A WWOX-binding molecule, transmembrane protein 207, is related to the invasiveness of gastric signet-ring cell carcinoma

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Using the PCR-based subtractive messenger RNA hybridization assay described in this paper, we isolated a ligherto uncharacterized gene, transmembrane protein 207 (TMEM207), which was selectively expressed in collagen gel-invading cultured signet-ring cell carcinoma KATO-III cells. TMEM207 has a C-terminal proline-rich PPxY motif, which binds to the WW-domain-containing oxidoreductase, WWOX. Enforced expression of TMEM207 significantly increased Matrigel invasion activity of KATO-III cells in vitro without affecting cell growth. In contrast, expression of TMEM207 with mutations in the PPxY motif did not significantly increase Matrigel invasion activity of KATO-III cells. Immunohistochemical staining showed that TMEM207 was strongly expressed in 7 of 30 gastric signet-ring cell carcinoma tissue specimens. Notably, TMEM207 expression was associated with the depth of cancer invasion and the presence of lymph node metastasis. The results of co-immunoprecipitation followed by western immunoblotting showed that TMEM207 is bound to WWOX in a PPxY motif-dependent manner. Small interfering RNA-mediated downregulation of WWOX also significantly increased Matrigel invasion activity of KATO-III cells. Notably, exogenous expression of TMEM207 impaired the WWOX-mediated repression of invasion of another cultured signet-ring cell carcinoma cell line, NUGC-4 cells. Recent studies have highlighted the fact that WWOX acts as a tumor suppressor factor in various malignant tumors, including gastric cancer. On the basis of these findings and the results of the present study, we think that overexpression of TMEM207 may facilitate invasive activity and metastasis of gastric signet-ring cell carcinoma, which possibly occur through binding to WWOX and attenuation of its function.

Introduction

Gastric signet-ring cell carcinoma (SRCC) is one of the major histopathological subtypes of gastric cancer, which is characterized by abundant intracytoplasmic mucin that displaces the nucleus to the periphery of the cell and exhibits a pathological behavior distinct from the behaviors of other histopathological types of gastric cancer (1-4).

Gastric SRCC is believed to arise from luminal neck cells and is confined to superficial mucosal tissue, at least in its initial stage (5). Although gastric SRCC is defined as poorly differentiated adenocarcinoma according to the World Health Organization classification method, a recent study clearly revealed that early intramucosal SRCC cells undergo terminal differentiation and exhibit a relatively indolent biological behavior (6). Indeed, gastric SRCC that does not invade the submucosal tissue has a lower rate of lymph node metastasis and a higher rate of favorable prognosis than the other histopathological types of gastric cancers (1,3,4). However, gastric SRCC cells gain invasive activity by mechanisms, which are yet to be clearly defined and finally show aggressive behavior, often manifested as robust infiltrative growth pattern and extensive spreading such as that observed in Krukenberg tumors, in which gastric SRCC spreads or metastasizes to the ovary. These advanced stage gastric SRCCs have been shown to have a significantly poorer prognosis than other types of gastric cancer (2,3). Therefore, it is important to determine how indolent mucosal SRCC progresses to advanced invasive cancer.

The molecular mechanisms underlying cancer invasion are exceedingly complex and can be distinct for each cancer type. For example, the homophilic adhesion molecule E-cadherin, which is ubiquitously expressed on epithelial cells (7), has an invasion-suppressing role in various cancers (8). Although loss of E-cadherin expression is found in gastric SRCC (9), recent advances revealed that mutation of the E-cadherin gene occurs as an early event and participates in tumor initiation rather than invasion in gastric SRCC. In hereditary gastric cancer, the loss of E-cadherin precedes the appearance of microscopic foci of intramucosal SRCC at the initial stages (10,11). The incidence of intramucosal SRCC after treatment with N-methyl-N-nitrosourea in mice lacking the E-cadherin gene is greater than that in wild-type mice (12). These findings indicate that the lack of E-cadherin expression may be related to an early event of gastric SRCC.

The WW-domain-containing oxidoreductase (WWOX) protein is well characterized as a tumor suppressor molecule (reviewed in ref. 13); it contains tandem N-terminal WW domains. The WWOX gene is located in the human chromosome region 16q23.3-24.1, which is a common chromosome fragile site designated FRAD16D (14). These chromosome fragile sites are highly susceptible to damage by environmental carcinogens. In fact, the WWOX gene frequently shows loss of heterozygosity, and hypermethylation of the promoter region has been detected in various cancers, including gastric cancer (15,16). Moreover, several cancers, such as breast cancer, overexpress aberrant transcripts (17), which lack the coding region for the enzymatic domain. These abnormal WWOX molecules are believed to act as dominant forms that abolish the tumor suppressor function of the intact WWOX molecule.

Through its WW domain, WWOX binds to proline–tyrosine rich motifs (PPxY, where P is proline, Y is tyrosine and x is any amino acid) of the partner molecules. This report states that a hitherto uncharacterized PPxY motif-containing molecule—transmembrane protein 207 (TMEM207)—may be related to the invasive phenotype of gastric SRCC, and this relationship is putatively mediated through binding to WWOX. Interestingly, enforced expression of TMEM207 facilitated the invasion of cultured WWOX-expressing gastric SRCC cells in Matrigel. Although WWOX was expressed in half of the invasive gastric SRCC specimens, coexpression of TMEM207 was related to the invasion status of gastric SRCC. These findings may indicate that TMEM207 is aberrantly expressed in invasive gastric SRCCs and binds to WWOX, which increases tumor invasion.

Materials and methods

Cell culture and collagen gel invasion assay

KATO-III and NUGC-4 cells, which are both derived from gastric SRCC and exhibit features of SRC (18,19), were obtained from the Japanese Cell Research Bank (Osaka, Japan). Cos7 cells were maintained in our laboratory. Cos7 cells are monkey kidney cells transfected with the SV40 large T antigen that allows for episomal replication of transfected plasmids, containing the SV40 origin of replication. The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco Life Technologies, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum and 50 µg/ml gentamycin (Gibco Life Technologies).

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Collagen gel-based cell invasion assays were performed using collagen solutions (Nitta Gelatin, Osaka, Japan) as described in a previous study (20). Briefly, 100 µl of a collagen solution (0.24%) was pipetted into a transwell with a diameter of 6.5 mm and an 8 µm pore filter (Corning, Corning, NY). Subsequently, 100 µl of 2 x 10^5 cells were added on the collagen layer with serum-free medium. One day later, DMEM supplemented with 10% foetal bovine serum and recombinant hepatocyte growth factor (final concentration, 20 µg/ml; Sigma–Aldrich, St Louis, MO) was placed below the filter. Cells were then allowed to invade the collagen matrix through the membrane.

After 48 h, the cells were collected from below or from the surface of the collagen gel from 10 wells of the 48-well plates. The collagen gel assay was repeated in triplicate to obtain invasive and non-invasive cell fractions from independent experiments.

Subtractive hybridization and sequence analysis
PCR-Select™ cDNA subtraction assays (Takara, Ohtsu, Japan) were performed using complementary DNA (cDNA) samples from collagen gel-invasive and non-invasive KATO-III cells. The PCR-Select cDNA subtraction kit was used according to the manufacturer’s protocols and procedures, as described in a previous study (21). Poly(A)^+ RNA was extracted from collagen gel-invasive and non-invasive KATO-III cells by using RNAzol (Invitrogen, Carlsbad, CA) and an Oligo-dT miRNA purification kit (Takara). Poly(A)^+ RNA was converted to first-strand cDNA by incubation with reverse transcriptase and the cDNA synthesized was digested with Rsal to generate blunt-ended fragments. Only double-stranded cDNA from collagen gel-invasive KATO-III cells was ligated using the following oligonucleotides: 5’-CTAATAGCCTACTATAGGTCCTAGCGGCCGCC-GCGGAGGT-3’ (adapter 1) or 5’-CTAATAGCCTACTAGGTCCTAGCGGCC-GCGGAGGT-3’ (adapter 2R). For the first hybridization, each adapter-ligated cDNA was hybridized with an excess amount of cDNA from non-invasive KATO-III cells. For the second hybridization by combination with each of the first hybridized solutions, cDNAs obtained from collagen gel-invasive KATO-III cells were amplified by PCR using the first primer, 5’-CTAATAGCCTACTATAGGTCCTAGCGGCCGCC-GCGGAGGT-3’ and second nested primer 1, 5’-ACGCTTGGTCGCGGCCGAGGT-3’. For the second hybridization, each adapter-ligated cDNA was hybridized with an excess amount of cDNA from collagen gel-invasive KATO-III cells. For the second hybridization by combination with each of the first hybridized solutions, cDNAs obtained from collagen gel-invasive KATO-III cells were amplified by PCR using the first primer, 5’-CTAATAGCCTACTATAGGTCCTAGCGGCCGCC-GCGGAGGT-3’ and second nested primer 1, 5’-ACGCTTGGTCGCGGCCGAGGT-3’. These cDNA fragments, which were predominantly expressed in collagen gel-invasive cells, were subcloned into the pmD20-T vector (Takara), transfected into JM109 cells (Promega, Madison, WI) and sequenced using the ABI 310 autosequencer (PerkinElmer, Norwalk, CT). Subtractive hybridization assays were repeated twice by using independently collected KATO-III cell fractions.

Protein sequences were obtained from the database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). For alignment with the sequence of the protein family, a conserved motif was first identified by using a modified version of the GeneWorks program (Teijin System Technology, Kanagawa, Japan). Then, the protein motif of TMEM207 was aligned against the entire sequence obtained from the National Center for Biotechnology Information database.

Reverse transcription–PCR and quantitative real-time RT–PCR
Reverse transcription–PCR (RT–PCR) was performed as described in a previous study (22). Total cellular RNA was prepared from cell lysates by using RNAzol. cDNA synthesis from total RNA and the subsequent PCR were incorporated into primer dimmers or non-specific amplicons during the real-time PCR runs. The incorporation of PCR products was analyzed by polyacrylamide gel electrophoresis in preliminary experiments. Single bands of the expected size were obtained in all instances. Furthermore, analysis of SYBR green dissociation curves after completion of 40 PCR cycles revealed the presence of single amplicons for each primer pair. Samples were amplified as follows: one cycle at 95°C for 10 min, 40 cycles at 95°C for 10 s and 60°C for 30 s. Expression levels of the target molecules were calculated using the comparative cycle time (Ct) value, which was normalized to the GAPDH PCR Ct value by subtracting the GAPDH Ct value from the target PCR Ct value to obtain ΔCt. From the ΔCt values, the target messenger RNA expression level (adjersed for GAPDH) was calculated using the following equation: relative messenger RNA expression = 2^-ΔCt target - ΔCt GAPDH.

Plasmid preparation and transfection
The entire coding sequence of the human TMEM207 cDNA was amplified by PCR using a primer pair obtained from kidney cDNA (Takara), i.e. 5’-GCCACATGTACAGATCATGCAGTTCAGC-3’ (sense) and 5’-TCAGCCTGGTTTCTAACTTTTCTACTAGG-3’ (antisense) and subcloned into the pTarget vector (Promega), which contains the SV40 origin of replication. After verification by sequencing on the ABI 310 autosequencer (PerkinElmer), the full-length vector was designated as the TMEM207 expression vector. For the substitution of two amino acids (modification of the PPPY motif to RSPY), TMEM207 cDNA was amplified by PCR using the primers 5’-GCCACATGTACAGATCATGCAGTTCAGC-3’ (sense) and 5’-TCAGCTGGTTTCTAACTTTTCTACTAGG-3’ (antisense), subcloned into the pTarget vector and verified by sequencing. The obtained vector was designated as the PMUT-MEM207 expression vector.

The entire coding sequence of the human TMEM207 cDNA was also amplified by PCR using a primer pair obtained from small intestine cDNA (Takara), i.e. 5’-GCCATGGCAGCGCTGCGCTACGCGGGGCTG-3’ (sense) and 5’-TTACCGCGAGTTCTGCTCCAAAGCGGCTTGC-3’ (antisense), subcloned into the pTarget vector and verified by sequencing. For stable expression of WWOX and TMEM207 in NUGC-4 cells, we used a bicistronic vector (pTargeT vector; Takara–Clonetech). The coding region of the WWOX cDNA was excised from the pTarget vector by digestion with NheI and SalI and cloned into NheI–XhoI site of the PMUT-MEM207 expression vector. For coexpression of WWOX and TMEM207, the coding region of the WWOX cDNA was excised from the pTarget vector by digestion with XhoI and NotI and cloned into the SalI–NotI site of the PMUT vector, which contained the WWOX cDNA.

Cos7 cells were transiently transfected with an expression vector or the empty vector by using diethylaminoethyl–dextran and harvested after 48 h. KATO-III and NUGC-4 cells were transfected using the respective plasmids by using N-[1-(2,3-dioleoyloxy)propyl]-N,N, and TMEM207, the coding region of the WWOX cDNA was excised from the pTarget vector by digestion with XhoI and NotI and cloned into SalI–NotI site of the PMUT vector, which contained the WWOX cDNA.

Cos7 cells were transiently transfected with an expression vector or the empty vector by using diethylaminoethyl–dextran and harvested after 48 h. KATO-III and NUGC-4 cells were transfected using the respective plasmids by using N-[1-(2,3-dioleoyloxy)propyl]-N,N-trimethylammonium methysulfate transfection reagents (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer’s protocol as described previously (22). Briefly, cells were seeded in 10 cm culture dishes. On the next day, culture medium was replaced by 5 ml Opti-MEMI medium (Invitrogen). Then, 5 µg of plasmid and BglII-linearized wPPPY TMEM207 expression vector, Scal-linearized PMUT-MEM207 expression vector or BglII-linearized control empty vector was dissolved in 25 µl medium containing 20 mM Hepes, pH 7.4 and 150 mM NaCl. After 15 min of incubation with 15 µg N-[1-(2,3-dioleoyloxy)propyl]-N,N-trimethylammonium methysulfate reagent, the plasmids were added to the medium. In some experiments, cells were transfected with the BglII-linearized WWOX expression vector. For stable coexpression of TMEM207 and WWOX, the BglII-linearized pRES vector, which contains the entire cDNA coding region of both TMEM207 and WWOX, was transfected into NUGC-4 cells.

Colonies resistant to G418 (at a final concentration of 600 µg/ml; Gibco BRL) were selected 3–4 weeks later and subcultured as described previously (22).

Antibodies
Rabbit-specific antibody to human recombinant TMEM207 (amino acids 77–130) was purchased from Sigma–Aldrich. A conventional rabbit antibody to the synthesized peptide VNVDHQPGNG (amino acids 40–50 of TMEM207) was generated in our laboratory. Briefly, the peptide, which was conjugated with keyhole limpet hemocyanin, was administered with complete Freund’s adjuvant in the first immunization and with incomplete Freund’s adjuvant in the subsequent booster injections. The antibody was purified by affinity chromatography using the same peptide. Rabbit anti-WWOX antibodies were purchased from Sigma–Aldrich and GenWay Biotech (San Diego, CA). Normal rabbit IgG antibody was prepared using Protein A-Sepharose beads (Pharmacia Biotech, Piscataway, NJ).

Western immunoblotting
Western immunoblotting was performed according to the previously described method (23), which was modified in accordance with the proposal by Towbin et al. (24). Immunoreactivity of the cells was assessed using a western immunoblotting detection kit (Bio-Rad). Briefly, equal amounts of proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto a polyvinylidene difluoride membrane (Millpore Co., Bedford, MA). After blocking with bovine serum albumin, the membranes were incubated with specific rabbit antibodies or normal rabbit IgG. In some experiments, the proteins were electrophoresed, electroblotted and reacted with an
anti-β-actin antibody (Sigma-Aldrich) in parallel. However, in some experiments, membranes were stripped and reprobed with an anti-β-actin antibody.

Cell proliferation and Matrigel invasion assay
Cell proliferation was evaluated by counting the number of viable cells as described previously (25). Briefly, 1 × 10⁶ cells were cultured on standard 35 mm tissue culture dishes (BD Falcon, Franklin Lakes, NJ) in triplicate. The number of live cells was determined after 24 and 48 h.

The invasiveness of the cultured cells was determined using 24-well BD BioCoat Matrigel Invasion Chamber Plates (BD Falcon) according to the manufacturer’s protocol as described previously (22). Briefly, 1 × 10⁶ cells were placed in the upper compartment of an invasion chamber. After 24 and/or 48 h of incubation with DMEM containing 10% (lower chamber) or 0% (upper chamber) fetal bovine serum, non-invading cells were gently removed from the filter by scrubbing with a cotton-tipped swab. The cells on the lower surface of the filter were counted under a microscope.

These experiments were repeated three times by using independently isolated clones and control clones. The Matrigel invasion assay was also performed using KATO-III cells, which were treated with small interfering RNAs (siRNAs). Experiments were performed in triplicate, and mean and standard deviation values were calculated.

Immunohistochemical staining
Previously obtained pathological tissue specimens of 30 gastric adenocarcinomas, of which the SRCC component comprised at least 50%, were used in this study. We immunostained the tissue specimens obtained from areas of deepest invasion. All tissue specimens were surgically obtained, fixed in 10% buffered formalin and embedded in paraffin. Staining was performed with rabbit anti-TMEM207, rabbit anti-WWOX (GenWay Biotech) or control antibodies by using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Immunohistochemical staining was performed as described previously (22,23,25).

The 5 × 5 mm² regions of stained tissues where SRCC showed the deepest invasion were sequentially examined. Immunoreactivity was based on examination of five high-power (×400) microscopic fields or the total tumor (when the tumor was <5 fields) for each case. Tumors that expressed strong immunoreactivity in >5% of SRCC cells were considered positive.

In some experiments, a commercially available antibody to TMEM207 (Sigma–Aldrich) was preadsorbed with lysates of TMEM207-expressing Cos7 cells or control Cos7 cells. In some immunohistochemical staining experiments, an antibody to the TMEM207 peptide was also preadsorbed with the immunized peptide to verify specificity.

Immunoprecipitation
Cos7 cells were cotransfected with the WWOX expression vector and wtTMEM207- or PPPY-mutated TMEM207 expression vector. At 48 h after transfection, the cells were solubilized in CelLytic M Cell Lysis Reagent (Nakarai Tesque, Kyoto, Japan); the solubilization procedure was performed in accordance with the manufacturer’s instructions. We also prepared cell lysates from WWOX- and TMEM207-coexpressing NUGC-4 transfectants.

The soluble fraction of cell lysates was obtained by centrifugation, incubated with rabbit anti-TMEM207 peptide antibody, anti-WWOX antibody or control antibody for 2 h at 4°C and mixed with protein A-Sepharose beads for 30 min at 4°C. Subsequently, the beads were washed four times with CellLytic M Cell Lysis Reagent containing a protease inhibitor cocktail (Nakarai Tesque, Kyoto, Japan); the solubilization procedure was performed in accordance with the manufacturer’s instructions. We also prepared cell lysates from WWOX- and TMEM207-coexpressing NUGC-4 transfectants.

The soluble fraction of cell lysates was obtained by centrifugation, incubated with rabbit anti-TMEM207 peptide antibody, anti-WWOX antibody or control antibody for 2 h at 4°C and mixed with protein A-Sepharose beads for 30 min at 4°C. Subsequently, the beads were washed four times with CellLytic M Cell Lysis Reagent containing a protease inhibitor cocktail. Thereafter, the immunocomplexes were eluted by boiling for 3 min in sodium dodecyl sulfate sample buffer containing 10 mM dithiothreitol and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by western immunoblotting.

siRNA-mediated RNA interference
The detailed procedure for siRNA silencing of a target gene has been described previously (20). In this study, we employed siRNA (Qagen) with sense strand 5′-CAUGAUUACAGAAUATT-C3′ and antisense strand 5′-UAUAUCUGUAACUGAGGGG-3′ to target 5′-CCCATGATTACGAT-ATT3′ for silencing of the WWOX gene according to the method described by Bouteille et al. (26). A green fluorescent protein siRNA duplex with the target sequence 5′-CGGAAUGGCAGCCGAGGAU-3′ was used as non-silencing control. siRNAs were transfected into KATO-III cells by using lipofectamineTM RNAiMAX (Invitrogen) in accordance with the manufacturer’s instructions. At 48 h after transfection, the cells were harvested and used for subsequent studies.

Statistical analysis
Statistical analysis was performed by using Student’s t test for unpaired observations. The χ² test was used to analyze the relationship between TMEM207 or WWOX expression and the clinical characteristics of gastric cancer tissue specimens. Findings with P < 0.05 were considered significant.

Results
Isolation of TMEM207 as a selectively expressed gene in KATO-III cells with high invasive activity
In the collagen gel invasion assay, ~1% of the inoculated KATO-III cells migrated through the collagen gel and were collected as cell fractions showing high invasive activity. In two independent subtraction assays, a total of seven cDNA fragments were isolated; however, cDNAs other than those of TMEM207 were isolated in one subtraction assay. Subsequent RT–PCR experiments did not confirm any differential expression of these genes, except of TMEM207 in invasive and non-invasive KATO-III cells. The results of screening are summarized in Supplementary Table 1S, available at Carcinogenesis Online.

The subsequent RT–PCR assay confirmed that the expression of the TMEM207 transcript in collagen gel-invasive KATO-III cells was higher than that in non-invasive KATO-III cells (Figure 1A). Quantitative RT–PCR also showed that the TMEM207 messenger RNA expression level was significantly higher in invasive KATO-III cells than in non-invasive KATO-III cells (Figure 1B).

Homology between TMEM207 and epidermal growth factor receptor-coamplified and overexpressed protein
TMEM207 is a poorly characterized molecule; therefore, we first evaluated the protein motif of TMEM207. A proline-rich PPxY motif (PPPY) was found near the C-terminus of the deduced amino acid sequence of TMEM207. In addition, a typical signal peptide and a putative single transmembrane domain were found in the TMEM207 amino acid sequence.

The protein homology search in which we focused on the presence of the signal peptide, single transmembrane domain and PPxY motif at the C-terminal side showed significant homology between TMEM207 and epidermal growth factor receptor-coamplified and overexpressed protein (Ecop) (Supplementary Figure 1S is available at Carcinogenesis Online). Recent studies have shown that in tobacco-related human squamous cell carcinoma, the Ecop gene is amplified and the Ecop molecule acts as an oncogenic factor (27,28). Furthermore, the PPxY motif may recruit WW domain-containing proteins such as WWOX, which are known to be involved in various forms of carcinogenesis (13).

These data encouraged further examination of TMEM207 in gastric SRCC.

Enforced TMEM207 expression increased Matrigel invasion activity of KATO-III cells
Next, we investigated whether TMEM207 expression increased the invasive activity of KATO-III cells in vitro. Enforced expression of TMEM207 in KATO-III cells was achieved by transfection with an expression vector, which contained the entire protein-coding region of TMEM207 cDNA (Figure 1C).

No significant differences in cell growth were observed between the TMEM207-overexpressing and empty vector-transfected KATO-III cells (Figure 1D). In contrast, enforced expression of TMEM207 caused a significant increase in Matrigel invasion activity of KATO-III cells (Figure 1E). In parallel, we also investigated whether the PPxY motif is important for TMEM207-induced Matrigel invasion activity. Alteration of the PPxY sequence significantly decreased Matrigel invasion activity of TMEM207 without affecting cell growth (Figure 1D and E).

This result confirmed that TMEM207 expression increased the invasive activity of KATO-III cells in vitro. A PPxY motif at the C-terminal side appeared to be important for TMEM207-induced invasive activity.

Expression of TMEM207 in gastric cancer tissues
Subsequently, immunohistochemical staining was performed to examine the TMEM207 expression in gastric cancer tissue specimens.
A commercially available antibody (Sigma–Aldrich) to a large region of the recombinant TMEM207 peptide (amino acids 77–130 of 146 amino acids) was purchased. Although western immunoblotting supported the specificity of this antibody (Figure 1C), we also prepared an antibody to a shorter region of the TMEM207 peptide in this study. The computer-aided analysis showed that the sequence of the immunized peptide contained a region of high-predicted immunogenicity and no significant homologies with other known eukaryotic proteins in the public database. Notably, the result of immunohistochemical staining using the anti-TMEM207 peptide was similar to that using the commercially available antibody. Furthermore, the results of western immunoblotting verified the specificity of this anti-TMEM207 peptide antibody (Supplementary Figure 2S is available at Carcinogenesis Online).

A representative staining is shown in Figure 2. Immunoreactivity with the anti-TMEM207 antibody was found in 7 of the 30 gastric SRCC specimens. Immunoreactivity was found in both cell surface and cytoplasm of SRCC cells (Figure 2). Immunoreactivity was significantly related to the degree of tissue invasion and presence of lymph node metastasis (Table I). In contrast, the control antibodies showed no significant staining. Preadsorption entirely diminished the immunoreactivity of the antibodies to TMEM207 (data not shown).

We concluded that TMEM207 was expressed in gastric SRCC, especially in carcinomas showing high invasion and metastatic activity.

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**Table I.** Analysis of the correlation between the presence of TMEM207 and pathological characteristics of 30 cases of gastric SRCC

<table>
<thead>
<tr>
<th>Pathological characteristics</th>
<th>TMEM207 expression</th>
<th>$\chi^2$ value</th>
<th>$P$ value</th>
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<td>Invasive status</td>
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<td></td>
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</tr>
<tr>
<td>pT1</td>
<td>0 9 0</td>
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</tr>
<tr>
<td>pT2–pT4</td>
<td>7 14 33</td>
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<tr>
<td>Lymph node metastasis</td>
<td>Absent 19 9.5</td>
<td>6.3 0.0012 significant</td>
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**TMEM207 binds to WWOX in a PPxY motif-dependent manner**

In this study, we further determined whether TMEM207 could bind to WWOX. Immunoprecipitation followed by western immunoblotting revealed that wild-type TMEM207 is bound to WWOX. Alteration of the PPxY motif significantly decreased the binding of TMEM207 to WWOX. A representative result is shown in Figure 3.

This result indicates that TMEM207 can bind to the tumor suppressor factor WWOX through a C-terminal PPxY motif.

**Downregulation of WWOX increases Matrigel invasion activity in vitro**

Although a recent study has shown that repression of WWOX expression may play a role in stomach carcinogenesis (16), the expression status and pathobiological property of WWOX in gastric SRCC remains unknown.

We performed western immunoblotting to determine whether KATO-III cells expressed WWOX. WWOX protein was expressed in KATO-III cells (Figure 4A). In our experiments, siRNA-mediated downregulation did not cause any significant changes in KATO-III cell growth (data not shown). In contrast, siRNA-mediated silencing of the WWOX gene appeared to increase Matrigel invasion activity of KATO-III cells (Figure 4B).

**Expression of WWOX in gastric cancer tissues**

Immunohistochemical staining showed that immunoreactivity (tested with the anti-WWOX antibody used in this study) was observed in 15 of 30 gastric SRCC tissue specimens. Preadsorption of the antibody with WWOX-expressing Cos7 cells entirely diminished the immunoreactivity (data not shown). Lack of immunoreactivity (revealed with an anti-WWOX antibody) was significantly related to invasion status or lymph node metastasis (Table II). We found four cases, which expressed both TMEM207 and WWOX (immunostaining of two representative cases is shown in Figure 4C). Interestingly, all of these four cases were invasive SRCC.

**Enforced expression of TMEM207 impaired the repressive effect of WWOX on Matrigel invasion activity of NUGC-4 cells**

Using another gastric SRCC cell line, NUGC-4, we further evaluated the effect of TMEM207 expression on Matrigel invasion activity.

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**Table II.** Analysis of the correlation between the lack of WWOX expression and pathological characteristics of 30 cases of gastric SRCC

<table>
<thead>
<tr>
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<th>WWOX expression</th>
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<th>P value</th>
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Fig. 3. TMEM207 bound to WWOX in a PPxY motif-dependent manner. Direct binding between wild-type TMEM207 and WWOX was demonstrated by co-immunoprecipitation assay followed by western immunoblotting. In contrast, alteration of the PPxY motif to RSPY significantly decreased binding of TMEM207 to WWOX. Cos7 cells were cotransfected with a WWOX expression vector and wild-type TMEM207- or PPxY-mutated TMEM207 expression vector. At 48 h after transfection, the cells were solubilized, incubated with a rabbit anti-TMEM207 or anti-WWOX antibody and mixed with protein A-Sepharose beads. After extensive washing, immunocomplexes were eluted by boiling in sodium dodecyl sulfate sample buffer containing 10 mM dithiothreitol and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by western immunoblotting using an anti-WWOX or anti-TMEM207 antibody. Results of immunoblotting using the input total cell lysates with anti-TMEM207 or anti-WWOX antibody are also shown. IgG antibody fragments released from the beads, i.e. heavy chains (H) and light chains (L), are shown too.

Fig. 4. Repression of WWOX expression increased invasion activity of KATO-III cells. (A and B) Western immunoblotting demonstrated that original KATO-III cells expressed the WWOX protein. Silencing of the WWOX gene by using siRNA significantly downregulated WWOX expression and increased Matrigel invasion activity of KATO-III cells (*) and "** P < 0.01). (C) Representative immunohistochemical staining of invasive SRCC, which demonstrated immunoreactivity with an anti-WWOX and anti-TMEM207 antibody. Invasive SRCC cells in submucosal tissues and invasive SRCC involving the muscularis propria exhibited immunoreactivity with anti-WWOX and anti-TMEM207 antibodies. Negative staining using a control antibody was shown as Mock. GFP, green fluorescent protein.
Western immunoblotting indicated significant expression of TMEM207 or WWOX in NUGC-4 cells. We prepared WWOX-expressing, WWOX- and TMEM207-coexpressing and control empty-IRES plasmid-transfected NUGC-4 cells (Figure 5A). In the present study, we did not find any significant differences in cell growth between control NUGC-4, WWOX-expressing NUGC-4 and WWOX- and TMEM207-coexpressing NUGC-4 transfectants (data not shown). Notably, enforced expression of WWOX reduced Matrigel invasion activity of NUGC-4 cells, whereas coexpression of TMEM207 significantly impaired the WWOX-mediated repression of invasion activity of NUGC-4 cells (Figure 5B). Binding of WWOX and TMEM207 was also found in WWOX- and TMEM207-coexpressing NUGC-4 cells (Figure 5C).

These data indicated that the oncogenic effect of TMEM207 might be related to a WWOX-mediated molecular mechanism in gastric SRCC.

Discussion

In the present study, by using a subtractive hybridization assay, we identified TMEM207 as a gene that is more prominently expressed in collagen gel-invasive gastric SRCC KATO-III cells than in non-invasive KATO-III cells. Enforced TMEM207 expression significantly increased Matrigel invasion activity of KATO-III cells without affecting cell growth. Immunohistochemical staining also showed that TMEM207 was expressed in highly invasive gastric SRCC but not in intramucosal or non-invasive SRCC. Notably, TMEM207 was significantly expressed in gastric SRCC with lymph node metastasis in comparison with its expression in gastric SRCC without lymph node metastasis. These findings indicate that TMEM207 may increase invasive activity and subsequently contribute to lymph node metastasis by inducing invasion into lymphatic vessels.

However, many advanced gastric SRCCs lack TMEM207; thus, it is likely that molecular mechanisms other than TMEM207 expression may also be sufficient to initiate invasion in gastric SRCC. Consistent with this speculation, a comparative genomic hybridization study showed an increase in the DNA copy number on chromosome 3q28, where the TMEM207 gene is located in ~8% of gastric cancers (29).

In the present study, four of seven TMEM207-expressing SRCC specimens also expressed WWOX. Both TMEM207 and WWOX immunoreactivity was observed in sections of these invasive SRCCs (Figure 4B); therefore, it is likely that TMEM207 may attenuate the tumor suppression function of WWOX in these cases as suggested by the *in vitro* data using NUGC-4 transfectants.

In contrast, of seven TMEM207-expressing invasive SRCC specimens were negative for WWOX immunoreactivity. The commercially available antibody (GenWay Biotech) used for the present immunohistochemical staining is generated against a peptide representing the residues RLAAFVTVDNPTKPPTQR of WWOX. Notably, truncated WWOX transcripts are frequently observed in various cancers. Truncated WWOX proteins, which were not detected by the antibody used in this study, could also interact with TMEM207. Alternatively, TMEM207 might bind to and attenuate the function of other WW domain-containing tumor suppressor molecules in one of seven TMEM207-expressing and WWOX-negative SRCC specimens.

Here, by using the Matrigel invasion assay, we showed that downregulation of WWOX expression facilitates KATO-III cell invasion. In contrast, enforced expression of WWOX impairs Matrigel invasion activity of NUGC-4 cells. These data are consistent with those of Maeda et al. (30), who very recently reported that lack of WWOX expression was clinicopathologically associated with the depth of invasion and lymph node metastasis in gastric carcinoma.

The WWOX molecule has two N-terminal WW domains and a C-terminal short-chain dehydrogenase/reductase domain. The WW domains, including that of WWOX, have been shown to bind to proline-rich sequences, PPXY motifs (13) (often PPPY, such as in the transcription factor activator protein-2 (31)), to mediate protein–protein interactions. The deduced amino acid sequence of TMEM207 is composed of a typical signal peptide, a putative transmembrane domain and a PPXY motif (PPPXY) at the C-terminal side (Supplementary Figure 1S is available at Carcinogenesis Online). Exogenous expression of PPPY-mutated TMEM207, which lacked the binding ability to WWOX, did not increase Matrigel invasion activity of KATO-III cells. Furthermore, enforced expression of TMEM207 abolished the WWOX-mediated repression of Matrigel invasion activity of NUGC-4 cells. These present data may indicate that binding to TMEM207 might alter the function of WWOX and lead to gastric SRCC invasion, if the gastric SRCC still expresses WWOX.
Ecop, which shows homology with TMEM207 at various sites (Supplementary Figure 1S is available at Carcinogenesis Online), including the PPXY motif at the C-terminal side, is overexpressed, upregulates nuclear factor kappa B transcriptional activity and promotes resistance to apoptosis in tobacco-related squamous cell carcinoma (27,28). TMEM207 and Ecop might constitute a novel family of oncoproteins, which show an overlapping pathobiological property of attenuating WWOX function.

At present, the physiological function of TMEM207 is unknown. The molecular structure suggests that TMEM207 is a type I transmembrane protein. In fact, fluorescence-activated cell sorting analysis showed that TMEM207 was expressed on the cell surface of KATO-III-transfectants (data not shown). However, immunohistochemical staining also showed that TMEM207 was localized not only on the cell surface but also in the cytoplasm of SRCC cells. Moreover, in metastatic goblet cells of the stomach, the immunoreactivity of anti-TMEM207 antibody was mainly localized in the cytoplasm (Figure 2). TMEM207 might be processed in the rough endoplasmic reticulum and/or Golgi apparatus like many other surface membrane proteins. Alternatively, it is more likely that TMEM207 is also integrated in the cytoplasmic membranes, for example, of a secretory vesicle or an organelle related to endosomal sorting of mucins. The PPXY sequence was also found in many viral proteins, e.g. human T-cell leukemia virus type 1, Marburg virus, Moloney murine leukemia virus and Mason–Pfizer monkey virus. These PPXY sequences are thought to interact with the endosomal sorting machinery to assist budding (32). In order to elucidate the biological property of TMEM207, we recently generated transgenic mice that overexpress TMEM207 in various secretory systems. Studies are now being undertaken to elucidate the mechanism responsible for the high incidence of spontaneous tumors found in these TMEM207-overexpressing mice.

In conclusion, we isolated a hitherto uncharacterized TMEM207, as an invasion-associated molecule in gastric SRCC. TMEM207 could be a molecular target for the development of a new therapeutic approach for patients with gastric SRCC.

Supplementary material

Supplementary Table 1S and Figures 1S and 2S can be found at http://carcin.oxfordjournals.org/.

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References


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