Original Paper

TM4SF1 Regulates Pancreatic Cancer Migration and Invasion *In Vitro* and *In Vivo*

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Key Words

TM4SF1 • Invasion • Migration • Pancreatic cancer • Matrix metalloproteinase

Abstract

Background/Aims: The cell surface protein transmembrane 4 L6 family member 1 (TM4SF1) has been detected in various tumors and plays a major role in the development of cancer. We aimed to investigate the effects of TM4SF1 on the migration and invasion of pancreatic cancer *in vitro* and *in vivo* and explore its related molecular mechanisms. **Methods:** qRT-PCR and immunohistochemical analyses were used to measure the expression of TM4SF1 in pancreatic cancer tissues and adjacent tissues. TM4SF1 was silenced using siRNA and shRNA to investigate the role of this protein in the proliferation and metastasis of pancreatic cancer cells. MTS and Transwell assays were used to examine the effect of TM4SF1 on pancreatic cancer cell lines. The expression and activity of MMP-2 and MMP-9 were determined by qRT-PCR, western blots and gelatin zymography. *In vivo*, orthotopic pancreatic tumor models were used to examine the formation of metastasis. **Results:** qRT-PCR and immunohistochemical analyses showed that TM4SF1 was highly expressed in pancreatic cancer tissues compared with the adjacent tissues. In *in vitro* experiments the silencing of TM4SF1 reduced cell migration and invasion and down-regulated the expression and activity of MMP-2 and MMP-9. However, no significant difference in cell proliferation was detected after silencing TM4SF1. Additionally, knocking down TM4SF1 decreased the formation of lung and liver metastases in orthotopic pancreatic tumor models. **Conclusion:** Our results demonstrate that the expression of TM4SF1 is higher in pancreatic cancer tissues and pancreatic cancer cell lines than controls. Knockdown of TM4SF1 inhibited the migration and invasion of pancreatic cancer cells by regulating the expression and activity of MMP-2 and MMP-9, which suggests that TM4SF1 may play a significant role in metastasis in pancreatic cancer.

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Introduction

Pancreatic cancer is one of the leading causes of cancer death worldwide. Pancreatic cancer is characterized by difficulties in early diagnosis, local invasion and rapid progression, which are associated with the poorest overall survival rate among human cancers [1, 2]. High invasion and metastasis are the main causes of cancer mortality. At the time of diagnosis, most patients have already lost the opportunity for surgical resection and for effective therapeutic treatment. Therefore, understanding the mechanisms of pancreatic cancer underlying its high propensity for metastasis and developing effective treatments targeting metastasis are needed to improve the quality of life and survival of patients [3-6].

In a previous study, we identified various genes that were overexpressed in pancreatic cancer cells by analyzing microarray-generated expression profiles of pancreatic cancer tissues compared with those of normal pancreatic tissues and chronic pancreatitis tissues [7]. This microarray analysis indicated that transmembrane 4 L6 family member 1 (TM4SF1) was expressed at a high level in pancreatic cancer tissues, suggesting that TM4SF1 may be an important regulator in pancreatic cancer development.

TM4SF1, also known as tumor-associated antigen L6, is overexpressed in epithelial cell cancers and regulates cancer cell motility and invasion. The *TM4SF1* gene maps to chromosomal region 3q21-3q25. TM4SF1 is abundantly expressed on the plasma membrane and intracellular vesicles [8]. One study indicated that TM4SF1 was necessary for nanopodia formation and interacted with myosin-10 and β -actin, which regulated the formation of filopodia during cell polarization and motility [9]. TM4SF1 was also highly expressed in activated endothelial cells (EC), including cultured ECs and ECs lining the blood vessels that carry metastatic cells. After stimulating vascular endothelial growth factor (VEGF)-A or thrombin, TM4SF1 interacted with integrins to mediate angiogenesis [10]. Therefore, TM4SF1 is not only involved in metastasis but also regulates the development of angiogenesis, making it a potential target for anti-angiogenesis and anti-tumor therapy.

Matrix metalloproteinases (MMPs), a group of zinc-dependent endopeptidases, have been extensively studied in cancer cell migration and invasion research and are known to degrade various types of extracellular matrix (ECM) proteins and influence the VEGF-mediated development of vasculature and metastasis. From a mechanistic view point, the expression and activity of MMPs is closely related to angiogenesis and metastasis in cancers. Among the MMPs, MMP2 and MMP9 have been identified as markers of metastasis, including pancreatic cancer metastasis. We evaluated the association of TM4SF1 with these two genes in pancreatic cancer metastasis [11-16].

In this study, we demonstrate that TM4SF1 is expressed at a high level in pancreatic cancers and pancreatic cancer cell lines. Silencing TM4SF1 inhibited cancer cell migration and invasion by regulating MMP2 and MMP9 *in vitro*. In an animal study, cells lacking TM4SF1 had a reduced capability to form pancreatic cancer metastases. These results suggest that TM4SF1 acts as an oncogene in pancreatic cancer and is a major target for its treatment or diagnosis.

Materials and Methods

Pancreatic tissue samples and cell lines

Slides of paraffin-embedded normal pancreatic tissues, chronic pancreatitis tissues or pancreatic adenocarcinoma tissues were obtained from the Department of Pathology at The University of Texas MD Anderson Cancer Center and Xin Hua Hospital, which is affiliated with Shanghai Jiao Tong University School of Medicine. BxPC-3, Capan-1, Capan-2, MOH, CFPAC-1, HPAF2 and HPAC cell lines were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO₂ atmosphere incubator. HS766T, PNS1, Panc-48, Panc-3, Panc-28, MIA PaCa-2, PANC-1, AsPC-1, L3.6pl and SU.86.86 cell lines were cultured in Dulbecco's modified Eagle's medium. Human pancreatic duct epithelial (HPDE) cells provided by Dr. Tsao (University of Toronto) were cultured in keratinocyte serum-free medium.

Immunohistochemical staining

Unstained 5 µm sections of clinical samples were deparaffinized with xylene and rehydrated with ethanol. Antigen retrieval was performed using Borg Decloaker. A primary rabbit polyclonal antibody against TM4SF1 (Sigma) was diluted 1:100 in 5% normal horse serum/1% normal goat serum in phosphate-buffered saline (PBS). Tissue slides were developed with a 3, 3'-diaminobenzidine substrate, counterstained with hematoxylin and mounted. The immunohistochemical stains were analyzed using an Olympus microscope. Images of the sections were captured using a chilled charge-coupled device camera (Photometrics) and the Smart Capture software program (Digital Scientific). The staining results were evaluated by a gastrointestinal pathologist (Dr. Huamin Wang, MD Anderson).

Total RNA extraction and quantitative reverse transcription-polymerase chain reaction

Total RNA from pancreatic adenocarcinomas, adjacent normal pancreatic tissues, chronic pancreatitis tissues, pancreatic cancer cell lines, and HPDE was isolated, and the quality of the RNA was determined as previously described [17]. The primers designed for human TM4SF1 were as follows: forward, 5'-GGT TCT TTT CTG GCA TCG TAG GAG GTG-3'; reverse, 5'-CTG GCC GAG GGA ATC AAG ACA TAGTG-3'. The primers designed for human 18S ribosomal RNA, which was used as a loading control, were as follows: forward, 5'-GAG CGG TCG GCG TCC CCC AAC TTC-3'; reverse, 5'-GCG CGT GCA GCC CCG GAC ATC TAA-3'. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed using SYBR Premix Ex Taq II (TaKaRa).

Small interfering RNA transfection

AsPC-1, MIA PaCa-2, and PANC-1 cell lines were transfected with TM4SF1 siRNA (siTM4SF1) in 6-well plates at a final concentration of 10 nmol/L using the HiPerFect transfection reagent (Qiagen) according to the manufacturer's instructions. The cells were incubated for 48-96 hours.

Cell growth

Cell growth was analyzed using the MTS reagent (Promega) according to the manufacturer's protocols. AsPC-1, MIA PaCa-2, and PANC-1 cells were transiently transfected with siControl or siTM4SF1 for 24 hours (n = 1000) and were then plated on 96-well plates and grown in 2% serum-containing medium. Cell numbers were estimated at 0, 24, 48, and 72 hours by adding MTS to the wells 1 hour before photometric analysis, as previously described [18].

Cell migration and invasion

To study cell migration and invasion, Transwell Polycarbonate Membrane Inserts (Corning) and Biocoat Matrigel invasion chambers (BD Biosciences) were used. Briefly, pancreatic cancer cells (2×10^5) transiently transfected with siControl or siTM4SF1 for 48 hours were suspended in 100 µL of serum-free media and added to the upper chambers, and 2% serum-containing medium was added to the lower chambers. The cells were allowed to invade the membrane for 24 hours. The invading cells were analyzed as previously described [18].

Western blotting analysis

Whole cell lysates were extracted with RIPA buffer for 20 min. The total protein concentration was determined using a BCA protein assay kit. Extracts containing 50 µg of protein were electrophoresed via 10% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with rabbit anti-TM4SF1 (Abcam, 1:2000 dilution), rabbit anti-MMP2 (Santa Cruz Biotechnology, 1:500 dilution) and rabbit anti-MMP9 (Abcam, 1:500 dilution) overnight at 4°C, followed by incubation with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody for 2 hours at room temperature. The signals were detected using an ECL kit (Millipore) according to the manufacturer's instruction.

Gelatin zymography

After transfection, the medium was changed to serum-free RPMI 1640 or DMEM. The cells were cultured for 24 hours, and the conditioned media were collected. The media were electrophoresed on 10% precast polyacrylamide gels containing 0.1% gelatin (Sigma). The enzymatic activity of MMP-2 and MMP-9 was measured using a gelatin zymography kit (Life Technology) according to the manufacturer's protocol.

Lentivirus-mediated short hairpin RNA knockdown of TM4SF1 expression

Lentiviral vectors containing TM4SF1 shRNA or control shRNA (Open Biosystems) were cotransfected into 293T cells with packaging vectors as previously described. For lentivirus infection, pancreatic cancer cells were seeded in 6-well plates and infected with shTM4SF1- or shControl-expressing virions. Stable pancreatic cancer cell clones expressing the above shRNAs were selected and maintained in medium containing 4 µg/ml puromycin according to the manufacturer's instructions and were screened by quantitative RT-PCR as previously described [13].

Animal studies

For *in vivo* tumor experiments, 5-week-old male athymic nude nu/nu mice were divided into shControl and shTM4SF1 groups, with seven mice each. TM4SF1 was knocked down by lentiviral transfection in MIA PaCa-2 cell lines, and stable shRNA-expressing clones were selected. Stably transfected cells or control cells were injected into the pancreases of mice. The tumor sizes were recorded weekly using bioluminescence imaging. Six weeks after the injection, the mice were sacrificed, and each pancreas was removed. The weight of the tumors was recorded. The liver and lungs were inspected for metastases using bioluminescence imaging and Living Image Software.

Statistical analysis

The data were analyzed with SPSS 19.0 statistical software. Experimental data are presented as the means from independent experiments. Statistically significant differences were determined using Dunnett's multiple comparison test, a Mann-Whitney U test and Kruskal-Wallis test or a two-tailed unpaired Student's t-test. A P-value less than 0.05 was considered significant.

Results

TM4SF1 is overexpressed in pancreatic tumors and cancer cell lines

To identify the specific cells expressing TM4SF1 in the present study, we conducted immunohistochemical staining of pancreatic tissues. We detected faint expression of TM4SF1 in normal pancreatic ducts but not in acinar cells or islets (Fig. 1A). In pancreatic tumors, TM4SF1 expression was high. These data confirmed that TM4SF1 is highly expressed in the neoplastic cells of pancreatic adenocarcinomas [19]. We used quantitative RT-PCR to compare the mRNA levels of TM4SF1 in pancreatic tumor, normal pancreatic tissue, and chronic pancreatitis tissue samples (Fig. 1B). TM4SF1 mRNA expression was 5- to 10- fold higher ($P < 0.05$) in pancreatic tumors than in the other tissues. We further investigated TM4SF1 mRNA expression in 18 pancreatic cancer cell lines and HPDE cells. The mRNA expression of TM4SF1 in all the pancreatic cancer cell lines was higher than that in HPDE cells ($P < 0.05$, Fig. 1C).

*TM4SF1 expression levels correlate with pancreatic cancer cell migration, invasion, and survival rates *in vitro**

To investigate the functional role of TM4SF1 in pancreatic cancer cells, we transiently transfected AsPC-1, MIA PaCa-2, and PANC-1 cells with siControl or siTM4SF1 and confirmed the silencing of TM4SF1 in the cells using qRT-PCR. The assay showed that the mRNA expression of TM4SF1 decreased by more than 70% with siTM4SF1 (Fig. 2A). We then analyzed the transfected cells for changes in proliferation, migration and invasion *in vitro*. The proliferation of TM4SF1-silenced cells over 72 hours was not significantly different compared to the siControl-transfected cells based on the MTS assay ($P > 0.05$, Fig. 2B). However, silencing of TM4SF1 markedly reduced the migration and invasion of pancreatic cancer cells (Fig. 3A and 3B). The mean reduction in the migration of AsPC-1, MIA PaCa-2, and PANC-1 cells was 63.65%, 61.77% and 69.33%, respectively. The mean reduction in the invasion of AsPC-1, MIA PaCa-2, and PANC-1 cells was 64.74%, 66.41% and 57.83% ($P < 0.05$).

Fig. 1. TM4SF1 was overexpressed in pancreatic tumors and cancer cell lines. (A) Immunohistochemical analysis showing the location of TM4SF1 in samples of pancreatic adenocarcinoma and adjacent benign pancreatic tissue at a magnification of $\times 200$. The normal tissue samples exhibited only slight staining for TM4SF1. In contrast, the adenocarcinoma sample showed strong staining. (B) qRT-PCR analysis of the TM4SF1 mRNA expression in normal pancreatic tissue, chronic pancreatitis tissue and pancreatic adenocarcinoma samples. The expression of TM4SF1 was higher in pancreatic adenocarcinomas than normal pancreatic and chronic pancreatitis tissues. (C) the mRNA expression of TM4SF1 was higher in the cancer cell lines than in HPDE cells.

Fig. 2. *In vitro* proliferation of AsPC-1, MIA PaCa-2, and PANC-1 cells was not significantly altered after transfection with siTM4SF1. (A) qRT-PCR analysis of mRNA expression after silencing TM4SF1 expression in AsPC-1, MIA PaCa-2 and PANC-1 cells. (B) MTS assays showed that the cell growth rates of siControl-transfected cells and siTM4SF1-transfected cells were not significantly different. * $P < 0.05$ vs control group.

TM4SF1 mediates MMP2 and MMP9 expression and activation

Previous studies have demonstrated that MMPs are major ECM-degrading enzymes and that the high expression and activation of MMPs may contribute to the development of cancer metastasis [20]. Thus, we determined whether TM4SF1 could lead to increased expression and activity of MMPs during the process of metastasis in pancreatic cancer. Compared with the siControl groups, the siTM4SF1 groups showed significantly down-regulated mRNA and protein levels of MMP2 and MMP9 in pancreatic cancer cells ($P < 0.05$, Fig. 4A and 4B). In addition, the results of gelatin zymography indicated that the secretion of MMP2 and MMP9 was significantly decreased in TM4SF1 siRNA-transfected cells compared to control siRNA-transfected cells ($P < 0.05$, Fig. 4C and 4D).

Fig. 3. Pancreatic cancer cell migration and invasion were decreased following transient silencing of TM4SF1. (A) Effect of siRNA silencing of TM4SF1 on the migration of AsPC-1, MIA PaCa-2, and PANC-1 cells. After transiently transfecting cells with siControl or siTM4SF1, cells were added to the upper compartment of a migration or invasion chamber, and the number of cells invading the lower chamber was estimated in eight random fields at $\times 100$ magnification after 24 hours. The rate of migration of cells with transiently silenced TM4SF1 expression was markedly reduced. (B) siRNA silencing of TM4SF1 expression on the invasion of AsPC-1, MIA PaCa-2, and PANC-1 cells. * $P < 0.05$ versus control.

TM4SF1 silencing reduces metastasis of pancreatic tumors in vivo

To further elucidate the role of TM4SF1 in pancreatic cancer, we transfected MIA PaCa-2 cell lines with shRNA constructs to stably knock down the expression of TM4SF1. We confirmed shRNA silencing using qRT-PCR (Fig. 5A) and determined that the mRNA expression of TM4SF1 decreased by approximately 90% with shTM4SF1-1. After injecting MIA PaCa-2 cells stably expressing shControl or shTM4SF1-1 into nude mice, orthotopic tumors developed (Fig. 5B). The tumor weights of the shTM4SF1 group were not significantly different than those of the shControl group. We also evaluated pancreatic tumor metastases to the liver and lung. TM4SF1 silencing reduced the incidence of metastasis to both sites (lungs, $P = 0.007$ and liver, $P = 0.0265$ [Mann-Whitney U test]) (Fig. 5D).

Discussion

The morbidity and mortality of pancreatic cancer is increasing rapidly and is considered to be a common cause of cancer-related death. The characteristics of pancreatic cancer include local invasion, early metastasis and chemoresistance. Because most patients are diagnosed at advanced stages, the successful surgical resection rate and the 5-year survival rate are low. Gemcitabine has been used as a first-line chemotherapy drug for pancreatic cancer, but the survival time of patients is still no more than 6 months for metastatic disease and 9-12 months for locally advanced disease. To improve patients' survival, a better understanding of the molecular mechanisms that control pancreatic cancer metastasis is urgently needed [21-24].

Fig. 4. Silencing of TM4SF1 decreased the expression and activity of MMP2 and MMP9. (A) the mRNA expression of MMP2 and MMP9 measured by qRT-PCR in AsPC-1 and PANC-1 cells after different treatments. (B) Western blot analysis of the protein expression of MMP2 and MMP9 after silencing TM4SF1 compared with a negative control. (C) Gelatin zymography of activated MMP2 and MMP9 in the conditioned media of si-Control-transfected and siTM4SF1-transfected cells. (D) densitometric analysis of MMP2 and MMP9 enzymatic activity. *P < 0.05 vs control.

In previous studies, TM4SF1, a membrane-associated glycoprotein, has been found to be overexpressed in various human malignant tumors and is considered to be a major mediator of cancer metastasis. TM4SF1 was expressed in human lung, breast, colon, renal, prostate and ovarian cancers [25, 26]. In squamous cell lung cancer, the expression of TM4SF1 promoted invasion and was significantly correlated with an early postoperative relapse and shorter survival. Coimmunoprecipitation analysis showed that CD13 was associated with TM4SF1 and formed a complex on lung cancer cells that enhanced cell migration [27]. For the first time, Allili demonstrated that TM4SF1 expression was higher in prostate cancer tissues than benign prostatic tissues and that TM4SF1 directly targeted the androgen receptor to

Fig. 5. Reduction in pancreatic cell metastasis *in vivo* after TM4SF1 silencing. (A) the mRNA expression of TM4SF1 in MIA PaCa-2 cells after knocking down TM4SF1 using shRNA. (B) growth of orthotopic tumors using MIA PaCa-2 cells that stably expressed shControl or shTM4SF1. (C) the tumor weights of the shControl and shTM4SF1 groups were not significantly different. (D) the incidences of metastasis to the lung and liver were calculated. TM4SF1 silencing significantly reduced the incidence of lung and liver metastases. * $P < 0.05$ versus control by Mann-Whitney U test, $P = 0.007$ for lung metastasis, $P = 0.0265$ for liver metastasis (for seven animals).

promote cell migration [28]. Using two highly motile breast cancer cell lines, TM4SF1 was found to be associated with tetraspanin-enriched microdomains (TERM) and regulate the surface expression of CD63 and CD82. They also found that TM4SF1 was ubiquitinated and that ubiquitination was essential for cell migration [8]. Additionally, the nanopodia were long and thin, and the F-actin-negative membrane projections had TM4SF1 clusters in TM4SF1-enriched microdomains (TMED) in EC and cancer cells. As a molecular organizer, TM4SF1 interacted with myosin-10 and β -actin and was involved in nanopodia formation and cell polarization and migration [9, 29].

The role of TM4SF1 in cancer angiogenesis and its potential as a therapeutic target in cancers have been demonstrated. In an *in vitro* study, endothelial cells lost the ability to polarize, migrate and proliferate after silencing TM4SF1. TM4SF1 was highly expressed by the endothelial lining of angiogenic blood vessels and affected the capacity of endothelial cells to form new blood vessels *in vivo*. Due to its location on the cellular surface of tumor-associated endothelium, TM4SF1 may be a promising therapeutic target in an antibody-drug conjugate (ADC) approach. The mouse anti-human TM4SF1 monoclonal antibody 8G4 attached to microtubules by binding myosin-X and was transported through the cytoplasm. This antibody lost its attachment with the membranes when it passed through the nuclear pores and entered the nucleoplasm. These results demonstrate that TM4SF1 is an important target in ADC cancer therapy [30-32].

Overall, we suggest that TM4SF1 is an important molecular target for metastasis and treatment of pancreatic cancer. In pancreatic cancer cell lines, miR-141 down-regulated the expression of TM4SF1 to inhibit cell migration and invasion by directly targeting the 3'-untranslated region of the mRNA of this gene [19]. Nevertheless, the molecular mechanisms of TM4SF1 in pancreatic cancer metastasis remain unclear. The aim of this study was to evaluate the carcinogenic role of TM4SF1 and determine its molecular mechanism of

action in pancreatic cancer. Our study showed that the expression of TM4SF1 was highly increased in pancreatic cancer tissues and pancreatic cancer cell lines. Surprisingly, we did not observe any effect of TM4SF1 on the proliferation of pancreatic cancer cells *in vitro* and *in vivo*. However, we found that silencing TM4SF1 reduced pancreatic cancer cell invasion and metastasis *in vivo*. These data suggest that TM4SF1 overexpression is common in pancreatic cancer and may be associated with cancer metastasis.

Previous studies have also demonstrated that degradation of extracellular matrix components is an important step in metastasis in cancers by promoting cancer cells to break through the basement membrane and metastasize to distant tissues [33]. It has generally been shown that MMP2 and MMP9 are bio-markers for cancer invasion and metastasis because they degrade ECM components in pancreatic cancer. MMPs also regulated the tumor microenvironment, which affected cell growth, inflammation or angiogenesis [34-38]. Furthermore, we demonstrated that down-regulation of TM4SF1 led to suppression of the mRNA and protein levels of MMP2 and MMP9, which indicated that TM4SF1 might affect cancer cell migration and invasion by enhancing the expression of MMP2 and MMP9. In addition, inhibition of TM4SF1 reduced the secretion of MMP2 and MMP9.

These results suggest that TM4SF1 induced pancreatic cancer metastasis through MMP2 and MMP9 regulation. However, the mechanism underlying the down-regulation of MMP2 and MMP9 after silencing TM4SF1, which affects cancer cell migration and invasion, remains to be explored.

Based on the above results, we conclude that TM4SF1 is overexpressed in pancreatic cancer and can influence cancer cell migration and invasion via MMP2 and MMP9 regulation and may provide a novel therapeutic target for the treatment of pancreatic cancer.

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Disclosure Statement

The authors declare that they have no competing interests.

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