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Expression of TMEFF2 in Human Pancreatic Cancer Tissue and the Effects of TMEFF2 Knockdown on Cell, Proliferation, and Apoptosis in Human Pancreatic Cell Lines

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The TMEFF2 gene encodes the transmembrane protein with EGF like and two follistatin-like domains 2 and has been reported to be a tumor suppressor gene, but its role remains unknown in pancreatic cancer. This study aimed to investigate the expression of TMEFF2 in human pancreatic cancer tissue and the effects of knockdown of TMEFF2 on cell, proliferation, and apoptosis in human pancreatic cell lines.

Material/Methods: Thirty-five samples of human pancreatic tissue and adjacent normal pancreatic tissue, and five human pancreatic cancer cell lines, CAPAN1, ASPC1, BXPC3, SW1990, and CFPAC were studied. RNA expression, protein expression, cell proliferation, and apoptosis were studied using real-time polymerase chain reaction (RT-PCR), Western blot, the cell counting kit-8 (CCK-8) assay, and flow cytometry, respectively. A co-immunoprecipitation assay evaluated protein interactions.

Results: TMEFF2 expression was down-regulated in pancreatic cancer tissue compared with normal pancreas. In human pancreatic cancer cell lines, overexpression of TMEFF2 suppressed cell proliferation and enhanced apoptosis, suppressed the expression of p-STAT3, MCL1, VEGF and increased the expression of the tyrosine-specific protein phosphatase, SHP-1. The co-immunoprecipitation assay showed that TMEFF2 interacted with SHP-1. Knockdown of expression of TMEFF2 resulted in the increased expression of p-STAT3, MCL1, and VEGF, increased cell proliferation and decreased cell apoptosis, which were reversed by overexpression of SHP-1.

Conclusions: In pancreatic cancer, TMEFF2 exerted as a tumor suppressor effect by regulating p-STAT3, MCL1, and VEGF via SHP-1.

MeSH Keywords: Myeloid Cell Leukemia Sequence 1 Protein • Pancreatic Neoplasms • Protein Tyrosine Phosphatase, Non-Receptor Type 6 • Vascular Endothelial Growth Factor A

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Background

Worldwide, pancreatic cancer has a high rate of morbidity and mortality as this type of cancer is difficult to diagnose in the early stage [1]. Although recent progress has been made in the treatment of pancreatic cancer, chemotherapy is still a first-line therapy, which is associated with side effects due to generalized cytotoxicity [2]. Therefore, more effective and less toxic treatments are required for patients with pancreatic cancer.

Transmembrane protein with EGF and two follistatin motifs 2 (TMEFF2) has been identified as a transmembrane protein found in the brain and prostate, but the role of the TMEFF2 gene in human pancreas and pancreatic cancer remains unclear. Recently published studies have shown that TMEFF2 acts as a tumor suppressor gene in several types of human cancer [3–6]. However, other studies have not supported this finding [7]. These conflicting data suggest a complex role for TMEFF2 in human cancer. Also, one member of the protein tyrosine phosphatase (PTP) family, the Src homology phosphatase-1 (SHP-1), plays a key role in T-cell signaling by regulating the phosphorylation with its catalytic protein tyrosine phosphatase (PTP) domain and two Src homology-2 (SH2) domains, and its reduced expression results in abnormal cell growth, indicating that SHP-1 acts as a cancer suppressor gene in humans [8–10]. In gastric cancer, TMEFF2 interacts with SHP-1 to suppress the tumor progression and its abnormal methylation has been shown to be associated with tumorigenesis [4,11]. The tumor-associated protein, signal transducer and activator of transcription 3 (STAT3), has also been reported to be a negative regulatory factor of TMEFF2 that could also influence the activation of STAT3 in gastric cancer, and which may be associated with SHP-1 expression [12]. However, in pancreatic cancer, the roles of SHP-1 and STAT3 and their interaction with TMEFF2 remain unclear.

As a member of the Bcl-2 family, myeloid cell leukemia-1 (Mcl-1), encoded by the MCL1 gene, has been shown to have anti-apoptotic activity in several human cancers, including pancreatic cancer [13–15]. The expression of vascular endothelial growth factor (VEGF) results in the development of blood vessels that supply solid tumors to facilitate their progression [16]. Therefore, the expression of VEGF is an important factor required for the development of solid tumors and is a potential target for cancer therapy [17,18]. The findings from previously published studies have shown that MCL1 and VEGF are downstream targets of the STAT3 signaling pathway [19,20]. However, whether or not MCL1 and VEGF are regulated by STAT3 in pancreatic cancer remains unclear.

Therefore, this study aimed to investigate the expression of TMEFF2 in human pancreatic cancer tissue and the effects of knockdown of TMEFF2 on cell, proliferation, and apoptosis in human pancreatic cell lines.

Material and Methods

Cell lines and tissue samples

Control human HEK293T cells and five human pancreatic cancer cell lines, CAPAN1, ASPC1, BXPC3, SW1990, and CFPAC, were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were maintained in Dulbecco's minimum essential medium (DMEM) (SH30243.01; Hyclone, Logan, UT, USA) containing 10% fetal calf serum (FCS) (16000-044; Gibco, Thermofisher Scientific, Waltham, MA, USA) and 1% penicillin and streptomycin (Solarbio Science & Technology Co., Ltd., Beijing, China). The cells were cultured in an incubator (Thermofisher Scientific, Waltham, MA, USA) at 37°C with 5% CO_2. Thirty-five pairs of pancreatic cancer tissues and their adjacent normal samples were obtained and stored in liquid nitrogen. This study was approved by the local Ethics Committee. Written informed consent was given by the patients who provided tissue samples.

Overexpression of TMEDD2 and knockdown using lentivirus

A lentiviral plasmid (pLVX-puro) containing the full-length TMEFF2 was constructed. After co-transfection into HEK293 cells with helper plasmids, the target lentivirus and control lentivirus (vector) were collected. Lentivirus was used to transfect CAPAN1 and ASPC1 cells.

The full-length SHP-1 sequence was constructed into a pCDNA3.1(+) vector at sites of Hind III and EcoR I and then confirmed by sequencing.

Three TMEFF2 short-hairpin RNAs (shRNAs) included siTMEFF2-1, siTMEFF2-2 and siTMEFF2-3, and a control shRNA (siNC) were constructed into lentiviral vectors (pLKO.1) (Addgene, Watertown, MA, USA). Together with package plasmids, the constructed plasmids were transfected into HEK293 cells. After 48 hours, the supernatant containing target lentivirus was collected and the supernatant was used to infect BXPC3 cells. Then, siNC with the pCDNA3.1 control vector, siTMEFF2 lentivirus, the SHP-1 overexpressed vector, and siTMEFF2 lentivirus with the SHP-1 overexpressed vector were used to transfect BXPC3 cells. The sequences used are listed in Table 1.

Cell counting kit-8 (CCK-8) assay

Cell proliferation rates were analyzed by the cell counting kit-8 (CCK-8) assay. Briefly, each well of a 96-well culture plate was seeded with approximately 5×10^3 target cells and maintained at 37°C overnight. After transfection for 12, 24, and 48 hours, 100 µL of DMEM with 10% CCK-8 medium (SAB Biotech, College Park, MD, USA) without FCS was added. The culture

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Table 1. Primers used for TMEFF2 overexpression, knockdown, and SHP-1 overexpression.

Primer	Primer sequence	Size (bp)
TMEFF2 (AB 017269.1)	Forward: 5'-CGGAATTCATGGTGCTGTGGGAGTCC-3' (EcoR I) Reverse: 5'-CGGGATCCTTAGATTAACCTCGTGGACGC-3' (BamH I)	1125
siTMEFF2-1 (AB 017269.1)	Forward: 5'-CCGGTGCTGGAATTGCTCTGGTTACTCGAGTAACCAGAGCAATTCCAGCTTTTTG-3' Reverse: 5'-AATTCAAAAAGCTGGAATTGCTCTGGTTACTCGAGTAACCAGAGCAATTCCAGCA-3'	525–543
siTMEFF2-2 (AB 017269.1)	Forward: 5'-CCGGTGGAGACATCCACCTGTGATCTCGAGATCACAGGTGGATGTCTCCTTTTTG-3' Reverse: 5'-AATTCAAAAAGGAGACATCCACCTGTGATCTCGAGATCACAGGTGGATGTCTCCA -3'	838–856
siTMEFF2-3 (AB 017269.1)	Forward: 5'-CCGGTGCCATCTTGCAGGTGTGATCTCGAGATCACACCTGCAAGATGGCTTTTTG-3' Reverse: 5'-AATTCAAAAAGCCATCTTGCAGGTGTGATCTCGAGATCACACCTGCAAGATGGCA -3'	1225–1243
SHP-1 (NM_002831.5)	Forward: 5'-CCCAAGCTTATGGTGAGGTGGTTTCACCC-3' (Hind III) Reverse: 5'-CGGAATTCTCACTTCCTCTTGAGGGAACC-3' (EcoR I)	1788

Table 2. Primers used for real-time polymerase chain reaction (RT-PCR).

Primer	Primer sequence	Size (bp)
TMEFF2 (AB 017269.1)	Forward: 5'-CTGGACACTTTGCGAGGGC-3' Reverse: 5' GGTGGGCGTTTGGCAGTC-3'	121
GAPDH (NM_001256799.2)	Forward: 5'-AATCCCATCACCATCTTC-3' Reverse: 5'-AGGCTGTTGTCATACTTC-3'	218
MCL1 (NM_001197320.1)	Forward: 5'-CAAAGCCAATGGGCAGGTC-3' Reverse: 5'-TACGCCGTCGCTGAAAAC-3'	188
VEGF (AY047581.1)	Forward: 5'-TGACGAGGGCCTGGAGTG-3' Reverse: 5'-CACAGGGATTTTCTTGTCTTGC-3'	167
GAPDH (NM_001256799)	Forward: 5'-CACCCACTCCTCCACCTTTG-3' Reverse: 5'-CCACCACCCTGTTGCTGTAG-3'	110

plates were incubated for 1 hour, and the optical density (OD) at 450 nm was read.

Cell apoptosis assay

Target cells were collected by centrifuging and were washed with PBS. A BD Accuri C6 fluorescence-activated cell sorting (FACS) instrument (BD Biosciences, Franklin Lakes, NJ, USA) and an Annexin V- fluorescein isothiocyanate (FITC) C1063 Apoptosis Detection Kit (Beyotime, Shanghai, China) were used to analyze apoptosis in the cells.

RNA isolation and real-time polymerase chain reaction (RT-PCR)

Total RNA from cell or tissue samples was extracted with TRIzol reagent (1596-026; Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. A RevertAid First Strand cDNA Synthesis Kit (K1622; Fermentas, Thermofisher Scientific, Waltham, MA, USA) was used to reverse transcribe RNA to cDNA. RT-PCR was performed using a SYBR Green PCR kit (K0223; Thermofisher Scientific, Waltham, MA, USA) and the signal was detected using an ABI 7300 detection system (Applied Biosystems, Foster City, CA, USA). GAPDH was used as an internal control. The primer sequences used are shown in Table 2.

Protein extraction and Western blot

Target cells were washed with PBS and then lysed in ice-cold RIPA buffer with added protease and phosphatase inhibitors. The proteins were collected by centrifuging at 4°C and the concentrations were measured using a BCA Protein Assay Kit (PICPI23223; Thermofisher Scientific, Waltham, MA, USA). A 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was used to separate the protein samples. Then, the target bands were transferred onto a nitrocellulose (NC) membrane (HATF00010; Merck Millipore, Burlington, MA, USA). After blocking the membranes for 1 hour with dried skimmed milk powder, the NC membrane was incubated with primary antibodies to TMEFF2, MCL1, VEGF, SHP-1, STAT3 and P-STAT3 (Abcam, Cambridge, MA, USA) and GAPDH (Cell Signaling Technology, Danvers, MA, USA), washed and incubated in secondary horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (Beyotime, Shanghai, China). Enhanced chemiluminescence (ECL) reagents (WBKLS0100; Merck Millipore,

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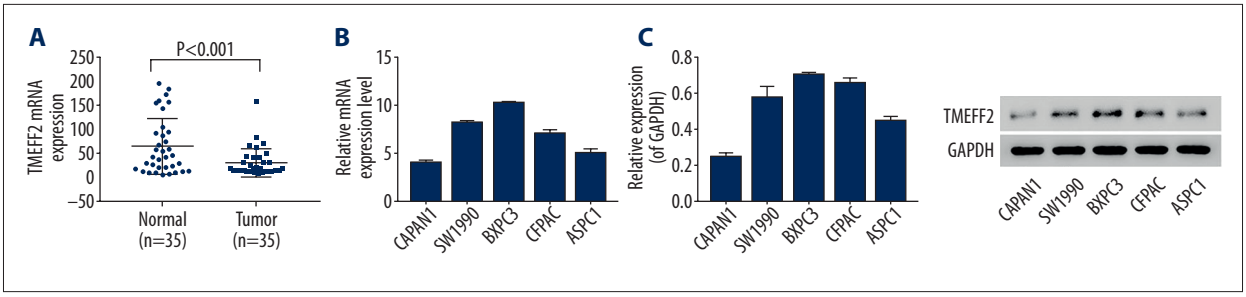

Figure 1. TMEFF2 was downregulated in pancreatic cancer tissue and cell lines. (**A**) Reduced mRNA expression levels of TMEFF2 were measured in pancreatic cancer tissues (n=35) compared with the adjacent normal pancreatic tissues by real-time polymerase chain reaction (RT-PCR). (**B**) mRNA expression levels of TMEFF2 were measured in five pancreatic cancer cell lines, CAPAN1, ASPC1, BXPC3, SW1990, and CFPAC by RT-PCR. (**C**) Protein expression levels of TMEFF2 were measured in five pancreatic cancer cell lines, CAPAN1, ASPC1, BXPC3, SW1990, and CFPAC by Western blot.

Burlington, MA, USA) were used to visualize the target bands and the densities were analyzed using Image J software.

The co-immunoprecipitation assay

Proteins were isolated in RIPA lysis buffer from cell samples. The primary antibody to TMEFF2 and SHP-1, or IgG (30000-0-AP; Proteintech, Manchester, UK) was added followed by incubation on ice for 2 hours. Immunoprecipitation was performed using Protein A/G PLUS-Agarose conjugate overnight (Thermofisher Scientific, Waltham, MA, USA). The protein complexes were collected by centrifuging and then washed with RIPA lysis buffer. Western blot was performed to analyze the final results.

Statistical analysis

Data were analyzed using GraphPad Prism software version 7.0 (GraphPad Software, La Jolla, CA, USA) and presented as the mean ± standard deviation (SD). Each experiment was performed in triplicate and the differences between groups were determined by analysis of variance (ANOVA). A P-value <0.05 was considered to be statistically significant.

Results

Downregulation of TMEFF2 expression was associated with pancreatic cancer

Analysis of the 35 pairs of pancreatic cancer and adjacent normal tissues showed that the mRNA expression levels of TMEFF2 were significantly downregulated in tumor tissues compared with adjacent normal tissues (Figure 1A). Five different pancreatic cancer cell lines CAPAN1, SW1990, BXPC3, CFPAC and ASPC1 were studied to analyze the expression level of endogenous TMEFF2. As shown in Figure 1B and 1C, CAPAN1 and ASPC1 cells showed lower expression levels of TMEFF2; BXPC3 cells showed higher expression levels of TMEFF2. Therefore, in the remaining studies, TMEFF2 was overexpressed in CAPAN1 and ASPC1 cells, but TMEFF2 was knocked down in BXPC3 cells.

TMEFF2 overexpression inhibited cell proliferation and increased apoptosis

Lentivirus that expressed TMEFF2 or control lentivirus (vector) were used to transfect CAPAN1 and ASPC1 human pancreatic carcinoma cells. As shown in Figure 2A–2D, the expression of TMEFF2 was significantly upregulated in ASPC1 and CAPAN1 cells, at both the mRNA and protein levels. The effect of TMEFF2 overexpression was studied in the human pancreatic cancer cells. The findings from the cell counting kit-8 (CCK-8) assay showed significantly reduced levels of cell proliferation in cells that overexpressed TMEFF2 (Figure 2E, 2H). Flow cytometry showed that upregulation of TMEFF2 increased cell apoptosis (Figure 2F, 2G).

TMEFF2 overexpression regulated expression levels of SHP-1, STAT3, MCL1, and VEGF

For ASPC1 and CAPAN1 human pancreatic cancer cells, the expression levels of SHP-1 and STAT3 were associated with TMEFF2 expression. As shown in Figure 3A and 3B, overexpression of TMEFF2 reduced the p-STAT3 expression level but increased the expression level of SHP-1. The expression level of MCL1 and VEGF were measured by real-time polymerase chain reaction (RT-PCR) (Figure 3C, 3E) and Western blot (Figure 3D, 3F). There was significant downregulation of MCL1 and VEGF when TMEFF2 was upregulated, compared with the control, which indicated that TMEFF2 might have a complex downstream signaling pathway.

Correlation between expression of TMEFF2, SHP-1, and STAT3

SHP-1 has previously been shown to be a direct target for TMEFF2 as a tumor suppressor in gastric cancer [4]. To determine whether

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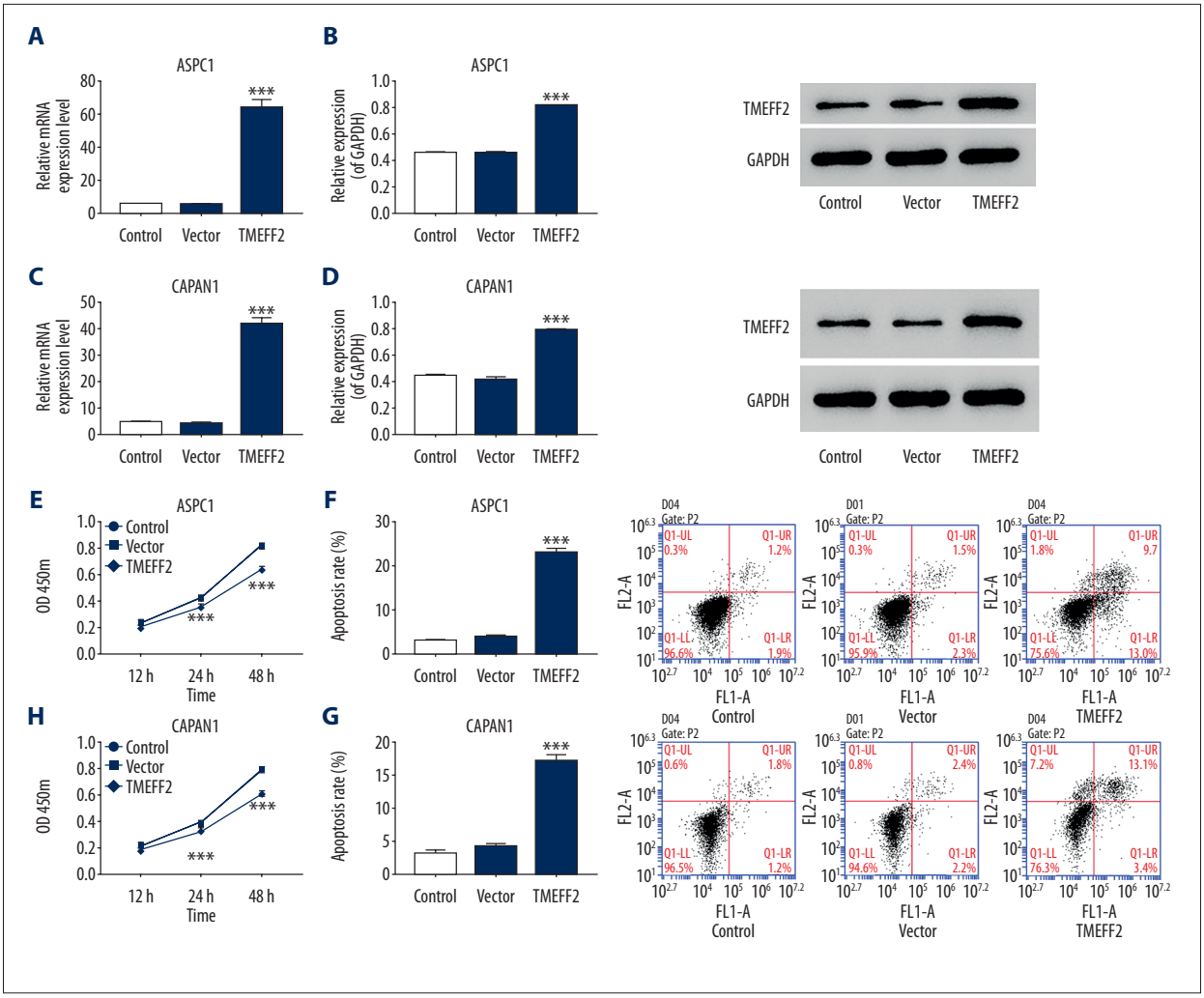

Figure 2. Overexpression of TMEFF2 inhibited cell proliferation and enhanced cell apoptosis in pancreatic cell lines. mRNA and protein expression levels of TMEFF2 were measured in ASPC1 pancreatic cancer cells (**A, B**) and CAPAN1 pancreatic cancer cells (**C, D**) pancreatic cancer cells after being transduced with lentivirus expressing TMEFF2, or control vector. Cell proliferation and cell apoptosis were measured by the cell counting kit-8 (CCK-8) assay or flow cytometry in ASPC1 pancreatic cancer cells (**E, F**) and CAPAN1 pancreatic cancer cells (**G, H**). Control, original ASPC1 or CAPAN1 cells; vector, cells transduced with lentivirus containing control vector; TMEFF2, cells transduced with lentivirus expressing TMEFF2. *** P <0.001 *vs.* control.

SHP-1 was a target for TMEFF2 in pancreatic cancer, a co-immunoprecipitation assay was performed. As shown in Figure 4A, in BXPC3 pancreatic cancer cells, TMEFF2 interacted with SHP-1 and so TMEFF2 was silenced in BXPC3 cells. As shown in Figure 4B and 4C, transfection with siTMEFF2-2 showed a relatively increased suppressive effect on TMEFF2 in BXPC3 cells. Therefore, siTMEFF2-2 was chosen for the subsequent experiments. BXPC3 cells were also transfected with the SHP-1 expression vector and an enhanced expression level of SHP-1 shown in BXPC3 cells when compared with the control groups (Figure 4D, 4E).

The associations between TMEFF2, SHP-1, and STAT3 were investigated. As shown in Figure 4F, TMEFF2 knockdown suppressed the expression of SHP-1 and enhanced the expression of p-STAT3. However, overexpression of SHP-1 showed the opposite effect and its overexpression could reduce the increased expression of p-STAT3 caused by TMEFF2 knockdown. These findings supported that SHP-1 was a directed target of TMEFF2 and TMEFF2 that could regulate p-STAT3 through SHP-1.

TMEFF2 regulated cell proliferation and apoptosis through complex downstream signaling pathways

The effect of TMEFF2 on cell proliferation and apoptosis was investigated in BXPC3 human pancreatic carcinoma cells. The results from the CCK-8 assay (Figure 5C) showed that knockdown of TMEFF2 induced cell proliferation. Overexpression

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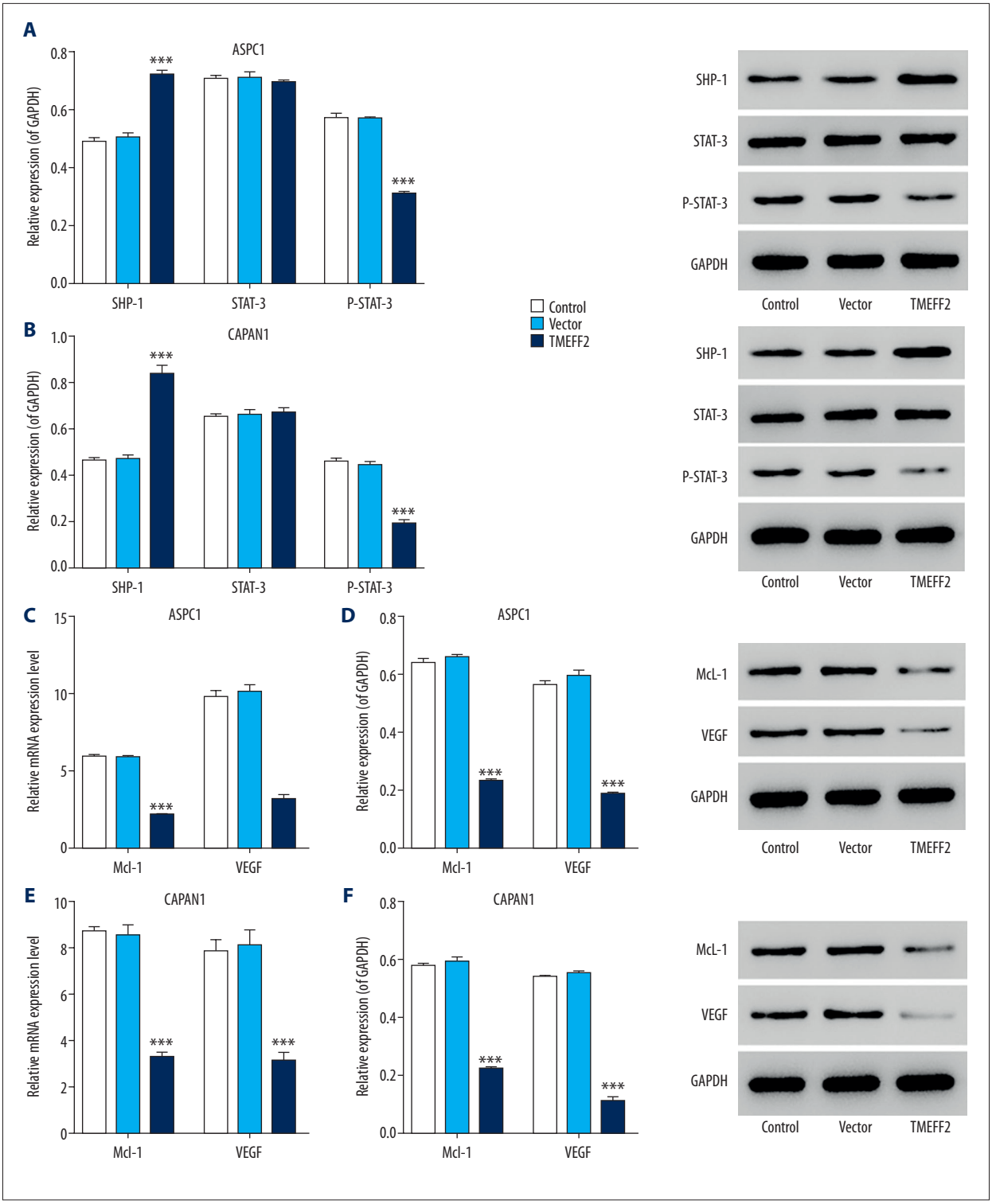

Figure 3. SHP-1, STAT3, MCL1, and VEGF were regulated by TMEFF2. Protein expression levels of SHP-1, STAT3 and p-STAT3 were measured by Western blot in ASPC1 pancreatic cancer cells (**A**) and CAPAN1 pancreatic cancer cells (**B**), which overexpressed TMEFF2. The mRNA and protein expression level of MCL1 and VEGF were measured by real-time polymerase chain reaction (RT-PCR) and Western blot in ASPC1 pancreatic cancer cells (**C, D**), and CAPAN1 pancreatic cancer cells (**E, F**), which overexpressed TMEFF2. Control, original ASPC1 or CAPAN1 pancreatic cancer cells; vector, cells transduced with lentivirus containing control vector; TMEFF2, cells transduced with lentivirus expressing TMEFF2. *** P<0.001 *vs.* control.

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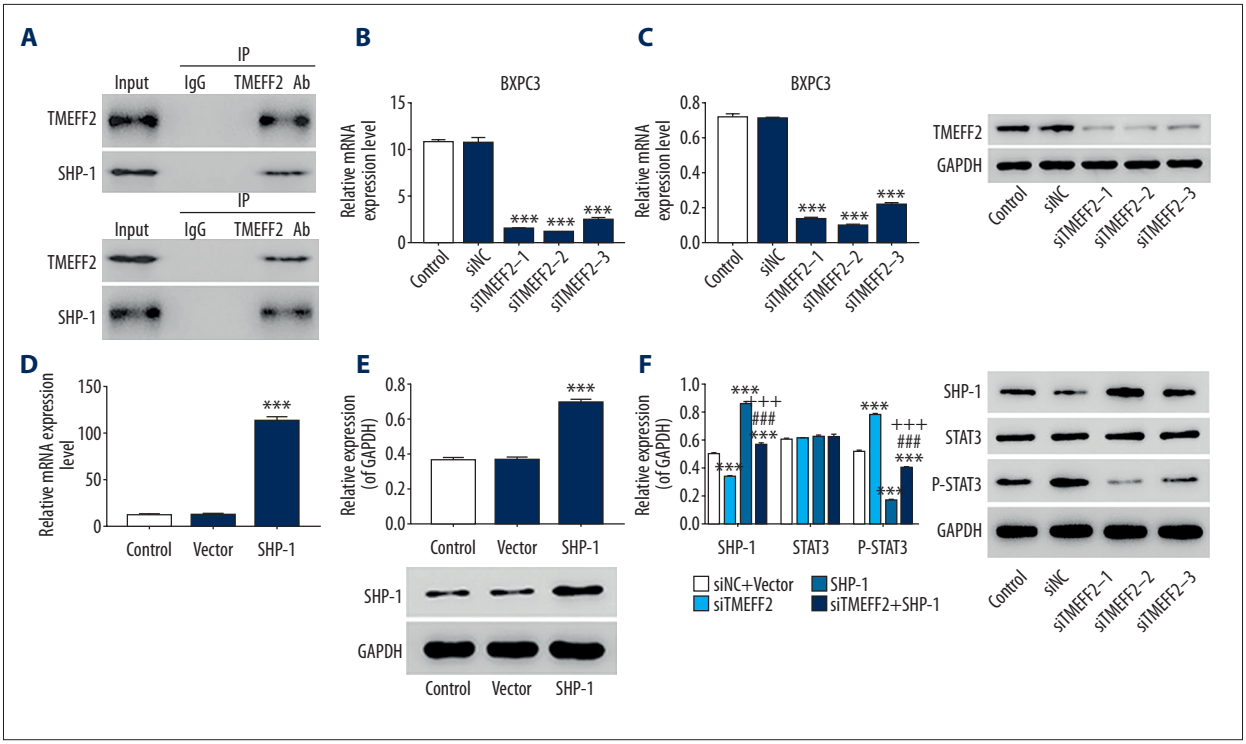

Figure 4. TMEFF2 regulated STAT3 via SHP-1. (**A**) The interactions between TMEFF2 and SHP-1 were measured using a co-immunoprecipitation assay. The mRNA (**B**) and protein (**C**) expression level of TMEFF2 were measured in BXPC3 pancreatic cancer cells after transfection with lentivirus containing short-hairpin RNAs (shRNAs) (siTMEFF2-1, -2 and -3) or control shRNA (siNC). The efficiency of SHP-1 overexpression was measured by real-time polymerase chain reaction (RT-PCR) (**D**) and Western blot (**E**) in BXPC3 pancreatic cancer cells, and (**F**) shows the protein expression level of SHP-1, STAT3, and p-STAT3 measured in BXPC3 pancreatic cancer cells transfected by control shRNA together with control vector (siNC + vector), lentivirus expressing siTMEFF2-2 (siTMEFF2), SHP-1 overexpression vector (SHP-1) and siTMEFF2-2 lentivirus with SHP-1 overexpression vector (siTMEFF2 + SHP-1). *** P<0.001 *vs.* control or siNC + vector; ### P<0.001 *vs.* siTMEFF2; +++ P<0.001 *vs.* SHP-1.

of SHP-1 suppressed cell proliferation, but there is no significant change of proliferation rate in BXPC3 cells when TMEFF2 was silenced. Similar results were found from flow cytometry. As shown in Figure 5D, there was a significant reduction in the levels of cell apoptosis in cells that underwent TMEFF2 knockdown, but there was an increase in apoptosis in cells expressing SHP-1. However, overexpression of SHP-1 in cells with TMEFF2 knockdown induced an increase in cell apoptosis.

The relationship between TMEFF2, SHP-1, MCL1, and VEGF were investigated. As shown in Figure 5A and 5B, TMEFF2 knockdown increased the expression levels of MCL1 and VEGF. Overexpression of SHP-1 reduced the expression level of both MCL1 and VEGF. Overexpression of SHP-1 abolished the effects of TMEFF2 knockdown on MCL1 and VEGF. These results indicated that VEGF and MCL1 were regulated by the TMEFF2/SHP-1 pathway in BXPC3 human pancreatic carcinoma cells, which indicated a combined effect on regulating cell proliferation and apoptosis through the TMEFF2/SHP-1 pathway.

Discussion

This study aimed to investigate the expression of the transmembrane protein with EGF and two follistatin motifs 2 (TMEFF2) in human pancreatic cancer tissue and the effects of knockdown of TMEFF2 on cell migration, proliferation, and apoptosis in five human pancreatic cancer cell lines, CAPAN1, ASPC1, BXPC3, SW1990, and CFPAC. The results showed that expression levels of TMEFF2 were downregulated in pancreatic cancer when compared with normal adjacent pancreatic tissue. TMEFF2 directly interacted with SHP-1 and regulated pancreatic cancer through signal transducer and activator of transcription 3 (STAT3), myeloid cell leukemia-1 (MCL-1), and vascular endothelial growth factor (VEGF).

Pancreatic cancer is associated with high mortality and there are limited treatment options [2]. Previously published studies have been shown that TMEFF2 acts as a tumor suppressor gene in several human cancers [4,21]. However, few previous studies have investigated the role of TMEFF2 and its downstream pathways in pancreatic cancer. The findings of the presents

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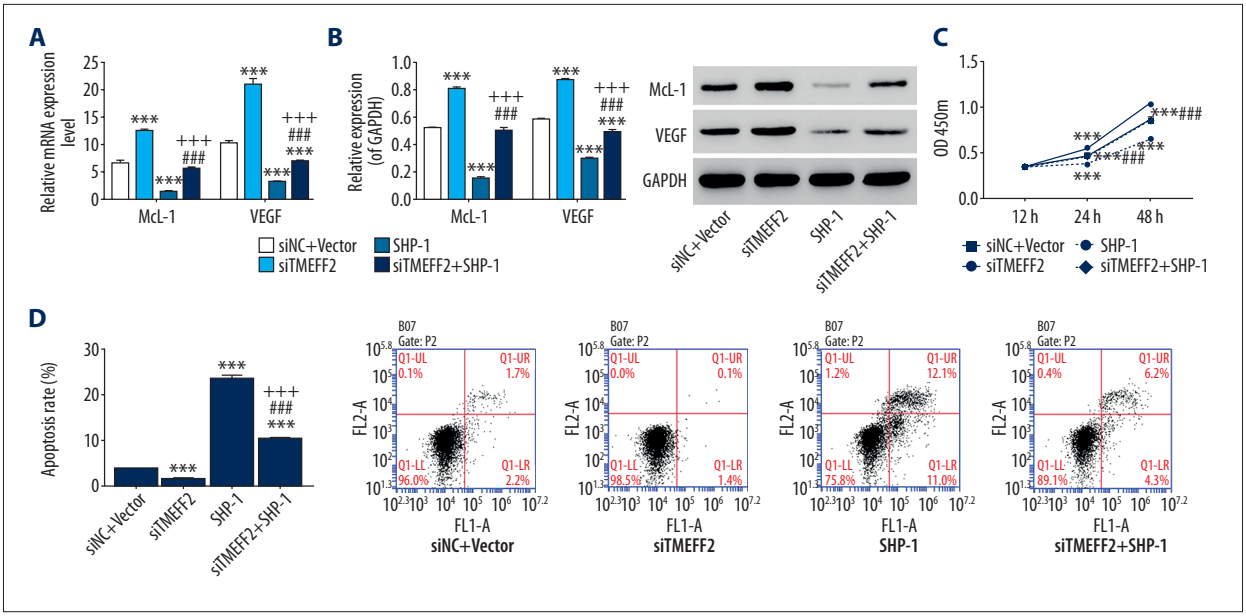

Figure 5. Effects of TMEFF2 knockdown and SHP-1 overexpression. (**A**) mRNA and (**B**) protein expression level of MCL1 and VEGF followed by (**C**) cell proliferation and (**D**) cell apoptosis were analyzed in BXPC3 cells, which were transfected with control shRNA together with control vector (siNC + vector), lentivirus expressing siTMEFF2-2 (siTMEFF2), SHP-1 overexpression vector (SHP-1) and siTMEFF2-2 lentivirus together with SHP-1 overexpression vector (siTMEFF2 + SHP-1). *** P<0.001 *vs.* control or siNC + vector; ### P<0.001 *vs.* siTMEFF2; +++ P<0.001 *vs.* SHP-1.

study showed that pancreatic cancer tissue had lower mRNA expression level for TMEFF2 when compared with normal pancreatic tissue, which supported the view that TMEFF2 might be acting as a tumor suppressor gene in pancreatic cancer. TMEFF2 was overexpressed in two human pancreatic cancer cell lines, ASPC1 and CAPAN1, which inhibited tumor cell proliferation and increased cell apoptosis when compared with controls. These findings also supported the tumor suppressor activity of TMEFF2 in human pancreatic cancer.

The tumor-associated protein, signal transducer and activator of transcription 3 (STAT3) and the Src homology phosphatase-1 (SHP-1) have been reported to be downstream effectors of TMEFF2 in gastric cancer [4,12]. Previously published studies have reported that SHP-1 suppressed the JAK/STAT pathway by increasing the expression levels of interleukin-5 (IL-5) [22]. The findings of the present study showed that overexpression of TMEFF2 increased the levels of SHP-1 and reduced the levels of p-STAT3 when compared with the controls, indicating that SHP-1 and p-STAT3 were regulated by TMEFF2. However, the relationship between SHP-1 and p-STAT3 remains unclear. Therefore, in this study, the interaction between TMEFF2 and SHP-1 in BXPC3 cells were investigated. The results from the co-immunoprecipitation assay showed that TMEFF2 could interact with SHP-1. Also, the results from Western blot showed that TMEFF2 knockdown resulted in an increased level of p-STAT3, while SHP-1 overexpression inhibited the effect of TMEFF2 knockdown and suppressed p-STAT3.

Myeloid cell leukemia-1 (Mcl-1) and vascular endothelial growth factor (VEGF) are proteins that confer an advantage to the growth of pancreatic cancer [17,23]. Previous studies have shown that in cholangiocarcinoma cells Mcl-1 protein and MCL1 gene expression were regulated by the STAT3 pathway [24], and VEGF was also shown to be regulated by STAT3 in colorectal cancer [25]. However, another study showed that STAT3 and VEGF were independent inhibitors of tumor progression in nasopharyngeal carcinoma [26]. In the present study, the findings showed that the expression levels of MCL1 and VEGF were suppressed by the overexpression of TMEFF2, which indicated that in pancreatic cancer, MCL1 and VEGF were regulated by TMEFF2. Together with previous findings that showed that STAT3 was regulated by TMEFF2 through SHP-1, it might be proposed that MCL1 and VEGF were also regulated by the TMEFF2/SHP-1 pathway. Therefore, TMEFF2 was silenced and SHP-1 was overexpressed in BAPC3 cells. Further results showed that both MCL1 and VEGF were upregulated by knockdown of TMEFF2. However, when SHP-1 was overexpressed, increased expression of MCL1 and VEGF due to TMEFF2 knockdown were suppressed. These findings indicated that MCL1 and VEGF were downstream effectors of SHP-1, but the relationship between MCL1, VEGF, and STAT3 remain unclear and further studies are needed on their role in pancreatic cancer.

Previously published studies have reported that downregulation of p-STAT3, MCL1, and VEGF suppressed cell proliferation and induced apoptosis in several types of cancer, including

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pancreatic cancer [19,27,28]. In the present study, the results of the cell counting kit-8 (CCK-8) showed that cell proliferation increased in TMEFF2 silenced cells, but overexpression of SHP-1 suppressed enhanced cell proliferation. Similar results were found from flow cytometry. SHP-1 overexpression increased the rate of cell apoptosis, which was suppressed by TMEFF2 knockdown. Consistent with previous findings, the expression of p-STAT3, MCL1, and VEGF were associated with cell proliferation and apoptosis in BAPC3 human pancreatic cancer cells.

Conclusions

The expression levels of TMEFF2 were downregulated in pancreatic cancer when compared with normal adjacent pancreatic tissue. TMEFF2 directly interacted with SHP-1 and regulated pancreatic cancer through STAT3, MCL-1, and VEGF in human pancreatic cancer cells *in vitro*. In human pancreatic cancer cells, TMEFF2 exerted as a tumor suppressor effect by regulating p-STAT3, MCL1, and VEGF through SHP-1.

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