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Identification of Novel Transmembrane Proteins in Scirrhous Type Gastric Cancer by *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 · TM9SF3 · Prognosis · Transmembrane 9 superfamily member 3

Abstract

Objective: Scirrhous type gastric cancer is highly aggressive and has a worse prognosis because of its 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 scirrhous type gastric cancer. ***Methods:*** We generated *Escherichia coli* ampicillin secretion trap (CAST) libraries from 2 human scirrhous type gastric cancer tissues and compared with a normal stomach CAST library. By sequencing 2,880 colonies from scirrhous CAST libraries, we identified a list of candidate genes. ***Results:*** We focused on TM9SF3 gene because it has the highest clone count and immunohistochemical analysis demonstrated that 46 (50%) of 91 gastric cancer cases were positive for TM9SF3 and it was observed frequently in scirrhous type gastric cancer. TM9SF3 expression showed a significant correlation with the depth of invasion, tumor stage and undifferentiated type of gastric cancer. There was a strong correlation between TM9SF3 expression and poor survival prognosis of patients, validated in two separate cohorts, by immunostaining or qRT-PCR. 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 scirrhous type gastric cancer.

Introduction

Gastric cancer (GC) is a major cause of death from malignant disease all over the world and develops as a result of multiple genetic and epigenetic alterations [1]. Generally, GCs have been classified into 2 histological types: an intestinal and a diffuse type by Lauren [2], or a differentiated type and an undifferentiated type by Nakamura et al. [3], based on the tendency toward gland formation. Among undifferentiated type GCs, scirrhous type GC has a worse prognosis than other types of GC, 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 type GC with gastrointestinal series 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 type GC and identified several candidate GC-associated genes.

To identify potential molecular markers for 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 (SAGE) [7], [8] and identified several genes including regenerating islet-

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derived family, member 4 (REG4, which encodes REGIV) [9], [10], olfactomedin 4 (OLFM4) [11], palate, lung and nasal epithelium carcinoma-associated protein (PLUNC) [12], and GJB6 (encoding connexin 30) [13]. Recent study on REGIV revealed that it also acts as a potential biomarker for peritoneal dissemination of gastric cancer [14]. Genes encoding transmembrane or secreted proteins specifically expressed in cancers are ideal biomarkers for cancer diagnosis and potential therapeutic targets. Our recent study of *Escherichia coli* (E. coli) ampicillin secretion trap (CAST) analysis on 2 GC cell lines identified several candidate genes encoding transmembrane proteins. Among them, Desmocollin 2 (DSC2) 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 type 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], however, detailed function and expression of the TM9SF3 gene in majority of human cancers has not been elucidated. TM9SF3 was reported as one of the genes overexpressed in chemotherapy resistant breast cancer cell lines by oligonucleotide microarray analysis [17].

This is the first study of CAST analysis on surgically resected scirrhous type 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 pattern of expression and localization of TM9SF3 expression in GC, using surgicallly 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

CAST library construction was performed 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 type GC, we generated CAST libraries from 2 human scirrhous type GC tissues. These 2 samples were obtained during surgery at Hiroshima University Hospital; one is 55-year old, female patient with Stage IIA (T3N0M0) and the other is 62-year old, female patient with Stage IIIB (T4N2M0). They were collected according to their enormous amount of accessible cancerous region, which was diagnosed by 2 pathologists. The 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 BamHI and EcoRI 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 tissues from 111 patients (Hiroshima cohort) who had undergone surgical excision for GC at the Hiroshima University Hospital or affiliated hospitals, including 20 cases with their corresponding lymph node metastasis. For quantitative reverse transcription-PCR (RT-PCR) analysis, 9 GC samples and corresponding non-neoplastic mucosa samples were obtained during surgery at the Hiroshima University Hospital. In Yokohama cohort, 227 GC cases from patients underwent 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 ethics committee of Yokohama City University Medical Center approved the guidelines. Noncancerous samples were purchased from Clontech (Palo Alto, CA, USA). The 338 cases were histologically classified as differentiated type (papillary adenocarcinoma or tubular adenocarcinoma) and undifferentiated type (poorly differenttiated adenocarcinoma, signet ring cell carcinoma or mucinous adenocarcinoma), according to Japanese Classification of Gastric Carcinomas [20]. Tumor staging was according to 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, CA, USA) as described previously [21]. We calculated the ratio of target gene mRNA expression levels between GC tissue (T) and corresponding non-neoplastic mucosa (N). T/N ratios > 2 fold were considered to represent overexpression. β-actin (ACTB gene) was used as housekeeping internal control. Western blot was performed as described previously [22].

Immunohistochemical Evaluation

Immunostaining was performed with Dako Envision+ Mouse Peroxidase Detection System (Dako Cytomation, Carpinteria, CA, USA). Antigen retrieval with Proteinase K (Dako) for 5 minutes at room temperature. After peroxidase activity was blocked with 3% H_2O_2-methanol for 10 minutes, sections were incubated with mouse polyclonal anti-TM9SF3 (Abcam/ab52889) antibody at 1:50 dilution for 1 hour at room temperature, followed by incubations with Envision+ anti-mouse

peroxidase for 1 hour. For color reaction, sections were incubated with DAB for 10 minutes, counterstained with 0.1% hematoxylin. When each specimen had more than 10% of cancer cells stain, the immunostaining was considered positive according to median cut-off values rounded off to the nearest 5% (range 0-80) for TM9SF3.

RNA Interference (RNAi)

To knockdown the endogenous TM9SF3, RNAi was performed. siRNA oligonucleotides for TM9SF3 and a negative control were purchased from Invitrogen (Carlsbad, CA, USA). Primer sequences for 3 siRNAs are listed in the Supplementary table. 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; 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 (Yasuda Women's University) [26], respectively. All cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical Co, Ltd, Tokyo, Japan) containing 10% fetal bovine serum (BioWhittaker, Walkersville, MD) in a humidified atmosphere of 5% CO and 95% air at 37°C. The MKN-28 cells were seeded at a density of 2000 cells per well in 96-well plates. Cell growth was monitored after day 0, 1, 2 and 4 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 μm pore diameter; Chemicon, Temecula, CA, USA) coated with Matrigel. Medium containing 10% serum was added in the bottom chamber using 24-well plate format. On day 1 and 2, non- invading cells in the upper chamber were removed by clean cotton swab and the cells attached on the lower surface of the insert were stained with Cell Stain (Chemicon, Temecula, CA, USA), and the invading cells were counted with 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 *P* value of less than 0.05 was considered statistically significant. Statistical analyses were performed using JMP software (version 9.0.2; SAS institute, Carey, NC).

Results

Establishment of CAST Libraries

To identify genes that encode transmembrane proteins expressed in scirrhous type GC, we generated CAST libraries from 2 scirrhous type 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 evaluated the subcellular localization of the gene products using GeneCards (accessed at http://www.genecards.org/index.shtml). While unifying 2,880 colonies from 2 scirrhous type 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 type GC, we focused on transmembrane proteins expressed in the cancer tissue library.

Analysis of GC Specific Gene Expression in comparison with Normal Tissue through CAST Method

To determine genes expressed specifically 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 CAST library but not once in the normal stomach CAST library. In total, 42 candidate genes were obtained, as listed in Table 1. We focused to 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.

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

Genes expressed at high levels in tumors and very low levels in normal tissues 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

Table 1. List of candidate genes specifically expressed in scirrhous type gastric cancer

SYMBOL	DESCRIPTION	CLONE NO.
TM9SF3	Homo sapiens transmembrane 9 superfamily member 3 (TM9SF3), mRNA.	55
CD74	Homo sapiens CD74 molecule, major histocompatibility complex, (CD74), transcript variant 2, mRNA.	50
SGCB	Homo sapiens sarcoglycan, beta (43kDa dystrophin-associated glycoprotein) (SGCB), mRNA.	22
ITGB6	Homo sapiens integrin, beta 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, beta 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 metallopeptidase domain 9 (meltrin gamma) (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 alpha FG-GAP repeat containing 3 (ITFG3), mRNA.	4
FZD3	Homo sapiens frizzled homolog 3 (Drosophila) (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 alpha (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 metallopeptidase domain 17 (ADAM17), mRNA.	2
CD55	Homo sapiens CD55 molecule, decay accelerating factor for complement (CD55), transcript variant 1, 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 beta 9 (PCDHB9), mRNA.	2
SLC38A2	Homo sapiens solute carrier family 38, member 2 (SLC38A2), mRNA.	2
SLC4A4	Homo sapiens solute carrier family 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

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 organs including the stomach. High TM9SF3 expression was observed in 4 out of 9 GC tissues (44%) (Fig. 1a). To validate the CAST data, TM9SF3 expression in GC was investigated by quantitative RT–PCR in an additional 227 GC samples and corresponding non-neoplastic mucosa. We calculated the ratio of target gene mRNA expression levels between GC tissue (T) and corresponding non-neoplastic mucosa (N). T/N ratios > 2-fold 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, relationship between clinicopathologic parameters and TM9SF3 in GC, we performed immunohistochemical (IHC) analysis of TM9SF3 using a commercially available antibody. TM9SF3 expression was detected in 46 (50%) of 91 GCs and it showed a diffuse staining of cancer cells from superficial to deep layer of both early GC and advanced GC (Fig. 2a, b). Histologically, TM9SF3 was observed more frequently in the undifferentiated type of GC than in differentiated GC ($p = 0.0213$) (Table 2). In high power field, it showed membranous pattern of staining in GC tissues and sometimes we observed its cytoplasmic accumulation (Fig. 2c). In corresponding non-neoplastic gastric mucosa, TM9SF3

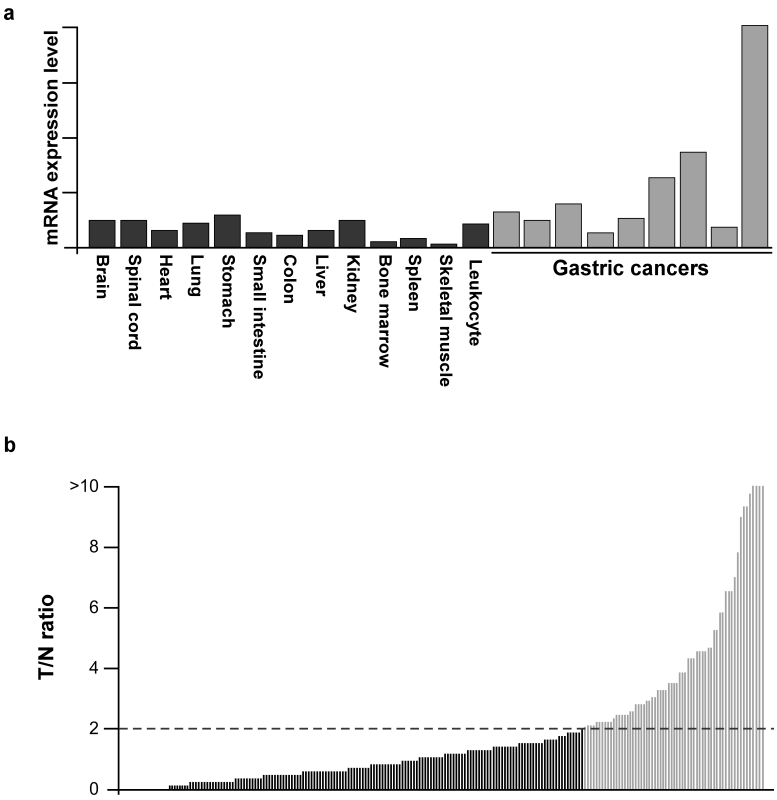

Fig. 1. Quantitative RT–PCR analysis of TM9SF3. **(a)** TM9SF3 mRNA expression level in 13 normal tissues and nine GC samples (arbitrary units). **(b)** T/N ratio of TM9SF3 mRNA level between GC tissue (T) and corresponding non-neoplastic mucosa (N) in 227 GC cases (Yokohama-cohort). T/N ratio > 2-fold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

was scarcely expressed (Fig. 2d) and it showed positive staining of cancer cells invading lymphatic vessel (Fig. 2e). Next, we examined the relationship between TM9SF3 expression and clinicopathological parameters. TM9SF3 staining showed a significant correlation with the depth of invasion ($p = 0.0065$), lymph node metastasis ($p = 0.0101$) and TNM stage ($p = 0.0065$). Furthermore, we grouped scirrhous type and non-scirrhous type within undifferentiated type GC and it showed strong correlation between scirrhous type GC and TM9SF3 expression ($p = 0.0156$). There was no significant association between TM9SF3 expression and other parameters (age, gender or M grade).

Relationship between Expression of TM9SF3 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 in the negative cases ($p = 0.0130$) (Fig. 3a). According to the immunostaining result, we analyzed on the group of undifferentiated type GC cases and it revealed poor survival probability in TM9SF3 positive GC cases ($p = 0.0131$) (Fig. 3b). Moreover, there was a tendency between scirrhous type GC with TM9SF3 expression and poor prognosis ($p = 0.0695$) (Fig. 3c) and then, we performed a validated analysis on Yokohama cohort (n = 227, analyzed by qRT-PCR), which displayed a significant correlation between survival probability and TM9SF3 mRNA level upregulation in scirrhous type GC ($p = 0.0231$) (Fig. 3d). This validation study mentioned that our immnunostaining data gave a uniform consistency with a separate cohort. In this cohort, TM9SF3 in scirrhous type GC is frequently overexpressed than corresponding non-neoplastic gastric mucosa, however, there was no correlation between clinicopathological features (age, TNM grade, tumor stage and histology) and TM9SF3 expression (data not shown). Taken together, it was concluded that TM9SF3 positive GC has poor survival probability and especially in which scirrhous type GC showed significant worse prognosis.

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 deposits. 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 metastasis, divided by the total number of cases. Concordance rates of TM9SF3 were 75% (15 of 20 gastric cancer

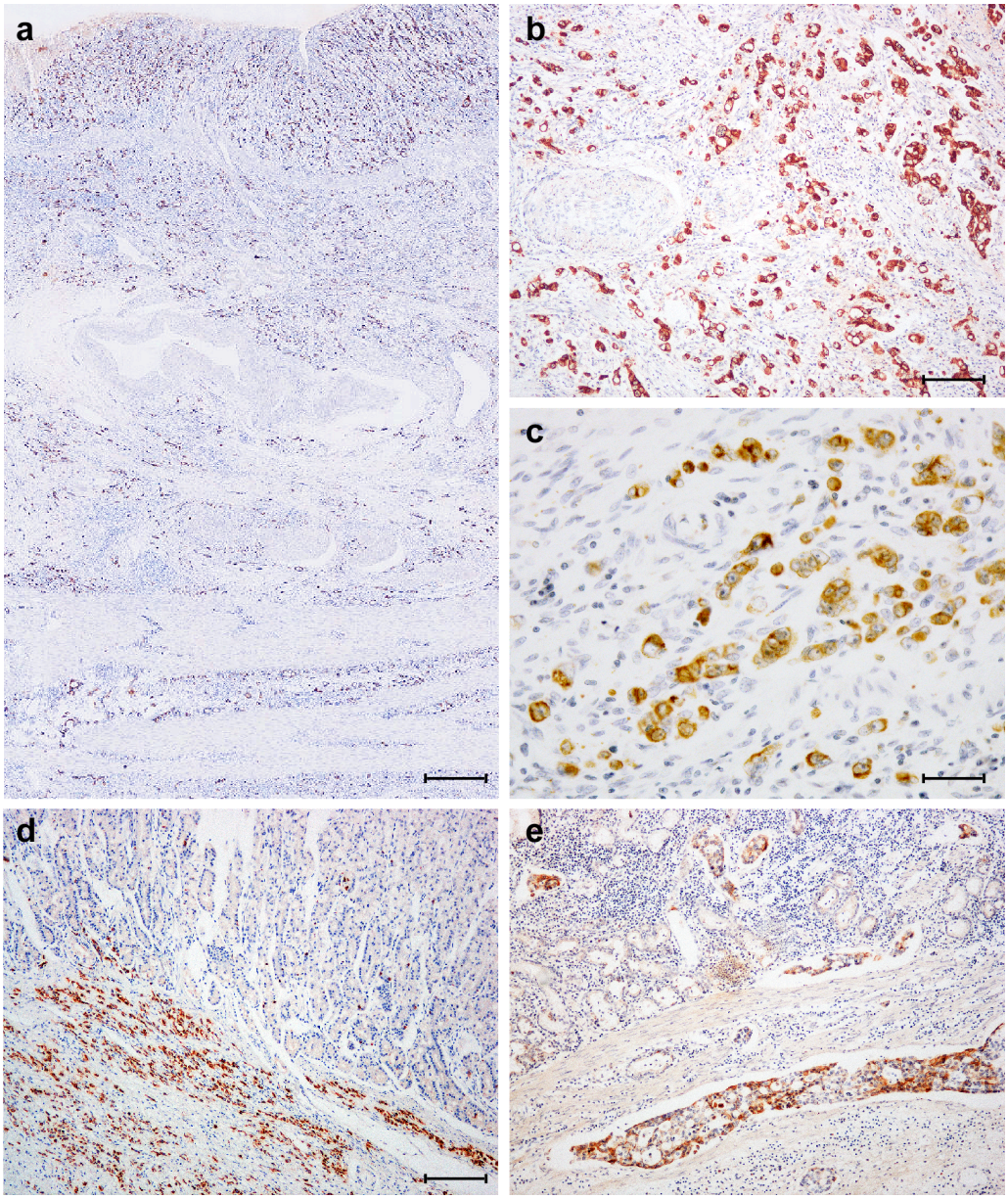

Fig. 2. Immunohistochemical staining of TM9SF3 in GC tissues. **(a and b)** TM9SF3 was detected in cancer cells from superficial to deep layer of undifferentiated type GC tissue. (x40 magnification; bar = 500 µm in **a**) **(c)** TM9SF3 expression was observed as membranous and cytoplasmic staining in cancer cells, but not in the surrounding stromal cells. (x200 magnification; bar = 100 µm) **(d)** In non-cancerous epithelium, adjacent to gastric cancer cells, TM9SF3 showed a few or no expression. **(e)** Expression of TM9SF3 was observed in GC cells in lymphatic vessel (x100 magnification; bar = 200 µm in **b**, **d**, **e**).

cases) (Supplementary Fig. 1 a and b).

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

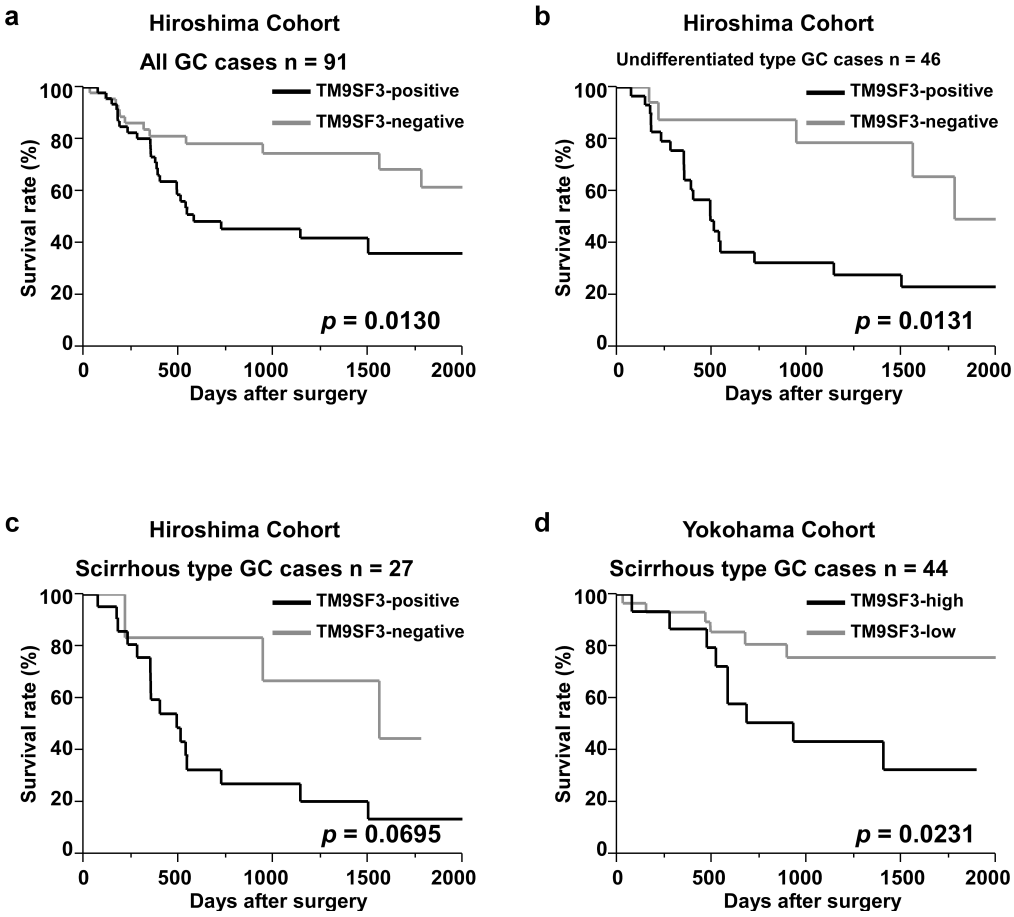

Fig. 3. Cancer specific survival in two separate cohorts; Hiroshima cohort (n = 91, immunostaining) and Yokohama cohort (n = 227, qRT-PCR). *P* value (log-rank test) is shown in the right lower quadrant of each panel. **(a)** Patient prognosis of positive TM9SF3 expression in all GC cases, using immunohistological data. **(b)** Analysis of undifferentiated type GC cases. **(c and d)** Kaplan-Meier plots of the cancer-specific mortality of scirrhous type GC cases in the Hiroshima and Yokohama cohorts, respectively.

prognosis in highly expressed GC cases, suggesting that TM9SF3 may be associated with cancer cell growth and invasion ability. However, the biological signifcance of TM9SF3 in GC has not been studied. Initially, we investigated TM9SF3 expression on 9 GC cell lines (Fig. 4a) and found strong expression 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 were confirmed by Western blot (Fig. 4b). To investigate the possible proliferative effect of TM9SF3, we performed an MTT assay 2 days after TM9SF3-siRNAs 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

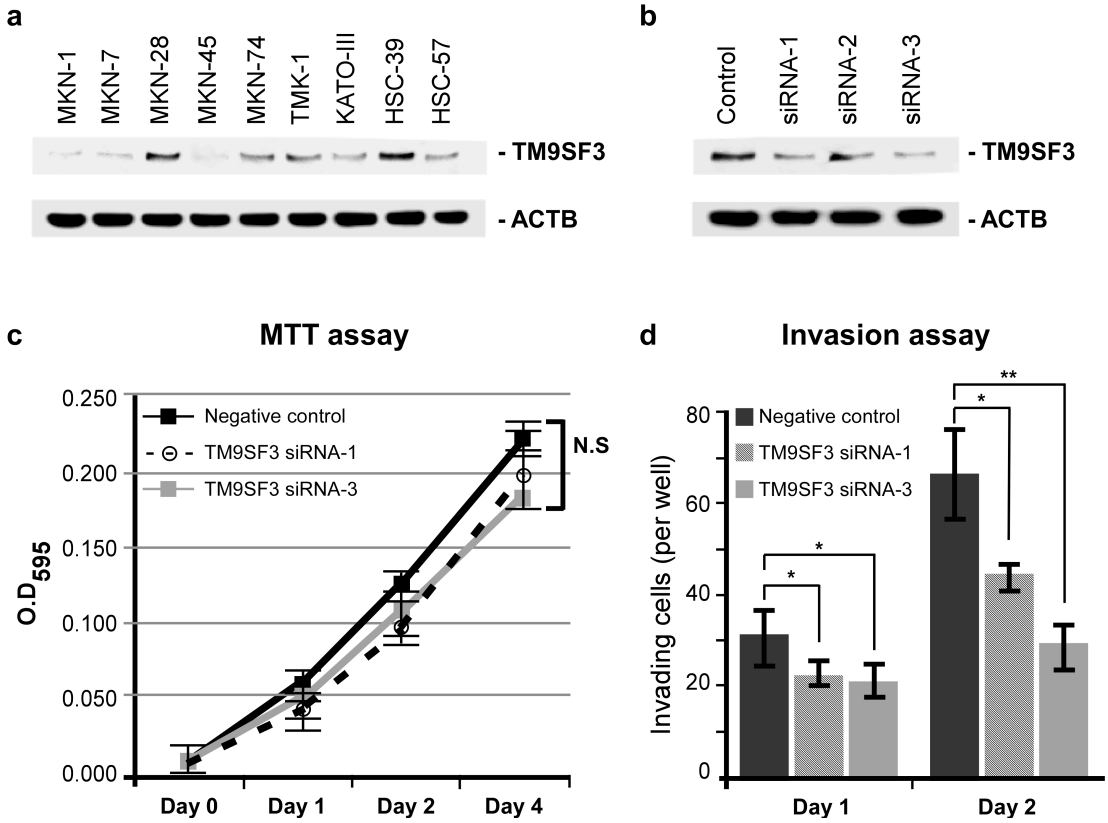

Fig. 4. Effect of TM9SF3 downregulation on cell growth and cell invasion. **(a)** The anti-TM9SF3 antibody detected at ~46 kD band on western blot of nine GC cell lines. β-actin was used as a loading control. **(b)** Western blot analysis of TM9SF3 in MKN-28 GC cells transfected with negative control siRNA or TM9SF3 siRNAs (siRNA 1–3). **(c)** Cell growth was assessed by an MTT assay on 96-well plates in MKN-28 cells. Bars and error bars show mean and s.d. of three 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-hour incubation, invading cells were counted. Bars and error bars show mean and s.d., respectively of three different experiments. O.D., optical density. N.S., not significant. (*, $P < 0.05$; **, $P < 0.008$).

TM9SF3 knockdown GC cells compared with negative control siRNA-transfected GC cells (Fig. 4d). These data verify that TM9SF3 is associated with invasion of cancer cells, but not with cancer cell growth in vitro.

Discussion

In the present study, we generated CAST libraries from 2 scirrhous type GC tissues, and identified several genes that encode transmembrane proteins present in scirrhous type GC. This is the first article analyzing surgically resected GC tissue samples by 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 scirrhous 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 the high concordance rate. With regard to the TM9SF3 upregulation, this could be explained by gain of DNA copy numbers in chromosome 10q24, which was reported in gastric cancer [31], [32], where 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 which is one of the members of the TM9SF family.

TM9SF members are characterized by a large non-cytoplasmic domain and nine putative transmembrane domains [16]. This family 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 response [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 involving in tumor cannibalism and aggressive phenotype of metastatic melanoma cells [35]. Using rat and Chinese Hamster models, Sugasawa 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 member, TM9SF2 has been found to be localized in endosomal or lysosomal compartment [37]. It is consistent with our result that TM9SF3 showed cytoplasmic accumulation as well as membranous staining pattern.

Based on our results, TM9SF3 expression was significantly correlated with tumor progression. In scirrhous type 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 (FGF-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, TGFβ, platelet-derived growth factor (PDGF) and FGF2, all of which are key mediators of fibroblast activation and tissue fibrosis [41]. Thus, the growth-promoting factors from GC cells and tumor-specific fibroblasts mutually augment each other's proliferation. Likewise, our present data also demonstrated that TM9SF3 positive scirrhous type GC cases had worse prognosis than negative cases, in both sets of 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 trans- porter in these cancer-stromal cell interactions although the precise function of TM9SF3 is unclear yet. Further investigations are indeed needed to illuminate these hypotheses. On the other hand, in Yokohama cohort, investigated on mRNA level, there was no statistically significant correlation with clinicopathologic parameters including TNM grade and tumor stage. It reflects that mRNA level, actually depends on the amount of tissue obtained and it was difficult to acquire tissue from deeper part of all GC samples.

During in vitro biochemical analyses of TM9SF3, a basement membrane-coated cell invasion assay showed that transient knockdown of TM9SF3 re- sulted in suppression of invasive capacity of GC cells. We speculate that human TM9SF3 might be involved 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 scirrhous type GC specific, 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. Recent study on invasion front of GCs showed that molecular expression of MMP-7, laminin-gamma2 and EGFR was associated with T grade, N grade and tumor stage [42]. However, GC is well known for its intra-tumoral heterogeneity and so, it is difficult to target the whole tumor mass because of such heterogeneous expression of tumor markers. Targeted therapy towards all malignant tumor cells is quite difficult and still required to identify. Here, TM9SF3 stained at both 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 type 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 the scirrhous type GC. Furthermore, using CAST method, we could identify unknown target genes and novel biomarkers for cancer diagnosis and management. In subsequent study, it might be interesting to examine on a large number of GC samples to study the chemotherapy resistance GC and novel candidates involving towards its molecular mechanism.

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Disclosure Statement

The authors have no conflict of interest to disclose.

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Supplementary information

Additional supplementary information can be found in online version of this article.