



Original article

Microsatellite instability is linked to loss of hMLH1 expression in advanced gastric cancers: lack of a relationship with the histological type and phenotype

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Abstract

Background. It has been suggested that the prevalence of microsatellite instability (MSI) is high in intramucosal differentiated gastric cancers with gastric foveolar phenotypic expression, and that these tumors are prone to lose their glandular structures and progress to undifferentiated-type lesions. To test this hypothesis, we examined the relationships among human MutL homologue 1 (hMLH1) expression (which is linked to MSI), the phenotype, and the histological type in patients with advanced and intramucosal gastric cancer.

Methods. We analyzed hMLH1 expression by immunohistochemistry in 70 advanced and 30 intramucosal gastric cancers with histological evaluation and assessment of the phenotype, and Cdx2 expression determined by immunohistochemistry. The MSI status was also examined in 20 cases.

Results. Thirteen (18.6%) advanced and 5 (16.7%) intramucosal gastric cancers were judged to be hMLH1-negative. In the advanced cases, no association was observed between the histological type and the phenotype and loss of hMLH1. In the intramucosal cases, MUC5AC expression was observed in all 5 hMLH1-negative differentiated-type cancers. However, no hMLH1-negative lesions were detected in the intramucosal undifferentiated cancers (0/14; $P < 0.05$ vs differentiated types). In the advanced cases, MSI-positivity (MSI+) and loss of hMLH1 expression did correlate ($P < 0.0001$), while no association was observed between MSI+, histological type, and phenotype.

Conclusion. Our data support the hypothesis that, phenotypically, some MSI-positive differentiated gastric cancers of gastric foveolar phenotypic expression may easily change, from gastric to intestinal phenotypic expression, also changing, histologically, from differentiated to undifferentiated type with progression.

Key words Microsatellite instability · hMLH1 · Phenotype · Intramucosal gastric cancers · Advanced gastric cancers

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Introduction

Microsatellite instability (MSI) is characterized by length mutations in tandem oligonucleotide repeats. This type of mutation occurs in a large subset of human tumors [1], and is believed to be caused by altered DNA mismatch repair (MMR) [2]. With regard to MMR genes, it is known that hypermethylation of the hMLH1 MMR gene promoter diminishes the expression of this enzyme in human tumors [3]. Such hypermethylation of hMLH1 is strongly associated with MSI in gastric cancers [4], and loss of hMLH1 expression is, in fact, extremely frequent in MSI-positive gastric cancer [5].

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, independent of the histological classification [6–15]. Endoh et al. [16,17] demonstrated a high prevalence of MSI in differentiated-type gastric tumors showing distinctive features of gastric foveolar epithelium, which are prone to loss of their glandular structure and progression to undifferentiated-type tumors [18], whereas Shibata et al. [19] found no relation between MSI and the phenotype in early differentiated gastric carcinomas. However, to our knowledge, there have been no studies analyzing the links between MSI and the stage and histological types of gastric cancer, in terms of gastric and intestinal phenotypic expression.

In this study, we therefore analyzed hMLH1 expression by immunohistochemistry, in 70 advanced and 30 intramucosal gastric cancers. Histological evaluation was done by hematoxylin and eosin (H&E) staining and assessment of the phenotype was carried out by immunohistochemistry. In addition, the MSI status was examined in 20 cases to allow comparisons of the phenotypic expression along with cancer progression.

Patients, materials, and methods

Samples and tissue collection

We examined 70 primary advanced and 30 intramucosal gastric cancers surgically resected at Aichi Cancer Center Hospital between 1994 and 2000 [12,13]. Of the 70 patients with advanced gastric cancers, 41 were men and 29 were women; they ranged in age from 32 to 84 years (mean, 61.8 ± 10.5 years). The lesions comprised 32 differentiated and 38 undifferentiated-type cancers, the former found in patients ranging in age from 52 to 84 years (mean, 62.7 ± 10.1 years) and the latter in individuals aged from 32 to 83 years (mean, 61.1 ± 10.9 years). Of the 30 patients with intramucosal gastric cancers, 20 were men and 10 were women; they ranged in age from 23 to 74 years (mean, 59.5 ± 11.2 years). The lesions comprised 16 differentiated and 14 undifferentiated-type cancers, the former found in patients ranging in age from 44 to 74 years (mean, 62.8 ± 8.1 years) and the latter in individuals aged from 23 to 72 years (mean, 55.9 ± 13.2 years). Histological classification was made according to the *Japanese classification of gastric carcinomas* [20]. In the patients with advanced disease, the cancers had invaded the subserosa (ss) or the serosa and the peritoneal cavity (se), including the adjacent organs (si). Informed consent for our study was obtained from all participating patients before surgery.

Within 30–40 min after removal of the stomach, the carcinoma tissues and the adjacent non-neoplastic mucosa were carefully sampled, cut into 5-mm squares on ice, and frozen at -80°C for later DNA extraction. Portions of the sampled tissues were fixed in 10% buffered formalin and then processed to paraffin sections for the confirmation of carcinoma and adjacent non-neoplastic mucosa in hematoxylin-eosin stained sections.

Immunohistochemistry

Immunohistochemical staining of the paraffin sections was carried out with monoclonal antibodies against the following antigens: hMLH1 (G168-15; 1:200, PharMingen, San Diego, CA, USA); Cdx2 (CDX2-88; 1:50, BioGenex, San Ramon, CA, USA); MUC5AC (CLH2; 1:500, Novocastra Laboratories, Newcastle upon Tyne, UK); MUC6 (CLH5; 1:500, Novocastra Laboratories); MUC2 (Ccp58; 1:500, Novocastra Laboratories); and villin (12; 1:20000, Transduction Laboratories, Lexington, KY, USA). With regard to gastric phenotypic markers, we used normal gastric mucosa and normal ileum as positive and negative controls, and used these in reverse for the intestinal phenotype. The precise procedures for immunohistochemical techniques were as previously described [8,11–13,21,22]. Briefly, 4- μm -thick consecutive sections were deparaf-

finized and hydrated through a graded series of alcohols. After the inhibition of endogenous peroxidase activity by immersion in 3% H_2O_2 /methanol solution, antigen retrieval was conducted, for the 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 treatment with 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 tetrahydrochloride (DAB). Nuclear counterstaining was accomplished with Mayer's hematoxylin.

Two independent pathologists (T.M. and T.T.) judged the histology and immunohistochemical staining for the phenotypic markers, Cdx2 and hMLH1. The results of staining for each antibody were evaluated in terms of the percentage of positively stained cancer cells, with 10% and above considered positive, as previously described [11–13,22,23].

Classification of cancers

MUC5AC and MUC6 are markers of the gastric-epithelial cell phenotype, whereas MUC2 and villin are typical of the intestinal-epithelial cell phenotype [8,11–13,21]. Gastric cancers in which more than 10% of the section area consisted of either 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 null type (N type) [11–13].

Microsatellite analysis

We screened for MSI using the primer set BAT-26, which amplifies 26 adenine-monomer repeats in the fifth intron of the *hMSH2* gene. It is more than 99.5% sensitive for detecting MSI-positive (MSI+) tumors, including gastric cancers [16,17,23–27]. Genomic DNAs from frozen tissues were isolated with a DNeasy Tissue Kit (Qiagen, Hilden, Germany). Primers specific for each locus were used to amplify the DNA pairs prepared from tumors and adjacent non-neoplastic mucosa by polymerase chain reaction (PCR). PCR was performed with Titanium Taq PCR kits (Clontech Laboratories, Palo Alto, CA, USA) for 1 cycle of 95°C for 2 min, followed by 30 cycles consisting of a denaturation step at 94°C for 45 s, an annealing step at 57°C

for 45s, and an elongation step at 72°C for 60s, in the presence of 0.2mCi of [³²P]-dCTP, according to the manufacturer's instructions. A final elongation at 72°C for 7 min followed. PCR products were electrophoresed in a denaturing 6% polyacrylamide gel, containing 8M urea, at 90 W for 1.5 h [22,23,28,29]. The gel was then dried on filter paper and subjected to imaging [30]. Bands of abnormal molecular weights of tumor DNA, not observed in background mucosa, were designated as MSI + [16,26].

Statistical analysis

The data were analyzed by Fischer's exact test or the χ^2 test for differences between groups. Survival curves after surgery were drawn using the Kaplan-Meier method. Statistical comparison of survival was performed using the log-rank test. *P* values of less than 0.05 were considered statistically significant.

Results

Classification of gastric cancers histologically and phenotypically

The 70 advanced gastric cancers were classified histologically as 32 differentiated and 38 undifferentiated lesions. The former were clearly subclassified as 8G, 7GI, 10I, and 7N types phenotypically, using the gastric and intestinal epithelial phenotypic markers (Table 1). Similarly, the 38 undifferentiated cancers could be clearly classified as 8G, 11GI, 8I, and 11N types, on the basis of their phenotype (Table 1).

In the 30 intramucosal cases, the cancers were classified histologically as 16 differentiated and 14 undifferentiated lesions. The former were clearly subclassified phenotypically as 2G, 8GI, 5I, and 1N types (Table 2). Similarly, the 14 undifferentiated cancers were clearly classified as 8G, 3GI, 1I, and 2N types on the basis of their phenotypic expression (Table 2).

The expression of hMLH1 in advanced gastric cancers

Fifty-seven (81.4%) and 13 (18.6%) advanced gastric cancers were judged to be hMLH1-positive and hMLH1-negative, respectively (Table 1). Histologically, the hMLH1-negative lesions were divided into 9 differentiated and 4 undifferentiated types, with no statistically significant link to differentiation, although a trend was suggested ($P = 0.072$). Phenotypically, the hMLH1-negative cases were classified as 4G, 1GI, 5I, and 3N types (Figs. 1 and 2), again with no significant association with differentiation ($P = 0.32$).

Table 1. Histological and phenotypic classification and hMLH1 expression in 70 advanced gastric carcinomas

Histological classification ^b	Phenotypic classification ^a						Total
	G+GI		I+N		I	N	
	G	GI	GI	I	I	N	
Differentiated	20% (3/15)	25.0% (2/8)	14.3% (1/7)	35.3% (6/17)	40.0% (4/10)	28.6% (2/7)	28.1% (9/32)
Undifferentiated	10.5% (2/19)	25.0% (2/8)	0% (0/11)	10.5% (2/19)	12.5% (1/8)	9.1% (1/11)	10.5% (4/38)
Total	14.7% (5/34)	25.0% (4/16)	5.5% (1/18)	22.2% (8/36)	27.8% (5/18)	16.7% (3/18)	18.6% (13/70)

^aPercentage (number) of hMLH1-negative cases

^bClassified based on structure of carcinomas. "Differentiated" includes tubular and papillary types, while "undifferentiated" consists of signet-ring cell and poorly differentiated types

Table 2. Histological and phenotypic classification and hMLH1 expression in 30 intramucosal gastric carcinomas

Histological classification ^b	Phenotypic classification ^a						Total
	G+GI			I+N			
	G	GI	I	N			
Differentiated	50% (5/10) ^{***}	37.5% (3/8)	0% (0/5)	0% (0/1)	31.3% (5/16)*		
Undifferentiated	0% (0/11)	0% (0/3)	0% (0/1)	0% (0/2)	0% (0/14)		
Total	23.8% (5/21)	27.3% (3/11)	0% (0/9)	0% (0/3)	16.7% (5/30)		

* $P < 0.05$, compared with undifferentiated counterpart; ** $P = 0.09$, compared with I+N counterpart; *** $P < 0.05$, compared with undifferentiated counterpart

^a Percentage (number) of hMLH1-negative cases

^b Classified based on structure of carcinomas. "Differentiated" includes tubular and papillary types, while "undifferentiated" consists of signet-ring cell and poorly differentiated types

The expression of hMLH1 in intramucosal gastric cancers

Twenty-five (83.3%) and 5 (16.7%) intramucosal gastric cancers were judged to be hMLH1-positive and hMLH1-negative, respectively (Table 2). Histologically, all 5 hMLH1-negative lesions were differentiated type, with none of the hMLH1-negative lesions being undifferentiated (** $P < 0.05$ differentiated vs undifferentiated type; Table 2). In the differentiated cases, the expression of MUC5AC, a gastric foveolar phenotypic marker, was judged to be positive in all 5 hMLH1-negative lesions, and positivity for MUC5AC tended to be higher in the G+GI phenotypes than in the I+N counterpart, although the difference did not reach statistical significance (** $P = 0.09$; Table 2). In other words in the G+GI phenotypes, there were significantly more hMLH1-negative cases of the differentiated type than of the undifferentiated type (* $P < 0.05$; Table 2).

MSI status in advanced gastric cancers

To cast light on the mismatch repair system, BAT-26, a microsatellite indicator of mononucleotide repeats, was used as a marker for MSI + (Fig. 3). We analyzed the MSI status in 5 hMLH1-negative and 15 hMLH1-positive cases. Data on MSI status, hMLH1 expression, and Cdx2 expression are shown in Table 3. All 5 MSI + cases demonstrated loss of hMLH1 expression. ($P < 0.0001$). No association was observed between MSI and the histological type or the phenotype. The expression of MUC5AC as a gastric foveolar phenotypic marker was judged to be positive in cases 1 and 4. Less than 10% of the section area exhibited MUC5AC in cases 2, 3, and 5. Cdx2 nuclear staining was not associated with the MSI status.

Postoperative survival analysis of patients with gastric cancer with reference to hMLH1 expression and MSI status

On Kaplan-Meier analysis, the patients with advanced gastric cancers who were hMLH1-negative had a better outcome than those in hMLH1-positive group, but the difference was not significant ($P = 0.14$; Fig. 4). The MSI status was not linked with patient survival (data not shown).

Discussion

Our present data demonstrate that a strong association was observed between MSI + and loss of hMLH1 in advanced gastric cancers, as previously described [4,5], but there was no link between MSI, histological type,

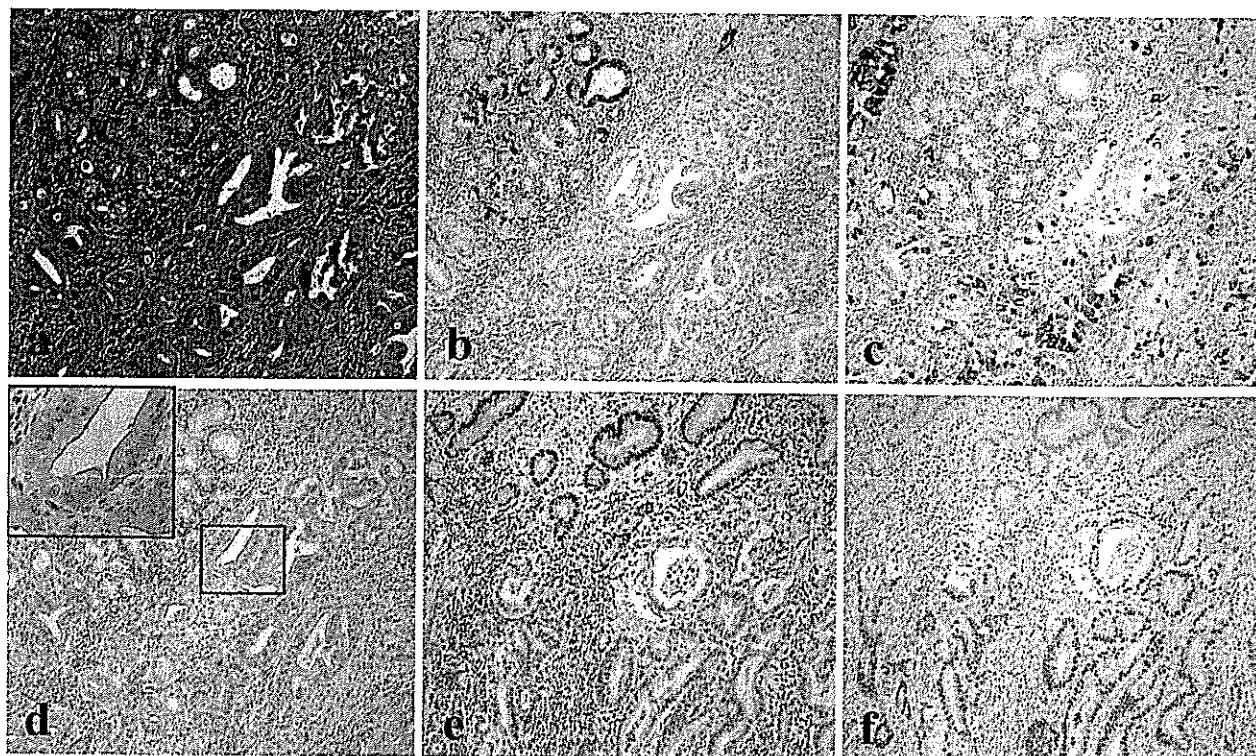


Fig. 1a-f. Moderately differentiated adenocarcinoma of intestinal phenotypic expression (case 3). **a** H&E staining. **b** MUC5AC is present in the cytoplasm of normal gastric mucosa, but not in cancer cells. **c** MUC2 is positive in the cytoplasm of cancer cells, but not in normal gastric mucosa. **d** Villin is positive on the luminal surface of cancer cells, but

not in normal gastric mucosa. *Inset*, higher magnification of the cancer cells. **e** Note, human MutL homologue 1 (hMLH1) nuclear staining is observed in the normal gastric mucosa, but not in carcinoma cells. **f** Cdx2 nuclear staining is apparent in the cancer cells, but not in normal gastric mucosa. (a-f Original $\times 100$; *Inset*, magnification of red square is $\times 560$)

Table 3. MSI status, hMLH1 expression, and Cdx2 expression in gastric cancers from 20 patients

Case number	Age (years)	Sex	Depth ^a	Histological type	Phenotype	Cdx2	hMLH1	MSI
Case 1	52	M	ss	Differentiated	G	-	-	+
Case 2	52	M	ss	Differentiated	I	+	-	+
Case 3	52	F	ss	Differentiated	I	+	-	+
Case 4	59	F	se	Undifferentiated	G	-	-	+
Case 5	72	M	ss	Undifferentiated	N	-	-	+
Case 6	74	M	se	Undifferentiated	G	-	+	-
Case 7	64	F	se	Differentiated	G	-	+	-
Case 8	55	M	ss	Differentiated	GI	+	+	-
Case 9	65	F	ss	Differentiated	I	+	+	-
Case 10	66	M	ss	Differentiated	I	+	+	-
Case 11	53	F	ss	Differentiated	I	+	+	-
Case 12	54	F	ss	Undifferentiated	G	-	+	-
Case 13	67	M	se	Undifferentiated	G	-	+	-
Case 14	67	M	si	Undifferentiated	G	-	+	-
Case 15	70	F	ss	Undifferentiated	GI	+	+	-
Case 16	43	F	si	Undifferentiated	GI	+	+	-
Case 17	56	F	se	Undifferentiated	GI	+	+	-
Case 18	53	M	si	Undifferentiated	I	+	+	-
Case 19	67	M	ss	Undifferentiated	N	-	+	-
Case 20	59	M	si	Undifferentiated	N	-	+	-

G, gastric phenotype; GI, gastric and intestinal phenotype; I, intestinal phenotype; N, null phenotype; MSI, microsatellite instability

^aThe cancers had invaded the subserosa (ss) or the serosa and the peritoneal cavity (se), including the adjacent organs (si)

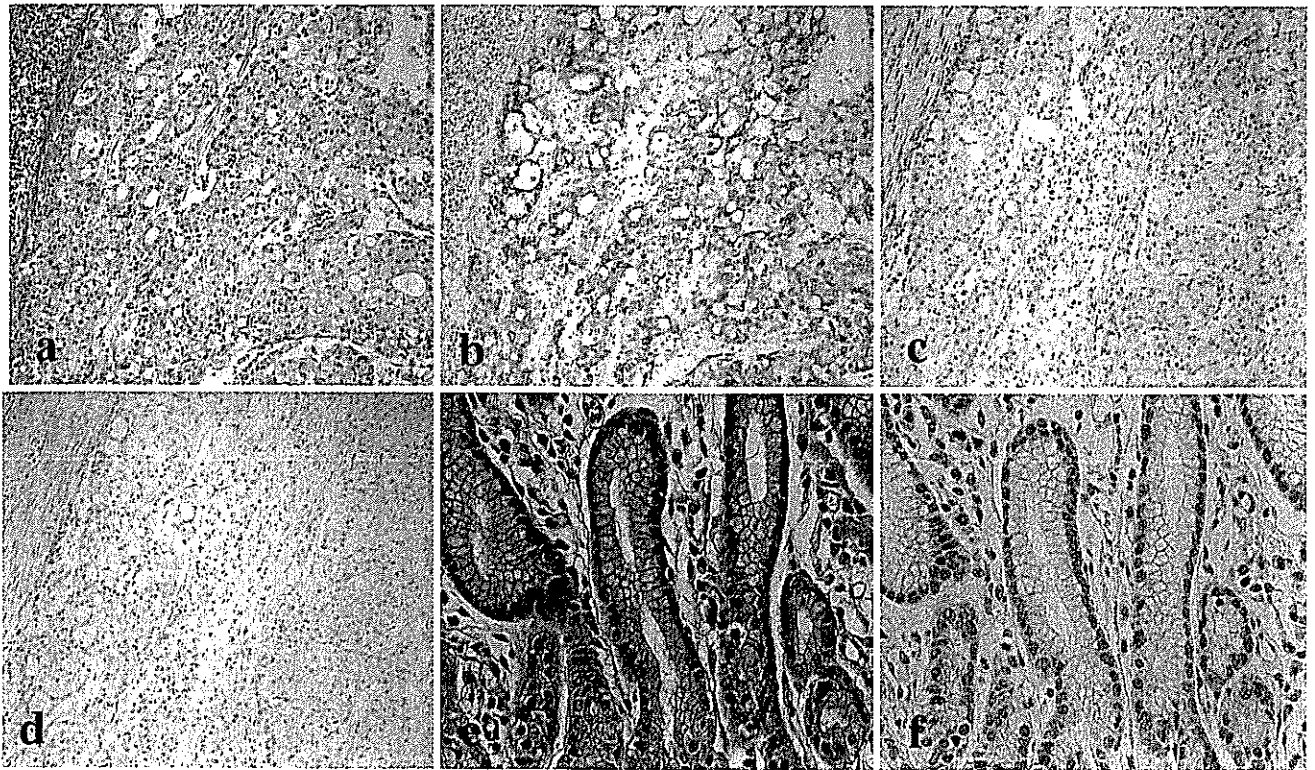


Fig. 2a-f. Poorly differentiated adenocarcinoma featuring gastric foveolar phenotypic expression (case 4). **a** H&E staining of tumor areas. **b** MUC5AC is present in the cytoplasm of cancer cells. **c** No hMLH1 nuclear staining is observed in cancer cells. **d** No Cdx2 nuclear staining is apparent in the cancer cells. **e** H&E staining of surrounding normal gastric mucosa. **f** hMLH1 nuclear staining is observed in the normal gastric mucosa. **a, b, c, and d,** $\times 100$; **e and f** $\times 320$

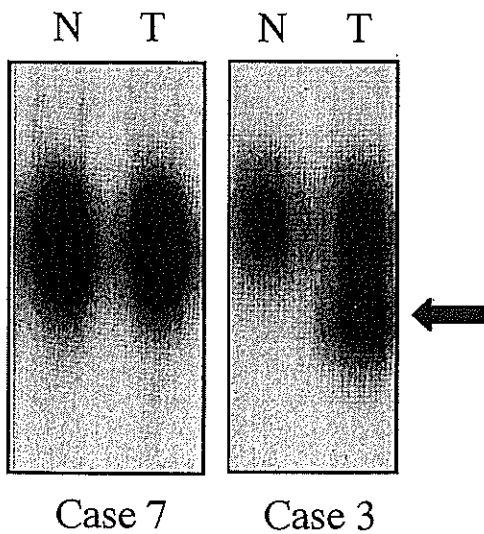


Fig. 3. Microsatellite instability (MSI) analysis, case 3, mobility shift indicating MSI is indicated by the *arrow*: case 7 shows no mobility shift. *N*, normal DNA; *T*, tumor DNA

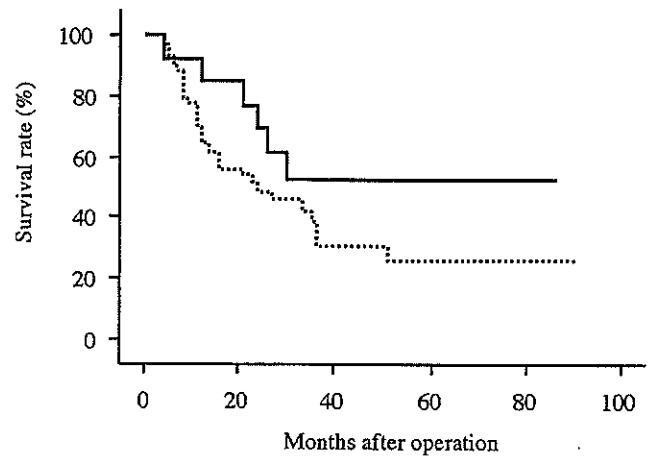


Fig. 4. Postoperative survival curves of patients with advanced gastric cancer with reference to hMLH1 expression. The hMLH1-negative patients (*continuous line*; $n = 13$) had a better outcome than the hMLH1-positive group (*dotted line*; $n = 57$), but the difference was not significant ($P = 0.14$)

and phenotype in the advanced gastric cancers. Shibata et al. [19], similarly, found no relationship between MSI and phenotype in differentiated gastric carcinomas, although Endoh et al. [16,17] reported an association with gastric foveolar phenotypic expression. This different result may be due to differences between the studies in the stages of gastric cancers examined. We separately evaluated intramucosal cancers and advanced cases invading beyond the subserosa in depth, while most of the cases analyzed by Endoh et al. [16,17] were intramucosal lesions. In fact, in their study, of 17 early differentiated gastric cancers of the gastric foveolar type, 12 (70.6%) were judged to be MSI +, while in 6 advanced differentiated gastric cancers of the gastric foveolar type, only 1 case (16.7%) was judged to be MSI +, suggesting the lack of a relationship between MSI + and phenotype in advanced cases [17]. Regarding the I type, they found that, in 11 differentiated gastric cancers, only 1 case with submucosal invasion (9.1%) was MSI + [17]. Our data showed that, in the differentiated advanced gastric cancers, 2 (25%) and 1 (14.3%) of the 8G and 7GI types, respectively, were judged to be hMLH1-negative, while in 10 differentiated advanced gastric cancers of I type, 4 cases (40%) were hMLH1-negative (Tables 1 and 3). In addition, of our 2G and 8GI intramucosal differentiated-type gastric carcinomas, 2 (100%) G and 3 (37.5%) GI cases had loss of hMLH1. All 5 hMLH1-negative cases had MUC5AC expression, suggesting an association between gastric foveolar phenotypic expression and MSI. No hMLH1-negative lesions were observed in the intramucosal differentiated gastric cancers of I type. It is well-known that human gastric cancers at an early stage, independent of the histological type, mainly consist of gastric-phenotype malignant cells, while their advanced counterparts tend to have more intestinal-phenotype malignant cells, suggesting a phenotypic shift from gastric to intestinal phenotypic expression during the course of tumor progression [6,8,10,14,31,32]. Thus, our data support the hypothesis that some MSI-positive differentiated gastric cancer cells featuring gastric foveolar phenotypic expression may easily change, phenotypically from gastric to intestinal phenotypic expression with progression. However, further studies of a larger number of intramucosal and submucosal/advanced differentiated gastric cancer cases may be needed to clarify any association between the phenotype and MSI/loss of hMLH1.

Our data also demonstrated no relationship between MSI + and histological type in the advanced gastric cancers, while a significant association was observed between differentiated type and loss of hMLH1 in the intramucosal gastric cancers. We need to consider changes in the features of gastric cancer cells with pro-

gression, from the viewpoint of the histological type. Regarding the histological type, Endoh et al. [18] have presented evidence that certain differentiated-type gastric carcinomas possess the features of gastric foveolar epithelium, and these are prone to progress to undifferentiated-type carcinomas through the loss of E-cadherin function. Kushima and Hattori [9] have also shown a close relationship between differentiated-type carcinomas with a predominant gastric foveolar epithelial phenotype and progression to undifferentiated type. Of 38 advanced undifferentiated gastric cancers in our study, 4 were judged to be hMLH1-negative (Table 1). Of 5 MSI + cases, 2 were undifferentiated type (Table 3). However, in 14 intramucosal undifferentiated gastric cancers, no hMLH1-negative lesions were observed. We therefore consider the possibility that some MSI-positive differentiated gastric cancers of gastric foveolar phenotypic expression may change from differentiated to undifferentiated type histologically with progression.

Hinoi et al. [33] have reported that loss of Cdx2 expression and MSI are prominent features of large-cell minimally differentiated carcinomas of the colon. However, we could find no relationship between the MSI status and Cdx2 expression in advanced gastric cancers. Similarly, no association was observed between the expression of hMLH1 and Cdx2. Also, we and others have noted a strong association between Cdx2 nuclear staining and intestinal phenotypic expression, independent of the histological type, in gastric cancers [11–13,34]. Thus, we consider that altered DNA mismatch repair may have no influence on the intestinal phenotype, including Cdx2 expression, in gastric cancers.

Our data have shown that, in patients with advanced gastric cancer the hMLH1-negative patients had a better outcome than the hMLH1-positive group, but the difference was not significant ($P = 0.14$; Fig. 4). Gastric cancers of microsatellite mutator phenotype had a good prognosis, shown by multivariate analysis [35]. A univariate analysis showed that gastric cancers of microsatellite mutator phenotype showing altered loci had a better prognosis than those showing two or fewer altered loci [36]. Thus, altered DNA mismatch repair may be linked with the prognosis of gastric cancers.

Intestinal metaplasia (IM) is widely thought to be a precancerous lesion for differentiated-type gastric cancers. However, previous studies of the phenotypic expression of individual intestinal metaplastic or stomach cancer cells have pointed to several contradictions to the prevailing paradigm [6–10,13,37,38]. With regard to MSI analysis, Tamura et al. [39] have shown that the majority of differentiated adenocarcinomas of the stomach may develop through a de-novo pathway, from the viewpoint of microsatellite alterations. Endoh et al. [16]

also made it clear that the genetic backgrounds of differentiated-type tumors were quite different among different cellular phenotypes. Frequent MSI and infrequent *p53* mutation are observed in tumors of gastric foveolar phenotypic expression, and conversely, infrequent MSI and considerable *p53* mutations are detected in tumors with a distinct intestinal cellular phenotype [16]. MSI occurs not only in gastric IM of patients with gastric carcinoma, but also in IM of cancer-free individuals [28]. However, Jin et al. [26] have reported that instability of BAT-26, a sensitive marker for the high-frequency MSI phenotype, was not found in intestinal metaplastic mucosa adjacent to any of a series of gastric carcinomas. Thus, further studies are clearly warranted to clarify whether IM is, indeed, a precancerous lesion.

In conclusion, our data support the hypothesis that, phenotypically, some MSI-positive differentiated gastric cancers of gastric foveolar phenotypic expression may, easily change from gastric to intestinal phenotypic expression, also changing, histologically from differentiated to undifferentiated type with progression.

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Original Article

Colonic and small-intestinal phenotypes in gastric cancers: Relationships with clinicopathological findings

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The clinicopathological significance of colonic and small-intestinal phenotypes has hitherto remained unclear in gastric cancers. The purpose of the present study was therefore to examine 86 gastric carcinomas histologically and phenotypically using several phenotypic markers, including colon-specific carbonic anhydrase 1 (CA1) and sucrase as small-intestine specific marker. Of 86 gastric cancers, sucrase and CA1 expression was observed in 12 (14.0%) and only in two cases (2.3%), respectively, associated with other intestinal markers such as villin and mucin core protein (MUC)2. In the sucrase cases, expression appeared independent of the stage. However, CA1 expression was observed only in two advanced cases. No association was observed between colonic and small-intestinal phenotypes, and lymph node metastasis and postoperative survival in the advanced gastric cancer cases with intestinal phenotypic expression. *Cdx2* appeared to be linked to upregulation of both CA1 and sucrase. In conclusion, the data suggest that colonic phenotype occurs rarely in gastric carcinogenesis. Colonic and small-intestinal phenotypes appear with expression of several intestinal phenotypic markers under the control of *Cdx2* and presumably other related transcription factors.

Key words: carbonic anhydrase 1, colonic phenotype, gastric cancers, small intestinal phenotype, sucrase

Human gastric cancers can be divided into two major groups according to histology: the intestinal and diffuse types according to Lauren,¹ which, respectively, correspond closely to the differentiated and undifferentiated types of Nakamura *et al.*² and Sugano *et al.*³ Although these classifications have been widely used, they are inadequate for studies of histogenesis of gastric carcinomas and phenotype expression at

the cellular level, because of the confusion of intestinal phenotypic cancer cells with diffuse structures and the presence of a gastric phenotype with the Lauren intestinal type.⁴ 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.^{5–14} Thus division into gastric (G), gastric-and-intestinal mixed (GI), intestinal (I), and null (N) phenotypes is possible, independent of the histological classification.^{4,15,16}

With regard to the distribution of intestinal epithelial cell markers such as villin and mucin core protein (MUC)2 in the normal human alimentary tract, most of them are present in both the colon and small intestine. However, carbonic anhydrase 1 (CA1) is found in epithelial cells located in the upper part of colonic mucosa, but not in the small intestine.^{17,18} Conversely, sucrase is detectable on the luminal surfaces of mature small intestinal absorptive cells, and rarely in colon.¹⁴ Therefore, regarding intestinal phenotypic expression, CA1 represents a colonic phenotype, while sucrase is representative of a small-intestinal phenotype. We and others have previously demonstrated a correlation between prognosis and phenotypic markers in gastric cancers.^{8,12,19–23} However, the separate clinicopathological significance of colonic and small-intestinal phenotypes has yet to be clarified in detail for gastric cancers. Indeed, to our knowledge, there have been no reports of expression of CA1 in gastric carcinomas.

The *caudal-related homeobox gene (Cdx) 2* is believed to be important for the maintenance of intestinal epithelial cells.^{24,25} Expression of *Cdx2* mRNA increases gradually from the duodenum to the proximal colon and decreases from the ascending colon to the rectum in the normal human alimentary tract.^{26,27} *Cdx2* plays an important role in the transcription of *CA1* and *sucrase* genes,^{28–30} and we and others have previously shown a strong association between this and intestinal phenotypic expression in gastric cancers.^{8,15,16,31} *Cdx2* nuclear staining has been observed immunohis-

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tochemically in gastric cancer cells having sucrase expression.¹⁵ However, whether there is a link between Cdx2 and CA1 expression has hitherto remained unclear.

In the present study we therefore examined a series of gastric cancers with reference to the expression of CA1 and sucrase, also performing a histological evaluation and determining expression of other phenotypic markers and Cdx2 by immunohistochemistry. We also evaluated the relationship between colonic and small-intestinal phenotypes and clinicopathological findings, including tumor stage, lymph node metastasis, and postoperative survival.

MATERIALS AND METHODS

Samples and tissue collection

We examined 86 primary gastric cancers surgically resected at Aichi Cancer Center Hospital between 1994 and 2001. Histological classification was made according to the Japanese Classification of Gastric Carcinomas.³² Out of the total, 22, 25, 31, and eight were T1, T2, T3, and T4 according to TNM classification. The lesions were histologically divided into 43 differentiated and 43 undifferentiated types. The group consisted of 47 men and 39 women, mean age 61.6 ± 11.0 years (mean \pm SD). All specimens were fixed in 10% buffered formalin. 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 and eosin for histological examination.

Immunohistochemistry

Expression of CA1, sucrase, villin, MUC2, Cdx2, MUC5AC, and MUC6 in carcinoma cells was examined by immunohistochemistry (Table 1). The antisucrase antibody was kindly provided by Dr Kazuyuki Hirano, Laboratory of Pharmaceutics, Gifu Pharmaceutical University, Gifu, Japan.¹⁴ MUC5AC and MUC6 are markers of the gastric epithelial cell phenotype, whereas MUC2 and villin are typical of the intestinal epithelial cell phenotype.^{4,8,15,16,27} Because villin and MUC2

are observed in both normal colon and small intestine, we defined these as general markers of the intestinal phenotype, CA1 and sucrase being markers for colonic and small-intestinal phenotypes. The precise procedures for immunohistochemical techniques are as previously described.^{4,8,14-16,27} Briefly, 4 μ m-thick consecutive sections were deparaffinized and hydrated through a graded series of alcohols. After inhibition of endogenous peroxidase activity by immersion in 3% H₂O₂/methanol solution, antigen retrieval was conducted for detection of binding of the aforementioned antibodies with 10 mmol/L 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 solution (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% H₂O₂ and 0.05% 3,3'-diaminobenzidine tetrachloride (DAB). Nuclear counterstaining was accomplished with Mayer's hematoxylin.

The phenotypes of gastric cancer cells were determined using two gastric (MUC5AC and MUC6) and two intestinal (villin and MUC2) phenotypic markers.^{8,15,16} The decisions as to the phenotypes of gastric cancerous areas were made as previously described.^{8,15,16} The lesions were phenotypically divided into four types: G, GI, I, and N phenotypes.

Immunohistochemical staining was evaluated by two independent pathologists (TM and TT). The results for CA1, sucrase, Cdx2, and other phenotypic markers were evaluated with reference to the percentage of positively stained cancer cells. A result was considered positive if $\geq 10\%$ of the cells were stained.

Statistical analysis

The data were analyzed using Fischer's exact test or χ^2 test for differences between the groups. Survival curves after surgery were drawn using the Kaplan-Meier method. Statistical comparison of survival was performed using the log-rank test.⁸ $P < 0.05$ was considered statistically significant.

Table 1 Antibodies for immunohistochemistry

Antigen	Clone/Type	Dilution	Source
Carbonic anhydrase 1 Sucrase	Polyclonal	1/1000	Chemicon International, Temecula, CA, USA
	Polyclonal	1/3000	Dr Kazuyuki Hirano, Laboratory of Pharmaceutics, Gifu Pharmaceutical University, Gifu, Japan
MUC2	Ccp58	1/500	Novocastra Laboratories, Newcastle upon Tyne, UK
Villin	12	1/20000	Transduction Laboratories, Lexington, KY, USA
Cdx2	CDX2-88	1/50	BioGenex, San Ramon, CA, USA
MUC5AC	CLH2	1/500	Novocastra Laboratories, Newcastle upon Tyne, UK
MUC6	CLH5	1/500	Novocastra Laboratories, Newcastle upon Tyne, UK

RESULTS

Expression of MUC5AC, MUC6, MUC2, villin and Cdx2 in gastric cancers

Expression of MUC5AC, MUC6, MUC2, and villin was demonstrated in 45 (52.3%), 24 (27.9%), 39 (45.3%), and 41 (47.7%) of 86 gastric cancers, respectively. Taking into account the combinations of expression of these four markers, the 86 gastric cancers were divided phenotypically into 18 G, 31 GI, 27 I, and 10 N types, independent of the histological classification (Table 2). Fifty-eight cases (66.3%) were thus judged to be positive for intestinal expression.

Totals of 63 (73.3%) and 23 (26.7%) gastric cancers were judged to be Cdx2 positive and Cdx2 negative, respectively (Table 2). Cdx2 nuclear staining was clearly observed in gastric cancer cells positive for intestinal phenotypic markers, without any association with histological classification (Figs 1,2). The cancer areas with Cdx2-positive nuclear staining were in perfect accord with those demonstrating intestinal phenotypic expression, regardless of the gastric phenotypic expression ($P < 0.0001$; Table 2). However, four G- and one N-type intramucosal cancers were judged Cdx2 positive, with nuclear staining also detected outside areas with intestinal phenotypic expression.

Expression of CA1 and sucrase in gastric cancer cells

In the normal human alimentary tract, expression of CA1 was clearly detected in epithelial cells located in the upper part

of colonic mucosa, but not in the small intestine. Sucrase, on the other hand, was present on the luminal surfaces of mature small intestinal absorptive cells, but not in the colon (Fig. 3).

Totals of two (2.3%) and 84 (97.7%) gastric cancers were judged to be CA1 positive and CA1 negative, respectively (Table 3). Most gastric cancer cells with cytoplasmic CA1 staining were detected in cancerous areas with expression of the intestinal phenotypic markers villin and MUC2 (Fig. 1). Regarding the intramural localization of tumor cells with CA1 expression, the expression of CA1 was observed in the whole tumor tissue of each case. Of the two CA1-positive cancers, one was classified histologically as differentiated, and the other as undifferentiated. No MUC5AC expression was observed in the cancerous areas harboring CA1 protein (Fig. 1).

With regard to sucrase, totals of 12 (14.0%) and 74 (86.0%) gastric cancers were judged to be sucrase positive and sucrase negative, respectively (Table 3). Most gastric cancer cells with sucrase expression on the luminal surface were also detected in cancerous areas with expression of villin and MUC2 (Figs 1,2). Regarding the intramural localization of tumor cells with sucrase expression, the expression of sucrase was also observed in the whole tumor tissue of each case. No statistical significance was observed for the relationship between sucrase expression and the histological classification of gastric cancers ($P = 0.53$). In one case, gastric cancer cells featuring expression of both CA1 and sucrase were observed in a cancerous area lacking gastric phenotypic expression (Fig. 1; Table 3).

Table 2 Correlations between clinicopathological findings and the phenotype classification in 86 gastric cancers

Clinicopathological findings	G type (n = 18)	GI type (n = 31)	I type (n = 27)	N type (n = 10)	P
Age (years) (mean \pm SD)	59.4 \pm 13.9	59.1 \pm 10.8	64.3 \pm 9.2	65.6 \pm 8.5	NS
Gender					
Male (n = 47)	10	16	15	6	NS
Female (n = 39)	8	15	12	4	
Histological classification					
Differentiated (n = 43)	10	13	17	3	NS
Undifferentiated (n = 43)	8	18	10	7	
Depth of invasion					
T1 (n = 22)	8	9	3	2	NS
T2 (n = 25)	4	7	12	2	
T3 (n = 31)	5	13	9	4	
T4 (n = 8)	1	2	3	2	
Lymph node metastasis					
Positive (n = 54)	10	19	19	6	NS
Negative (n = 32)	8	12	8	4	
Cdx2					
Positive (n = 63)	4	31	27	1	<0.0001
Negative (n = 23)	14	0	0	9	

G type, gastric phenotype; GI, gastric and intestinal mixed phenotype; I type, intestinal phenotype; N type, null phenotype; NS, not significant.

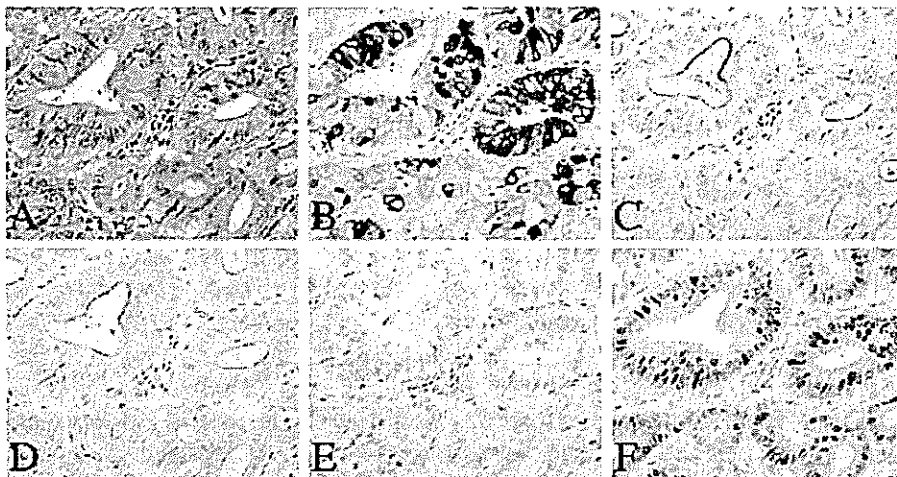


Figure 1 Advanced moderately differentiated tubular adenocarcinoma with both colonic and small-intestinal phenotypes (fourth case in Table 3). (A) HE staining; (B) expression of carbonic anhydrase 1 (CA1) is apparent in the cytoplasm of cancer cells; (C) expression of sucrase is evident on the luminal surfaces of cancer cells; (D) villin is positive on the luminal surfaces of cancer cells; (E) no MUC5AC expression is apparent in the cytoplasm of cancer cells; (F) Cdx2 nuclear staining is detectable in the tumor cells. Original magnification, $\times 320$.

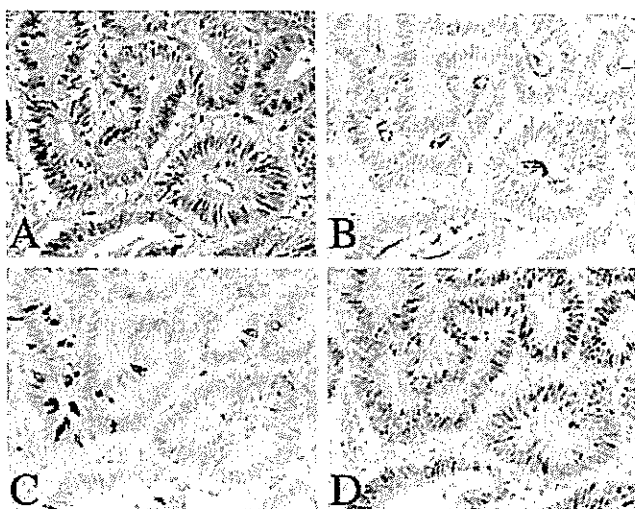


Figure 2 An intramucosal well differentiated tubular adenocarcinoma of small-intestinal phenotype (second case in Table 3). (A) HE staining; (B) expression of sucrase is evident on the luminal surfaces of cancer cells; (C) MUC2 is partially detectable in the cytoplasm of cancer cells; (D) Cdx2 nuclear staining is detectable in the tumor cells. Original magnification, $\times 320$.

Relationship between CA1, sucrase and Cdx2 expression in gastric cancer cells

The gastric cancer cells with cytoplasmic CA1 expression had Cdx2 nuclear staining in both cases (Fig. 1). The gastric cancer cells with sucrase expression in the luminal surface also had Cdx2 nuclear staining in all 12 cases, without exception (Figs 1,2).

Relationship between CA1, sucrase and clinicopathological findings in gastric cancers

Data for the relationship between CA1, sucrase and tumor stages are summarized in Table 4. The expression of sucrase

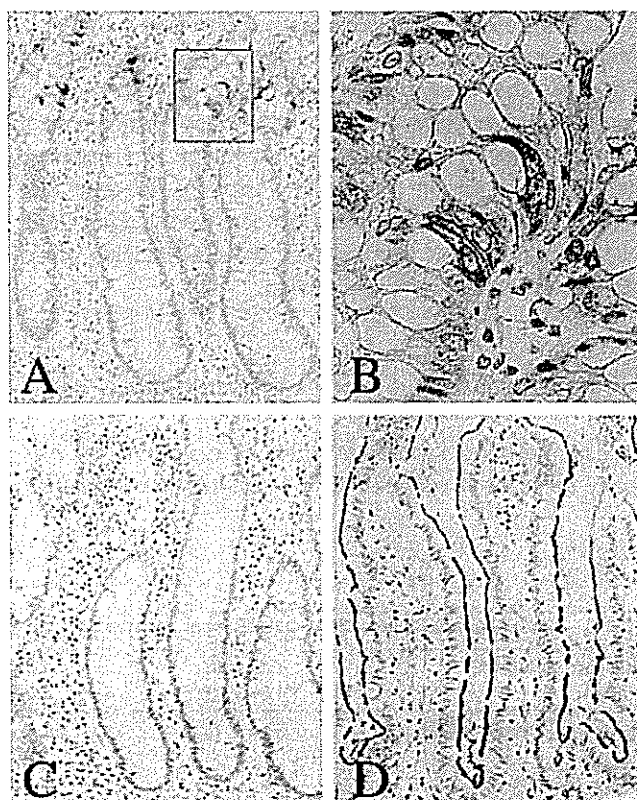


Figure 3 Expression of carbonic anhydrase 1 (CA1) and sucrase in the normal human alimentary tract. (A) Cytoplasmic staining of CA1 is detected in epithelial cells located in the upper part of colonic mucosa; (B) higher magnification of the red square in (A); (C) note the lack of sucrase expression in the luminal surfaces of epithelial cells in the colon; (D) sucrase is apparent on the luminal surfaces of absorptive cells in the small intestine. Original magnification, A,C,D, $\times 160$; B, $\times 640$.

Table 3 Clinicopathological findings in 13 cases of gastric cancers with CA1 or sucrase expression

Age (years)	Sex	Depth	Histological type	LN metastasis	Phenotype	CA1	Sucrase	Cdx2	MUC5AC
64	M	T1	Differentiated	-	GI	-	+	+	+
67	M	T1	Differentiated	-	I	-	+	+	-
68	M	T1	Differentiated	-	I	-	+	+	-
52	F	T2	Differentiated	-	I	+	+	+	-
65	F	T2	Differentiated	+	I	-	+	+	-
83	F	T2	Undifferentiated	+	I	-	+	+	-
62	F	T2	Undifferentiated	+	I	-	+	+	-
58	M	T3	Differentiated	+	GI	-	+	+	+
84	F	T3	Differentiated	-	GI	-	+	+	+
50	F	T3	Undifferentiated	+	I	-	+	+	-
53	M	T4	Undifferentiated	+	I	+	-	+	-
65	M	T4	Undifferentiated	+	I	-	+	+	-
53	F	T4	Undifferentiated	+	GI	-	+	+	+

CA1, carbonic anhydrase 1; F, female; GI, gastric and intestinal mixed phenotype; I, intestinal phenotype; LN, lymph node; M, male.

Table 4 Expression of CA1 and sucrase in 86 gastric cancers

CA1	Sucrase	Stage				Total
		T1	T2	T3	T4	
+	+	0	1	0	0	1
+	-	0	0	0	1	1
-	+	3	3	3	2	11
-	-	19	21	28	5	73
Total		22	25	31	8	86

CA1, carbonic anhydrase 1.

Table 5 Relationship between CA1, sucrase, and LN metastasis in 46 advanced gastric cancers with intestinal phenotypic expression

CA1	Sucrase	LN metastasis		Total
		+	-	
+	+	0	1	1
+	-	1	0	1
-	+	7	1	8
-	-	30	6	36
Total		38	8	46

CA1, carbonic anhydrase 1; LN, lymph node.

was observed in three early gastric cancers as well as in later stages, while CA1 expression was detected only in two advanced lesions (Fig. 2).

Fifty-four (62.8%) gastric cancers were judged to feature lymph node metastasis, while 32 (37.2%) did not (Table 2). Of the 31 GI- and 27 I-type gastric cancers, 12 early gastric cancers (nine GI and three I types) had no lymph node metastasis. The relationship between CA1, sucrase, and lymph node metastasis in 46 advanced gastric cancers with the intestinal phenotypic expression (22 GI and 24 I types) is summarized in Table 5. No significant link was apparent for either CA1 or sucrase expression.

Similarly, there was no significant relationship between CA1 (data not shown) or sucrase and patient survival on Kaplan-Meier analysis of the 46 advanced cases with intestinal phenotypic expression (22 GI and 24 I types; Fig. 4).

DISCUSSION

Our data provide clear evidence, for the first time (to our knowledge), that only a small percentage of gastric cancers are CA1 positive with immunohistochemical staining. Regarding the expression of sucrase, approximately one-tenth of the examined lesions were positive. Gastric cancer cells with cytoplasmic CA1 were detected in a small propor-

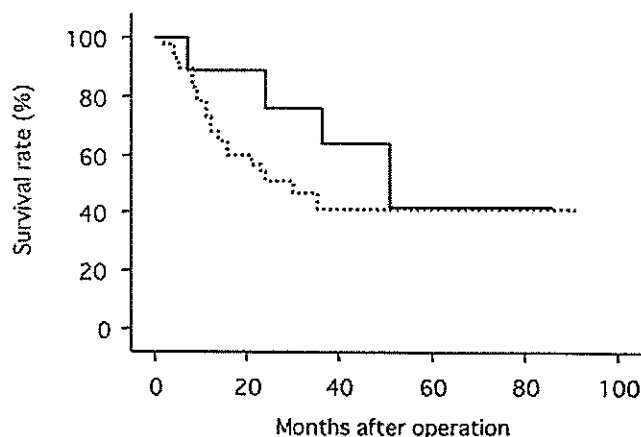


Figure 4 Postoperative survival curves of gastric cancer patients with reference to sucrase expression. The difference between (-) sucrase positivity (n = 9) and (···) sucrase negativity (n = 37) was not significant for 46 advanced gastric cancer cases (22 GI and 24 I types; P = 0.32). GI type, gastric and intestinal mixed phenotype; I type, intestinal phenotype.

tion of cancerous areas having an intestinal phenotype expression. Similarly, gastric tumor cells were positive for sucrase on their luminal surfaces. Sucrase expression was observed in tumors of all stages, while CA1 expression was detected not in the early gastric cancers but in advanced

cases (Table 4). We and others have previously demonstrated that human gastric cancers at an early stage, independent of the histological type, mainly consist of gastric phenotype malignant cells, while their advanced counterparts tend to have a more intestinal phenotype with progression.^{5,11,13,14} Further studies of a larger number of early and advanced gastric cancer cases may be needed to clarify any association between colonic/small-intestinal phenotypes and the alternation from gastric to intestinal phenotypic expression with progression.

We and others have previously demonstrated that the advanced gastric cancers with intestinal phenotypic expression have a relatively good prognosis compared with those without intestinal phenotypic expression,^{8,12} and Cdx2 is a useful prognostic marker.⁹ In the present study we analyzed the relationship between sucrase, CA1, and postoperative survival in 46 advanced gastric cancers with intestinal phenotypic expression (22 GI and 24 I types). However, there was no significant link between patient survival and the small-intestinal or colonic phenotypes. We also could not demonstrate any relationship between lymph node metastasis and sucrase and CA1 expression in the 46 advanced cases, in line with earlier findings for Cdx2 expression and metastasis.¹⁶ Further studies of a larger number of gastric cancer cases may be needed to clarify any significance for these phenotypes with regard to clinicopathological parameters.

We here demonstrated no expression of MUC5AC as a gastric foveolar phenotypic marker in cancerous areas positive for CA1 protein (Fig. 1). In a preliminary study, CA1 expression was found to correlate inversely with MUC5AC expression in intestinal metaplastic (IM) glands of pyloric mucosa, as assessed by immunohistochemistry and relative quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR; Tanaka *et al.*, unpublished data, 2005). With regard to MUC5AC expression, Sox2 is localized in the nuclei of positive cells in pyloric and GI-type IM (GI-IM) glands, but not in solely I-type IM (I-IM).²⁷ Similarly, Sox2 is strongly associated with MUC5AC expression in gastric cancer cells.^{33,34} Sox2, as a gastric-specific transcription factor, and Cdx1/2, as intestinal-specific transcription factors, play important roles in determining gastric and intestinal phenotypic expression in IM and gastric cancers.^{15,26,27,34} Indeed, the balance of gastric- and intestinal-specific transcription factors may be the determinant of phenotypes of IM glands and gastric cancer cells, as well as in the normal alimentary tract.^{27,34}

In the latter, the areas with expression of CA1, sucrase, and other intestinal phenotypic markers are all under the control of Cdx2, believed to be important for the maintenance of intestinal epithelial cells.^{24,25} Yamamoto *et al.* have shown that Cdx2 interacts with the *MUC2* promoter and activates *MUC2* transcription, thus playing an important role in the differentiation of goblet cells.³⁵ We have previously demon-

strated concurrent expression of several intestinal phenotypic markers in the same gastric cancerous areas.¹⁶ Gastric cancer cells with cytoplasmic CA1 were detected in a small proportion of cancerous areas having an intestinal phenotype expression. Similarly, gastric tumor cells were positive for sucrase on their luminal surfaces. We and others also have previously shown a strong association between Cdx2 and intestinal phenotypic expression in gastric cancers.^{8,15,16,31} Therefore, we consider that intestinalization of gastric cancer cells may encompass several phenotypic markers regulated by Cdx2.

In the present study, Cdx2 nuclear staining was observed in 63 (73.3%) of 86 gastric cancers. In series of colorectal cancers, Cdx2 expression was detected in 80–90% of tumor cases.^{36,37} Several reports have pointed to a tumor suppressor potential of Cdx2 in human colorectal tumorigenesis,^{25,38–40} and we and others have previously shown that Cdx2 might suppress the invasion of gastric cancer cells.^{8,41} With regard to CA1 expression, 16 (16.7%) of 96 colorectal cancer cases showed CA1-positive cancer cells,⁴² while only two (2.3%) of 86 of the present gastric cancer cases were judged to be CA1 positive. However, gastric cancer cells with CA1 expression exhibited Cdx2 nuclear staining, in line with the finding by Drummond *et al.* that Cdx2 plays an important role in the intestine-specific expression of CA1.²⁸ *In vitro* in HeLa cells, Cdx2 exerts a positive regulatory effect by binding to a motif 87 bp upstream of the *CA1* TATA box.²⁹ With regard to the expression of sucrase, this has been noted in approximately 10–50% of colorectal carcinomas,^{43–45} while 12 (14.0%) of 86 of the present gastric cancer cases were judged to be sucrase-positive. The immunoreactivity of this protein was strongly associated with Cdx2 expression even in gastric cancers, as previously described.¹⁵ Suh *et al.* have shown that intestine-specific transcription of sucrase–isomaltase, a gene that is expressed exclusively in differentiated enterocytes, is dependent on binding of Cdx2 to an evolutionarily conserved promoter element in the sucrase–isomaltase gene.³⁰ To our knowledge there have been no reports of expression of Cdx2, CA1 and sucrase in small intestinal carcinomas. We consider that ectopic expression of Cdx2 is an important factor for the expression of both CA1 and sucrase in gastric cancerous mucosa.

In conclusion, our data suggest that the colonic phenotype occurs rarely in gastric carcinogenesis. Colonic and small intestinal phenotypes appear with expression of several intestinal phenotypic markers under the control of Cdx2 and presumably other related transcription factors.

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Original Article

Coexistence of gastric- and intestinal-type endocrine cells in gastric and intestinal mixed intestinal metaplasia of the human stomach

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Intestinal metaplasia (IM) in the human stomach has previously been classified into a gastric and intestinal mixed (GI-IM) and a solely intestinal phenotype (I-IM). The phenotypes of mucous and endocrine cells were evaluated in 3034 glandular ducts associated with chronic gastritis. In the pyloric region, the relative expression of gastric endocrine cell markers, such as gastrin and somatostatin, decreased gradually from glandular ducts with only gastric mucous cell phenotype (G type) to GI-IM toward I-IM, while that of the intestinal endocrine cell markers, glicentin, gastric inhibitory polypeptide (GIP), and glucagon-like peptide-1 (GLP-1) was inversely correlated. In the fundic region, gastrin-positive cells emerged in the pseudo-pyloric and GI-IM glands, whereas I-IM glands did not possess any gastrin-positive cells, suggesting the presence of a distinct pathway of intestinalization. Double staining revealed coexistence of gastrin- and GLP-1-positive cells in the same gland and occasionally in the same cell in GI-IM glands. These results suggest that the phenotypes of endocrine cells are in line with those for mucous counterparts and support the concept that all of the different types of mucous and endocrine cells in normal and IM glands might be derived from a single progenitor cell in each gland.

Key words: endocrine cell, gastric and intestinal mixed intestinal metaplasia, pseudopyloric metaplasia, stomach

Intestinal metaplasia (IM) is common in the human stomach. It occurs as a result of *Helicobacter pylori* (*H. pylori*) infection and consequent chronic gastritis.^{1,2} The presence of IM has been thought to increase the risk of gastric cancer.^{3–7} But

many questions remain regarding its pathogenesis to neoplasias. Several classifications of IM have been suggested by pathologists. Kawachi *et al.* first proposed division into complete and incomplete types on the basis of morphology.⁸ Jass and Filipe described three grades of IM with classical mucin staining.⁹ Although these classifications are generally accepted, they are based upon only intestinal properties and do not consider gastric properties that are still preserved in association.¹⁰ We have therefore proposed a new classification of IM on the basis of cellular differentiation status using both gastric and intestinal mucous cell markers with division into gastric and intestinal mixed phenotype IM (GI-IM) and solely intestinal phenotype IM (I-IM).¹¹ Downregulation of *Sox2*, as well as ectopic expression of *Cdx* genes, are important factors for the development of IM.¹² Experimentally, the alternation of phenotypes of IM can be clearly observed on sequential observation in X-ray-treated rats¹³ and *H. pylori*-infected gerbils.¹⁴ However, in the human there are many unsolved problems regarding the differences between GI-IM and I-IM.

Regarding the cellular differentiation of endocrine cells in the gastrointestinal tract, gastrin and somatostatin are predominantly detectable in the pyloric glands of the stomach, while a product of glicentin, gastric inhibitory polypeptide (GIP), and glucagon-like peptide-1 (GLP-1) are characteristic of the small intestine and colon.^{15–18} Therefore, gastrin and somatostatin could be gastric endocrine cell markers, while glicentin, GIP, and GLP-1 could be intestinal ones. Glicentin is an enteroglucagon with tropic actions on the intestinal epithelial cells,^{19,20} while GIP exhibits a glucose-dependent insulinotropic effect,²¹ and GLP-1 influences gastric motility and sensation.²² Several studies have shown that change of endocrine cells is observed in IM in the pyloric mucosa.^{20,23,24} However, it is still not clear what exactly occurs in phenotypes of endocrine cells in association with those of gastric mucous

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cells, particularly in GI-IM. Furthermore, there has not been reported intestinalization of mucous epithelium in association with the phenotypic change of endocrine cells in the fundic regions.

In the present study we therefore focused on the intestinalization of endocrine cells in the light of our classification of IM through GI-mixed toward I-IM in both pyloric and fundic mucosa.

MATERIALS AND METHODS

Samples

A total of 25 stomachs, resected due to gastric cancer at Aichi Cancer Center Hospital, were investigated. Of the 25 cases, seven were of early gastric cancer and 18 were of advanced gastric cancer. They were divided histologically into two well-differentiated, 13 moderately differentiated, eight poorly differentiated, and two signet-ring cell carcinomas. The group consisted of 17 men and eight women, aged 63.6 ± 9.0 years. Eleven samples excluding invasion were taken from the fundus and 14 from the pylorus, which were >5 mm distant from gastric cancers. All samples were fixed in 10% buffered formalin and embedded in paraffin. Serial sections were cut at 4 μ m, one being stained with hematoxylin and eosin (HE) for routine histological assessment. Control samples of normal gastrointestinal tract were taken from stomach, duodenum, jejunum, ileum, and colon in three cases surgically resected at Aichi Cancer Center Hospital. Of the three cases, one was from a 68-year-old man, and two were from women (37 years old and 61 years old, respectively). Two pathologists evaluated the histological slides independently (TT and KI).

Mucin histochemistry and immunohistochemistry

For mucin histochemistry, we adopted paradoxical concanavalin A staining (PCS) for identifying class III mucin in mucous neck cells and pyloric glandular epithelium.^{25,26}

Expression of human gastric mucin (HGM) and mucin core protein (MUC)2, chromogranin A, gastrin, somatostatin, glicentin, GIP, and GLP-1 was examined using specific antibodies (Table 1). The precise procedures for immunohistochemical demonstration were as previously described.^{11,27-29}

Phenotypic classification of glandular ducts in the stomach with reference to mucous cell markers

All of the straight glandular ducts from 25 stomach sections were evaluated and divided histologically and phenotypically into six types: pyloric glandular duct (P), fundic glandular duct (F), pseudopyloric glandular duct in fundic mucosa (pseudo-P), gastric and intestinal mixed phenotype IM (GI-IM), solely intestinal phenotype IM without Paneth cells (I-IM-Pa(-)), and solely intestinal phenotype IM with Paneth cells (I-IM-Pa(+)) using the gastric and intestinal mucous cell markers.^{11,27} P ducts and pseudo-P ducts, being positive for PCS, have surface mucous cells stained with HGM, but are negative for an intestinal mucous cell marker, MUC2. The F duct similarly stains with HGM in surface mucous cells and PCS in mucous neck cells, again being negative for MUC2. The GI-IM exhibits staining for at least one gastric mucous cell marker as well as MUC2 intestinal mucous cell marker, whereas I-IM are positive for MUC2, with no staining for gastric mucous cell markers. Regarding I-IM, we distinguish between lesions with and without Paneth cells. GI-IM and I-IM-Pa(-) belong to the incomplete IM category while I-IM-Pa(+) corresponds to complete-type IM. We have judged the phenotypes of 1030 fundic and 2004 pyloric glandular ducts in areas of chronic gastritis. Ducts with irregular shape or branching were excluded from the analysis.

Phenotypic classification of endocrine cells in the stomach with reference to endocrine cell markers

The endocrine cells of glandular ducts were identified as positive for chromogranin A. Gastrin and somatostatin are markers of the gastric endocrine cell, whereas glicentin, GIP,

Table 1 Antibodies for immunohistochemistry

Antibodies	Clonality	Dilution	Source
Anti-human chromogranin A (344-374)	Polyclonal	1:1000	Y
Anti-human gastrin 34 (1-15)	Polyclonal	1:2000	Y
Anti-human somatostatin	Polyclonal	1:200	D
Anti-human glicentin (1-32)	Polyclonal	1:2000	Y
Anti-human gastric inhibitory polypeptide	Polyclonal	1:5000	Y
Anti-human glucagon-like peptide-1 (7-36)	Polyclonal	1:5000	Y
Anti-human gastric mucin (clone 45M1)	Monoclonal	1:200	N
Anti-human MUC2 (clone Ccp58)	Monoclonal	1:100	N

D, DakoCytomation, Glostrup, Denmark; N, Novocastra, Newcastle upon Tyne, UK; Y, Yanaihara Institute, Fujinomiya, Japan.

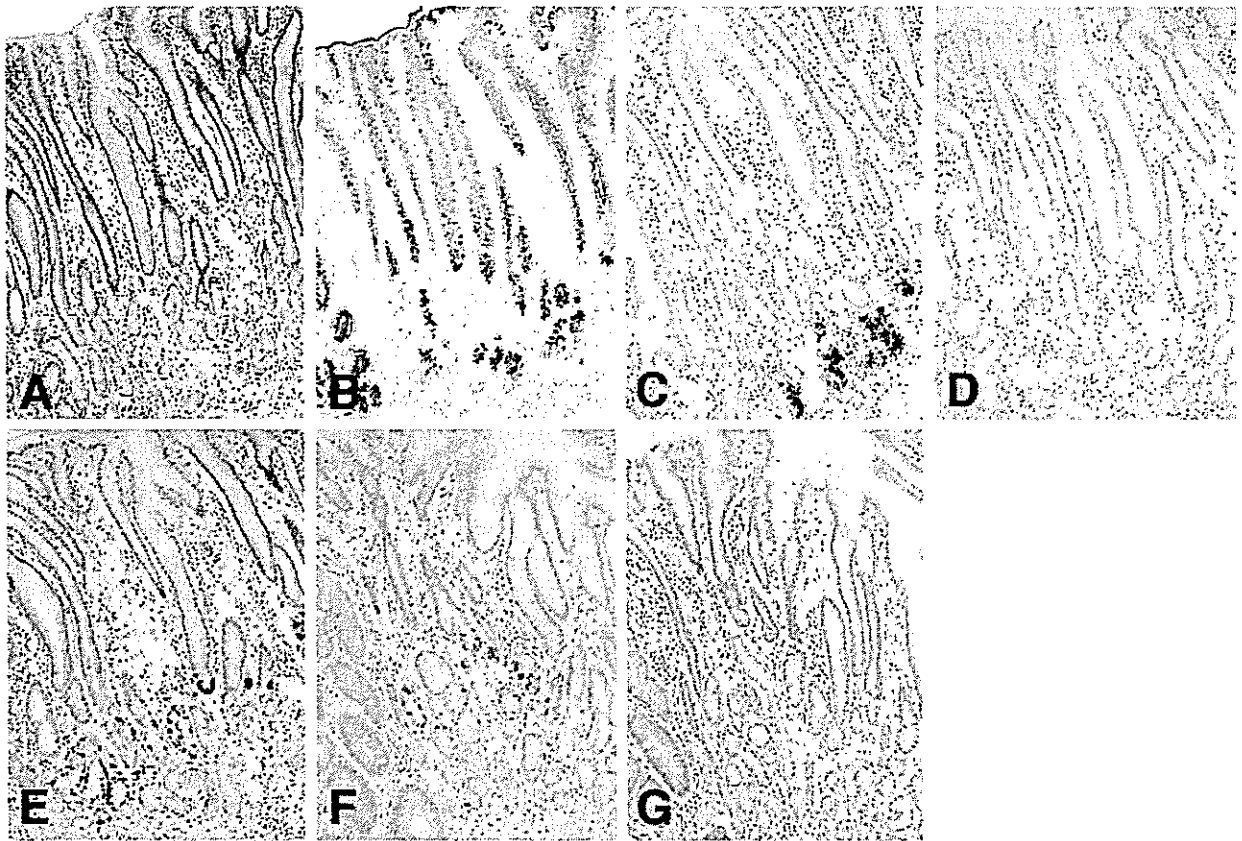


Figure 1

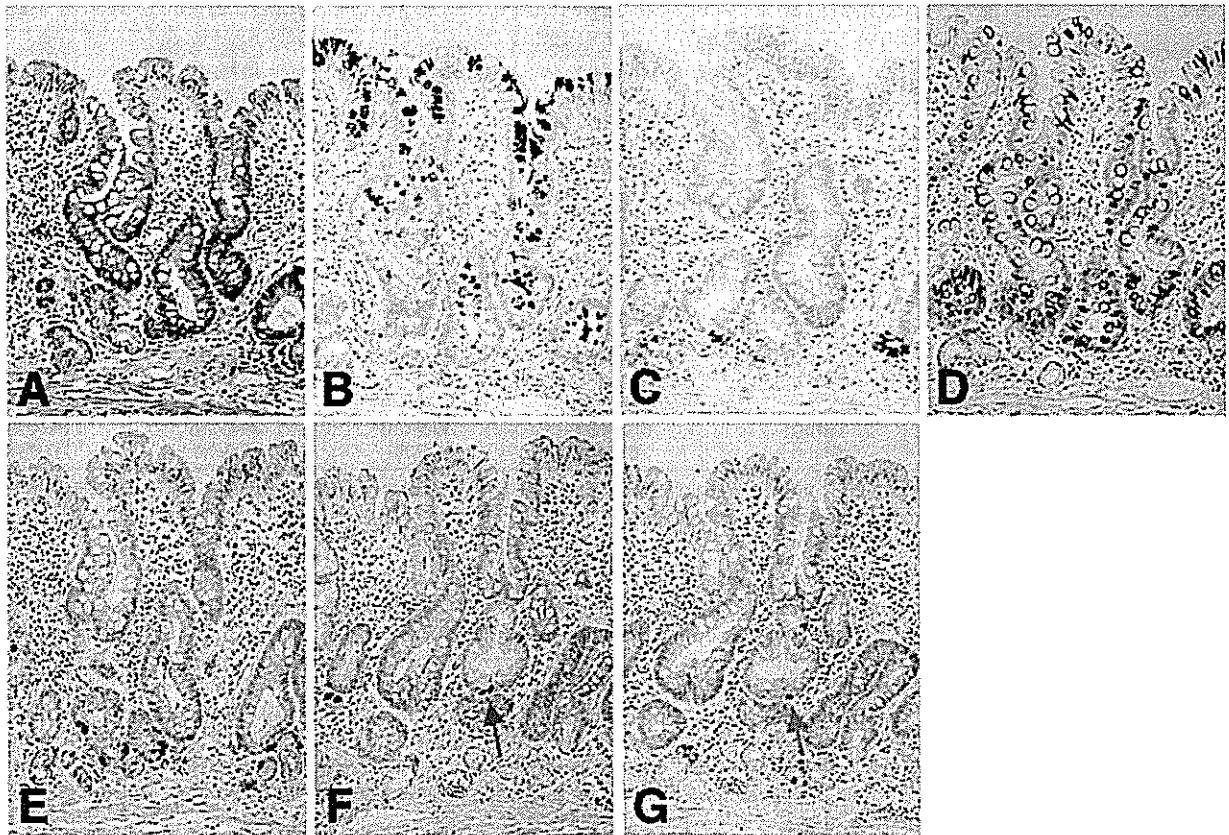


Figure 2