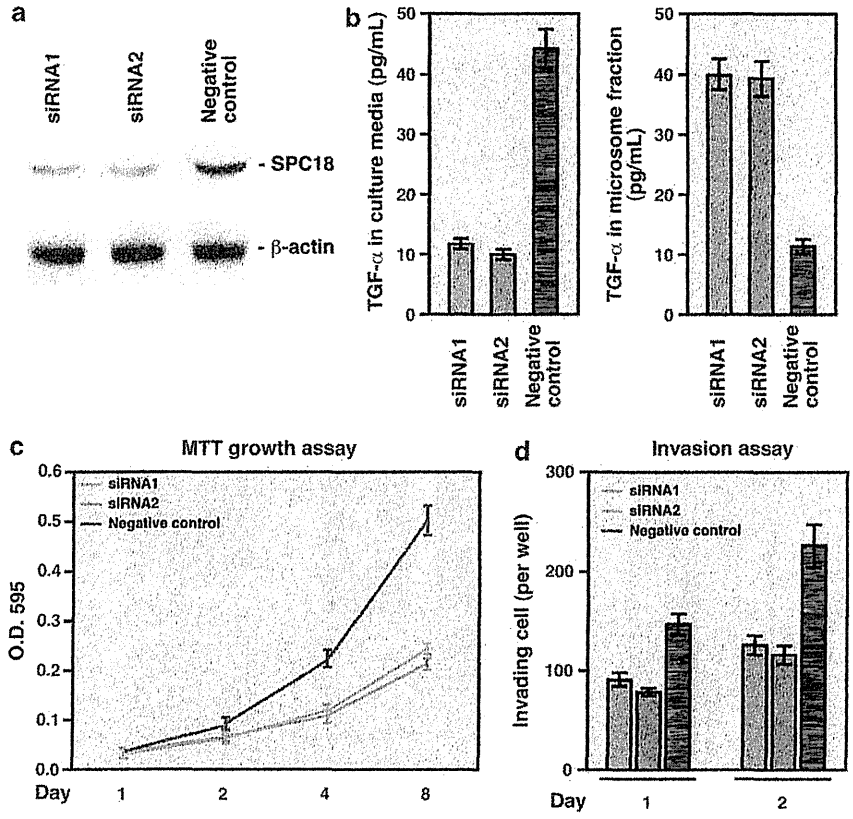

Figure 4. (**a**) Western blot analysis of SPC18 in MKN-45 cells transfected with the SPC18 siRNA (siRNA1 and 2) and negative control siRNA. (**b**) TGF-α protein levels in the culture media or microsome fraction of MKN-45 cells transfected with SPC18 siRNA (siRNA1 and 2) and negative control siRNA. Bars and error bars indicate mean and s.d., respectively, of the three different experiments. (**c**) Effect of SPC18 knockdown on MKN-45 cell viability. Cell viability was assessed using MTT assay at days 1, 2, 4 and 8 after seeding on 96-well plates. Bars and error bars indicate mean and s.d., respectively, of three different experiments. (**d**) Effect of SPC18 knockdown on cell invasion in MKN-45 cells. MKN-45 cells transfected with SPC18 siRNA (siRNA1 and 2) or negative control siRNA were incubated in Boyden chambers. After 1 and 2 days, invading cells were counted. Bars and error bars indicate mean and s.d., respectively, of three different experiments.

Taken together, these results indicate that SPC18 contributes to cancer cell progression via TGF-α secretion.

DISCUSSION

In the present study, we built a small and focused oligonucleotide array on which 394 genes were selected based on our SAGE data and previously reported array data, and found that *SEC11A*, which encodes SPC18 protein, is overexpressed in GC. Although growth factors such as TGF-α and EGF have an important role in cancer cell growth, the possible effects of alteration to cellular secretion mechanisms on the secretion of these oncogenic factors have not been investigated. We showed that expression levels of SPC18 varied in association with tumor stage in both the Hiroshima and Chiba cohorts. Forced expression of SPC18 in the GC cell line promoted cancer cell growth *in vitro* and *in vivo*, and stimulated cancer cell invasion. Furthermore, forced expression of SPC18 induced TGF-α secretion. Knockdown of SPC18 by RNAi inhibited TGF-α secretion. Taken together, these results indicate that SPC18 is associated with GC cell progression rather than GC pathogenesis.

TGF-α is biosynthesized as a larger transmembrane protein termed pro-TGF-α (TGF-α precursor).[19,20] TGF-α precursor contains an extracellular domain of ~100 amino acids that includes the NH_2-terminal signal sequence and the 50-amino acid TGF-α, a hydrophobic transmembrane domain, and a 35-residue cytoplasmic domain.[21] The NH_2-terminal signal sequence allows translocation of the nascent polypeptide chain through the membrane of the ER.[19,20] Three proteolytic events contribute to the complete maturation of the TGF-α precursor. The first event involves the removal of the signal peptide by signal peptidases. This occurs upon the precursor peptide's entry into the secretory route in the ER. In the present study, SPC18 inhibition by RNAi did not change mRNA expression levels of TGF-α in the MKN-45 cells, although SPC18 inhibition reduced TGF-α protein levels in culture media, suggesting that SPC18 overexpression does not upregulate TGF-α expression at the transcriptional level. We also confirmed that the levels of TGF-α in microsome fraction from the NKN-45 cells transfected with SPC18 siRNA1 or SPC18 siRNA2 were significantly higher than those from MKN-45 cells transfected with negative control siRNA. It is likely that SPC18 cleaves the signal peptide of TGF-α within the ER, and that the overexpression of SPC18 enhances secretion of TGF-α.

SPC reportedly has five distinct subunits[11] of which both SPC18 and SPC21 are presumed to have catalytic activity.[12] Although overexpression of SPC18 has frequently been found in GC tissue samples, overexpression of SPC21 was not detected in this study. These results led us to question whether overexpression of SPC18 alone is sufficient to induce high signal peptidase activity in SPC. We confirmed that the knockdown of SPC21 by RNAi inhibits TGF-α secretion in MKN-45 cells (data not shown), indicating that SPC21 is required for maximal signal peptidase activity of the SPC. In contrast, TGF-α secretion can be induced in MKN-1 GC cells by forced expression of SPC18, without SPC21 overexpression (data not shown), suggesting a requirement for further investigation to elucidate possible additional functions of SPC18, as well as

interactions between SPC18 with other subunits of the SPC and other molecules involved in cellular secretion processes.

The oligonucleotide array analysis in this study found that expression of six genes was significantly higher in GC at stage III/IV than GC at stage I/II. Among these genes, *MMP7*, which encodes matrilysin, has been reported to be associated with cancer cell invasion.[14] *TDGF1*, which encodes cripto protein, has been reported to be involved in cancer cell proliferation, migration, epithelial-to-mesenchymal transition and in stimulation of tumor angiogenesis.[22] However, the significance of *DDOST*, *NDUFB7* and *SUPT4H1* remains unclear. *DDOST* has been reported to be associated with cancer cell metastasis by microarray analysis.[6] *NDUFB7*and *SUPT4H1* were reported to be overexpressed in GC in our SAGE analysis.[2] To further our understanding of the role of these genes in GC, detailed expression analysis using methods such as qRT–PCR or immunohistochemical analysis should be performed.

In summary, we found that SPC18 is overexpressed in GC. We also showed that SPC18 contributes to malignant progression by promotion of TGF-α secretion in GC. SPC18 expression was not an independent prognostic indicator, and, as a result, SPC18 may not be suitable as a biomarker to identify patients with poor prognosis. In contrast, knockdown of SPC18 by RNAi inhibits TGF-α secretion, indicating that secretion of other growth factors promoting cancer cell growth may be inhibited by SPC18 knockdown. Specific inhibitors of SPC18 may constitute promising anticancer drugs.

MATERIALS AND METHODS

Tissue samples

In all, 1164 primary tumors were collected from patients diagnosed with GC. The samples were obtained during surgery at the Hiroshima University Hospital or an affiliated hospital. We confirmed microscopically that the tumor specimens were predominantly (>80%) cancer tissue. Samples were frozen immediately in liquid nitrogen and stored at −80 °C until use. Twenty-five GC samples and corresponding non-neoplastic mucosal samples were obtained for use in our oligonucleotide array analysis.

For qRT–PCR analysis, the noncancerous samples of heart, lung, stomach, small intestine, colon, liver, pancreas, kidney, bone marrow, peripheral leukocytes, spleen, skeletal muscle, brain and spinal cord were purchased from Clontech (Palo Alto, CA, USA). In total, fifty-one GC samples and corresponding non-neoplastic mucosa samples were used for qRT–PCR analysis.

For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from 1088 patients who had undergone surgical excision of GC. Of these patients, 99 had been treated at the Hiroshima University Hospital, Hiroshima, Japan (Hiroshima cohort); the remaining 989 were treated at the National Cancer Center Hospital East, Chiba, Japan (Chiba cohort). Immunohistochemical analysis was performed using whole paraffin-embedded blocks for the Hiroshima cohort and tissue microarray for the Chiba cohort.[23] This study was approved by the Ethical Committee for Human Genome Research of Hiroshima University and the institutional review board of the National Cancer Center.

Cell lines, expression vector, transfection and TGF-α treatment

MKN-1 and MKN-45 GC cell lines were provided by Dr Toshimitsu Suzuki.[24] All cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% fetal bovine serum (BioWhittaker, Walkersville, MD, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. For constitutive expression of the *SEC11A* gene, cDNA was PCR amplified and subcloned into pcDNA 3.1 (Invitrogen Corp., Carlsbad, CA, USA) in-frame with a NH_2-terminal (pcDNA-V5-SPC18) V5 epitope tag. Transient transfection was carried out with FuGENE6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA). After 24 h of serum starvation, 100 nM concentration of TGF-α (Sigma, St Louis, MO, USA) was added.

Oligonucleotide array construction and data analysis

The 70-nucleotide oligonucleotides were synthesized with amino-modified 5′ termini (Oligator Human Refset, Illumina, San Diego, CA, USA). The oligonucleotides were dissolved in 40 μl of Solution I (Takara Bio Inc., Shiga, Japan), and then spotted in triplicate onto glass slides (Hubble Slide, Takara Bio Inc.) using a GMS 417 Arrayer (Affymetrix, Santa Clara, CA, USA). Slides were fixed in 0.2% SDS for 2 min and in 0.3 N NaOH for 5 min, then dehydrated with 100% cold ethanol for 3 min and finally air-dried. The array contained 394 genes, including GC-related genes identified by our previous SAGE analysis,[2] known genes related to development and progression of GC,[6–8] and genes associated with sensitivity to anticancer drugs.[9] A list of the genes on the array is available upon request. Preparation of labeled probe, hybridization, detection and data analysis were performed as described previously.[25]

Antibodies

Rabbit polyclonal antibodies were raised against His-tagged recombinant SPC18 produced in bacteria and purified with nickel resin (Qiagen, Valencia, CA, USA). Specificity of the anti-SPC18 antibody was evaluated by ELISA. Immunoreactive sera were affinity-purified with the His-tagged recombinant SPC18 protein. An anti-V5 monoclonal antibody was purchased from Invitrogen. An anti-Ki67 antibody (Dako Cytomation, Glostrup, Denmark) was used to measure proliferative activity. To investigate EGFR phosphorylation, an anti-EGFR antibody and anti-phospho-EGFR (Tyr1068) were purchased from Cell Signaling Technology (Beverly, MA, USA). To investigate AKT phosphorylation, an anti-AKT antibody and anti-phospho-AKT (Ser473) were purchased from Cell Signaling Technology.

Xenograft model

SCID mice (CLEA) were injected subcutaneously with MKN-1 cells transfected with pcDNA-V5-SPC18 ($n=5$) or MKN-1 cells transfected with pcDNA 3.1 control vector ($n=5$; 10^7 cells in 0.1 ml of PBS). Tumors were measured with calipers on days 12, 15, 19, 22, 26, 29, 33, 37, 42 and 48 post injection, by which time they had become palpable and visible. Tumor volumes were calculated using the equation: $\text{width}^2 \times \text{length} \times 0.5$. Subcutaneous tumors were surgically excised, weighed and photographed, and a portion of each tumor was placed in 10% formalin for paraffin embedding and subsequent immunohistochemical analysis. Animal protocols were approved by the committee for Ethics of Animal Experimentation and were in accordance with the Guidelines for Animal Experiments in the National Cancer Center.

The following were used in this study: qRT–PCR analysis, western blot analysis, immunohistochemical analysis, cell viability and *in vitro* invasion assays, RNAi, measurement of TGF-α and EGF, and statistical methods.

Detailed information is described in the Supplementary Data.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Gastric Cancer
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ORIGINAL ARTICLE

Clinicopathological significance of MMP-7, laminin γ2 and EGFR expression at the invasive front of gastric carcinoma

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Abstract
Background For several types of cancer, including gastric cancer (GC), tumor cells at the invasive front are considered to have a more aggressive behavior compared with those in the more central region. The aim of the present study was to analyze the expression of MMP-7, laminin γ2 and EGFR in a large number of GCs and to investigate how these expression patterns correlate with clinicopathologic parameters, infiltrative patterns, histology or mucin phenotype.
Methods We immunohistochemically examined the expression of MMP-7, laminin γ2 and EGFR using a tissue microarray analysis of 790 GCs, and evaluated their clinicopathological significance.
Results MMP-7, cytoplasmic laminin γ2, extracellular laminin γ2 and EGFR expression were observed in 25, 25, 8 and 21 % of the 790 GC cases, respectively. Expression of MMP-7, cytoplasmic laminin γ2 and EGFR was associated with advanced T grade, N grade and tumor stage. Extracellular laminin γ2 expression was not associated with any clinicopathologic parameters, infiltrative patterns, histology or mucin phenotype. Furthermore, we investigated the correlations of MMP-7, laminin γ2 and EGFR expression. MMP-7 expression was significantly more frequent in positive expression of cytoplasmic laminin γ2 than negative cases, and EGFR expression was significantly more frequent in positive expression of cytoplasmic laminin γ2 and MMP-7.
Conclusions Molecular expression of MMP-7, laminin γ2 or EGFR, and their combinations, may be associated with GC tumor aggressiveness. Assessment of expression of these molecules at the invasive front of primary tumors is clinically significant in predicting the malignant behavior of GC.

Keywords Gastric cancer · Invasive front · MMP-7 · Laminin gamma 2 · EGFR

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Introduction

Gastric cancer (GC) is one of the most common malignancies worldwide and develops as a result of multiple genetic and epigenetic alterations [1]. Advances in diagnostic tools and treatments have led to excellent long-term survival for early-detected GC [2]. However, despite improvements in diagnostic and therapeutic strategies, the prognosis of advanced GC with extensive invasion and metastasis remains poor. Several discrete steps can be discerned in the biological cascades of metastasis [3], and several molecules have been suggested to be involved in mediating GC aggressiveness [4]. The histological features of GC may differ widely from area to area within the same tumor due to tumor heterogeneity. The most useful clinicopathologic features and molecular signatures can be deduced from the invasive front of the tumor, where the most transformed and presumably most aggressive cells reside. In addition to classification by histology (the Lauren classification, the Japanese Classification of Gastric Carcinoma, and so on), GCs may also be classified into four phenotypes by their mucin expression profile: G type (gastric phenotype), I type (intestinal phenotype), GI type (gastric and intestinal mixed phenotype) and N type (neither gastric nor intestinal

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phenotype). Although G-type tumors are associated with poor patient outcome and greater malignant potential in the incipient phase of invasion and metastasis compared with other types [5], there is little understanding of whether or not mucin phenotypic classification could be used for evaluating tumor aggressiveness at the invasive front of GCs.

In carcinomas, the basement membrane, a specialized form of extracellular matrix (ECM) that separates the tumor from the stroma and acts as a mechanical barrier against cancer cell invasion, must first be degraded to allow these cells to migrate [6]. Degradation of ECM components is mostly controlled by proteolytic enzymes called matrix metalloproteinases (MMPs). MMPs have been shown to be overexpressed in several kinds of carcinomas, and to be associated with tumor invasion, metastasis or progression [7]. MMP-7, also known as matrilysin, is a member of the MMP gene family and has proteolytic activity against a wide spectrum of substrates such as collagens, proteoglycans, elastin, laminin, fibronectin, and casein [8–10]. MMP-7 is often overexpressed at the invasive front in various types of human cancer and is associated with cancer progression [11, 12]. Previous reports have suggested that MMP-7 expression also correlates with tumor invasion and metastasis in advanced GC [13]. Laminins are a family of high–molecular weight ECM proteins, also involved in cellular adhesion, growth and differentiation [14]. Laminins consist of α, β, and γ chains, and there are at least 12 isoforms. Laminin-5, which consists of α3, β3, and γ2 chains, is localized in epithelial basement membranes, functions as a ligand for integrins, and plays an important role in cell migration and adhesion [15, 16]. Some studies have reported that laminin γ2 is expressed at the invasive front in tumor cells, while others demonstrated that loss of laminin γ2 in the epithelium-stroma interface is an immunohistochemical marker of malignancy in epithelial lesions [17–21]. Laminin γ2 expression patterns are divided into two distinct types, cytoplasmic staining and extracellular staining. Okada et al. [22] reported that stromal laminin γ2 expression is associated with poor prognosis and destructive growth of gallbladder adenocarcinoma. In GC, it has been also reported that cytoplasmic laminin γ2 staining is associated with advanced lymph node metastasis and tumor stage [23]. It has been reported that MMP-7 expression is correlated with laminin γ2 expression in colorectal and biliary tract cancer [21, 24]. However, little is known about the association between MMP-7 and laminin γ2 at the invasive front of GC. In addition, it has also been reported that the laminin γ2 chain is cleaved by membrane-type 1 MMP (MT1-MMP, MMP-14) and MMP-2 [25] and that the cleaved γ2 chains bind epidermal growth factor receptors (EGFR) on cancer cell surfaces and transmit intracellular signals that promote cell growth and mobility [26]. Furthermore, it has been reported that laminin γ2 expression is correlated with EGFR in oral [27–29] and esophageal [19] squamous cell carcinoma and lung adenocarcinoma [30].

Although MMP-7, laminin γ2 and EGFR are representative molecules recognized as independent prognostic markers, there is little understanding of the correlations with some of the possible combinations, and the relationship between the combination of markers and clinicopathologic factors. The aims of the present study were to analyze the expression of MMP-7, laminin γ2, EGFR or their combinations at the invasive front in a large number of GCs and to investigate how these expression patterns correlate with clinicopathologic parameters, infiltrative patterns, histology or mucin phenotype. Because the functional and biological properties of GCs may reflect the tumor's ability to produce these molecules, it would be of interest to determine which factors are best correlated with tumor aggressiveness.

Materials and methods

Samples of GCs at the invasive front and tissue microarray (TMA) construction

We randomly selected a total of 1019 GCs from the surgical pathology files of the Hiroshima University Hospital and its affiliated hospitals. Among those, 229 cases (22 %) were intramucosal GCs and were excluded from the present study, leaving 790 GCs (78 %) diagnosed with pathologically proven invasive GCs (507 men and 283 women; age range, 31–93 years). Surgically resected specimens were routinely fixed in 10 % buffered formalin and examined macroscopically. All sections contained tumor tissue and surrounding non-neoplastic tissue and were embedded in paraffin. Additional consecutive 5-μm sections were cut from a selected tissue block and stained with hematoxylin and eosin (HE). Tumor staging was performed according to the Union Internationale Contre le Cancer (UICC) system [31]. There were 248 T1 and 542 T2–T4 in these 790 cases. Nodal metastasis and distant metastasis were present in 428 patients and 8 patients (54 and 1 %, respectively). Tumor staging revealed 352 stage I and 438 stage II–IV cases. The 790 GC cases were histologically classified as 436 intestinal type and 354 diffuse type, according to the Lauren classification system [32]. Using the Japanese Classification of Gastric Carcinoma, tumor infiltration patterns (INFs) were classified into three subgroups according to the pattern of tumor infiltration into the surrounding tissue: INFa, INFb and INFc. The INFa group exhibits expanding growth and a distinct border with the surrounding tissue, INFc describes infiltrating growth and an indistinct border with the surrounding tissue, while INFb falls between the two (Fig. 1a–f). In accordance with the Ethical Guidelines for Human Genome/Gene Research

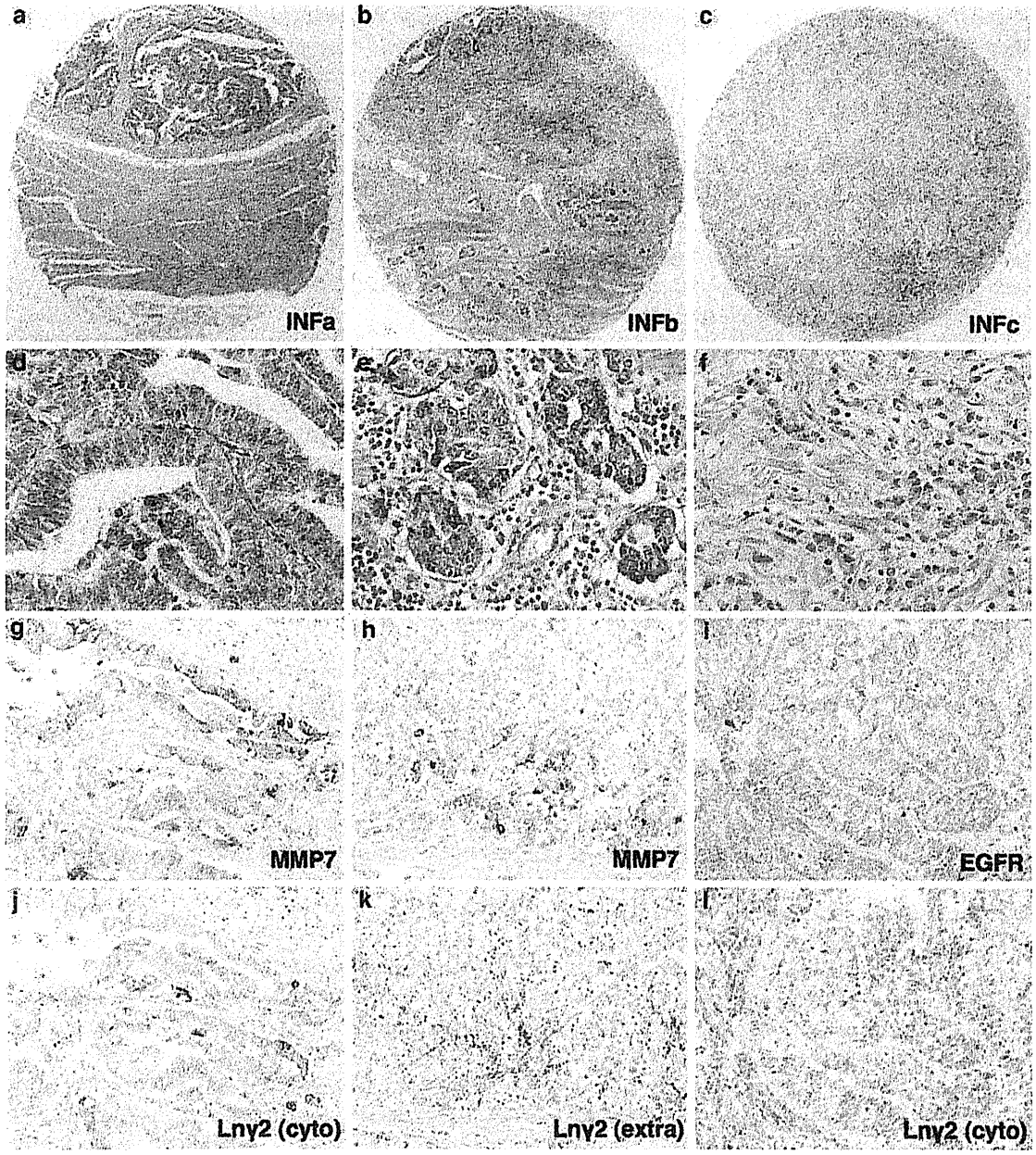

Fig. 1 Infiltration pattern (**a–f**) at the invasive front of gastric cancer (GC) and immunostaining of MMP-7, laminin γ2 and EGFR (**g–l**). Tumor infiltration patterns (INFs) were classified into three subgroups according to the pattern of tumor infiltration into the surrounding tissue: INFa (**a**, **d**), INFb (**b**, **e**) and INFc (**c**, **f**). Immunohistochemically, MMP-7 was often coexpressed with cytoplasmic laminin γ2 (**g**, **j**), but rarely coexpressed with extracellular laminin γ2 (**h**, **k**). EGFR expression was also colocalized with cytoplasmic laminin γ2 (**i**, **l**)

enacted by the Japanese Government, tissue specimens were collected and used after approval by the Ethical Review Committee of the Hiroshima University School of Medicine and by the ethical review committees of collaborating organizations. The two most representative portions to be sampled for the TMAs were carefully selected from different intratumoral areas in each case and marked on the HE-stained slide. One invasive front area and one superficial area as its control were selected.

The invasive front of GCs varies in complexity from smooth to highly complex when the front splits up into small cell clusters or even single cancer cells. In this study, we defined the invasive front of GCs as tumor cells or clusters at the perpendicularly deepest site of tumor invasion. A 2-mm-diameter tissue core of each donor block was punched out and transferred to a recipient block with a maximum of 48 cores using a tissue microarrayer (AZUMAYA KIN-1, Tokyo, Japan). Five-μm-thick sections were cut from the

recipient block and transferred to slide glasses. HE staining was performed on TMA for confirmation of the tumor tissue.

Immunohistochemistry

For immunostaining of all markers except EGFR, a Dako LSAB Kit was used according to the manufacturer's recommendations. The antibodies and their conditions used in the current study are shown in Table 1. After peroxidase activity was blocked with 3 % H_2O_2-methanol for 10 min, the sections were incubated with normal goat serum (Dako Corporation, Carpinteria, CA) for 20 min to block nonspecific antibody binding sites. The sections were incubated with the primary antibodies for 1 h at room temperature, followed by incubations with biotinylated anti-mouse immunoglobulin G and peroxidase-labeled streptavidin for 10 min each. For immunostaining of EGFR, a Dako EGFR pharmDx™ assay detection system (Dako Corporation, Carpinteria, CA) was used. Staining was completed with a 10-min incubation with the substrate-chromogen solution. The sections were counterstained with 0.1 % hematoxylin. Appropriate positive and negative control samples were also stained.

Evaluation of positive cases and cutoff-point thresholds

For the TMAs, staining was considered positive if any tumor cells were stained appropriately. The percentage of reactive cells necessary for a positive result reflects the viewpoint and opinion of the authors. Immunostaining results were evaluated independently by two investigators (KS and MM), and when the evaluations differed, a decision was made by consensus while investigators reviewed the specimen with a multihead microscope. Neoplastic tissue was evaluated semiquantitatively at magnifications of ×100 and ×400. The cytoplasmic staining of MMP-7, MUC5AC, MUC6 and MUC2, cytoplasmic and extracellular staining of laminin γ2, and the membranous staining of EGFR and CD10 were classified according to the percentage of stained cells within carcinomatous areas. The extracellular staining of laminin γ2 was characterized by the laminin γ2-positive staining in the stroma adjacent to the cancer cell nests. The expression of each molecule was classified as 0 % (score 0), 1–9 % (score 1), 10–49 % (score 2) or >50 % (score 3) of staining. When each specimen had more than 10 % (score 2 and 3) of cancer cells or stromal positively stained, the immunostaining was considered positive according to median cut off values rounded off to the nearest 5 %.

Mucin phenotypes of GCs

790 GCs were evaluated according to the criteria [33] for classification of G type and I type. GCs in which more than 10 % of the cells displayed the gastric (MUC5AC and/or MUC6) or intestinal epithelial cell phenotype (MUC2 and/or CD10) were considered G type or I type, respectively. Those sections that showed both G and I type were classified as GI type, and those that lacked both G and I type were classified as N type.

Statistical methods

Associations between clinicopathologic variables and immunostaining for MMP-7, laminin γ2 or EGFR were analyzed by the chi-square test. A p-value less than 0.05 was considered statistically significant. Hierarchical clustering analysis was performed using the WARD clustering algorithms. Statistical analyses were performed using JMP software (version 10.0.2; SAS Institute, Carey, NC).

Results

Staining patterns of MMP-7, laminin γ2 and EGFR at the invasive front and the control regions of GCs and their correlation with clinicopathologic parameters

We performed immunostaining of MMP-7, laminin γ2 and EGFR at the invasive front and the control regions of GCs. The median percentage of positive cancer cells was 9

Table 1 Antibodies and conditions used

Antibody	Clone	Dilution	Source	Pretreatment
MMP-7	141-7B2	1:100	Daiichi Fine Chemical, Japan	Autoclave
Laminin γ2	D4B5	1:50	Chemicon, USA	Protease XXIV
EGFR	2-18C9	Diluted	DAKO, USA	Proteinase K
MUC5AC	CLH2	1:50	Novocastra, UK	MW
MUC6	CLH5	1:50	Novocastra, UK	MW
MUC2	Ccp58	1:50	Novocastra, UK	MW
CD10	56C6	1:50	Novocastra, UK	MW

Autoclave indicates heating to 121 °C in an autoclave for 40 min. Protease XXIV indicates pretreatment by Protease XXIV (Sigma, St Louis, MO) for 15 min at room temperature. For immunostaining of EGFR, a Dako EGFR pharmDx™ assay detection system (Dako Corporation, Carpinteria, CA) was used. MW indicates microwaving (500 W) in citrate buffer (pH 6.0) for 15 min

(range 0–85) for MMP-7, 8 (range 0–70) for laminin γ2, and 8 (range 0–65) for EGFR.

At the invasive front of GCs, MMP-7 expression was detected in 195 (25 %) of the 790 cases (score 0: 122 cases, score 1: 473 cases, score 2: 177 cases, score 3: 18 cases) and was seen exclusively in the cytoplasm (Fig. 1g, h). Two laminin γ2 staining patterns (cytoplasmic staining and extracellular staining) have been reported in GCs [18, 23]. Laminin γ2 cytoplasmic expression was detected in 195 (25 %) (score 0: 156 cases, score 1: 439 cases, score 2: 182 cases, score 3: 13 cases) (Fig. 1j, l), and laminin γ2 extracellular expression was detected in 60 (8 %) (score 0: 302 cases, score 1: 428 cases, score 2: 54 cases, score 3: 6 cases) (Fig. 1k). EGFR membranous expression was detected in 162 (21 %) (score 0: 214 cases, score 1: 414 cases, score 2: 152 cases, score 3: 10 cases) of the 790 cases.

Next, we investigated the relationship between their expressions and clinicopathologic parameters including age, sex, T grade, N grade, M grade and tumor stage (Table 2). Expression of MMP-7 was associated with advanced T grade ($p = 0.0207$), N grade ($p < 0.0001$) and tumor stage ($p < 0.0001$). Expression of cytoplasmic laminin γ2 was associated with advanced T grade ($p = 0.0003$), N grade ($p < 0.0001$) and tumor stage ($p < 0.0001$). Expression of EGFR was associated with advanced T grade ($p < 0.0001$), N grade ($p < 0.0001$) and tumor stage ($p < 0.0001$). However, extracellular laminin γ2 expression was not associated with any clinicopathologic parameters.

Table 2 Relationship between MMP-7, laminin γ2, EGFR expression and clinicopathological characteristics at the invasive front of 790 gastric cancers

	MMP-7			LNγ2 (cyto)			LNγ2 (extra)			EGFR		
	Positive	Negative	*p*-value*	Positive	Negative	*p*-value*	Positive	Negative	*p* value*	Positive	Negative	*p*-value*
Age												
≤65 years	94 (25 %)	287	NS	89 (23 %)	292	NS	34 (9 %)	347	NS	70 (18 %)	311	NS
>65 years	101 (25 %)	308		106 (26 %)	303		26 (6 %)	383		92 (22 %)	317	
Sex												
Female	71 (25 %)	212	NS	60 (21 %)	223	NS	20 (7 %)	263	NS	53 (19 %)	230	NS
Male	124 (24 %)	383		135 (27 %)	372		40 (8 %)	467		109 (21 %)	398	
T grade												
T1	48 (19 %)	200	0.0207	41 (17 %)	207	0.0003	16 (6 %)	232	NS	26 (10 %)	222	<0.0001
T2/3/4	147 (27 %)	395		154 (28 %)	388		44 (8 %)	498		136 (25 %)	406	
N grade												
N0	48 (13 %)	314	<0.0001	60 (17 %)	302	<0.0001	25 (7 %)	337	NS	36 (10 %)	326	<0.0001
N1/2/3	147 (34 %)	281		135 (32 %)	293		35 (8 %)	393		126 (29 %)	302	
M grade												
M0	192 (25 %)	590	NS	193 (25 %)	589	NS	58 (7 %)	724	NS	160 (20 %)	622	NS
M1	3 (38 %)	5		2 (25 %)	6		2 (25 %)	6		2 (25 %)	6	
Stage												
I	59 (17 %)	293	<0.0001	61 (17 %)	291	<0.0001	22 (6 %)	330	NS	40 (11 %)	312	<0.0001
II/III/IV	136 (31 %)	302		134 (31 %)	304		38 (9 %)	400		122 (28 %)	316	
INF												
a	25 (20 %)	97	NS	29 (24 %)	93	NS	10 (8 %)	112	NS	18 (15 %)	104	NS
b	113 (27 %)	302		112 (27 %)	303		31 (7 %)	384		103 (25 %)	312	
c	57 (23 %)	196		54 (21 %)	199		19 (8 %)	234		41 (16 %)	212	
Histology												
Intestinal type	113 (26 %)	323	NS	110 (25 %)	326	NS	36 (8 %)	400	NS	95 (22 %)	341	NS
Diffuse type	82 (23 %)	272		85 (24 %)	269		24 (7 %)	330		67 (19 %)	287	
Mucin type												
G type	53 (23 %)	182	NS	57 (24 %)	178	NS	19 (8 %)	216	NS	33 (14 %)	202	NS
GI type	37 (37 %)	62		40 (40 %)	59		7 (7 %)	92		32 (32 %)	67	
I type	32 (19 %)	135		32 (19 %)	135		11 (6 %)	156		37 (22 %)	130	
N type	73 (25 %)	216		66 (23 %)	223		23 (8 %)	266		60 (21 %)	229	

LNγ2 laminin-5 γ2 chain, *cyto* cytoplasmic pattern, *extra* extracellular pattern, *NS* not significant

* Chi-square test

Table 3 Relationship between MMP-7, laminin γ2, EGFR expression and clinicopathological characteristics at the control regions of 790 gastric cancers

	MMP-7			LNγ2 (cyto)			LNγ2 (extra)			EGFR		
	Positive	Negative	*p* value*	Positive	Negative	*p* value*	Positive	Negative	*p* value*	Positive	Negative	*p* value*
Age												
≤65 years	55 (14 %)	326	NS	60 (16 %)	321	NS	18 (5 %)	363	NS	30 (8 %)	351	NS
>65 years	61 (15 %)	348		85 (21 %)	324		23 (6 %)	386		39 (10 %)	370	
Sex												
Female	45 (16 %)	238	NS	49 (15 %)	234	NS	12 (4 %)	271	NS	22 (8 %)	261	NS
Male	71 (14 %)	436		96 (19 %)	411		29 (6 %)	478		47 (9 %)	460	
T grade												
T1	41 (17 %)	207	NS	32 (13 %)	216	0.0074	8 (3 %)	240	NS	21 (8 %)	227	NS
T2/3/4	75 (14 %)	467		113 (21 %)	429		33 (6 %)	509		48 (9 %)	494	
N grade												
N0	56 (13 %)	306	NS	52 (14 %)	310	0.0096	14 (4 %)	348	NS	30 (8 %)	332	NS
N1/2/3	60 (14 %)	368		93 (22 %)	335		27 (6 %)	401		39 (9 %)	389	
M grade												
M0	115 (25 %)	667	NS	143 (18 %)	639	NS	40 (5 %)	742	NS	69 (9 %)	713	NS
M1	1 (13 %)	7		2 (25 %)	6		1 (13 %)	7		0	8	
Stage												
I	59 (17 %)	293	NS	53 (15 %)	299	0.0336	14 (4 %)	338	NS	32 (9 %)	320	NS
II/III/IV	57 (13 %)	381		92 (21 %)	346		27 (6 %)	411		37 (8 %)	401	

LNγ2 laminin-5 γ2 chain, *cyto* cytoplasmic pattern, *extra* extracellular pattern, *NS* not significant

* Chi-square test

In contrast, we performed immunostaining of MMP-7, laminin γ2 and EGFR at the superficial areas of GCs. MMP-7 expression was detected in 116 (15 %) of the 790 cases. Laminin γ2 cytoplasmic expression was detected in 145 cases (18 %), and laminin γ2 extracellular expression was detected in 41 cases (5 %). EGFR expression was detected in 69 (9 %) of the 790 GC cases. Expression of cytoplasmic laminin γ2 was associated with advanced T grade ($p = 0.0074$), N grade ($p = 0.0096$) and tumor stage ($p = 0.0336$), whereas MMP-7, extracellular laminin γ2 and EGFR expression were not associated with any clinicopathologic parameters (Table 3).

Correlation of MMP-7, laminin γ2 and EGFR expression with infiltrative patterns, histology and mucin phenotypes at the invasive front of GCs

We analyzed the relationships between expression of these molecules and infiltrative patterns, histology and mucin phenotypes at the invasive front of GC. Infiltrative patterns of 790 GCs included 122 INFa, 415 INFb and 253 INFc, and tumor histology was classified into 436 intestinal type and 354 diffuse type. The distribution of each mucin phenotype included 235 G type, 99 GI type, 167 I type and 289 N type. However, expression of MMP7, laminin γ2 and EGFR was not associated with infiltrative patterns, histology and mucin phenotypes (Table 2).

Association of expression among MMP-7, laminin γ2 and EGFR

We next investigated the correlations among the expression of MMP-7, laminin γ2 and EGFR. First, we investigated between MMP-7 and laminin γ2 expression. MMP-7 was often coexpressed with cytoplasmic laminin γ2 (Fig. 1g, j), but rarely coexpressed with extracellular laminin γ2 (Fig. 1h, k). MMP-7 expression was significantly more frequent with positive expression of cytoplasmic laminin γ2 than negative cases ($p < 0.0001$). However, positive expression of MMP-7 showed no significant correlation with expression of extracellular laminin γ2 (Table 4). We then investigated the association between laminin γ2 and EGFR expression. EGFR expression was significantly more frequent with positive expression of cytoplasmic laminin γ2 and MMP-7 than negative cases ($p < 0.0001$, Fig. 1i, l). No significant association between extracellular laminin γ2 and EGFR expression was detected. Hierarchical clustering of these molecules also showed virtually identical expression of MMP-7, cytoplasmic laminin γ2 and EGFR in one

Table 4 Relationships among MMP-7, laminin γ2 and EGFR expression in 790 gastric cancers

	LNγ2 (cyto)		*p* value*	LNγ2 (extra)		*p* value*	EGFR		*P* value*
	+	−		+	−		+	−	
MMP-7									
+	110 (56 %)	85	<0.0001	18 (9 %)	177	NS	103 (53 %)	92	<0.0001
−	85 (14 %)	510		42 (7 %)	553		59 (10 %)	536	

	LN γ2 (extra)		*p* value*	EGFR		*p* value*
	+	−		+	−	
LNγ2 (cyto)						
+	17 (9 %)	178	NS	82 (42 %)	113	<0.0001
−	43 (7 %)	552		80 (13 %)	515	

	EGFR		*p* value*
	+	−	
LN γ2 (extra)			
+	12 (20 %)	48	NS
−	150 (21 %)	580	

LNγ2 laminin-5 γ2 chain, *cyto* cytoplasmic pattern, *extra* extracellular pattern, *NS* not significant

* Chi Square test

cluster, and that of extracellular laminin γ2 in another cluster (Fig. 2). This indicates significant associations of expression among these molecules.

Combined expressions of MMP-7, cytoplasmic laminin γ2 and EGFR at the invasive front and the control regions of GCs and their correlation with clinicopathologic parameters

At the invasive front of GCs, combined expressions of MMP-7, cytoplasmic laminin γ2 and EGFR were detected in 60 (8 %) of the 790 cases. At the control regions of GCs, their combined expression was detected in 5 (1 %) of the 790 cases. Combined expression at the invasive front was associated with advanced T grade ($p = 0.0004$), N grade ($p < 0.0001$) and tumor stage ($p < 0.0001$), whereas combined expression at the control regions was not associated with any clinicopathologic parameters (Table 5).

Discussion

In GC, various predictive factors, such as tumor size, gross appearance, cancer differentiation, depth of invasion, histological growth pattern, lymphatic invasion and venous invasion are responsible for the clinical outcomes of patients [34–40]. For several types of cancer, tumor cells at the invasive front are considered to have more aggressive behavior compared with those in the more central region [41–43] and are characterized by a dynamic process referred to as epithelial mesenchymal transition (EMT) [44, 45]. EMT is considered to be a transient and reversible process, and represents only one of the several steps required for tumor progression via invasion and metastatic spread [46], because it has also been implicated in the fundamental steps of tumorigenesis, such as invasion and metastasis [47]. In this study, we used the TMA method to examine expression of each molecule in GCs. It is well recognized that TMA is efficient for screening molecular alterations in a large number of tumor cases. However, major drawbacks of TMA analysis occur when the characteristics of sampled tissue do not always represent those of whole tumor. Although minute TMAs cannot ensure representative areas of donor specimen, we used 2-mm-diameter needles, which are large enough to evaluate the morphological appearance if representative regions are carefully selected with HE slides [48]. In terms of the possible diversity of histological components or molecular abnormalities in GCs, several previous reports have shown an excellent concordance between the results obtained from TMAs and those from full sections [49, 50]. Analyses using area-specific four-point TMAs clearly demonstrated

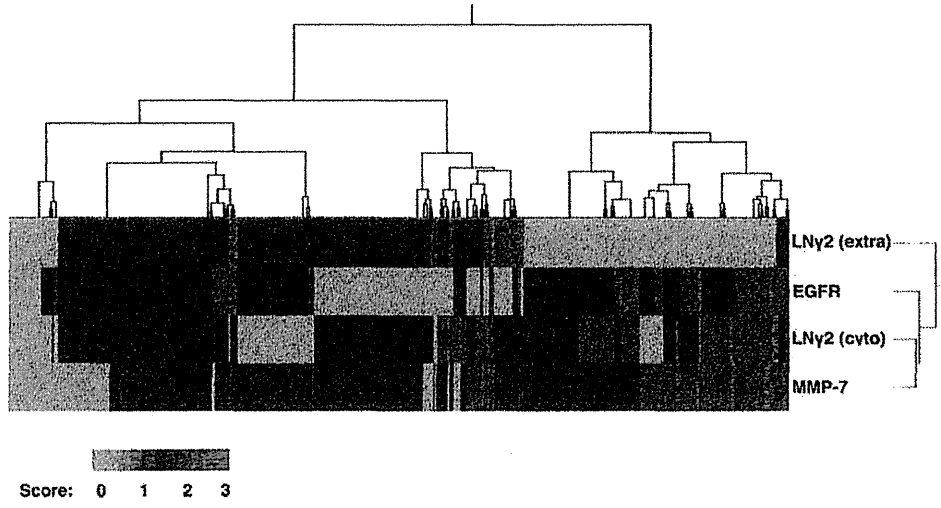

Fig. 2 Hierarchical clustering analysis of the immunohistochemical data of 790 gastric cancers to assess similarity among MMP-7, laminin γ2 and EGFR. The branch length represents the similarity between results obtained in this study. Each column represents a patient. Each row represents a marker staining as indicated on the right side. MMP-7, cytoplasmic laminin γ2 and EGFR clustered together, while extracellular laminin γ2 was in a second cluster. *LNγ2* laminin-5 γ2 chain, *cyto* cytoplasmic pattern, *extra* extracellular pattern

Table 5 Combined expressions of MMP-7, cytoplasmic laminin γ2 and EGFR at the invasive front and the control regions of GCs and their correlation with clinicopathologic parameters

	Invasive front			Control		
	All markers expression	Not all markers expression	*p*-value*	All markers expression	Not all markers expression	*p*-value*
Age						
≤65 years	23 (6 %)	358	NS	1 (0.3 %)	380	NS
>65 years	37 (9 %)	372		4 (1 %)	405	
Sex						
Female	17 (6 %)	266	NS	1 (0.4 %)	282	NS
Male	43 (8 %)	464		4 (0.8 %)	503	
T grade						
T1	7 (3 %)	241	0.0004	3 (1 %)	245	NS
T2/3/4	53 (10 %)	489		2 (0.4 %)	540	
N grade						
N0	5 (1 %)	357	<0.0001	1 (0.3 %)	361	NS
N1/2/3	55 (13 %)	373		4 (1 %)	424	
M grade						
M0	60 (8 %)	722	NS	5 (0.6 %)	777	NS
M1	0	8		0	8	
Stage						
I	7 (2 %)	345	<0.0001	3 (0.9 %)	349	NS
II/III/IV	53 (12 %)	385		2 (0.5 %)	436	

NS not significant

* Chi-square test

that laminin γ2 in the invasive front largely influenced the clinical aggressiveness of colon cancer and its tendency to metastasize [51].

The present study demonstrated that MMP-7, cytoplasmic laminin γ2 and EGFR at the invasive front of GC play a pivotal role in tumor progression and regional lymph node metastasis, whereas all these molecules except cytoplasmic laminin γ2 at the control regions were not associated with any clinicopathologic parameters. In particular, cytoplasmic expression of laminin γ2 in GCs might be a potent predictive factor for tumor aggressiveness as previously reported in pancreatic ductal adenocarcinomas [52]. Laminin 5 reportedly plays an important role in EMT through down-regulation of E-cadherin and translocation of β-catenin into the nuclei [53]. Preferential expression of laminin γ2 in carcinoma cells at the invasive front and its

correlation with tumor progression suggest that this molecule plays a role in the acquisition of a migrating and invading epithelial cell phenotype that is a prerequisite for malignancy [17, 23, 24]. It is known that activation of cancer-related genes in carcinoma cells affects their associated stromal cells. Certain stromal cell populations lying close to carcinoma cells may be induced to assist the invasion process by signals released by the cancer cells, stimulating the synthesis of gene products that facilitate cancer cell invasion and migration [54]. Interactions of carcinoma cells with stromal cells or with the surrounding extracellular matrix at the invasive front may result in accumulation of laminin γ2 at the invasive front. The laminin γ2 chain has been revealed to contain an epidermal growth factor (EGF)-like domain [26], and once the γ2 chain is physiologically processed by some stimulating factors such as MMP or bone morphogenetic protein-1 (BMP-1) [55, 56], the EGFR of β4 integrin would be stimulated, inducing the disruption of hemidesmosomes and tumor cell migration. The present study revealed that the combined expressions of MMP-7, laminin γ2 and EGFR at the invasive front were also associated with advanced T grade, N grade and tumor stage. However, each molecule was not significantly associated with infiltration pattern, histology and mucin phenotype. In invasive GCs, the cytoplasmic expression of laminin γ2 was reportedly detected in budding cells or dissociating cells, and its extracellular expression has been frequently detected in differentiated types [18]. There may therefore be some inconsistency between these results and the previous reports. Histologically, GCs demonstrate marked heterogeneity at both architectural and cytological levels, often with co-existence of several histologic elements. In this study, we defined the invasive front of GCs as tumor cells or clusters at the perpendicularly deepest site of tumor invasion, and punched out a 2-mm-diameter tissue core of each donor block. However, GCs containing minute amounts of tumor budding or dedifferentiation were presumably included in intestinal type GC. We also reported the significant association between the undifferentiated type of GC and N mucin phenotype [57]. However, expression of MMP7, laminin γ2 or EGFR was not associated with any mucin phenotypes. At the invasive front of GCs, meanwhile, it is suggested that aggressive GC cells with expression of these molecules do not always show tumor budding or dedifferentiation as shown in Fig. 1.

In conclusion, we clarified that expression of MMP-7, laminin γ2 or EGFR molecules, and their combinations, might be associated with tumor aggressiveness in GC. Assessment of the expression of these molecules at the invasive front of primary tumors may be clinically useful to predict the malignant behavior of GC.

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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 surgicalllly 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 differentiated 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

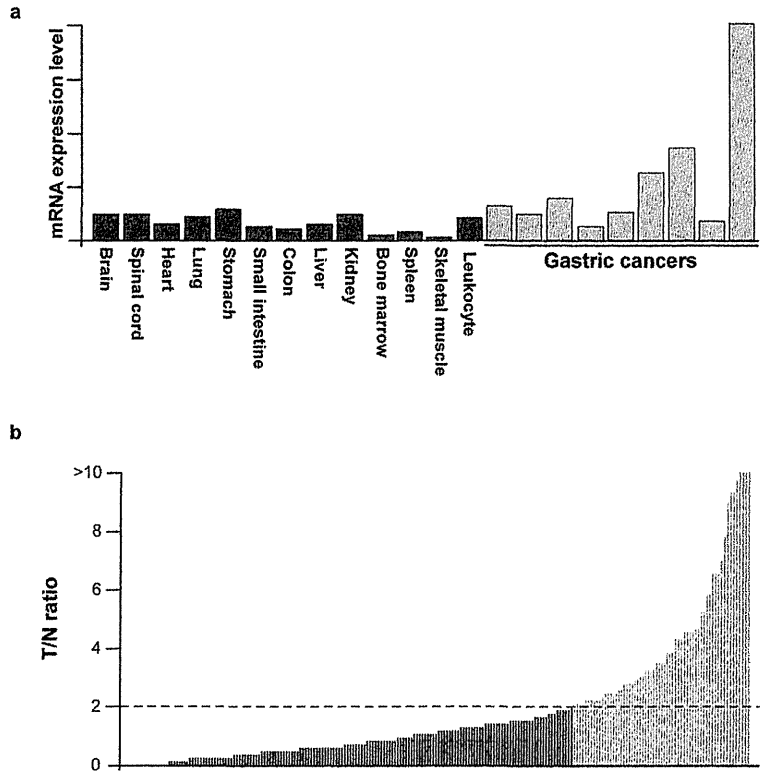

Fig. 1. Quantitative RT–PCR analysis of TM9SF3. **(a)** TM9SF3 mRNA expression level in 13 normal tissues and nine GC samples (arbitrary units). **(b)** T/N ratio of TM9SF3 mRNA level between GC tissue (T) and corresponding non-neoplastic mucosa (N) in 227 GC cases (Yokohama-cohort). T/N ratio > 2-fold 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

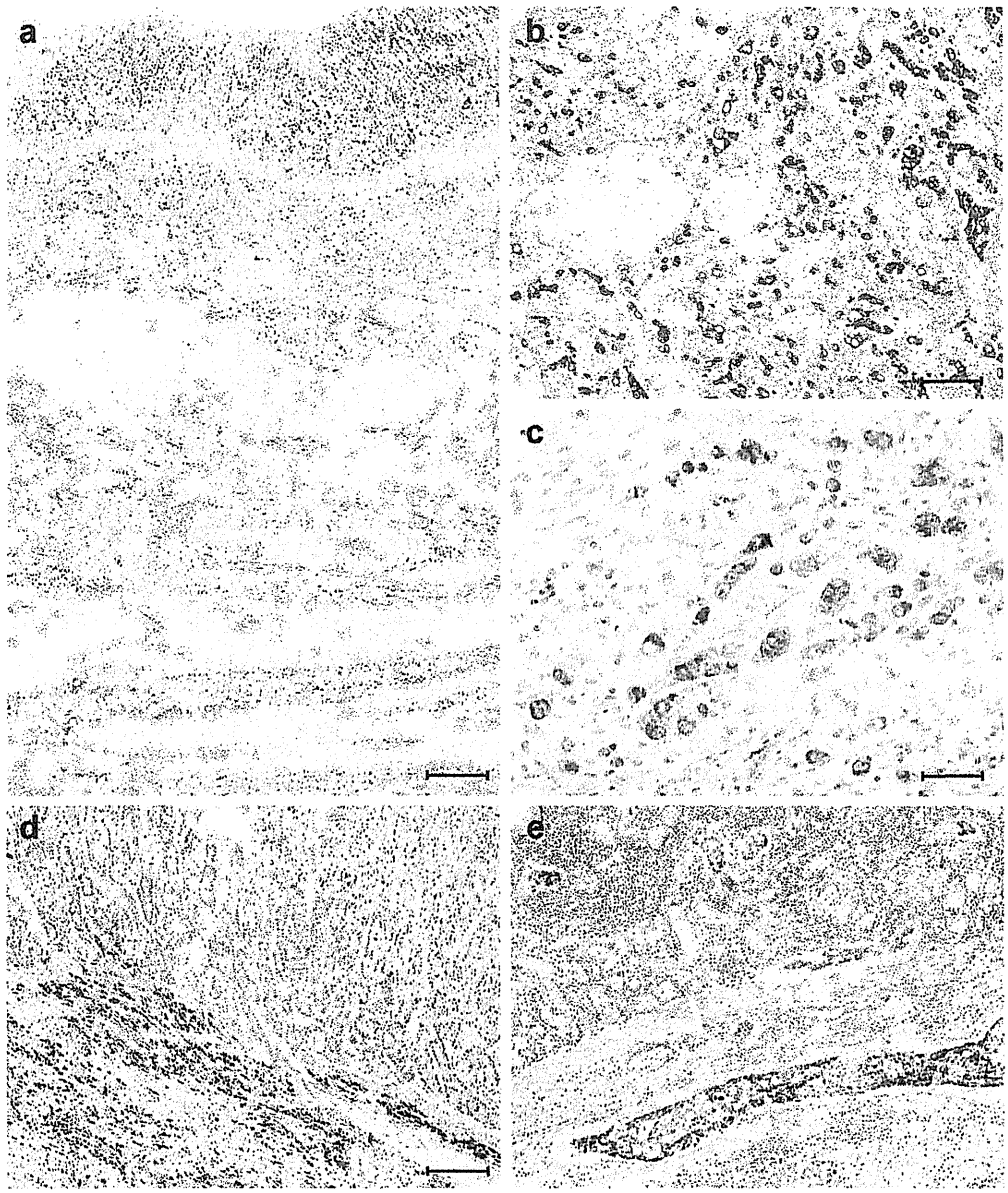

Fig. 2. Immunohistochemical staining of TM9SF3 in GC tissues. **(a and b)** TM9SF3 was detected in cancer cells from superficial to deep layer of undifferentiated type GC tissue. (x40 magnification; bar = 500 μm in **a**) **(c)** TM9SF3 expression was observed as membranous and cytoplasmic staining in 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