Identification of Novel Transmembrane Proteins in Scirrhus-Type Gastric Cancer by the *Escherichia coli* Ampicillin Secretion Trap (CAST) Method: TM9SF3 Participates in Tumor Invasion and Serves as a Prognostic Factor

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Key Words

CAST · Gastric cancer · Scirrhus-type gastric cancer ·
TM9SF3 · Transmembrane 9 superfamily member 3

Abstract

Objective: Scirrhus-type gastric cancer (GC) is highly aggressive and has a poor prognosis due to rapid cancer cell infiltration accompanied by extensive stromal fibrosis. The aim of this study is to identify genes that encode transmembrane proteins frequently expressed in scirrhus-type GC. **Methods:** We compared *Escherichia coli* ampicillin secretion trap (CAST) libraries from 2 human scirrhus-type GC tissues with a normal stomach CAST library. By sequencing 2,880 colonies from scirrhus CAST libraries, we identified a list of candidate genes. **Results:** We focused on the TM9SF3 gene because it has the highest clone count, and immunohistochemical analysis demonstrated that 46 (50%) of 91 GC cases were positive for TM9SF3, which was observed frequently in scirrhus-type GC. TM9SF3 expression showed a significant correlation with the depth of invasion, tumor stage and un-

differentiated GC. There was a strong correlation between TM9SF3 expression and poor patient outcome, which was validated in two separate cohorts by immunostaining and quantitative RT-PCR, respectively. Transient knockdown of the TM9SF3 gene by siRNA showed decreased tumor cell-invasive capacity. **Conclusion:** Our results indicate that TM9SF3 might be a potential diagnostic and therapeutic target for scirrhus-type GC.

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Introduction

Gastric cancer (GC), which develops following multiple genetic and epigenetic alterations, is a major cause of death from malignant disease all over the world [1]. Generally, GCs have been classified into two histological types: an intestinal/diffuse type (Lauren [2]) and a differentiated/undifferentiated type (Nakamura et al. [3]), based on its tendency to gland formation. Among undifferentiated GCs, scirrhus-type GC has a worse progno-

sis than other types of GCs, reflecting rapid proliferation, progressive invasion and a high frequency of metastasis to the peritoneum [4]. Histologically, scirrhous cancer cells show diffuse infiltration of a broad region of the gastric wall, without severely affecting the mucosal lining of the stomach. Because of such pathological features, early clinical diagnosis of scirrhous GC with gastrointestinal imaging or endoscopy remains difficult despite recent advances in the diagnosis and treatment of other GCs [5]. Actually, there are no good biomarkers for this type of GC yet and, therefore, we performed gene expression profiling using scirrhous GC and identified several candidate GC-associated genes.

To identify potential molecular markers of GC and to better understand the development of GC at the molecular level, comprehensive gene expression analysis is useful. We previously performed several large-scale gene expression studies using array-based hybridization [6] and serial analysis of gene expression [7, 8], and identified several genes, including REG4 (regenerating islet-derived family member 4, which encodes REGIV) [9, 10], OLFM4 (olfactomedin 4) [11], PLUNC (palate, lung and nasal epithelium carcinoma-associated protein) [12] and GJB6 (encoding connexin 30) [13]. A recent study on REGIV revealed that it also acts as a potential biomarker of peritoneal GC dissemination [14]. Genes encoding transmembrane or secreted proteins specifically expressed in cancers are ideal biomarkers of cancer diagnosis and potential therapeutic targets. Our recent study of *Escherichia coli* ampicillin secretion trap (CAST) analysis on 2 GC cell lines identified several candidate genes encoding transmembrane proteins. Among them, DSC2 (desmocollin 2) expression was associated with GC of the intestinal mucin phenotype with CDX2 expression [15].

Here, we identified several genes that encode transmembrane proteins expressed in scirrhous GC tissue. Among these genes, we focused on the TM9SF3 gene because this gene is frequently overexpressed in GC and the most detected clone in our study. Moreover, there is no reported study of TM9SF3 expression in GC. TM9SF3 encodes transmembrane 9 superfamily member 3, which is one of the members of the TM9SF family also known as nonaspanins [16], but the detailed function and expression of the TM9SF3 gene has not been elucidated in the majority of human cancers. TM9SF3 overexpression was reported in chemotherapy-resistant breast cancer cell lines by oligonucleotide microarray analysis [17].

This is the first study of CAST analysis on surgically resected scirrhous GC tissue. The present study also represents the first detailed analysis of TM9SF3 expression

in human GC and examines the relationship between TM9SF3 staining and clinicopathological characteristics, including tumor stage, TNM grading and histological type. We clarified the TM9SF3 expression pattern and localized its expression in GC using surgically resected GC samples by immunohistochemical analysis. Furthermore, the biological role of TM9SF3 was examined in GC cell lines using an siRNA knockdown system on cancer cell growth and invasion.

Materials and Methods

CAST Library Construction

A CAST library was constructed as described previously [18]. CAST is a survival-based signal sequence trap that exploits the ability of mammalian signal sequences to confer ampicillin resistance to a mutant β -lactamase lacking the endogenous signal sequence [19]. For *E. coli* to survive the antibiotic challenge, the signal sequence and translation initiator ATG codon must be cloned in frame with the leaderless β -lactamase reporter. In this study, to identify genes that present in scirrhous GC, we generated CAST libraries from 2 human scirrhous GC tissues. These 2 tissue samples were obtained during surgery at the Hiroshima University Hospital from a 55-year-old female patient with stage IIA (T3N0M0) and a 62-year-old female patient with stage IIIB (T4N2M0). The samples were collected according to their enormous amount of accessible cancerous region, which was diagnosed by two pathologists. RNA was obtained from the tumor core in the greater curvature of the stomach, without necrosis area, for each case. Each cDNA library was generated and ligated into the pCAST vector, along with *Bam*HI and *Eco*RI sites, for restrictive regulation of reverse transcription and directional cloning. Then, the surviving ampicillin-resistant clones were picked up and sequenced in 96-well format.

Tissue Samples

In total, 338 primary tumor samples were collected from patients diagnosed with GC. For immunohistochemical analysis, we used archival formalin-fixed paraffin-embedded tissue from 111 patients (Hiroshima cohort) who had undergone surgical excision for GC at the Hiroshima University Hospital or affiliated hospitals (including 20 patients with lymph node metastasis). For quantitative reverse transcription-PCR (RT-PCR) analysis, 9 GC samples and corresponding nonneoplastic mucosa samples were obtained during surgery at the Hiroshima University Hospital. In the Yokohama cohort, 227 GC tissue samples from patients subjected to surgery at the Gastroenterological Center, Yokohama City University Medical Center, and at the Department of Surgery, Yokohama City University, from January 2002 through July 2007, were used for mRNA analysis. Informed consent was obtained, and the Ethics Committee of the Yokohama City University Medical Center approved the guidelines. Noncancerous samples were purchased from Clontech (Palo Alto, Calif., USA). The 338 cases were histologically classified as differentiated (papillary adenocarcinoma or tubular adenocarcinoma) and undifferentiated GC (poorly differentiated adenocarcinoma, signet ring cell carcinoma or mu-

cinous adenocarcinoma) according to the Japanese classification of gastric carcinomas [20]. Tumor staging was according to the International Union against Cancer TNM classification of malignant tumors.

Quantitative RT-PCR and Western Blot

Quantitative RT-PCR was performed with an ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, Calif., USA) as described previously [21]. We calculated the ratio of target gene mRNA expression levels between GC tissue (T) and corresponding nonneoplastic mucosa (N). T/N ratios >2 were considered to represent overexpression. β -Actin (ACTB gene) was used as housekeeping internal control. Western blotting was performed as described previously [22].

Immunohistochemical Evaluation

Immunostaining was performed with the Dako Envision+ Mouse Peroxidase Detection System (Dako Cytomation, Carpinteria, Calif., USA). Antigen retrieval was performed with proteinase K (Dako) for 5 min at room temperature. After blocking peroxidase activity with 3% H₂O₂-methanol for 10 min, sections were incubated with mouse polyclonal anti-TM9SF3 (Abcam/ab52889) antibody at 1:50 dilution for 1 h at room temperature, followed by incubation with Envision+ anti-mouse peroxidase for 1 h. For color reaction, sections were incubated with DAB for 10 min (counterstained with 0.1% hematoxylin). Specimens with >10% cancer cell immunostaining were considered positive according to median cutoff values rounded off to the nearest 5% (range 0–80) for TM9SF3.

RNA Interference

To knock down endogenous TM9SF3, RNA interference was performed. siRNA oligonucleotides for TM9SF3 and a negative control were purchased from Invitrogen (Carlsbad, Calif., USA). Primer sequences for 3 siRNAs are listed in the online supplementary table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000357821). Transfection was done using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer's protocol.

Cell Lines, Cell Growth and in vitro Invasion Assays

Nine cell lines derived from human GC were used. The TMK-1 cell line was established in our laboratory from a poorly differentiated adenocarcinoma [23]. Five GC cell lines of the MKN series, MKN-1 (adenosquamous cell carcinoma); MKN-7, MKN-28 and MKN-74 (well-differentiated adenocarcinoma), and MKN-45 (poorly differentiated adenocarcinoma), were kindly provided by Dr. Toshimitsu Suzuki (Fukushima Medical University School of Medicine) [24]. KATO-III, HSC-39 (signet ring cell carcinoma) and HSC-57 (well-differentiated adenocarcinoma) cell lines were kindly provided by Dr. Morimasa Sekiguchi (University of Tokyo) [25] and Dr. Kazuyoshi Yanagihara [26], respectively. All cell lines were maintained in RPMI 1640 (Nissui, Tokyo, Japan) containing 10% fetal bovine serum (BioWhittaker, Walkersville, Md., USA) in a humidified atmosphere of 5% CO and 95% air at 37°C. The MKN-28 cells were seeded at a density of 2,000 cells per well in 96-well plates. Cell growth was monitored after 0, 1, 2 and 4 days for MTT assay, as mentioned elsewhere [27]. Modified Boyden chamber assays were carried out to examine invasiveness. Cells were plated at 200,000 cells per well

in RPMI 1640 medium plus 1% serum in the upper chamber of a Transwell insert (8- μ m pore diameter; Chemicon, Temecula, Calif., USA) coated with Matrigel. Medium containing 10% serum was added in the bottom chamber using a 24-well plate format. On day 1 and 2, noninvading cells in the upper chamber were removed using a clean cotton swab and the cells attached on the lower surface of the insert were stained with Cell Stain (Chemicon); invading cells were counted using an ordinary light microscope.

Statistical Methods

Correlations between clinicopathological parameters and TM9SF3 expression were analyzed by Fisher's exact test and log-rank test for Kaplan-Meier analysis. A value of $p < 0.05$ was considered statistically significant. Statistical analyses were performed using JMP software (version 9.0.2; SAS Institute, Cary, N.C., USA).

Results

Establishment of CAST Libraries

To identify genes that encode transmembrane proteins expressed in scirrhous GC, we generated CAST libraries from 2 scirrhous GC tissues and used a previously established normal stomach CAST library [15] to compare gene expression profiles. In this fashion, we detected and sequenced 1,440 ampicillin-resistant colonies from each scirrhous CAST library. Then, these sequences were compared to those deposited in the public databases using BLAST (accessed at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>), and the subcellular localization of the gene products was evaluated using GeneCards (accessed at <http://www.genecards.org/index.shtml>). While unifying 2,880 colonies from 2 scirrhous GC tissues, 711 colonies were human-named genes, including 323 genes which were cloned in frame and upstream of the leaderless β -lactamase, in which 48 genes encoded secreted proteins, 130 genes encoded transmembrane proteins and the remaining 145 genes encoded proteins that were neither secreted nor transmembrane proteins. Because the purpose of this study is to identify genes that encode transmembrane proteins specifically present in scirrhous GC, we focused on transmembrane proteins expressed in the cancer tissue library.

Analysis of GC-Specific Gene Expression in

Comparison with Normal Tissue by the CAST Method

To determine genes specifically expressed in GC, we compared the gene list from two GC tissue CAST libraries to the normal stomach CAST library. We selected genes that were detected at least twice in each GC tissue

Table 1. List of candidate genes specifically expressed in scirrhous-type GC

Symbol	Description	Clone No.
TM9SF3	Homo sapiens transmembrane 9 superfamily member 3 (TM9SF3), mRNA	55
CD74	Homo sapiens CD74 molecule, major histocompatibility complex, class II invariant chain (CD74), transcript variant 2, mRNA	50
SGCB	Homo sapiens sarcoglycan, β (43-kDa dystrophin-associated glycoprotein) (SGCB), mRNA	22
ITGB6	Homo sapiens integrin, β 6 (ITGB6), mRNA	21
TSPAN8	Homo sapiens tetraspanin 8 (TSPAN8), mRNA	16
CD63	Homo sapiens CD63 molecule (CD63), transcript variant 1, mRNA	14
SLCO2A1	Homo sapiens solute carrier organic anion transporter family, member 2A1 (SLCO2A1), mRNA	10
ENPP4	Homo sapiens ectonucleotide pyrophosphatase/phosphodiesterase 4 (putative function) (ENPP4), mRNA	7
SERINC3	Homo sapiens serine incorporator 3 (SERINC3), transcript variant 1, mRNA	7
ATP4B	Homo sapiens ATPase, H^+/K^+ exchanging, β polypeptide (ATP4B), mRNA	6
CD68	Homo sapiens CD68 molecule (CD68), transcript variant 1, mRNA	6
SLC12A2	Homo sapiens solute carrier family 12 (sodium/potassium/chloride transporters), member 2 (SLC12A2), mRNA	6
SLC16A7	Homo sapiens solute carrier family 16, member 7 (monocarboxylic acid transporter 2) (SLC16A7), mRNA	6
ADAM9	Homo sapiens ADAM metalloproteinase domain 9 (meltrin γ) (ADAM9), transcript variant 1, mRNA	5
ATP8B1	Homo sapiens ATPase, class I, type 8B, member 1 (ATP8B1), mRNA	5
CDH17	Homo sapiens cadherin 17, LI cadherin (liver-intestine) (CDH17), transcript variant 1, mRNA	4
CLCC1	Homo sapiens chloride channel CLIC-like 1 (CLCC1), transcript variant 2, mRNA	4
CLDN7	Homo sapiens claudin 7 (CLDN7), transcript variant 1, mRNA	4
ITFG3	Homo sapiens integrin α FG-GAP repeat containing 3 (ITFG3), mRNA	4
FZD3	Homo sapiens frizzled homolog 3 (<i>Drosophila</i>) (FZD3), mRNA	3
GPNMB	Homo sapiens glycoprotein (transmembrane) nmb (GPNMB), transcript variant 2, mRNA	3
HLA-DRA	Homo sapiens major histocompatibility complex, class II, DR α (HLA-DRA), mRNA	3
LMBR1	Homo sapiens limb region 1 homolog (mouse) (LMBR1), mRNA	3
PKD2	Homo sapiens polycystic kidney disease 2 (autosomal dominant) (PKD2), mRNA	3
PROM1	Homo sapiens prominin 1 (PROM1), transcript variant 1, mRNA	3
TFRC	Homo sapiens transferrin receptor (p90, CD71) (TFRC), mRNA	3
TRPM7	Homo sapiens transient receptor potential cation channel, subfamily M, member 7 (TRPM7), mRNA	3
ADAM17	Homo sapiens ADAM metalloproteinase domain 17 (ADAM17), mRNA	2
CD55	Homo sapiens CD55 molecule, decay accelerating factor for complement (Cromer blood group) (CD55), mRNA	2
DRAM2	Homo sapiens DNA-damage regulated autophagy modulator 2 (DRAM2), mRNA	2
DSC2	Homo sapiens desmocollin 2 (DSC2), transcript variant Dsc2a, mRNA	2
ENTPD1	Homo sapiens ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1), transcript variant 1, mRNA	2
ITLN1	Homo sapiens intelectin 1 (galactofuranose binding) (ITLN1), mRNA	2
MS4A6A	Homo sapiens membrane-spanning 4-domains, subfamily A, member 6A (MS4A6A), transcript variant 2, mRNA	2
PCDH18	Homo sapiens protocadherin 18 (PCDH18), mRNA	2
PCDHB9	Homo sapiens protocadherin β 9 (PCDHB9), mRNA	2
SLC38A2	Homo sapiens solute carrier family 38, member 2 (SLC38A2), mRNA	2
SLC4A4	Homo sapiens solute carrier family 4, sodium bicarbonate cotransporter, member 4 (SLC4A4), transcript variant 2, mRNA	2
TAOK3	Homo sapiens TAO kinase 3 (TAOK3), mRNA	2
TMBIM4	Homo sapiens transmembrane BAX inhibitor motif containing 4 (TMBIM4), mRNA	2
TNFSF13B	Homo sapiens tumor necrosis factor (ligand) superfamily, member 13b (TNFSF13B), transcript variant 1, mRNA	2
ZDHHC14	Homo sapiens zinc finger, DHHC-type containing 14 (ZDHHC14), transcript variant 1, mRNA	2

CAST library but not once in the normal stomach CAST library. In total, 42 candidate genes were spotted, as listed in table 1. We focused on TM9SF3 because it had the highest number of clones counted in our candidate list; moreover, there is no detailed functional analysis of TM9SF3 in human cancers yet. Here, we used bulk cancer tissue samples, which contain both cancer cells and stromal components. Actually, some of the genes were

derived from stromal cells. For instance, CD74 is associated with macrophage migration-inhibitory factor [28] and CD68 is a marker for the various cells of the macrophage lineage [29]. High on the list, sarcoglycan is well known for connecting the muscle fiber cytoskeleton to the extracellular matrix [30]. These results suggested that CAST is a robust and reliable technique to identify novel genes.

Fig. 1. Quantitative RT-PCR analysis of TM9SF3. **a** TM9SF3 mRNA expression level in 13 normal tissues and 9 GC samples (arbitrary units). **b** T/N ratio of TM9SF3 mRNA level in 227 GC cases (Yokohama cohort). T/N ratio >2 was considered to represent overexpression. Upregulation of the TM9SF3 gene was observed in 28% of the total cases.

Messenger RNA Expression of TM9SF3 in Systemic Normal Organs and GC Tissue

Genes expressed at high levels in tumors and very low levels in normal tissue are ideal diagnostic markers and therapeutic targets. To confirm whether the TM9SF3 gene is cancer specific, quantitative RT-PCR was performed in 9 GC tissue samples and in 13 kinds of normal tissue (liver, kidney, heart, colon, brain, bone marrow, skeletal muscle, lung, small intestine, spleen, spinal cord, stomach and peripheral leukocyte). TM9SF3 expression was detected at low levels, or lesser extent, in normal organ tissue, including the stomach. High TM9SF3 expression was observed in 4 of 9 GC tissues (44%; fig. 1a). To validate CAST data, TM9SF3 expression in GC was investigated by quantitative RT-PCR in an additional 227 GC samples and corresponding nonneoplastic mucosa. We calculated the ratio of target gene mRNA expression levels between GC tissue and corresponding nonneoplastic mucosa, and T/N ratios >2 were considered to represent overexpression. TM9SF3 mRNA was upregulated in 63 of 227 cases (28%; fig. 1b).

Immunohistochemical Analysis of TM9SF3 in GC

To analyze tissue localization, pattern of distribution and the relationship between clinicopathologic parameters and TM9SF3 in GC, we performed immunohistochemical analysis of TM9SF3 using a commercially available antibody. TM9SF3 expression was detected in 46 (50%) of 91 GCs showing diffuse staining of cancer cells from superficial to deep layers of both early- and advanced-stage GC (fig. 2a, b). Histologically, TM9SF3 was observed more frequently in undifferentiated than in differentiated GCs ($p = 0.0213$; table 2). In high-power fields, it showed a membranous pattern of staining in GC tissue and sometimes we observed its cytoplasmic accumulation (fig. 2c). In corresponding nonneoplastic gastric mucosa, TM9SF3 was scarcely expressed (fig. 2d) and showed positive staining of cancer cells invading lymphatic vessels (fig. 2e). Next, we examined the relationship between TM9SF3 expression and clinicopathological parameters. TM9SF3 staining showed a significant correlation with depth of invasion ($p = 0.0065$), lymph node metastasis ($p = 0.0101$) and TNM stage ($p = 0.0065$). Furthermore, we grouped undifferentiated GC into scirrhous and non-

Fig. 2. Immunohistochemical staining of TM9SF3 in GC tissues. **a, b** TM9SF3 was detected in cancer cells from superficial to deep layers of undifferentiated GC tissue. **c** TM9SF3 expression was observed as membranous and cytoplasmic staining in cancer cells but not in the surrounding stromal cells. **d** In noncancerous epithelium, adjacent to GC cells, TM9SF3 showed weak or no expression. **e** Expression of TM9SF3 was observed in GC cells in lymphatic vessels. **a** $\times 40$; bar = 500 μm . **b, d, e** $\times 100$; bar = 200 μm . **c** $\times 200$; bar = 100 μm .

scirrhous types, and a strong correlation between scirrhous GC and TM9SF3 expression was found ($p = 0.0156$). There was no significant association between TM9SF3 expression and other parameters (age, gender or M grade).

Relationship between TM9SF3 Expression and Patient Prognosis

We also examined the relationship between TM9SF3 expression and survival prognosis in 91 GC cases. The prognosis of patients with positive TM9SF3 expression was significantly worse than that of the TM9SF3-negative cases ($p = 0.0130$; fig. 3a). According to the immunostaining results, in the group of undifferentiated GC cases, the probability of survival was poor in TM9SF3-positive GC cases ($p = 0.0131$; fig. 3b). Moreover, there was a tendency

of scirrhous GC with TM9SF3 expression to poor prognosis ($p = 0.0695$; fig. 3c). In addition, a validated analysis of the Yokohama cohort ($n = 227$, analyzed by quantitative RT-PCR) displayed a significant correlation between survival probability and TM9SF3 mRNA level upregulation in scirrhous GC ($p = 0.0231$; fig. 3d). This validation study confirmed our immunostaining data in a separate cohort. In this cohort, TM9SF3 is frequently overexpressed in scirrhous GC compared to corresponding nonneoplastic gastric mucosa; however, there was no correlation between clinicopathological features (age, TNM grade, tumor stage and histology) and TM9SF3 expression (data not shown). The results lead to the conclusion that survival probability is poor in TM9SF3-positive GC, and particularly scirrhous GC patients showed a significantly dismal prognosis.

Table 2. Relationship between TM9SF3 expression and clinico-pathological parameters in 91 GC cases by immunohistochemistry

	TM9SF3 expression		p value
	positive	negative	
Age			
≤65 years (n = 49)	26 (53%)	23	NS
>65 years (n = 42)	20 (48%)	22	
Gender			
Male (n = 51)	21 (41%)	30	NS
Female (n = 40)	25 (63%)	15	
T grade			
T1 (n = 28)	8 (29%)	20	0.0065
T2/T3/T4 (n = 63)	38 (60%)	25	
N grade			
N0 (n = 25)	7 (28%)	18	0.0101
N1/N2/N3 (n = 66)	39 (59%)	27	
M grade			
M0 (n = 76)	37 (49%)	39	NS
M1 (n = 15)	9 (60%)	6	
Stage			
Stage I (n = 28)	8 (29%)	20	0.0065
Stage II/III/IV (n = 63)	38 (60%)	25	
Histology			
Differentiated (n = 45)	17 (38%)	28	0.0213
Undifferentiated (n = 46)	29 (63%)	17	
Scirrhou (n = 27)	18 (67%)	9	0.0156
Nonscirrhou (n = 19)	5 (26%)	14	

p values were calculated by Fisher's exact test. Histology was determined according to the Japanese Classification of Gastric Carcinomas. NS = Nonsignificant.

TM9SF3 Expression in Primary and Lymph Node Metastatic Sites

Immunostaining of corresponding lymph node metastatic sites was performed to confirm the distribution of TM9SF3 in metastatic tissue. Compared with the positive rate and staining pattern of TM9SF3 in primary tumors, concordance rates were calculated as a combination of both positive and negative cases in primary and metastatic tissue divided by the total number of cases. Concordance rates of TM9SF3 were 75% (15 of 20 GC cases; on-line suppl. fig. 1).

Role of TM9SF3 Downregulation on Cell Growth and Invasion in GC

TM9SF3 staining showed a significant correlation with depth of invasion, lymph node metastasis and worse prognosis in GC cases with increased TM9SF3 expression, suggesting that TM9SF3 may be associated with cancer cell growth and invasion ability. However, the bio-

logical significance of TM9SF3 in GC has not been studied. Initially, we investigated TM9SF3 expression in 9 GC cell lines (fig. 4a) and increased expression was detected in HSC-39 and MKN-28 cell lines. HSC-39 is derived from signet ring cell carcinoma of the stomach and is an ideal cell line for this study. Unfortunately, it is a floating cancer cell line and difficult to transfect and process for experimental procedures, and so we utilized MKN-28 cells for the following analyses. Gene silencing in MKN-28 cells was confirmed by Western blotting (fig. 4b). To investigate the possible proliferative effect of TM9SF3, we performed an MTT assay 2 days after TM9SF3-siRNA and negative control siRNA transfection. There was no significant difference between TM9SF3 siRNA-transfected MKN-28 cells and negative control siRNA-transfected cells (fig. 4c). Next, to determine the possible role of TM9SF3 in the invasiveness of GC cells, a Transwell invasion assay was performed in the MKN-28 GC cell line. Invasion ability was significantly downregulated in TM9SF3-knockdown GC cells compared with negative control siRNA-transfected GC cells (fig. 4d). These data verify that TM9SF3 is associated with cancer cell invasion but not with cancer cell growth in vitro.

Discussion

In the present study, we generated CAST libraries from 2 scirrhou GC tissues and identified several genes that encode transmembrane proteins present in scirrhou GC. This is the first article analyzing surgically resected GC tissue samples using the CAST method. We emphasized on transmembrane proteins for their central role as putative novel biomarkers and therapeutic targets, and observed that TM9SF3 showed the highest clone count in the candidate list of the scirrhou CAST library. Both quantitative RT-PCR analysis and immunohistochemistry revealed that TM9SF3 was frequently overexpressed in GC. The distribution of TM9SF3 in metastatic lymph nodes also showed a high concordance rate. With regard to TM9SF3 upregulation, this could be explained by the gain of DNA copy numbers in chromosome 10q24, which was reported in GC [31, 32], where the TM9SF3 gene is located. In addition, we observed a significant correlation between TM9SF3 expression and poor survival prognosis in two validation studies.

TM9SF3 encodes transmembrane 9 superfamily member 3, a member of the TM9SF family. TM9SF members are characterized by a large noncytoplasmic domain and nine putative transmembrane domains [16]. This family

Fig. 3. Cancer-specific survival in two separate cohorts: the Hiroshima cohort (n = 91, immunostaining) and the Yokohama cohort (n = 227, quantitative RT-PCR). p values (log-rank test) are shown in the right lower quadrant of each panel. **a** Patient prognosis of positive TM9SF3 expression in all GC cases (n = 91) using immu-

nohistological data. **b** Analysis of undifferentiated type GC cases (n = 46). **c, d** Kaplan-Meier plots of the cancer-specific mortality of scirrhous GC cases in the Hiroshima (n = 27) and Yokohama cohorts (n = 44), respectively.

is highly conserved through evolution, and four members are reported in mammals (TM9SF1–TM9SF4), suggesting an important biological role for these proteins. However, except for the recently characterized genetic studies in *Dictyostelium* and *Drosophila* showing that TM9SF members are required for adhesion and phagocytosis in innate immune responses [33], the biological functions of TM9SF proteins remain largely unknown. Recent studies have demonstrated that human TM9SF1 plays a role in the regulation of autophagy [34] and human TM9SF4 is associated with tumor cannibalism and the aggressive phenotype of metastatic melanoma cells [35]. Using rat and Chinese hamster models, Sugawara et al. [36] have reported that TM9SF3, also known as SMBP, was the first member of TM9SF with functional ligand binding properties. In addition, TM9SF proteins have been found as endosomal or Golgi-like distribution [16], and one of the

TM9SF family members, TM9SF2, has been found to be localized in the endosomal or lysosomal compartment [37]. Consistent with our results, TM9SF3 showed cytoplasmic accumulation as well as a membranous staining pattern.

Our results revealed a significant correlation between TM9SF3 expression and tumor progression. In scirrhous GC, MMP-2 produced from stromal fibroblasts is activated by MT1-MMP expressed by GC cells and affects cancer progression in a paracrine manner [38]. Also, fibroblast growth factor-7 from gastric fibroblasts also affected the growth of scirrhous GC cells [39]. Reciprocally, most fibroblasts were partially regulated by cancer cell-derived growth factors [40], such as transforming growth factor- β , platelet-derived growth factor and fibroblast growth factor-2, all of which are key mediators of fibroblast activation and tissue fibrosis [41]. Thus, the growth-

Fig. 4. Effect of TM9SF3 downregulation on cell growth and cell invasion. **a** The anti-TM9SF3 antibody detected a ~46-kDa band on Western blot in 9 GC cell lines. β -Actin (ACTB) was used as a loading control. **b** Western blot analysis of TM9SF3 in MKN-28 GC cells transfected with negative control siRNA or TM9SF3 siRNAs (siRNA-1 to siRNA-3). **c** Cell growth was assessed by an MTT assay on 96-well plates in MKN-28 cells. Means and SD of

3 different experiments. **d** Effect of TM9SF3 knockdown on cell invasion in MKN-28 cells. MKN-28 GC cells transfected with negative control siRNA or TM9SF3 siRNA-1 and siRNA-3 were incubated in Boyden chambers. After 24 and 48 h of incubation, invading cells were counted. Means and SD of 3 different experiments. OD = Optical density; NS = nonsignificant. * $p < 0.05$; ** $p < 0.008$.

promoting factors from GC cells and tumor-specific fibroblasts mutually augment proliferation. Likewise, our present data also demonstrated that TM9SF3-positive scirrhous GC cases had a worse prognosis than TM9SF3-negative cases in the two separate cohorts. Here, we suggest that TM9SF3 could establish robust malignant behavior of scirrhous GC cells by acting like a receptor, channel or small molecule transporter in these cancer-stromal cell interactions although the precise function of TM9SF3 is unclear yet. Indeed, further investigations are needed to elucidate these hypotheses. On the other hand, in the Yokohama cohort, at the mRNA level, there was no statistically significant correlation with clinicopathologic parameters, including TNM grade and tumor stage. It reflects that the mRNA level actually depends on the amount of tissue obtained and it was difficult to sample tissue from the deeper part of all GC.

During in vitro biochemical analyses of TM9SF3, a basement membrane-coated cell invasion assay showed that transient knockdown of TM9SF3 resulted in suppression of the invasive capacity of GC cells. We speculate that human TM9SF3 might participate in an invasive mechanism of GC cells. The next crucial step will be to elucidate how TM9SF3 is involved in the tumor invasion process and whether it is specific for scirrhous GC, in which cancer-stromal interactions have been especially evident. In general, tumor cells at the invasion front are considered to have more aggressive and malignant behavior. A recent study on invasion in GC showed that molecular expression of MMP-7, laminin- γ_2 and epidermal growth factor receptor was associated with T grade, N grade and tumor stage [42]. However, GC is well known for its intratumoral heterogeneity and, thus, it is difficult to target the whole tumor mass because of such heterogeneous expression of tumor markers. Targeted therapy

to all malignant tumor cells is quite difficult and still required to identify. Here, TM9SF3 staining was found in both the mucosal region and invasion front of tumor mass and, thus, it might be a useful therapeutic target for GC.

Taken together, TM9SF3 is a promising prognostic marker for cancer diagnosis of the stomach, especially in scirrhous GC. Evaluating the molecular mechanism of TM9SF3 involvement in tumor-stroma interactions might improve our understanding of GC carcinogenesis and tumor progression. TM9SF3 expression may be a key factor mediating the biological behavior of scirrhous GC. Furthermore, using the CAST method, we could identify unknown target genes and novel biomarkers for cancer diagnosis and management. Further studies including a large number of GC samples on chemotherapy resistance of GC and novel candidates involved in its molecular mechanism are warranted.

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Disclosure Statement

The authors have no conflict of interest to disclose.

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