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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

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

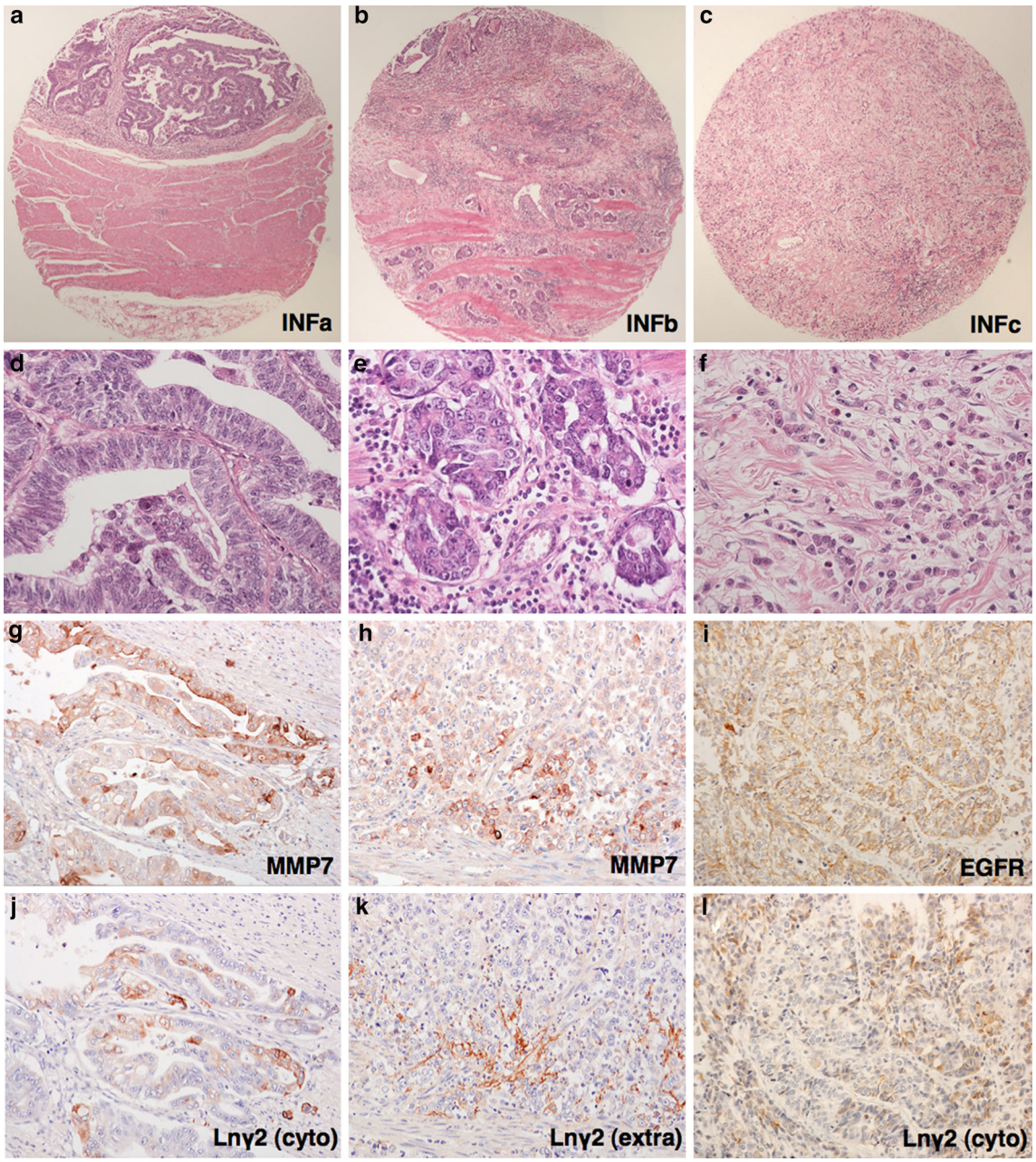

Fig. 1 Infiltration pattern (**a**–**f**) at the invasive front of gastric cancer (GC) and immunostaining of MMP-7, laminin γ2 and EGFR (**g**–**l**). Tumor infiltration patterns (INFs) were classified into three subgroups according to the pattern of tumor infiltration into the surrounding tissue: INFa (**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^{TM} 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 $\times 100$ and $\times 400$. The cytoplasmic staining of MMP-7, MUC5AC, MUC6 and MUC2, cytoplasmic and extracellular staining of laminin $\gamma 2$, 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 $\gamma 2$ was characterized by the laminin $\gamma 2$-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 $\gamma 2$ 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 $\gamma 2$ 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 $\gamma 2$ 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 $\gamma 2$	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^{TM} 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												
Tl	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
Ml	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

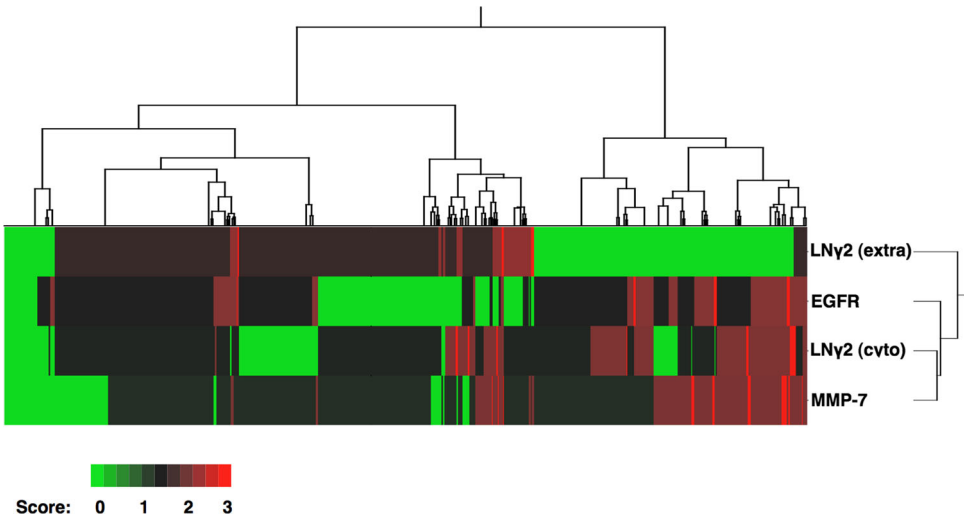

Fig. 2 Hierarchical clustering analysis of the immunohistochemical data of 790 gastric cancers to assess similarity among MMP-7, laminin γ2 and EGFR. The branch length represents the similarity between results obtained in this study. Each column represents a patient. Each row represents a marker staining 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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