INTRODUCTION

Colorectal cancer (CRC) is the third leading cause of cancer-related mortality in the world. Despite recent progress in chemotherapeutic options, including mAb therapeutics, the prognosis for metastatic CRC is still very poor. Of the mAb therapies for CRC, mAbs against vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) are clinically available. Although EGFR is expressed in several normal tissues, the mAb can preferentially accumulate in CRC tumor tissues as a result of the enhanced permeability and retention effect of tumor tissue. However, because EGFR is expressed at a high level in normal skin tissue, skin toxicity is common, and cessation of mAb treatment is sometimes inevitable even if the therapy is effective in patients. In the case of anti-VEGF mAb, some patients receiving the mAb have life-threatening side-effects including serious bleeding and gastrointestinal perforation.

In this context, we need to find a new CRC-specific molecule and develop mAb against the molecule in order to produce an effective antibody therapy in the treatment of metastatic CRC with minimal side-effects.

To find a CRC-specific molecule, comprehensive expression analysis is usually carried out between CRC cells and their normal counterpart, mucoepithelial cells. However, in reality, obtaining pure live normal mucoepithelial cells is more difficult than obtaining CRC cells even if a laser micro-dissection method is used. We have reported that many mucoepithelial cells are exfoliated in stool, some of which...
contain intact mRNA. Based on the results, we previously developed a method for obtaining almost pure normal mucoepithelial cells from lavage solution following colonoscopies of healthy examinees. We then identified several new CRC-specific molecules after comprehensive expression analyses between the pure mucoepithelial cells and the CRC cell lines. TMEM180 was one of them and is a predicted 11-pass transmembrane protein (UniProtKB, http://www.uniprot.org/uniprot/). In the present study, we developed a mAb against TMEM180 and evaluated the potential of using its mAb for CRC therapy.

2 | MATERIALS AND METHODS

2.1 | Collection of human samples

Human exfoliated colonocytes in a saline mucosal wash fluid were obtained from two healthy donors at the time of their colonoscopy examination. Cell purification and processing methods were conducted as previously described.

The experimental protocols and procedures were approved by the institutional review board of the National Cancer Center, Japan. All methods were carried out in accordance with the relevant guidelines and regulations.

2.2 | Cells and cell culture

Human colon cancer cell lines of SW480, LoVo, DLD-1, HT-29, HCT116, and Colo320 and hematopoietic cell lines of K562, Ramos, RL and Raji were purchased from ATCC. TMEM180 gene knockdown SW480 cells were established using shRNA vector (MISSION TRC clones TRC0000243137; Sigma-Aldrich, St Louis, MO, USA) according to our previously reported protocol. For overexpression of the TMEM180 gene, plasmid pCMV-TMEM180 (OriGene, Rockville, MD, USA) was transfected into DLD-1 cells (DLD-1-OE) using Lipofectamine LTX (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s protocol.

Growth and survival of the cells were evaluated using the WST-8 assay (CCK-8; Dojindo, Tokyo, Japan). Reaction signals were evaluated by measuring the absorbance at 450 nm and using a microplate reader (SpectraMax Paradigm; Molecular Devices, San Jose, CA, USA).

To evaluate the cellular response to hypoxia, DLD-1 and SW480 cells were cultured in DMEM (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% FBS (Life Technologies, Carlsbad, CA, USA) and a 1% penicillin-streptomycin-amphotericin B suspension (Wako Pure Chemical Industries) under normoxic (37°C, 5% CO2, 21% O2) or hypoxic (37°C, 5% CO2, 1% O2) conditions. BIONIX2 hypoxic cell culture kit (Sugiyama-Gen Co., Ltd., Tokyo, Japan) was also used to generate hypoxic conditions according to the manufacturer’s instructions.

2.3 | DNA microarray

DNA microarray analysis was conducted using 10 μg total RNA from colon cancer cells or exfoliated colonocytes and the Affymetrix GeneChip Human Genome U133 plus 2.0 arrays; the analysis was conducted according to standard Affymetrix protocols. In the microarray analysis, 91 genes encoding membrane proteins, which were highly expressed in CRC cells but not in the colonocytes from healthy donors, were selected from 38 500 genes contained in the chip array.

2.4 | Real-time quantitative RT-PCR

RNA extraction and qRT-PCR were conducted as previously described. PCR consisted of 10 μL TaqMan Fast Universal PCR Master Mix (Thermo Fisher Scientific), 1 μL TaqMan primer/probe mixture (Thermo Fisher Scientific) and 9 μL template cDNA diluted to a total volume of 20 μL. Real-time PCR was carried out in an Applied Biosystems 7500 Fast System (Thermo Fisher Scientific). Relative quantification of the total RNA in each sample was conducted using the comparative Ct (threshold cycle) method. Relative expression of each gene was normalized against expression of GAPDH or ACTB.

2.5 | In situ hybridization

In situ hybridization was conducted as previously described. Deparaffinized sections of human tissue (Genostaff Co., Ltd., Tokyo, Japan) were fixed with 4% paraformaldehyde (PFA; Wako Pure Chemical Industries). After treatment with 7 μg/mL proteinase K (Roche, Basel, Switzerland), the sections were refixed with 4% PFA and acetylated with 0.25% acetic anhydride. After the tissue was dehydrated, hybridization was done with digoxigenin-labeled RNA probes (475-bp fragment of TMEM180, GeneBank accession number NM_024789, nucleotide positions 1314-1789). After the tissue was washed, RNase treatment was conducted. The sections were then rewarshed and treated with 0.5% blocking reagent (Roche), followed by treatment with 20% heat-inactivated sheep serum (Sigma-Aldrich). After incubation with an alkaline phosphatase (AP)- conjugated anti-DIG antibody (Roche) for 2 hours, the sections were visualized using a nitro-blue tetrazolium and 5-bromo-4-chloro-3′-indolyolphosphate (NBT/BCIP) solution (Roche).

2.6 | Antibodies

Hybridoma cells producing anti-TMEM180 monoclonal antibodies (mAbs) were established using myeloma cells (p3x63) and lymph node cells from a rat with immunizing recombinant human extradomain (355-400 aa, UniProtKB entry number Q14CX5) of TMEM180- or TMEM180-positive tumor exosomes. The TMEM180-positive tumor exosomes were purified from the culture supernatant of DLD-1-OE cells using hydroxyapatite and gel filtration (Superdex 200 pg; GE Healthcare, Tokyo, Japan). Initially, the anti-TMEM180 mAb (rat IgM) was obtained using recombinant human extradomain of TMEM180. Expression level of TMEM180 in the exosome was confirmed by ELISA with the mAb. Finally, the anti-TMEM180 mAb (rat IgM, clone 669) was obtained using TMEM180-positive tumor exosomes. Heavy-chain variable and the kappa light-chain
variable-region cDNAs were humanized and cloned into the vector for human IgG1 expression. The vectors were transfected into CHO cells (Riken BioResource Center, Ibaraki, Japan). A stable clone (humanized IgG, clone 669) was isolated. The procedure was conducted according to standard protocols.14

2.7 Exosome isolation, immunogold electron microscopy and ELISA

DLD-1 cells (6.8 × 10^6) were plated on a 15-cm dish and grown overnight. FBS-supplemented medium was depleted by washing twice with PBS. The cells were then incubated with serum-free culture medium at 30 mL/dish for 24 hours. After collection and filtration through a 0.22-μm filter, the exosome-containing supernatant was obtained. This supernatant was preserved at 4°C with a protease inhibitor (Wako Pure Chemical Industries) in each experiment.

For immunogold electron microscopy, isolated exosomes were adsorbed on nickel grids with supported films. Anti-TMEM180 mAb, anti-CD9 (Ancell Corporation, Bayport, MN, USA) or CD63 mAb (Ancell Corporation) was added to the grids, which were then incubated at room temperature. After the grids were washed with 1% BSA/PBS, they were incubated at room temperature with gold-conjugated goat anti-rat/mouse IgG polyclonal antibody (British BioCell International Ltd., Crumlin, UK) and washed with 1% BSA/PBS. After glutaraldehyde fixation, negative staining was carried out using a 2% solution of phosphotungstic acid (pH 7.0). The samples were then dried and visualized with transmission electron microscopy (JEM-1400Plus; JEOL Ltd., Tokyo, Japan). Images were acquired with a CCD camera (VELETA; Olympus Soft Imaging Solutions GmbH, Münster, Germany).

For sandwich ELISA, anti-TMEM 180 mAb or anti-CD9 mAb (Ancell Corporation) as a capture antibody was coated onto 96-well plates. Isolated exosomes from DLD-1 or K562 cells were added to each plate and incubated for 1 hour at room temperature. After washing, the plates were incubated with HRP-labeled anti-TMEM180 mAb as a detection antibody for 1 hour at room temperature. After washing, absorbance at 450 nm was measured by Spectra Max paradigm (Molecular Devices, San Jose, CA, USA).

2.8 Immunohistochemistry

Human specimens were purchased from BioChain Institute, Inc (Newark, CA, USA). Immunohistochemistry (IHC) was carried out as previously described.15 Anti-TMEM180 antibody or anti-EGFR mAb (cetuximab, Merck, Darmstadt, Germany) was labeled with HRP using Peroxidase Labeling Kit-SH (Dojindo). Tissues were then incubated with the HRP-conjugated anti-TMEM180 mAb or anti-EGFR mAb for 1 hour at room temperature. After the tissue was washed with PBS, the reaction was visualized using diaminobenzidine (DAB) (Dako, Santa Clara, CA, USA), and the slides were counterstained with hematoxylin.

For cell staining, cultured cells were fixed with 4% PFA/PBS. After permeabilization with 0.1% Triton X-100/PBS and blocking with 5% skimmed milk, the cells were stained with R-phycocerythrin-labeled anti-TMEM180 using R-Phycoerythrin Labeling Kit-SH (Dojindo) as the primary antibody. The cells were then incubated with goat anti-mouse IgG antibody conjugated to Alexa Fluor 488 (1:200; Thermo Fisher Scientific) as a secondary antibody and DAPI solution for nuclear staining. Images were acquired using a BZ-9000 digital high-definition microscopic system (Keyence Co., Osaka, Japan). Fluorescence intensity of TMEM180 from at least 1500 DAPI-positive cells was analyzed using ImageJ (NIH). Representative results were from at least two independent experiments.

2.9 Western blotting

Cells were lysed with buffer containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.1% SDS, 1% Triton X-100 and Complete Protease Inhibitor Cocktail (Roche). The lysate was boiled at 95°C for 5 minutes for denaturing and inactivation of the endogenous proteases. The samples were separated using SDS-PAGE (4%-15% Mini-PROTEAN TGX Precast Gels; Bio-Rad, Hercules, CA, USA) and transferred onto PVDF membranes (Bio-Rad). The membranes were blocked with TBS containing 5% skimmed milk and 0.1% Tween 20 (Sigma-Aldrich). The blots were then incubated with anti-hypoxia-inducible factor 1-alpha (anti-HIF-1α) (1:500; GeneTex, Inc., Irvine, CA, USA) or β-actin (1:1000; Epitomics, Inc., Burlingame, CA, USA) antibodies as the primary antibody at room temperature for 2 hours or at 4°C overnight. The membranes were washed three times with TBS containing 0.1% Tween 20 (TBS-T) and then incubated with HRP-conjugated anti-mouse (1:1000; R&D Systems, Minneapolis, MN, USA) or anti-rabbit IgG (1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA) as the secondary antibody. Can Get Signal solution (Toyobo, Osaka, Japan) was used to reduce background noise. After the membranes were washed with TBS-T, proteins were visualized using ECL prime (GE Healthcare). The images were acquired using a ChemiDoc imager (Bio-Rad).

2.10 Flow cytometry

Flow cytometry was conducted as previously described method.10 In vitro cultured cells were stained with anti-TMEM180 mAbs (clone 669) as a first antibody and an Alexa Fluor 488/647-conjugated anti-mouse/human-Ig polyclonal antibody (Thermo Fisher Scientific) as a secondary antibody. Stained cells were analyzed using a Guava easyCyte 10HT (Merck Millipore Co., Burlington, MA, USA) or Aria flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Dead cells, which were stained using propidium iodide (PI) (Thermo Fisher Scientific), were excluded from the analysis. The data were analyzed using the FlowJo program (Tree Star, Inc., San Carlos, CA, USA).

2.11 Antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity assay

51Cr release was conducted to evaluate antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) activities. Here, 5 × 10^5 DLD-1, SW480 or K562 cells were labelled for 2 hours at 37°C with 3.7 MBq of ^51Cr (100 μCi).
For ADCC evaluation, cells were placed in 96-well plates at $1 \times 10^5$ or $1 \times 10^4$ cells/well. The cells were exposed to anti-TMEM180 mAb or anti-EGFR mAb at 40-fold the number of natural killer (NK) cells (Chemicals Evaluation and Research Institute, Tokyo, Japan) as an effector target ratio and used as effector cells.

For CDC evaluation, cells were placed in 96-well plates at $5 \times 10^4$ cells/well. The cells were exposed to anti-TMEM180 mAb or anti-EGFR mAb with complement human sera (Chemicals Evaluation and Research Institute Japan).

The cells were then incubated at 37°C, 5% CO₂ for 4 hours. Cytotoxicity (%) = ([test sample release – background release]/[maximum release – background release]) × 100. All samples were run in triplicate.

2.12 Animal model and antitumor activity

Female BALB/c nude mice (5 weeks old) were purchased from Charles River Laboratories Japan Inc (Kanagawa, Japan).

To evaluate the antitumor activity of anti-TMEM180 mAb, mice were inoculated s.c. in the flank with $5 \times 10^6$ DLD-1 or SW480 cells. Length (L) and width (W) of tumor masses were measured every 4 days, and tumor volume was calculated using (L × W²)/2. When the mean tumor volume reached approximately 100-200 mm³, the tumors were randomly divided into groups that consisted of five mice each. PBS, anti-TMEM180 mAb or anti-EGFR mAb cetuximab was given on day 0 by i.p. injection. We selected an i.p. injection because it has been well used in similar experiments of antibody therapeutics. Moreover, as it was reported in other references, we confirmed that there was no clear difference in the biodistribution and tumor accumulation between i.p. injection and i.v. injection of the mAb (data not shown).

All animal procedures were carried out in compliance with the Guidelines for the Care and Use of Experimental Animals that were established by the Committee for Animal Experimentation of the National Cancer Center. These guidelines meet the ethical standards required by law and comply with the guidelines for the use of experimental animals in Japan.

2.13 Database analysis

Number of hypoxia response element consensus sequences (ACGTG or GCCGTG at 3000 bp upstream (NC_000010.11, location: 102457895-102460895) from exon 1 of the TMEM180 gene was analyzed. The sequence was obtained from NCBI gene databases (https://www.ncbi.nlm.nih.gov/gene/). TMEM180 gene expression in normal tissues and cancer tissues was analyzed using the microarray gene expression dataset from RefEx (http://refex.dbcls.jp/) and Oncomine (http://www.oncomine.org/), respectively. Comparison of TMEM180 gene expression between 286 colorectal adenocarcinoma patient samples and 41 normal tissue samples using The Cancer Genome Atlas (TCGA) cancer genomics data was carried out using UALCAN (http://ualcan.path.uab.edu/index.html).

2.14 Generation of TMEM180 KO mice

TMEM180 gene KO mice were generated using CRISPR/Cas9-mediated genome editing technology. Briefly, guide RNAs (gRNAs) at exon 2 of mouse TMEM180 genome immediately downstream of the ATG start codon were designed using CRISPRdirect. Two gRNAs (gS01(5′-AGCTGTGGTGTACGGCTCGTTGG-3′), gS03(5′-TGTGGTCCCTGCTGACGTGGG-3′)) were selected in an in vitro digestion assay system using the target TMEM180 PCR product. These two gRNAs were inserted into the BbsI restriction site in the px330 plasmid and constructed as a px330-TMEM180 genome editing vector.

Pronuclear stage ova of C57BL/6J (Clea Japan, Tokyo, Japan) were prepared by in vitro fertilization and injection of 1.67 ng/μL px330-TMEM180 DNA vector into the pronucleus according to standard protocols. The injected ova were transferred into the oviduct of the pseudopregnant Jcl:MCH (Clea Japan) female on that day.

Two viable mice from gS01 and 14 viable mice from gS03 were obtained independently by cesarean section. Genotyping of mouse tail DNA by PCR [forward (5′-CTAAACAGCACAGTCTGCCC-3′), reverse (5′-CTAAAAACGACACGCTGCCC-3′)] and purified PCR products were sequenced using the forward primer. Results of the sequence analysis are shown in Table S1.

2.15 Statistical analysis

Significant differences between the groups were determined using Student’s t test for ELISA data, ANOVA to compare tumor size and body weight, and chi-squared test. All analyses were carried out using SPSS software version 20 (IBM, Armonk, NY, USA).

3 RESULTS

3.1 Identification and characterization of TMEM180 as a new CRC marker

We previously developed a method to obtain almost pure normal mucoepithelial cells from the lavage solution following colonscopies of healthy examinees using immune beads with anti-EpCAM mAb. Moreover, the previous study reported a comprehensive DNA microarray analysis comparing CRC cell lines (SW480, LoVo, DLD-1, HT-29 and HCT116) and normal mucoepithelial cells. We reanalyzed these microarray data and found that TMEM180 was highly expressed in five CRC cell lines but not in the normal colonocytes obtained from lavage of two healthy volunteers (Figure 1A). In quantitative RT-PCR (qPCR) analysis, carried out as a first validation, TMEM180 was expressed at high levels in CRC tissue samples (Figure 1B). In situ hybridization (ISH) was then conducted for the final validation (Figure 1C,D and Figure S1). All of these methods indicated that TMEM180 was a new CRC marker (Figure 1).
3.2 | First production of anti-TMEM180 antibody with a recombinant TMEM180 peptide

To evaluate the potential of TMEM180 as a new target for the diagnosis and/or therapy of CRC, we attempted to produce a mAb. However, the TMEM180 gene is predicted to encode an 11-pass transmembrane protein with limited extracellular domain expression. First, we immunized rats with a recombinant extracellular domain of the TMEM180 protein and obtained an IgM mAb. During the evaluation process, we unexpectedly noticed that the TMEM180 protein was present in the culture supernatant of SW480 and DLD-1 CRC cells. We collected the supernatant and subjected it to hydroxyapatite and gel filtration chromatography. We found that a high concentration of the TMEM180 protein existed in the void fraction (Figure S2A).

As a result of the detection of TMEM180 in the culture supernatant, we hypothesized that TMEM180 coexisted with exosomes or other extracellular microparticles. To test whether TMEM180 exists on tumor-derived exosomes, we conducted immune electron microscopy for the enriched exosomes with CD9 or CD63 mAbs and the TMEM180 mAb. The images clearly indicated that TMEM180 was present on the tumor exosome, similar to CD9 and CD63 (Figure S2B). In the sandwich ELISA, both TMEM180-positive and CD9-positive exosomes were detected (Figure S2C). We concluded that the tumor exosomes released from DLD-1 cells contain TMEM180.

3.3 | Second production of anti-TMEM180 antibody with TMEM180-positive exosomes and validation of a CRC-specific target

We judged that purifying the full-length TMEM180 protein while maintaining the native structure necessary for producing a useful mAb recognizable for the live cell would be the most difficult step. Moreover, we thought that several mAbs with a higher affinity could be obtained by antigens with native structure. Therefore, we immunized rats again with TMEM180-positive tumor exosomes purified from the supernatant of DLD-1 cells. The newly obtained anti-TMEM180 mAb (IgM, clone 669) was able to recognize most CRC cells, but not hematopoietic cells (Figure S3A,B).

We then converted the rat IgM to humanized IgG1 in order to evaluate the potential for therapeutic application of the mAb. First, we examined the expression of TMEM180 in CRC and normal major tissues (colon, skin, liver, brain, kidney, lung and heart) by IHC. Cetuximab...
YASUNAGA et al. (an anti-EGFR mAb) was used as a reference as it is currently widely used for CRC patients. We found that nine of 37 CRC tissues (24.3%) were TMEM180-strong positive and 43.2% (16/37) were TMEM180-weak positive. Regarding EGFR, seven of 37 CRC tissues (18.9%) were alternate EGFR-strong positive and 35.1% (13/37) were EGFR-weak positive. However, there was no significant difference in the expression level between TMEM180 and EGFR (Figure 2A,B). IHC with the mAb did not show staining in major organs including the brain, heart, lung, liver, kidney, colon and skin (Figure 2C). In contrast, IHC with cetuximab showed strongly positive staining in the skin, moderately positive staining in the liver and colon and weakly positive staining in the brain, kidney and lung (Figure 2C).

According to a database, the expression of TMEM180 at the mRNA level is very low not only in normal organs but also in their respective cancer tissues (Figure S4A,B). However, clinical samples used in these datasets would contain various stromal cells, such as fibroblasts or blood cells. Therefore, the expression level of TMEM180 was underestimated in the regular methods used in the database. By contrast, our unique comprehensive expression analysis using pure CRC cells and normal colonocytes without stroma cells could increase the expression level of TMEM180 to enable detection stably as a new CRC marker.

### 3.4 Diagram and functional role of TMEM180

TMEM180 is classified in the major facilitator superfamily and appears to function as a cation symporter (Figure 3A). To address the functional role of TMEM180, we established two TMEM180 gene knockdown (KD) SW480 cell lines: KD1 cells with the lowest expression of TMEM180 protein, and KD2 cells with intermediate TMEM180 protein expression (Figure 3B). We investigated the uptake of glutamine and arginine in the cation symport mechanism because the utilization of both amino acids was strongly increased in tumor cells.31,32 We found that SW480-WT cells were capable of growing in a serum-free medium with glutamine and arginine. By contrast, both KD1 and KD2 could not grow in the same medium (Figure 3C). These findings suggest TMEM180 has an important role in the uptake or metabolism of glutamine and arginine in tumor growth and proliferation.
3.5 | Anti-TMEM180 mAb is highly effective in the targeted inhibition of CRC tumors

Anti-TMEM180 mAb showed little to no direct cytotoxicity in DLD-1 and SW480 cells in vitro (Figure S5A). We next evaluated ADCC and CDC in DLD-1 and SW480 cells. Cetuximab was also used in this study as a reference. Anti-TMEM180 mAb appeared to have ADCC and CDC in both cell lines (Figure S5B,C).

Because of in vitro ADCC activity of both mAbs, we evaluated the in vivo antitumor activity of the anti-TMEM180 mAb and cetuximab in mice bearing DLD-1 or SW480 xenografts. Preliminary experiments showed that cetuximab exerted statistically significant antitumor effects in both tumor xenografts. In the case of anti-TMEM180 mAb, the antitumor effect was significant in DLD-1 xenografts. Strikingly, all SW480 xenografts were completely eradicated by the anti-TMEM180 mAb. We then confirmed these results using a dosage of 25 or 12.5 mg/kg given twice a week for a total of eight injections for both mAbs in mice bearing DLD-1 or SW480 xenografts, respectively. In mice with the DLD-1 xenografts, both anti-TMEM180 mAb and cetuximab showed a significant antitumor effect compared to the control treatment (Figure 4A). In mice with the SW480 xenografts, cetuximab showed a significant antitumor effect at 25 mg/kg but not at 12.5 mg/kg. In contrast, anti-TMEM180 mAb elicited a striking antitumor effect, eradicating the tumor at both 25 and 12.5 mg/kg (Figure 4A). There was no significant difference in the treatment-related body weight loss among the five groups (Figure 4B).

3.6 | Biological characteristics of TMEM180 expression in CRC cells and tissues

Using a public database to analyze the promoter region of the TMEM180 gene, we found that there were 10 hypoxia response element consensus sequences\textsuperscript{22,23} in the gene (Figure 5A). Moreover, HIF-1α expression was elevated in both DLD-1 and SW480 cells in hypoxic conditions (Figure 5B). We therefore evaluated the change in TMEM180 expression in response to the same treatment. Hypoxia significantly increased TMEM180 mRNA expression in SW480 cells and also tended to increase expression in DLD-1 cells (Figure 5C). Protein expression level of TMEM180 was significantly increased in both DLD-1 and SW480 cells after hypoxia treatment (Figure 5D,E). Therefore, TMEM180 expression in CRC cells is regulated by hypoxia.
In the present study, we identified a new CRC-specific molecule, TMEM180, by taking advantage of our original method, and succeeded in establishing a mAb against TMEM180 followed by humanizing it. The expression profile of TMEM180 differed from the expression profile of EGFR, and TMEM180 mAb staining was almost absent in normal tissues, unlike staining with the EGFR mAb. We also found that TMEM180 was expressed at levels as high as the levels of other existing markers such as EGFR and CD44 in cancers and at extremely low levels compared to the levels of other molecules in normal tissues (Figure S6). We, therefore, think that the tumor specificity of TMEM180 is substantially higher than that of other molecules.

More importantly, anti-TMEM180 mAb had in vitro ADCC and CDC activity and significant in vivo antitumor activity in mice bearing TMEM180-positive CRC cell xenografts. Anti-TMEM180 mAb treatment suppressed DLD-1 tumor growth equally to cetuximab treatment. Surprisingly, SW480 tumors were completely eradicated by anti-TMEM180 mAb treatment but not by cetuximab treatment. This remarkable antitumor effect is now under evaluation.

In further experiments, we found that the TMEM180 gene had 10 hypoxia response element consensus sequences, which may be HIF-1α binding sites controlled by hypoxia. Recent studies have indicated that not only hypoxia but also chemotherapy induces HIF-1α expression followed by T-cell anergy and increased dissemination of cancer cells. HIF-1α is currently considered a key factor of cancer stem cell function. In cancer tissues, unlike in normal tissues, angiogenesis and hypoxia associated with the necrosis caused by cancer-induced blood coagulation are repeated alternately during clinical progression. However, HIF-1α is upregulated in many types of cancer besides CRC. Therefore, another transcription factor specific in CRC might regulate TMEM180 expression in conjunction with HIF-1α. Further molecular and translational studies are needed to elucidate the detailed biological role of TMEM180 and the antitumor mechanism of its targeting therapy.

Safety of a drug should also be considered for therapeutic use in clinical practice. According to the database, TMEM180 mRNA is hardly expressed in any normal tissues (Figure S4A). Also, the expression of TMEM180 is extremely low in all organs compared to the expression of other known CRC markers. In the present study, IHC also showed that there was no TMEM180 expression in all major organs studied. Finally, we succeeded in obtaining a TMEM180 gene KO mouse that...
Our mAb is now being processed according to Good Manufacturer Practice principles for testing in a first-in-human clinical trial. We think that there are several concerns to be clarified before entering a clinical trial. We must investigate further antitumor activity using other CRC cell lines and PDX tumors. We will make progress on the functional analysis of TMEM180 including the involvement in glutamine and arginine metabolism as a cation transporter after obtaining an F2 generation of the KO mice. We hope that a future clinical trial will verify the significance of the mAb for the treatment of CRC.

**CONFLICTS OF INTEREST**

Yasuhiro Matsumura is co-founder, shareholder and Board Member of RIN Institute Inc., the company that owns the anti-TMEM180 antibody. Masahiro Yasunaga is shareholder of RIN Institute Inc. Shinji Saijou, Shingo Hanaoka, Takahiro Anzai and Ryo Tsumura declare no conflict of interest for this article.

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