Abstract. Serine/threonine-protein kinase-1 (SMG-1) belongs to the phosphatidylinositol 3-kinase-related kinase family. Altered expression of SMG-1 contributes to human carcinogenesis and cancer progression. The present study detected the expression levels of SMG-1 in normal and cancerous pancreatic tissues and then assessed the effects of SMG-1-knockdown in pancreatic cancer cell lines in vitro. A pancreatic cancer tissue array and pancreatic cancer cell lines were used to detect the expression levels of SMG-1 and a lentivirus expressing either SMG-1 or negative control short hairpin (sh)RNA were used to knockdown the expression of SMG-1 in the pancreatic cancer cell lines. Western blot, cell proliferation, Cell Counting kit-8, Transwell tumor cell migration and invasion assays, and flow cytometric analysis of cell apoptosis with or without gemcitabine or cisplatin treatment were performed to assess the tumor cells. The protein expression of SMG-1 was higher in the pancreatic cancer tissues and was associated with an advanced tumor stage. Knock down of the expression of SMG-1 inhibited tumor cell proliferation and induced the chemosensitivity of pancreatic cancer cells in vitro.

Introduction

Pancreatic cancer is a significant health problem worldwide (1), with an estimated 279,000 cases diagnosed globally in 2008 (2). The majority of patients are diagnosed at an advanced stage, and the 5-year survival rate of patients following surgery is poor, with 213,000 pancreatic cancer-associated mortalities worldwide despite advancements in surgery, radiation therapy and chemotherapy (3). Therefore, it is important to identify the molecular mechanisms underlying the development and progression of pancreatic cancer in order to develop novel strategies for its early detection and treatment.

The present study investigated serine/threonine-protein kinase-1 (SMG-1), which belongs to the phosphatidylinositol 3-kinase-related kinase (PIKK) family (4,5). Functionally, SMG-1 has been linked to the nonsense-mediated decay (NMD) of mRNA, as a member of the mRNA surveillance complex (4). SMG-1 also has NMD-independent functions (4), including cellular stress responses. Previous studies (6,7) have demonstrated that hSMG-1 is an important regulator of the cell cycle checkpoints by regulating the synthesis and proteolysis of p21. SMG-1 is also considered to be a potential cancer susceptibility gene. The catalogue of somatic mutations in the COSMIC cancer database reveals that mutations in SMG-1 are associated with breast, kidney and stomach cancer (www.sanger.ac.uk/genetics/CGP/cosmic) (8). Previous investigations have demonstrated that SMG-1 is widely expressed in multiple tissues and cell lines (5,9). It has been observed that SMG-1 regulates the G1/S checkpoint of the cell cycle via a p53-dependent and a p53-independent pathway, and depletion of the SMG-1 protein increases the cell growth of colorectal cancer cells, indicating that SMG-1 is a tumor suppressor gene (10). Another study demonstrated that the
SMG-1-induced activation of the p53 pathway is associated with the chemoprotective effects of temop (11). However, additional studies have revealed that the SMG-1 promoter hypermethylation-induced downregulation of the expression of SMG-1 is associated with improved survival rates in patients with human papaloma virus (HPV)-positive head and neck squamous cell carcinoma (12). SMG-1 antagonizes tumor necrosis factor-α-induced apoptosis in osteosarcoma cells (13). A kinome-wide screen identified SMG-1 as an essential kinase for the survival of multiple myeloma and SMG-1 knockdown with small interfering (si)RNA reduced the survival of myeloma cell lines (14). Furthermore, SMG-1 mRNA has been observed to be upregulated in acute myeloid leukemia (15). These studies indicated that SMG-1 may have different roles in cancer progression.

The present study detected the protein expression of SMG-1 in pancreatic cancer tissue specimens and subsequently assessed the effects of SMG-1 knockdown on the sensitivity of pancreatic cancer cells to chemotherapeutic agents in vitro.

Materials and methods

Immunohistochemical analysis of the expression of SMG-1 in pancreatic cancer tissue. The present study was approved by the ethics committee of the Second Affiliated Hospital of Nanjing Medical University (Nanjing, China). A pancreatic cancer tissue microarray (TMA) was obtained from Alenabio (cat. no. PA2082; Xian, China). The TMA contained 94 cases of pathologically diagnosed pancreatic adenocarcinoma with certain additional clinicopathological data from the patients. A pathologist inspected all the specimens and confirmed the diagnosis of pancreatic adenocarcinoma, mucinous adenocarcinoma, adenosquamous carcinoma, squamous cell carcinoma, acinic cell carcinoma and neuroendocrine carcinoma, five cases of normal pancreatic tissue and five cases of distant normal pancreatic tissue. The age of the tissue donors ranged between 39 and 78 years, with a mean age of 57.5±9.9 years, and a female to male ratio of 1.6. The tumor tissue was obtained from patients at Tongxu People’s Hospital (Kaifeng, China).

For immunostaining of the SMG-1 protein, the TMA sections were deparaffinized in xylene (Xilong Chemical Co., Ltd., Shenzhen, China) twice and rehydrated in a series of ethanol (100% for 5 min, 95% for 5 min, 75% for 5 min) followed by ultrapure water. Staining was performed by incubating the sections with mouse monoclonal anti-SMG-1 antibody (1:300; cat. no. SAB1404950-100UG; Sigma-Aldrich, St. Louis, MO, USA), at 4°C overnight. The intensity of the SMG-1 staining was scored by a pathologist, in a blinded manner, as negative (no signal), weak (weak intensity in <50% of tumor cells), moderate (strong intensity in <50% of tumor cells) and strong (strong intensity in the majority of tumor cells).

Cell lines and culture. The COLO-357, BxPc-3, Capan-1, Capan-2, SW1990 and PANC-1 human pancreatic cancer cell lines were obtained from the Shanghai Cell Bank (Shanghai, China) and cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 µg/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. All reagents were obtained from Wisent (St. Bruno, PQ, Canada).

SMG-1 short hairpin (sh)RNA recombinant lentiviral vectors and lentivirus. A lentiviral vector-mediated shRNA against SMG-1 was designed and constructed by GenePharma (Shanghai, China). Four pairs of shRNAs, each targeting different regions of the SMG-1 transcript (GenBank accession no. NM_015092), and one negative control shRNA were constructed. The target mRNA sequences of SMG-1 were as follows: sh-SMG-1 #1 (no. 5252), 5'-GCAGAAAGGTGGTTGCAATG-3'; sh-SMG-1 #2 (no. 6931), 5'-GCTCGACACATTTCTGTAACA-3'; sh-SMG-1 #3 (no. 7512), 5'-GGGTGGTAACCTGGAGTGAGG-3' and sh-SMG-1 #4 (no. 8877): 5'-GGAACAGCTGCTGACAGTCTTA-3'. The scrambled sequence, 5'-ACTACCGTTGTATAGGTG-3', was used as a negative control. These lentiviral vectors were then used by GenePharma to produce the lentivirus.

Lentivirus infection of pancreatic cancer cell lines. To knock down the expression of SMG-1, the SW1990 and PANC-1 pancreatic cancer cell lines were infected with a lentivirus. Briefly, the cells were seeded into six-well plates (Corning Costar, Inc., Corning, NY, USA) at a density of 40% and grown for 24 h at 37°C. The cells were then infected with a lentivirus containing the shRNA targeting SMG-1 (SW1990 at an multiplicity of infection (MOI) of 10 and PANC-1 at an MOI of 15, based on pre-experimental data), according to the manufacturer’s instructions. The lentiviral infection efficiency was confirmed by the immunofluorescence density of the enhanced green fluorescent protein reporter gene, and the RNA interference (i) efficiency was determined by analyzing the mRNA and protein expression of SMG-1. Based on these investigations, SMG-1 shRNA lentivirus construct #1 was selected for the subsequent experiments (data not shown).

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The cells were washed with phosphate-buffered saline (PBS; Beyotime Institute of Biotechnology, Haimen, China) and the total RNA was isolated from the cells using RNAiso Plus (Takara Bio, Inc., Dalian, China), according to the manufacturer’s instructions. Primer-Script RT Master mix (Takara Bio, Inc.) was used to synthesize cDNA from the RNA samples. qPCR was performed using SYBR Green (Roche Diagnostics, Indianapolis, IN, USA) on a 7500 Real-Time-PCR System (Applied Biosystems, Foster City, CA, USA). The qPCR amplification was performed on 10 ng cDNA (total reaction volume, 20 µl) as follows: An initial cycle of 95°C for 10 min, 40 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, and then a final extension at 72°C for 5 min. The mRNA expression of β-actin was used as an internal control for determining the relative mRNA expression of SMG-1. The cycle threshold (Ct) comparative ΔΔCt method was used to calculate the relative mRNA expression of SMG-1, and the fold-changes were analyzed by 2^ΔΔCt (16). The primers used for RT-qPCR were as follows: SMG-1, forward 5'-TTATCGCCAAAGAAACACC-3' and reverse 5'-AGGAATCTTGGGCCTTTTGT-3' and β-actin, forward 5'-CTCCATCTGGCGGCAGGTCC-3' and reverse 5'-GCTGTACCTTCCACCGTTCC-3'. All the experiments were performed in biological triplicate.
were performed in triplicate and repeated three times with independent RNA samples.

Protein extraction and western blotting. The total cellular protein was extracted from the SW1990 and PANC-1 cells using radioimmunoprecipitation buffer (Beyotime Institute of Biotechnology), supplemented with 1% phenylmethylsulfonylfluoride (Beyotime Institute of Biotechnology). The protein concentration was estimated with a (bicinchoninic acid) BCA kit (Nanjing KeyGen Biotech. Co., Ltd., Nanjing, China). Following quantification, the protein samples were separated by 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Beyotime Institute of Biotechnology) and transferred onto polyvinylidenedifluoride membranes (Beyotime Institute of Biotechnology). The membranes were blocked with 5% non-fat dry milk (Yili Industrial Group Co., Ltd., Inner Mongolia, China) in Tris-buffered saline (TBS) and incubated with the following primary antibodies: mouse monoclonal anti-SMG-1 (1:300; cat. no. SAB1404950-100UG; Sigma-Aldrich) or mouse monoclonal anti-GAPDH (1:500; cat. no. AG019; Beyotime Institute of Biotechnology, Jiangsu, China) at 4˚C overnight. The following day, the membranes were washed with TBS-Tween-20 (TBS-T; Beyotime Institute of Biotechnology) and further incubated with a secondary horseradish peroxidase-coupled goat anti-mouse antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 2 h at room temperature. The membranes were washed three times with TBS-T and the color was developed using an electrochemiluminescence kit (Pierce Biotechnology, Inc., Rockford, IL, USA). The membranes were then exposed to X-ray film (GelDoc XR system; Bio-Rad Laboratories, Inc., Hercules, CA, USA) to visualize the signals. The target protein expression of GAPDH was used as an internal control for determining the relative expression levels of SMG-1.

Cell proliferation assay. The cells were infected with either the negative control lentivirus or the SMG-1 shRNA lentivirus for 3 days at 37˚C and then seeded into 96-well culture plates (Costar, Cambridge, UK) at a density of 2x10^4 cells/well. The cells were incubated at 37˚C for up to 5 days. Cell proliferation was detected daily using a Cell Counting kit-8 (CCK-8; Nanjing KeyGen Biotech., Co., Ltd.), according to the manufacturer’s instructions, for 5 days. Briefly, 10 µl CCK-8 solution was added to each well and the optical density was detected using a microplate reader (Sunrise™; Tecan, Grödig, Austria) at 450 nm, with a reference wavelength of 650 nm. Each assay was performed in triplicate and repeated independently three times.

Tumor cell migration and invasion assay. The tumor cell migration and invasion capacities were measured using a Transwell chamber assay with or without Matrigel coating. The Transwell chambers were 6.5 mm in diameter and had a 8 µm pore size (Corning Costar, Inc.). The SW1990 and PANC-1 cells were seeded into the upper chamber (5.0x10^4 cells per Transwell) pre-coated with or without 1 mg/ml Matrigel (BD Biosciences, San Jose, CA, USA), and the lower wells were filled with 500 µl 10% FBS-DMEM. Following incubation for 24 h at 37˚C, the non-invading cells were removed using cotton swabs and the cells that had invaded into the underside of the membrane were stained with 0.1% crystal violet (Beyotime Institute of Biotechnology) for 15 min at 37˚C. The membranes were washed with PBS and the invading cells were counted under an inverted microscope (Eclipse Ti-E; Nikon Corporation, Tokyo, Japan). All the experiments were performed in triplicate and repeated once.

Flow cytometric analysis of cell cycle distribution and apoptosis. The cell cycle progression and apoptosis were assessed by flow cytometry using a BD FACSCalibur (BD Biosciences). The SW1990 and PANC-1 cells were grown and infected with sh-SMG-1 or with a control lentivirus at 37˚C for 12 h, and then treated with 10 mg/ml gemcitabine or cisplatin (Jiangsu Hansoh Pharmaceutical Co., Ltd., Jiangsu, China). Cell cycle analysis was conducted using PI/RNase Staining Buffer (BD Pharmlingen, San Diego, CA, USA). For cell cycle analysis, the cells were collected, washed twice with PBS and fixed with 70% ethanol at -20˚C overnight. The cells were then washed twice with PBS and resuspended in 500 µl PBS containing 0.2% Triton-X-100, 10 mM EDTA, 100 µg/ml RNase A and 50 µg/ml propidium iodide. The samples were incubated at room temperature for 30 min.

Figure 1. Differential protein expression levels of SMG-1 in pancreatic cancer from a tissue microarray (hematoxylin and eosin stain) with immunohistochemical staining. Representative images reveal (A) strong, (B) moderate, (C) weak and (D) negative protein staining of SMG-1 in the pancreatic cancer tissue. Magnification, x200. SMG-1, serine/threonine-protein kinase-1.
Apoptosis analysis was conducted using Annexin V-FITC Apoptosis Detection kit (BD Pharmingen). For the detection of apoptosis, the cells were collected and washed twice with PBS, prior to suspending in 100 µl 1X binding buffer and staining with 5 µl annexin-V 647 and 5 µl 7-aminoactinomycin D at room temperature for 15 min in the dark. The samples were analyzed using a flow cytometer (BD FACSCalibur; BD Biosciences). All the experiments were performed in triplicate and repeated once.

Statistical analysis. All data are expressed as the mean ± standard deviation. Student’s t-test was used to analyze the differences between groups using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). The differences among the strips from the western blot analysis were inferred by comparing the gray level of the strips using ImageJ software, version 1.43 (SeekBio, Huzhou, China). P<0.05 was considered to indicate a statistically significant difference.

Results

Differential protein expression of SMG-1 in pancreatic cancer tissues. The present study first assessed the protein expression levels of SMG-1 in normal and cancerous pancreatic tissues. Immunohistochemical staining detected SMG-1 in the nuclei and cytoplasm, but was more predominant in the nuclei of the tumor cells (Fig. 1). According to the expression of SMG-1, the pancreatic cancer cases were divided those exhibiting high (moderate/strong) and low (negative/weak) expression levels. In total, 51.1% (48/94) of the pancreatic cancer tissues expressed a high level of SMG-1 protein, whereas 48.9% (46/94) expressed a low level of SMG-1 protein (Table I). By contrast, 2/5 distant pancreatic tissues and 1/5 normal pancreatic tissues expressed...
a high level of SMG-1 protein. These results were consistent with those in the Human Protein Atlas (http://www.protein-atlas.org/ENSG00000157106). Since the TMA was obtained from a company with limited clinicopathological data, the expression of SMG-1 was correlated with the expression of SMG-1 among pancreatic cancer tissue, distant pancreatic tissue and normal pancreatic tissue, which revealed that the expression of SMG-1 was associated with an advanced tumor stage, but not with age and gender (Table I).

Expression and knockdown of SMG-1 in pancreatic cancer cell lines. The expression levels of SMG-1 in six different pancreatic cancer cell lines were assessed by western blot analysis. The data revealed that all six pancreatic cancer cell lines expressed high protein levels of SMG-1, and these levels were highest in the PANC-1 and SW1990 cells (Fig. 2A).

Thus, PANC-1 and SW1990 cells were selected for the subsequent knock down of SMG-1 expression using the shRNA lentivirus. The data revealed that SMG-1 shRNA lentivirus #1 significantly reduced the protein expression levels of SMG-1 in the two cell lines (Fig. 2B) compared with the negative control shRNA lentivirus.

Knockdown of SMG-1 inhibits the proliferation of pancreatic cancer cells. As shown in Fig. 3A, knockdown of SMG-1 significantly inhibited the proliferation of PANC-1 and SW1990 cells compared with negative control lentivirus-infected tumor cells (P<0.05, vs. control). Flow cytometric analysis revealed that the knockdown of SMG-1 reduced the S/G2 phase of the cell cycle compared with the control cells in the SW1990 cells (61.6±2.25, vs. 44.3±3.5%, P<0.05) and PANC-1 cells (61±4, vs. 49.7±3.5%, P<0.05; Fig. 3B), while the G1 phase of the cell cycle was increased in the SW1990 cells (38.3±2.25, vs. 52.3±3.5%, P<0.05) and PANC-1 cells (39±4, vs. 50.3±3.5%, P<0.05; Fig. 3B). These observations demonstrated that loss of the expression of SMG-1 inhibited the proliferation of pancreatic cancer cells via the induction of the G1 phase of the cell cycle.

Effect of SMG-1 knockdown on the regulation of pancreatic cancer cell invasion and migration. To determine whether SMG-1 knockdown affected the invasion and migration of the SW1990 and PANC-1 cells, Matrigel invasion and Transwell assays were performed. Infection with the SMG-1 shRNA lentivirus #1 had no significant effect on the tumor cell invasion and migration capacities (Fig. 4A and B) compared with the cells infected with the control lentivirus.

SMG-1 knockdown increases the chemosensitivity of pancreatic cancer cells to treatment with gemcitabine and cisplatin. Following infection of the pancreatic cancer cells with SMG-1 shRNA and negative control lentiviruses for 72 h, the cells were treated with 10 mg/ml gemcitabine or 10 mg/ml cisplatin for an additional 24 h. Flow cytometric analysis revealed that the levels of apoptosis in the SW1990 cells treated with gemcitabine/cisplatin increased, between 16.55±1.26 and 21.97±1.55% in the gemcitabine-treated cells compared with control cells, and between 9.28±0.34 and 16.93±0.66% in the cisplatin-treated cells (Fig. 4C and D). Similarly, the PANC-1 cells demonstrated similar responses when treated with gemcitabine (8.94±0.59, vs. 12.35±0.66% and cisplatin (10.8±0.7, vs. 15.74±0.89%; Fig. 4C and E).
Discussion

The present study detected the protein expression levels of SMG-1 in pancreatic cancer and normal tissues, and found that the expression levels of SMG-1 were increased in pancreatic cancer tissue compared with normal tissue. This finding is consistent with data from the Human Protein Atlas. It was also revealed that the protein expression of SMG-1 was associated with an advanced tumor stage. Subsequently, the expression of SMG-1 was knocked down in pancreatic cancer cell lines and phenotypic changes in the tumor cells were observed. The data demonstrated that knock down of SMG-1 inhibited the proliferation of pancreatic cancer cells and increased tumor cell chemosensitivity. However, the tumor cell invasion and migration capacities remained unaltered.

SMG-1 is the newest member of the PIKK family (4,5) due to its homology with CeSMG-1 (5). SMG-1 is involved in NMD of mRNA, and previous studies have demonstrated that abnormal SMG-1 function is involved in human cancer (5,8,17). Other investigations have revealed that SMG-1 is important in human carcinogenesis and cancer progression (12,18,19), and may be a tumor suppressor gene (5,8,9,20,21). These studies demonstrated that SMG-1 is a stress-responsive enzyme and regulates the cell cycle G1/S checkpoint, while silencing of SMG-1 increases tumor cell growth (10,21). By contrast,
downregulation of the expression of SMG-1 is associated with improved prognosis in head and neck cancer (12).

In the present study, high protein expression levels of SMG-1 were found in pancreatic cancer tissues and cell lines. In addition, the protein expression of SMG-1 was associated with an advanced tumor stage, although only limited clinicopathological data was available from the TMA company. These findings were consistent with data from the Human Protein Atlas.

Previous studies have demonstrated that SMG-1 is involved in multiple biological processes, including cell proliferation, apoptosis and stress responses (6,13,20,22,23). The present study utilized an shRNA technique to knock down the expression of SMG-1 to assess the functions of SMG-1 in pancreatic cancer cell lines (24). The SMG-1 shRNA lentivirus #1 significantly reduced the expression of SMG-1 in two pancreatic cancer cell lines (Fig. 2B). SMG-1 knockdown inhibited cell proliferation and increased the chemosensitivity of the cells to gemcitabine and cisplatin treatment in vitro. Previous studies have reported that the loss of SMG-1 function significantly increases the rate and extent of apoptotic tumor cell death induced by chemotherapy, irradiation or cytokine treatment (12,13). Another study demonstrated that SMG-1-depleted human cells exhibit an increased level of spontaneous DNA damage (7).

Since the majority pancreatic cancer patients (~75%) are diagnosed at an advanced stage, chemotherapy is a tentative treatment option (25). SMG-1 shRNA may assist in treating patients with chemotherapy-resistant tumors. A previous study demonstrated that human papillomavirus (HPV) head and neck cancer cells and tissues express SMG-1 at lower levels compared with HPV-negative cancer tissues, and depletion of SMG-1 in HPV-negative head and neck cancer cells increases the sensitivity to radiation and chemotherapy (12).

Another study reported that the sensitivity of lung cancer cells to gemcitabine and cisplatin increased following silencing of the expression of SMG-1 using siRNA (26). The present study demonstrated similar results in pancreatic cancer tissues and cell lines. However, this was only proof-of-principle and further studies are required to fully elucidate the role of SMG-1 protein in the development and progression of pancreatic cancer, by investigating the in vivo effects of SMG-1 knockdown on the chemosensitivity of pancreatic cancer cells.

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