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Original Article

SphK1 is involved in the progression of human pancreatic cancer

Du-Xiong Cai, Hai-Mei Long, Ying-Jie Su

Department of Gastroenterology, The First Affiliated Hospital of Hainan Medical University, Haikou, People's Republic of China

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Abstract: Sphingosine kinase 1 (SphK1) expression is elevated in various cancers and is associated with shorter survival times for patients. However, the effect of SphK1 in pancreatic cancer (PC) remains unclear. In this study, we investigate the effect of SphK1 in human PC tissues and PC cells line, SW1990. The SphK1 expression in PC tissues was measured by qRT-PCR and western blotting. The cultured SW1990 cells were divided into three groups: PMA group, DMS group and control group. Cell proliferation was determined colony formation assay, and cell apoptosis was detected by flow cytometry. RT-PCR and Western blot were used to evaluate the mRNA and protein expression of Sphk1, respectively. The Sphk1 expression levels in PC tissues were significantly increased compared with adjacent normal pancreatic tissues ($P < 0.01$). Sphk1 expression levels in PMA group was significantly increased compared with these in the DMS group and control group ($P < 0.05$), and expression of Sphk1 in DMS group was significantly lower than control group ($P < 0.01$). PMA significantly enhanced cell proliferation and suppressed cell apoptosis, whereas DMS suppressed cell proliferation and enhanced cell apoptosis. Cell colony formation rate and apoptosis rate for the control group, PMA group and DMS group were as follows: colony formation rate: 1.29% ± 0.27%, 2.12% ± 0.18% and 0.75% ± 0.16%; apoptosis rate: 16.7%, 9.7% and 31.7% (all $P < 0.05$ vs the control group). These data suggest that SphK1 has an important role in PC and presents an attractive therapeutic target for the treatment for PC.

Keywords: Sphingosine kinase 1 (SphK1), pancreatic cancer (PC), cell proliferation, apoptosis

Introduction

Pancreatic cancer (PC) is a malignant tumor with a very poor prognosis, and the 5-year survival is 1.2-6% [1]. Although surgical resection of PC is the first-choice treatment, only 10%-15% are eligible for surgery and about 80% of patients have to undergo radiotherapy or chemotherapy [2]. Therefore, there is an urgent need to identify a novel effective therapeutic target.

Sphingosine kinase 1 (SphK1), the key enzyme of sphingosine 1 phosphate (S1P) synthesis, is a bioactive lipid with oncogenic functions that promoting tumor cell growth and invasion [3]. There is mounting evidence from in vitro and in vivo that SphK1 play an important role in oncogenesis, cell survival, proliferation, transformation, and angiogenesis [4]. SphK1 is considered an oncogenic kinase which is overexpressed in many human cancers, including breast cancer, prostate cancer, ovarian cancer, and lung cancer [5]. SphK1 mRNA levels have been found significantly higher in various tumor tissues such as those of breast, colon, lung, and ovary, than in normal tissues [6]. Moreover, the upregulation of SphK1 is associated with poor prognosis in many types of human cancers, such as glioblastoma, breast cancer, and gastric cancer [7]. A remarkable correlation between shorter overall survival times and high SphK1 expression of gastric cancer suggested that SphK1 could be a prognostic marker in many cancers [8].

SphK1 expression has been involved in the resistance of chemotherapeutics, radiation and targeted agents, which suggested that SphK1 may be considered as a novel therapeutic target. Pharmacological or biological inhibitors of SphK1 decrease S1P levels led to

increased chemotherapy and radiation sensitivity in malignant tumor cells, and then induce tumor cell apoptosis [9]. Sphingosine Kinase Inhibitor II (SKI-II) as an inhibitor of SphK1 has anti-proliferative activity in various cancer cell lines [10]. BML-258, a water-soluble sphingosine analogue, is a SphK1-selective inhibitor that is effective for tumor cell both in vitro and in vivo [11]. Moreover, FTY720 is a novel competitive SphK1 inhibitor which is an effective orally bioavailable anti-cancer agent, and inhibits tumor cells proliferation and migration [12]. These results indicated that SphK1 is a novel therapeutic target in many human cancers, and inhibitors of SphK1 in combination with chemotherapeutic or radiation treatments can synergistically act to induce tumor cell apoptosis.

In the present study, we aimed to investigate the clinical implications and prognostic significance of SphK1 expression and to investigate the *in vitro* and *in vivo* effects of targeting SphK1 with pharmacological inhibitors in PC.

Materials and methods

Clinical tissue samples and pancreatic cancer cell lines

A total of 20 freshly-frozen primary PC and matched adjacent non-tumor tissues were collected from patients who underwent pancreatic surgical resection at the First Affiliated Hospital of Hainan Medical University between January 2015 and December 2015. All the PC tissue specimens were identified as pancreatic ductal adenocarcinoma, and the pathological information was retrieved from the Pathology Department. All cases are not receiving any previous cancer-associated treatment, and had a history of any other types of cancer before surgery. All patients underwent pretreatment evaluation, including chest X-ray, magnetic resonance imaging (MRI) and pelvis to evaluate the tumor stage. All the samples were stored at -80°C following surgery until RNA extraction. The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Hainan Medical University, and informed consent was signed from all patients prior to tissue collection. Human pancreatic cancer cell lines SW1990 were purchased from Chinese Academy of Sciences (Shanghai, China) and were cultured in DMEM (Gibco, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Wisent Biotec, Co. Ltd) in a humidified 5% CO_2 atmosphere at 37°C.

Cell culture

The human pancreatic cancer cells, SW1990, were cultured in DMEM medium (Gibco, Carlsbad, CA) supplemented with streptomycin (100 μg/ml) and penicillin (100 U/ml), and 10% FBS. The cells were grown at 37°C with 5% CO_2. After 12 h, cells were stimulated by the addition of 100 nM PMA (phorbol 12-myristate 13-acetate) or 50 μM DMS (N, N-dimethylsphingosine) for 24 hours.

Colony formation assay

The cells were seeded in new 6-well plates in triplicate and cultured in medium with 100 nM PMA or 50 DMS μM, grown for one week, fixed with 10% methanol for 15 min and stained with 0.5% crystal violet for 20 min. The dishes were washed and dried, and the number of colonies with more than 50 cells was counted to obtain a cloning efficiency for 100 nM PMA or 50 DMS μM.

Analysis of cell apoptosis

Flow cytometric analysis was performed by staining the cells with Annexin V-FITC and propidium iodide (PI). Cells (2×10^5/well) were plated in 6-well plates and incubated overnight. 100 nM PMA or 50 DMS μM was added and the control group was supplemented with equal volumes of PBS. After cultured 24 h, the control or treated cells were resuspended in Annexin V-binding buffer, stained with fluorescein-conjugated Annexin V and PI (Annexin V-FITC kit; Becton Dickinson, Franklin Lakes, NJ, USA) and incubated at room temperature for 15 min. The cells were analyzed by BD FACSCanto II flow cytometer (BD Biosciences). The results were obtained from at least three independent experiments with triplicated wells each time.

Quantitative RT-PCR (qRT-PCR)

Total RNA of cells and tissue samples were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The extracted RNA was reverse transcribed into cDNA using PrimeScript™ RT Master Mix (TaKaRa, Kyoto, Japan) according to the manufacturer's instructions. The PCR components were set up as follows: 1 μl cDNA product, 12.5 μl master mix, 10 pmol/μl forward primer, 10 pmol/μl reverse primer and DEPC added to a final volume of 25 μl. PCR was per-

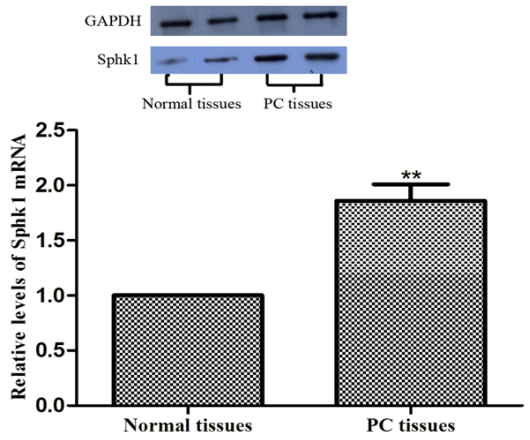

Figure 1. SphK1 expression in PC and normal tissues by western blot and RT-PCR analysis. The results demonstrated that the expression levels of SphK1 were significantly increased in human PC tissues compared with the adjacent normal pancreatic tissues ($P < 0.01$).

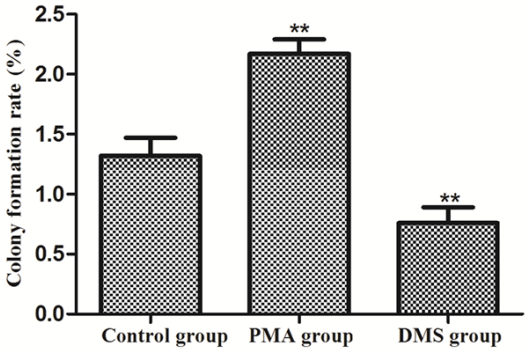

Figure 2. The effect of DMS and PMA on human pancreatic cancer SW1990 cell colony formation. Compared with control group, treatment with DMS suppressed colony formation, but PMA treatment promoted colony formation. There were significant differences among these groups ($P < 0.05$).

formed with 40 cycles of denaturation: 5 min at 94°C, 30 sec at 90°C, 40 sec at 52°C, and extension 30 sec at 75°C. The primers were designed using primer 5 software. Data analysis was conducted according to the $2^{-\Delta\Delta Ct}$ methods using the threshold cycle (Ct) values for target genes and GAPDH, as an endogenous gene. All samples were performed in triplicate.

Western blot analysis

Cells and tissue samples were washed with cold PBS, supplemented with 100 μl/well cell lysis buffer (Beyotime Institute of Biotechnology) and placed on ice for 15 min. Samples were formed by centrifugation at 14,000× g for 10 min and protein concentration were determined by the BCA method. The proteins were separated by 12% SDS-PAGE and then transferred onto PVDF membranes (Millipore, Bedford, MA, USA). The blots were blocked with 5% non-fat milk and then probed with primary antibodies (1:1,000 dilution) against the SphK1 and β-actin protein at 4°C overnight. After washing, the membranes were incubated with secondary antibody (1:10,000 dilution) at room temperature. Antibodies were diluted in TBS containing 0.05% (v/v) Tween-20 and 5% BSA. Proteins were analyzed using the near infrared laser imaging system.

Statistical analysis

Data are presented as the mean ± standard deviation. The differences were analyzed using the Student's t-test. All the analyses were performed using SPSS software, version 17.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

SphK1 expression in PC and normal tissues

SphK1 expression levels in human PC tissues and adjacent normal pancreatic tissues were examined by Western blot and RT-qPCR (**Figure 1**). The expression levels of SphK1 were significantly increased in human PC tissues compared with the adjacent normal pancreatic tissues ($P < 0.01$). The results indicated that the up-regulation of SphK1 may be involved in human pancreatic carcinogenesis.

Effect of DMS and PMA on cell colony formation

To determine the effects of DMS and PMA on the ability of single cell proliferation, colony formation assay was performed. Colony formation efficiency was calculated with the number of visible colonies divided by the number of plated cells. Compared with control group, treatment with DMS suppressed colony formation, but PMA treatment promoted colony formation (**Figure 2**). There were significant differences among these groups ($P < 0.05$).

Effect of DMS and PMA on cell apoptosis in human pancreatic cancer SW1990 cell

The SW1990 cells were treated with 100 nM PMA or 50 DMS μM for 24 h, stained with

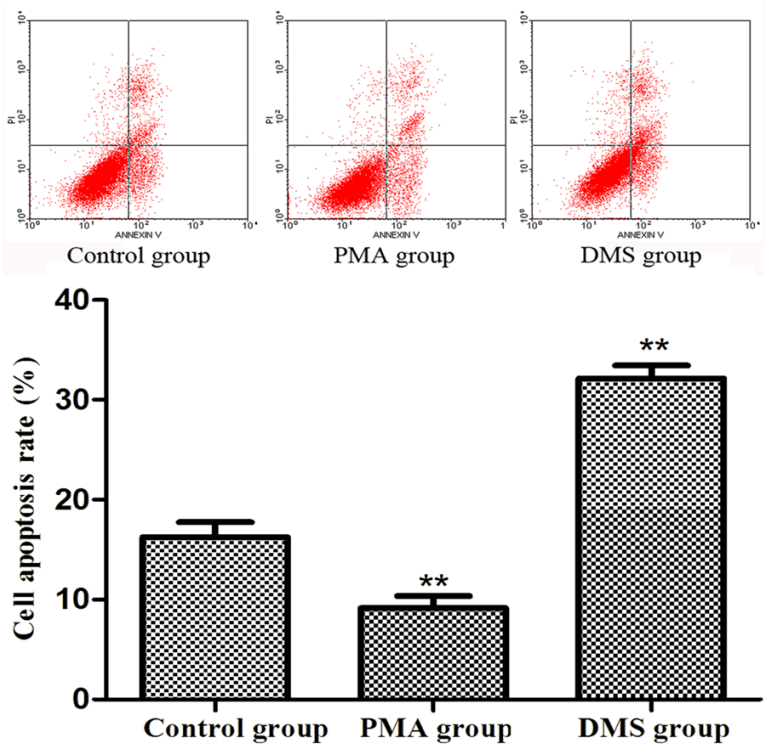

Figure 3. Effect of DMS and PMA on cell apoptosis in human pancreatic cancer SW1990 cell. The percentage of apoptotic cells in DMS group was increased compared with the control control and PMA group ($P < 0.01$), and there was a significant reduction in the percentages of apoptotic cells in the PMA group compared with control control ($P < 0.05$).

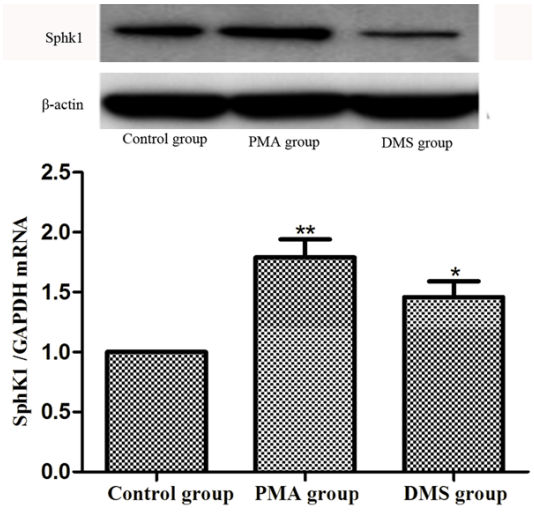

Figure 4. Effect of PMA and DMS on SphK1 gene in human pancreatic cancer SW1990 cell. The results revealed that the mRNA and protein levels of SPHK1 were markedly downregulated following treatment with DMS compared with PMA and control group ($P < 0.05$). In addition, the mRNA and protein levels of SphK1 in PMA group were higher than that control group ($P < 0.01$).

Annexin V-FITC and PI, and analyzed by flow cytometry (**Figure 3**). FITC single-positive cells represent early apoptotic cells, FITC/PI double-positive cells represent apoptotic cells and PI single-positive cells represent dead cells. The percentages of apoptotic cells increased with the DMS treatment, but the percentages of apoptotic cells decreased with the PMA treatment. The percentage of apoptotic cells in DMS group was increased compared with the control and PMA group ($P < 0.01$), and there was a significant reduction in the percentages of apoptotic cells in the PMA group compared with control ($P < 0.05$).

Effect of PMA and DMS on SphK1 gene in human pancreatic cancer SW1990 cell

To investigate the effects of DMS and PMA on SphK1 gene expression, we analyzed the mRNA and protein levels of SphK1 in SW1990 cells by RT-PCR and Western blot (**Figure 4**). The results revealed that the mRNA and protein levels of SPHK1 were markedly downregulated following treatment with DMS compared with PMA and control group ($P < 0.05$). In addition, the mRNA and protein levels of SphK1 in PMA group were higher than that control group ($P < 0.01$).

Discussion

In this study, we determined the roles of SphK1 in PC pathogenesis and the underlying mechanisms. The SphK1 expression was overexpressed at the PC tissues, as we observed a significant increased SphK1 mRNA and protein levels in the PC group compared with the normal pancreatic tissues, which suggesting a novel biomarker that contributes to PC pathogenesis and aggressive biology. We also demonstrated that the changes of SphK1 activity with specific SphK1 inhibitor or with specific SphK1 agonist might represent a novel strategy for the treatment of PC.

SphK1 as an oncogene in tumorigenesis is over-expression in various types of cancers, including breast cancer, ovarian cancer, prostate cancer, lung cancer, and stomach cancer. It is reported that high expression of SphK1 is related to poor prognosis in patients with certain cancers, such as glioblastoma, breast cancer, and gastric cancer [12, 13]. However, little is known about the role of SphK1 in the development of PC. We found that SphK1 is upregulated in PC tissues, and the expression of SphK1 in PC tissues was significantly higher than that in normal pancreatic tissues. The data showed that high expression of SphK1 might be involved in the development of PC.

Numerous studies have shown that SphK1 is found to enhance the proliferation ability of cancer cells. High expression of SphK1 inhibits the apoptosis of tumor cells has been extensively demonstrated [14]. Gucluler et al. showed that overexpression of SphK1 enhances tumor formation of human breast cancer MCF-7 cells in nude mice [15]. Over-expression of SphK1 contributes to cellular resistance to chemotherapy drugs was also observed [16]. In contrast, inhibition of SphK1 could attenuate lung cancer cell growth in vitro as well as in vivo [17, 18]. We further demonstrated that blocking SphK1 with pharmacological inhibitors significantly impaired PC cells survival and inhibited their proliferation. DMS is an inhibitor of SphK1, and the ability of DMS to prevent the development of cancer has been widely investigated in preclinical models. DMS could inhibit the growth and migration of various tumor cells [19]. In addition, DMS could act as a SphK1 inhibitor to exhibit the antiproliferative effect in various cancers in a manner independent of S1P receptors. In the present study, we demonstrated for the first time that inhibition of SphK1 by DMS markedly suppressed the ability of cell growth of SW1990 cells and an increase in tumor cells apoptosis in PC compared with control group, and it also has capacity to inhibit the expression of SphK1 in SW1990 cells. These results provide new insights into the alterations of SphK1 expression and activity that are associated with the development and progression of PC. We suggest that therapeutic targeting of SphK1 is a potential therapeutic strategy for the treatment of PC.

There is mounting evidence suggesting that activation of SphK1 is critical for it to produce S1P that mediates its biological effects [20]. Different external stimuli, particularly growth factors and chemoattractants, such as phorbol 12-myristate 13-acetate (PMA) involves extracellular signal-regulated kinase (ERK) 1/2-mediated phosphorylation of SphK1 on Ser225, cause a rapid activation of SphK1 [21]. This phosphorylation site can control the catalytic activity of SphK1 and induce translocation of SphK1. SphK1 is a cytosolic enzyme, which is translocation to the plasma membrane [22], where its substrate sphingosine resides allows precise temporal and localized formation of S1P that subsequently activates appropriate cell surface S1P receptors [23]. PMA increase expression of SphK1 by transcriptional upregulation, chronically increasing production of S1P perhaps to increase cells capability to grow and move. Phosphorylation of SphK1 has been shown to induce conformational changes that enhance its binding to membrane acidic phospholipids such as phosphatidylserine which can prolong its retention at the plasma membrane [24]. This phosphorylation-dependent localization of SphK1 to the plasma membrane appears essential for cell proliferation and survival [25]. These results suggested that PMA can activate SphK1 that increase cells proliferation and migration. In the current study, the PMA treated-SW1990 cells, the SphK1 levels were markedly higher than those in the control group and DMS group, and the apoptosis rate of PMA group was lower than that in the DMS group and control group.

Precious researches have clearly demonstrated that SphK1 acts as an oncogene and overexpression of SphK1 can induce tumor growth and inhibit tumor cells apoptosis, resulting in drug resistance in multiple cancers [26, 27]. Based on the results of Annexin FITC and PI staining analysis, we confirmed that DMS significantly induced apoptosis in SW1990 cells, but PMA significantly decreased the apoptosis of SW1990 cells. All of the results suggested that SphK1 may regulate PC cell proliferation and apoptosis.

In conclusion, we demonstrated that overexpression of SphK1 is significantly associated with the development of PC. Expression of SphK1 represents a novel biomarker for the prognosis of patients with PC. Our findings also indicate that inhibition of SphK1 with pharma-

cological inhibitors results in potent antitumor activity in PC in vitro.

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Disclosure of conflict of interest

None.

Address correspondence to: Du-Xiong Cai, Department of Gastroenterology, The First Affiliated Hospital of Hainan Medical University, 31# Longhua Road, Haikou 570102, People's Republic of China. Tel: +86-98-66528102; E-mail: xie_reproduction@126.com

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