Table 2. Histopathological response in gerbils

Group	No.	Antrum						Corpus						Anti-*Hp* IgG titer (A.I)	Serum gastin levels (pg/mL)
		Mucosal thickness (mm)	Neutrophils	Mono-nuclear cells	Hyperplasia	Intestinal metaplasia	BrdU labeling index (%)	Mucosal thickness (mm)	Neutrophils	Mono-nuclear cells	Hyperplasia	Intestinal metaplasia	BrdU labeling index (%)		
A	20	0.82 ± 0.25*	2.1 ± 0.6*	3.0 ± 0.2*	2.9 ± 0.3*	2.3 ± 0.7*	24.6 ± 5.9*	1.04 ± 0.26*	1.3 ± 0.3*	2.4 ± 0.3*	2.4 ± 0.2*	1.1 ± 0.4*	14.1 ± 3.3*	288.1 ± 44.7*	1235.1 ± 373.1*
a1	6	0.35 ± 0.09†	2.9 ± 0.2‡	3.0 ± 0.0‡	1.3 ± 0.3†	0.0 ± 0.0	20.2 ± 6.3‡	0.64 ± 0.07†	1.0 ± 0.1†	1.0 ± 0.3‡	1.1 ± 0.2‡	0.0 ± 0.0	4.5 ± 1.5	14.3 ± 3.5‡	ND
B	10	0.48 ± 0.20	1.7 ± 0.3	2.3 ± 0.2	1.8 ± 0.6	0.7 ± 0.4	13.4 ± 4.2	0.77 ± 0.29	1.2 ± 0.3	1.6 ± 0.6	1.6 ± 0.5	0.3 ± 0.3	9.6 ± 2.1	175.8 ± 23.1	323.6 ± 114.4
b1	7	0.30 ± 0.11	2.4 ± 0.2	2.4 ± 0.4	1.4 ± 0.2	0.0 ± 0.0	10.4 ± 3.8	0.54 ± 0.06	1.0 ± 0.0	1.1 ± 0.2	1.1 ± 0.3	0.0 ± 0.0	4.4 ± 1.0	8.8 ± 1.4	ND
C	13	0.60 ± 0.17	1.4 ± 0.3	2.1 ± 0.3	1.8 ± 0.3	0.6 ± 0.2	8.3 ± 1.8	0.77 ± 0.21	1.1 ± 0.0	1.5 ± 0.4	1.4 ± 0.2	0.5 ± 0.4	9.3 ± 2.3	137.1 ± 19.6	395.0 ± 68.3
c1	7	0.19 ± 0.02	1.0 ± 0.1	1.0 ± 0.1	0.7 ± 0.3	0.0 ± 0.0	7.2 ± 1.8	0.45 ± 0.08	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.0 ± 0.0	3.5 ± 1.3	0.8 ± 0.3	ND
D	16	0.20 ± 0.04	0.0 ± 0.0	0.4 ± 0.1	0.0 ± 0.0	0.0 ± 0.1	4.4 ± 1.9	0.42 ± 0.04	0.0 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	3.0 ± 0.8	1.4 ± 0.3	206.2 ± 18.6
E	5	0.62 ± 0.13§	1.9 ± 0.2¶	3.0 ± 0.0§	2.8 ± 0.3¶	2.7 ± 0.3§	20.4 ± 4.8§	1.02 ± 0.18§	1.5 ± 0.4¶	2.4 ± 0.2¶	2.0 ± 0.4¶	1.1 ± 0.4¶	13.7 ± 1.6§	498.4 ± 74.0§	807.6 ± 117.0§
F	5	0.41 ± 0.09	1.6 ± 0.2	2.5 ± 0.4	2.3 ± 0.3	1.0 ± 0.0	10.2 ± 2.4	0.86 ± 0.05	1.2 ± 0.3	2.0 ± 0.0	1.7 ± 0.6	0.7 ± 0.3	5.9 ± 2.1	115.2 ± 36.5	396.3 ± 61.6
G	5	0.34 ± 0.08	1.3 ± 0.3	2.0 ± 0.0	1.9 ± 0.4	0.6 ± 0.2	4.5 ± 1.6	0.71 ± 0.22	1.0 ± 0.1	1.4 ± 0.2	1.0 ± 0.0	0.5 ± 0.1	3.9 ± 1.6	102.0 ± 37.5	375.2 ± 89.1
H	6	0.19 ± 0.03	0.0 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	2.1 ± 0.4	0.44 ± 0.06	0.0 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	2.7 ± 0.9	1.3 ± 0.4	244.7 ± 8.8

*P < 0.05 to group B and C; †P < 0.05 to group b1; ‡P < 0.05 to group b1 and c1; §P < 0.05 to group F and G; ¶P < 0.05 to group G; ND, not determined; *Hp, H.pylori*; BrdU, bromodeoxyuridine

Fig. 2. Histopathological findings of pyloric mucosa after inoculation of *H. pylori*. (a) Marked lymphoid follicle formation in a group a1 gerbil (H&E; × 25); (b) Mild gastritis in a group c1 gerbil (H&E; × 25); (c) Marked gastritis with heterotopic proliferative glands (HPGs) at 70 weeks post-infection (group A, H&E; × 25); (D) Moderate gastritis in a group C gerbil (H&E; × 25).

Fig. 3. Histology of adenocarcinomas. (a) Well differentiated adenocarcinoma in glandular stomach at 70 weeks post-*H. pylori* infection in a group A gerbil (H&E; × 80). (b) Signet ring-cell carcinoma at 70 weeks post-infection in a group A gerbil (H&E; × 400).

Expression of cytokine mRNAs in gastric mucosa of gerbils. IL-1β, TNF-α, COX-2 and iNOS mRNA levels were very low in uninfected gerbils at 77 weeks of age. Infection with the H. pylori resulted in significant increase in transcripts at 20 and 70 weeks in long-term infection gerbils compared with middle and short-term infection gerbils (Fig. 4). The mRNA levels at 70 week post-infection were greater than at 20 weeks post-infection. In H. pylori-infected gerbils, IL-1β, TNF-α, COX-2, and iNOS mRNA levels correlated significantly with chronic active inflammation scores in the gastric mucosa.

Anti-*H. pylori* IgG titers and gastrin levels. The serum anti-H. pylori IgG titers and gastrin levels were significantly higher in the long-term infection group than in the middle and short-term groups (Table 2).

Discussion

At 20 and 70 weeks post-infection, histologic examination revealed more severe neutrophil and mononuclear cells infiltration and

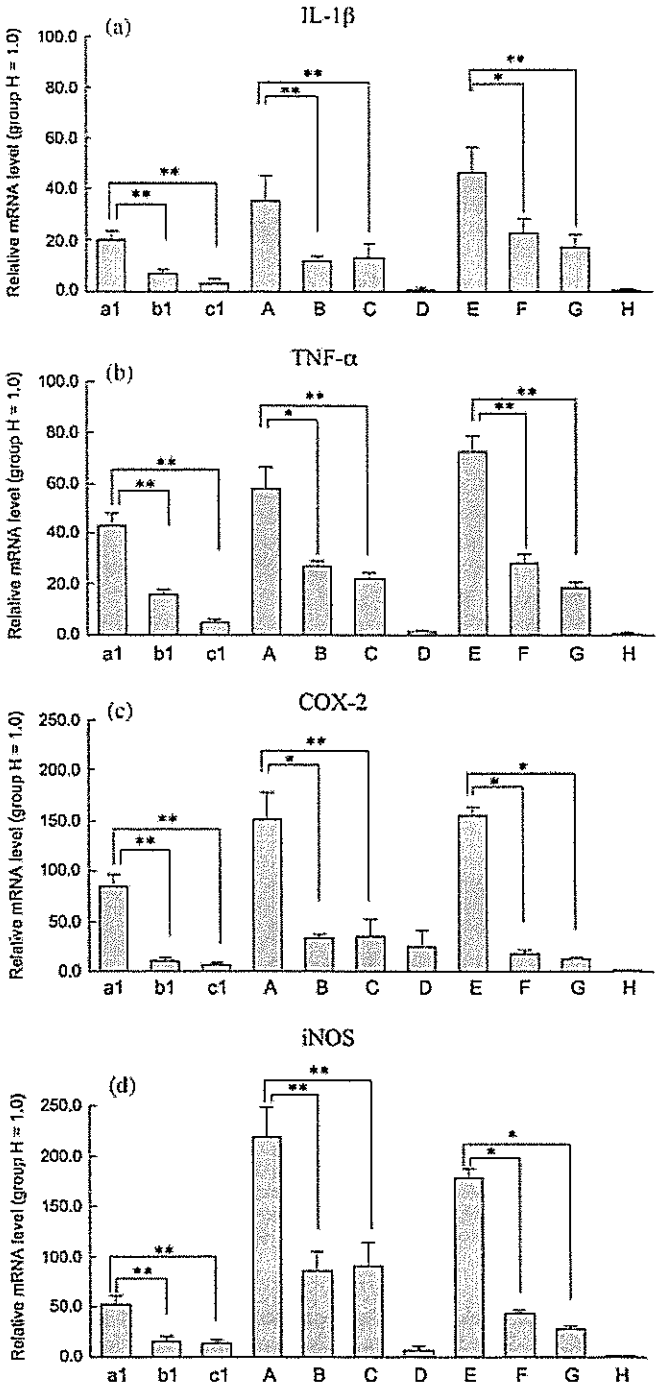

Fig. 4. Relative expression levels of IL-1β, TNF-α, COX-2 and iNOS mRNAs in glandular stomachs of gerbils. (a) IL-1β; (b) TNFα; (c) COX-2; (d) iNOS. Values were set at 1.0 in group H and expressed as mean ± SE relative values. Note increase in relative IL-1β, TNFα, COX-2 and iNOS mRNA expression levels in group A as compared to groups B and C; in group a1 as compared to groups b1 and c1; in group E as compared to groups F and G. $*P < 0.05$ and $**P < 0.01$, by the Bonferroni multiple-comparison test.

submucosal lymphoid follicle formation in the antrum mucosa in early/long-term groups than in the middle and short-term H. pylori-infected groups, and this correlated with the yield of adenocarcinomas. The results thus provide support for the conclusion that infiltration of polymorphonuclear neutrophils and mucosa-associated lymphoid tissue, as well as damage to the epithelial cells, characterizes the first phase of gastric carcinogenesis in this model.[20] Neutrophil infiltration is an almost invariable finding in H. pylori-associated gastritis and is topographically related to H. pylori colonization. It is now evident that inflammatory cells have powerful effects on gastric mucosal injury, neutrophils being recruited to the gastric mucosa and generate reactive oxygen and nitrogen species and proteases.[21] However, neutrophils are not able to kill the H. pylori that live in the gastric mucus, and compounds produced by activated neutrophils themselves are potentially harmful for normal tissue. Active neutrophil infiltration in gastric H. pylori infection may contribute to the increased levels of mutation also observed in Big Blue transgenic mice.[22]

The type of host immune response against H. pylori is considered crucial for the outcome of the infection.[23] One possible mechanism involves up-regulation of inflammatory responses through increase of synthesis and release of pro-inflammatory mediators, such as iNOS and COX-2, via effects on nuclear factor κB (NF-κB).[24,25] A predominant H. pylori-specific Th1 response, characterized by high TNF-α, IL-1β and interferon-γ (IFN-γ) production is associated with gastritis and peptic ulcer.[26] We found mRNA levels of mucosal IL-1β and TNF-α mRNA levels were significantly higher in gerbils with long-term H. pylori-infection than in those with middle and the short-term infection. Yamaoka et al. reported that IL-1β and IFN-γ play important roles in the acute phase of pyloric inflammation in H. pylori-infected gerbils.[12] H. pylori stimulates NF-κB activation and chemokine interleukin-8 (IL-8) expression in gastric epithelial cells. Epithelial IL-8 can be induced not only by direct stimulation of H. pylori, but also following exposure to the endogenous proinflammatory mediators IL-1 and TNF-α in the gastric mucosa.[27,28]

COX-2 and iNOS may play important roles in stomach tumor growth and progression. COX-2 is frequently undetectable in normal tissues but is induced by cytokines, growth factors, reactive oxygen species and tumor promoters,[29,30] and catalyzes the committed step in the conversion of arachidonic acid to protumorigenic eicosanoids, such as prostaglandin E2, which are involved in the maintenance of tumor integrity.[30,31] Several reports have documented H. pylori up-regulation of COX-2 mRNA expression and release of prostaglandin E2 from a gastric cancer cell line, as well as in the gastric mucosa of gerbil models and in humans.[32–34] In the present study, the fact that long-term H. pylori infection significantly up-regulated COX-2 expression is of clear interest.

A number of previous findings also suggest that iNOS contributes to H pylori-associated gastric carcinogenesis in mice and man. Increased iNOS activity has been observed in patients with chronic gastritis and gastric adenocarcinoma patients,[35] nitric oxide (NO) endogenously produced by this family of enzymes causing mutations and DNA deamination.[36,37] Our present observations suggest that COX-2 and iNOS are both induced in H. pylori-positive gastritis and thus may modulate the inflammation and alterations in epithelial cell growth that occur with this disease. The increase in proliferative activity in the mucosa is another feature of interest. At 20 and 70 weeks post-infection, BrdU-labeled cells represented almost 20–25% of the antrum epithelial cells in long-term infected gerbils (groups a1 and A). Early inoculation and long-term H. pylori infection thus appeared to increase epithelial cell apoptosis and proliferation, and this would be expected to lead to increased susceptibility to carcinogens.

Immunohistochemical study showed increased expression of iNOS and COX-2 genes in H. pylori gastritis in humans: iNOS protein was detected in epithelium, endothelium, and lamina propria inflammatory cells, and COX-2 protein localized to mononuclear and fibroblast cells in the lamina propria.[24] Thus, iNOS as well as other inflammatory cytokines may be expressed

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Table 3. Incidence of gastric carcinoma

Groups	Age colonized	Duration of colonization	Age sacrificed	No. of animals	Carcinoma		
					Dif.	Undif.	Incidence (%)
A *Hp* 20 w + MNU	7 w	70 w	77 w	20	12	1	13 (65.0)*
a1 before MNU treatment	7 w	20 w	27 w	6	0	0	0
B *Hp* 8 w + MNU	19 w	58 w	77 w	10	2	0	2 (20.0)
b1 before MNU treatment	19 w	8 w	27 w	7	0	0	0
C *Hp* 2 w + MNU	25 w	52 w	77 w	13	2	1	3 (23.1)
c1 before MNU treatment	25 w	2 w	27 w	7	0	0	0
D MNU	(-)	(-)	77 w	16	0	0	0
E *Hp* 70 w	7 w	70 w	77 w	5	0	0	0
F *Hp* 58 w	19 w	58 w	77 w	5	0	0	0
G *Hp* 52 w	25 w	52 w	77 w	5	0	0	0
H Control	(-)	(-)	77 w	6	0	0	0

*$P < 0.05$ between groups A, B and C, $P < 0.05$ to group B and group C, $P < 0.001$ to group D, by Fisher's exact test. *Hp, H. pylori* infection (i.g.); Dif, differentiated adenocarcinoma; Undif, undifferentiated adenocarcinoma. MNU, *N*-methyl-*N*-nitorosourea.

not only in inflammatory cells but also in epithelial cells in the H. pylori-infected Mongolian gerbils.

Group A showed lower anti-H. pylori IgG titer than that of the MNU untreated group (group E), although not statistically significant ($P < 0.08$). This tendency was not consistent between H. pylori + MNU and H. pylori alone groups (Groups B vs. F; Groups C vs. G) in anti-H. pylori IgG titers and gastrin levels (Table 2). Thus, the effects of MNU on mucosal atrophy could not be considered significant.

Of note, gerbils infected with H. pylori developed an antral-predominant gastritis, which progressed to corpus gastritis at 70 weeks. This pattern is typical of what is seen in humans living in regions of high gastric cancer incidence, and explains why H. pylori-related gastric cancer in gerbils develops most often in the antrum.[38] In the present study, we examined the inflammation status at two experimental timing points: 20 and 70 weeks. Among the 20-week groups (Groups a1, b1, and c1), Group a1 showed the highest inflammatory and proliferative responses, which might affect MNU-induced tumorigenesis. Thus, it could be concluded that the severity of H. pylori-induced inflammation may play important roles in gastric carcinogenesis.

Long-term H. pylori infection was here accompanied by significantly increased anti-H. pylori serum IgG antibody titer, highlighting the importance of the immune response of the host in the development of H. pylori-related gastric lesions. The results also suggest that the anti-H. pylori serum IgG antibody titer may be used as a marker of the severity of the H. pylori-infected chronic active gastritis.[18] A previous study demonstrated that acid secretion is decreased in gerbils infected with H. pylori.[39] In the present case, long-term H. pylori infection induced chronic pangastritis and may have suppressed parietal cell function, causing compensatory gastrin release corresponding with a lower gastric acid secretion. Furthermore, increased gastrin levels could contribute to gastric barrier dysfunction in H. pylori infection.[40] Wang et al. found that chronic hypergastrinemia can synergize with H. pylori infection and contribute to eventual parietal cell loss and progression to gastric cancer in insulin-gastrin (INS-GAS) transgenic mice.[41]

In summary, our study shows that the severity of chronic gastritis induced by pathogen H. pylori is linked with glandular gastric carcinogenesis. The gastric mucosal injury demonstrated in the long-standing H. pylori-infected gerbil clearly can lead to increased susceptibility to carcinogenic substances and also contribute to immune responses, perpetuation of mucosal inflammation and cancer development. This phenomenon is not only dependent on the timing and age at H. pylori infection, but also the period of infection.[42] The pathological changes present at the early stage of H. pylori infection seems to persist and become aggravated during the life span of the animals, thus contributing to the multifactorial processes underlying gastric neoplasia.

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Histology and Histopathology

Cellular and Molecular Biology

Gastric and intestinal phenotypic correlation between exocrine and endocrine components in human stomach tumors

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Summary. We have previously suggested that an origin of a stomach cancer is from a progenitor cell specializing toward exocrine cell (Exo-cell) lineages. To clarify whether our hypothesis is correct or not, we analyzed the expression of Exo-cell and endocrine cell (End-cell) markers in a series of lesions for comparison. We evaluated chromogranin A (CgA) expression in 37 early and 73 advanced stomach cancers, in 30 stomach adenomas, in 8 carcinoid tumors, and in 4 endocrine cell carcinomas (ECCs) with assessment of gastric and/or intestinal (G/I) phenotypes in both Exo-cell and End-cell by immunohistochemistry. CgA expression was observed in 10.8% of the early and 16.4% of the advanced stomach cancers, respectively. The End-cell G/I phenotypes were in line with the Exo-cell counterparts in the CgA-positive stomach cancerous areas, and there was strong association between Cdx2 expression and the intestinal End-cell markers. All of the adenoma cases had the intestinal Exo-cell phenotypic expression, with the positive link between Exo-cell and End-cell G/I phenotypes. All stomach carcinoids had CgA expression but no expression of Exo-cell markers. In conclusion, most stomach cancers might develop from a progenitor cell specializing towards Exo-cell lineages, but some cases possessed both Exo-cell and End-cell markers with maturely differentiated phenotypes. In such cases, Exo-cell and End-cell phenotypes were found to correlate strongly, suggesting the possibility of histogenesis from "cancer stem cells".

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Key words: Stomach cancers, Endocrine cell, Phenotypes, Progenitor cell, Cancer stem cells

Introduction

Gastrointestinal stem cells have the capacity for long-term self-replication and the ability to give rise to all other epithelial cell lineages (Schier and Wright, 2005). We have previously shown that each epithelial gland in the alimentary tract is derived from a single stem cell, based on clonality analysis using a strain specific antibody in C3H/HeN⇔BALB/c chimeric mice (Tatematsu et al., 1994, 1996; Tsukamoto et al., in press). The stem cell gives rise to two kinds of progenitor cell directly: (i) progenitor cell specializing toward exocrine cell (Exo-cell) lineages; and (ii) progenitor cells specializing toward endocrine cell (End-cell) lineages (Tatematsu et al., 2003, 2005).

Regarding the histogenesis of stomach cancer, if the cancer originated from the stem cell, the mixture of differentiation toward both Exo-cell and End-cell lineages should be observed more frequently and homogenously in the whole stomach cancerous tissues, although in fact it is observed rarely. Stomach epithelial tumors are divided into two major types: Exo-cell type (adenomas and carcinomas) and End-cell type [carcinoid tumors and endocrine cell carcinomas (ECC)]. Therefore, we have suggested the hypothesis that the origin of stomach cancers is from a progenitor cell specializing towards an Exo-cell lineage (Tatematsu et al., 2005). However, there have been several reports that chromogranin A (CgA), an End-cell differentiation marker, was immunohistochemically found in about 15-70% of human stomach cancers, although with

differences in anti-CgA antibodies and criteria of CgA positivity among the reports, suggesting the possession of both Exo-cell and End-cell differentiation in the stomach cancers (Park et al., 1992; Blumenfeld et al., 1996; Waldum et al., 1998; Qvigstad et al., 2000; Tzaneva, 2002; Naritomi et al., 2003). The existence of CgA positive stomach cancer cells indicates a concept that stomach cancer may occur from a stem cell harboring the ability to differentiate toward both Exo-cell and End-cell lineages. Recently, several reports have demonstrated the existence of malignant cells possessing self-renewal properties similar to normal stem cells in human myeloid leukemias (Bonnet and Dick, 1997), breast cancers (Al-Hajj et al., 2003) and brain tumors (Singh et al., 2004). There may be the possibility that the cancer stem cells appear secondarily in the stomach cancerous tissue, and they produce both Exo-cell and End-cell types, being similar to normal stem cells.

Human stomach cancers have been classified by Lauren into two major groups, the "intestinal" and "diffuse" types (Lauren, 1965), which respectively nearly correspond to the "differentiated" and "undifferentiated" types of Nakamura et al. (1968) and Sugano et al. (1982). However, the above-mentioned classifications are inadequate for studies of histogenesis of gastric carcinomas and phenotype expression at the cellular level, because they confuse an intestinal phenotype with a "diffuse" structure and a gastric phenotype with the "intestinal" type of Lauren (Tatematsu et al., 2003). The phenotypic expression of stomach cancer cells of each histological type can be clearly classified into gastric and intestinal epithelial cell types by immunohistochemistry using gastric and intestinal epithelial cell markers such as MUC5AC, MUC6, MUC2, and villin (Tatematsu et al., 2003). In contrast, gastric and intestinal differentiation of endocrine cells in stomach cancers has not been fully evaluated.

To clarify whether our hypothesis is correct or not, the present study was conducted to analyze CgA expression by immunohistochemistry in a series of early and advanced stomach cancers with histological evaluation by hematoxylin and eosin (H&E) staining. The relations of gastric and intestinal differentiation between Exo- and End-cells were immunohistochemically evaluated within multiple areas within each stomach cancer case, and for comparison, adenoma cases and small numbers of carcinoids and ECCs, were similarly assessed.

Materials and methods

Samples and tissue collection

A total of 110 primary stomach cancers surgically resected at Aichi Cancer Center Hospital between 1994 and 2000 (Mizoshita et al., 2004a,b; Tsukamoto et al., 2005) were examined, 37 early and 73 advanced lesions, found in patients ranging in age from 43 to 78 years (mean±SD, 59.8±8.8 years) and 32 to 84 years (62.1±10.2 years), respectively. Histological classification was made into differentiated and undifferentiated adenocarcinomas according to the Japanese Classification of Gastric Carcinomas (Japanese Gastric Cancer Association, 1998). Early cases localized in mucosa (m) or in submucosa (sm). In the advanced cases, the cancers had invaded the muscularis propria (mp), the subserosa (ss), or the serosa and the peritoneal cavity (se), including adjacent organs (si).

We examined 12 primary endocrine tumors (8 carcinoids and 4 ECCs) surgically resected, too. The endocrine tumors were diagnosed by the presence of at least one of a number of endocrine markers, including CgA, synaptophysin, or CD56 and were found in 7 men and 5 women ranging in age from 39 to 66 years (52.0±10.8 years).

We also evaluated 30 stomach adenomas obtained by endoscopic mucosal resection or submucosal dissection. The stomach adenomas were found in 17 men and 13 women ranging in age from 46 to 79 years (64.7±10.2 years). Adenoma cases having a cancerous component were excluded from this study.

All specimens were fixed in 10% buffered formalin. Carcinomas with adjacent non-neoplastic mucosa were cut serially into 5 mm slices in parallel with the lesser curvature and embedded in paraffin, and then stained with H&E for histological examination.

Immunohistochemistry

Immunohistochemical staining was carried out with antibodies against the following antigens: Cdx2 (BioGenex, San Ramon, CA, USA); MUC5AC (Novocastra Laboratories, Newcastle upon Tyne UK); MUC6 (Novocastra Laboratories); MUC2 (Novocastra Laboratories); and villin (Transduction Labolatories, Lexington, KY, USA); CgA (Dako, Glostrup, Demmark); Gastrin (Yanaihara Institute, Fujinomiya, Japan); Somatostatin (Dako); glucagon-like peptide-1 (GLP-1) (Yanaihara); gastric inhibitory polypeptide (GIP) (Yanaihara); Glicentin (Yanaihara). With regard to gastric phenotypic markers, we used normal gastric mucosa and normal ileum as positive and negative controls, or vice versa, for intestinal phenotypic ones. The precise procedures for immunohistochemical techniques were as previously described (Mizoshita et al., 2003, 2004a,b; Tatematsu et al., 2003; Tsukamoto et al., 2004, 2005; Otsuka et al., 2005). Briefly, 4 μm-thick consecutive sections were deparaffinized and hydrated through a graded series of alcohol. After inhibition of endogenous peroxidase activity by immersion in 3% H_2O_2 /methanol solution, antigen retrieval was conducted by heating in 10 mM citrate buffer (pH 6.0) in a microwave oven for 10 min at 98°C. Sections were incubated with primary antibodies, thoroughly washed in phosphate-buffered saline (PBS), then incubated with biotinylated secondary antibodies, followed by avidin-biotinylated horseradish peroxidase complexes

(Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA). Finally, binding was visualized by incubation with 0.01% H_2O_2 and 0.05% 3.3'-diaminobenzidine tetrachloride (DAB). Nuclear counterstaining was accomplished with Mayer's hematoxylin.

Two independent pathologists (T.T. and T.M.) judged the histology and immunohistochemical staining of the Exo-cell and End-cell markers including Cdx2. The result for CgA staining was evaluated with reference to the percentage of positively stained tumor cells. A result was considered positive if at least 10% of tumor cells were stained. When less than 10% of tumor cells were stained, immunostaining was considered negative.

Classification of tumors

MUC5AC and MUC6 are markers of the gastric Exo-cell phenotype, whereas MUC2 and villin are typical of the intestinal Exo-cell phenotype (Mizoshita et al., 2003, 2004a,b; Tatematsu et al., 2003; Tsukamoto et al., 2005). Similarly, gastrin and somatostatin are markers of the gastric End-cell phenotype, whereas GLP-1, GIP, and glicentin are typical of the intestinal End-cell phenotype (Otsuka et al., 2005).

In the CgA-positive tumor cases, expression of both Exo-cell and End-cell markers was evaluated in tumorous areas having CgA cytoplasmic staining. Firstly, we examined the expression of the End-cell markers in the CgA-positive tumors. Stomach tumorous areas were classified as endocrine-gastric (e-G type) or endocrine-intestinal (e-I type), respectively, with at least one gastric or intestinal End-cell phenotype, and endocrine-gastric-and-intestinal mixed phenotype (e-GI type) when both gastric and intestinal markers were present. Those showing neither gastric nor intestinal phenotypic expression were grouped as endocrine-null type (e-N type). Then, stomach tumorous areas positive for at least one gastric or intestinal Exo-cell marker were classified as of gastric (G type) or intestinal (I type) phenotype, respectively. Those which exhibited both phenotypes were classified as gastric-and-intestinal mixed (GI type), while those showing neither were grouped as null (N type).

Statistical analysis

The data were analyzed by Fisher's exact or χ^2 test for differences between groups. The P-values <0.05 were considered statistically significant.

Results

Expression of CgA in the early and advanced stomach cancers

Totals of 16 (14.5%) and 94 (85.5%) stomach cancers were judged to be CgA-positive and CgA-negative, respectively (Table 1). In the early cases, totals of 4 (10.8%) and 33 (89.2%) lesions were judged to be CgA-positive and CgA-negative, respectively. In the advanced cases, totals of 12 (16.4%) and 61 (83.6%) lesions were judged to be CgA-positive and CgA-negative, respectively. With the histological classification, the CgA-positive rates in cases of the differentiated, and undifferentiated types were 12.5% (7/56) and 16.7% (9/54), respectively, the difference being not significant. There were no significant differences between CgA-positive and CgA-negative groups with reference to age and sex. No lymph node metastasis was observed with the 37 early cases. There was also no significant difference between CgA-positive and CgA-negative groups with reference to lymph node metastasis in the advanced cases. On Kaplan-Meier analysis of the advanced cases, the 5-year survival rates in patients of the CgA-positive and CgA-negative groups were 38.2% and 43.6%, respectively, the difference not being significant (data not shown).

Relations between expression of Exo-cell and End-cell markers in 4 early and 12 advanced CgA-positive stomach cancers

We examined the expression of End-cell markers in 4 early and 12 advanced CgA-positive stomach cancers. Ten (62.5%) cases had the expression of at least one End-cell marker, while 6 cases had no expression of End-cell markers. In 10 CgA-positive cases with End-cell marker expression, we evaluated the expression of the Exo-cell markers (Table 2). In 2 cases (Cases 4 and

Table 1. Correlations between clinicopathological findings and the chromogranin A expression in 37 early and 73 advanced stomach cancers.

Clinicopathological findings	CgA (+) (n=16)	CgA (-) (n=94)	P- value
Age			
Years (mean ± s.d.)	64.0 ± 10.2	60.9 ± 9.7	NS
Sex			
Male (n=67)	12	55	NS
Female (n=43)	4	39	
Histological classification[a]			
Differentiated (n=56)	7	49	NS
Undifferentiated (n54)	9	45	
Depth			
early (n=37)	4	33	NS
advanced (n=73)	12	61	
Lymph node metastasis			
Positive (n=63)	10	53	NS
Negative (n=47)	6	41	

CgA, chromogranin A; NS, not significant; [a]: Classified based on structure of elements. "Differentiated" includes tubular and papillary types, while "Undifferentiated" consists of signet-ring cell and poorly differentiated types.

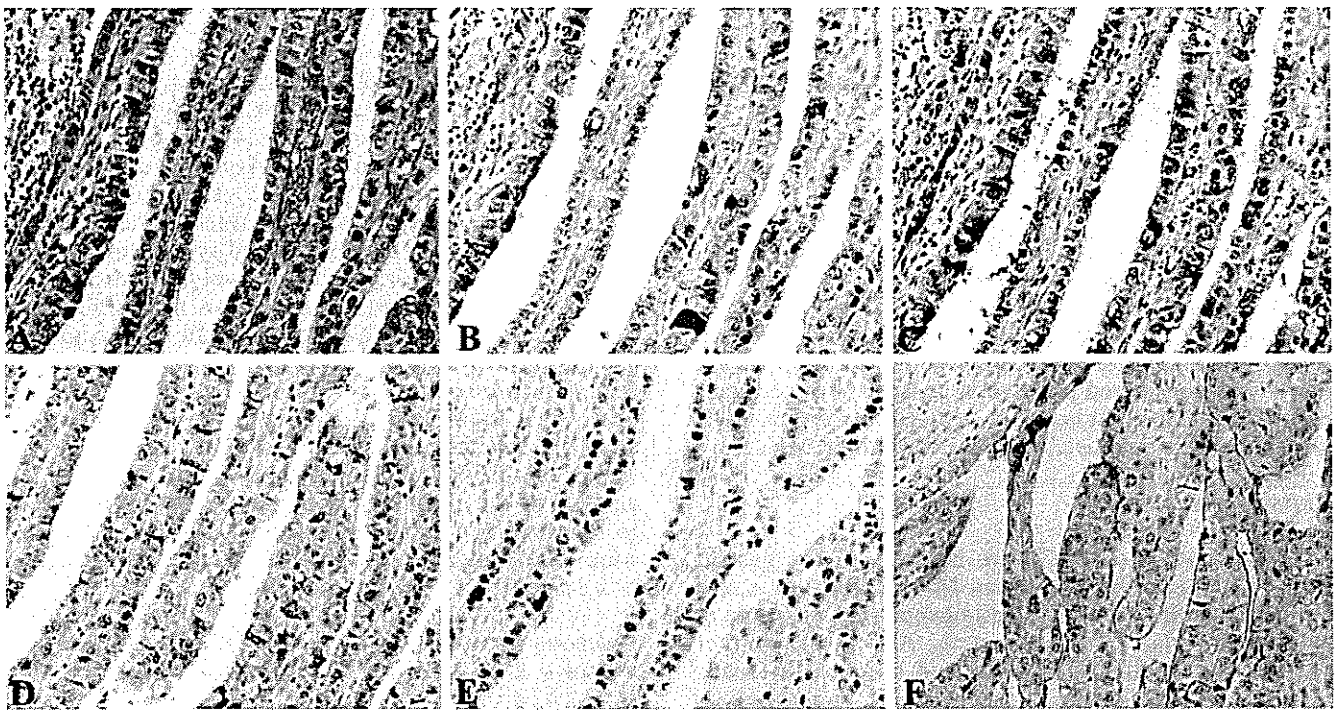


Fig. 1. A stomach cancerous area of e-I type (Case 6f). A. H&E staining. B. CgA cytoplasmic staining observed in some tumor cells. C. GIP is present in the cytoplasm of some cancer cells. D. Glicentin is evident in the cytoplasm of some cancer cells. E. Cdx2 nuclear staining in the tumor cells. F. Villin is positive on the luminal surfaces of cancer cells. CgA, chromogranin A; GIP, gastric inhibitory polypeptide. x 200

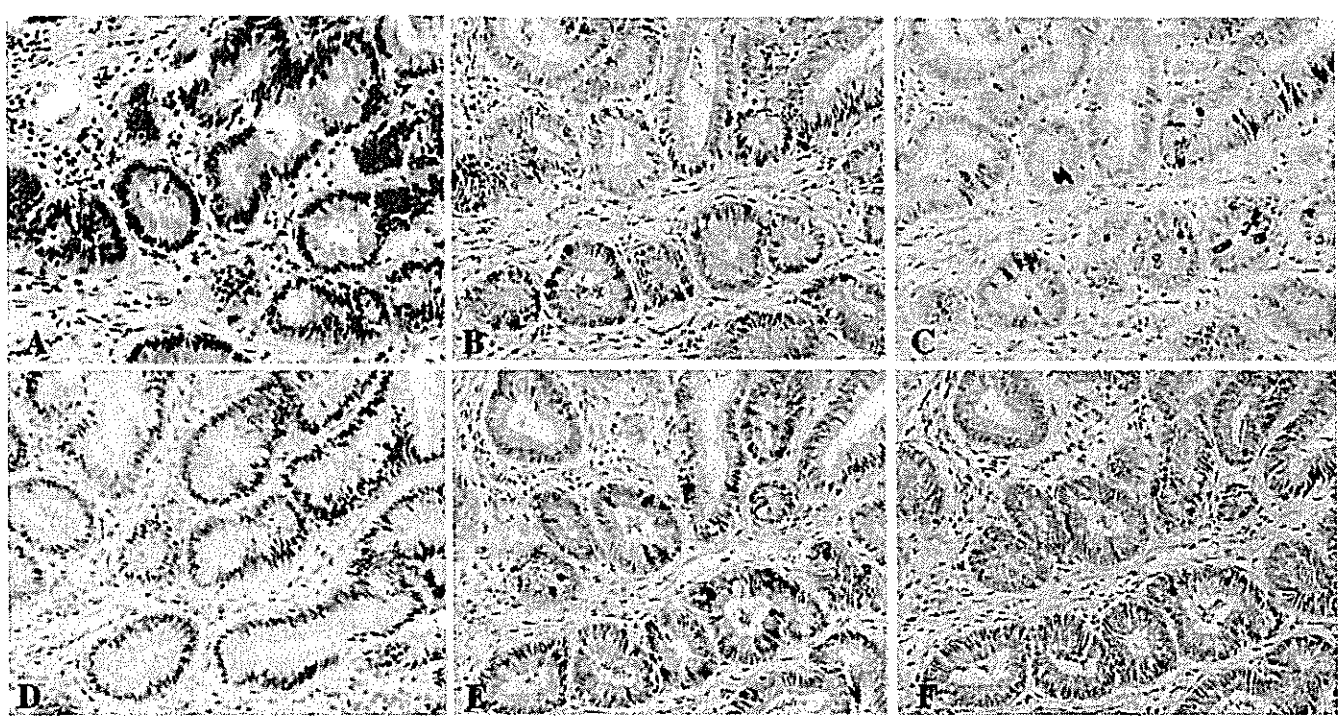


Fig. 2. A stomach adenoma case of e-I type (Adenoma 13). A. H&E staining. B. CgA expression is apparent in the cytoplasm of tumor cells. C. MUC2 is present in the cytoplasm of some adenoma cells. D. Cdx2 is positive in the nuclei of adenoma gland cells. E. GLP-1 is evident in the cytoplasm of some adenoma cells. F. GIP is present in the cytoplasm of some adenoma cells. CgA, chromogranin A; GLP-1, glucagon-like peptide-1; GIP, gastric inhibitory polypeptide. x 200

5) of 10 tumors with End-cell marker expression, the staining of the markers were observed uniformly all over the whole tumor tissues, while 8 cases had heterogeneity of both Exo-cell and End-cell marker expression within a whole tumor. Therefore, we evaluated the relation between Exo-cell and End-cell phenotypes in the 23 small cancerous areas covering more than 500 μm in diameter with CgA cytoplasmic expression in these 8 cases (Cases 1, 2, 3, 6, 7, 8, 9, and 10).

With regard to the gastric End-cell markers, expression of gastrin was detected in 11 cancerous areas with CgA expression in 5 cases, while somatostatin

Table 2. Relations between exocrine and endocrine cell markers with reference to phenotypic classification in 25 cancerous areas.

Cases	Regions	Phenotype 1[a] (Exocrine cell differentiation)	MUC5AC	MUC6	MUC2	Villin	Cdx2[b]	Phenotype 2 (Exocrine cell differentiation)	Gastrin	Somatostatin	GLP-1	GIP	Glicerin
Cancer 1	a	G	■	■				e-G	■				
Cancer 1	d	G	■	■				e-G	■				
Cancer 1	e	G	■					e-G	■				
Cancer 2	a	G	■					e-G	■				
Cancer 2	b	G		■			■	e-I					■
Cancer 3	a	GI	■		■	■	■	e-GI	■		■	■	
Cancer 3	b	GI	■			■	■	e-GI	■	■		■	
Cancer 3	c	GI	■		■	■	■	e-GI	■			■	
Cancer 3	d	GI	■			■	■	e-GI		■		■	
Cancer 3	e	GI	■		■	■	■	e-GI	■			■	
Cancer 4	whole	GI	■		■	■	■	e-GI		■	■	■	
Cancer 5	whole	GI	■	■	■		■	e-GI		■	■	■	
Cancer 1	b	GI	■		■		■	e-I				■	
Cancer 6	a	I				■	■	e-G	■				
Cancer 1	c	I			■		■	e-I					■
Cancer 6	f	I				■	■	e-I				■	■
Cancer 6	e	I				■	■	e-I					■
Cancer 7	a	I			■		■	e-I			■		
Cancer 8	a	N						e-G	■				
Cancer 6	b	N						e-GI	■		■		
Cancer 8	b	N						e-I			■	■	
Cancer 9	a	N					■	e-I					■
Cancer 6	d	N						e-I				■	
Cancer 6	c	N					■	e-I			■		
Cancer 10	a	N					■	e-I				■	

a: P=0.0004, comapared with Phenotype 2 by c2 test; b: P=0.006, comapared with Phenotype 2 by χ^2 test. Positive gastric and intestinal markers are filled with red or blue, respecitvely; e-G type, gastric endocrine cell phenotype; e-GI type, gastric-and-intestinal endocrine cell phenotype; e-I type, intestinal endocrine phenotype; G type, gastric exocrine cell phenotype; GI type, gastric-and-intestinal exocrine cell phenotype; I type, intestinal exocrine cell phenotype; N type, null exocrine phenotype.

expression was observed in 4 cancerous areas of 4 cases. Regarding the intestinal End-cell markers, expression of glicentin was detected in 5 areas of 4 cases. Expression of GIP was observed in 12 cancerous areas of 7 cases, while GLP-1 expression was detected in 7 lesions in 6 cases. The total of 25 cancerous areas (10 cancer cases) were divided phenotypically into 6 e-G, 8 e-GI, and 11 e-I types. In 15 (60.0%) cancerous areas (areas 1a, 1c, 1d, 1e, 2a, 3a, 3b, 3c, 3d, 3e, 4 whole, 5 whole, 6e, 6f, and 7a), the phenotypes of End-cell markers were in line with those of the Exo-cell counterparts, and strong association was observed between the Exo-cell and End-cell markers from the viewpoint of phenotypic expression in the remainder (Fig. 1, Table 2, P=0.0004). Cdx2 expression was also strongly associated with the presence of intestinal End-cell markers such as glicentin, GLP-1, and GIP (Table 2, P=0.006). When the multiple areas within tumors were compared, the phenotypes of both Exo- and End-cell markers of Cancer Case 3 coincided well among areas a-e. However, those of areas b and c were different from areas a, d, and e in Case 1. This discrepancy was also observed in Case 6 (areas a, f, and e vs. areas b, d, and c). In 4 cancerous areas (Cases 6a, 6b, 6d, and 8b), Cdx2 expression was not in line with the intestinal End-cell marker expression. However, regarding Case 6a, Cdx2 nuclear staining was observed in the cancerous area of e-G type exhibiting villin expression.

Table 3. Relations between exocrine and endocrine cell markers with reference to phenotypic classification in 14 CgA-positive adenomas.

Cases	Phenotype 1[a] (Exocrine cell differentiation)	MUC5AC	MUC6	MUC2	Villin	Cdx2	Phenotype 2 (Endocrine cell differentiation)	Gastrin	Somatostatin	GLP-1	GIP	Glicentin
Adenoma 1	GI		■	■	■	■	e-G		■			
Adenoma 2	GI		■	■	■	■	e-G		■			
Adenoma 3	GI	■	■	■	■	■	e-GI		■	■	■	
Adenoma 4	GI		■		■	■	e-GI		■		■	
Adenoma 5	GI	■	■		■	■	e-I			■	■	
Adenoma 6	I			■	■	■	e-GI		■	■	■	
Adenoma 7	I			■	■	■	e-GI		■	■	■	
Adenoma 8	I			■	■	■	e-I			■	■	
Adenoma 9	I				■	■	e-I			■		
Adenoma 10	I			■	■	■	e-I			■		
Adenoma 11	I			■	■	■	e-I			■		
Adenoma 12	I			■	■	■	e-I			■	■	
Adenoma 13	I			■	■	■	e-I			■	■	
Adenoma 14	I				■	■	e-I			■	■	

[a]: P=0.031, comapared with Phenotype 2 by χ^2 test; Positive gastric and intestinal markers are filled with red or blue, respecitvely; e-G type, gastric endocrine cell phenotype; e-GI type, gastric-and-intestinal endocrine cell phenotype; e-I type, intestinal endocrine phenotype; GI type, gastric-and-intestinal exocrine cell phenotype; I type, intestinal exocrine cell phenotype.

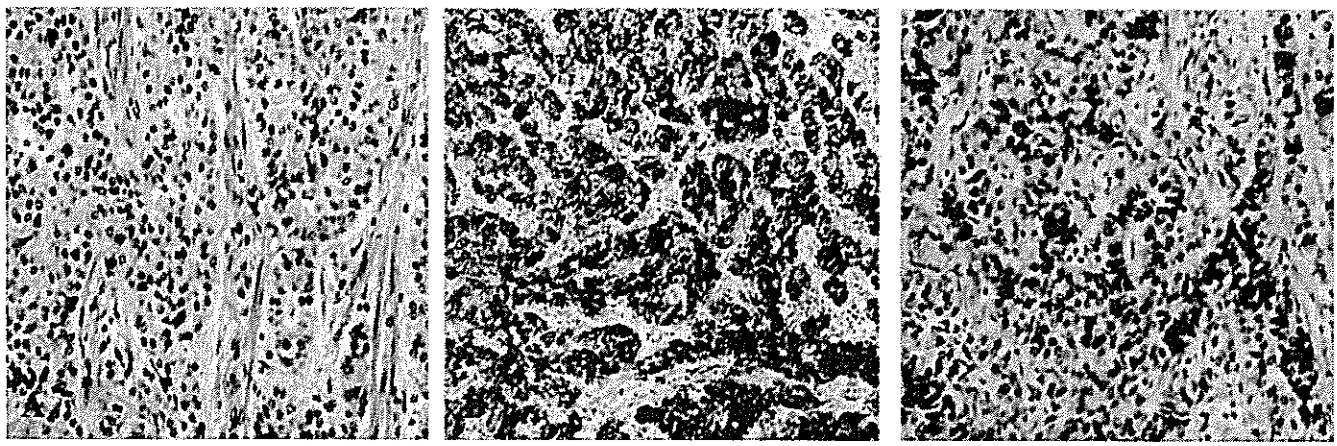


Fig. 3. A stomach carcinoid tumor with expression of gastrin (Carcinoid case No.4). A. H&E staining. B. CgA expression is apparent in the cytoplasm of tumor cells. C. Gastrin is present in the cytoplasm of carcinoid cells. CgA, chromogranin A. x 200

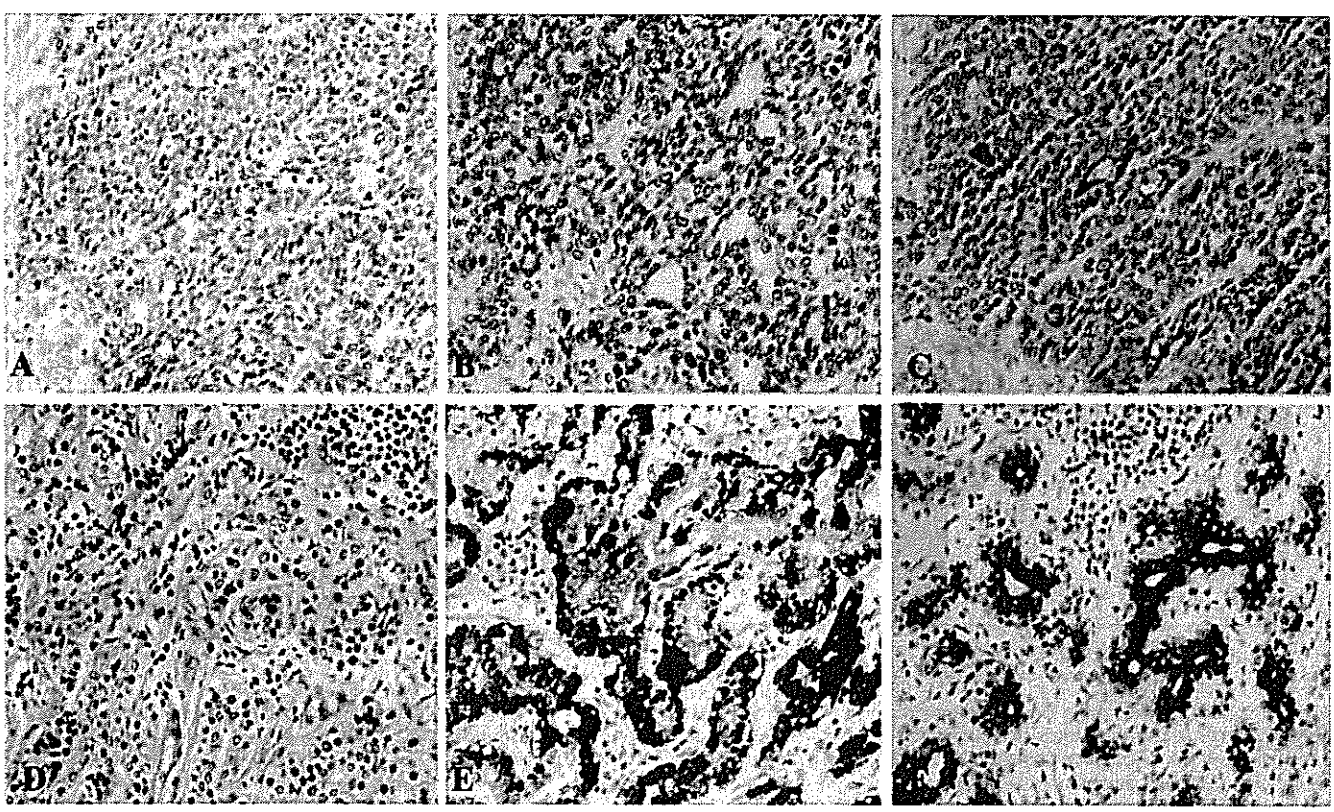


Fig. 4. Two cases of ECCs featuring expression of Exo-cell markers. A-C. ECC1 in Table 4. A. H&E staining. B. Lack of CgA cytoplasmic staining in the cancer cells. Positive for synaptophysin and CD56 (data not shown). C. Villin is positive on the luminal surfaces of cancer cells. D-F. ECC2 in Table 4. D. H&E staining. E. CgA expression is evident in the cytoplasm of cancer cells. F. MUC6 is positive in the cytoplasm of cancer cells. ECC, endocrine cell carcinoma; CgA, chromogranin A; Exo-cell, exocrine cell. x 200

Relations between expression of Exo-cell and End-cell markers in 14 CgA-positive stomach adenomas

Totals of 15 (50%) and 15 (50%) stomach adenomas were judged to be CgA-positive and CgA-negative, respectively. We examined the expression of End-cell markers in the 15 CgA-positive adenomas. Of the 15 cases, 14 CgA-positive cases had the expression of at least one End-cell marker, while 1 case exhibited no End-cell marker expression. Therefore, we also analyzed expression of Exo-cell markers in the above-mentioned 14 cases. The lesions were divided by the End-cell marker expression into 2 e-G, 4 e-GI, and 8 e-I types (Table 3). They were also classified by the Exo-cell marker expression as 5 GI and 9 I types. The phenotypes of End-cell markers in 2 e-GI and 7 e-I types were in line with those of the Exo-cell counterparts. Strong association was observed between the Exo-cell and End-cell markers from the viewpoint of phenotypic expression in adenoma cases (Fig. 2, Table 3, P=0.031). Cdx2 expression was present in all stomach adenoma cases exhibiting the intestinal Exo-cell phenotypic expression.

Expression of CgA, Exo-cell and End-cell markers in 8 carcinoid tumors and 4 ECCs of the stomach

Data for expression of Exo-cell and End-cell markers in the End-cell tumors are summarized in Table 4. Eight carcinoid tumors (100%) and 1 ECCs (25%)

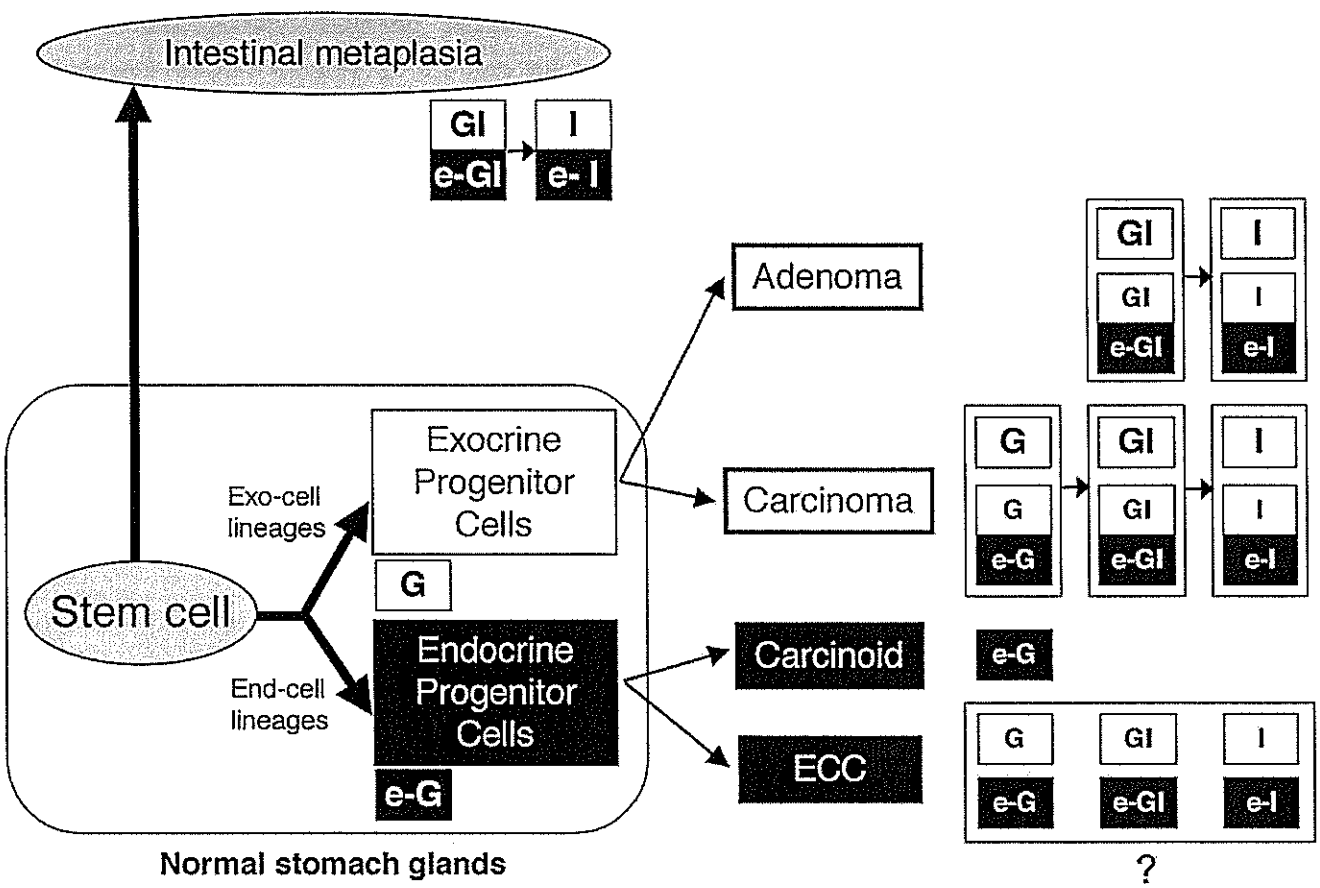
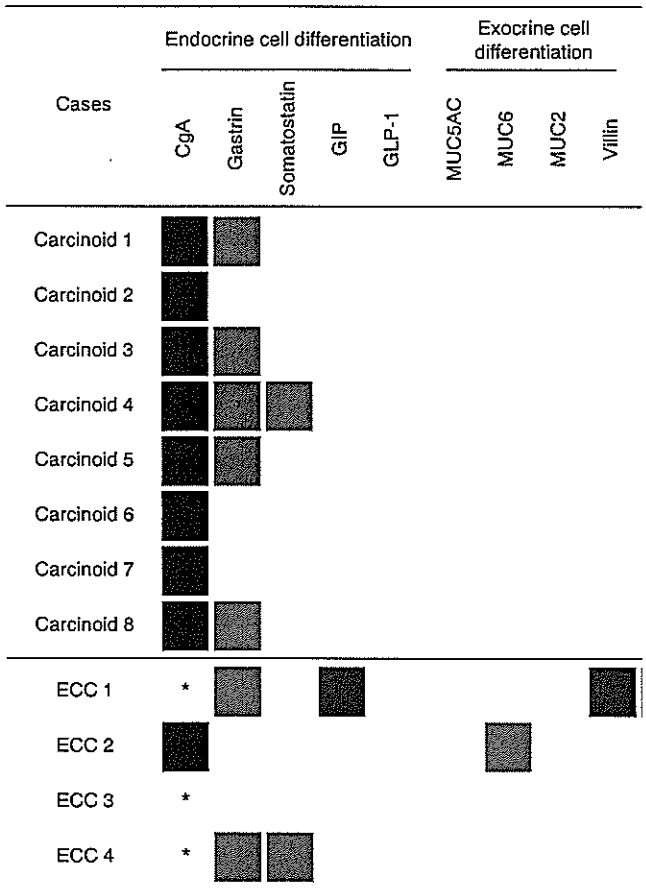


Fig. 5. Schematic illustration of the hypothesis for tumorigenesis pathways in stomach from the viewpoint of exocine and endocrine phenotypic expression. IM is due to abnormal stem cell differentiation in the *Helicobacter pylori* infected stomach. Stomach cancer and adenoma originate from a progenitor cell specializing towards Exo-cell lineages in the gastric mucosa. Most stomach cancers have no phenotypic expression of End-cell. Some of them have End-cell phenotypic expression, preserving the link between End-cell and Exo-cell phenotypes. Almost half of adenomas have no phenotypic expression of End-cell. The remainder of them have End-cell phenotypic expression, again keeping the link between End- and Exo-cell phenotypes. Stomach carcinoid and ECC occur from a progenitor cell specializing towards End-cell lineages in the gastric mucosa, some of the latter may possess Exo-cell lineage. IM, intestinal metaplasia; ECC, endocrine cell carcinoma; Exo-cell, exocrine cell; End-cell, endocrine cell; G, exocrine gastric type; GI, exocrine gastric-and-intestinal-mixed type; I, exocrine intestinal type; e-G, endocrine gastric type; e-GI, endocrine gastric-and-intestinal-mixed type; e-I, endocrine intestinal type.

Table 4. Expression of endocrine and exocrine cell markers in the carcinoids and the endocrine cell carcinomas of the stomach.



CgA positive cases are filled in black. Positive gastric and intestinal markers are filled with red or blue, respecitvely; *: ECC cases 1, 3, and 4 are positive for synaptophysin and/or CD56.

were judged to be CgA-positive. Of the 8 carcinoids, 5 and 1 demonstrated expression of gastrin (Carcinoid cases 1, 3, 4, 5, and 8) and somatostatin (Carcinoid case 4) respectively, while none of the intestinal End-cell markers and no Exo-cell markers were observed. Of the 4 ECCs, expression of gastrin, somatostatin, and GIP was found in 2 (ECC cases 1 and 4), 1 (ECC case 4), and 1 (ECC case 1), respectively. Regarding Exo-cell markers, MUC6 and villin were positive in ECC cases 2 and 1, respectively (Figs. 3, 4, Table 4).

Discussion

We summarized with a schematic illustration of the hypothesis for pathways of carcinoma, adenoma, carcinoid, and ECC in the stomach in Fig. 5. In the human stomach, we have documented clear evidence that the phenotypes of End-cells are strongly associated with those of Exo-cells in intestinal metaplasia (IM) (Otsuka et al., 2005). Especially, expression of both gastric and intestinal End-cell markers is observed in the End-cells of gastric-and-intestinal-mixed Exo-cell phenotype IM (GI-IM) at the cellular level, as well as the glandular level (Otsuka et al., 2005). Intestinalization progresses from GI to solely intestinal phenotype IM (I-IM) in the non-cancerous mucosa of human stomach (Tatematsu et al., 2003). Moreover, IM glands have both End-cells and Exo-cell lineages. Thus, it is evident that IM is due to abnormal stem cell differentiation in the Helicobacter pylori infected stomach (Tatematsu et al., 2003).

In the present study, 85.5% (94/110) of the stomach cancer cases had no CgA expression. Therefore, most of the stomach cancers, which had a tendency to differentiate into not End-cells but solely Exo-cells, were thought to be compatible with our hypothesis that stomach cancer originates from a progenitor cell specializing towards Exo-cell lineages (Tatematsu et al., 2005). However, this cannot explain the other cancer cases having the remarkable expression of CgA or the link between Exo-cell and End-cell phenotypes. This result suggests the necessity to consider the concept of cancer stem cells in stomach cancer. The existence of cancer stem cells in human myeloid leukemias (Bonnet and Dick, 1997), breast cancers (Al-Hajj et al., 2003), and brain tumors (Singh et al., 2004) was demonstrated. The similar concept may be introduced into stomach cancer. The cancer stem cells, which possess self-renewal properties and the ability to produce both Exo-cell and End-cell types like normal stem cells, may appear secondarily in some stomach cancer cases. Should stomach cancers have originated from stem cell itself in stomach glands, most stomach cancers could have both End-cell and Exo-cell phenotypic carcinoma cells. However, actually, most stomach cancers have no tendency to differentiate into End-cells in the present study. Furthermore, Cancer cases 1 and 6 showed several areas harboring both Exo- and End-cell markers, which did not coincide with each other in terms of gastric and intestinal phenotypes. Thus, we consider that most stomach cancers occur from a progenitor cell specializing towards Exo-cell lineages, and the cancer stem cells appear secondarily in some of them.

Our data have demonstrated the evidence that all of the adenoma cases had the intestinal Exo-cell phenotypic expression as GI or I types, and no G type adenoma was detected. It is well-known that human stomach cancers at an early stage, independent of the histological type, mainly consist of G type malignant cells, while their advanced counterparts tend to have more I type malignant cells, suggesting a phenotypic shift from gastric to intestinal phenotypic expression during the course of tumor progression (Yamachika et al., 1997; Yoshikawa et al., 1998; Egashira et al., 1999; Bamba et al., 2001; Tatematsu et al., 2003, 2005). Most stomach cancers develop independently of adenomas (Hattori, 1986; Hirohashi and Sugimura, 1991; Ogasawara et al., 1994; Sakurai et al., 1995; Tahara and Yokozaki, 1996),

which is also supported by our present data of the conflict of Exo-cell marker expression between the stomach adenomas and cancers. On the other hand, the origin of adenomas may be from a progenitor cell specializing towards Exo-cell lineages in IM glands, considering the similarity of Exo-cell phenotypes between adenoma and IM. IM is widely thought to be a precancerous lesion for differentiated type stomach cancers. However, previous studies on phenotypic expression and microsatellite instability (MSI) of individual intestinal metaplastic or stomach cancer cells have pointed to several contradictions in the prevailing paradigm (Hattori, 1986; Tatematsu et al., 1990, 2003, 2005; Kushima and Hattori, 1993; Tamura et al., 1995; Endoh et al., 2000; Kawachi et al., 2003; Tatematsu et al., 2003; Mizoshita et al., 2004b, 2005). Therefore, we consider that the pathway of adenoma and IM occurrence may be different from that of stomach carcinogenesis, essentially. In addition, half of the adenoma cases had expression of CgA, and this percentage was much higher than that of the stomach cancers. The link between Exo-cell and End-cell phenotypes was also observed in adenoma cases, being similar to stomach cancers. There may be the possibility that the tumor stem cells appear more easily in the stomach adenomas than in the stomach cancerous tissues.

Our study showed that all the examined stomach carcinoid tumors had expression of CgA but no Exo-cell phenotype, and 75% (6/8) of carcinoid cases were classified as e-G type. Thus, we consider that stomach carcinoids originate from a progenitor cell specializing towards End-cell lineages in the stomach glandular ducts (Tahara et al., 1975; Bordi et al., 1991; Tatematsu et al., 2005). Regarding the ECCs cases, there was no clear tendency. However, 50% (2/4) of ECC cases had the Exo-cell phenotypic expression. ECCs of the stomach may arise from endocrine precursor cell clones occurring in preceding adenocarcinoma components as the Exo-cell types (Tahara et al., 1975; Nishikura et al., 2000, 2003). The presence of both Exo-cell and End-cell components in the ECCs may be explained by the hypothesis of cancer stem cells, being similar to stomach cancers.

We demonstrated the clear evidence that Cdx2 nuclear staining is strongly associated with intestinal End-cell phenotypic expression in stomach cancer cases. La Rosa et al. (2004) have previously suggested Cdx2 to be a sensitive and specific marker of midgut End-cells and we have presented evidence that Cdx2 is strongly associated with intestinal Exo-cell phenotypic expression of the alimentary tract (Mizoshita et al., 2001). Tsukamoto et al. (2004) also earlier showed Sox2 and Cdx1/2 to be gastric and intestinal specific transcription factors, respectively. In isolated pyloric and intestinal metaplastic glandular ducts, the phenotypes of Exo-cells were found to be strongly associated with these specific transcription factors (Tsukamoto et al., 2004). The phenotypes of malignant cells in human stomach cancers were also found to be strongly associated with these specific transcription factors, independent of the histological type (Mizoshita et al., 2003, 2004a,b; Tsukamoto et al., 2005). Thus, we consider that Cdx2 is important for expression of intestinal End-cell markers even in stomach cancer cells as well as intestinal Exo-cell phenotypic expression.

In conclusion, most stomach cancers might develop from a progenitor cell specializing towards Exo-cell lineages, but some cases possessed both Exo-cell and End-cell markers with maturely differentiated phenotypes. In such cases, Exo-cell and End-cell phenotypes were found to correlate strongly, suggesting the possibility of histogenesis from "cancer stem cells" occurring secondarily.

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Down regulation of gastric and intestinal phenotypic expression in Epstein-Barr virus-associated stomach cancers

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Summary. Aims: We have previously demonstrated the importance of gastric and intestinal phenotypic expression for stomach carcinogenesis. In this study, we focused on Epstein-Barr virus (EBV)-associated stomach cancers, with special attention to Cdx2.

Methods and Results: We evaluated the expression of gastric and intestinal phenotypic markers by immunohistochemistry in 35 EBV-positive [EBV (+)] and 75 EBV-negative [EBV (-)] stomach cancers in Colombia. The lesions were divided phenotypically into gastric (G), gastric-and-intestinal mixed (GI), intestinal (I), and null (N) phenotypes. In the EBV (+) cases, the lesions were divided phenotypically into 9 G (25.7%), 1 GI (2.9%), 3 I (8.6%), and 22 N (62.9%) types. Similarly, the EBV (-) lesions were also classified phenotypically as 15 G (20.0%), 19 GI (25.3%), 24 I (32.0%), and 17 N (22.7%) types. The proportion of N type EBV (+) lesions was higher than for their EBV (-) counterparts ($P<0.0001$). The expression of Cdx2 and MUC2 was also found to be significantly lower in EBV (+) than in EBV (-) stomach cancers ($P=0.0001$; $P<0.0001$). Cdx2 expression in the intestinal metaplastic glands present in non-neoplastic mucosa surrounding EBV (+) lesions was also significantly lower than in EBV (-) tumors ($P=0.016$) despite no evidence of EBV infection.

Conclusions. EBV (+) stomach cancers are characterized by low expression of intestinal phenotype markers, including Cdx2, and only occasional gastric phenotypic expression.

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Key words: Stomach cancer, Epstein-Barr virus, N type, Cdx2, MUC2

Introduction

Epstein-Barr virus (EBV) is a ubiquitous human herpes virus implicated in the etiology of many human malignancies, such as Burkitt's lymphoma (zur Hausen et al., 1970), nasopharyngeal carcinoma (Raab-Traub, 1992), Hodgkin's disease (Weiss et al., 1989), lymphoproliferative disorders in immunodeficiency patients (Hanto et al., 1981), and stomach cancer (Fukayama et al., 1998). EBV-associated stomach cancer account for about 10% of all gastric neoplasms (Shibata and Weiss, 1992; Tokunaga et al., 1993), although Helicobacter pylori (H. pylori) infection is a more important factor for stomach carcinogenesis. There are differences in the proportions of EBV-associated stomach cancers from country to country (Takada, 2000), and the rate in Colombia is significantly higher than in places with heavy gastric cancer burdens, such as Japan, China and Korea (Carrascal et al., 2003). The lesions due to EBV infection resemble nasopharyngeal lymphoepitheliomas and are named lymphoepithelioma-like carcinomas, and specific antigens such as EBV-determined nuclear antigen-1 (EBNA-1) and EBV-encoded small RNA-1 (EBER-1) point to the presence of the virus (Burke et al., 1990; Yanai et al., 1997a,b). Stomach cancers associated with EBV infection were more common in the upper stomach (cardia and fundus), and histologically are most often of undifferentiated type (Yanai et al., 1997). Each EBV-associated stomach cancer appears of monoclonal origin arising from a single EBV-infected cell (Imai et al., 1994). However, there are many obscure points with regard to the

relations between EBV infection and stomach carcinogenesis.

Gastric and intestinal phenotypic expression is important for the histogenesis of stomach cancer (Tatematsu et al., 2003). Several reports have indicated that it is possible to analyze the phenotypic expression of each gastric cancer cell using gastric and intestinal epithelial cell markers (Egashira et al., 1999; Kawachi et al., 2003; Mizoshita et al., 2003; Tsukamoto et al., 2005). Thus, division into gastric (G), gastric-and-intestinal mixed (GI), intestinal (I), and null (N) phenotypes is possible, independent of the histological classification (Tajima et al., 2001; Tatematsu et al., 2003; Inada et al., 2004; Mizoshita et al., 2004a). However, the relation between EBV infection and phenotypic expression has yet to be clarified in detail in stomach cancers associated with the virus. Several authors have demonstrated a correlation between EBV infection and phenotypic marker expression (Lee et al., 2004; Nakamura et al., 2005), but concrete conclusions have yet to be drawn.

In the present study, we therefore evaluated the expression of gastric and intestinal phenotypic markers by immunohistochemistry in 110 stomach cancers in Colombia, along with adjacent non-neoplastic mucosa. The EBV infection status was also evaluated by in situ hybridization in these lesions.

Materials and methods

Samples and tissue collections

The study subjects were stomach carcinoma patients newly diagnosed during the period between September 2000 and June 2003 in the following four reference hospitals in Colombia: Instituto de los Seguros Sociales "Rafael Uribe Uribe", Hospital Universitario del Valle, Hospital San Juan de Dios in Cali, and Instituto Nacional de Cancerologia in Bogota. We examined EBER-1 expression among formalin-fixed paraffin-embedded blocks of 368 cases with gastric carcinomas, and found that 42 cases were positive (Koriyama et al., manuscript submitted). We selected paraffin-embedded blocks of 35 cases with gastric carcinomas, mainly surgically resected tumors, for the present analysis. Seventy-five EBER-1-negative cases were selected matched for gender, age (5-year category), histology [differentiated (well and moderately differentiated) and undifferentiated (poorly differentiated and signet-ring cell) types in majority area], and area (Bogota or Cali) (Table 1). The Institutional Review Board of the Faculty of Health, Universidad del Valle, Cali, Colombia, approved this study and all subjects gave informed consent.

The patient group comprised 84 men and 26 women, aged 59.0±12.5 years (mean ± standard deviation). All specimens were fixed in 10% buffered formalin. Classification was made according to the Japanese Classification of Gastric Carcinomas (Japanese Gastric Cancer Association, 1998) in spite of widely used Lauren's classification (Lauren, 1965), which is inadequate for the studies of histogenesis of stomach cancers and phenotypic expression at the cellular level, because it confuses intestinal phenotypic cancer cells with "diffuse" structure and gastric phenotypes with the "intestinal" (glandular or tubular) morphology. Carcinomas with adjacent non-neoplastic mucosa were serially cut into 5-mm slices in parallel with the lesser curvature and embedded in paraffin, and then sectioned and stained with hematoxylin-eosin (HE) for histological examination.

In situ hybridization of EBER-1

EBER-1 in situ hybridization was performed with a kit according to the manufacturer's instructions (Dako, Glostrup, Denmark). Paraffin sections 4 μm thick were deparaffinized, rehydrated, predigested with proteinase K for 15 min at room temperature and hybridized with a fluorescein-conjugated EBV oligonucleotide probe (EBER PNA Probe/Fluorescein) for 90 min at 55°C. After washing with 0.1M TBS (pH 10) for 25 min at 55°C, hybridization signals were detected by serial incubation with anti-fluorescein isothiocyanate rabbit polyclonal antibody (Anti-FITC/AP), and then with biotinylated Mouse IgG as secondary antibody, followed by the avidin biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA). Finally, immune complexes were visualized by incubation with 0.01% H_2O_2 and 0.05% 3,3'-diaminobenzidine tetrachloride (DAB). Nuclear counterstaining was accomplished with Mayer's hematoxylin. From the results, EBER-positive and EBER-negative lesions were defined as EBV-positive [EBV (+)] and EBV-negative [EBV (-)](Fukayama et al., 2001).

Histological and immunohistochemical examination

Immunohistochemical staining was carried out with monoclonal antibodies against the following antigens:

Table 1. Correlations between clinicopathologic findings and EBV infection in 110 stomach cancers.

Clinicopathologic findings	EBV (+) (n=35)	EBV (-) (n=75)	P-value
Age			
Years (mean±SD)	58.9±13.6	59.1±12.0	P=0.88
Sex			
Male(n=84)	28	56	P=0.63
Female(n=26)	7	19	
Histological classification [a]			
Differentiated type (n=44)	13	31	P=0.835
Undifferentiated type (n=66)	22	44	

SD: standard deviation. [a]: Classified based on structure of elements. "Differentiated type" includes tubular and papillary types, whereas "Undifferentiated type" consists of signet-ring cell and poorly differentiated types.

MUC5AC (CLH2, 1:500; Novocastra Laboratories, Newcastle upon Tyne, UK); MUC6 (CLH5, 1:500; Novocastra Laboratories); MUC2 (Ccp58, 1:500; Novocastra Laboratories); villin (12, 1:20,000; Transduction Laboratories, Lexington, KY, USA); and Cdx2 (Caudal-related homeobox gene 2) (CDX2-88, 1:100; BioGenex, San Ramon, CA, USA).

For gastric and intestinal phenotypic markers, we used normal gastric mucosa and ileum as controls. The precise procedures for immunohistochemical techniques were as previously described (Tatematsu et al., 2003; Mizoshita et al., 2003, 2004b; Tsukamoto et al., 2005). Briefly, 4 μm-thick consecutive sections were deparaffinized and hydrated through a graded series of alcohols. After inhibition of endogenous peroxidase activity by immersion in 3% H_2O_2/methanol solution, antigen retrieval was conducted for detection of binding of the above-mentioned antibodies with 10 mM citrate buffer, pH 6.0, in a microwave oven for 10 min at 98°C. Sections were incubated with primary antibodies, thoroughly washed in phosphate-buffered saline (PBS), then incubated with biotinylated secondary antibody, followed by the avidin biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA). Finally, immune complexes were visualized by incubation with 0.01% H_2O_2 and 0.05% DAB. Nuclear counterstaining was accomplished with Mayer's hematoxylin.

Three independent pathologists (N.H., T.M., and T.T.) judged the histology and immunohistochemical staining for the phenotypic markers and Cdx2. Reactivity for the phenotypic markers and Cdx2 was scored according to the percentage of positively stained tumor cells in the section areas on a 4-point-scale: score 0, <10%; score 1, 10-33%; score 2, 34-66%; score 3, 67-100%. A result was considered positive (+) with a score of 1 or more.

Phenotypic classification of cancers

The phenotypes of stomach cancer cells were determined using two gastric (MUC5AC and MUC6) and two intestinal (villin and MUC2) phenotypic markers. The decisions as to the phenotypes of stomach cancerous areas in which 10% or more of the section area consisted of at least one gastric or intestinal epithelial cell phenotype were classified as gastric (G type) or intestinal (I type) phenotype cancers, respectively. Those which showed both gastric and intestinal phenotypes were classified as gastric and intestinal mixed phenotype (GI type) cancers, while those showing neither gastric nor intestinal phenotype expression were grouped as unclassified (N type) (Tatematsu et al., 2003; Mizoshita et al., 2003; Tsukamoto et al., 2005).

Evaluation of the background gastritis of stomach cancer

Inflammatory response in non-neoplastic surrounding mucosa [of 26 EBV (+) and 57 EBV (-) stomach cancers] were scored according to the Updated Sydney System (Dixon et al., 1996). The degree of gastric mucosal inflammation including mononuclear cell infiltration, neutrophils infiltration, glandular atrophy, and intestinal metaplasia were classified into four grades as follows: 0 = none, 1 = mild, 2 = moderate and 3 = marked.

Expression of gastric and intestinal phenotypic markers and Cdx2 in intestinal metaplastic glands in non-neoplastic surrounding mucosa of EBV (+) and EBV (-) stomach cancers

Intestinal metaplastic glands were observed in non-neoplastic surrounding mucosa of 9 EBV (+) and 26 EBV (-) stomach cancers. The expression of gastric and intestinal phenotypic markers and Cdx2 was also evaluated in intestinal metaplastic glands of both EBV (+) and EBV (-) cases (Mizoshita et al., 2004b, Tatematsu et al., 2005). Reactivity for the phenotypic markers and Cdx2 was scored according to the percentage of positively stained epithelial cells in the intestinal metaplastic glands on a 4-point-scale: score 0, <10%; score 1, 10-33%; score 2, 34-66%; score 3, 67-100%.

Statistical analysis

The data were analyzed by the Fisher's exact test, c2 test or Mann-Whitney U test for differences between EBV (+) and EBV (-) groups. P-values <0.05 were considered as statistically significant.

Results

Relations between EBV infection and expression of gastric and intestinal phenotypic markers, and Cdx2, in stomach cancers

Data for comparisons between EBV (+) and EBV (-) lesions for phenotypic marker and Cdx2 expression in cancerous tissues are summarized in Table 2. The average scores for MUC2 and Cdx2 expression were significantly lower in EBV (+) than in EBV (-) cases ($P<0.0001$ and $P=0.0001$, respectively), independently of whether differentiated ($P<0.005$ and $P<0.02$, respectively) or undifferentiated ($P<0.01$ and $P<0.005$, respectively). Regarding the other phenotypic markers, there were no significant differences between the two groups.

Comparison of phenotypes between EBV (+) and EBV (-) stomach cancers

Data for comparisons between EBV (+) and EBV (-) lesions are summarized in Table 3. In the EBV (+) cases, the lesions were divided phenotypically into 9 G (25.7%), 1 GI (2.9%), 3 I (8.6%), and 22 N (62.9%) types. Similarly, the EBV (-) lesions were also classified phenotypically as 15 G (20.0%), 19 GI (25.3%), 24 I

(32.0%), and 17 N (22.7%) types. There was a significant difference in the proportions of each phenotype between EBV (+) and EBV (-) lesions (P<0.0001).

Comparison of phenotypic markers in differentiated and undifferentiated regions in EBV (+) and EBV (-) stomach cancer cases

To further analyze the expression of gastric and intestinal phenotypic markers, the phenotypes were compared in mixed structure cases containing differentiated and undifferentiated regions (Table 4). Six EBV (+) cases consisted of 2 adenocarcinomas with differentiated predominance and 4 tumors with larger undifferentiated areas. Among them, 3 cases lacked the phenotypic markers in the undifferentiated regions (3/6=50%). For EBV (-) cases, 2 cases were differentiated region dominant and 7 were undifferentiated predominant, none of them lost the

Table 2. Correlations between EBV infection and the expression of the phenotypic markers, and Cdx2 in the stomach cancer cases.

	The average scores of each marker[a]				
	MUC5AC	MUC6	MUC2	villin	Cdx2
EBV (+) (n=35)	0.51±0.16	0.029±0.029	0.057±0.040	0.086±0.063	0.20±0.099
Differentiated (n=13)	0.615±0.266	0.077±0.077	0.077±0.077	0.231±0.166	0.231±0.166
Undifferentiated (n=22)	0.455±0.194	0±0	0.045±0.045	0±0	0.182±0.125
EBV (-) (n=75)	1.013±0.15	0.16±0.063	1.033±0.13	0.23±0.070	1.060±0.13
Differentiated (n=31)	1.000±0.2236	0.226±0.101	0.903±0.169	0.484±0.153	1.355±0.2
Undifferentiated (n=44)	1.023±0.191	0.114±0.081	1.125±0.166	0.045±0.032	0.852±0.156
P-values between EBV (+) and (-) cases[b]	P= 0.098	P= 0.58	P< 0.0001	P= 0.39	P= 0.0001
P-values between EBV (+) and (-) differentiated adenocarcinomas	NS	NS	P< 0.005	NS	P< 0.02
P-values between EBV (+) and (-) undifferentiated adenocarcinomas	NS	NS	P< 0.01	NS	P< 0.005

[a]: Each score is average ± standard error (SE); [b]: Each P-value is analyzed by Mann-Whitney U test. NS, not significant.

Table 3. The phenotype classification in EBV (+) and EBV (-) stomach cancers.

	Phenotypic classification[a]				
	G type	GI type	I type	N type	total
EBV (+) (n=35)	9 (25.7%)	1 (2.9%)	3 (8.6%)	22 (62.9%)	35 (100%)
Differentiated	3	1	2	7	13
Undifferentiated	6	0	1	15	22
EBV (-) (n=75)	15 (20.0%)	19 (25.3%)	24 (32.0%)	17 (22.7%)	75 (100%)
Differentiated	4	10	11	6	31
Undifferentiated	11	9	13	11	44
Total	24 (21.8%)	20 (18.2%)	27 (24.5%)	39 (35.5%)	110 (100%)

[a]: P< 0.0001 among G, GI, I , and N types between EBV (+) and (-) cases (χ^2 test).

Table 4. Correlation between EBV infection and the expression of the phenotypic markers, and Cdx2 in intestinal metaplasia.

	The average scores of each marker[a]				
	MUC5AC	MUC6	MUC2	villin	Cdx2
EBV (+) (n=9)	1.000±0.441	0	2.333±0.441	2.286±0.421	0.556±0.377
EBV (-) (n=26)	1.769±0.256	0.231±0.139	2.808±0.136	2.350±0.244	1.654±0.192
P-value[b]	P=0.15	P=0.61	P=0.50	P=0.80	P=0.016

[a]: Each score is average±standard error (SE); [b]: Each P-value is analyzed by Mann-Whitney U test.