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SIGNIFICANCE OF THE ASSOCIATION BETWEEN HEPARIN-BINDING EPIDERMAL GROWTH FACTOR-LIKE GROWTH FACTOR AND CD9 IN HUMAN GASTRIC CANCER

Yoko MURAYAMA[1]*, Jun-ichiro MIYAGAWA[1], Yasuhisa SHINOMURA[1], Shuji KANAYAMA[1], Koji ISOZAKI[1], Katsumi YAMAMORI[1], Hitoshi MIZUNO[2], Shingo ISHIGURO[4], Tatsuya KIYOHARA[1], Yoshiji MIYAZAKI[1], Naoyuki TANIGUCHI[3], Shigeki HIGASHIYAMA[5] and Yuji MATSUZAWA[1]

[1]*Department of Internal Medicine and Molecular Science, Graduate School of Medicine, Osaka University, Yamadaoka, Suita, Osaka, Japan*
[2]*Department of Surgery, Graduate School of Medicine, Osaka University, Yamadaoka, Suita, Osaka, Japan*
[3]*Department of Biochemistry, Graduate School of Medicine, Osaka University, Yamadaoka, Suita, Osaka, Japan*
[4]*Department of Pathology, Osaka Medical Center for Cancer and Cardiovascular Disease, Nakamichi, Higashinari-ku, Osaka, Japan*
[5]*Department of Molecular Biochemistry, School of Allied Health Science, Osaka University, Faculty of Medicine, Yamadaoka, Suita, Osaka, Japan*

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a member of the EGF family. Juxtacrine activity of proHB-EGF (the membrane-anchored form of HB-EGF) has been shown to be significantly potentiated when it is coexpressed with CD9 *in vitro*. The purpose of our study was to investigate the issue of whether proHB-EGF and CD9 are coexpressed in gastric cancer. HB-EGF gene expression and protein production in human gastric cancers was investigated, and EGF receptor and CD9 expressions were also evaluated. HB-EGF mRNA levels in gastric cancers were elevated, compared with normal gastric tissues, especially in the intestinal type. ProHB-EGF immunoreactivity was detected primarily in the cytoplasm and plasma membrane of gastric cancer cells. Of 66 patients, 40 (60.6%) exhibited proHB-EGF immunoreactivity and the level of its expression was significantly associated with tumor status ($p < 0.01$) and histological differentiation ($p < 0.001$). In addition, proHB-EGF mRNA was detected at high levels in the intestinal type by *in situ* hybridization. CD9 immunoreactivity was found to be preserved in 26 of 36 patients (72.2%) and CD9 protein expression was inversely associated with lymph node status ($p < 0.05$). A significant correlation between its expression and histological differentiation ($p < 0.01$) was found, and the association of CD9 with proHB-EGF was increased in the intestinal type, as evidenced by an immunoprecipitation method. These results indicate that the coexpression of proHB-EGF and CD9 may be involved in the tumorigenesis and/or proliferation of gastric cancers in a juxtacrine manner.

Key words: *gastric cancer; HB-EGF; CD9; juxtacrine manner*

Human gastrointestinal cancers express a variety of growth factors, hormones, cytokines and their receptors, thus regulating their growth in an autocrine and/or paracrine manner.[1–3] This excessive production of growth factors may well be the reason why cancer cells are able to circumvent physiological constraints in terms of cell growth. Gastric cancers frequently coexpress epidermal growth factor receptor (EGFR) and one or more of its ligands, including EGF, transforming growth factor-α (TGF-α), heparin-binding epidermal growth factor-like growth factor (HB-EGF), amphiregulin (AR), betacellulin (BTC) and heregulins (HRGs).[4–9] In addition to EGFR, HB-EGF, BTC and HRG are also able to bind directly to ErbB4.[10] This suggests that the expressions of this receptor family and its ligands play a role in the biological activities associated with human gastric cancers. However, little information is available concerning the relationship between EGF family expression and various histological types of gastric cancers.

HB-EGF, a member of the EGF family, was first identified as a 20–22 kDa glycoprotein that is produced by macrophages and macrophage-like cells.[11,12] When HB-EGF binds to EGF receptors, it induces EGF receptor autophosphorylation, as well as a variety of other biological activities, including proliferation and migration. Secreted mature HB-EGF is a potent mitogen for NIH-3T3 cells, bovine aortic smooth muscle cells (BASMC),[11–13] rat hepatocytes,[14] human keratinocytes[15,16] and rat gastric epithelial cells.[17] In addition, in a previous study, we demonstrated that HB-EGF is present in human gastric mucosa.[18] Some studies have shown that HB-EGF is over-expressed in the case of pancreatic, gastric, colon cancer and hepatocellular carcinomas.[5,19–21] Furthermore, HB-EGF has been shown to play an important role in tumorigenesis and the proliferation of human gastric cancer.[5]

HB-EGF is synthesized as a membrane-anchored form (proHB-EGF) and is then further processed to a soluble form (soluble HB-EGF).[22] ProHB-EGF on the cell surface is capable of stimulating growth in adjacent cells through cell-cell contact,[23,24] via the so-called juxtacrine mechanism.[25] *In vitro*, the juxtacrine growth factor activity of proHB-EGF is increased by approximately 30-fold when co-expressed with CD9.[23] Other tetraspans have no effect on the HB-EGF activities in hematopoietic cells.[26] Therefore, CD9 is generally thought to function as a co-factor of proHB-EGF in juxtacrine stimulation. CD9 is a 24 to 27 kDa glycoprotein and belongs to the transmembrane 4 superfamily of membrane proteins (TM4SF), which includes CD37, CD53, CD63, CD81, CD82 and CD151.[27] CD9 is a major cell surface protein in pre-B cells, platelets and activated T lymphocytes,[28,29] and is also expressed in nonhematopoietic tissues.[30–32] However, the precise physiological functions of CD9 remain unknown. Thus, based on these experimental results, an understanding of the relationship between HB-EGF and CD9 expression in gastric cancers or/and non-cancerous tissues represents an important clinical issue. Our hypothesis is that cancer cells might employ HB-EGF and CD9 for their biological malignancy. In the present study, we

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*Correspondence to: Department of Internal Medicine and Molecular Science, Graduate School of Medicine, Osaka University 2-2 B5, Yamadaoka, Suita, Osaka 565-0871, Japan. Fax: +81-6-6879-3739.
E-mail: yokom@imed2.med.osaka-u.ac.jp

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report on a study of the expression of HB-EGF and CD9 in various histological types of gastric cancers.

MATERIAL AND METHODS

Clinical characteristics of patients and surgical specimens

Gastric cancer tissues of various histological types, which had been obtained surgically ($n = 60$, from 28 men and 32 women, 32–82 years old), as well as endoscopic biopsy samples ($n = 5$, 2 men and 3 women, 26–78 years old) were examined. None of the patients had received preoperative irradiation or chemotherapy. Informed consent was obtained from each patient prior to endoscopy or surgery. Normal gastric mucosal samples were located at least 8 cm from the margin of the cancers. Fresh tissue samples were divided into 3 parts. One portion was immediately frozen in liquid nitrogen after surgical removal and stored at −80°C for use in future RNA extraction and the immunostaining of frozen sections. Another portion was fixed with 10% phosphate-buffered formalin for immunohistochemistry, and the remaining tissue was fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for *in situ* hybridization. However, the frozen tissues intended for RNA extraction and immunostaining from some patients were not of sufficient quality for our study, and those samples were only processed for formalin-fixed, paraffin-embedded tissue for use in histological evaluation. Histologically, gastric cancers could be were categorized into 33 intestinal types and 33 diffuse types based on the Lauren system of classification.[33] Intestinal type tumors were classified as well- or moderately- differentiated tubular adenocarcinoma according to the criteria of the Japanese Research Society for Gastric Cancer.[34] Diffuse type tumors were classified as either signet-ring or poorly-differentiated types.

Northern hybridization

The preparation of human HB-EGF cDNA has been described previously.[18] Total RNA was extracted from human gastric cancers (n = 12) and normal gastric tissues (n = 12) by using the guanidine thiocyanate/acid phenol method.[35] Forty micrograms of total RNA was denatured and fractionated on 1% agarose-formaldehyde gels and transferred to nylon membranes (Hybond-N; Amersham, Buckinghamshire, England) by using a vacuum blotting system (VacuGene XL; Pharmacia BiotechAB, Uppsala, Sweden). After fixation at 80°C for 2 hr, the membranes were prehybridized, hybridized and then washed under high stringency conditions. The human HB-EGF cDNA was radiolabeled with ^{32}P by random priming. Blots were hybridized with labeled HB-EGF and β-actin probes were exposed to XAR film (Kodak, Rochester, NY) for 1 day and 3 hr, respectively, at −70°C by using intensifying screens.

The hybridization signals were quantified by densitometry and NIH imaging. The relative intensities of HB-EGF mRNA/β-actin mRNA were estimated by assessing the ratio of the protected HB-EGF band to the β-actin protected band.

Anti-HB-EGF antibody

Antibodies that recognize proHB-EGF were produced by immunizing rabbits with synthetic peptides H-1 (human HB-EGF precursor C-terminal residues: cytoplasmic domain). Details of the anti-HB-EGF antibodies have been described previously.[18]

Anti-CD9 antibody

Antibodies that recognize CD9, and which can be used for immunohistochemistry, were kindly provided by Dr. Mekada (Department of Cell Biology, Research Institute for Microbial Diseases, Osaka University, Japan). This monoclonal antibody was isolated by immunizing BALA/c mice with Vero cell membranes. Immunoprecipitation and immunoblotting studies revealed that this antibody binds to a 24–27 kDa membrane protein,[36] which is identical to CD9.

Immunohistochemistry

The procedure for proHB-EGF and EGF-receptor immunostaining has been described previously.[18] Since CD9 antigenicity to our antibody is not preserved in formalin-fixed, paraffin-embedded tissues, frozen sections were used. Frozen sections of 6 μm thick cut on a cryostat were mounted on poly-L-lysine-coated slides and air dried. In each case, one additional section was stained with hematoxylin and eosin to verify the presence of cancer cells. To detect CD9, the sections were fixed with acetone at 4°C for 10 min, washed with PBS, immersed in 3% H_2O_2 to quench endogenous peroxidase and then incubated with 10% nonimmune goat serum for 30 min at room temperature. A 3-step immunoperoxidase protocol, consisting of incubation with a mouse anti-CD9 antibody diluted 1/100 for 2 hr at room temperature, followed by incubation with biotinylated goat anti-mouse immunoglobulins (Dako Co., Carpinteria, CA) diluted 1/500 for 10 min at room temperature and incubation with peroxidase-labeled streptavidin (Dako) for 10 min was used. Reactions were visualized with 3,3′-diaminobenzidine tetrahydrochloride in the presence of 0.05% H_2O_2. The sections were then counterstained with methyl green, dehydrated and mounted. For a negative control, the primary antiserum was replaced with normal mouse IgG (Dako).

Probe preparation

Sense and anti-sense oligonucleotide primers, which were specific for the coding regions of the EGF-receptor genes, were synthesized by conventional technology. The following oligonucleotide primers were used: EGF-receptor, sense primer, 5′-GCCAACGCCACAACCACCGC-3′ (101 through 120), and antisense primer, 5′ - GCCCTTCGCACTTCTTACAC -3′ (1161 through 1180). Total RNA (5 μg) from gastric mucosa was used as a template; single-strand cDNA was synthesized with anti-sense primers using reverse transcriptase (GibcoBRL, Grand Island, NY) and random hexanucleotides. Sequencing of the obtained cDNA was performed as described previously,[37] and the sequence was found to be identical to that of the human EGF-receptor cDNA, which has been described previously.[38] The cDNA obtained by RT-PCR was subcloned into the EcoRV site of pBluescript SK(−) plasmid, linearized with XbaI and transcribed with T3 RNA polymerase to generate an antisense (cRNA) probe. Alternatively, the cDNA clone was linearized with EcoRI and transcribed with T7 RNA polymerase to generate a sense probe. Similarly, a 742 bp fragment of human HB-EGF cDNA (227–969),[39] subcloned as described above, was then either linearized with HindIII and transcribed with T7 RNA polymerase to generate a 742 bp antisense probe or linearized with XbaI and transcribed with T3 RNA polymerase to generate a sense probe.

In situ *hybridization of HB-EGF and EGF-receptor mRNA*

The tissue localizations of the messenger RNAs that encode the HB-EGF and EGF-receptor were analyzed using *in situ* hybridization. Serial sections (5 μm thick) were cut, and odd-numbered sections were used for the *in situ* hybridization. Even-numbered sections were stained with either hematoxylin and eosin or immunostained for the proHB-EGF and EGF receptor. Details of the *in situ* hybridization technique have been described previously.[40] Digoxigenin-labeled singled-stranded RNA probes were prepared using the DIG RNA Labeling Kit (Boehringer Mannheim Biochemica, Mannheim, Germany), according to the manufacturer's instructions. Hybridization of HB-EGF mRNA and EGF-receptor mRNA was performed at 50°C for 16 hr, and the signals were detected by using a Nucleic Acid Detection Kit (Boehringer Mannheim Biochemica). Controls included *(i)* hybridization with sense probes, *(ii)* ribonuclease treatment prior to hybridization and *(iii)* omission of the anti-sense RNA probe and the anti-digoxigenin antibody.

Evaluation of proHB-EGF and CD9 staining

The intensity of proHB-EGF immunostaining in cancer cells was compared with that in normal epithelial cells (parietal cells) in

the same sample. Cancer cells with a degree of proHB-EGF immunostaining that was comparable to that of normal epithelial cells were defined as proHB-EGF-positive. We semiquantitatively estimated the positivity of proHB-EGF in each case, as follows: The cases were classified into 4 categories: (2+) positive immunoreactivity was observed in more than 50 % of the cells, (1+) positive immunoreactivity was observed in 10–50% of the cells, (±) positive immunoreactivity was observed in less than 10% of the cells and (−) no staining was observed. For statistical analyses, we regarded as positive (+) for proHB-EGF when they were classified as (1+) or (2+), and negative when classified as (±) or (−) according to previous report.[41] The criteria for the evaluation of CD9 immunostaining has been described previously.[42–44] When more than 50% of the cancer cells in a given specimen were positively stained, the samples were classified as CD9- positive (+); and when this value was less than 50%, as CD9-negative (−).

Immunoprecipitation and western blotting

The tissues from human gastric cancers (n=12) and the corresponding normal gastric mucosa (n = 12) were homogenized and subsequently lysed in lysis buffer [20 mmol/L Tris (pH 8.0), 137 mmol/L NaCl, 10% Glycerol, 1% 10 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS), 10 mmol/L EDTA, 100 mmol/L NaF, 1 mmol/L Phenylmethylsulfonyl fluoride (PMSF), 0.25 TIU/mL of Aprotinin, 10 μg/mL of Leupeptin and 2 mmol/L Sodium orthovanate (Na_3VO_4, pH 8.0;Sigma Chemical Co., St. Louis, MO)] and clarified by centrifugation at 15,000g for 15 min. The supernatant was incubated with anti-proHB-EGF antibody overnight at 4°C with gentle rocking. The mixtures were further incubated with protein G-Sepharose (Sigma) for 2 hr at 4°C with gentle rocking. Lysates were centrifuged and protein G-Separose beads were washed 3 times with lysis buffer to remove unbound proteins, resuspended in nonreducing sample buffer (25 mmol/L Tris HCl, 2% SDS, 10% glycerol, and 0.05% bromophenol blue, pH 6.8), boiled for 5 min and electrophoresed on an SDS-PAGE (15%) (PAGEL;ATTO; Tokyo, Japan). The proteins were electrophoretically transferred to polyvinylidene difluoride membranes (PVDF) (Immobilon; Millipore, Bedford, MA). The filters were incubated with blocking buffer (5% skimmed milk). To determine if proHB-EGF protein is associated with CD9 protein in gastric cancer, the membranes were incubated with anti-CD9 antibody for 1 hr at room temperature. After washing with Tris-buffered saline containing 0.1% Tween 20 (TBST) 3 times at 10 min intervals, the membranes were incubated with peroxidase-conjugated goat anti-mouse IgG antibodies (American Qualex, San Clemente, CA) for 1 hr at room temperature. The membranes were then washed 3 times with TBST and treated with ECL detection reagents for 1 min at room temperature. The membranes were exposed to scientific imaging films (Fuji X-ray film RX-U).

Statistical analysis

Data are expressed as the mean (SEM). Statistical analyses were performed with Fisher's exact test and χ^2 analysis. A p value of less than 0.05 ($p < 0.05$) was considered to be statistically significant.

RESULTS

HB-EGF gene expression in human gastric cancer

The expression of HB-EGF mRNA in human gastric cancer tissues was first demonstrated by using Northern hybridization. Total RNA samples were obtained separately from gastric cancer tissues and corresponding normal mucosa. The 2.5 kb HB-EGF transcript was clearly detectable at various levels (Fig. 1*a*). A 1.5-fold increase in HB-EGF mRNA levels was found in all gastric cancers compared with normal gastric tissues ($p < 0.05$; Fig. 1*b*). Furthermore, a 2.1-fold increase in HB-EGF mRNA levels was detected in the intestinal type of human gastric cancer tissues ($p < 0.01$; Fig. 1*b*).

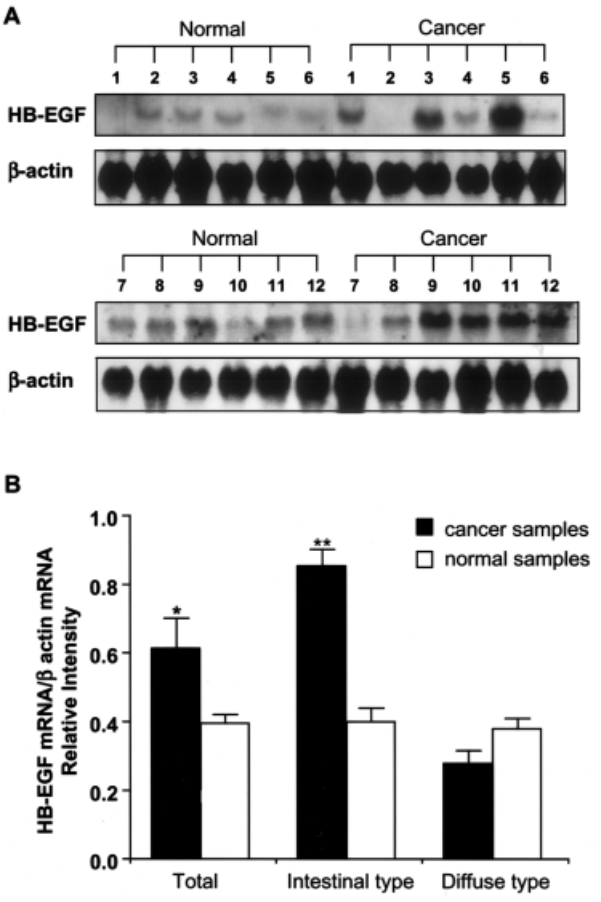

FIGURE 1 – Detection of HB-EGF mRNA in gastric cancers. *(a)* Total RNA (40 μg/ lane) samples, obtained separately from human gastric cancer tissues (T) and the corresponding normal mucosa (N) were separated on 1% agarose-formaldehyde gels prior to transfer to nylon membranes. Blots were probed under high stringency for HB-EGF and the β-actin transcripts. The membrane was hybridized with ^{32}P-labeled HB-EGF probe and β-actin probe as an internal control. Lanes 1–12: normal human gastric mucosa; Lanes 1, 3, 5, 9, 10, 11 and 12: intestinal types of gastric cancer; lanes 2, 4, 6, 7 and 8: diffuse types of gastric cancer. See Material and Methods for details. (*b*) Quantitation of HB-EGF mRNA in gastric cancers. Each band was quantified by densitometry and NIH imaging. The relative intensity between HB-EGF and β-actin signals was calculated. Data are expressed as the mean ± SEM. *$p < 0.05$, ** $p < 0.01$ by comparison with normal samples.

Immunostaining of proHB-EGF and EGF-receptor in gastric cancer tissues

Sixty-six cases of gastric cancer were studied (intestinal type, 33 cases; diffuse type, 33 cases) according to the Lauren classification of gastric cancer.[33] We previously reported that proHB-EGF and EGF-receptor immunoreactivity were both present in normal gastric mucosa.[18] Therefore as an initial step, we immunohistochemically examined the expression of proHB-EGF and EGF receptors in 2 histologically distinct types of gastric cancer tissues. ProHB-EGF was present primarily in the cytoplasm and plasma membrane of cancer cells (Fig. 2), and were especially in cancer cells forming tubular structure in the intestinal type of tumors (Fig. 2*a*). Immunoreactivity to proHB-EGF in diffuse type tumors was weak when compared with levels in the intestinal type (Fig. 2*b*). Overexpression of the EGF-receptor was readily detected in cancer cells, as shown in Figure 2. EGF-receptors are localized primarily in tumor cell membranes and are often found in the cytoplasm of intestinal

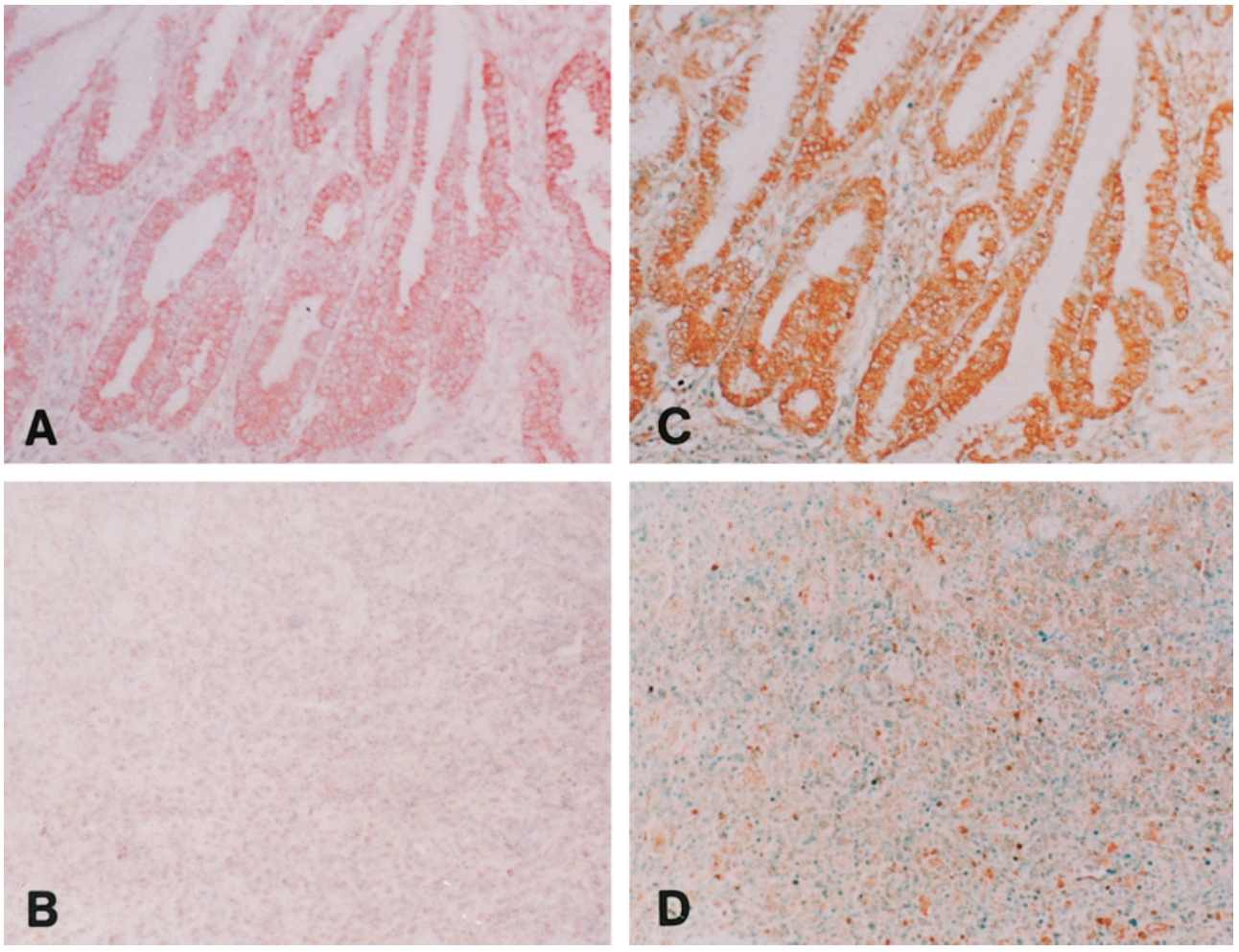

FIGURE 2 – Immunohistochemical staining of the proHB-EGF and EGF-receptor in intestinal-type and diffuse-type of gastric cancers. ProHB-EGF was present primarily in the cytoplasm and membranes of cancer cells of the intestinal type (*a*). ProHB-EGF staining of the diffuse type was weak, compared with that of the intestinal type (*b*). The EGF-receptor is localized in cancer cell membranes and is often accompanied by cytoplasmic staining in the intestinal type (*c*). In the diffuse type, cancer cells exhibited only weak membrane staining (*d*). Original magnification ×210.

type cells (Fig. 2*c*). In the diffuse type, the cancer cells exhibited only weak membrane staining (Fig. 2*d*). Moreover, the epithelial cells of the intestinal metaplasia exhibited a positive cytoplasmic staining for proHB-EGF throughout the mucosa, while normal columnar epithelial cells of the gastric pits were negative. Of the 18 cases examined, 15 (83.3%) were positive for proHB-EGF (data not shown).

In situ *hybridization of HB-EGF and EGF-receptor mRNA*

We next examined the expression of HB-EGF mRNA and EGF-receptor mRNA in gastric cancer tissues by *in situ* hybridization. Sections were stained with hematoxylin and eosin (Fig. 3*a*). As shown in Figure 3*c*, HB-EGF mRNA was localized in the cytoplasm of intestinal-type tumor cells and was found to be expressed at high levels. EGF-receptor mRNA was expressed in the cytoplasm of cancer cells (Fig. 3*d*). Control *in situ* hybridizations of the gastric cancer tissues with sense probes for HB-EGF mRNA was negative (Fig. 3*b*). These results reveal that HB-EGF mRNA is expressed in cancer cells of the intestinal type.

Relationship between proHB-EGF expression and various clinicopathological factors

The relationship between proHB-EGF protein expression and clinicopathological factors are shown for 66 gastric cancers in Table I. ProHB-EGF-immunoreactivity was detected in 40 of the 66 (60.6%) cases. No statistically significant relationship was found between protein expression and age, sex, lymph node status or pathological status. In contrast, proHB-EGF protein expression was significantly associated with tumor status ($p < 0.01$). The immunoreactivity of proHB-EGF tended to be stronger in deeply invasive cancer cells than in cancer cells of more superficial layers. Moreover, a significant correlation between proHB-EGF protein expression and the histological differentiation ($p < 0.001$) was found. The intestinal type of gastric cancer expressed the proHB-EGF protein more frequently than the diffuse type (87.9% *vs.* 33.3%; $p < 0.001$). The frequency of proHB-EGF positives was higher in well-differentiated tubular adenocarcinomas (87.5%, 14 of 16 cases) and moderately-differentiated tubular adenocarcinomas (88.2%, 15 of 17 cases) than in poorly-differentiated tubular adenocarcinomas (40.0%, 8 of 20 cases) or signet-ring cell cancers (23.1%, 3 of 13 cases). ProHB-EGF staining appeared to be stronger for the intestinal type of gastric cancers (Table I).

Immunostaining of CD9 in gastric cancer tissues

CD9 expression was examined in 36 gastric cancers (intestinal type, 18 cases; diffuse type, 18 cases). The staining patterns of CD9 for various histological types of gastric cancers are shown in

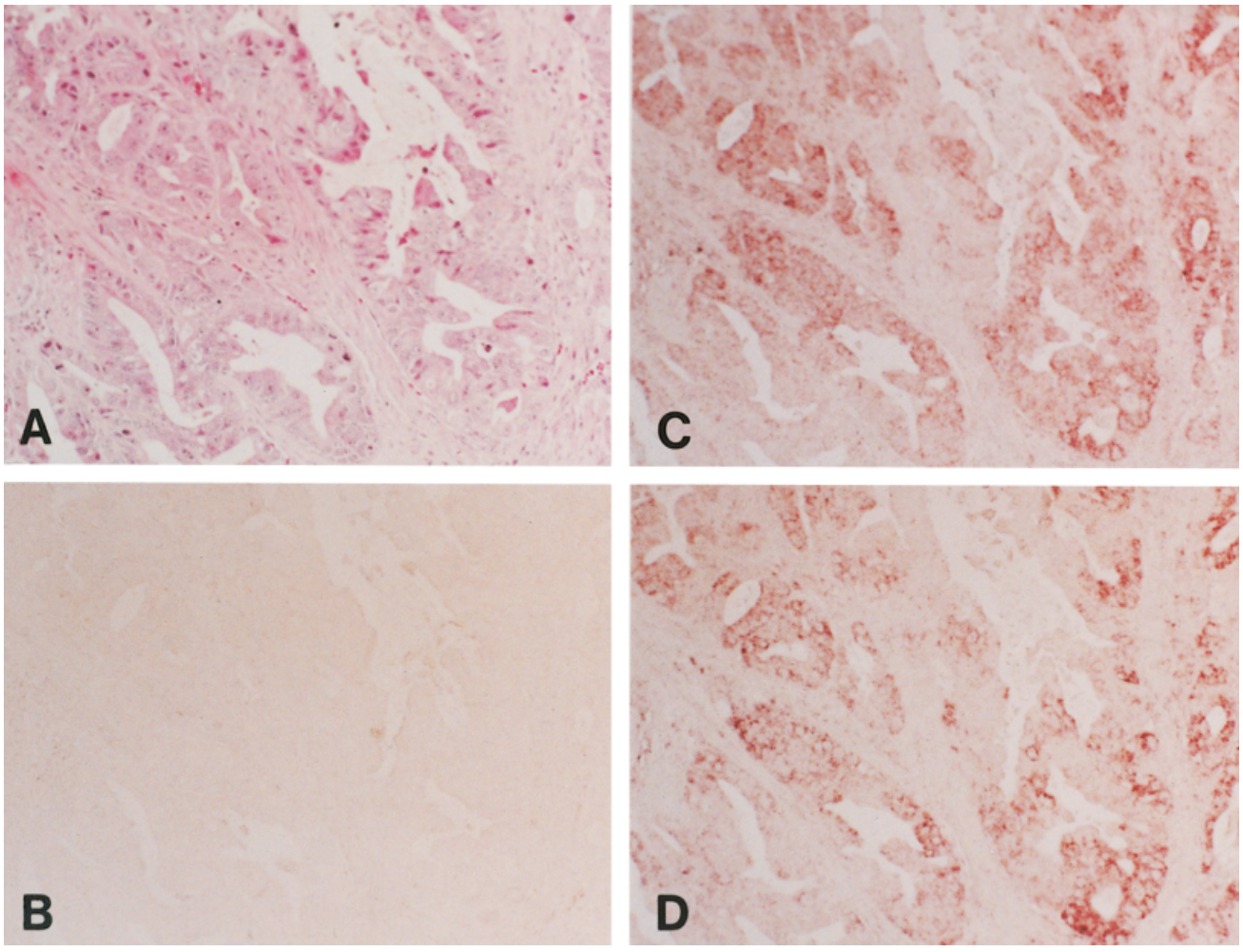

Figure 3 – Localization of the HB-EGF mRNA and the EGF-receptor mRNA in the intestinal type of gastric cancer by *in situ* hybridization. Sections were stained with hematoxylin and eosin (*a*). A adjacent section that was hybridized with the sense probe for HB-EGF mRNA was negative (*b*). A section that was hybridized with the HB-EGF antisense RNA probe shows specific hybridization in the cytoplasm of the cancer cells (*c*). A section that was hybridized with the EGF-receptor antisense RNA probe shows specific hybridization in the cytoplasm of the cancer cells (*d*). Original magnification ×210.

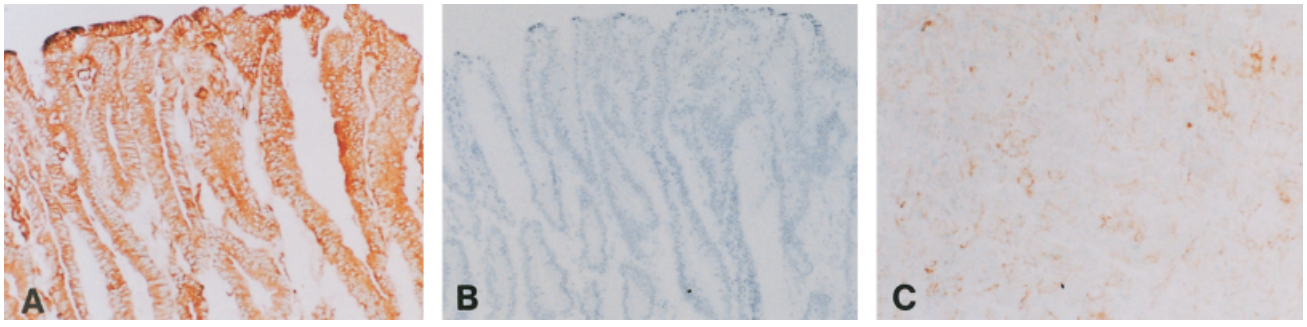

Figure 4 – Immunohistochemical staining of CD9 in gastric cancers. Strong CD9 staining was observed along the cell junctions in the intestinal type of gastric cancer (*a*). CD9 staining in the diffuse type of gastric cancer was reduced (*c*). No reaction product was detected when preimmune sera from mice in which the primary antibodies were raised was used (*b*). Original magnification ×210.

Figure 4. In the intestinal type, CD9 staining was intense and appeared at the surface of the membrane (Fig. 4*a*), while in the diffuse type, CD9 staining was reduced (Fig. 4*c*). Sections that had been incubated with preimmune mouse serum instead of the CD9 antibody exhibited no staining (Fig. 4*b*). In most of these tumors, CD9 expression was observed mainly in the membranous portion of the cancer cells. In contrast to cells that were present in the form of tubules or clusters, sparsely infiltrating cells were stained faintly or at undetectable levels. The relationship between CD9 protein expression and various prognostic factors are shown in Table II. CD9 immunoreactivity was preserved in 26 of 36 (72.2%) gastric cancers. No statistically significant relationships between protein

TABLE I – RELATIONSHIP OF PROHB-EGF EXPRESSION AND CLINICOPATHOLOGICAL FACTORS IN 66 PATIENTS WITH GASTRIC CANCERS

	All patients (*n*)	Results of immunoreactivity Number of patients (%)		*p* value
		ProHB-EGF(+)	ProHB-EGF(−)	
Age (mean ± SD, years)	65.5 ± 1.2	66.5 ± 4.0	64.5 ± 1.8	NS[1]
Sex				
Male	44	26	18	NS
Female	22	14	8	
Histological differentiation				
Intestinal	33	29 (87.9)	4 (12.1)	$p < 0.001$
Diffuse	33	11 (33.3)	22 (66.7)	
Nodal status				
N0	23	13 (56.5)	10 (43.5)	NS
N1	23	15 (65.2)	8 (34.8)	
N2	20	12 (60.0)	8 (40.0)	
Tumor status				
T1	24	10 (41.7)	14 (58.3)	$p < 0.01$
T2	20	10 (50.0)	10 (50.0)	
T3	22	20 (90.9)	2 (9.0)	
Pathological status				
IA	14	6 (42.9)	8 (64.3)	NS
IB	14	8 (57.1)	6 (42.9)	
II	12	6 (50.0)	6 (50.0)	
IIIA	15	11 (73.3)	4 (26.7)	
IIIB	11	9 (81.8)	2 (18.2)	

[1] NS, not significant.

TABLE II – RELATIONSHIP OF CD9 EXPRESSION AND CLINICOPATHOLOGICAL FACTORS IN 36 PATIENTS WITH GASTRIC CANCERS

	All patients (*n*)	Results of immunoreactivity Number of patients (%)		*p* value
		CD9(+)	CD9(−)	
Age (mean ± SD, years)	63.8 ± 1.8	64.0 ± 4.0	63.5 ± 1.5	NS[1]
Sex				
Male	26	18	8	NS
Female	10	8	2	
Histological differentiation				
Intestinal	18	17 (94.4)	1 (5.6)	$p < 0.01$
Diffuse	18	9 (50.0)	9 (50.0)	
Nodal status				
N0	16	15 (93.8)	1 (0.6)	$p < 0.05$
N1	11	7 (63.6)	4 (36.4)	
N2	9	4 (50.0)	5 (55.6)	
Tumor status				
T1	12	9 (75.0)	2 (16.7)	NS
T2	13	8 (72.7)	5 (38.5)	
T3	11	9 (69.2)	3 (27.3)	
Pathological status				
IA	7	6 (85.7)	1 (14.3)	NS
IB	9	8 (88.8)	1 (11.1)	
II	8	6 (75.0)	2 (25.0)	
IIIA	7	3 (42.9)	4 (57.1)	
IIIB	5	3 (60.0)	2 (40.0)	

[1] NS, not significant.

expression and age, sex, tumor status or pathological status were found. In contrast, CD9 protein expression was inversely associated with lymph node status ($p < 0.05$). Moreover, a significant correlation between CD9 protein expression and histological differentiation ($p < 0.01$) was found. Intestinal types of gastric cancers were positive for CD9 immunoreactivity in 17 of 18 (90.0%) cases. No CD9 immunoreactivity was observed in 1 of the 18 (5.6%) cases. Diffuse types of gastric cancers were CD9 positive in 9 of 18 (50.0%) cases and CD9 immunoreactivity was not observed in 9 (50.0%) cases. Thus, the intestinal type of gastric cancer is more likely to express CD9 protein than the diffuse type (90.0% *vs.* 50.0%; $p < 0.01$).

The association of CD9 with proHB-EGF in gastric cancers

In order to further investigate the issue of whether CD9 is able to associate with proHB-EGF in human gastric cancers, immunoprecipitation experiments were performed. As shown in Figure 5, proHB-EGF was coprecipitated with CD9. The association bands of proHB-EGF and CD9 were more intense in 7 of 7 intestinal types among human gastric cancer tissues but was not observed in 5 diffuse types (Fig. 5). This association was also demonstrated, but more faintly, in normal gastric mucosa (data not shown).

Relationship between proHB-EGF and CD9 expression in gastric cancers

The correlation between proHB-EGF immunoreactivity and CD9 immunoreactivity in gastric cancers was found to be significant ($p < 0.01$) (Table III). The frequency of proHB-EGF immunoreactivities in cases, which were also positive for CD9 immunoreactivity, was 76.9% and that of proHB-EGF immunoreactivities in the negative cases with CD9 immunoreactivity was 30.0%. Of the 20 cases in

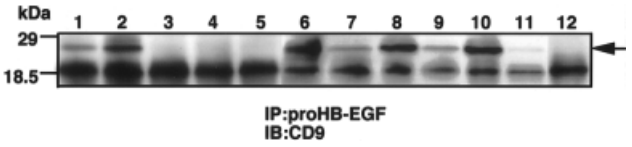

FIGURE 5 – Association of CD9 and proHB-EGF in gastric cancers. Detergent lysates were prepared from samples that were obtained separately from gastric cancer tissues and the corresponding normal mucosa, and the cell lysates were immunoprecipitated with the anti-proHB-EGF antibody. Immunoprecipitated proteins were separated by SDS-PAGE (15%), and transferred to a nitrocellulose membrane. The blot was reacted with anti-CD9 antibody and the proteins were visualized using ECL chemiluminescence system. Lanes 1, 2, 6, 7, 8, 9 and 10: intestinal types of gastric cancer; lanes 3, 4, 5, 11 and 12: diffuse types of gastric cancer. The sizes (kDa) of the predicted products are indicated. The arrow shows the association of CD9 with proHB-EGF. See Material and Methods for details.

which both proHB-EGF and CD9 immunoreactivities were positive, 19 cases were of the intestinal type of gastric cancers.

DISCUSSION

The data presented herein demonstrate the expression of HB-EGF mRNA and the corresponding protein in various histological types of gastric cancers, as evidenced by northern hybridization, immunohistochemistry and *in situ* hybridization. The localization of CD9 in gastric cancers is also verified. ProHB-EGF protein was immunohistochemically detected in approximately 60% (40/66) of the human gastric cancer tissues examined. This is consistent with a previous report which concluded that HB-EGF immunoreactivity was detectable in 72% (5/7) of gastric cancer samples.[5] The present study demonstrates that proHB-EGF expression is significantly associated with tumor status ($p < 0.01$). Thus, these results suggest that proHB-EGF plays an important role in the development or progression of human gastric cancers. Furthermore, the present study demonstrates that strong proHB-EGF immunoreactivity is detectable in the intestinal type and that the positivity of proHB-EGF immunoreactivity is significantly higher in the intestinal type of gastric cancers than in the diffuse type. High levels of HB-EGF mRNA expression in the intestinal type of gastric cancer tissues were found. It has been reported that 93% of intestinal-type gastric cancers exhibited strong TGF-α immunoreactivity, and only 30 % of the diffuse-type were stained.[6] The relative absence of TGF-α may be a significant issue in the development of the poorly differentiated types of gastric carcinoma, and this might be also the case with HB-EGF expression. Moreover, proHB-EGF-positive gastric cancer cells were also positive for EGF receptors. The expressions of HB-EGF mRNA and EGF-receptor mRNA in gastric cancer tissues have also been clearly demonstrated via the use of *in situ* hybridization. These observations suggest that HB-EGF plays a role in tumourigenesis of human gastric cancer in a paracrine, autocrine and/or juxtacrine manner.

TM4SF proteins have the ability to act as "molecular facilitators", which mediate the formation of large molecular complexes, allowing them to function more efficiently.[27] CD9 is one of the best characterized of the TM4SF members. The precise physiological functions of CD9 remain unknown. However, several studies have suggested that CD9 may be involved in cell signaling,[45,46] cell adhesion,[28,29] cell motility,[47] tumor cell metastasis.[42–44,48–51] Clinical studies on the expression of CD9 in human cancer tissues have shown a the relationship between the reduced expression of CD9 and the aggressive behavior of the tumor.[42–44,48–51] The present study demonstrates that CD9 protein levels are inversely associated with lymph node metastasis ($p < 0.05$). These results are consistent with previous findings, in which a correlation of CD9 gene expression with good prognosis in patients with the breast cancer and non-small cell lung cancer was reported.[42,48,49]

TABLE III – RELATIONSHIP BETWEEN PROHB-EGF AND CD9 EXPRESSION IN GASTRIC CANCERS

CD9 immunoreactivity	ProHB-EGF immunoreactivity[1]		
	(+)	(−)	Total
(+)	20 (76.9%)	6 (23.0%)	26
(−)	3 (30.0%)	7 (70.0%)	10
Total	23	12	36

[1]Correlation between ProHB-EGF immunoreactivity and CD9 immunoreactivity was significant in χ^2 test ($p < 0.01$).

Thus, it is possible that the reduced CD9 expression is also strongly associated with an increased risk of recurrence in gastric cancer. Interestingly, CD9 protein expression was found to be associated with histological differentiation in the case of gastric cancer ($p < 0.01$). The present study demonstrates that the positivity of CD9-expressing cells is significantly higher in intestinal types of gastric cancers than in the diffuse types. It is highly possible that diffuse type of gastric cancers are associated with peritoneal dissemination, lymph node metastasis and poor behavior. The loss of CD9 expression also may be associated with reduced cell-cell adhesion or a disorganized glandular morphology. Although the precise mechanism for the downregulation of CD9 remains unclear, CD9 expression is clearly reduced in the diffuse type. These data suggest that CD9 expression in gastric cancer tissues may, in part, be associated with poor behavior or their histological type based on morphological characteristics.

Membrane-anchored growth factors exert mitogenic activity via 2 mechanisms, namely, the paracrine mode and the juxtacrine mode. ProHB-EGF is a membrane-anchored protein and has juxtacrine growth activity. Juxtacrine signaling is one of the important pathways for cell-cell communication in tumor cells, in which signaling molecules, which are anchored in the cell membrane, bind to and activate receptors on the surface of immediately neighboring cells. CD9 is thought to function as a cofactor for proHB-EGF in juxtacrine stimulation. However, in previous studies, the association between proHB-EGF and CD9 in gastric cancer tissues has not been reported. As a result, we examined the issue of whether proHB-EGF and CD9 are associated. In the co-precipitation study performed here, the association of proHB-EGF and CD9 was found to be increased in the intestinal type of gastric cancer tissues. We further demonstrated that the correlation between proHB-EGF immunoreactivity and CD9 immunoreactivity was significant ($p < 0.01$), and that the coexpression of proHB-EGF and CD9 was typically recognized in intestinal type of gastric cancers. We also confirmed that anti-CD9 antibody inhibited the growth of the human gastric cancer cell line MKN-28 from well-differentiated adenocarcinoma *in vitro*, similar to the neutralizing antibodies to HB-EGF (Murayama *et al*., unpublished observation). This finding suggests that an interaction between proHB-EGF and CD9 is important in the growth of intestinal types of gastric cancer. It has been previously demonstrated that the coexpression of CD9 with proHB-EGF or proAR up-regulated their juxtacrine growth factor activities in keratinocytes.[52] Based on our results, CD9 could form a complex with proHB-EGF and might cooperate with proHB-EGF to stimulate human gastric cancer growth in a juxtacrine manner. It has also been reported that a hepatoma cell line (AH66tc) that stably produces proHB-EGF was resistant to apoptosis. Interestingly, this was not observed by the mature form.[53] The same phenomenon has been reported in the renal epithelial cell line (NRK52).[54] Thus, the coexpression of proHB-EGF and CD9 might contribute to the survival of gastric cancer cells, although further study will be necessary to clarify the co-operative intracellular signaling by proHB-EGF and CD9.

In conclusion, both proHB-EGF and CD9 are coexpressed in gastric cancers, especially of the intestinal type, and may be involved in tumorigenesis and/or maintenance, probably in a juxtacrine manner.

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