

# Therapeutic effect of a TM4SF5-specific peptide vaccine against colon cancer in a mouse model

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**Molecular-targeted therapy has gained attention because of its high efficacy and weak side effects. Previously, we confirmed that transmembrane 4 superfamily member 5 protein (TM4SF5) can serve as a molecular target to prevent or treat hepatocellular carcinoma (HCC). We recently extended the application of the peptide vaccine, composed of CpG-DNA, liposome complex, and TM4SF5 peptide, to prevent colon cancer in a mouse model. Here, we first implanted mice with mouse colon cancer cells and then checked therapeutic effects of the vaccine against tumor growth. Immunization with the peptide vaccine resulted in robust production of TM4SF5-specific antibodies, alleviated tumor growth, and reduced survival rate of the tumor-bearing mice. We also found that serum levels of VEGF were markedly reduced in the mice immunized with the peptide vaccine. Therefore, we suggest that the TM4SF5-specific peptide vaccine has a therapeutic effect against colon cancer in a mouse model. [BMB Reports 2014; 47(4): 215-220]**

## INTRODUCTION

Vaccines usually represent biological reagents preventing diseases resulting from virulent bacteria or viruses by activating and thereby training the immunological defense system before infection in humans and animals. The vaccine concept has been broadened, however, to include prophylactic and therapeutic reagents to treat intractable diseases such as cancers and degenerative diseases (1, 2).

To enhance the efficacy of vaccines by stimulating the immune system, investigators usually use adjuvants such as alu-

minum salts, complete and incomplete Freund's adjuvants, organic adjuvants, and CpG-DNA (3). CpG-DNA is a sequence containing unmethylated CpG dinucleotides flanked by specific base sequences (4-6). CpG-DNA has immunostimulatory activities including activation of antigen-presenting cells, induction of Th1 responses, and immunoglobulin (Ig) isotype switching (7-9). It has accordingly been studied as an adjuvant for production of antibodies and efficacious induction of immunological responses *in vivo* (7-9). Previously, we isolated natural phosphodiester bond CpG-DNA (specifically, MB-ODN 4531(O)) from *Mycobacterium bovis* genomic DNA and confirmed its stimulating activity to induce optimal innate immune responses (10). We further enhanced the adjuvant activities of natural CpG-DNA by encapsulation in a special liposome complex composed of DOPE : CHEMS (1 : 1 ratio) (Lipoplex(O)) (11). With the aid of this novel adjuvant formulation, we confirmed that complexes of B cell epitope peptide and Lipoplex(O) significantly induced peptide-specific IgG production (12-14).

Cancer is one of the most critical causes of death worldwide. However, the efficacy of prevention and therapy of cancer remains limited as carcinogenesis is a multi-step process involving mutations in many different genes and signaling pathways (2). Therefore, understanding the main molecular signatures of cancers and screening proper targets of therapy are very important to treat cancers. Recently, molecular-targeted therapy involving tumor specific antigens or tumor-associated antigens has gained attention because these approaches are expected to have high efficacy and low side effects (15, 16).

The transmembrane 4 superfamily member 5 protein (TM4SF5), a member of the tetraspanin family with four hydrophobic transmembrane domains, was implicated in cancer (17-22). TM4SF5 was previously implicated in hepatocellular carcinoma (HCC) through involvement in epithelial-mesenchymal transition and uncontrolled cell proliferation (18,19). Previously, we proved that the transmembrane 4 superfamily member 5 protein (TM4SF5) can serve as a molecular target for hepatocellular carcinoma (HCC): a peptide vaccine targeting TM4SF5 had preventive or therapeutic effects against hepatocellular carcinoma in a mouse model (20). The peptide vac-

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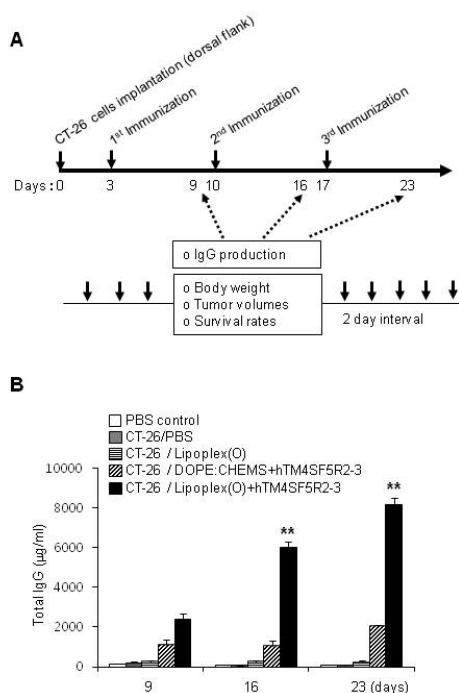
cine also induced long-term memory function in mice to enable production of TM4SF5-specific antibodies that enhanced survival after implantation of HCC cells (21). Furthermore, we confirmed that TM4SF5 can act as a tumor specific antigen to prevent colon cancer; the TM4SF5-specific peptide vaccine prevented growth of colon tumors in a mouse model (22).

In this study, we investigated the therapeutic effect of the TM4SF5-specific peptide against colon cancer in a mouse model and confirmed that immunization with a complex of TM4SF5 peptide and Lipoplex(O) reduced the growth of tumors derived from pre-implanted colon cancer cells in mice.

## RESULTS

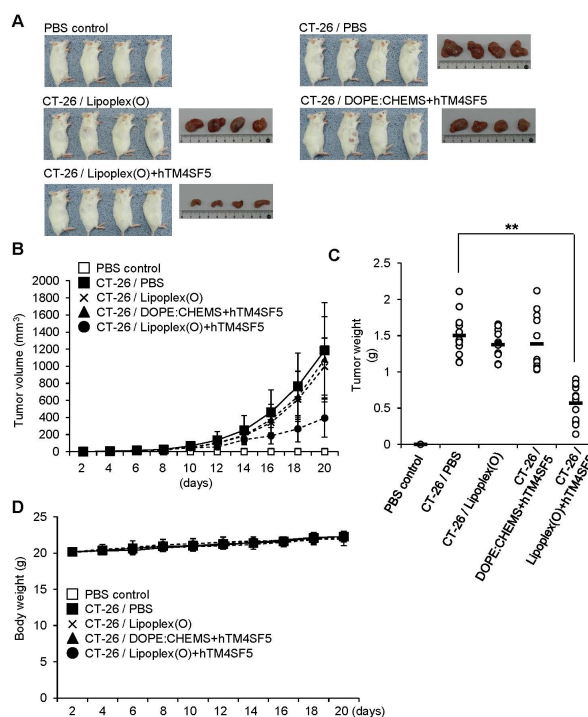
### Production of IgG by immunization with a complex of TM4SF5 peptide and Lipoplex(O) in the CT-26 cells implanted mice

To check the therapeutic effect of the peptide vaccine, mice were first implanted with CT-26 cells and then immunized with a complex of TM4SF5 peptide and Lipoplex(O) three times at 7



**Fig. 1.** Production of IgG by immunization with a complex of TM4SF5 peptide and Lipoplex(O). (A) Experimental schedule and parameters to check the mouse phenotypes. BALB/c mice were implanted with CT-26 cells, and after three days the mice were immunized with PBS, TM4SF5 peptide encapsulated in DOPE:CHEMS, or TM4SF5 peptide and Lipoplex(O) complex at 7 day intervals (n=12 per group). (B) Amount of IgG during the experimental periods. The sera were collected, and titers of the peptide-specific total IgG were assayed with an ELISA kit (n=3 per group). \*\*P < 0.01 (vs PBS control).

day intervals from three days after transplantation. The whole experimental schedule is shown in Fig. 1A. As a negative control, three groups of mice were injected with PBS, Lipoplex(O), or TM4SF5 peptide encapsulated in DOPE : CHEMS. When we checked immune response by measuring TM4SF5-specific IgG amounts, immunization with a complex of TM4SF5 peptide and Lipoplex(O) induced robust production of anti-TM4SF5 antibodies after two iterations of immunization and the amounts were further increased after the third immunization (Fig. 1B). A slight increase of anti-TM4SF5 antibodies was also found in the mice immunized with TM4SF5 peptide encapsulated in DOPE:CHEMS. However, the amount was much lower compared to the group immunized with TM4SF5 peptide and Lipoplex(O). Therefore, we confirmed that CpG-DNA is essential for robust antibody production.



**Fig. 2.** Suppression of tumor growth by a vaccine containing TM4SF5 peptide and Lipoplex(O) complex in the CT-26 cell implanted mice. BALB/c mice were injected with PBS control or CT-26 cells. CT-26 cell implanted mice were immunized with PBS, TM4SF5 peptide encapsulated in DOPE:CHEMS, or a complex of TM4SF5 peptide and Lipoplex(O). Tumor formation in the mice was compared (n=12 per group). (A) Macroscopic appearance of colon tumor tissues. Four representative mice out of 12 mice are shown. (B) Tumor volumes were calculated as (length × width<sup>2</sup>)/2. (C) Tumor growth was measured by tumor weight. (D) Body weights were measured at the indicated time intervals. \*\*P < 0.01.

### Therapeutic efficacy of the vaccine against colon cancer

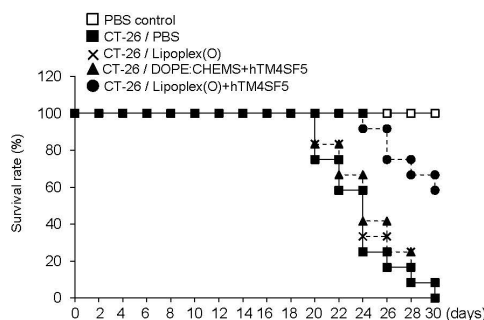
To evaluate the therapeutic anti-tumor activity of the vaccine, we checked the physical phenotype of mice at 20 days after challenge with CT-26 cells followed by three immunizations. The results regarding tumor size (Fig. 2A), tumor volume (Fig. 2B), and tumor weight (Fig. 2C) revealed that the tumor growth was significantly suppressed in the mice immunized with a complex of TM4SF5 peptide and Lipoplex(O). There was no significant effect in the mice immunized with Lipoplex(O) or TM4SF5 peptide encapsulated in DOPE : CHEMS, which is in agreement with the weak production of TM4SF5-specific antibodies (Fig. 1B). Immunization did not affect the body weight of the mice during the experiment, suggesting that there were no significant side effects (Fig. 2D).

### Enhanced survival of the tumor-bearing mice by immunization with a complex of TM4SF5 peptide and Lipoplex(O)

To further evaluate the therapeutic efficacy of the vaccine, we assessed the survival rate of the mice bearing colon cancer cell derived tumors until 30 days after implantation with CT-26 cells. As shown in Fig. 3, most of the unimmunized control mice (11 out of 12 mice) died by day 30 after implantation. In contrast, more than 60% of the mice immunized with a complex of TM4SF5 peptide and Lipoplex(O) survived until day 30. Survival of the mice injected with Lipoplex(O) or TM4SF5 peptide encapsulated with DOPE:CHEMS was similar to the survival rate of the mice injected with PBS after CT-26 implantation (Fig. 3). Therefore, we can conclude that the vaccine composed of TM4SF5 peptide and Lipoplex(O) showed a prominent suppressive effect against colon cancers.

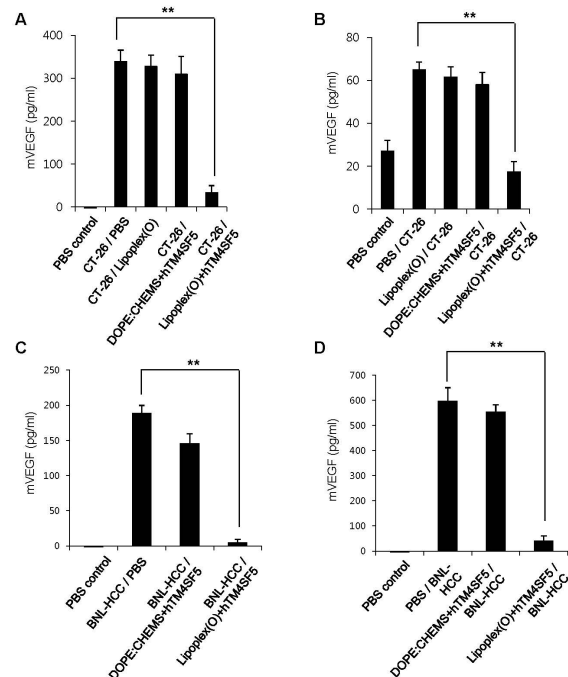
### Decreased serum levels of mouse VEGF in the mice immunized with a complex of TM4SF5 peptide and Lipoplex(O)

As VEGF is expressed in and released from cancer cells, serum levels of VEGF can be an indicator of tumor growth. To under-



**Fig. 3.** Survival rate of tumor-bearing mice. BALB/c mice were implanted with CT-26 cells and then immunized with PBS, TM4SF5 peptide encapsulated in DOPE:CHEMS, or the TM4SF5 peptide and Lipoplex(O) complex (n = 12 per group). The survival rate was recorded for 30 days after implantation.

stand the effect of peptide vaccine in terms of VEGF expression, we measured amounts of VEGF in the sera of the tested mice. As shown in Fig. 4A, there was a drastic increase of VEGF expression in the sera of the mice implanted with CT-26 cells. However, when the mice were immunized with TM4SF5 peptide and Lipoplex(O) after implantation, the serum levels of VEGF fell significantly to nearly basal levels (Fig. 4A). This was also observed when the mice were pre-immunized and then challenged with CT-26 cells to assess the preventive effects (Fig. 4B). Furthermore, similar results were obtained with a mouse HCC model using BNL-HCC cells in preventive or therapeutic experimental settings (Figs. 4C and 4D). Therefore, we suggest that immunization with a complex of TM4SF5 peptide and Lipoplex(O) inhibits growth of HCC and colon tumors and there-



**Fig. 4.** Decreased serum levels of VEGF in the mice immunized with a complex of TM4SF5 peptide and Lipoplex(O). Sera were obtained from the mice after implantation with CT-26 cells on day 23 (A, B) or BNL-HCC cells on day 30 (C, D) and the amounts of VEGF were measured with an ELISA kit (n = 5 per each group). (A) BALB/c mice were implanted with CT-26 cells and then immunized with the TM4SF5 peptide and Lipoplex(O) complex or indicated combinations. (B) BALB/c mice were immunized with the TM4SF5 peptide and Lipoplex(O) complex or indicated combinations, and then implanted with CT-26 cells. (C) BALB/c mice were implanted with BNL-HCC cells and then immunized with the TM4SF5 peptide and Lipoplex(O) complex or indicated combinations. (D) BALB/c mice were immunized with the TM4SF5 peptide and Lipoplex(O) complex or indicated combinations, and then implanted with BNL-HCC cells. \*\*P < 0.01.

by down-regulates serum VEGF expression from the tumors.

## DISCUSSION

Prevention is the best medicine; however the efficacy of preventive vaccines against cancer is very limited (2, 23). Furthermore, most cancers are examined only after some period after onset. Therefore, therapeutic approaches for cancer are practically necessary for most real cases. Previously, we confirmed TM4SF5 as a target for an anti-cancer vaccine against HCC and colon cancer in a mouse model (20, 22). The TM4SF5-specific peptide vaccine was effective against HCC in prophylactic and therapeutic settings (20). For colon cancer, we recently confirmed that the vaccine has a preventive effect (22). Here, we suggest that the vaccine has therapeutic effects against colon cancer in a mouse model.

When we previously examined the therapeutic efficacy of the peptide vaccine in a mouse HCC model, we implanted BALB/c mice with mouse HCC cells of the BNL-HCC cell line and waited until the tumor size reached 5 mm. We then immunized the mice with the peptide vaccine three times at 10 day intervals to check the therapeutic effects of the vaccine (20). We first employed the same protocol for the mice implanted with CT-26 cells. However, the experiment failed as the mice died before we completed the entire immunization protocol because of the rapid growth of CT-26 cells. Therefore, we modified the experimental procedure to start immunization of the mice with the TM4SF5-specific peptide vaccine three days after CT-26 cells implantation. The immunization interval was also reduced from 10 days to 7 days. In this modified setting, we found that the immunization induced robust production of TM4SF5-specific antibody (Fig. 1) and the growth of tumors derived from the implanted CT-26 cells was suppressed by immunization with the peptide vaccine (Figs. 2 and 3). The tumor volume and tumor weight decreased, and the survival rate of tumor-bearing mice increased. Therefore, we carefully conclude that the TM4SF5-specific peptide vaccine has therapeutic effects against colon cancer to some extent. Considering the differences in HCC and colon cancer models, it is likely that the vaccine immunization protocols have to be optimized depending on the cancer tissue types.

Vascular endothelial growth factor (VEGF) is a cytokine that stimulates vasculogenesis and angiogenesis (24). VEGF expression can be induced by cells in a hypoxic condition similar to that inside solid tumors through stabilization of transcription factor HIF-1 $\alpha$  (25). Overexpression of VEGF has been correlated with poor prognosis of cancer patients (26). In this study, we found that the expression level of serum VEGF was drastically increased after implantation with CT-26 cells and significantly reduced by immunization with the peptide vaccine before and after cancer cell implantation (Fig. 4). Therefore, the peptide vaccine negatively regulates expression of VEGF in the sera. Consistent results were obtained when we performed the same experiments in a HCC mouse model.

Reduced expression of VEGF may be mainly resulted from the suppressed tumor growth by the peptide vaccine. Considering that TM4SF5 regulates angiogenic activity of HCC cells through the induction of VEGF (27), functional interruption of TM4SF5 by the TM4SF5-specific antibodies, in turn, may further reduce VEGF expression.

Efficacious cancer vaccines require induction of tumor specific immune responses without side effects. The peptide vaccine composed of TM4SF5 B cell epitope and Lipoplex(O) revealed preventive and therapeutic effects in HCC and colon cancer models without apparent body weight loss, suggesting the absence of prominent side effects. Further studies on the mechanisms involved in the vaccination effects and evaluation of the safety and efficacy of TM4SF5-specific antibodies in a mouse model may provide important information for future application in humans.

## MATERIALS AND METHODS

### Synthesis of CpG-DNA and a B cell epitope peptide

MB-ODN 4531(O), a natural phosphodiester bond CpG-DNA, consists of 20 bases containing three CpG motifs (underlined): AGCAGCGTTCGTCGGCCT (10). The B cell epitope peptide of human TM4SF5 (TM4SF5R2-3, <sup>138</sup>NRTLWDRCEAPPRV<sup>151</sup>) was selected and produced as previously described (12).

### Preparation of B cell epitope and CpG-DNA co-encapsulated in DOPE:CHEMS complexes

Liposome complexes consisting of TM4SF5 B cell epitope and CpG-DNA (MB-ODN 4531(O)) co-encapsulated with DOPE:CHEMS were prepared as reported previously (12). Briefly, DOPE and CHEMS were mixed in 10% ethanol at a molar ratio of 1 : 1, evaporated with nitrogen gas to make a solvent-free lipid film, and resuspended in a mixture containing equal volumes of water-soluble MB-ODN 4531(O) (50  $\mu$ g) and peptide (50  $\mu$ g), followed by vigorous stirring at room temperature for 30 min. After adjusting the pH to 7.0, the complex of peptide and Lipoplex(O) was sonicated lightly for 30 s with a sonicator (Sonifier 450, Branson Ultrasonics). After the complex was filtered with a 0.22  $\mu$ m filter, it was freeze-thawed three times with liquid nitrogen.

### Cell culture

The mouse colon cancer cell line CT-26 was obtained from the Korean Cell Line Bank. CT-26 cells were maintained in a DMEM medium containing 10% fetal bovine serum (FBS; Hyclone), 2 mM glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. Cells were cultured at 37°C in an atmosphere containing 5% CO<sub>2</sub>.

### Colon cancer mouse model

Four-week-old BALB/c mice were inoculated subcutaneously in the dorsal right flank with PBS or 5  $\times$  10<sup>6</sup> of CT-26 cells in a 50% Matrigel solution (HBSS/Matrigel, 1 : 1 v/v, BD

Biosciences), as previously described (22, 28). The CT-26 implanted mice were then injected intraperitoneally with PBS, TM4SF5 peptide encapsulated in DOPE : CHEMS, or a complex of TM4SF5 peptide (50 µg/mouse) and Lipoplex(O) three times at 7 day intervals. The tumor size was measured at 2 day intervals with calipers in three dimensions, and tumor volumes were calculated as width<sup>2</sup> × length/2. The mice were sacrificed 20 days after cancer cell implantation, and the tumors were surgically excised and weighed. Mice were sacrificed when the tumor size reached 2,000 mm<sup>3</sup> or the mice lost >20% of their initial body weight in accordance with the Guide for the Care and Use of Laboratory Animals of the National Veterinary Research & Quarantine Service of Korea to minimize suffering from a large tumor burden. The protocol was approved by the Institutional Animal Care and Use Committee of Hallym University (Permit Number: Hallym 2011-89). After the vaccination, the survival rate was recorded for 30 days.

#### Antigen-specific Ig enzyme-linked immunosorbent assays (ELISA)

Mouse sera were achieved by orbital bleeding. To determine the amounts and titers of total IgG, 96-well immunoplates (Nalgen Nunc International) were coated with 5 µg/ml of TM4SF5 peptide and then blocked with 0.05% of Tween-20 in PBS (PBST) containing 1% BSA. Total IgG levels were measured as previously described (12).

#### Measurement of serum VEGF using ELISA

To measure the amounts of mouse VEGF, mouse sera were collected from blood samples obtained by a heart punch method before sacrifice. A mouse VEGF Sandwich ELISA kit was purchased from RayBiotech (Norcross, GA, USA). The mouse anti-VEGF capturing antibody-coated 96 well plates were incubated with diluted sera (1 : 20) for 2 h at room temperature. After washing, biotinylated anti-VEGF detection antibodies were added to the wells and incubated for 1 h at room temperature. The plates were washed, and streptavidin-horse radish peroxidase conjugate solution was added followed by incubation for 45 min at room temperature. All wells were washed and treated with a TMB substrate solution. A Spectra Max 250 microplate reader (Molecular Devices, Sunnyvale, CA, USA) was used to measure the absorbance at 450 nm.

#### Statistics

Results are expressed as mean ± standard deviation. Statistical significance between two samples was evaluated using the Student's t test (29, 30). A P value of <0.05 was taken as statistically significant. A survival analysis was performed using the Kaplan-Meier method and the results were compared with those of a logrank test.

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