Article

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Somatostatin Inhibited the EMT of Pancreatic Cancer Cells by Mediating the TGF-β/Smad Signaling Pathway

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Background: Pancreatic cancer (PC), a commonly recognized malignancy, arises within the digestive tract. Somatostatin (SOM) is a regulatory peptide that acts on secretion *in vivo*. Several studies have shown that SOM has inhibitory effects on various cancers. This work aims to probe the inhibitory effect, and mechanism of SOM action, on the epithelial–mesenchymal transition (EMT) of PC cells.

Methods: First, the effects of SOM and transforming growth factor-β (TGF-β) on the proliferation of PC cells was determined by Cell Counting Kit-8 (CCK-8) assay. Next, we assessed the impact of SOM and TGF-β on the metastasis and apoptosis of PC cells using transwell assays and flow cytometry. Finally, we evaluated the effects of SOM and TGF-β on the expression of EMT-related proteins, apoptosis-related proteins, and proteins related to the TGF-β/Smad signaling pathway in PC cells using western blot analysis.

Results: SOM suppressed the growth and metastasis of PC cells, and facilitated their apoptosis ($p < 0.05$). Moreover, SOM reversed pro-apoptotic effects of TGF-β ($p < 0.05$). Specifically, SOM increased the expression of Cysteine-aspartic acid protease 3 (Caspase-3) and Bcl-2-associated X protein (Bax) proteins while reducing the expression of B-cell lymphoma 2 (Bcl-2) protein ($p < 0.05$). SOM also reversed the TGF-β-induced EMT process. The TGF-β1, Smad2, and Smad3 proteins in PC cells treated with SOM were significantly down-regulated ($p < 0.05$).

Conclusions: SOM suppressed the EMT progression in PC cells through its regulation of the TGF-β/Smad signaling pathway.

Keywords: pancreatic cancer; somatostatin; TGF-β/Smad; EMT

Introduction

Pancreatic cancer (PC), an aggressive malignancy originating predominantly from the ductal epithelium of the pancreas within the digestive system, stands as one of the most lethal forms of cancer worldwide [1,2]. The early clinical symptoms of PC are not obvious, and about 80% of patients lose the opportunity for surgery when they are diagnosed. Only a small number of patients can undergo radical resection, and the prognosis of patients is poor [3]. Chemotherapy, radiotherapy, or other comprehensive treatments have limited effects on patients with advanced PC. Therefore, there is an urgent need to understand the potential molecular mechanisms of the occurrence and progression of PC, find new targets for tumor treatment, and offer more technical assistance for the treatment of PC.

Somatostatin (SOM), also known as a growth hormone release inhibitor, is cleaved from a 116 amino acid peptide in two active forms, one with 14 amino acids and the other with 28 amino acids [4]. SOM is widely distributed and has anti-tumor effects. The physiological function of somatostatin is mediated by five G protein-coupled receptors which have similar structural features and signal transduction mechanisms. However, the somatostatin receptor expression subtypes vary among different tumors. At present, several somatostatin analogs, such as lanreotide, octreotide, and pasireotide, have been developed and commonly used in clinical practice. Pancreatic cancer is a hormone-sensitive tumor, and it has been reported that SOM has an inhibitory effect on PC [5]. However, the mechanisms of SOM action in PC have not been elucidated.

Epithelial–mesenchymal transition (EMT) is a vital pathway for tumor cell invasion, metastasis, anti-apoptosis, and other invasive behaviors [6,7]. One of the most typical features of EMT is the loss of E-cadherin (E-ca) on the cell surface accompanied by the increased expression of N-cadherin (N-ca). Unilateral loss of E-cadherin reduces the adhesion of tumor cells, which is conducive to the metastasis of tumor cells from the primary lesion. The acquisition of high N-cadherin expression can promote and enhance the distant metastasis of tumor cells. Through these mechanisms, the EMT process enhances the capacity for tumor metastasis.

Transforming growth factor-β (TGF-β), in premalignant tumors, functions to regulate cell growth and differentiation. However, with the further development and deterioration of tumors, many tumor cells will produce TGF-β through autocrine and paracrine pathways, which makes tumor cells resistant to the effect of TGF-β. The higher level of TGF-β in tumor tissue will then promote EMT of tumor cells and enhance the ability of the tumor to infiltrate and metastasize to distant sites [8].

TGF-β/Smad is the classical EMT signaling pathway [9]. TGF-β mainly promotes EMT by activating the classical Smad pathway to enhance signal transduction.

In this study, the Capan-1 cell line was used to probe the function of SOM on PC cell viability, migration, invasion, and apoptosis, and to investigate the potential mechanism of action of SOM on TGF-β-triggered EMT.

Materials and Methods

Cell Culture

We purchased Human PC cell lines (Capan-1) (iCell-h038) and culture medium from iCell Bioscience Inc., (Saibaikang Biotechnology Co., Ltd., Shanghai, China). The melted cells were added to 5 mL of complete medium containing RPMI-1640 (iCell-128-0002), 10% fetal bovine serum (FBS; iCell-0500) and P/S double antibiotic (iCell-15140-122) (iCell Bioscience Inc., Shanghai, China), centrifuged for 5 min (1000 rpm/min), the supernatant was discarded, and after adding complete medium, the cells were cultured in a specific culture environment (37 °C, 5% CO_2). All cell lines used in this study had completed mycoplasma detection and STR identification.

Cell Counting Kit-8 (CCK-8) Assay

Capan-1 cell suspension (2×10^5 cells /mL) was prepared and placed in a 96-well plate. The culture plate was placed in an incubator for 24 hours, different concentrations of SOM were added for drug intervention, and the culture was continued for 48 hours. Cell Counting Kit-8 (CCK-8) solution (CA1210, Solarbio, Beijing, China) was added to each well and remained in the incubator for co-incubation for 2 h. The absorbance (A) at 450 nm was measured using a microplate reader (muLISKANMK3, Thermo Fisher Scientific, Waltham, MA, USA). Cell viability = [(A drug treatment group – A control group)/(A control group – A blank group)] $\times$ 100%.

Transwell Assay

Cell migration assay: First, Capan-1 cells were digested with trypsin (T1300, Solarbio, Beijing, China) and adjusted to 2.5×10^5 cells/mL. Then, 500 µL of medium containing 10% FBS was added to the lower transwell chamber (3422, Corning, New York, NY, USA). Next, 300 µL Capan-1 cells and serum-free medium were added to the upper chamber and incubated at 37 ℃ for 24 h. The above samples were rinsed with PBS, fixed with 4% paraformaldehyde (P0099, Beyotime, Shanghai, China) and rinsed with PBS. 0.1% crystal violet staining solution (G1064, Solarbio, Beijing, China) was added for 10 min, and the sample was rinsed with water. Finally, images were collected under a fluorescence microscope (CKX53, Olympus, Tokyo, Japan).

Cell invasion assay: Matrigel (356231, BDMatrigel, New York, NY, USA) was first melted and diluted to 1 mg/mL with serum-free medium. 100 µL Matrigel was added to the upper chamber and allowed to dry naturally. The steps of the cell migration assay were then repeated. The number of cells was determined by direct counting.

Flow Cytometry

First, Capan-1 cells ((1–10) $\times$ 10^5 cells) were collected and washed twice by centrifugation with PBS. Then, Capan-1 cells were resuspended in 500 µL of 1$\times$ Binding Buffer, and 5 µL of Annexin V-FITC and 10 µL of PI (AP101, Hangzhou Lianke Biotechnology Co., Ltd., Hangzhou, China) were added to each tube. Next, the mixture was mixed and incubated in the dark for 5 min. Subsequent analysis was performed by flow cytometry (BECKMAN, Beckman Coulter Co., Ltd., Miami, FL, USA).

Western Blot

First, Capan-1 cells were collected, lysate was added, cells were lysed for 45 min, and centrifuged for 15 min (12,000 rpm/min). Protein concentration was determined using a BCA protein concentration kit (P0012, Beyotime, Shanghai, China). Subsequently, protein separation was performed by gel electrophoresis. The target protein was transferred to the PVDF membrane (IPVH00010, Millipore, Billerica, MA, USA) in the transfer cell. 5% TBST was added to the PVDF membrane. Then β-actin (1:1000 dilution; cat no. AC004, ABclonal, Inc., Guangzhou, China), Cysteine-aspartic acid protease 3 (Caspase-3) (1:1000 dilution; cat no. A2156, ABclonal, Inc., Guangzhou, China), Bcl-2-associated X protein (Bax) (1:1000 dilution; cat no. A12009, ABclonal, Inc., Guangzhou, China), B-cell lymphoma 2 (Bcl-2) (1:1000 dilution; cat no. A0208, ABclonal, Inc., Guangzhou, China), E-cadherin (1:1000 dilution; cat no. A20798, ABclonal, Inc., Guangzhou, China), N-cadherin (1:1000 dilution; cat no. A19083, ABclonal, Inc., Guangzhou, China), Vimentin (1:1000 dilution; cat no. A19607, ABclonal, Inc., Guangzhou, China), TGF-β (1:1000 dilution; cat no. A2124, ABclonal, Inc., Guangzhou, China), Smad2 (1:1000 dilution; cat no. A19114, ABclonal, Inc., Guangzhou, China), and Smad3 (1:1000 dilution; cat no. A19115, ABclonal, Inc., Guangzhou, China) antibodies were added and incubated at 4 °C for 12 h. Horseradish peroxidase (1:1000 dilution; cat no. ZB-2305, ZB-2301, Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China)-labeled secondary antibodies were diluted with

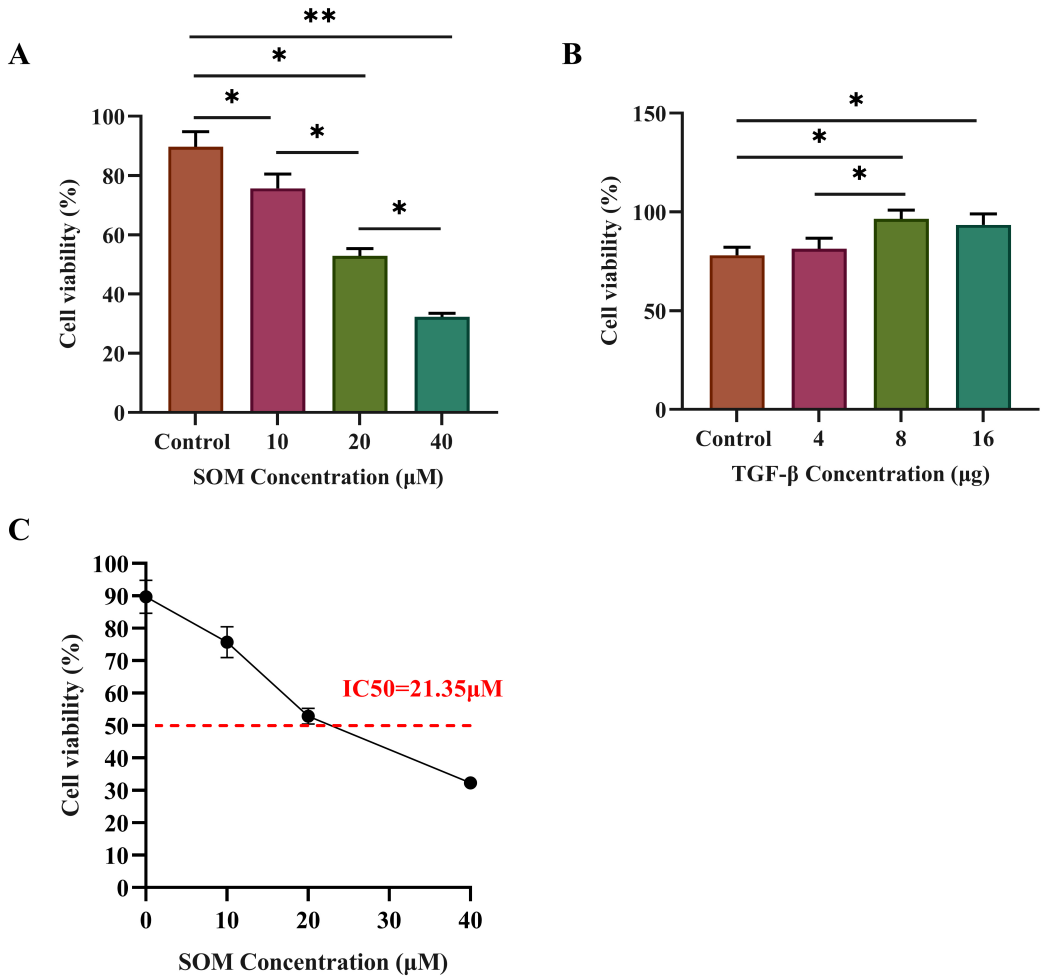

Fig. 1. Determination of Pancreatic cancer (PC) cell viability by Cell Counting Kit-8 (CCK-8) assay. (A) Somatostatin (SOM) inhibited the viability of PC cells (n = 6). (B,C) TGF-β facilitated the viability of PC cells (n = 6), *$p < 0.05$, **$p < 0.01$. TGF-β, transforming growth factor-β.

blocking solution and added to PVDF membranes. Next, the above samples were exposed through a chemiluminescence instrument (5260Muti, Shanghai Tianneng Life Science Co., Ltd., Shanghai, China). Finally, the gray value of protein bands was analyzed by Image J software (version 1.0, National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

We utilized GraphPad Prime software version 8.0 (GraphPad Software, Inc., San Diego, CA, USA, https://www.graphpad-prism.cn/) for statistical analysis. Mean differences between two groups were analyzed using a *t*-test, while comparisons among multiple groups were conducted using one-way analysis of variance (ANOVA), with post-hoc tests performed using Tukey's method. The data are presented as mean ± standard deviation (SD), and statistical significance was determined at a significance level of $p < 0.05$.

Results

Influence of SOM and TGF-β on PC Cell Viability in Vitro

The optimal time of action of SOM and TGF-β on PC cells was 72 h through pre-experiments. Hence, the action time for the cell viability assay was 72 h. In Fig. 1A, SOM exhibited a dose-dependent suppression of Capan-1 cell viability ($p < 0.05$). The 50% inhibitory concentration (IC50) of SOM on PC cells was determined to be 21.35 μM (Fig. 1C). In contrast, TGF-β facilitated the viability of PC cells (Fig. 1B). We observed that the viability of PC cells that were treated by TGF-β (8 μg and 16 μg) was higher than that in the group without any intervention ($p < 0.05$). Nevertheless, there was no obvious difference in the viability of PC cells between the 8-μg group and the 16-μg group. Therefore, we chose a dose of 20 μM for SOM and 8 μg for TGF-β for next study.

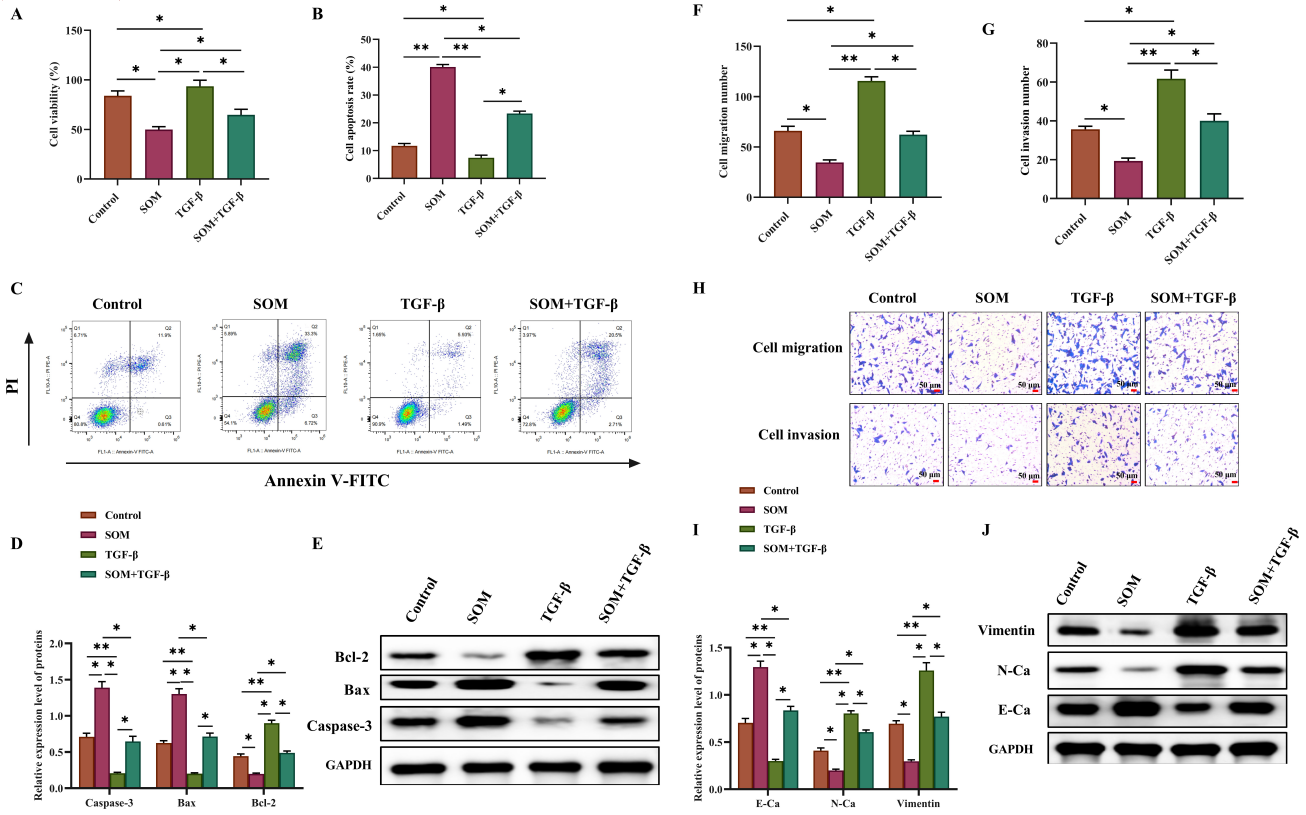

Fig. 2. The impact of SOM/TGF-β on the viability, metastasis, and apoptosis of PC cells. (A) Effect of SOM/TGF-β on PC cell viability (n = 6). (B,C) Effects of SOM/TGF-β on PC cell apoptosis. (D,E) Effects of SOM/TGF-β on apoptosis-related proteins in PC cells (n = 6). (F–H) Effects of SOM/TGF-β on PC cell metastasis (n = 3). (H) Scale bar: 50 µm. (I,J) Effects of SOM/TGF-β on EMT-related proteins in PC cells (n = 6). *$p < 0.05$, **$p < 0.01$. EMT, epithelial–mesenchymal transition; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; E-Ca, E-cadherin; N-Ca, N-cadherin; Caspase-3, Cysteine-aspartic acid protease 3.

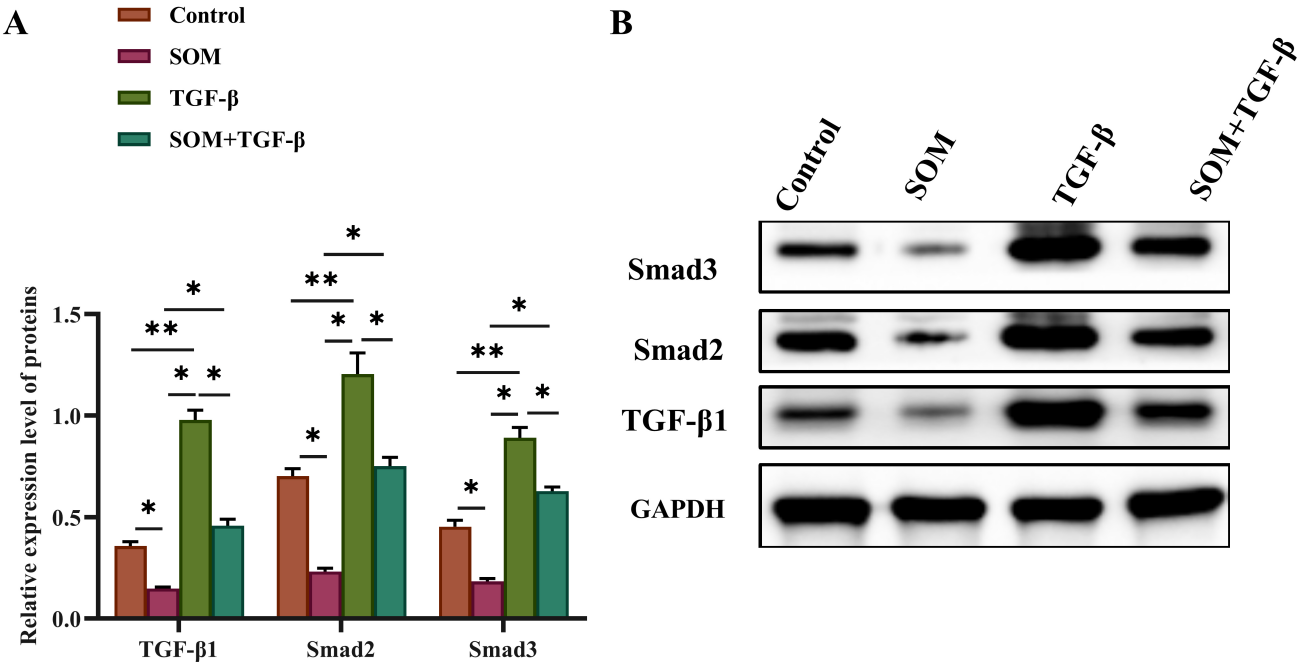

Fig. 3. Effect of SOM on TGF-β1, Smad2, and Smad3 proteins. (A,B) The effects of SOM on the levels of TGF-β1, Smad2, and Smad3 (n = 6), *$p < 0.05$, **$p < 0.01$.

Effect of SOM/TGF-β on Viability, Apoptosis, and Metastasis of PC Cells

Next, we further probed the function of SOM/TGF-β on PC cell viability and apoptosis. As shown in Fig. 2A–C, TGF-β exhibited significant pro-proliferation and anti-apoptosis effects on PC cells, while the addition of SOM reversed the proliferative effect of TGF-β on PC ($p < 0.05$). Subsequently, we assessed the influence of SOM/TGF-β on proteins related to apoptosis through western blot analysis (Fig. 2D,E). TGF-β promoted the expression of the Bcl-2 protein and inhibited the expression of Caspase-3 and Bax proteins ($p < 0.05$). SOM treatment resulted in a substantial increase in the protein levels of Caspase-3 and Bax, while simultaneously suppressing the expression of Bcl-2 ($p < 0.05$). In the SOM+TGF-β group, the addition of SOM reversed the TGF-β-mediated increase in Bcl-2 expression and the suppressive effect of TGF-β on Caspase-3 and Bax expression ($p < 0.05$). Results presented in Fig. 2F–H indicated that SOM had an anti-metastasis effect on PC cells ($p < 0.05$). TGF-β induced the migration and proliferation of PC cells, and the addition of SOM reversed the TGF-β-triggered metastasis ($p < 0.05$, $p < 0.01$). We also analyzed the effect of SOM/TGF-β on the EMT process of PC cells. As seen in Fig. 2I,J, TGF-β had a significant inhibitory effect on E-cadherin expression and simultaneously promoted the expression of N-cadherin and vimentin ($p < 0.05$, $p < 0.01$). Nevertheless, when SOM was introduced, it countered the TGF-β-induced EMT. In the SOM+TGF-β group, there was a marked elevation in the expression of E-cadherin, accompanied by a substantial reduction in N-cadherin and vimentin levels as compared to the group that received TGF-β alone ($p < 0.05$).

SOM Effectively Dampened the TGF-β1/Smad Signaling Pathway Activation

Next, we investigated the influence of SOM on TGF-β/Smad signaling (Fig. 3A,B). Compared with other groups, the application of TGF-β resulted in a substantial increase in the levels of TGF-β1, Smad2, and Smad3 proteins within the PC cells ($p < 0.05$, $p < 0.01$). Conversely, SOM suppressed the levels of TGF-β1, Smad2, and Smad3 proteins ($p < 0.05$).

Discussion

Somatostatin is a synthetic peptide hormone with a half-life period of less than 3 minutes. It has been proven to have a good therapeutic effect in the treatment of digestive system diseases such as acute pancreatitis and esophageal variceal bleeding [10,11]. Recently, SOM has been shown to have inhibitory effects in various cancers, including prostate cancer [12], medullary thyroid cancer [13], gastric cancer [14], and PC [15]. However, there is a lack of conclusive evidence for the anticancer effect of SOM in PC. The present study focused on the anticancer effect of SOM on PC cells and revealed for the first time the relevant mechanism of SOM on PC anti-proliferation and anti-metastasis. SOM inhibited the viability of the Capan-1 cell line in a manner dependent on the dosage. The IC50 of SOM for PC cells was determined to be 21.35 μM.

The progression of apoptosis is highly regulated by different and various genes. As the key mediator of programmed cell death, caspase is responsible for both the initiation and execution of apoptosis [16,17]. Therefore, the detection of cleaved caspase-3 can be used to indicate whether cells are undergoing apoptosis and the severity of the apoptosis process [18].

The apoptotic pathway is intricately controlled by members of the Bcl-2 family of proteins [19]. The Bcl-2 family of proteins can be principally categorized into two groups: those that promote apoptosis, known as pro-apoptotic proteins (e.g., Bax), and those that inhibit apoptosis, referred to as anti-apoptotic proteins (e.g., Bcl-2) [20]. In our current investigation, we initially demonstrated through flow cytometry that the apoptosis rate of PC cells increased following SOM treatment. SOM inhibited the expression of Bcl-2 and enhanced the expression of Bax in PC cells. Furthermore, SOM also triggered the activation of Caspase-3 in PC cells.

Cancer cells lose their intercellular junctions and cell polarity capacity through the EMT process, detangle from the extracellular matrix, and acquire enhanced motility and an enhanced invasive phenotype [21]. The EMT process, also known as the cadherin transition process, is marked by the loss of E-cadherin and the gain of mesenchymal N-cadherin and vimentin [22]. The inhibitory effects of SOM on the metastasis of Capan-1 cells were confirmed by the transwell assay. SOM led to a decrease in the expression levels of N-cadherin and vimentin while simultaneously increasing the expression level of E-cadherin.

Prior studies have established that the TGF-β/Smad signaling pathway plays a pivotal role in facilitating the EMT of tumor cells [23]. TGF-β1, an inflammatory cytokine, is a promoter and supporter of EMT and can activate TGF-β1-induced EMT through Smad-mediated transcriptional regulation [24]. It has been found that inhibition of TGF-β1/Smad2 signaling can reverse the EMT phenotype and metastasis of tumor cells [25,26]. In our study, SOM significantly inhibited TGF-β1-induced metastasis and anti-apoptosis abilities of PC cells. Furthermore, in response to TGF-β1 stimulation, the expression of mesenchymal markers N-cadherin and vimentin increased, and the levels of the anti-apoptotic protein Bcl-2 also increased. However, SOM inhibited these changes. Finally, we confirmed that SOM treatment led to a substantial reduction in the protein concentrations of TGF-β1, Smad2, and Smad3.

These findings suggested that SOM may prevent the development of EMT by regulating TGF-β/Smad signaling in pancreatic cancer.

Conclusions

SOM restrained the viability and metastatic behavior induced by TGF-β in human PC cells, and triggered apoptosis in these cells. Additionally, SOM inhibited EMT in PC cells by modulating the TGF-β/Smad signaling pathway.

Availability of Data and Materials

Data to support the findings of this study are available on reasonable request from the corresponding author.

Author Contributions

XZ, WTW and LL designed the research study. XZ and LL performed the research. WTW and LL provided help and advice on the experiments. XZ and WTW analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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