Targeting TM4SF5 with anti-TM4SF5 monoclonal antibody suppresses the growth and motility of human pancreatic cancer cells

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Received February 22, 2019; Accepted August 5, 2019

DOI: 10.3892/ol.2019.11134

Abstract. Pancreatic cancer is one of the most lethal cancers. Transmembrane 4 superfamily member 5 protein (TM4SF5) is one of the candidate molecular targets used for the prevention and treatment of TM4SF5-expressing cancers, including hepatocellular carcinoma, colon cancer and pancreatic cancer. Recently, a previous study reported the preventive effects of a peptide vaccine, which targeted TM4SF5, in a mouse pancreatic cancer model. The present study investigated the implication of TM4SF5 and the suppressive effect of anti-human TM4SF5 monoclonal antibody (anti-hTM4SF5 antibody) in human pancreatic cancer cell lines *in vitro*. Treatment with anti-hTM4SF5 antibody reduced cell viability, modulated the expression of EMT markers Vimentin and E-cadherin, and decreased cell motility in human pancreatic cancer cells that endogenously expressed TM4SF5. When TM4SF5 was exogenously overexpressed in the TM4SF5-negative cell line, the cells indicated increased cell viability and motility compared with control cells, and the phenotype was reversed by anti-hTM4SF5 antibody treatment. Therefore, the results of the current study demonstrated that the high expression of TM4SF5 is a tumorigenic factor in human pancreatic cells and anti-hTM4SF5 antibody treatment exhibits a suppressive effect in TM4SF5-expressing pancreatic cancer cells.

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Abbreviations: EMT, epithelial-mesenchymal transition; HCC, hepatocellular carcinoma; PDAC, pancreatic ductal adenocarcinoma; TAA, tumor-associated antigen; TM4SF5, transmembrane 4 superfamily member 5 protein

Key words: transmembrane 4 superfamily member 5 protein, pancreatic cancer, antibody, anticancer activity, proliferation, motility

Introduction

According to the reports of National Cancer Institutes (USA), pancreatic cancer was the tenth and eleventh most prevalent case of cancer in men and women from 2010 to 2014 and the fourth leading cause of death from cancer in men and women from 2011 to 2015. The case and death rates show an interesting tendency (1): Pancreatic cancer has a higher death rate than incidence rate. This is because early diagnosis is difficult, and the cancer is mostly found after it has already progressed to an advanced stage in many cases (2). Most pancreatic cancers are pancreatic ductal adenocarcinoma (PDAC) (3). Pancreatic cancer begins in minimally dysplastic epithelium and progresses to invasive carcinoma by accumulation of mutations; activation of KRAS oncogene, inactivation of tumor-suppressor genes such as CDKN2A and TP53, and deletion of SMAD4 (4).

Tetraspanins form protein complexes with other tetraspanins, integrins, and other membrane proteins, and the protein complexes form tetraspanin-enriched microdomains (TEM) by binding to membrane cholesterols and anchorage to the actin cytoskeleton (5). The tetraspanins are involved in regulation of cell differentiation, migration, proliferation, and tumor progression (6,7). Transmembrane 4 superfamily member 5 (TM4SF5), one of the tetraspanins, was first reported in 1998, and the expression of TM4SF5 was reported in human cancer such as hepatocellular carcinoma (HCC), colon cancer, and pancreatic cancer (8,9). The molecular function of TM4SF5 was intensively investigated in HCC (9-13). TM4SF5 induces the epithelial-mesenchymal transition (EMT), enhances translocation of $p27^{kip1}$ into cytosol, and reduces RhoA activity (9-11). The cytosolic $p27^{kip1}$ inhibits RhoA activity, and this leads to uncontrolled cell growth and tumorigenesis by loss of contact inhibition (9,11). In addition, TM4SF5 accelerates G1/S phase by controlling cytosolic $p27^{kip1}$ and RhoA activity (12). TM4SF5 induces vascular endothelial growth factor (VEGF) expression and secretion, leading to an increase of angiogenic activity (13). On the other hand, treatment of the TM4SF5-targeted monoclonal antibody reverses cellular events induced by TM4SF5 expression; anti-TM4SF5 antibody induces translocation of $p27^{kip1}$ into nucleus, increased RhoA

activity, reduced EMT, and suppression of tumor growth and metastasis (10,14,15).

Since 1975, when the procedure of effectively producing monoclonal antibodies (mAbs) was developed (16), antibodies were used for imaging and therapy. In therapy, murine mAb triggered the stimulation of the patient's immune system. In the early 1990s, a technique for cloning IgG genes was developed and, as a result, IgG genes could be expressed in eukaryotic cells (17). This development of antibody technology has resulted in the production of antibodies such as chimeric antibodies and humanized antibodies (18). The chimeric antibodies include 70% of human sequences and the Fc portion is fully human. In order to humanize more of the parts of the murine mAb, all parts except the complementarity-determining region (CDR) portion are replaced with human sequences. As a result, humanized antibodies include 85-90% of human sequences and have lower immune responses than chimeric antibodies (18). Anti-cancer therapy using mAb targeting surface antigens expressed on tumor cells is an efficacious strategy to treat cancer (19).

Previously, we found that TM4SF5-targeted monoclonal antibody and peptide vaccination has preventive and therapeutic effects on hepatocellular carcinoma and colon cancer models (10,14,15,20,21). Regarding the implication of TM4SF5 as a target of anticancer strategy in pancreatic cancer, we revealed immunization with TM4SF5 peptide vaccine prevented tumor growth derived from TM4SF5-expressing mouse pancreatic cancer cells in an allograft mouse model (22). In this study, we confirmed the implication of TM4SF5 expression and anti-growth/motility effect of antibody targeting TM4SF5 in human pancreatic cancer cells.

Materials and methods

Cell culture. Mia-PaCa-2 and PANC-1 cells were maintained in Dulbecco's modified Eagle's media (DMEM; Hyclone) with 10% fetal bovine serum (FBS; Hyclone), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37˚C under a humidified atmosphere of 5% CO_2. ASPC-1, Capan-1, and Capan-2 cells were maintained in Roswell Park Memorial Institute medium (RPMI-1640; Hyclone) with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. CFPAC-1 cells were maintained in Iscove's modified Dulbecco's medium (IMDM; Hyclone) with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. H6c7 cells were maintained in Keratinocyte-SFM (Invitrogen; Thermo Fisher Scientific, Inc.) with 100 U/ml penicillin, and 100 μg/ml streptomycin.

Reverse transcription (RT) PCR. Total RNA was isolated with the TRI Reagent® according to the manufacturer's instructions (MRC). Then, 2 μg of total RNA was reverse-transcribed in the first-strand synthesis buffer containing 6 μg/ml oligo(dT) primer, 50 U M-MLV reverse transcriptase, 2 mM dNTP, 10 mM DTT, and 40 U RNaseOUT™ recombinant ribonuclease inhibitor (Invitrogen; Thermo Fisher Scientific, Inc.). The reaction was carried out at 37˚C for 50 min and heat inactivated at 70˚C for 15 min. One microliter of the synthesized cDNA solution was subjected to a semi-quantitative PCR of 25 (for GAPDH) or 30 (for TM4SF5) cycles consisting of denaturation for 40 sec at 95˚C, annealing for 40 sec at 58˚C, and extension for 40 sec at 72˚C. The primer sequences used were as follows: GAPDH, 5'-TCCACCACCCTGTTGCTGTA-3' (sense) and 5'-ACCACAGTCCATGCCATCAC-3' (anti-sense) (product size 452 bp); human TM4SF5, 5'-AGCTTGCAAGTC TGGCTCAT-3' (sense) and 5'-GCTGGATCCCACACAGTA CT-3' (anti-sense) (product size 401 bp).

Packaging and transduction of control and TM4SF5-encoding retroviruses. The human TM4SF5 cDNA was amplified from pcDNA3.1-hTM4SF5 (14) by PCR using the following primer set: hTM4SF5 5' primer, 5'-GAATTCGCCACCATGGAA CAAAAACTCATCTCAGAAGAGGATCTGGGTGCAATG TGTACGGGAAAA-3' and hTM4SF5 3' primer, 5'-CTC GAGTCAGTGAGGTGTGTCCTG-3'. The cDNA fragments were cloned into the expression vector pLXSN (Clontech Laboratories, Inc.) using the *Xho*I and *Eco*RI sites. GP2-293, a cell line derived from 293 cells, was obtained from Clontech and used as a packaging cell line for preparation of the retroviruses. GP2-293 cells were maintained in DMEM containing 10% FBS in a 5% CO_2 incubator at 37˚C. Retroviral vectors pLXSN or pLXSN-hTM4SF5 along with pVSV-G (Clontech Laboratories, Inc.) encoding the pseudo-envelope protein gene were transfected into the cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Twelve hours later, the medium was exchanged with fresh culture medium supplemented with 10 mM sodium butyrate (Sigma-Aldrich; Merck KGaA). After 48 h, the supernatant of the culture medium was taken and filtrated through a filter with a 0.45 μm pore size. The retrovirus supernatants were concentrated using Centricon centrifugal filters (Millipore) and stored at -80˚C. The viral supernatant was applied to Mia-PaCa-2 cells along with 8 μg/ml of polybrene (Sigma-Aldrich; Merck KGaA). Twenty-four hours later, G418 (Sigma-Aldrich; Merck KGaA) was added at a concentration of 1 mg/ml, and the G418-resistant transfected Mia-PaCa-2 cells were selected by culture of the cells in the presence of G418 for 2 weeks.

Western blot analysis. Harvested cells were lysed in a lysis buffer (pH 8.0, 20 mM Tris-HCl, 137 mM NaCl, 10% glycerol, 10 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40, protease inhibitor cocktail, and phosphatase inhibitor). Proteins were resolved by SDS-polyacrylamide gel electrophoresis and electro-transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). The membranes were blocked with 5% dry milk in phosphate buffered saline-Tween-20 (PBS-T; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, and 0.05% Tween-20) and probed with an appropriate primary antibody. The monoclonal anti-GAPDH antibody was purchased from Santa Cruz Biotechnology, Inc. The anti-E-cadherin and anti-Vimentin polyclonal antibodies were purchased from Cell Signaling Technology, Inc. Immunoreactive proteins were visualized by horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (Santa Cruz Biotechnology, Inc.) and an ECL solution (ATTO).

Humanized anti-hTM4SF5 monoclonal antibody. The humanized anti-hTM4SF5 monoclonal antibody (anti-hTM4SF5 antibody), hEC2-C-2, was established based on the mouse monoclonal antibody mEC2-C obtained by immunization with

the cyclic peptide hTM4SF5EC2-C as an antigen (14). The anti-hTM4SF5 antibody recognizes structural epitope of the second extracellular loop region of TM4SF5. To obtain humanized antibody with intact IgG format, VH and Vk encoding genes originated from mEC2-C were synthesized (Bioneer). The synthesized genes were then inserted into the modified pcDNA 3.4 expression vector (Invitrogen; Thermo Fisher Scientific, Inc.) carrying the human IgG1 constant regions (CH1-hinge-CH2-CH3) or human kappa chain constant region (CL) for mammalian cell expression in HEK 293F cells. The antibodies were purified using Protein A affinity chromatography following the manufacturer's protocol after 5-7 days of cell culture.

Immunostaining and confocal microscopy. The cells were fixed with 4% paraformaldehyde and blocked with 3% BSA containing 0.1% Triton X-100. Cells were treated with anti-hTM4SF5 antibody (3 μg/ml) for 3 h. After extensive washing with PBS, the samples were incubated with Alexa Flour 488-conjugated goat anti-human IgG (Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h. The nuclei were stained with Hoechst 33258 (Sigma-Aldrich).

Immunoprecipitation. Whole cell lysates were lysed in a lysis buffer. After anti-hTM4SF5 antibody was conjugated to protein A-agarose beads (Roche Diagnostics), the whole cell lysates were immunoprecipitated with the antibody-conjugated agarose beads. Immunoprecipitated proteins were washed with PBS and processed for a standard western blot analysis using the mouse anti-hTM4SF5 monoclonal antibody (mEC2-CF, 1 μg/ml), which we previously reported (23).

Cell proliferation assay. The cell proliferation ELISA, BrdU colorimetric kit (Roche Diagnostics), was used to measure the cell proliferation according to the manufacturer's instructions. Cells were treated with normal IgG or anti-hTM4SF5 antibody (10 μg/ml) for 3 days. The BrdU solution was added to each well, and then the plates were incubated for 4 h at 37˚C. After fixation of the cells, anti-BrdU antibody conjugated with peroxidase was added to each well for 90 min at room temperature. A colorimetric assay was developed with a substrate solution, and the absorbance at 370 nm with a reference wavelength of 492 nm was measured using a microplate reader (Bio-Rad Laboratories, Inc.).

In vitro wound-healing assay. Cells were placed in a 6-well plate, cultured overnight to 80 ~90% confluence in a medium containing serum, and the monolayer was wounded with a pipette tip (3 wounds/well). Normal IgG or anti-hTM4SF5 antibody (10 μg/ml) was added to the medium for the indicated periods. The cells were fixed with 4% paraformaldehyde (Biosesang) for 20 min and stained with 0.01% crystal violet (Sigma-Aldrich; Merck KGaA) for 20 min. The wound-healing activity of cells was calculated by the following formula: The cells migrating into wound (%)=[(the wounded area at 0 day-cell-free space in the wounded area)/wounded area at 0 day x100]. The percent ratio of migrated area to wounded area was measured at 3 points per each wound (9 points/well in total) under a microscope (Nikon).

In vitro cell migration and invasion assays. Trans-well chambers with 8 μm porosity (Corning Incorporated) were used for these assays. For the migration assays, the lower side of the trans-well chamber membranes was coated with gelatin (10 μg/well; Sigma-Aldrich; Merck KGaA). For the invasion assays, a Matrigel invasion chamber (Corning Incorporated) was used. Cells were suspended in serum-free medium with normal IgG or anti-hTM4SF5 antibody (10 μg/ml) and placed on the top of the trans-well chamber. DMEM medium containing 10% FBS was placed in the lower chamber. After incubation for 48 h, the cells that invaded the lower surface of the filters were fixed, stained with crystal violet, and counted under a microscope (Nikon). The migrated and invaded cells were calculated by the following formula: The migrated or invaded cells (%)=(the total area-cell-free space in the total area)/the total area x100.

Statistics. The results are shown as the mean ± standard error of the mean (SEM) from at least three independent experiments. Statistical significance of the differences between two samples was evaluated using Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Validation of the TM4SF5 expression in human pancreatic cancer cell lines. Previously, we checked TM4SF5 expression in human pancreatic cancer tissues by immunohistochemistry (10,22). To validate the function of TM4SF5 in human pancreatic cancer cells, we first checked the mRNA levels of TM4SF5 in non-cancerous human pancreatic duct epithelial cell line H6c7 and several human pancreatic cancer cell lines such as ASPC-1, Capan-1, Capan-2, CFPAC-1, Mia-PaCa-2, and PANC-1 by RT-PCR. As shown in Fig. 1A, TM4SF5 mRNA was expressed in ASPC-1, Capan-1, Capan-2, and CFPAC-1 cells but barely expressed in Mia-PaCa-2, PANC-1, and H6c7 cells. Next, we investigated expression of TM4SF5 protein using immunostaining and confocal microscopy in Capan-2 and PANC-1 cells as a representative TM4SF5-positive and TM4SF5-negative cell line, respectively. In accordance with the mRNA expression, TM4SF5 protein was detected in Capan-2 cells but not in PANC-1 cells (Fig. 1B). Expression of TM4SF5 protein in the cells couldn't be verified by western blot analysis because the anti-TM4SF5 antibody has very low sensitivity when used for western blot analysis.

Suppression of human pancreatic cancer cell growth by treatment with the anti-hTM4SF5 antibody. The tetraspanins, including TM4SF5, regulates tumor growth and metastasis by interaction with integrins in HCC (11,24-26). Previously, we confirmed that immunization with the TM4SF5 peptide vaccine suppressed growth of TM4SF5-expressing mouse pancreatic cancer cells *in vivo* (22). Therefore, we first checked the growth of TM4SF5-expressing human pancreatic cancer cells after treatment with anti-hTM4SF5 antibody interrupting the function of TM4SF5. To check the cell growth, we measured the proliferation rate of human pancreatic cancer cells using the BrdU incorporation assay. The proliferation rates were significantly decreased by the treatment with anti-hTM4SF5 antibody compared to normal IgG treatment

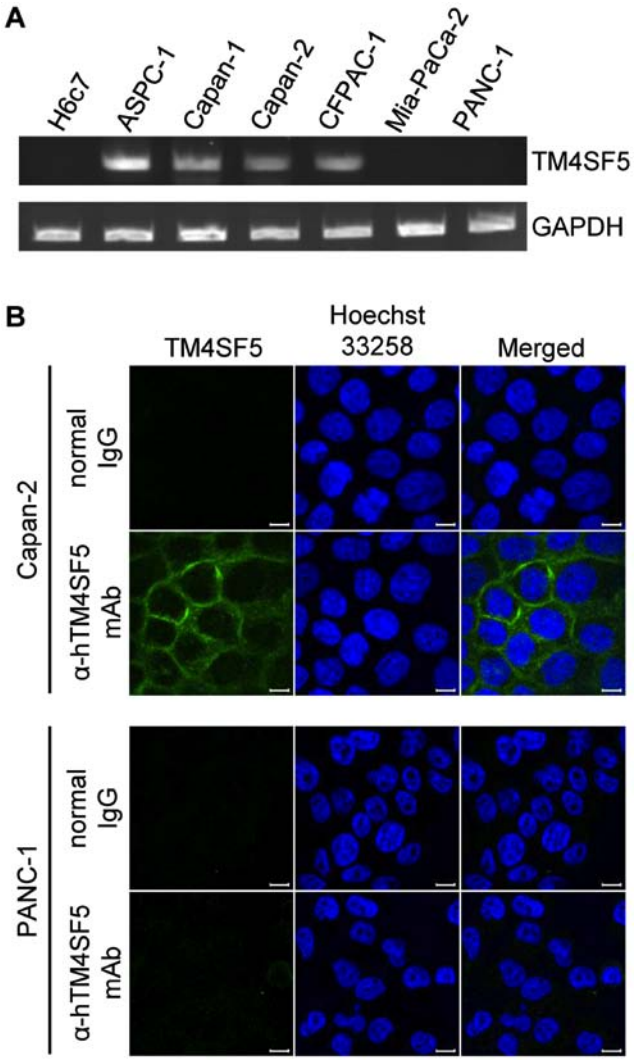

Figure 1. Validation of TM4SF5 expression in non-cancerous human pancreatic duct epithelial cells and human pancreatic cancer cells. (A) The expression of TM4SF5 mRNA was analyzed using RT-PCR. GAPDH was used as a loading control. (B) The expression of TM4SF5 protein was detected using immunostaining and confocal microscopy using humanized anti-hTM4SF5 monoclonal antibody (anti-hTM4SF5 mAb). Normal IgG was used as a control. Scale bar, 10 μm. TM4SF5, transmembrane 4 superfamily member 5 protein; RT, reverse transcription.

in TM4SF5-positive cell lines (ASPC-1, Capan-1, Capan-2, and CFPAC-1). However, there was no difference between the treatment groups in TM4SF5-negative cell lines (Mia-PaCa-2 and PANC-1) (Fig. 2). These data revealed that anti-hTM4SF5 antibody suppresses growth of TM4SF5-expressing human pancreatic cells.

Suppression of human pancreatic cancer cell motility by treatment with the anti-hTM4SF5 antibody. Previously, we reported that targeting of TM4SF5 inhibits motility of HCC and colon cancer cells *in vitro* and *in vivo* (10,14,15). Therefore, we checked the motility of human pancreatic cancer cells using wound healing assay and transwell migration/invasion assay after treatment with the anti-hTM4SF5 antibody. As shown in Fig. 3A, the wound healing activity was significantly decreased by the treatment with the anti-hTM4SF5 antibody compared to normal IgG in the TM4SF5-positive cell line Capan-2. In contrast, the anti-hTM4SF5 antibody treatment had no effect in the TM4SF5-negative cell line PANC-1. The transwell migration and invasion activities were reduced by the anti-hTM4SF5 antibody treatment, but not by the normal IgG treatment, in Capan-2. However, anti-hTM4SF5 antibody had no effect in PANC-1 (Fig. 3B and C). Similar results were obtained in other TM4SF5-positive cell lines (ASPC-1 and CFPAC-1) and TM4SF5-negative cell line Mia-PaCa-2 (Fig. S1). Therefore, these results have shown that the anti-hTM4SF5 antibody inhibits the motility of TM4SF5-expressing pancreatic cancer cells *in vitro*.

Molecular change of EMT markers by anti-hTM4SF5 antibody treatment. In our previous studies, molecular levels of the EMT markers were changed by targeting TM4SF5 with antibody in HCC and colon cancer cells (10,15). Therefore, we checked the expression of EMT markers after anti-hTM4SF5 antibody treatment in human pancreatic cancer cells. Vimentin and E-cadherin are mesenchymal and epithelial markers, respectively, and their levels of expression change during EMT (27,28). In the TM4SF5-positive cell lines Capan-2 and ASPC-1, the expression level of E-cadherin was increased at 5 days after treatment with anti-hTM4SF5 antibody compared to the normal IgG (Fig. 4). Similar results were obtained in another TM4SF5-positive cell line CFPAC-1 (Fig. S2). In ASPC-1 cells, the expression level of Vimentin was decreased by the treatment with anti-hTM4SF5 antibody. There was no expression of Vimentin in Capan-2 (Fig. 4) and CFPAC-1 (Fig. S2) cells irrespective of antibody treatment. TM4SF5-negative cell lines PANC-1 (Fig. 4) and Mia-PaCa-2 (Fig. S2) did not express or only slightly expressed E-cadherin and commonly expressed Vimentin. The expression levels of E-cadherin and Vimentin were not changed by treatment with the anti-hTM4SF5 antibody or the normal IgG in the cells. These data suggest that the anti-hTM4SF5 antibody can inhibit EMT associated with tumor progression in TM4SF5-positive pancreatic cancer cells.

Establishment of the TM4SF5-overexpressing human pancreatic cancer cells. To validate the effects of TM4SF5 expression in human pancreatic cancer cells directly, we established a human pancreatic cancer cell line stably expressing TM4SF5 using TM4SF5-negative Mia-PaCa-2 cells. For exogenous expression of TM4SF5 in TM4SF5-negative pancreatic cancer cells, we used a retroviral system. We established control Mia-PaCa-2 cells transduced with the parental pLXSN vector (Mia-PaCa-2-mock) and TM4SF5-expressing Mia-PaCa-2 cells transduced with the recombinant vector pLXSN-hTM4SF5 (Mia-PaCa-2-hTM4SF5). To validate expression of TM4SF5, we detected TM4SF5 mRNA levels by RT-PCR (Fig. 5A). The TM4SF5 mRNA was detected in the clones A1 and A5 of Mia-PaCa-2-hTM4SF5 cells, but not in the clones of Mia-PaCa-2-mock cells. As we obtained similar results from the two clones, we further analyzed the clone A5 of Mia-PaCa-2-hTM4SF5 for the remaining investigation. The expression level of TM4SF5 protein was also checked by immunoprecipitation followed by western blot analysis (Fig. 5B). TM4SF5 protein was detected in the Mia-PaCa-2-hTM4SF5 cells, but not in the Mia-PaCa-2-mock cells.

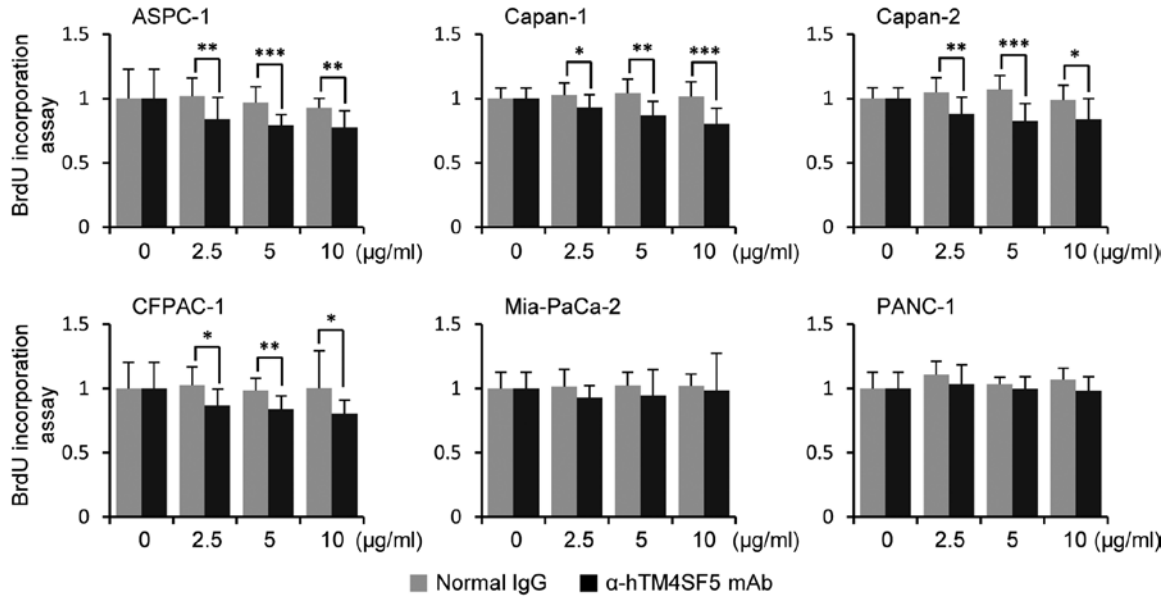

Figure 2. The effect of anti-hTM4SF5 mAb on the growth of human pancreatic cancer cells. Cell growth was measured using a BrdU incorporation assay. Values are the means ± SEM. *P<0.05, **P<0.01, ***P<0.005 vs. each normal IgG control. TM4SF5, transmembrane 4 superfamily member 5 protein.

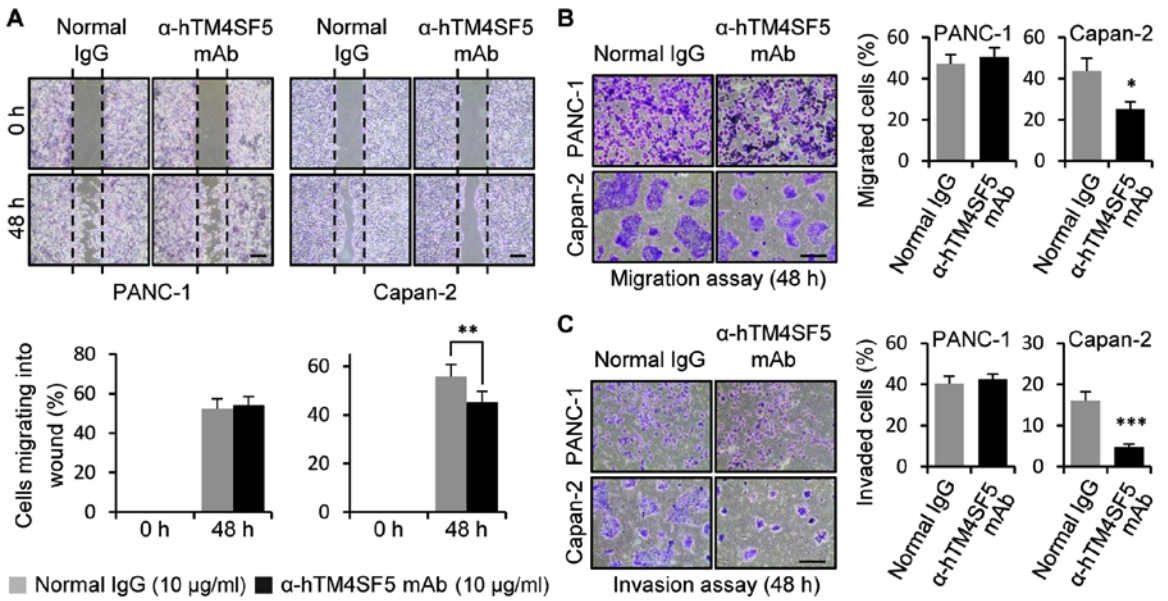

Figure 3. Motility change of human pancreatic cancer cells by anti-hTM4SF5 mAb treatment. The motility properties of Capan-2 and PANC-1 cells were compared after treatment with normal IgG or the anti-hTM4SF5 mAb. (A) Wound healing assay. A monolayer culture of Capan-2 and PANC-1 was wounded with a pipette tip, and the migration of cells into the wound area was examined at the indicated time points. Scale bar, 200 μm. (B) Migration and (C) invasion activity, the migrated and invaded cells on the lower sides of the transwell chambers were counted after incubation with the indicated materials. Scale bar, 100 μm. The percentage of wound healed, migrated or invaded cells was measured and compared. Values are the means ± SEM. *P<0.05, **P<0.01, ***P<0.005 vs. normal IgG control. TM4SF5, transmembrane 4 superfamily member 5 protein.

To analyze the cellular change induced by TM4SF5 expression, we checked cell growth and motility of the Mia-PaCa-2-hTM4SF5 and Mia-PaCa-2-mock cells. First, we measured the change of cell growth using a BrdU incorporation assay. The cell growth was increased in Mia-PaCa-2-hTM4SF5 cells compared to Mia-PaCa-2-mock cells (Fig. 5C). Next, we measured the change of cell motility using a wound healing assay and a transwell migration/invasion chamber. The cell migration into the wound area was increased in Mia-PaCa-2-hTM4SF5 cells compared to Mia-PaCa-2-mock cells (Fig. 5D), and the rate of cell migration and invasion through the transwell chamber also increased in Mia-PaCa-2-hTM4SF5 cells (Fig. 5E). Therefore, we conclude that expression of TM4SF5 enhanced cell growth and motility in human pancreatic cancer cells.

Suppression of cell growth and motility by anti-hTM4SF5 antibody treatment in TM4SF5-overexpressing pancreatic cancer cells. To check whether the down-regulation of cell proliferation and motility by the treatment with

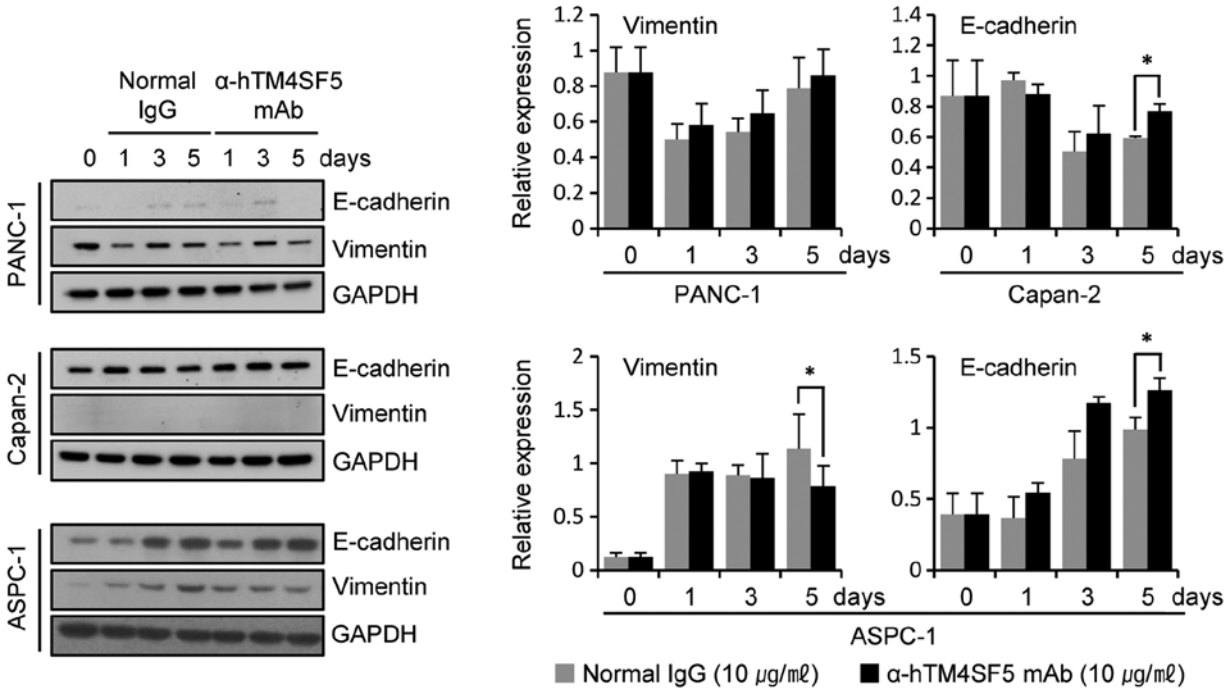

Figure 4. Changes in EMT marker expression in human pancreatic cancer cells after anti-hTM4SF5 mAb treatment. The expression of E-cadherin and Vimentin in Capan-2, PANC-1, and ASPC-1 cells were analyzed using western blot analysis after treatment with normal IgG or the anti-hTM4SF5 mAb at the indicated time points. The relative intensities of E-cadherin and Vimentin bands are indicated as a graph after normalization with GAPDH. P-values are evaluated using a ratio paired t-test. Values are the means ± SEM. *P<0.05 vs. normal IgG control. EMT, epithelial-mesenchymal transition; TM4SF5, transmembrane 4 superfamily member 5 protein.

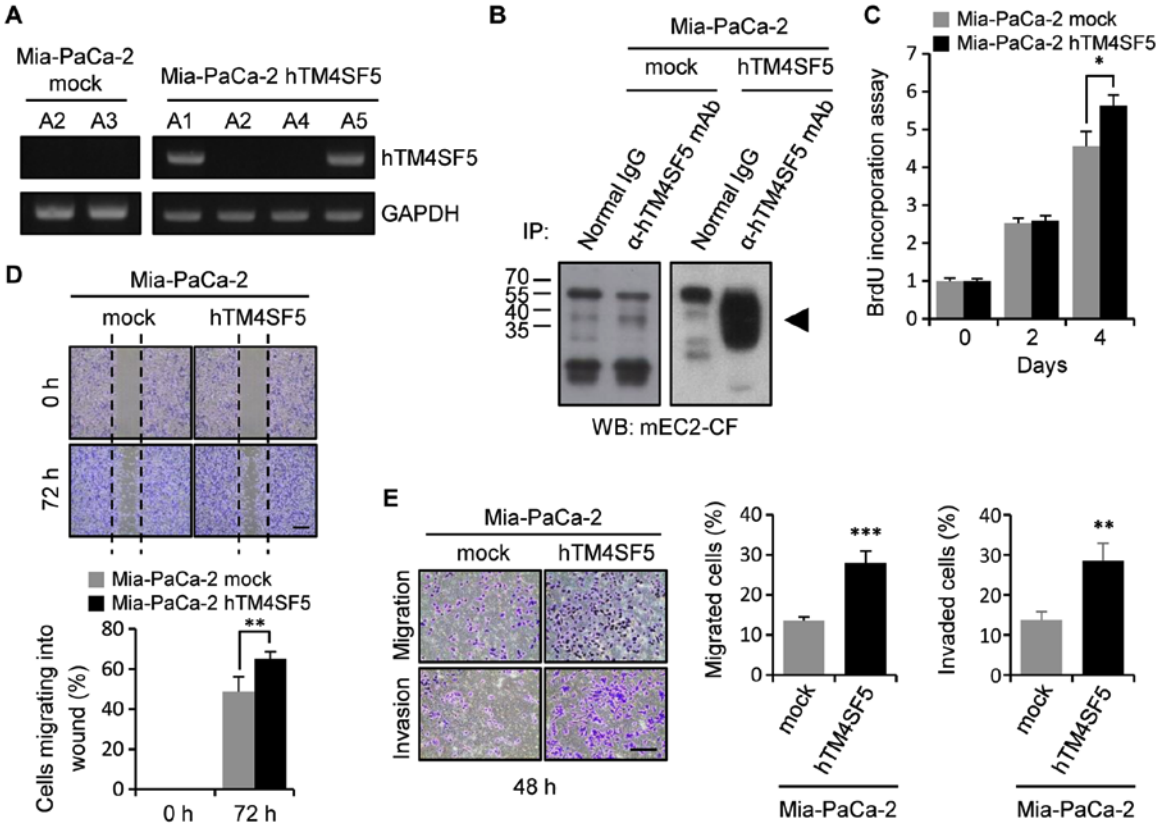

Figure 5. Establishment and characterization of TM4SF5-overexpressing human pancreatic cancer cells. (A) The expression of TM4SF5 mRNA in cells was determined using RT-PCR. GAPDH was used as a loading control. (B) The expression levels of TM4SF5 protein in cells were verified by immunoprecipitation and western blot analysis using the humanized anti-hTM4SF5 mAb (hEC2-C-2) and mouse anti-hTM4SF5 monoclonal antibody (mEC2-CF). Arrow head, TM4SF5. (C) The cell growth was measured using a BrdU incorporation assay. (D) Wound healing assay. Scale bar, 200 μm. (E) The migratory and invasive properties. The percentage of wound healed, migrated or invaded cells was measured and compared. Values are the means ± SEM. *P<0.05, **P<0.01, ***P<0.005 vs. Mia-PaCa-2-mock. TM4SF5, transmembrane 4 superfamily member 5 protein; RT, reverse transcription.

anti-hTM4SF5 antibody observed in human pancreatic cancer cells naturally expressing TM4SF5 (Figs. 2-4) also occurs in TM4SF5-overexpressing pancreatic calls, we measured cell proliferation and motility after treatment with anti-hTM4SF5 antibody in the Mia-PaCa-2-hTM4SF5 cells. First, we measured the cell proliferation using a BrdU incorporation assay. The cell proliferation was significantly reduced in the Mia-PaCa-2-hTM4SF5 cells by treatment with anti-hTM4SF5 antibody compared to normal IgG but was not changed in the Mia-PaCa-2-mock cells (Fig. 6). Next, we measured migration activity. As shown in Fig. 7A, the wound healing activity was decreased by the anti-hTM4SF5 antibody treatment compared to the normal IgG treatment in the Mia-PaCa-2-TM4SF5 cells. The rate of migration/invasion was also decreased by anti-hTM4SF5 antibody treatment compared to the normal IgG control in the Mia-PaCa-2-hTM4SF5 cells (Fig. 7B and C). However, there was no difference induced by the antibody treatment in Mia-PaCa-2-mock cells. These results show that anti-hTM4SF5 antibody reduced cell proliferation and motility of the TM4SF5-overexpressing pancreatic cancer cells as it reduced these parameters of the TM4SF5-positive pancreatic cancer cells.

Modified expression of EMT markers after anti-hTM4SF5 antibody treatment in TM4SF5-overexpressing pancreatic cancer cells. Because the cell motility was increased by TM4SF5 transduction (Fig. 5D and E) and decreased by anti-hTM4SF5 antibody treatment in the TM4SF5-overexpressing pancreatic cancer cells (Fig. 7), we next checked whether expression of EMT markers (E-cadherin and Vimentin) in the TM4SF5-overexpressing pancreatic cancer cells was changed after anti-hTM4SF5 antibody treatment. The expression level of Vimentin was decreased by anti-hTM4SF5 antibody treatment in the Mia-PaCa-2-hTM4SF5 cells compared to normal IgG treatment. In contrast, the Vimentin expression level was not changed in Mia-PaCa-2-mock cells by anti-hTM4SF5 antibody treatment. The E-cadherin was not detected in Mia-PaCa-2-mock and Mia-PaCa-2-hTM4SF5 cells (Fig. 8). These data suggest that anti-hTM4SF5 antibody can influence mesenchymal-epithelial transition (MET) in a TM4SF5-overexpressing human pancreatic cancer cell model.

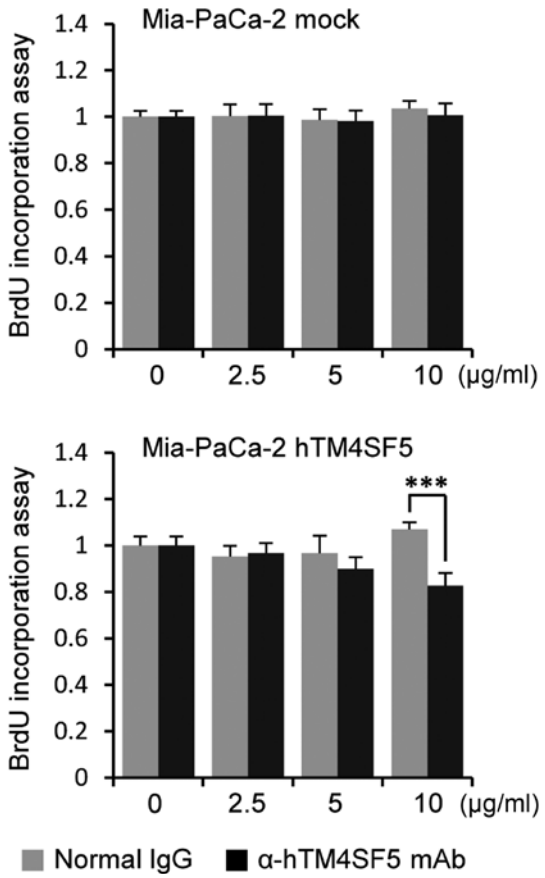

Figure 6. The effect of anti-hTM4SF5 mAb on the growth of TM4SF5-overexpressing human pancreatic cancer cells. The cell growth was measured using a BrdU incorporation assay. Values are the means ± SEM. ***P<0.005 vs. each normal IgG control. TM4SF5, transmembrane 4 superfamily member 5 protein.

Discussion

Previously, we found that TM4SF5 can be a target for therapy and prevention of HCC and colon cancer (10,14,15,20,21). Ahn *et al* also produced TM4SF5-targeted chimeric antibodies using phage display method and showed that TM4SF5-targeting antibodies had anti-cancer activity in TM4SF5-expressing HCC and colon cancer (29). Because expression of TM4SF5 in pancreatic cancer was previously reported (8,10), here we investigated expression and function of TM4SF5 in human pancreatic cancer cell lines and confirmed anti-cancer effects of the antibody targeting TM4SF5 on TM4SF5-expressing cells to evaluate its possible application to pancreatic cancer.

Treatment of TM4SF5-expressing human pancreatic cancer cells with anti-hTM4SF5 antibody significantly suppressed cell growth (Figs. 2 and 6) and motility (Figs. 3, 7 and S1). Furthermore, the expression of EMT markers was changed by treatment of anti-hTM4SF5 antibody (Figs. 4, 8 and S2). Taken together, these results show that high expression of TM4SF5 can endow the human pancreatic cells with oncogenic properties and that anti-hTM4SF5 antibody has therapeutic effects in pancreatic cancer cells, suggesting possible application of the anti-hTM4SF5 antibody in treating pancreatic cancer. From a practical perspective, the anti-hTM4SF5 antibody can be applied to antibody-drug conjugates (ADC). The use of ADCs is an emerging strategy for anticancer therapy that combines antibody-mediated targeted treatment with cytotoxic chemotherapy drugs (30). The ADCs induce specific targeting and therapeutic effects through antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) (31).

E-cadherin and Vimentin are typical EMT markers. Loss of E-cadherin expression induced or contributed to drug resistance of colon cancer and breast cancer (32,33). In addition, Vimentin expression was shown to be involved in the drug resistance of colon cancer (34). EMT marker expression is correlated with conventional drug resistance also in pancreatic cancer cells, and suppression of mesenchymal marker ZEB-1 induces an increase of E-cadherin and overcoming of drug resistance (35,36). Based on our results, E-cadherin was not or very weakly detected in PANC-1 and Mia-PaCa-2, and Vimentin was not or very

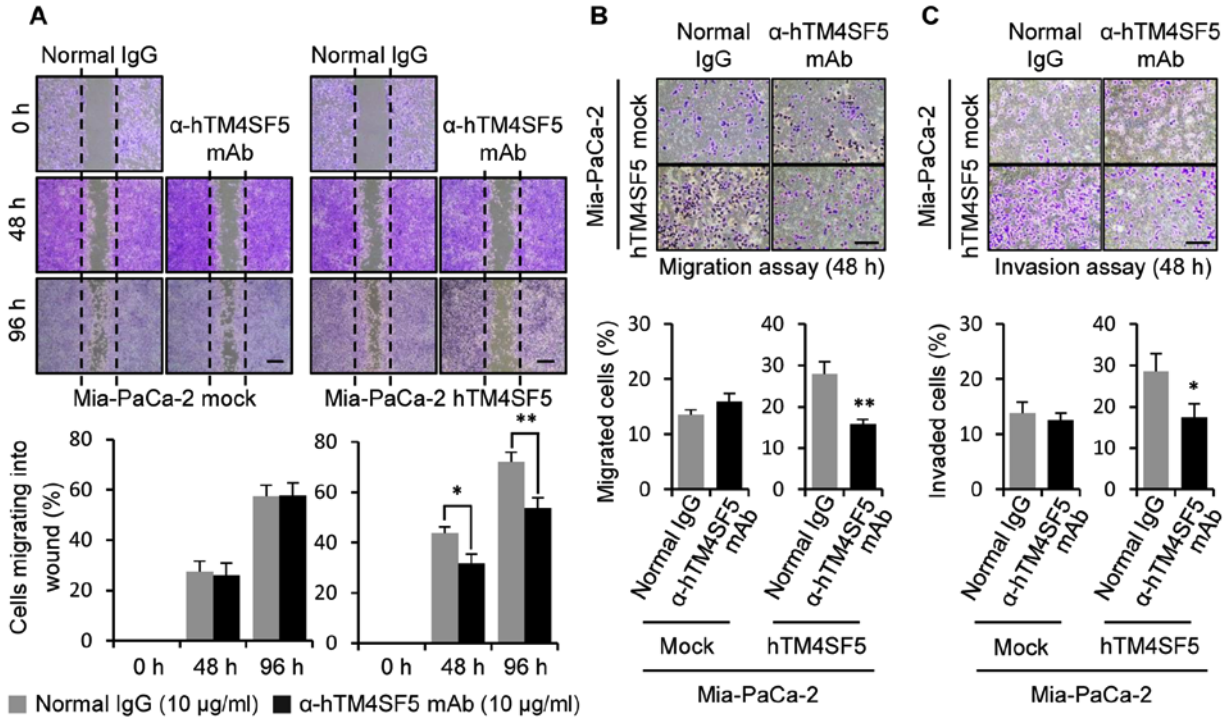

Figure 7. Motility change of TM4SF5-overexpressing human pancreatic cancer cells by the anti-hTM4SF5 mAb treatment. The motility properties of the Mia-PaCa-2-mock and Mia-PaCa-2-hTM4SF5 cells were compared after treatment with normal IgG or the anti-hTM4SF5 mAb. (A) Wound healing assay. Scale bar, 200 μm. (B) Migratory and (C) invasive properties. Scale bar, 100 μm. The percentage of wound healed, migrated or invaded cells was measured and compared. Values are the means ± SEM. *P<0.05, **P<0.01 vs. normal IgG control. TM4SF5, transmembrane 4 superfamily member 5 protein.

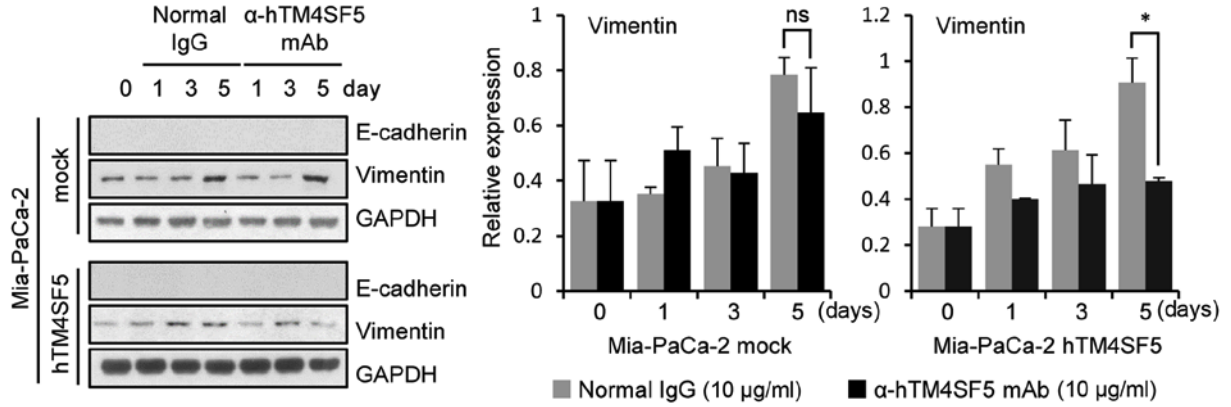

Figure 8. Change of EMT marker expression in TM4SF5-overexpressing human pancreatic cancer cells after the anti-hTM4SF5 mAb treatment. The expression levels of E-cadherin and Vimentin were analyzed using western blot analysis at the indicated time points after treatment with normal IgG or the anti-hTM4SF5 mAb. GAPDH was used as a loading control. Values are the means ± SEM. *P<0.05 vs. normal IgG control. EMT, epithelial-mesenchymal transition; TM4SF5, transmembrane 4 superfamily member 5 protein.

weakly detected in Capan-2 and CFPAC-1. These expression patterns in these cell lines have been reported by many groups and are associated with cellular phenomenon (37-41). In terms of anti-cancer drug resistance, anti-cancer drug sensitive cells (BxPC-3, HPAC, ASPC-1, and CFPAC-1) expressed E-cadherin, whereas the less sensitive cells (PANC-1 and Mia-PaCa-2) expressed Vimentin (39). In terms of invasive properties, E-cadherin was expressed in low invasive cells such as BxPC-3, CFPAC-1, and SW1990, and Vimentin was expressed in highly-invasive cells such as PaTu8988 (37). In addition, E-cadherin was expressed in cells showing epithelial characteristics (Capan-2 and BxPC-3), and Vimentin was expressed in mesenchymal-like cells (Mia-PaCa-2) in terms of cell shape (41). Based on our investigation, all the TM4SF5-expressing cells we examined in detail (ASPC-1, Capan-2, and CFPAC-1) expressed E-cadherin. Furthermore, the TM4SF5-positive cells were responsive to the suppressive effects of anti-TM4SF5 antibody. Considering regulation of EMT marker expression by the anti-hTM4SF5 antibody (Figs. 4 and 8) and correlation between drug resistance and EMT properties, the anti-hTM4SF5 antibody treatment may enhance the efficacy of anti-cancer reagent in chemotherapy of pancreatic cancer patients.

For the treatment of pancreatic ductal adenocarcinoma (PDAC), Gemcitabine has been considered a first-line therapy. However, gemcitabine treatment provides only a slight effect and consequently the overall survival of patients is approximately 6 months (42). Therefore, investigators have explored various therapeutic strategies including the use of therapeutic antibodies (43-45). Various therapeutic antibody candidates bind to different targets: Cetucimab, anti-EGFR chimeric antibody (46); trastuzumab, humanized anti-ErbB2/HER2 antibody (47); tigatuzumab, humanized anti-death receptor 5 antibody (48); cixutumumab, anti-IGF-1R antibody (49); bevacizumab, humanized anti-VEGF-A antibody (50), and so on. The antibodies have been studied and used in clinical trials to treat pancreatic cancer (43-45,51). Despite these attempts, the clinical trials did not show adequate clinical outcomes, and pancreatic cancer remains a lethal disease. Therefore, continuous investigation and new target discovery are needed for the treatment of pancreatic cancer. Previously, we suggested TM4SF5 as an anti-cancer target of pancreatic cancer because vaccination with TM4SF5 peptide vaccine suppressed growth of TM4SF5-expressing tumors in a mouse pancreatic cancer model (22). However, the effect of peptide vaccine may have limitations because of low antigenicity and tumor heterogeneity (52). Therefore, therapeutic antibodies, which can be evaluated in detail and applied promptly in necessity, may have advantages in the aspect of practical application. Therefore, it was required to investigate the efficacy of the anti-hTM4SF5 antibody using human pancreatic cancer cells. In this study, we found that treatment of the anti-hTM4SF5 antibody suppressed the growth and motility of TM4SF5-expressing pancreatic cancer cells. In addition, TM4SF5 expression induced growth and motility of pancreatic tumor cells. Although effectiveness and safety of anti-TM4SF5 antibody *in vivo* have to be tested using animal model in the future, we believe that our approach and results may respectively provide a novel strategy and useful information to treat pancreatic cancer.

Acknowledgements

Not applicable.

Funding

The current study was supported by grants from the National Research Foundation (grant nos. NRF-2015R1A2A2A01007209 and NRF-2018R1A2B6002504) funded by the Ministry of Science and ICT in the Republic of Korea.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SP, HJK and YL conceived the study and its design, and wrote the manuscript. SP and JAP performed experiments including PCR, cell line establishment, proliferation assays, migration assays and western blot analysis. DK performed immunostaining and confocal analysis. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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