Monoclonal antibody targeting of the cell surface molecule TM4SF5 inhibits the growth of hepatocellular carcinoma

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Running Title: Anti-tumor Activity of an anti-TM4SF5 Antibody
Abstract

The cell surface transmembrane receptor TM4SF5 has been implicated in hepatocellular carcinoma (HCC) but its candidacy as a therapeutic target has not been evaluated. Building on a finding that immunization with a peptide vaccine targeting human TM4SF5 can exert prophylactic and therapeutic effects in a murine model of HCC, we developed a monoclonal antibody to characterize expression of TM4SF5 in HCC and target its function there as an anticancer strategy. We found that the antibody modulated cell signaling in HCC cells in vitro, reducing cell motility, modulating E-cadherin expression, altering p27kip1 localization and increasing RhoA activity. Using a mouse xenograft model of human HCC, we documented the in vivo efficacy of the antibody which suppressed tumor growth in either tumor prevention or treatment designs. Our work offers a preclinical proof of concept for TM4SF5 as a promising target for antibody therapeutics to treat HCC.

Key words: TM4SF5, antibody, therapeutics, HCC, mouse model
Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers and the second or third most frequent cause of cancer-related death worldwide (1, 2). HCC is also the most common type of liver cancer and is usually associated with prior hepatitis virus infection or liver cirrhosis. The primary treatment for HCC is surgical resection; however, the high frequency of recurrence and poor liver function following the removal of liver tissue are significant obstacles to recovery (3). Furthermore, HCC is frequently resistant to conventional chemotherapy and radiotherapy (4). Therefore, understanding the signaling pathways involved in HCC development and developing targeted therapies is likely to be an important strategy for the effective treatment of HCC (3, 5).

Tetraspanins, also known as transmembrane 4 superfamily (TM4SF) members, mediate signal transduction events that regulate cell differentiation, activation, growth and migration (6, 7). For example, TM4SF4 regulates endocrine pancreas differentiation and inhibits Rho family of GTPase-activated cell migration and actin organization in a Rho kinase (ROCK)-independent fashion (7). The diverse activities of tetraspanins seem to be related to their capacity to associate with various molecules, including integrins and other tetraspanins (8, 9). Lately, tetraspanins have also gained attention as both suppressors and promoters of metastasis depending on the particular tetraspanins (10). Therefore, tetraspanins have emerged as diagnostic and prognostic markers, as well as therapeutic targets for preventing tumor progression (10, 11).

TM4SF5 is highly expressed in colon carcinomas, pancreatic tumors, and HCC (12, 13). Lee et al. showed that TM4SF5 is involved in HCC development, specifically by inducing morphological elongation, epithelial-mesenchymal transition (EMT), uncontrolled cell
proliferation, and angiogenesis (13, 14). A synthetic inhibitor targeting TM4SF5, 4’-(p-toluenesulfonyl-amido)-4-hydroxychalcone (TSAHC), has been shown to inhibit HCC growth and metastasis in vitro and in vivo (15). Therefore, TM4SF5 seems to play an important role in HCC formation, and it is a rational molecular target for the clinical development of HCC therapeutics (16).

In previous studies, we produced a TM4SF5-specific monoclonal antibody by immunizing mice with a peptide corresponding to a B-cell epitope of human TM4SF5 (denoted the hTM4SF5R2-3 peptide); this peptide was coencapsulated with CpG-DNA in a phosphatidyl-β-oleoyl-γ-palmitoyl ethanolamine : cholesterol hemisuccinate (DOPE:CHEMS) liposome complex (called Lipoplex(O)) (17). We then confirmed that the antibody markedly delayed the growth of the Huh-7 human HCC cell line, which express endogenous TM4SF5 (18). We also showed that active immunization with the peptide vaccine had prevented the growth of tumors from subsequently transplanted HCC cells and suppressed the growth of tumors in mice bearing mouse HCC-cell derived tumors (18, 19).

Herein, we analyzed expression of TM4SF5 in human HCC tissue samples using an anti-TM4SF5 monoclonal antibody. We also assessed the anti-invasiveness and anti-migratory properties of the antibody in vitro in HCC cells. Finally, we measured in vivo anti-tumor activity of the antibody against mouse-derived and human-derived HCC tumors in mice and found that passive immunization, by intraperitoneal injection of this monoclonal antibody, inhibits the progression of tumors in these mouse models of HCC. Therefore, we provide direct evidence that anti-TM4SF5 monoclonal antibody can be a potential therapeutics to treat HCC.
Materials and Methods

Production of the mouse anti-human TM4SF5R2-3 monoclonal antibody

As described previously (17, 20), BALB/c mice were injected intraperitoneally with the hTM4SF5R2-3 peptide derived from human TM4SF5 (138NRTLWDRCEAPPRV151) and CpG-DNA coencapsulated in a DOPE:CHEMS complex four times, each at 10-day intervals. In accordance with the standard hybridoma purification protocols, we screened for hybridoma cells that produced the anti-hTM4SF5R2-3 peptide-specific monoclonal antibody (21). The anti-TM4SF5 monoclonal antibody (IgG2a) was purified from the ascitic fluid by protein A column chromatography.

Surface plasmon resonance (SPR) analysis

The binding affinity of the anti-hTM4SF5 monoclonal antibody to the human (hTM4SF5R2-3) and mouse TM4SF5R2-3 peptides (mTM4SF5R2-3) was measured using Biacore 3000 at 25°C. Biotinylated peptides were captured on the flow cell surface of a SA sensor chip coated with streptavidin. Injection of a buffer solution containing biotin served as a negative control. Data were evaluated using Biacore Bia evaluation software version 3.0 (Biacore). The data were analyzed using fitting models for the ligand-analyte interaction such as 1:1 (Langmuir) binding, 1:1 binding with drifting baseline, 1:1 binding with mass transfer, and bivalent analyte. Based on the results, 1:1 binding with mass transfer was selected as a best model. The parameters such as association rate (ka), dissociation rate (kd), the equilibrium dissociation constant (binding constant, $K_D$, $kd/ka$), and the chi square value were calculated using the software. The sensorgram data were extracted using the software and the graphs were reproduced using Microsoft Excel program to show the binding affinity.
Tissue microarrays and immunohistochemistry

For the HCC and normal tissue analysis, formalin-fixed, paraffin-embedded AccuMax tissue arrays were purchased from ISUABXIS with the approval of the Institutional Review Board in Hallym University. The following arrays were used: normal tissues (A103(9)), various human cancer tissues with corresponding normal tissues (A301(VI)) and human liver cancer tissues (A204, A204(II) and A204(III)). The tissue arrays were stained with the anti-hTM4SF5 monoclonal antibody (10 μg/ml), according to standard procedures. The percentages of cells expressing TM4SF5 were calculated as the number of TM4SF5-positive cells divided by the total number of cells in each tumor type.

Cell culture

The human HCC cell lines, Huh-7 and SNU-739, were obtained from the Korean Cell Line Bank and were maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS). The cell lines were characterized by the cell bank using DNA fingerprinting analysis, species verification test, mycoplasma contamination test, and viral contamination test. Mouse BNL 1ME A.7R.1 HCC cells (BNL-HCC cells) were obtained from ATCC (ATCC® TIB-75™) and maintained in DMEM medium containing 10% FBS. ATCC characterized the cell line with tests for morphology, post-freeze viability, interspecies determination (isoenzyme analysis), mycoplasma contamination, and bacterial and fungal contamination. All cells were cultured at 37°C in an atmosphere containing 5% CO₂. We made stocks for each cell line at early passages, and cultures were maintained until passage 20 (within 2 months) and then discarded.

In vitro cell migration and invasion assays
Trans-well chambers with 8 μm porosity were used for these assays. For migration assays, the lower side of the trans-well chamber membranes was coated with gelatin (10 μg/well). For invasion assays, the upper and lower sides of the chamber membranes were coated with Matrigel (1.2 mg/ml) (BD Biosciences) and gelatin, respectively. Huh-7 cells and SNU-739 cells were suspended (1.6~3.3 x 10^5 cells/ml) in serum-free medium with mouse IgG2a isotype control (IgG2a control; Bethyl Laboratories) or the anti-TM4SF5 monoclonal antibody (10 μg/ml) and placed on the top of the trans-well chamber. RPMI medium containing 10% FBS was placed in the lower chamber. After incubation for 12 h ~ 72 h, the cells that invaded to the lower surface of the filters were fixed, stained with crystal violet, and counted under a microscope (E-200, Nikon).

**In vitro wound-healing assays**

For wound-healing assays, 1x10^6 cells (Huh-7 cells or SNU-739 cells) were placed in a 6-well plate, cultured overnight to confluence in medium containing serum, and the monolayer was wounded with a pipette tip. PBS, IgG2a control, or the anti-hTM4SF5 monoclonal antibody (10 μg/ml) was added to the medium for the indicated times. The cells were fixed with 4% paraformaldehyde for 30 min and stained with Giemsa for 30 min. The number of cells that migrated into the wounded area was counted in three wells per experimental treatment and three wounds per well under a microscope (TS100, Nikon).

**Confocal microscopy**

Cells were cultured on glass cover slips in 12-well plates 18 h prior to treatment with the anti-hTM4SF5 monoclonal antibody (10 μg/ml). After treatment with the antibody for the indicated times, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.1%
Triton X-100, and stained with the anti-p27kip1 antibody (Rabbit monoclonal Ab, Cell Signaling Technology, Cat. No. 3686) or the anti-E-cadherin antibody (two different antibodies: rabbit monoclonal antibody for Fig. 3, Cell Signaling Technology, Cat. No. 3195 and rabbit polyclonal antibody for Supplementary Fig. S6, Abcam, Cat. No. ab15148), for 2 h. After extensive washing in PBS, the samples were incubated with Alexa Flour 488 (or Alexa Flour 546)-conjugated goat anti-rabbit IgG for 1 h. The nuclei were stained with Hoechst 33258, and the mounted samples were scanned with an LSM 710 (Carl Zeiss).

**Biodistribution imaging in vivo**

5 mg/ml of the anti-TM4SF5 monoclonal antibody and IgG2a control (or normal mouse IgG) in PBS solution was adjusted to contain 50 mM borate buffer (pH 8.5). Proteins were conjugated with DyLight 755 and purified using a DyLight 755 Antibody Labeling Kit (Thermo Scientific) in accordance with the manufacturer’s specifications. Fifty micrograms of DyLight 755-labeled anti-TM4SF5 monoclonal antibody or DyLight 755-labeled IgG2a control (or normal mouse IgG) was injected into the intraperitoneal cavity of BALB/c control mice or mice bearing BNL-HCC cell derived tumors. The distribution profiles of the anti-TM4SF5 monoclonal antibody were quantified by *in vivo* fluorescence using the real-time IVIS imaging system 200 (Xenogen Corp.) at the indicated time intervals. To determine the distribution of the DyLight 755-labeled anti-TM4SF5 monoclonal antibody in HCC tumor tissues, the tissues were removed aseptically at 72 h post injection. The tissues were frozen and cut into 4-μm-thick slices using Cryostat. The slices were stained with SYTOX Green dye for nuclei, and the mounted samples were scanned with an LSM 710.

**Animals**
Four-week-old male BALB/cAnCrj-nu/nu mice and BALB/c mice were obtained from Central Lab. Animal Inc. The mice were maintained under specific pathogen-free conditions. All procedures involving animal studies were in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Veterinary Research & Quarantine Service of Korea. The protocol was approved by the Institutional Animal Care and Use Committee of Hallym University (Permit Number: Hallym 2010-10, Hallym 2012-66, Hallym 2013-104). The mice were sacrificed under Zoletil 50+Rompun anesthesia, and all efforts were made to minimize suffering.

**Hepatocellular carcinoma mouse model**

For the xenograft assays, thirty BALB/cAnCrj-nu/nu mice were inoculated subcutaneously in the dorsal right flank with 5x10⁶ Huh-7 cells in 50% Matrigel as previously described (22). When tumors reached 5 mm in diameter, the mice were randomly divided into three treatment groups (eight mice/group): PBS, IgG2a control, and the anti-TM4SF5 monoclonal antibody. The antibodies (25 mg/kg) were injected twice weekly into the intraperitoneal cavity. Tumor diameters were measured using calipers at 4-day intervals, and tumor volumes were calculated using the formula, width² × length/2, as described previously (18). The mice were sacrificed 40 days after Huh-7 cells injection and the tumors were weighed. For the syngeneic transplanted tumor assay, thirty BALB/c mice were inoculated subcutaneously in the dorsal right flank with 5x10⁶ BNL-HCC cells containing 50% Matrigel and the effects of injected PBS, normal IgG control, and the anti-TM4SF5 monoclonal antibody were evaluated as described above. Tumor diameters were measured at 7-day intervals, and tumor volumes were calculated. BALB/c mice were sacrificed 10 weeks after antibody injection, and the tumors were weighed.
Histology and immunohistochemistry

For histopathological examinations, the tumors and organs were removed and fixed in a 4% buffered formalin solution overnight, embedded in paraffin using standard methods, and cut into 5-μm-thick sections. The deparaffinized sections were then stained with hematoxylin and eosin (H&E). To assess the expression of TM4SF5, the deparaffinized sections were stained with the anti-TM4SF5 monoclonal antibody (10 μg/ml), according to standard procedures using Histostain Plus kit. The samples were then counterstained with hematoxylin. All images were examined using a Nikon Eclipse E-200 microscope (Nikon).

Statistics

Results are expressed as mean ± standard deviation. Statistical significance between two samples was evaluated using the Student’s t test. A p-value of <0.05 was taken as statistically significant.
Results

Specificity and affinity of the anti-TM4SF5 monoclonal antibody

As previous data had shown that an anti-TM4SF5 monoclonal antibody reacted against mouse TM4SF5, we sought to explore its specificity towards the human protein. In order to do this, we isolated an anti-TM4SF5 monoclonal antibody (clone #2D4-18) through hybridoma technology using spleen cells obtained from mice that had been immunized with a complex of TM4SF5 B-cell epitope (hTM4SF5R2-3 peptide) and Lipoplex(O), without carriers. The monoclonal antibody was purified from the ascitic fluid to high purity (Supplementary Fig. S1A). We investigated the reactivity of this anti-TM4SF5 monoclonal antibody against the human and mouse TM4SF5R2-3 peptides using enzyme-linked immunosorbent assays (ELISAs), and the anti-TM4SF5 monoclonal antibody reacted with both the human and mouse TM4SF5R2-3 peptides (Supplementary Fig. S1B and S1C). The cross-reactivity of this anti-TM4SF5 monoclonal antibody was also confirmed by a competitive ELISA assay. Mouse TM4SF5R2-3 peptide efficiently inhibited the ability of the anti-TM4SF5 monoclonal antibody to bind the human TM4SF5R2-3 peptide (Supplementary Fig. S1B), and vice versa (Supplementary Fig. S1C). Our microarray data revealed that TM4SF4 and TM4SF5 genes are highly expressed in Huh-7 cells compared with control SNU-739 cells (GEO accession number GSE57106, Supplementary Fig. S2A). To further confirm the specificity of this antibody, we analyzed its reactivity with the synthesized peptides derived from other human TM4SF members such as TM4SF1, TM4SF4, TM4SF18, and TM4SF19 (Supplementary Fig. S2B and 2C). The anti-TM4SF5 monoclonal antibody reacted only with TM4SF5R2-3 peptide. Moreover, we quantitatively measured the binding affinity of the anti-TM4SF5 monoclonal antibody using a surface plasmon resonance
biosensor, Biacore. The mAb reacted with human and mouse TM4SF5R2-3 peptides with a similar equilibrium dissociation constant ($K_d$) of $\sim 2$ nM (Fig. 1A and 1B). Together, these data show that the anti-TM4SF5 monoclonal antibody used in this study reacts specifically with TM4SF5 with a high affinity.

**Expression of TM4SF5 in human tissues from various origin**

It has been reported that TM4SF5 mRNA is expressed in colon carcinoma, pancreatic tumors, and HCC, as determined by reverse transcriptase (RT)-PCR and Northern blotting (12). Here, we investigated the expression of the TM4SF5 protein in various human tissues including human HCC specimens by immunohistochemical staining with the anti-TM4SF5 monoclonal antibody to validate the presence of the target in HCC.

First, we evaluated the expression and distribution of TM4SF5 protein in 45 different types of normal human tissue by immunohistochemistry. As shown in Supplementary Fig. S3, TM4SF5 was not expressed in normal tissues.

Next, the expression of TM4SF5 protein from different tumor types was evaluated by immunohistochemistry. As shown in Fig. 1C-1F and Table 1, there was no expression of TM4SF5 in normal liver, however all of the HCC tissues expressed TM4SF5 (staining of $>11\%$ of the tumor cells present in all samples). Analysis of 105 HCC specimens showed that 30\% of HCC tissue samples expressed TM4SF5 in $>75\%$ of the tumor cells, and 45\% and 25\% of HCC were positive for TM4SF5 expression with 50-74\% and 11-49\% (Table 1). The levels of TM4SF5 immunostaining did not correlate with tumor grade or stage (Supplementary Fig. S4). In addition to HCC, TM4SF5 expression was also detected in colon cancer and pancreatic cancer tissues, in agreement with the previously published mRNA data (12) (Fig. 1G and 1H). These results confirm that this monoclonal antibody is useful for
TM4SF5 protein detection in human tissues. In contrast, prominent expression of TM4SF5 was not detected in other cancer types we tested (Supplementary Fig. S5). Considering a recent report that 45% of human esophageal cancer tissues showed expression of TM4SF5 in >50% of the tumor cells (23) and the fact that the cancer tissue array we used was composed of just one case per each cancer, detailed studies are required to clarify the implication of TM4SF5 in other cancer types.

**Effect of the anti-TM4SF5 monoclonal antibody on human HCC cell migration**

The tetraspanin superfamily, including TM4SF5, activates integrin-mediated signaling pathways that are pivotal for cell migration/invasion and tumor cell metastasis (10,13,14). Therefore, we evaluated the influence of the anti-TM4SF5 antibody on cell migration and invasion using human HCC cells of Huh-7 cell line expressing TM4SF5. As shown in Figs 2A and 2B, we found that the addition of the anti-TM4SF5 monoclonal antibody, but not PBS or IgG2a control, inhibited the migration of Huh-7 cells. In contrast, the antibody had little effect on the migration of SNU-739 cells that do not express TM4SF5. In addition, we performed a wound healing assay *in vitro*. As shown in Fig. 2C, the migration of Huh-7 cells into the wounded area was significantly reduced in the presence of the anti-TM4SF5 antibody but not PBS or IgG2a control. However, there was no difference in wound healing capacity in SNU-739 cells treated with IgG2a control or the anti-TM4SF5 antibody (Fig. 2C). These results confirm that targeting TM4SF5 with this antibody reduces HCC cell motility *in vitro*.

**Effect of the anti-TM4SF5 monoclonal antibody on E-cadherin expression and actin organization in HCC cells**

E-cadherin is one of membrane adhesion molecules involved in cell-cell interaction, and
reduced E-cadherin expression is associated with EMT and metastasis (24). As the anti-TM4SF5 antibody reduces mobility of HCC cells, we then investigated expression of E-cadherin after treatment of Huh-7 cells and SNU-739 cells with anti-TM4SF5 antibody. First, we monitored E-cadherin expression by immunostaining and confocal microscopy. As shown in Figs 3A and 3B, E-cadherin expression was enhanced by the anti-TM4SF5 antibody in Huh-7 cells but not SNU-739 cells. There was no change in the cells treated with PBS or IgG2a control (Fig. 3 and Supplementary Fig. S6). Accordingly, total E-cadherin protein expression increased following treatment of Huh-7 cells with the anti-TM4SF5 antibody, as determined by western blotting (Fig. 3C). There was no change in expression of E-cadherin in SNU-739 cells after antibody treatment.

Next, we monitored actin organization in Huh-7 cells and SNU-739 cells after treatment with PBS, IgG2a control, or anti-TM4SF5 antibody using Phalloidin staining as well as paxillin staining. PBS and IgG2a control-treated Huh-7 cells displayed aberrant actin bundling, but treatment with the anti-TM4SF5 antibody resulted in the formation of well defined stress fibers (Supplementary Fig. S7A). In contrast, the well-defined stress fibers in SNU-739 cells supporting polygonal morphology were unaffected by treatment (Supplementary Fig. S7A). Staining of the focal adhesion molecule paxillin also revealed that the number of focal adhesion sites increased specifically in Huh-7 cells after treatment with anti-TM4SF5 antibody (Supplementary Fig. S7B). Taken together, these results indicate that the anti-TM4SF5 monoclonal antibody modulates actin polymerization in TM4SF5 expressing cells.

The anti-TM4SF5 monoclonal antibody leads to nuclear translocation of p27kip1 and increased Rho activity in HCC cells
The cyclin-dependent kinase inhibitor p27kip1 negatively regulates cell proliferation through inhibition of G1/S cell cycle progression (25). When we treated the TM4SF5-expressing Huh-7 cells with anti-TM4SF5 antibody, we found that growth of Huh-7 cells were suppressed (18). Furthermore, previous study showed that p27kip1 is localized in the cytosol of HCC cells overexpressing TM4SF5 (26). Therefore, we examined expression and localization of p27kip1 in Huh-7 and SNU-739 cells. Immunostaining and confocal microscopy revealed that p27kip1 expression was not clearly detected in the nucleus of Huh-7 cells prior to treatment with the anti-TM4SF5 antibody. However, following treatment with the anti-TM4SF5 antibody, p27kip1 staining was clearly observed in the nucleus (Fig. 3D). In contrast, p27kip1 localization did not change after treatment with the anti-TM4SF5 antibody in SNU-739 cells (Fig. 3E). Western blotting showed no prominent change in the total levels of p27kip1 protein expression in either cell line (Fig. 3F). Therefore, treatment with the anti-TM4SF5 antibody induces the translocation of p27kip1 from the cytosol to the nucleus and the nuclear p27kip1 may suppress cell growth in Huh-7 cells.

RhoA is a small G protein involved in contact inhibition and regulates the actin cytoskeleton in the formation of stress fibers (27). Furthermore, RhoA has been reported to be negatively regulated by p27kip1 via a protein-protein interaction (28-30). Therefore, translocation of p27kip1 into the nucleus after treatment with anti-TM4SF5 antibody may reduce its interaction with RhoA and may enhance RhoA activity. As expected, the interaction between p27kip1 and RhoA decreased and RhoA activity increased following treatment of Huh-7 cells with the anti-TM4SF5 antibody (Supplementary Figs. S7C and S7D). Treatment of Huh-7 cells with normal IgG had no effect. There was no effect in SNU-739 cells after treatment with normal IgG or anti-TM4SF5 antibody. Taken together, these results suggest that the treatment of TM4SF5 expressing HCC cells with anti-TM4SF5 antibody induces
intracellular molecular events leading to growth inhibition and enhanced adhesion.

**Localization of the injected anti-TM4SF5 monoclonal antibody on HCC tumors in vivo**

Given that the anti-TM4SF5 monoclonal antibody can inhibit the growth, invasion and migration of tumor cell lines, we next investigated whether it had any effect on tumors in vivo. First, we sought to determine distribution of the anti-TM4SF5 monoclonal antibody after injection using a syngeneic transplanted tumor model established with mouse BNL-HCC cells. The anti-TM4SF5 monoclonal antibody and mouse IgG2a control were conjugated with DyLight 755 (a fluorescent dye) and injected into the intraperitoneal cavity of mice harboring BNL-HCC cell derived tumors. After 72 h, the distribution of the DyLight 755-labeled antibody was quantified by measuring the total photon flux (photons/sec) of the fluorescence. As shown in Fig. 4A and 4B, the DyLight 755-labeled anti-TM4SF5 monoclonal antibody was localized in the tumors, whereas DyLight 755-labeled IgG2a control was not detected in the mice. When we excised the tumor mass and analyzed microsections of the frozen tissue, we found that many of the tumor cells were stained with the DyLight 755-labeled anti-TM4SF5 antibody (Fig. 4C). In contrast, we could not detect any labeling in the control sections obtained from mice injected with DyLight 755-labeled IgG2a control.

Next, we injected the DyLight 755-labeled anti-TM4SF5 monoclonal antibody into the intraperitoneal cavity of control mice and mice harboring tumors to examine the time-dependent distribution of the DyLight 755-labeled antibody over 96 h. As shown in Supplementary Fig. S8, the highest fluorescence was detected in the abdominal region at 0 h, and the signal became gradually distributed throughout the whole body over 12 h. The fluorescence signal was focused in the tumor region between 24 and 96 h in the tumor-bearing mice. However, the fluorescence gradually disappeared in the normal mice possibly
because of secretion (Figure 4 and Supplementary Fig. S8). Therefore, the anti-hTM4SF5 monoclonal antibody can target tumor cells expressing TM4SF5 \textit{in vivo}.

The anti-TM4SF5 monoclonal antibody inhibits HCC tumor growth in a xenograft mouse model

To evaluate the efficacy of the anti-TM4SF5 monoclonal antibody against HCC tumors in mice, we determined the effect of the anti-TM4SF5 monoclonal antibody on the growth of HCC cells \textit{in vivo} using a xenograft mouse model, which involves human cells, before the syngeneic transplanted tumor model. We injected nude mice subcutaneously in the dorsal right flank with Huh-7 cells and allowed the tumors to grow. When the tumor reached 5 mm in diameter, the animals were treated with mouse IgG2a control or anti-TM4SF5 monoclonal antibody by intraperitoneal injection. Using both tumor volume and weight as measures, we observed that the antibody attenuated the progression of HCC tumors compared to PBS or IgG2a control-treated animals (Fig. 5A-C). However, antibody treatment did not affect the body weight of the mice during this experiment suggesting that the antibody has no prominent side effects (Fig. 5D). The expression of TM4SF5 in HCC xenograft tumor tissue was confirmed by immunohistochemistry using the anti-TM4SF5 antibody (Fig. 5E). Together, these experiments suggest that the anti-TM4SF5 monoclonal antibody can attenuate tumor grown in mouse xenograft models of HCC.

The anti-TM4SF5 monoclonal antibody inhibits HCC tumor growth in a syngeneic transplanted tumor model

To investigate whether the effect of the anti-TM4SF5 antibody on HCC tumor growth was maintained in a syngeneic transplanted tumor model, we used BNL-HCC cells. As
shown in Fig. 6A-C, treatment with the anti-TM4SF5 monoclonal antibody significantly suppressed the progression of HCC tumors compared to the mice treated with control IgG. The anti-hTM4SF5 antibody did not affect the body weight of mice during the experiment, suggesting that the antibody has no prominent side effects (Fig. 6D). Together, these experiments suggest that the anti-TM4SF5 monoclonal antibody can inhibit tumor growth in mouse syngeneic transplanted tumor models of HCC.
Discussion

In this study, we confirmed that the TM4SF5 protein is over-expressed in human HCC, colon, and pancreatic cancer tissues using a high affinity monoclonal antibody against TM4SF5. Interestingly, TM4SF5 expression was detected in all of the HCC tissue samples we examined, at various expression levels (Table 1). Therefore, we focused on HCC in this study; however, further investigation of TM4SF5 expression and function in colon and pancreatic cancer is warranted. In previous studies, we found that treatment with the anti-TM4SF5 monoclonal antibody inhibited the growth of human and mouse HCC cells and accordingly decreased the proportion of cells in S-phase (18). Here, we further investigated in vitro and in vivo effects of anti-TM4SF5 antibody and the molecular mechanisms underlying the functional effects.

Treatment of Huh-7 cells with the anti-TM4SF5 monoclonal antibody significantly inhibited cell motility in vitro, as determined by migration, invasion, and wound healing assays (Fig. 2). Furthermore, the expression of E-cadherin, stress fiber formation, and focal adhesion in Huh-7 cells was enhanced in response to the anti-TM4SF5 monoclonal antibody (Fig. 3). Taken together, we can conclude that anti-TM4SF5 antibody provoke multiple in vitro anti-cancer effects including reduced cell growth, reduced motility, and enhanced adhesion.

E-cadherin, a member of calcium-dependent adhesion molecules mainly expressed in epithelial cells, is known to be downregulated in the situation of EMT and metastasis (24). Enhanced expression of E-cadherin in response to anti-TM4SF5 antibody treatment suggests a possibility that TM4SF5 suppresses transcription of E-cadherin and the anti-TM4SF5 monoclonal antibody prevents the activity of TM4SF5. Tetraspanins are components of microdomains that involve many different molecules, such as extracellular matrix proteins,
adhesion molecules, cytokine receptors, and integrins (8, 9). Therefore, the anti-TM4SF5 monoclonal antibody may also contribute to the interaction of TM4SF5 with adhesion molecules, such as E-cadherin, by stabilizing the cell-cell contacts, as has previously been suggested for antibody-based immunotherapeutics targeting another tetraspanin, CD151 (11).

Fine regulation of RhoA activity is tightly associated with motility, and CDK inhibitors, such as p27kip1, p21WAF1, and p57, are known to modulate motility through interruption of the RhoA/ROCK signaling pathway (28-30). Treatment of Huh-7 cells with the anti-TM4SF5 monoclonal antibody induced the translocation of p27kip1 from the cytosol into the nucleus (Fig. 3). p27kip1 can play a role as a G1 checkpoint in the nucleus, which might contribute to the decrease in the S-phase population and the reduced tumor cell growth observed in response to the anti-TM4SF5 antibody (18). Furthermore, translocation of p27kip1 was accompanied by the reduced association of p27kip1 and RhoA and enhanced RhoA activity (Supplementary Fig. S7). Previously, Lee et al. demonstrated that over-expression of TM4SF5 induces loss of contact inhibition through epithelial-mesenchymal transition (EMT) (13). They revealed that TM4SF5 over-expression enhanced the expression of p27kip1 in the cytosol, reduced RhoA activity, and reduced E-cadherin expression. Therefore, treatment of TM4SF5-expressing cells with an anti-TM4SF5 monoclonal antibody reverses the molecular events induced by TM4SF5 over-expression. Taken together, our results suggest that the anti-TM4SF5 monoclonal antibody blocks tumorigenic TM4SF5 signaling, which inhibits tumor cell growth and modulates the adhesion and migration properties of TM4SF5-expressing cells in vitro. Our results also suggest a possible application of anti-TM4SF5 antibody for therapy against HCC.

To confirm the anti-tumor activity of the anti-TM4SF5 monoclonal antibody in vivo, we administered the anti-TM4SF5 antibody into mice bearing tumors composed of human or
mouse HCC cells. The anti-TM4SF5 monoclonal antibody localized to HCC tumors in mice (Fig. 4), and it significantly suppressed tumor growth (Fig. 5 and Fig. 6). Therefore, our results demonstrate that this anti-TM4SF5 monoclonal antibody has therapeutic effects in a mouse model of HCC and suggest that using this TM4SF5-specific monoclonal antibody could be investigated as a therapeutic strategy for HCC in humans.

The anti-tumor activity of the anti-TM4SF5 monoclonal antibody in vivo can be partially explained by the direct tumor suppressing activity of this antibody revealed in vitro. In addition, the antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) could play a role in vivo. ADCC involving antibodies and Fc receptors of cytotoxic cells such as NK cells, macrophages, and neutrophiles is known to be an important mechanism involved in the anti-tumor function of therapeutic antibodies such as trastuzumab and tituximab (31). Antigen-specific antibodies can trigger CDC through the classical complement pathway (32). Therefore, the immunological function of anti-TM4SF5 monoclonal antibody in mice has to be examined. Induction of the antibody-mediated effector function is highly dependent on the isotype of the antibody, and most of the potent therapeutic antibodies on the market are IgG1 (33) probably because IgG1 is the most abundant IgG subclass in human serum and has high affinity for Fc receptors on phagocytic cells in human (34). The anti-TM4SF5 monoclonal antibody used in this study is the isotype IgG2a. As IgG2a has extremely low affinity for Fc Receptors on phagocytic cells in human (34), further consideration of the isotype may be required for the future application of this antibody in humans. Considering that TM4SF5 is involved in EMT and metastasis (13-16), and that the treatment of TM4SF5-expressing cells with the anti-TM4SF5 monoclonal antibody reduced motility and enhanced expression of E-cadherin, the anti-TM4SF5 monoclonal antibody may have anti-metastatic activity and therefore may be able to
contribute to preventing metastasis in patients with primary HCC. Therefore, this topic warrants further investigation in the future.

In this study, we validated anti-TM4SF5 monoclonal antibody as an efficacious therapeutics in mouse syngeneic transplanted tumor model and xenograft model. Further studies on the immunological mechanisms of antibody action, production and \textit{in vitro} evaluation of humanized antibodies, and \textit{in vivo} evaluation of the humanized antibody in the context of safety and efficacy may provide important information for future application in humans.

Acknowledgements

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References


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Figure legends

Figure 1. Reactivity of the mouse anti-TM4SF5 monoclonal antibody with TM4SF5 peptides and TM4SF5 protein expressed in human tissues. A-B, Binding affinity of anti-TM4SF5 monoclonal antibody for the human TM4SF5R2-3 (A) and mouse TM4SF5R2-3 (B) peptides was analyzed using Biacore. Biotinylated peptides were immobilized on a CM4 sensor chip, and increasing amounts of antibody were applied. Kinetic parameters of binding reaction are shown under the sensorgrams. These results are representatives of at least three independent experiments. C-H, Immunohistochemical analysis of hepatocellular carcinoma (HCC) and other tissue arrays was performed using the anti-TM4SF5 monoclonal antibody. Scale bars; left panel, 200 μm. right panel, 50 μm. C, Normal liver tissue. D, Examples of HCC tissues with 11-49% of tumor cells expressing TM4SF5. E, Examples of HCC tissues with 50-74% of tumor cells expressing TM4SF5. F, Examples of HCC tissues with >75% of tumor cells expressing TM4SF5. G, Colon cancer tissue. H, Pancreatic cancer tissue.

Figure 2. Effects of the anti-TM4SF5 monoclonal antibody on HCC cell migration and invasion. The migratory and invasive properties of Huh-7 cells and SNU-739 cells were compared after treatment with phosphate-buffered saline (PBS), IgG2a control (IgG2a) or the anti-TM4SF5 monoclonal antibody. The number of migrated or invaded cells was counted and compared (graphs). A, Migration. After incubation with the indicated material for 12 h (SNU-739 cells) or 24 h (Huh-7 cells), the migrated cells on the lower sides of the membranes were counted. Scale bars, 100 μm. B, Invasion. After incubation with PBS, IgG2a control or the anti-TM4SF5 monoclonal antibody for 24 h (SNU-739 cells) or 72 h (Huh-7 cells), the invading cells on the lower sides of the membranes were counted. Scale bars, 100 μm.
μm. C, Wound-healing activity. A monolayer culture of Huh-7 and SNU-739 was wounded with a pipette tip, and the effect of PBS, IgG2a control or the anti-TM4SF5 monoclonal antibody was examined at the indicated time points. Scale bars, 100 μm. These results are representatives of three independent experiments. **p<0.01, mean ± SD.

**Figure 3.** Effects of the anti-TM4SF5 monoclonal antibody on E-cadherin expression and p27kip1 translocation. A-B, E-cadherin expression was examined by immunostaining and confocal microscopy after treatment with PBS, IgG2a control, or the anti-TM4SF5 monoclonal antibody for 4 days in Huh-7 cells (A) and SNU-739 cells (B). The secondary antibody control without the anti-E-cadherin antibody staining is shown as (-) E cadherin. C, Total levels of E-cadherin in Huh-7 (left) and SNU-739 (right) cells were examined by western blotting. The amounts of β-actin are shown as a protein loading control. D-E, The expression and location of p27kip1 were examined by immunostaining and confocal microscopy after treatment with IgG2a control or the anti-TM4SF5 monoclonal antibody for 12 h in Huh-7 cells (D) and SNU-739 cells (E). F, Total levels of p27kip1 protein in Huh-7 (left) and SNU-739 (right) cells were examined by western blotting. The amounts of β-actin are shown as a protein loading control. These results are representatives of three independent experiments. Scale bars, 10 μm.

**Figure 4.** Biodistribution of the anti-TM4SF5 monoclonal antibody in HCC tumor tissue. BALB/c mice were injected with BNL-HCC cells to generate tumor-bearing mice. DyLight 755-labeled IgG2a control or the anti-TM4SF5 monoclonal antibody was injected into the intraperitoneal cavity of the mice, and fluorescence was examined after 72 h. A-B,
Fluorescence of the mice was examined using the real-time IVIS imaging system 200. The intensity of fluorescence is indicated on the right. A, Whole body. B, The dissected mice. C, The dissected tumor tissue was frozen, and the microsection samples were stained with SYTOX Green dye (for nuclei) and analyzed by confocal microscopy. Scale bars, 20 μm. These are representative of three independent experiments.

**Figure 5.** Therapeutic efficacy of the anti-TM4SF5 monoclonal antibody against HCC tumor growth in a xenograft mouse model. A mouse xenograft model was established by the implantation of Huh-7 cells in BALB/cAnCrj-nu/nu mice. PBS, IgG2a control or the anti-TM4SF5 monoclonal antibody were injected into the mice when the tumors reached 5 mm in diameter, and tumor growth was monitored for 40 days (n=8 each). Four representative mice are shown. A, Macroscopic appearance of tumor tissues. B, Tumor volume. C, Individual tumor weights for each treatment group. Mean values are indicated as a horizontal bars. D, Individual body weights for each treatment group. E, Expression of TM4SF5 in the tumor tissues induced by Huh-7 cells. TM4SF5 expression was confirmed by immunohistochemical analysis using the anti-TM4SF5 monoclonal antibody. The TM4SF5 positive area is expressed as a brown color. H&E and IHC represent hematoxylin & eosin staining and immunohistochemistry, respectively. These results are representatives of three independent experiments. Scale bars, 10 μm. **p<0.01, mean ± SD.

**Figure 6.** Therapeutic efficacy of the anti-TM4SF5 monoclonal antibody against HCC tumor growth in syngeneic transplanted tumor model. A mouse tumor model was established by implantation of BNL-HCC cells in BALB/c mice. PBS, normal mouse IgG or the anti-TM4SF5 monoclonal antibody were injected intraperitoneally into the mice when the tumors...
reached 5 mm in diameter, and tumor growth was monitored for 70 days (n=8 each). Five representative mice are shown. A, Macroscopic appearance of tumor tissues. B, Individual tumor volumes. C, Individual tumor weights. Mean values are indicated as a horizontal bars. D, Individual body weights for each treatment group. These results are representatives of two independent experiments. *p<0.05, mean ± SD.
**Table 1. Immunohistochemical analysis of TM4SF5 expression in liver cancer tissues**

<table>
<thead>
<tr>
<th>HCC tissue sections (AccuMax Array)</th>
<th>n</th>
<th>TM4SF5 positive (%)</th>
<th>No. (%) cases of TM4SF5 staining</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>≥75%</td>
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<tr>
<td>A204</td>
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<tr>
<td>A204(II)</td>
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<td>A204(III)</td>
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<tr>
<td>total</td>
<td>105</td>
<td>100</td>
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</table>

The percentages in parentheses were calculated as the number of TM4SF5-positive samples for each quartile, divided by the total number of samples in each tumor type.
Figure 1.

A) hTM4SF5R2-3 peptide-biotin coating

- Anti-TM4SF Ab
  - 32 nM
  - 16 nM
  - 8 nM
  - 4 nM
  - 2 nM

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<td>480</td>
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1:1 binding with mass transfer

- $k_a$ (M$^{-1}$s$^{-1}$) $2.19 \times 10^7$
- $k_d$ (s$^{-1}$) 0.0619
- $K_D$ (M) $2.74 \times 10^{-9}$
- Fit ($\chi^2$) 7.14

B) mTM4SF5R2-3 peptide-biotin coating

- Anti-TM4SF Ab
  - 32 nM
  - 16 nM
  - 8 nM
  - 4 nM
  - 2 nM

<table>
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</table>

1:1 binding with mass transfer

- $k_a$ (M$^{-1}$s$^{-1}$) $2.34 \times 10^7$
- $k_d$ (s$^{-1}$) 0.0599
- $K_D$ (M) $1.87 \times 10^{-9}$
- Fit ($\chi^2$) 10.6

C) Normal liver

D) HCC 11-49%

E) HCC 50-74%

F) HCC ≥ 75%

G) Colon cancer

H) Pancreatic cancer
Figure 2.

A

**PBS Anti-TM4SF5 AbIgG2a**

**Migrated cell numbers (Huh-7 cells)**

**SNU-739 cells**

B

**PBS Anti-TM4SF5 AbIgG2a**

**Invaded cell numbers (Huh-7 cells)**

**SNU-739 cells**

C

**PBS Anti-hTM4SF5 AbIgG2a**

**Number of cells migrating into wound (Huh-7 cells)**

**SNU-739 cells**
Figure 3.

A. E-cadherin Nuclei Merged
B. E-cadherin Nuclei Merged
C. Huh-7 IgG2a Anti-TM4SF5 Ab 0 1 2 3 4 (days) E-cadherin β-actin
D. p27kip1 Nuclei Merged
E. SNU-739 IgG2a Anti-TM4SF5 Ab 0 1 2 3 4 (days) E-cadherin β-actin
F. Huh-7 IgG2a Anti-TM4SF5 Ab 0 1 3 6 12 (h) p27kip1 β-actin
Figure 4.

A

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<th>IgG2a-DyLight 755</th>
<th>Anti-TM4SF5 Ab-DyLight 755</th>
<th>IgG2a-DyLight 755</th>
<th>Anti-TM4SF5 Ab-DyLight 755</th>
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B

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C

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</tr>
<tr>
<td>Anti-TM4SF5 Ab-DyLight 755</td>
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</tr>
</tbody>
</table>
Figure 5.

A. Huh7/PBS  Huh-7 IgG2a  Huh7/Anti-TM4SF5 Ab

B. Tumor volume (mm$^3$)

C. Tumor weight (g)

D. Body weight (g)

E. Normal liver  Huh-7 tumor

H&E  IHC
Figure 6.

A

BNL-HCC/PBS  BNL-HCC/ normal IgG  BNL-HCC/ Anti-TM4SF5 Ab

B

PBS  BNL-HCC  BNL-HCC/ normal IgG  BNL-HCC/ Anti-TM4SF5 Ab

C

Tumor weight (g)

D

Body weight (g)
Monoclonal antibody targeting of the cell surface molecule TM4SF5 inhibits the growth of hepatocellular carcinoma

Sanghoon Kwon, Kyung-Chan Choi, Young-Eun Kim, et al.

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