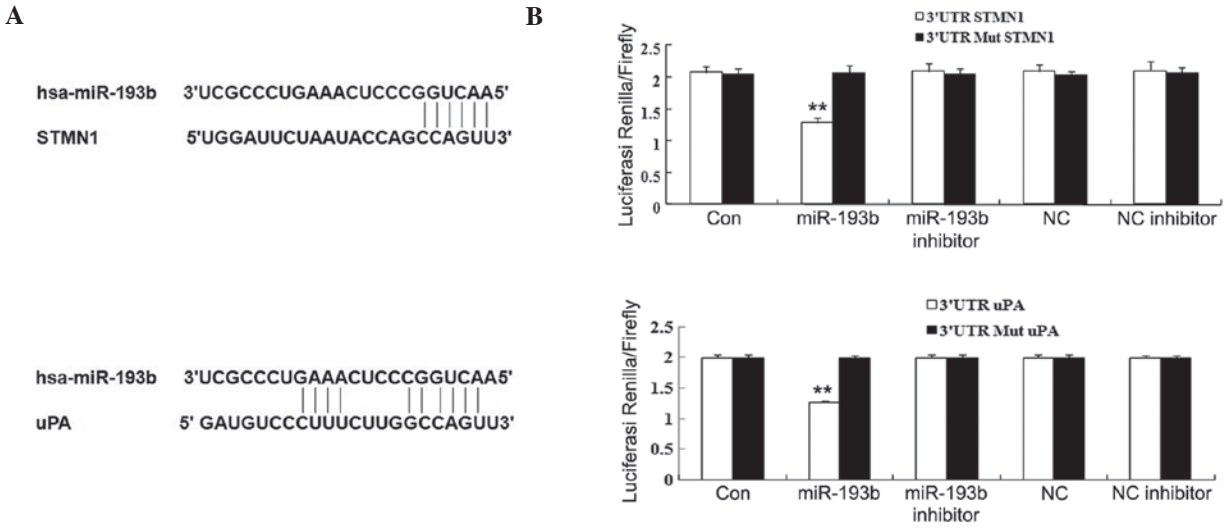

Figure 5. (A) miR-193b-binding sites on the 3'-UTR of stathmin 1 (*STMN1*) and urokinase-type plasminogen activator (*uPA*) genes. (B) The luciferase assay shows that the expression of STMN1 and uPA is decreased upon transfection of cells with miR-193b. The 3'-UTRs were subcloned into the psiCHECK™-2 luciferase reporter expression vector to obtain the pMIR-STMN1, -mut STMN1, -uPA and -mut uPA vectors. Panc-1 cells were cotransfected with each of these vectors and 50 nM miR-193b or 100 mM miR-193b inhibitor (Table II). Con, Panc-1 cells transfected with the pRL-TK; NC, Panc-1 cells transfected with the negative control miRNA (NC); and NC inhibitor (Table II), Panc-1 cells transfected with NC and the miR-193b inhibitor.

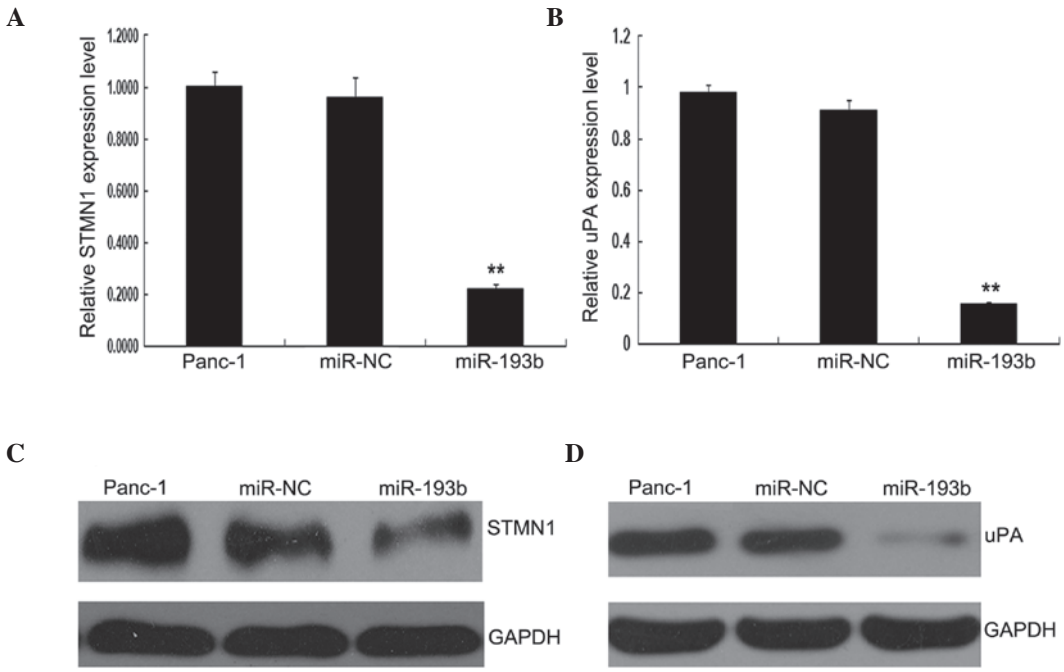

Figure 6. miR-193b inhibits the expression of stathmin 1 (STMN1) and urokinase-type plasminogen activator urokinase-type plasminogen activator (uPA), as shown by RT-qPCR and western blotting after transfection of Panc-1 cells with miR-193b. (A and B) Relative *STMN1* and *uPA* mRNA levels in Panc-1 cells 48 h after transfection with miR-193b or miR-NC. Bars denote standard deviation of the mean. $^{**}P<0.05$, compared to the miR-NC group or the Panc-1 control group (untransfected cells). (C and D) Western blots showing STMN1 and uPA protein expression in Panc-1 cells 48 h after transfection with miR-193b or miR-NC. Con, untransfected Panc-1 cells; miR-NC, Panc-1 cells transfected with negative control miRNA; miR-193b, Panc-1 cells transfected with miR-193b. GAPDH was used as the loading control.

Effects of miR-193b transfection on pancreatic cancer cell proliferation. Since miR-193b may be related to the development of human pancreatic cancer, we further investigated its effects on *in vitro* proliferation. The MTT assay was performed in Panc-1 cells transfected with the miR-193b, in which the miR is expected to be overexpressed. As shown in Fig. 2, the assay showed that in the miR-193b-transfected Panc-1 cells, the proliferation rate was lower than that observed in the control groups ($P<0.05$). These results suggested that miR-193b inhibits Panc-1 cell proliferation.

Effects of miR-193b transfection on cell apoptosis. Immortality is a common characteristic of cancer cells. To further explore the role of miR-193b on the apoptosis of pancreatic cells, we performed Annexin V/PI flow cytometry analysis on Panc-1 cells transfected with miR-193b and miR-NC. Fig. 3 shows

that the percentage of apoptotic Panc-1 cells was higher in the miR-193b (10.97±0.22%) than in the miR-NC (2.98±0.12%; P<0.01) group. Thus, these results suggested that miR-193b enhances Panc-1 cell apoptosis.

Effects of miR-193b transfection on cell migration and invasion. To address the potential effects of miR-193b on the migration and invasion of pancreatic cancer cells *in vitro*, we performed cell migration and invasion assays in the different cell lines. As shown in Fig. 4, Panc-1 cells transfected with miR-193b showed markedly reduced migratory and invasive activity than the miR-NC or the untransfected Panc-1 cells (P<0.01). Taken together, these results suggest that miR-193b may act as a potent suppressor of pancreatic cancer cell migration and invasion.

miR-193b directly represses the expression of STMN1 and uPA through binding to their 3'-UTRs. To investigate the mechanisms by which miR-193b inhibits pancreatic cancer growth and metastasis, we performed a bioinformatic analysis to identify the potential target genes of miR-193b. We found a number of human genes that contain potential miR-193b-binding sequences in their 3'-UTR regions. Among these, we focused on two oncogenes, *STMN1* and *uPA*. To confirm that these genes are direct targets of miR-193b, luciferase assays were used. The 3'-UTRs of the *STMN1* and *uPA* genes (Fig. 5A) were cloned downstream of the coding sequence of luciferase. The resulting constructs were cotransfected with miR-193b or miR-NC (Table II) into Panc-1 cells. The results showed that miR-193b, but not miR-NC, specifically decreases the luciferase reporter levels (Fig. 5B). Moreover, the inhibitory effects of miR-193b were eliminated upon seed-sequence-deletion mutation of the miR-193b-binding site within the 3'-UTRs of *STMN1* and *uPA*. These results indicated that *STMN1* and *uPA* are the direct downstream targets of miR-193b in pancreatic cancer.

To further investigate the regulatory roles of miR-193b on the *STMN1* and *uPA* genes, we transfected Panc-1 cells with miR-193b or miR-NC, and detected the expression of *STMN1* and *uPA* using RT-qPCR and western blotting assays. As shown in Fig. 6, both mRNA and protein expression levels of STMN1 and uPA were significantly decreased after miR-193b transfection, as compared to the miR-NC group or the untransfected cells (P<0.05). These data suggest that STMN1 and uPA expression is negatively regulated by miR-193b.

Discussion

The present study revealed, for the first time to the best of our knowledge, the roles of miR-193b in pancreatic cancer, as well as two of its target genes, *STMN1* and *uPA*. We found that the expression level of miR-193b is reduced in pancreatic caner tissues, as compared to the level of adjacent matched non-tumorous tissues and healthy pancreatic tissues. Additional assays demonstrated that transfection of cells with miR-193b, which is expected to increase its expression level, significantly inhibited cellular proliferation, migration and invasion, and markedly promoted apoptosis in pancreatic cancer cells. In addition, *STMN1* and *uPA* were identified as direct targets of miR-193b, and their expression in pancreatic cancer cells was found to be negatively regulated by miR-193b.

Accumulating evidence has suggested that miR-193b acts as a tumor suppressor in various types of cancer, such as breast, gastric, cervical, prostate cancer, etc. (19-22). For instance, Wu and colleagues reported that the expression level of miR-193b is significantly downregulated in endometrioid adenocarcinoma (9). Rauhala *et al* found that miR-193b is an epigenetically regulated tumor suppressor in prostate cancer (10). Furthermore, miR-193b was found to be significantly downregulated in melanoma tissues, and overexpression of miR-193b in melanoma cell lines repressed cell proliferation, probably through regulating cyclin D1 (23). However, the detailed role of miR-193b in pancreatic cancer remains unclear. In our study, we report that miR-193b is systematically downregulated in pancreatic cancer tissues. Moreover, we found that the expression level of miR-193b negatively correlates to the TNM stages of pancreatic cancer, indicating that downregulation of miR-193b may promote the development and progression of pancreatic cancer.

Recently, Ikeda and colleagues suggested that activation of the MAPK may play a role in aberrant expression of miR-193b, which is associated with pancreatic cancer (14). They used RT-qPCR to identify the MAPK-associated miRNAs in pancreatic cancer cells, and further found that overexpression of the MAPK-associated miR-193b exerts the most notable inhibitory effect on proliferation of cultured pancreatic cancer cells (14). Consistent with their findings, we also found that transfection of miR-193b inhibits pancreatic cancer cell proliferation. We further demonstrated that transfection of cells with miR-193b effectively promoted apoptosis and suppressed migration and invasion in pancreatic cancer cells. Considering published and our present findings, we suggest that miR-193b plays a tumor-suppressive role in pancreatic cancer.

To further investigate the molecular mechanism underlying the involvement of miR-193b in pancreatic cancer, we performed a luciferase reporter assay, and demonstrated that the genes *STMN1* and *uPA* are the direct targets of miR-193b, their expression levels being negatively regulated by this miR in pancreatic cancer cells. A number of studies have identified miR-193b targets in human malignancies, such as the genes *CCND1*, *NT5E*, *PLAU*, *STARD7*, *STMN1*, *uPA*, and *YWHAZ*, and showed that the tumor-suppressive role of miR-193b mainly involves inhibition of the expression of its targets in human cancer (12,14).

STMN1 encodes a cytosolic phosphoprotein, which plays crucial roles in the formation and the function of the mitotic spindle. By acting as a microtubule destabilizer, STMN1 participates in cellular biological processes such as cell division, motility, and differentiation (24). Recent studies have demonstrated that STMN1 is upregulated in multiple types of malignant tumors, including sarcoma, hepatocellular carcinoma, gastric, breast and prostate cancer, oral squamous cell carcinoma, and lung adenocarcinomas (25-28). Furthermore, *STMN1* has been suggested to act as an oncogene in human malignancies. For instance, STMN1 was recently reported to upregulate ovarian clear cell adenocarcinoma via the regulation of HIF-1α through the PI3K/Akt/mTOR pathway (28). Moreover, the oncogenic role of STMN1 has been revealed in pancreatic cancer. Jiang *et al* found that ectopic overexpression of STMN1 prevents transforming growth factor-β inducible early gene 1 (TIEG1)-mediated growth inhibition of pancreatic cancer cells, while small interfering RNAs targeting *STMN1* inhibited pancreatic cancer cell growth (26).

Wang and colleagues suggested that the therapeutic effect of gemcitabine in pancreatic cancer may be associated with the inhibition of STMN1 (30). In this study, we showed that transfection of miR-193b significantly reduced the expression of STMN1 in pancreatic cancer cells. Based on these findings and ours, we suggest that overexpression of miR-193b effectively inhibits pancreatic cancer growth *in vitro*, at least in part through the inhibition of STMN1 expression.

The second miR-193 target studied herein is the gene encoding uPA, which has been implicated in different physiological and pathophysiological processes, including cell adhesion and migration (31). In fact, the oncogenic role of uPA in pancreatic cancer is well established. Cantero *et al* demonstrated that the expression of the uPA protein, as well as of its receptor, is significantly increased in pancreatic cancer, which correlates to shorter postoperative survival (32). Moreover, upregulation of uPA was demonstrated to induce pancreatic cancer cell invasion (33). In this study, we found that miR-193b transfection markedly reduced uPA expression in pancreatic cancer cells, suggesting that the inhibition of pancreatic cancer growth by miR-193b *in vitro* may involve direct modulation of uPA expression. In addition, uPA was suggested to participate in the post-translational modification of STMN1 (34). Therefore, future studies need to focus on the molecular mechanism underlying miR-193b effects on pancreatic cancer, and specifically on the relationship among miR-193b, uPA and STMN1.

In conclusion, our study revealed that the expression of miR-193b is reduced in pancreatic cancer tissues, and that transfection with miR-193b has an inhibitory effect on pancreatic cancer cell proliferation *in vitro*, potentially via regulating the expression of the *STMN1* and *uPA genes*, which were identified as direct targets of miR-193b. Therefore, our study expands the current understanding on the molecular mechanism underlying the effects of miR-193b on pancreatic cancer cells, and suggests that miR-193 may constitute a promising therapeutic agent for suppressing pancreatic cancer growth and metastasis.

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References

1. Arora S, Bhardwaj A, Srivastava SK, Singh S, McClellan S, Wang B and Singh AP: Honokiol arrests cell cycle, induces apoptosis, and potentiates the cytotoxic effect of gemcitabine in human pancreatic cancer cells. PLoS One 6: e21573, 2011.
2. Niedergethmann M, Alves F, Neff JK, *et al*: Gene expression profiling of liver metastases and tumour invasion in pancreatic cancer using an orthotopic SCID mouse model. Br J Cancer 97: 1432-1440, 2007.
3. Garzon R, Calin GA and Croce CM: MicroRNAs in cancer. Annu Rev Med 60: 167-179, 2009.
4. Iorio MV and Croce CM: MicroRNAs in cancer: small molecules with a huge impact. J Clin Oncol 27: 5848-5856, 2009.
5. Croce CM and Calin GA: miRNAs, cancer, and stem cell division. Cell 122: 6-7, 2005.
6. Gregory RI and Shiekhattar R: MicroRNA biogenesis and cancer. Cancer Res 65: 3509-3512, 2005.
7. Shen J, Stass SA and Jiang F: MicroRNAs as potential biomarkers in human solid tumors. Cancer Lett 329: 125-136, 2013.
8. Nana-Sinkam SP and Croce CM: Clinical applications for microRNAs in cancer. Clin Pharmacol Ther 93: 98-104, 2013.
9. Wu W, Lin Z, Zhuang Z and Liang X: Expression profile of mammalian microRNAs in endometrioid adenocarcinoma. Eur J Cancer Prev 18: 50-55, 2009.
10. Rauhala HE, Jalava SE, Isotalo J, *et al*: miR-193b is an epigenetically regulated putative tumor suppressor in prostate cancer. Int J Cancer 127: 1363-1372, 2010.
11. Xu C, Liu S, Fu H, *et al*: MicroRNA-193b regulates proliferation, migration and invasion in human hepatocellular carcinoma cells. Eur J Cancer 46: 2828-2836, 2010.
12. Li XF, Yan PJ and Shao ZM: Downregulation of miR-193b contributes to enhance urokinase-type plasminogen activator (uPA) expression and tumor progression and invasion in human breast cancer. Oncogene 28: 3937-3948, 2009.
13. Hu H, Li S, Liu J and Ni B: MicroRNA-193b modulates proliferation, migration, and invasion of non-small cell lung cancer cells. Acta Biochim Biophys Sin (Shanghai) 44: 424-430, 2012.
14. Ikeda Y, Tanji E, Makino N, Kawata S and Furukawa T: MicroRNAs associated with mitogen-activated protein kinase in human pancreatic cancer. Mol Cancer Res 10: 259-269, 2012
15. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. Cell 115:787-98, 2003.
16. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ. MiR-Base: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res 34:D140–D144, 2006.
17. Krek A, Grün D, Poy MN, Wolf R, Rosenberg L,Epstein EJ, MacMenamin P, da Piedade I, Gunsalus KC, Stoffel M, Rajewsky N. Combinatorial microRNA target predictions. Nat Genet 37:495-500, 2005.
18. Chen J, Zhang X, Lentz C, Abi-Daoud M, Pare GC, Yang X, *et al*: miR-193b regulates mcl-1 in melanoma. Am J Pathol 176: 2162-2168, 2011.
19. Tahiri A, Leivonen SK, Lüders T, *et al*: Deregulation of cancer-related miRNAs is a common event in both benign and malignant human breast tumors. Carcinogenesis 35: 76-85, 2014.
20. Zhou H, Wang K, Hu Z and Wen J: TGF-β1 alters microRNA profile in human gastric cancer cells. Chin J Cancer Res 25: 102-111, 2013.
21. Cheung TH, Man KN, Yu MY, *et al*: Dysregulated microRNAs in the pathogenesis and progression of cervical neoplasm. Cell Cycle 11: 2876-2884, 2012.
22. Xie C, Jiang XH, Zhang JT, *et al*: CFTR suppresses tumor progression through miR-193b targeting urokinase plasminogen activator (uPA) in prostate cancer. Oncogene 32: 2282-2291, 2013.
23. Chen J, Feilotter HE, Pare GC, *et al*: MicroRNA-193b represses cell proliferation and regulates cyclin D1 in melanoma. Am J Pathol 176: 2520-2529, 2010.
24. Rana S, Maples PB, Senzer N and Nemunaitis J: Stathmin 1: a novel therapeutic target for anticancer activity. Expert Rev Anticancer Ther 8: 1461-1470, 2008.
25. Belletti B and Baldassarre G: Stathmin: a protein with many tasks. New biomarker and potential target in cancer. Expert Opin Ther Targets 15: 1249-1266, 2011.
26. Mistry SJ and Atweh GF: Role of stathmin in the regulation of the mitotic spindle: potential applications in cancer therapy. Mt Sinai J Med 69: 299-304, 2002.
27. Ke B, Wu LL, Liu N, Zhang RP, Wang CL and Liang H: Overexpression of stathmin 1 is associated with poor prognosis of patients with gastric cancer. Tumour Biol 34: 3137-3145, 2013.
28. Tamura K, Yoshie M, Miyajima E, Kano M and Tachikawa E: Stathmin regulates hypoxia-inducible factor-1α expression through the mammalian target of rapamycin pathway in ovarian clear cell adenocarcinoma. ISRN Pharmacol: 279593, 2013. doi: 10.1155/2013/279593.
29. Jiang L, Chen Y, Chan CY, *et al*: Down-regulation of stathmin is required for TGF-beta inducible early gene 1 induced growth inhibition of pancreatic cancer cells. Cancer Lett 274: 101-108, 2009.
30. Wang Y, Kuramitsu Y, Ueno T, *et al*: Proteomic differential display identifies upregulated vinculin as a possible biomarker of pancreatic cancer. Oncol Rep 28: 1845-1850, 2012.
31. Reichel CA, Kanse SM and Krombach F: At the interface of fibrinolysis and inflammation: the role of urokinase-type plasminogen activator in the leukocyte extravasation cascade. Trends Cardiovasc Med 22: 192-196, 2012.
32. Cantero D, Friess H, Deflorin J, *et al*: Enhanced expression of urokinase plasminogen activator and its receptor in pancreatic carcinoma. Br J Cancer 75: 388-395, 1997.
33. He X, Zheng Z, Li J, *et al*: DJ-1 promotes invasion and metastasis of pancreatic cancer cells by activating SRC/ERK/uPA. Carcinogenesis 33: 555-562, 2012.
34. Saldanha RG, Xu N, Molloy MP, Veal DA and Baker MS: Differential proteome expression associated with urokinase plasminogen activator receptor (uPAR) suppression in malignant epithelial cancer. J Proteome Res 7: 4792-4806, 2008.