Abstract. The objective of this study was to investigate the effect of silencing gene protein phosphatase 1H (PPM1H) on malignant phenotype of human pancreatic cancer cell line BxPC-3. In order to explore the function of PPM1H in pancreatic cancer cells, real-time PCR and western blotting were used to detect the expression of PPM1H in different pancreatic cancer cell lines. Human pancreatic cancer cell line BxPC-3 was treated with 10 ng/ml TGF-β1 and 200 ng/ml BMP2 for 72 h, respectively, and the mRNA and protein expression levels of PPM1H and EMT-related markers (E-cadherin, vimentin) were detected by real-time PCR and western blotting, respectively. Using exogenous RNA interference technology to silence the PPM1H gene, the expression of PPM1H and EMT-related markers at mRNA and protein levels were detected by real-time PCR and western blotting. The cell migration and invasion were measured using Transwell assays. Finally, cell counting kit-8 (CCK-8) and flow cytometry were used to determine the effect of PPM1H on cell proliferation and apoptosis of BxPC-3 cells. The expression levels of PPM1H in all of the examined pancreatic cancer cell lines (BxPC-3, MIA-PACA2, PANC-1, SW1990, PANC-03.27) were lower than that of normal pancreatic ductal epithelial cells (HPDE6-C7) at both mRNA and protein levels. Both TGF-β1 and BMP2 treatment induced EMT and downregulation of PPM1H in BxPC-3 cells. By using RNA interference to transiently knock down PPM1H expression in BxPC-3 cells, we found that the expression of E-cadherin was downregulated while vimentin was upregulated. The data suggested that silencing PPM1H gene can promote the invasion and metastasis of BxPC-3 cells. Cell proliferation and apoptosis detection demonstrated that silencing PPM1H gene can promote the proliferation and inhibit apoptosis of BxPC-3 cells. In conclusion, PPM1H is aberrantly expressed in human pancreatic cancer cell lines and can be downregulated when EMT is induced by cytokine stimulation. Silencing PPM1H gene can induce EMT in BxPC-3 cells, and promote the invasion and metastasis of BxPC-3 cells. Moreover, silencing PPM1H gene can promote the proliferation and inhibit apoptosis of BxPC-3 cells. PPM1H may be a new tumor-suppressor factor for pancreatic cancer and provides new insight into molecular targets for gene therapy of pancreatic cancer.

Introduction

Pancreatic cancer (PC) is one of the most common malignant tumors and remains a treatment-refractory cancer due to its early formation of metastases, poor prognostic and high mortality (1,2). The incidence and mortality of PC are increasing world wide. Currently, the most common treatments for PC patients are surgical resection followed by adjuvant chemotherapy and radiotherapy. Unfortunately, >80% of PC patients already have local invasion or distant metastasis when first diagnosed, and thus have lost the opportunity of surgical therapy. The overall 5-year survival rate of these patients is <5% (3-5). Even for those patients whose pancreatic cancer can be surgically resected, followed by adjuvant chemotherapy and radiotherapy, the overall 5-year survival rate is <25% (6,7). The high metastatic potential, strong growth and chemotherapy resistance are the main causes of high mortality of pancreatic cancer (8,9). However, the multiple metastasis mechanism of PC remains unclear. Therefore, understanding the key factors involved in these processes is urgently needed to explore new therapeutic targets of PC.

Accumulating evidence demonstrates that epithelial-mesenchymal transition (EMT) could promote cell proliferation, cell motility and aggression, as well as anti-apoptotic ability and chemotherapy resistance (10-14). The key step of EMT is that the expression of epithelial phenotype E-cadherin is downregulated, which plays an important role in the maintenance of cell interactions and epithelial cell polarity. Moreover, the expression of mesenchymal phenotype such as vimentin, N-cadherin, α-SMA, fibronectin are upregulated (15-17). The deletion of E-cadherin in pancreatic cancer is associated with...
the increase of tumor size, distant metastasis and pathological staging (18). Upregulation of mesenchymal phenotype could decrease the sensitivity of PC cells to chemotherapeutic drugs, and increase the resistance to chemotherapy (19,20).

Transforming growth factor-β1 (TGF-β1) and bone morphogenetic proteins 2 (BMP2) are both members of transforming growth factor-β superfamily, which are involved in cell proliferation, differentiation, apoptosis, metastasis and angiogenesis (21-25). Dysregulation of their pathway contributes to a variety of pathologies, including cancer, fibrosis and inflammation. In the early stage of tumor, TGF-β1 exhibits tumor suppressive effects by inhibiting cell cycle progression and promoting apoptosis. However, in the late stage TGF-β1 exerts tumor promoting effects by increasing tumor invasiveness and metastasis. BMP2 also has a similar dual effect. High expression of TGF-β1 and BMP2 in tumor tissue or blood of patients may be correlated with disease progression and poor prognosis. A huge number of studies indicated that both TGF-β1 and BMP2 may be involved in the process of EMT in PC cells through Smad signaling pathway or non-Smad signaling pathway so as to increase the ability of invasion and metastasis (26-30), and the latter may play a more important role. However, the concrete mechanism has not been elucidated yet.

Protein phosphatase 1H (PPM1H), a member of the PP2C family of serine/threonine protein phosphatases, which was originally identified as a negative regulator of neurite outgrowth, was comprehensively expressed in healthy human tissues (GeneCards: http://www.genecards.org/cgi-bin/carddisp.pl?gene=PPM1H&search=PPM1H). PPM1H was reported to control cell proliferation and differentiation (31-34). Recently, it has been indicated to participate in the process of cancer development (31-33). However, the role of PPM1H in cancer remains controversial. Previously, PPM1H was found to be downregulated in glioblastoma and renal cell carcinoma compared to normal brain and kidney but upregulated in colon adenocarcinoma and prostate adenocarcinoma compared to normal colon and prostate. It was also demonstrated that low PPM1H expression in breast cancer trend toward trastuzumab resistance and worse clinical outcome. These studies suggested that the role of PPM1H may differ depending on the cancer type and its exact mechanism of action in cancer remains largely unknown. Until now, no attempt was made to examine the role of PPM1H in pancreatic cancer.

In this study, we examined the expression of PPM1H in human PC cell lines. In addition, we explored the role of PPM1H in inducing EMT as well as promoting human PC cell migration. Furthermore, we determined the effect of PPM1H depletion on the proliferation and apoptosis of human PC cells.

Materials and methods

Cell lines and cell culture. The human pancreatic cancer cell lines PANC-1, BxPC-3, PANC-03.27, SW1990, MIA-PACA2 and the normal human pancreatic epithelial cell line HPDE6-C7 were used in this study. The cell lines PANC-1, BxPC-3 and HPDE6-C7 were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China), and the other cells were obtained from the American Type Culture Collection (ATCC). The cell lines BxPC-3, PANC-03.27, MIA-PACA2 and HPDE6-C7 were cultured in RPMI-1640 medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (Gibco) and the cell lines PANC-1 and SW1990 were cultured in DMEM medium (Gibco) containing 10% fetal bovine serum (Gibco). All of the cells were grown at 37°C in a humidified atmosphere containing 95% O₂ and 5% CO₂. BxPC-3 cells were grown to approximately 60% confluency in RPMI-1640 +10% FBS and were then serum-deprived overnight in RPMI-1640 medium. Cells were then treated with 10 ng/ml TGF-β1 or 200 ng/ml BMP2 (Peprotech Inc., Rocky Hill, NJ, USA) for 72 h. The morphology of cells was visualized with a phase contrast microscope (x200, Nikon, Japan) and imaged with digital photography.

siRNA transfection. siRNA targeting human PPM1H (5'-CACGCUUCUUUACCGAGA-3' and 5'-UCUCGGUGA AAGAAGCGUGG-3' duplex) was synthesized by Ribobio Co. (Guang Zhou, China). A scrambled duplex siRNA was used as the negative control. BxPC-3 cells were plated at 2x10⁵/well in 6-well plates and incubated until they reached 50% confluency. Cells were transfected with PPM1H siRNA or the negative control siRNA at a final concentration of 50 nM with Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. After 6 h of transfection, the medium was replaced with RPMI-1640 medium containing 10% fetal bovine serum. Cells were then incubated for 72 h for RNA isolation and protein extraction.

RNA isolation and quantitative RT-PCR (qRT-PCR). The total RNA from BxPC-3 cells was extracted using the TRIzol reagent (Takara Inc., Otsu, Japan) according to the manufacturer's instructions and was resuspended in nuclease-free water. Then, 500 ng of total RNA was reverse-transcribed in a final volume of 10 µl with PrimeScript™ II First-Strand cDNA Synthesis kit (Takara, Inc.). The reverse transcription reaction was processed at 37°C for 15 min, 85°C for 5S and 4°C for 1 min. qRT-PCR was performed using the SYBR Green Master Mix (Takara, Inc.) on StepOne Real-time PCR systems (Applied Biosystems, Foster City, CA, USA) in triplicate, and non-template controls were run for each assay under the same conditions. The PCR reaction was carried out with an initial denaturation step of 95°C for 30 sec followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. Primers used for real-time PCR were as follows: PPM1H (forward, 5’-GCATTCATGC TTCACTTTG-3’, reverse, 5’-GCATTCATGC TTCACTTTG-3’); β-actin (forward, 5’-GCCATCCTCTGTCC AGCCTCACTTGC-3’, reverse, 5’-GCCATCCTCTGTCC AGCCTCACTTGC-3’); E-cadherin (forward, 5’-CTGGTGCAGC AGCAGAGG-3’, reverse, 5’-CTGGTGCAGC AGCAGAGG-3’); Vimentin (forward, 5’-CTGGTGCAGC AGCAGAGG-3’, reverse, 5’-CTGGTGCAGC AGCAGAGG-3’); α-SMA (forward, 5’-GGTTCCATACCCCTTT CTGTG-3’, reverse, 5’-GGTTCCATACCCCTTT CTGTG-3’); β-actin (forward, 5’-GGTTCCATACCCCTTT CTGTG-3’, reverse, 5’-GGTTCCATACCCCTTT CTGTG-3’).

The analysis of qPCR was carried out using the 2⁻ΔΔCT method. β-actin was taken as the internal control.

Western blotting. Proteins were extracted from cultured cells and then quantitated using Pierce BCA Protein Assay kit (Boster Biological Engineering Co., Ltd., Wuhan, China). Equal amounts of protein from different samples were separated by
10% SDS/polyacrylamide gel in Tris-glycin buffer and transferred to PVDF membranes. The membranes were blocked for 1 h at room temperature in blocking buffer (5% skim milk in 0.5 Tween-20-TBST), then probed with PPM1H antibody (Abgent, diluted 1:1000 in TBST), E-cadherin antibody (ProteinTech, diluted 1:800 in TBST), vimentin antibody (ProteinTech, diluted 1:5000 in TBST), akt antibody (CST, diluted 1:1000 in TBST) and p-akt antibody (CST, diluted 1:1000 in TBST), respectively, overnight at 4˚C with GAPDH antibody (Promoter Biological Co., China, diluted 1:5000 in TBST) as control. Following washes with TBST, the membranes were incubated with HRP-conjugated goat anti-rabbit secondary antibody (Promoter Biological Co., diluted 1:5000 in TBST) for 1 h at room temperature. After extensive washing with TBST, the complex was detected by SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. Blot was scanned and densitometric analysis was done by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Figure 1. Expression of PPM1H in human normal pancreatic ductal epithelial cell line (HPDE6-C7) and different human pancreatic cancer cell lines (BxPC-3, MIA-PACA2, SW1990, PANC-1, PANC-03.27). (A) RT-PCR analysis of PPM1H in six cell lines. The relative mRNA levels with the use of control HPDE6-C7 were normalized to 1. Values are given as means and standard deviations from more than three independent experiments when compared to control cells. *P<0.05. (B) Western blot analysis of PPM1H in six cell lines. GAPDH is shown as a control.

Cell proliferation assay. Cell proliferation was analyzed using the Cell Count Kit-8 (CCK-8) assay. BxPC-3 cells were seeded in 96-well plates at a density of 10000 cells per well and transfected with PPM1H siRNA or the negative control siRNA when the cells reached 50% confluency, then incubated for 1, 2 or 3 days. At the indicated time point, CCK8 solution was added to each well and incubated for 1, 2, 3, 4 h. The absorbance value (optical density) of each well was measured at 450 nm. For each experimental condition, 6-wells were used. All experiments were performed thrice.

Flow cytometry. BxPC-3 cells were transfected with PPM1H siRNA or the negative control siRNA for 72 h, then were harvested and washed with ice-cold PBS. The cells were
trypsinized without EDTA and resuspended with binding buffer. Annexin V and PI staining were carried out using the Annexin V-FITC Apoptosis Detection kit (BD Biosciences, San Jose, CA, USA), according to the manufacturer’s instructions. After a 15-min incubation in a dark at room temperature, the cells were immediately analyzed by FACScan flow cytometer.

Statistical analysis. Data were presented as mean ± standard deviation (SD). Experiments were repeated at least three times. SPSS 17.0 software (IBM, Chicago, IL, USA) was used for data analysis. Group differences were analyzed by Student’s t-test or analysis of variance (ANOVA) according to the data type. P<0.05 was considered statistically significant.

Results

Expression of PPM1H in pancreatic cancer cell lines. The expression of PPM1H in human pancreatic cancer cell lines (BxPC-3, MIA-PACA2, SW1990, PANC-1, PANC-03.27) were analyzed by real-time PCR and western blotting. The real-time PCR results showed that PPM1H mRNA expression in PC cells were significantly lower than in normal pancreatic ductal epithelial cells (HPDE6-C7) (Fig. 1A). In addition, western blot data also showed lower expression of PPM1H in PC cell lines compared with HPDE6-C7 cells (Fig. 1B). These data indicated that the expression of PPM1H was down-regulated in PC cells.

Effect of TGF-β1 and BMP2 on EMT and PPM1H expression in PC cells. TGF-β1 and BMP2 were demonstrated to be involved in the process of EMT in tumor cells so as to increase their ability of invasion and metastasis. As showed by western blotting, after treatment with 10 ng/ml TGF-β1 or 200 ng/ml BMP2 for 72 h, the expression of E-cadherin in BxPC-3 cells was decreased, and the expression of vimentin was increased, indicating EMT of BxPC-3 cells were induced.

Figure 2. Effect of TGF-β1 and BMP2 on the expression of EMT-related makers and PPM1H in BxPC-3 cell line. (A) Effect of 10 ng/ml TGF-β1 on the expression of EMT related molecules and PPM1H in BxPC-3 cell line. (B) Effect of 200 ng/ml BMP2 on the expression of EMT related molecules and PPM1H in BxPC-3 cell line. Compared with control group. *P<0.05.
In addition, the expression of PPM1H was significantly reduced in TGF-β1 or BMP2 treated BxPC-3 cells (P<0.05, Fig. 2). These data suggested that PPM1H may participate in the regulation of EMT.

**PPM1H gene knockdown induces EMT in PC cells.** To investigate whether PPM1H could induce EMT in PC cells, PPM1H specific siRNA were used to silence PPM1H expression. EMT-related markers E-cadherin and vimentin were detected by real-time PCR and western blotting, respectively, in PPM1H siRNA (si-PPM1H) transfected BxPC-3 cells. The data showed in BxPC-3 cells that downregulated E-cadherin and upregulated vimentin expression were induced by PPM1H gene silencing (P<0.05, Fig. 3), indicating PPM1H gene silencing may cause EMT in PC cells.

**PPM1H knockdown promotes invasion and migration of PC cells.** Next, we used Transwell cell migration assay to examine the role of PPM1H in invasion and migration of PC cells. The cell migration rates were compared in PPM1H siRNA treated group and control group. The data showed that the cells penetrated the artificial basement membrane in siPPM1H group were two to three times more than in siCtrl group (P<0.05, Fig. 4), implicating that PPM1H depletion resulted in increased invasion and migration of PC cells.

**PPM1H knockdown promotes proliferation and inhibits apoptosis of PC cells.** To further determine the role of PPM1H in PC cells, we assessed the effect of PPM1H depletion on cell proliferation and apoptosis. It was shown that silencing PPM1H resulted in increased proliferation rate in BxPC-3 cells (P<0.05, Fig. 5A). We also found that the apoptosis rate was reduced nearly 20% in siPPM1H group compared to siCtrl group (P<0.05, Fig. 5B). These results demonstrated that PPM1H silencing could promote proliferation and inhibit apoptosis of PC cells.

**PPM1H knockdown promotes Akt phosphorylation of PC cells.** To explore the mechanisms involved in PPM1H function in PC cells, we tested Akt activity of BxPC-3 by western blotting. As shown in Fig. 6, silencing PPM1H can increase Akt phosphorylation. This result suggested that PPM1H may function through the Akt signaling pathway.

**Discussion**

The morbidity and mortality of pancreatic cancer have been gradually rising, while those of other common cancers have been declining (7). Although the detection and management of pancreatic cancer have achieved certain developments, only less than 5% of patients live 5 years after diagnosis (6,7). Since...
its malignant degree is high and the rate of resection is low, and pancreatic cancer responds poorly to most chemotherapeutic agents, it is urgent for us to understand the biological mechanisms that contribute to development and progression of pancreatic cancer.

EMT was initially considered to be an important feature of embryonic differentiation and morphology. And now, it has been demonstrated to be involved in the development of many diseases, such as inflammation, fibrosis and tumor (35). Accumulating evidence has indicated that EMT can promote an early stage tumor into aggressive malignant tumor by increasing the motility and invasiveness of the cells, this process was consistent with the acquisition of tumor stem cell phenotype (36-39). Some studies have shown that EMT can increase the invasive and anti-apoptosis capacity of cells, and may also affect its ability to resist chemotherapy. In recent years, EMT has been considered to play a critical role in the high drug-resistance and invasion of pancreatic cancer, therefore, the study of EMT in pancreatic cancer is helpful for the development of new drugs and new targets for molecular therapy.

Figure 4. Silencing PPM1H promotes invasion and migration ability of BxPC-3 cells. BxPC-3 cells were transfected with siPPM1H or siCtrl. After 72 h, cells (8x10^4) were loaded into the top chamber of 24-well Transwell plates and incubated for another 24 h. The migrated cells were stained with 0.1% crystal violet and the average number per field was quantified under high power (original magnification x200) of the phase contrast microscope. *P<0.05.

Figure 5. Effects of PPM1H silencing on cell proliferation and apoptosis of BxPC-3 cells. (A) Silencing PPM1H promoted cell growth as determined by CCK8 assay. Each bar represents the average ± SD of three independent experiments. (B) Silencing PPM1H inhibited the apoptosis of BxPC-3 cells by flow cytometry. Each bar represents the average ± SD of three independent experiments. *P<0.05.
Phosphorylation and dephosphorylation of proteins have important effect on the regulation of life activities of eukaryotic organisms, and the process of phosphorylation is co-regulated by protein kinase and protein phosphatase. Protein phosphatase is a kind of key enzyme that controls dephosphorylation of proteins. Its absence or dysfunction can lead to a variety of diseases. PPM1H (protein phosphatase, Mg2+/Mn2+ dependent 1H) is a member of the PP2C family of Ser/ Thr protein phosphatases, which is not sensitive to Okada acid. Most members of the PP2C family have been shown to act as inhibitors of cell growth and cellular stress signaling. These members include PP2Cα, PP2Cβ, and the recently identified PP2Cs, ILKAP, and PHLPP; ILKAP negatively affects proliferation and malignant transformation, and PHLPP promotes apoptosis and inhibits tumor growth. Based on these observations, it is reasonable to assume that, in general, type 2C phosphatases act as tumor suppressor proteins. In addition to the PP2C family, a number of the PPM1H (PPM1D/Wip1), which behaves as an oncogene (40-43).

At present, there are few studies on PPM1H in tumor, and the effect of PPM1H is controversial. As the result of this study shows that PPM1H was downregulated at both transcriptional and translational levels in five human pancreatic cancer cell lines compared with normal pancreatic ductal epithelial cell line (P<0.05), these results were different from the research of Sugura et al in colon cancer cell lines (31). These results suggested that PPM1H plays a complex role in cancer and the effect of PPM1H may vary among cancers of different organs or tissues. Based on this result, we speculated that PPM1H may act as a tumor suppressor in pancreatic cancer.

Both TGF-β1 and BMP2 can regulate EMT through Smad signaling pathway and non-Smad signaling pathway, the latter is considered to play a more important role in the process of inducing EMT (26-27,44-46). Our study demonstrated that both TGF-β1 and BMP2 can induce EMT in BxPC-3 cells, interestingly the expression of PPM1H was significantly downregulated after induction of EMT. It suggests that PPM1H may participate in the regulation of EMT. To further confirm the role of PPM1H in EMT of pancreatic cancer cells, we chose BxPC-3 cell line for PPM1H silencing in which endogenous PPM1H expression was at relatively high level. We used interference RNA (RNAi) technique to induce PPM1H gene silencing, and qRT-PCR and western blotting were used to detect the expression of PPM1H and EMT related molecules. After silencing PPM1H, the expression of epithelial mesenchymal phenotype E-cadherin was downregulated, while the expression of mesenchymal phenotype vimentin was upregulated in BxPC-3 cells (P<0.05). It was indicated that deletion of PPM1H gene can promote EMT independently in BxPC-3 cells. Then, we used Transwell cell invasion and migration assay to examine whether the invasion and metastasis capacity would be promoted when BxPC-3 cells were induced in EMT. The results showed that the invasion and metastasis of BxPC-3 cells were enhanced after silencing PPM1H (P<0.05), which illustrated that silencing PPM1H could enhance the invasion and metastasis of pancreatic cancer cells by promoting EMT.

The studies of Lee-Hoeflich et al have found that the loss of PPM1H gene can lead P27 suppressor gene to degrade so as to promote the proliferation of breast cancer cells (32). Hence, we speculated that PPM1H might suppress the proliferation of pancreatic cancer cells. We performed CCK8 assays to investigate the effect of PPM1H silencing on the proliferation of BxPC-3 cells. Our results showed that knockdown of PPM1H resulted in a more dramatic increase of the proliferation rate in BxPC-3 cells than that in control cells (P<0.05). PPM1H could suppress cell proliferation of BxPC-3 cells in vitro. In addition, we assessed that silencing PPM1H can inhibit the apoptosis of BxPC-3 cells by flow cytometry (P<0.05). Although we found that silencing PPM1H gene can promote proliferation and inhibits apoptosis of BxPC-3 cells, its specific mechanism still needs further exploration.

Since Smad4 is homozygously deleted in BxPC-3 cells (47), we suspected that PPM1H affect is through non-Smad pathway. In particular, it has been reported that mutation or deletion of Smad4 is found in approximately 50% of pancreatic tumors and is correlated with poor prognosis (48). Thus, it may be more important to investigate non-Smad pathways in detail in order to further understand invasion and metastasis of pancreatic cancer. In this study, we found that silencing PPM1H can increase Akt phosphorylation. The Akt signaling pathway is involved in many cellular processes during the occurrence and development of cancer, such as proliferation, apoptosis, invasion and metastasis (49,50). Dephosphorylation of Akt can make it inactive, and thus inhibit the various biological activities of Akt. This
result suggests that the PPM1H biological effects may be through the Akt signaling pathway.

Although we have demonstrated that silencing PPM1H can increase the invasion and metastasis of BxPC-3 cells, as well as promote its proliferation and inhibit apoptosis, we also need to explore the specific mechanisms of these effects. Whether silencing PPM1H can also promote BxPC-3 cells to acquire CSC characteristics so that it can induce chemotherapeutic resistance of BxPC-3 cells, and whether overexpression of PPM1H could reverse the above process is not known.

PPM1H presented low expression in pancreatic cancer cell lines, and silencing PPM1H can enhance the invasion and metastasis of BxPC-3 cells by mediating EMT. Silencing PPM1H also promotes proliferation and inhibits apoptosis of BxPC-3 cells. These effects may be produced by increasing AKT phosphorylation. This study suggests that PPM1H may be a novel tumor suppressor gene for pancreatic cancer. A better understanding of the function of PPM1H in pancreatic cancer is expected to provide new insight into the molecular targets of gene therapy for pancreatic cancer.

References