transcription repressor of ECPg. In the human stomach, immunohistochemical analysis revealed Sox2 localization in the foveolar epithelial cells as described here. However, expression of Sox2 protein in fundic and pyloric gland cells is relatively obscure. Considering the findings for cSox2 and ECPg in the chicken stomach, lower amounts of Sox2 in human fundic and pyloric glands than in foveolar epithelial cells might appear reasonable. In IM, Sox2 transcripts begin to decrease and gradually disappear as IM progresses from GI-mixed type to I type showing inverse correlation with Cdx1 and Cdx2. It seems possible either that Sox2 may negatively regulate Cdx1 and Cdx2 expression or vice versa. Like the relationship of MUC2/villin and Cdx1/2, Sox2 may possibly stimulate the expression of gastric differentiation markers including MUC5AC, but repress others such as MUC6 and pepsinogen as suggested in the chicken system (Sakamoto et al. 2000). Considering alternative factors that may regulate stomach differentiation, another candidate is GATA-5. In the chicken stomach, cGATA-5 is more strongly transcribed in glandular than in luminal epithelial cells, coinciding with the ECPg localization. Furthermore, the cGATA-5 protein specifically binds to the GATA binding sites of ECPg promoter and positively regulates its transcription (Sakamoto et al. 2000). Thus, GATA-5, whose expression pattern remains to be clarified, could be a possible gastric transcription factor especially for fundic and/or pyloric gland cells.

In conclusion, we described here for the first time the Sox2 distribution in the human, restricted to the stomach within the gastrointestinal tract as reported in the chicken. The expression patterns of Sox2 and Cdx1/Cdx2 appear inversely related and down-regulation of Sox2 could be an important mechanism in IM, in addition to ectopic expression of Cdx1/Cdx2 at the transcriptional and translational levels. It remains to be elucidated whether gastric and intestinal gene transcription is regulated independently or in a coordinated fashion. Reversal of the shift in expression of regulatory genes could hopefully play a role in repairing atrophic and metaplastic mucosa (Walker 2003) as a novel therapeutic approach in the future.

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ORIGINAL PAPER

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Expression of the intestine-specific transcription factors, Cdx1 and Cdx2, correlates shift to an intestinal phenotype in gastric cancer cells

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Abstract *Purpose*: It is well known that gastric cancers (GCs) at early stages, independent of the histological type, mainly consist of gastric phenotype malignant cells, while those at advanced stage tend to have a more intestinal phenotype with progression. However, the connection between this shift and expression of homeobox genes, which are important factors to maintain tissue character, has remained unclear. We therefore evaluated the expression of Cdx1/2 in relation to the phenotype of GCs. *Methods*: We analyzed the expression of Cdx1/2 mRNAs by Northern blotting and Cdx2 protein by immunohistochemistry in seventy advanced GCs, and evaluated phenotypically using mucin- and immunohistochemistry. *Results*: Seventy GCs were divided phenotypically into 16 gastric (G type), 18 gastric and intestinal mixed (GI type), 18 intestinal (I type), and 18 null (N type) phenotypes, independent of the histological classification. Cdx1 and Cdx2 mRNAs statistically demonstrated an increase with shift from G to I ($P=0.042$ and $P=0.0082$, respectively). Cdx2 nuclear staining was observed immunohistochemically in the intestinal phenotypic cancer cells, but could not be detected in those with only the gastric phenotype. *Conclusions*: These results show that Cdx1 and Cdx2 might be indispensable for intestinal phenotypic expression even in gastric cancer cells.

Keywords Intestinal phenotype · Gastric phenotype · Cdx1 · Cdx2

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Introduction

The phenotypic expression of malignant cells is widely thought to resemble that of the tissue of origin. Histologically, human gastric cancers present as two major groups, the "intestinal" and "diffuse" types of Lauren (Lauren 1965), which, respectively, nearly correspond to the "differentiated" and "undifferentiated" types of Nakamura et al. (Nakamura et al. 1968) and Sugano et al. (Sugano et al. 1982). Although these classifications have been widely used, they are not appropriate for studies of the histogenesis of gastric carcinomas and phenotype expression at the cellular level, because they confuse intestinal phenotypic cancer cells with a "diffuse" structure and the gastric phenotype with the "intestinal" type of Lauren (Tatematsu et al. 2003). It is also reported that gastric cancers at an early stage, independent of the histological type, mainly consist of gastric phenotype malignant cells, the intestinal phenotype increasing with progression (Yamachika et al. 1997; Bamba et al. 2001; Yoshikawa et al. 1998). Using gastric and intestinal epithelial cell markers, it is possible to analyze the phenotypic expression of each gastric cancer cell (Yoshikawa et al. 1998; Tatematsu et al. 1986; Tatematsu et al. 1990; Tatematsu et al. 1992). Stomach cancer cells can thereby now be clearly classified into gastric phenotype malignant cells, resembling pyloric gland cells and surface mucous cells, and intestinal phenotype malignant cells, like goblet and intestinal absorptive cells (Table 1). Caudal-related homeobox genes (Cdx) 1 and Cdx2 are believed to be

Table 1 Antibodies and cell types recognized. (*M* monoclonal, *P* polyclonal)

Antigen (clone)	Cell type	Source	Clonality	Dilution
MUC5AC (CLH2)	Gastric foveolar epithelial cell	Novocastra Laboratories, Newcastle, UK	M	1/500
HGM[a](45M1)	Gastric foveolar epithelial cell	Novocastra Laboratories, Newcastle, UK	M	1/200
MUC6 (CLH5)	Mucous neck cell, pyloric gland cell	Novocastra Laboratories, Newcastle, UK	M	1/500
Sialyl-Tn (TKH-2)	Goblet cell in small intestine	Dr. J.L. Werther, Gastrointestinal Research Laboratory, Division of Gastroenterology, Mount Sinai Medical Center, NY, USA	M	1/1,000
MUC2 (Ccp58)	Goblet cell in small intestine and colon	Novocastra Laboratories, Newcastle, UK	M	1/500
Sucrase	Microvilli of absorptive cell in small intestine and colon	Dr. Kazuyuki Hirano, Department of Pharmaceutical University, Gifu, Japan	P	1/3,000
Villin (12)	Microvilli of absorptive cell in small intestine and colon	Transduction Laboraties, Lexington, Ky., USA	M	1/20,000

[a]Human gastric mucin

important for the maintenance of intestinal epithelial cells (Silberg et al. 2002; Silberg et al. 2000; Soubeyran et al. 1999; Freund et al. 1998; Mallo et al. 1997; Mizoshita et al. 2001) and there have been several reports of their expression in gastric carcinomas (Silberg et al. 1997; Bai et al. 2000; Bai et al. 2002; Seno et al. 2002; Almeida et al. 2003). However, it has remained unclear whether this is directly linked with cellular differentiation. In this study, we therefore analyzed the expression of Cdx1/2 mRNAs by Northern blotting and Cdx2 protein by immunohistochemistry in 70 cases of advanced gastric carcinoma, evaluated histologically by hematoxylin and eosin (H&E) staining and phenotypically using mucin- and immunohistochemistry. The purposes were: 1) to examine the correlation between expression of the two homeobox genes and the histological classification of gastric cancer; 2) to assess the relationship between the two homeobox genes and the phenotypic expression of gastric cancer; and 3) to evaluate Cdx2 nuclear staining in individual gastric cancer cells.

Materials and methods

Samples and tissue collection

We examined 70 primary advanced gastric cancers surgically resected at Aichi Cancer Center Hospital between 1994 and 2000. Of the 70 patients, 41 were men and 29 were women; they ranged in age from 32 years 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 years to 84 years (mean, 62.7 ± 10.1 years) and the latter in individuals aged from 32 years to 83 years (mean, 61.1 ± 10.9 years). Histological classification was made according to the Japanese Classification of Gastric Carcinomas (Japanese Research Society for Gastric Cancer 1998). The cancers had invaded the subserosa (T2 for TNM classification) or the serosa and the peritoneal cavity (T3), including the adjacent organs (T4). Informed consent for our study was obtained from all participating patients before surgery.

Within 30–40 min after removal of the stomach, only carcinoma tissues were carefully sampled, cut into 5 mm squares on ice and frozen at −80 °C for later RNA extraction. Portions of the sampled tissues were fixed in 10% buffered formalin and then processed to paraffin sections for confirmation of carcinoma tissue. The samples for RNA extraction were strictly checked for the presence of the intestinal metaplasia (IM) and those that were positive were eliminated. Moreover, carcinomas with adjacent non-neoplastic mucosa were cut serially into 5-μm slices, in parallel with the lesser curvature at its maximum diameter, then fixed in 10% formalin solution and embedded in paraffin. They were cut into serial sections, the first of which was stained with H&E for histological diagnosis.

Northern blotting

Total RNAs from frozen tissues were isolated with TRIzol total RNA isolation reagent (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's instructions (Tsukamoto et al. 2000) and 20-μg aliquots were electrophoresed in 0.8% agarose-formaldehyde gels and transferred to Hybond-N (-) nylon membranes. A 535 bp NotI/EcoRI fragment of Cdx1 expressed sequence tag (GeneBank accession number AI911156, purchased from Incyte Genomics, Palo Alto, Calif., USA) corresponding to the 3'-untranslated region and a 468 bp EcoRI/ApaI fragment of Cdx2 cDNA (generous gift from Dr. Juan Lucio Iovanna) corresponding to the 5'-coding region (Mallo et al. 1997) without the homeobox domain, were used as probes and labeled with [α-^{32}P] dCTP using the Megaprime DNA labeling system (Amersham Biosciences, Buckinghamshire, England, UK). Membranes were hybridized with ^{32}P-labeled Cdx1 or Cdx2 cDNAs in QuikHyb solution (Stratagene, La Jolla, Calif., USA) for 1 h, and washed with 2 ×SSC and 0.1% SDS at room temperature, 0.1 × SSC and 0.1% SDS. They were then placed in contact with image plates at room temperature for 3 h. Each band was quantitatively analyzed by densitometry. We defined the average amounts of Cdx1 and Cdx2 mRNAs in the ascending colon as 1.0 in each case (Mizoshita et al. 2001) and then the relative amounts of Cdx1 and Cdx2 were determined in the 70 gastric cancer sample after normalizing them with reference to 36B4.

Immunohistochemistry and mucin histochemistry

Expression of MUC5AC, MUC6, MUC2, human gastric mucin (HGM), sialyl-Tn antigen, sucrase, and villin in carcinoma cells was examined by applying immunohistochemistry (Table 1). The TKH-2 antibody against sialyl-Tn antigen was generously donated by Dr. J. L. Werther, Gastrointestinal Research Laboratory, Mount Sinai Medical Center, N.Y., USA, and anti-sucrase antibody was kindly provided by Dr. Kazuyuki Hirano, Department of Pharmaceutics, Gifu Pharmaceutical University, Gifu, Japan. The precise procedures for immunohistochemical demonstration were as previously described (Yoshikawa et al. 1998; Inada et al. 1997; Inada et al. 2001). 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 achieved for the

detection of above-mentioned antibodies by heating in 10 m mol/l citrate buffer (pH 6.0) in a microwave oven for 10 min at 120 °C. Then, sections were incubated with primary antibodies. After thorough washing in phosphate-buffered saline (PBS), they were next incubated with biotinylated secondary antibody, and then with avidin-biotin horseradish peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, Calif., 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. We also examined expression of Cdx2 using anti-Cdx2 monoclonal antibody (BioGenex, San Ramon, Calif., USA) with the same methods (Seno et al. 2002; Almeida et al. 2003).

For mucin histochemistry, we adopted paradoxical concanavalin A staining (PCS) for identifying class III mucin in mucous neck cells (Katsuyama et al. 1985; Katsuyama and Spicer 1978).

The results for each gastric and intestinal differentiation marker and Cdx2 expression were evaluated with reference to the percentage of positively stained cancer cells. A result was considered positive if at least 10% of the cells were stained. When less than 10% of cancer cells were stained, the immunostaining was considered negative.

Classification of cancers

MUC5AC, HGM, MUC6, and PCS class III mucin are markers of the gastric epithelial cell phenotype, whereas MUC2, TKH-2, villin, and sucrase are typical of the intestinal epithelial cell phenotype (Yoshikawa et al. 1998; Tajima et al. 2001). Gastric cancers in which more than 10% of the section area consisted of only gastric or intestinal phenotype epithelial cells 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 or intestinal phenotype expression were grouped as unclassified (N type).

Statistical analysis

The unpaired *t*-test was applied to establish the significance of differences in Cdx1/2 mRNAs expression between the histological types. The Kruskal-Wallis test was applied to establish the significance of difference between Cdx1/2 mRNAs expression and the phenotypes. Incidences of Cdx2 nuclear staining with each histological type were assessed using Fischer's exact test. Trends in the incidences of Cdx2 nuclear staining with the four phenotypes were assessed by the χ^2 test for trend. $P < 0.05$ was regarded as statistically significant.

Results

Classification of gastric cancers histologically and phenotypically

The 70 gastric cancers were divided into 32 differentiated and 38 undifferentiated lesions histologically. The former were clearly sub-classified as eight G, seven GI, ten I, and seven N types phenotypically (Table 2). Similarly, the 38 undifferentiated cancers could be clearly classified as eight G, eleven GI, eight I, and 11 N types on the basis of their phenotypes. We found no statistical differences in phenotypic expression patterns between the differentiated and undifferentiated examples.

Table 2 Histological and phenotypical classification, and Cdx2 expression in 70 gastric carcinomas

Histological classification[b]	Phenotypic classification[a]				Total
	G	GI	I	N	
Differentiated	8 (0)	7 (7)	10 (10)	7 (0)	32 (17)
Undifferentiated	8 (0)	11 (11)	8 (8)	11 (0)	38 (19)
Total	16 (0)	18 (18)	18 (18)	18 (0)	70 (36)

[a]The number of Cdx2 positive cases are given in the parenthesis
[b]Classified based on structure of carcinomas. "Differentiated" includes tubular and papillary types, whereas "undifferentiated" consists of signet-ring cell and scirrhous types

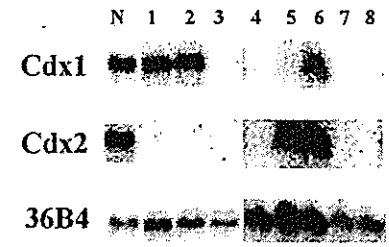

Fig. 1 Cdx1 and Cdx2 mRNA expression in human gastric cancer tissues. Cdx1 and Cdx2 mRNAs are apparent as 2.0-kb and 1.9-kb bands; 36B4, producing a 1.5-kb band, was used as the loading control. *Lanes N*: normal ascending colon, *1*: GI type undifferentiated adenocarcinoma, *2*: GI type undifferentiated adenocarcinoma, *3*: N type undifferentiated adenocarcinoma, *4*: N type differentiated adenocarcinoma, *5*: I type differentiated adenocarcinoma, *6*: I type differentiated adenocarcinoma, *7*: G type differentiated adenocarcinoma. *8*: G type differentiated adenocarcinoma

Expression of Cdx1 in cancers

The expression of Cdx1 mRNAs varied from case to case, both in the differentiated and undifferentiated types (Fig. 1and Fig. 2A). The range of scatter was greater in the latter. The average value for Cdx1 mRNA expression in differentiated lesions was 0.19 ± 0.28 (means $\pm$ SD), and for undifferentiated cancers was 0.29 ± 0.39, the difference not being statistically significant ($P = 0.23$).

Expression of Cdx2 in cancers

The range of scatter of Cdx2 mRNA values in undifferentiated cancers was narrower than in the differentiated type, opposite to the situation with the Cdx1 case. In addition, the ranges for both types were also narrower than for Cdx1 mRNA (Fig. 1 and 2B), with average values of 0.22 ± 0.24 and 0.13 ± 0.13 for the differentiated and undifferentiated types, respectively, no significant difference being present ($P = 0.081$).

Expression of Cdx1 in G, GI, I, and N type gastric cancers

The 70 gastric carcinomas were divided into 16 G, 18 GI, 18 I, and 18 N types. The average values for

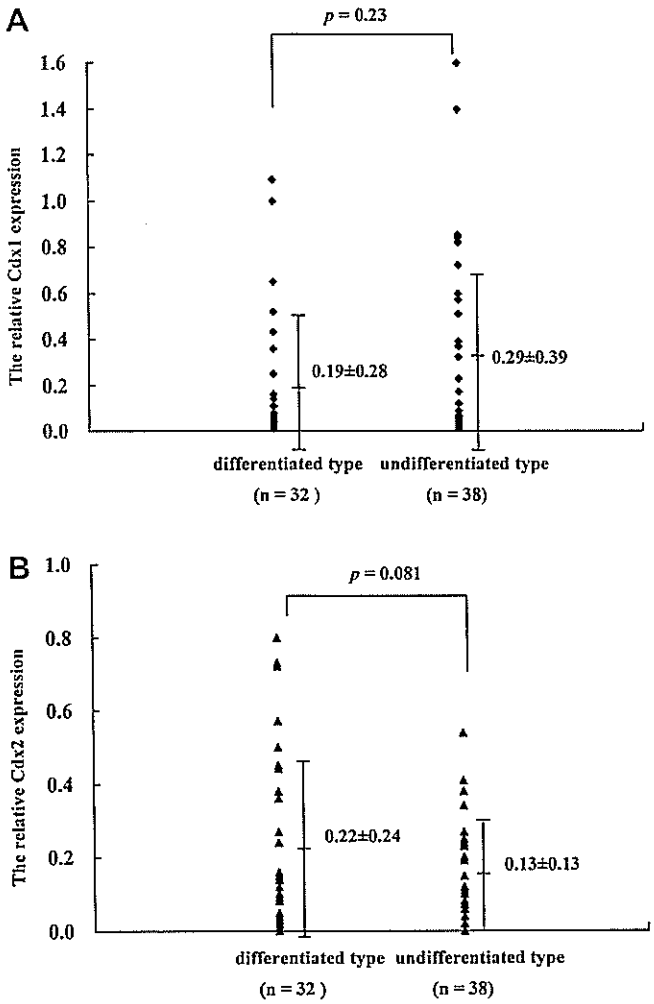

Fig. 2A, B The relative expression of Cdx1 and Cdx2 mRNAs with reference to the histological classification. A The relative amount of Cdx1 mRNA in the ascending colon as 1.0 in each case for the 70 gastric cancers. B The relative amount of Cdx2 mRNA in the ascending colon as 1.0 in each case for the 70 gastric cancers. Data are averages for the 32 differentiated and 38 undifferentiated type gastric cancers (means ± SD)

Cdx1 mRNA were 0.11 ± 0.20, 0.35 ± 0.40, 0.38 ± 0.45, and 0.11 ± 0.16 (means ± SD), respectively (Fig. 3A), the relative amounts significantly increasing from the G to the I type ($P = 0.042$). The relative amounts of Cdx1 mRNAs were also significantly greater in the GI and I types than in the N type ($P = 0.020$ and $P = 0.018$).

Expression of Cdx2 in G, GI, I, and N type gastric cancers

The average values for Cdx2 mRNA in G, GI, I, and N types were 0.084 ± 0.13, 0.20 ± 0.19, 0.26 ± 0.25, and 0.14 ± 0.13 (means ± SD), respectively (Fig. 3B), also demonstrating statistically significant increased in the I type ($P = 0.0082$).

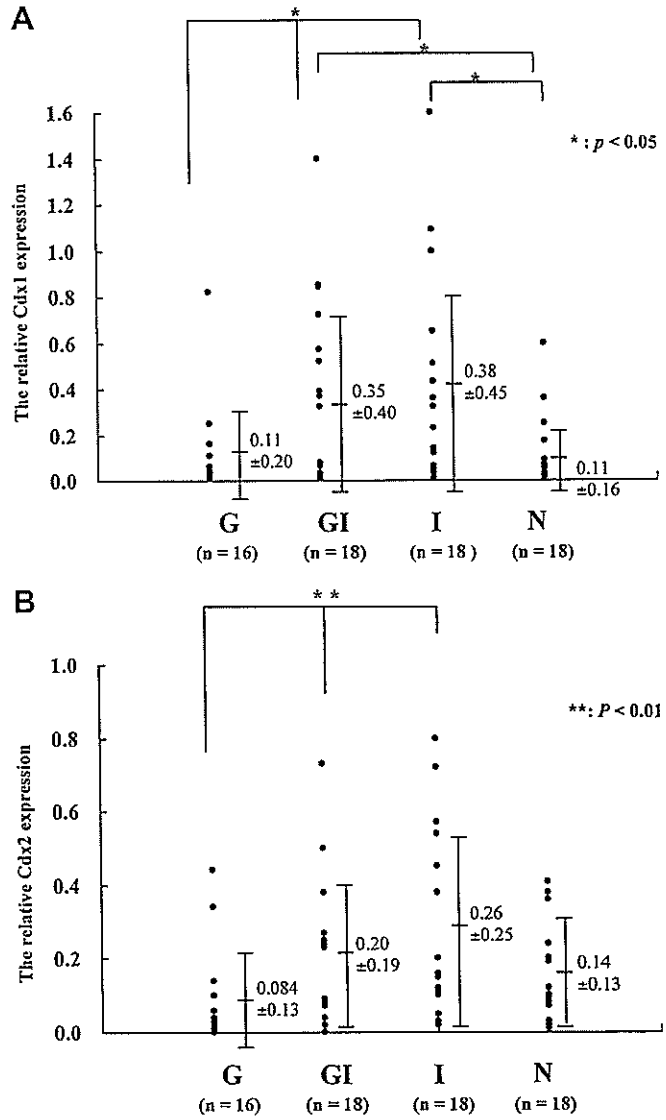

Fig. 3A, B The relative expression of Cdx1 and Cdx2 mRNAs with reference to the phenotype. A The expression of Cdx1 mRNAs correlated with the intestinal phenotypic expression in gastric cancers. Data values are averages for 16 G, 18 GI, 18 I, and 18 N types (means ± SD). The P value by statistical analysis for shift from the G to the I type is 0.042. The P values by statistical analysis for GI vs N, I vs N are 0.020 and 0.018. B The expression of Cdx2 mRNAs also correlated with the intestinal phenotypic expression in gastric cancers. Data values are averages for 16 G, 18 GI, 18 I, and 18 N types (means ± SD). The P value by statistical analysis from G to I types is 0.0082

Immunohistochemical staining of Cdx2 in gastric cancers

Cdx2 nuclear staining was detected in intestinal phenotype cancer cells, but not in the cancer cells with only gastric phenotypic expression even if adjacent intestinal metaplasia was positively stained (Fig. 4 and Fig. 5). In the GI and I type gastric cancers, the areas of Cdx2 positive nuclear staining were in perfect accord with those of intestinal phenotypic expression (Table 2). The

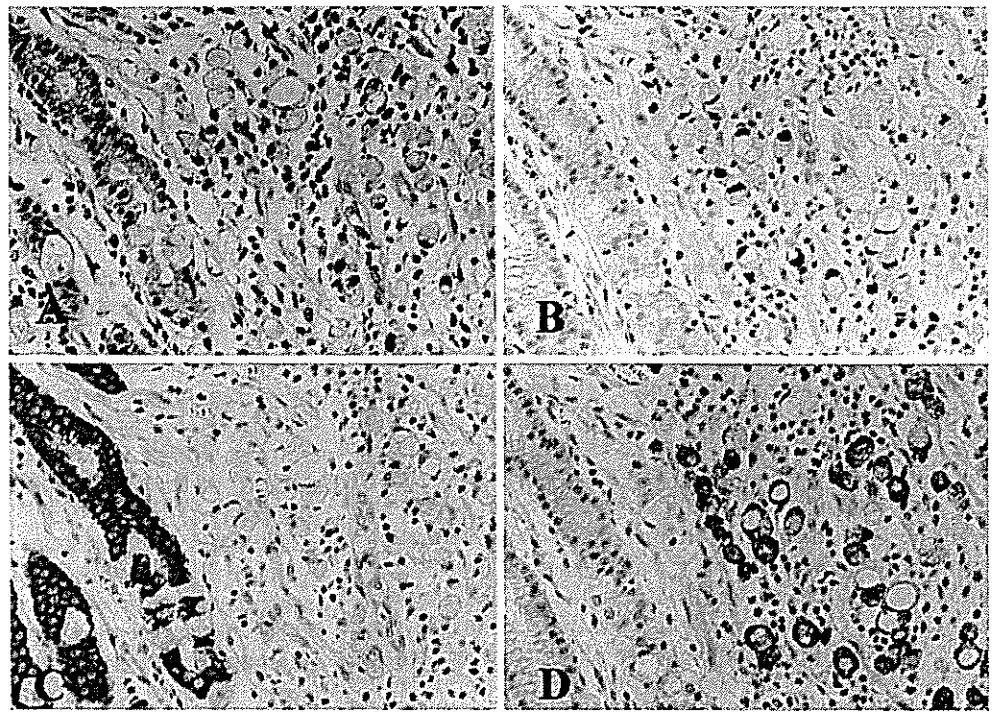

Fig. 4A–D Histology of I type undifferentiated carcinoma (signet-ring cell carcinoma). A H&E staining. B Cdx2 immunohistochemistry. Note that nuclear staining is observed in the carcinoma cells, but not in normal gastric mucosa. C MUC5AC immunostaining. MUC5AC is detected in the cytoplasm of normal gastric mucosa, but not in cancer cells. D MUC2 immunostaining. MUC2 is detected in the cytoplasm of cancer cells, but not in normal gastric mucosa. (Original magnification, ×200)

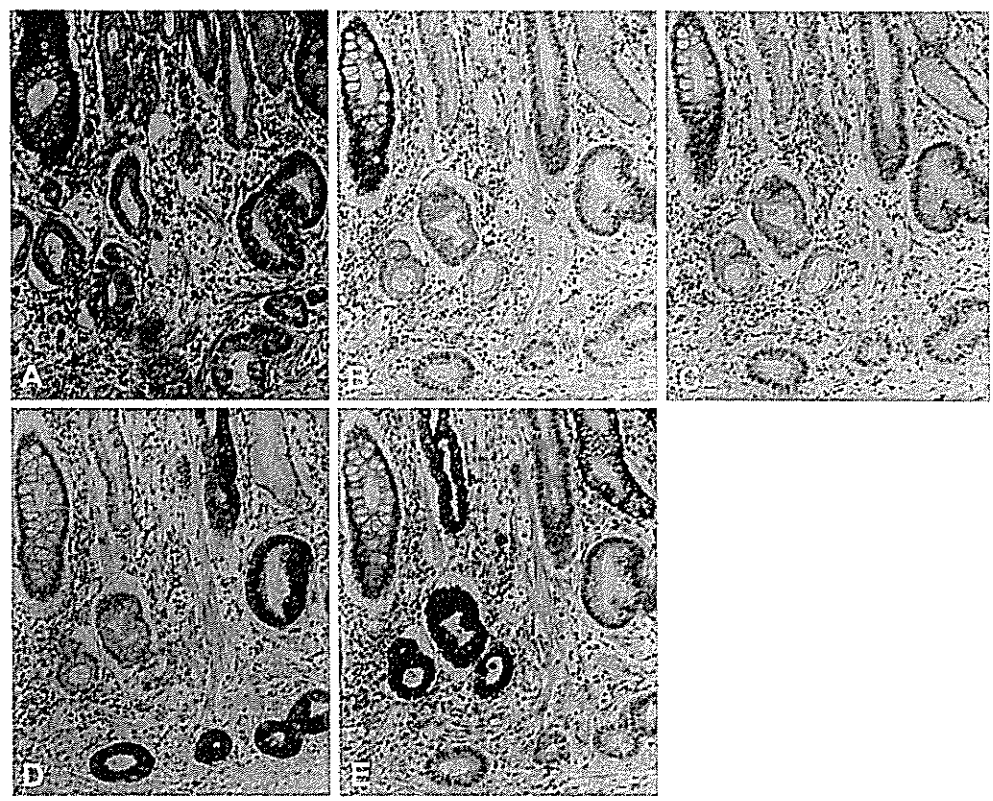

Fig. 5A–E Histology of G type (gastric foveolar phenotype) differentiated adenocarcinoma. A H&E staining. B Cdx2 immunohistochemistry. Cdx2 nuclear staining is observed in intestinal metaplasia, but not in cancer or normal pyloric gland cells. C MUC2 immunostaining. Detected in the cytoplasm of intestinal metaplastic gland, but not in cancer or normal pyloric gland cells. D MUC5AC immunohistochemistry. MUC5AC is detected in the cytoplasm of cancer cells, but not in pyloric gland cells or intestinal metaplastic gland. E MUC6 immunostaining. MUC6 is detected in the cytoplasm of pyloric gland cells, but not cancer cells or IM. (Original magnification, ×100)

G or N type gastric cancers occasionally had less than 10% of the section area exhibiting intestinal phenotypic expression, and these cases also had weak nuclear staining of Cdx2 in the affected areas, without exception. The GI and I types had significantly greater Cdx2 expression than the G and N types ($P<0.001$) (Table 2). No association was observed between the immunohistochemical staining of Cdx2 and the histological classification of gastric carcinomas ($P=0.79$) (Table 2).

Discussion

Our present data clearly demonstrate a correlation between Cdx1/2 expression and intestinal phenotypic expression of gastric cancers. Furthermore, Cdx2 was found to be expressed in intestinal phenotype cancer cells at the cell level. The results are in line with the finding by Almeida et al. (Almeida et al. 2003) of a strong association between the intestinal mucin MUC2 and Cdx1/2, but they also reported cases of incompatibility. We here showed that the intestinal phenotypic cancer cells exhibit nuclear staining of Cdx2, without exception, using four intestinal cellular differentiation markers, MUC2 included. Recently, Yamamoto et al. (Yamamoto et al. 2003) showed that Cdx2 may induce expression of MUC2 mucin in goblet cells. We consider that this function of Cdx2 in goblet cells might also apply to gastric cancers. Our results are also compatible with previous reports that Cdx2 plays an important role in the expression of sucrase-isomaltase, which has been employed as an intestinal differentiation marker (Suh et al. 1994). In addition, gastric phenotypic expression was defined using not only surface mucous cell markers such as MUC5AC and HGM, but also pyloric gland cell markers such as MUC6 and class III mucin. Cdx2 nuclear staining was not detected in any cancers cells with only gastric phenotypic expression. While the amounts of Cdx2 mRNAs did not directly correlate with the immunoreactivity in a few cases, we considered that this might be explained by the heterogeneous nuclear staining.

Our data revealed lower P values for Cdx2 than Cdx1 on Northern blotting analysis. Silberg et al. (Silberg et al. 2002) showed that gastric expression of Cdx2 alone was sufficient to induce intestinal metaplasia in the Cdx2-expressing transgenic mice. Similarly, Mutoh et al. (Mutoh et al. 2002) found that the ectopic expression of Cdx2 induced intestinal metaplasia in the stomach of Cdx2-expressing transgenic mice using H^+/K^+-ATPase promoter. With regard to analysis of Cdx1, to our knowledge, no one has reported establishment of transgenic mice. Taking into account all the available data, however, we consider that Cdx2 might be more important than Cdx1 for intestinal phenotypic expression in gastric cancers.

In the present study there was no statistically significant correlation between the expression of two homeobox genes and the histological classification of gastric cancer. This result is also compatible with the report of Almeida et al. (Almeida et al. 2003). However, it has also been reported that the histological type of gastric carcinoma is associated with higher Cdx2 expression in the "intestinal" type than the "diffuse" type of Lauren (Bai et al. 2002; Seno et al. 2002). First, we consider that this discrepancy could depend on the antibodies used. Seno et al. (Seno et al. 2002) used a polyclonal antibody raised against mouse Cdx2 protein, while Bai et al. (Bai et al. 2002) applied a polyclonal antibody of their own. On the other hand, we used a monoclonal antibody purchased from BioGenex. Almeida et al. (Almeida et al. 2003) utilized this same antibody and showed that Cdx2 correlates with intestinal phenotypic expression. In our study, the results for each gastric and intestinal differentiation marker and Cdx2 expression were evaluated with reference to the percentage of positively stained cancer cells. A result was only considered positive if at least 10% of the cells were stained. Sensitivity of Cdx2 immunohistochemistry between these different antibodies might thus have influenced the judgment of positivity. Second, we used several gastric and intestinal differentiation markers to judge the direction of differentiation, including MUC5AC, HGM, MUC6, MUC2, villin, and so forth. In contrast, Bai et al. and Seno et al. determined the phenotypes of their carcinomas based solely on their morphology.

Evaluation of the relation between intestinal phenotypic expression and Cdx1 at the mRNA level, demonstrated a clear link, as reported earlier (Almeida et al. 2003). Silberg et al. (Silberg et al. 1997) found heterogeneous nuclear staining of Cdx1 observed in individual gastric cancers. Considering our Cdx2 data, this Cdx1 result might also be explained by differences in phenotype of each cancer cell. Soubeyran et al. (Soubeyran et al. 1999) showed IEC/Cdx1 cells to have a differentiated phenotype including formation of complex junctions and glycocalix coated microvilli with expression of animopeptidase N and villin. Our data are compatible with their report of suggesting that Cdx1 is related to intestinal phenotypic expression.

We also tried to clarify the significance of Cdx1 and Cdx2 expression in the gastrointestinal tract. Previous studies suggested that Cdx2 is a tumor-suppressor gene with regard to colorectal carcinogenesis (Ee et al. 1995; Mallo et al. 1997; Mallo et al. 1998; Vider et al. 1997). Since Seno et al. (Seno et al. 2002) reported that Cdx2-positive gastric cancer patients survived significantly longer than their Cdx2-negative counterparts, a tumor-suppressor effect might also extend to gastric carcinogenesis. The role of Cdx1 in carcinogenesis remains controversial. Subraminian et al. (Subraminian et al. 1998) reported its expression to be strong in regenerating epithelial foci, but not in quiescent sterilized crypts after irradiation-induced damage. They also showed that no colonic tumor developed in Cdx1-deficient mice (Subraminian et al. 1995). It was reported that Cdx1 expression in IEC-6 cells induces phenotypic changes characteristic of differentiating enterocytes, suggesting an important role for Cdx1 in the transition from stem cells to proliferating/transit cells (Soubeyran et al. 1999). Further studies are clearly warranted to clarify the role of Cdx1 in gastric carcinogenesis.

In conclusion, the present results provide compelling evidence that Cdx1 and Cdx2 might be indispensable for intestinal phenotypic expression, even in gastric cancer cells.

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β-Catenin gene alteration in glandular stomach adenocarcinomas in *N*-methyl-*N*-nitrosourea-treated and *Helicobacter pylori*-infected Mongolian gerbils

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The goal of this study was to elucidate whether *β-catenin* gene mutations might contribute to glandular stomach carcinogenesis in *Helicobacter pylori (H.pylori)*-infected Mongolian gerbils. Firstly, exon 3 of gerbil β-catenin cDNA, a mutation hot spot, was cloned and sequenced and found to have 89.3% homology with the human form and 95.5% with the rat and mouse forms. Peptide sequence in this region was shown to be 100% conserved in these mammals. Then, 45 stomach adenocarcinomas induced with *N*-methyl-*N*-nitrosourea (MNU) plus *H. pylori* infection and 7 induced with MNU alone were examined for β-catenin expression by immunohistochemistry and for DNA mutations using a combination of microdissection and PCR-single strand conformation polymorphism analysis. One gastric cancer in the MNU+*H. pylori* group (2.2%) displayed nuclear (N) β-catenin localization, 3 (6.7%) showed cytoplasmic (C) distribution in local regions, and 41 (91.1%) demonstrated cell membrane (M) localization. Tumors induced by MNU alone showed only membranous β-catenin localization (7/7). Analysis of exon 3 of the *β-catenin* gene demonstrated all tumors with membrane or cytoplasmic staining as well as surrounding normal mucosa (S) to feature wild-type β-catenin. In contrast, the lesion with nuclear staining had a missense mutation at codon 34 [GAC (Gly)→GAA (Glu)] in exon 3 (1/1=100%, N vs. M, $P<0.05$; and N vs. S, $P<0.05$). In conclusion, these results suggest that β-catenin may not be a frequent target for mutation in stomach carcinogenesis in MNU+*H. pylori*-treated gerbils. (Cancer Sci 2004; 95: 487–490)

Abnormal expression of E-cadherin and β-catenin results in loss of epithelial cell-to-cell adhesion, leading to uncontrolled cell growth, and may therefore participate in gastric cancer development.[1,2] However, studies of mutations relevant to Wnt/β-catenin signaling in human stomach tumors have yielded conflicting results.[3–6] We previously reported the existence of *β*-catenin gene mutations in 18% of rat stomach cancers induced by N-methyl-N′-nitro-N-nitrosoguanidine (MNNG).[7] In contrast, no mutations were found in N-methyl-N-nitrosourea (MNU)-induced rat gastric carcinomas in another study.[8] Therefore, the clinicopathological significance of *β*-catenin gene mutation is unclear.

Recently, the Helicobacter pylori (H. pylori)-infected Mongolian gerbil has been established as an appropriate animal model for the study of gastric cancer development, with induction of adenocarcinomas by MNNG or MNU.[9–12] However, little information has thus far been generated regarding molecular events occurring in the gerbil model, partly because of the undefined genetic background.

In this study, stomach adenocarcinomas developing in H. pylori-infected or uninfected gerbils treated with MNU in the drinking water were utilized to examine β-catenin protein localization by immunohistochemistry and the mutational status of exon 3 of *β*-catenin gene by using DNA extracted from histologically distinct regions.

Materials and Methods

Tumor samples. Fifty-two gastric adenocarcinomas were collected from 50 gerbils treated with one of three experimental protocols. In experiment I, 28 7-week-old, specific-pathogen-free, male Mongolian gerbils (Meriones unguiculatus; MGS/Sea, Seac Yoshitomi, Ltd., Fukuoka) were inoculated with H. pylori (ATCC 43504, American Tissue Culture Collection, Rockville, MD), then starting 2 weeks thereafter, were given ad libitum drinking water containing 10 ppm of MNU (Sigma Chemical Co., St. Louis, MO) in light-shielded bottles for 20 weeks continuously. In experiment II, 15 gerbils received MNU in water at a concentration of 20 ppm on alternate weeks for a total of 5 weeks exposure and were inoculated with H. pylori one week after the completion of this carcinogen exposure. In experiment III, 7 gerbils received MNU only at a concentration of 10 ppm for 20 weeks continuously. All animals were sacrificed at the 70th experimental week. The excised stomachs were fixed in 10% formalin in phosphate buffer for 24 h and samples of tumors and background tissue were routinely processed for embedding in paraffin.

Histopathological analysis. Tissue sections were stained with hematoxylin and eosin (H&E) for histological diagnosis. Immunohistochemical staining with monoclonal anti-β-catenin antibody (clone 14, BD Transduction Laboratories, Lexington, KY) at 4°C overnight followed by the avidin-biotin complex method (Vector Laboratories, Inc., Burlingame, CA) was performed as described earlier.[13] Immunoreactivity of β-catenin was classified into "membranous (M)," "cytoplasmic (C)," and "nuclear (N)" according to the intracellular localization of the protein. Tumors were then classified into "M" with only membranous β-catenin staining, "C" if they harbored tumor cells with cytoplasmic β-catenin at least in part but without nuclear staining, and finally "N" if they possessed tumor cells with nuclear accumulation of β-catenin anywhere within the tumor, as described previously.[7]

Sequencing analysis of β-catenin exon 3. A segment of 224 bp from the genomic DNA in the normal gastric mucosa of Mongolian gerbils was amplified. The PCR product was prepared as the template and the nucleotide sequence was analyzed using a BigDye Terminator Cycle Sequencing Kit, v 3.1 (Applied Biosystems, Foster City, CA) with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequences of the forward (5′-GCTGACCTGATGGAGTTGGA-3′) and reverse (5′-GCTACTTGCTCTTGCGTGAA-3′) PCR primers were designed based on the similarity to those of human, mouse, and rat, as described.[13]

PCR-single strand conformation polymorphism (PCR-SSCP) analysis and direct sequencing. Tumor areas and surrounding stomach mucosa were microdissected from 10-μm-thick unstained serial

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paraffin sections under a stereoscopic microscope, then genomic DNA was extracted using the Pinpoint Slide DNA Isolation System (Zymo Research, Orange, CA) used in our previous work.[7] PCR-SSCP analysis of β-catenin exon 3 was performed with established methods.[13,14]

Results

β-Catenin localization. Fifty animals were observed to have 45 differentiated and 7 undifferentiated gastric adenocarcinomas. In the MNU+H. pylori group, immunostaining of β-catenin revealed that 41 of the demonstrated tumors had only membranous localization (41/45, 91.1%) and 3 had (3/45, 6.7%) cytoplasmic β-catenin staining. The majority of differentiated adenocarcinomas had preserved cellular and nuclear polarity, showing homogeneous low-grade morphology. In contrast, one small lesion showed heterogeneity with high-grade cytological and structural atypia within the tumor masses, where nuclear β-catenin accumulation was observed (1/45, 2.2%, see Fig. 1). On the other hand, all samples (7/7, 100%) in the MNU-only group showed membranous localization (Table 1).

β-Catenin exon 3 sequence of normal gerbil. Sequences of the 224 bp portion of β-catenin exon 3 cDNA in various animals including gerbil, human, rat, mouse, and Xenopus were aligned (Fig. 2). The nucleotide sequences of the Mongolian gerbil and human forms exhibited good homology (89.3%), the relation to mouse and rat being even closer (95.5%). Peptide sequences in this region matched completely in the mammals, and almost perfectly (95.9%) with that of Xenopus.

***β-Catenin* gene mutations.** Representative PCR-SSCP results are shown in Fig. 3. DNA samples from lesions with membranous and cytoplasmic staining showed similar DNA mobility to that of samples from the surrounding normal tissues and a wild-type control (lane 1). However, the example with nuclear β-catenin staining (lane 2) harbored a band (a) with abnormal mobility. Sequencing analysis confirmed this to be due to a GAC (Gly)→GAA (Glu) missense mutation at codon 34 (Fig. 4).

Discussion

We found only a single mutation at exon 3 of *β*-catenin gene in one of 45 cancers that developed in Mongolian gerbils infected with H. pylori and treated with MNU, and 7 cancers in gerbils given MNU alone. To our knowledge, this is the first report of any such mutation of the *β*-catenin gene in a gastric cancer in a Mongolian gerbil. In human and rat lesions, mutations at exon 3 of β-catenin are usually localized at glycogen synthase kinase (GSK)-3β phosphorylation sites (codons 29, 37, 41, and 47) and the adjacent codons (28, 32, 34, 39, and 48), where serine and threonine residues are physiologically phosphorylated. The mutation spectrum of β-catenin in the Mongolian gerbil gastric cancer, although only one was found in this study, was in line with reports on rodent tumors with mutation located at codon 34 of exon 3.[15,16] Our mutation of *β*-catenin gene was associated with nuclear staining of the protein. We have previously demonstrated such mutations to correlate with nuclear β-catenin in rat gastric cancers induced with MNNG.[7] Ikenoue et al.[17] have suggested that the *β*-catenin gene mutation status may be associated with a shift in the localization from the membrane to the nucleus. It is well known that mutations can prevent degradation of β-catenin protein in an APC (adenomatous polyposis coli)-dependent manner and cause activation of the β-

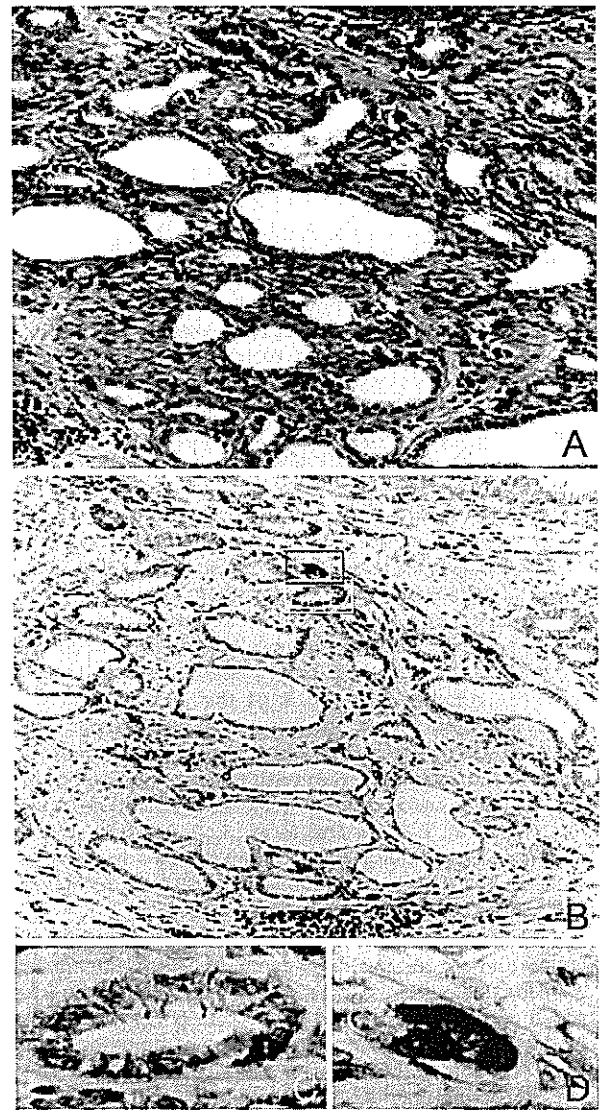

Fig. 1. Mongolian gerbil glandular stomach cancers induced by MNU and *H. pylori* infection. (A) H&E staining of a differentiated adenocarcinoma induced by MNU and *H. pylori* infection (×80). (B) Immunohistochemical analysis of β-catenin protein (×80). (C) Magnified yellow box in (B), representative results for tumor cells with membranous localization of β-catenin (×640). (D) Magnified red box in (B), representative results for tumor cells with nuclear accumulation of β-catenin (×640).

Table 1. β-Catenin localization and exon 3 mutation in Mongolian gerbils' stomach tumors

		β-Catenin localization		β-Catenin mutation	
		MNU+*H. pylori*	MNU only	MNU+*H. pylori*	MNU only
Gastric tumor	Nucleus	1/45 (2.2%)	0/7 (0%)	1/1 (100%)[1)]	0/7 (0%)[4)]
	Cytoplasm	3/45 (6.7%)	0/7 (0%)	0/3 (0%)	0/7 (0%)
	Membrane	41/45 (91.1%)	7/7 (100%)	0/41 (0%)[2)]	0/7 (0%)
Surronding normal tissue	Membrane	45/45 (100%)	7/7 (100%)	0/41 (0%)[3)]	0/7 (0%)

1) $P<0.05$ vs. *2)* and *3)*; $P=0.13$ vs. *4)* (Fisher's exact test).

Nucleotide sequence

```
          277                        300                                                  350
Gerbil    5'-GACCTGATGG AGTTGGACAT GGCCATGGAG CCGGACAGAA AGGCTGCTGT AAGCCACTGG CAGCAGCAGT CATACCTGGA
Human     5'-GATCTGATGG AGTTGGACAT GGCCATGGAG CCGGACAGAA AAGCGGCTGT TAGTCACTGG CAGCAACAGT CTTACCTGGA
Rat       5'-GACCTCATGG AGTTGGACAT GGCCATGGAG CCAGACAGAA AGGCCGCTGT CAGCCACTGG CAGCAGCAAT CTTACCTGGA
Mouse     5'-GACCTGATGG AGTTGGACAT GGCCATGGAG CCGGACAGAA AAGCTGCTGT CAGCCACTGG CAGCAGCAGT CTTACCTGGA
Xenopus   5'-GATCTGATGG AGCTGGACAT GGCCATGGAG CCAGACCGAA AGGCAGCAGT GAGCCACTGG CAGCAGCAGT CTTACCTGGA

                                                  400
          CTCTGGAATC CACTCTGGTG CCACCACCAC AGCTCCTTCC CTGAGTGGCA AGGGCAACCC TGAGGAAGAA GACGTGGACA
          CTCTGGAATC CATTCTGGTG CCACTACCAC AGCTCCTTCT CTGAGTGGTA AAGGCAATCC TGAGGAAGAG GATGTGGATA
          TTCTGGAATC CACTCTGGTG CCACCACCAC AGCTCCTTCC CTGAGTGGCA AGGGCAATCC TGAGGAAGAA GATGTGGACA
          TTCTGGAATC CATTCTGGTG CCACCACCAC AGCTCCTTCC CTGAGTGGCA AGGGCAACCC TGAGGAAGAA GATGTTGACA
          TTCTGGCATT CATTCTGGAG CTACCACCAC AGCTCCATCT TTGAGTGGCA AAGGAAACCC AGAGGATGAA GATGTGGATA

                    450                                                        500
          CCTCCCAAGT CCTCTATGAG TGGGAGCAAG GCTTTTCCCA GTCCTTCACG CAAGAGCAAG TAGC-3'
          CCTCCCAAGT CCTGTATGAG TGGGAACAGG GATTTTCTCA GTCCTTCACT CAAGAGCAAG TAGC-3'
          CCTCCCAAGT CCTTTATGAG TGGGAGCAAG GCTTTTCCCA GTCCTTCACG CAAGAGCAAG TAGC-3'
          CCTCCCAAGT CCTCTATGAA TGGGAGCAAG GCTTTTCCCA GTCCTTCACG CAAGAGCAAG TAGC-3'
          CCATCCAAGT TTTGTATGAG TGGGAGCAAG GCTTCTCTCA GTCCTTCACT CAAGATCAAG TGGC-3'
```

Amino acid sequence

```
                                        33  37  41  45
Gerbil    DLMELDMAME PDRKAAVSHW QQQSYLDSGI HSGATTTAPS LSQKGNPEEE DVDTSQVLYE WEQGFSQSFT QEQV
Human     DLMELDMAME PDRKAAVSHW QQQSYLDSGI HSGATTTAPS LSQKGNPEEE DVDTSQVLYE WEQGFSQSFT QEQV
Rat       DLMELDMAME PDRKAAVSHW QQQSYLDSGI HSGATTTAPS LSQKGNPEEE DVDTSQVLYE WEQGFSQSFT QEQV
Mouse     DLMELDMAME PDRKAAVSHW QQQSYLDSGI HSGATTTAPS LSQKGNPEEE DVDTSQVLYE WEQGFSQSFT QEQV
Xenopus   DLMELDMAME PDRKAAVSHW QQQSYLDSGI HSGATTTAPS LSQKGNPEDE DVDTQVLYE WEQGFSQSFT QDQV
```

Fig. 2. β-Catenin DNA sequences of Mongolian gerbils in comparison with other species. Top panel, nucleotide sequences of exon 3. Alignment of the 224 bp portion of β-catenin exon 3 cDNA sequences for the Mongolian gerbil, human, rat, mouse, and *Xenopus*: 89.3% oligonucleotide identities were observed between Mongolian gerbil and human, 95.5% with mouse and rat, and 84.4% with *Xenopus*. Bottom panel, the amino acid sequence in exon 3 of β-catenin, which is conserved perfectly among mammals.

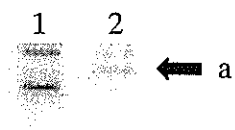

Fig. 3. PCR-SSCP analysis of β-catenin exon 3 in gerbil stomach adenocarcinomas. Lane 1, wild-type control; lane 2, adenocarcinoma sample with nuclear β-catenin staining showing a mobility shift. "a", abnormal band.

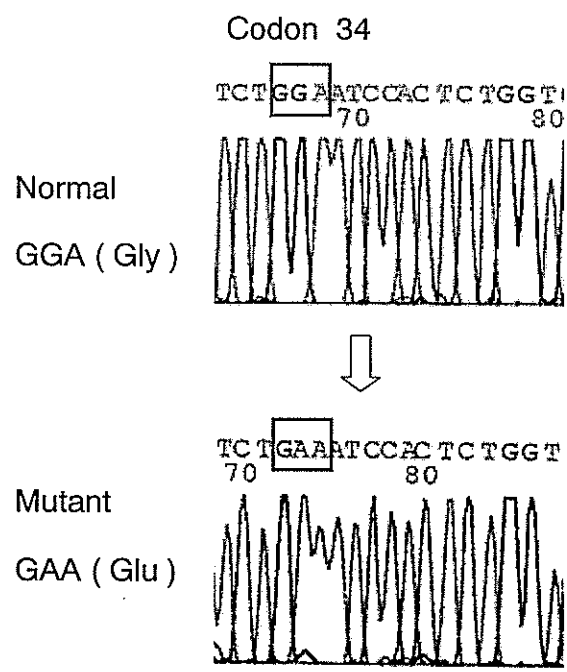

Fig. 4. Sequencing analysis of the *β-catenin* gene isolated from the gerbil stomach carcinoma illustrated in Fig. 3, showing codon 34. Top panel, wild type; bottom panel, mutant.

catenin/Tcf-4 signal transduction pathway in human and rodent models, including rats and mice. Since the sequence of β-catenin exon 3 was highly conserved among the mammals analyzed in this report, the physiological role of β-catenin and the oncogenic mechanism associated with its mutation could be quite similar in Mongolian gerbils as well.

In human stomach cancers, the reported incidences of mutations in exon 3 of *β*-catenin gene have ranged from 0 to over 30%, and loss of E-cadherin expression appears to correlate with poor differentiation and invasion into adjacent organs in adenocarcinomas.[18–21] We have previously revealed that β-catenin mutation occurs in the late stage progression of rat stomach cancers.[7] In addition, Saito et al.[22] detected no mutations in exon 3 of the *β*-catenin gene in 9 early-onset human gastric cancers while Clements et al.[19] found a significant number of stomach adenocarcinomas with β-catenin mutations and nuclear accumulation, including advanced stage lesions. Therefore, we consider that *β*-catenin gene mutations might be important for late-stage progression in gastric carcinogenesis. β-Catenin activation is usually confined to a small region within a stomach cancer, and thus the use of a microdissection technique to allow sampling of pure populations of tumor cells may prevent false-negative results.[19] The discrepancy in frequency with previous reports could be due to the techniques applied for extraction of DNA from tumor tissues.

In conclusion, mutation of the *β*-catenin gene exon 3 may not be a common event in the generation of stomach cancers in the Mongolian gerbil model with MNU exposure and H. pylori infection, but uncontrolled activation of the Wnt signaling pathway could contribute to stomach carcinogenesis in certain tumors. In this study, one β-catenin mutation was detected among

the H. pylori-infected gerbils, and there was no statistically significant significance between the MNU+H. pylori and MNU-alone groups (1/45 vs. 0/7, $P>0.05$). Thus, H. pylori infection may not enhance β-catenin gene alteration. It may help clarify the influence of H. pylori infection in stomach carcinogenesis to analyze more samples treated with MNU only and to compare the two groups in the future. H. pylori infection frequently causes chronic gastritis, and long-term infection increases the risk of gastric cancer. Yu et al.[23] earlier found that loss or downregulation of α-catenin mRNA in the gastric mucosa was associated with H. pylori infection, which is also known to accelerate E-cadherin methylation.[24] These results are suggestive of activation of the Wnt-catenin-Tcf signaling pathway with H. pylori infection in the stomach. β-Catenin was expressed on the membrane of the cancer cells in 48 of 52 (92%) gastric cancer tissues. Thus, other molecular mechanisms, including downregulation of E-cadherin, might have occurred in our model. Whether other genetic or epigenetic alterations occur in gastric cancer cells in cases lacking β-catenin mutations is an intriguing possibility warranting further research.[25,26]

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Pathology International 2004; 54: 392–400

Original Article

Immunohistochemically detectable Cdx2 is present in intestinal phenotypic elements in early gastric cancers of both differentiated and undifferentiated types, with no correlation to non-neoplastic surrounding mucosa

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It has previously been reported that Cdx2 is the useful prognostic and intestinal phenotypic marker in advanced gastric cancers (GC). In this study, Cdx2 expression and phenotype in early GC and non-neoplastic surrounding mucosa were examined. A total of 130 early GC (70 intramucosal and 60 submucosally invasive cancers) histologically and phenotypically were evaluated. The expression of Cdx2 was assessed by immunohistochemistry. The lesions were phenotypically divided into 44 gastric (G), 42 gastric and intestinal mixed (GI), 30 intestinal (I), and 14 null (N) types, independent of the histological classification. Most of the early GC were Cdx2-positive, nuclear staining being strongly associated with intestinal phenotypic expression. Early differentiated cancers tended to feature both Cdx2 and intestinal phenotypic expression, while their undifferentiated counterparts were more likely to demonstrate only gastric phenotypic expression ($P < 0.05$). The phenotypes of six intramucosal microcarcinomas did not correlate with those of adjacent normal glands. These data suggest that Cdx2 is expressed in the very early stage of gastric carcinogenesis in association with the shift from gastric to intestinal phenotypic expression. This appears to occur in differentiated cancers at an earlier stage than in undifferentiated ones, and may be linked to suppression of expansion of malignant cells.

Key words: Cdx2, early gastric cancers, histological type, immunohistochemistry, phenotype, stomach

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Human gastric carcinomas have been classified by Lauren into two major groups, the intestinal and diffuse types,[1] which respectively, nearly correspond to the differentiated and undifferentiated types of Nakamura *et al.*[2] and Sugano *et al.*[3] However, the above-mentioned classifications are inadequate for studies of histogenesis of gastric carcinomas and phenotype expression at the cellular level because they confuse intestinal phenotypic cancer cells with a diffuse structure and gastric phenotypic ones with the intestinal type of Lauren.[4] The phenotypic expression of gastric cancer (GC) 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.[4–11] It has also been suggested that intestinal-type carcinomas arise in intestinalized mucosa, while the diffuse-type develops from the gastric mucosa proper.[1,2,12,13] This hypothesis is based on morphological similarities between cancers and intestinal metaplasia (IM), and on the results of comparisons of carcinomas and surrounding mucosa.[9] However, previous studies on phenotypic expression of each IM or stomach cancer cells have pointed to several contradictions to this hypothesis.[6,7,9,14–20]

It is widely thought that the phenotypic expression of tumor cells resembles that of the tissue of origin. We have previously shown that GC at early stages, independent of the histological-type, mainly consist of gastric phenotypic cancer cells, and a shift from gastric to intestinal phenotypic expression is clearly observed with progression in experimental animal models.[14,15,21,22] We, and others, have also reported a similar phenomenon in human GC.[5,16,18,23–25] It is clearly of interest to determine what type of genes are associated with this phenotypic shift.

The *caudal*-related homeobox gene (Cdx) 2 is important for the maintenance of intestinal epithelial cells[26–29] and there have been several reports of Cdx2 expression in human gastric carcinomas and IM.[10,11,29–35] We, and others, have previously shown that Cdx2 is associated strongly with the intestinal phenotypic expression in GC.[10,11,33]

In this study, we analyzed Cdx2 expression by immunohistochemistry in 70 intramucosal cancers which are considered as dysplasia by Western pathologists, and 60 carcinomas with submucosal invasion with histological evaluation by hematoxylin–eosin (HE) staining and assessment of the phenotype by immunohistochemistry. The purpose was to evaluate the relation between Cdx2 expression and the phenotype in early GC and non-neoplastic surrounding mucosa.

MATERIALS AND METHODS

Samples and tissue collection

We examined 130 primary early GC surgically resected at Aichi Cancer Center Hospital, Nagoya, Japan, between 1995 and 1999. Histological classification was made according to the Japanese Classification of Gastric Carcinomas.[36] Out of the 130 early GC, 70 were intramucosal (m, T1 for TNM classification) and 60 demonstrated submucosal invasion (sm, T1 for TNM classification); patients in the former category ranged in age from 37 to 78 years (mean, 59.6 ± 8.4 years) and the latter from 32 to 84 years (mean, 60.7 ± 10.8 years). 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 HE for histological examination.

Immunohistochemistry

Immunohistochemical staining was carried out with monoclonal antibodies against the following antigens: Cdx2 (392M, 1:50; BioGenex, San Ramon, CA, USA); MUC5AC (CLH2, 1:500; Novocastra Laboratories, Newcastle, UK); MUC6 (CLH5, 1:500; Novocastra Laboratories); MUC2 (Ccp58, 1:500; Novocastra Laboratories); and villin (12, 1:20 000; Transduction Laboratories, Lexington, KY, USA). 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.[5,10,11,37,38] Briefly, 4 m-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 for detection of binding of the above-mentioned antibodies with 10 mmol/L citrate buffer (pH 6.0) in a microwave oven for 10 min at 120∝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% H_2O_2 and 0.05% 3,3 -diaminobenzidine tetrachloride (DAB). Nuclear counterstaining was accomplished with Mayer's hematoxylin.

The results of each antibody staining were evaluated in terms of the percentage of positively stained cancer cells, with >10% considered positive, as previously described.[9–11]

Classification of cancers

MUC5AC and MUC6 are markers of the gastric epithelial cell phenotype, while MUC2 and villin are typical of the intestinal epithelial cell phenotype.[5,8–11,39,40] Two independent pathologists (TT and KI) judged the histology and immunohistochemical staining of the phenotypic markers including Cdx2. Gastric cancers in which more than 10% 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 that 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).

Comparison of phenotypic expression between microcarcinomas and non-neoplastic surrounding mucosa

Typical findings for mucin and brush border elements in normal and metaplastic gastric mucosa are shown in Figs 1 and 2. Included in the 70 cases were six intramucosal microcarcinomas (defined as lesions less than 3.0 mm across).[9] There were no microcarcinomas with submucosal invasion. Non-neoplastic glandular ducts were divided histologically and phenotypically into four types: pyloric glandular duct, fundic glandular duct, gastric and intestinal mixed phenotype (GI) IM, solely intestinal phenotype (I) IM.[4,37] Pyloric glandular ducts are stained by MUC5AC in surface mucous cells and MUC6 in glandular cells, but are negative for intestinal epithelial cell markers. The fundic glandular duct similarly stains with MUC5AC in surface mucous cells and MUC6 in mucous neck cells, again being negative for intestinal epithelial cells

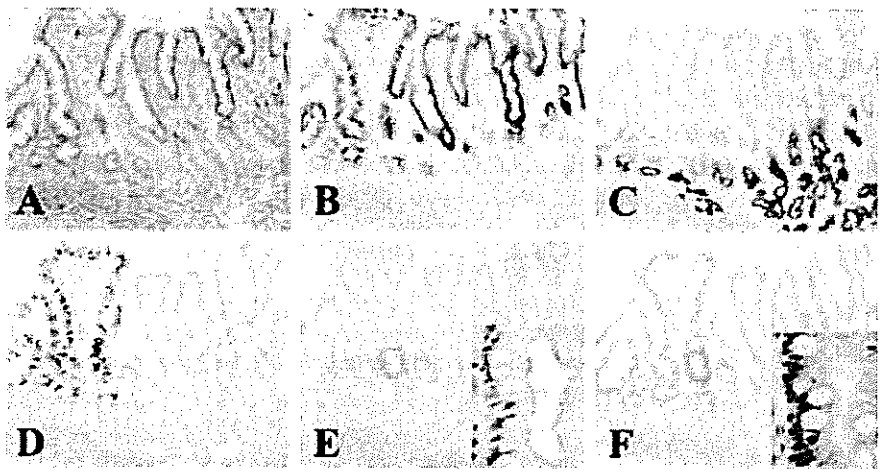

Figure 1 Pyloric glandular ducts (right), and the gastric and intestinal mixed phenotype intestinal metaplasia (IM) (left). (A) HE staining. (B) MUC5AC is positive in the cytoplasm of normal gastric foveolar epithelia and IM. (C) MUC6 is present in the cytoplasm of the normal pyloric glands and IM. (D) MUC2 is evident in the cytoplasm of IM, but not in the normal gastric mucosa. (E) Villin is weakly and partially positive at the luminal surface of IM, but not the normal gastric mucosa. Inset, higher magnification of the IM gland. (F) Cdx2 nuclear staining is apparent in the IM, but not in the normal gastric mucosa. Inset, higher magnification of the IM gland. (Original magnification, 80; red squares are magnified to insets at 1000.)

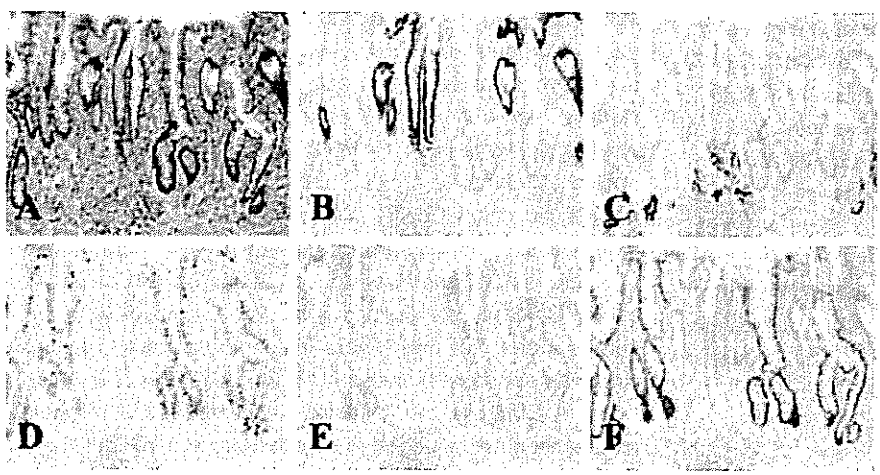

Figure 2 Fundic glandular ducts and solely intestinal phenotype intestinal metaplasia (IM). (A) HE staining. (B) MUC5AC is positive in the cytoplasm of the normal gastric foveolar epithelia, but not in IM. (C) MUC6 is present in the cytoplasm of the mucous neck cells, but not in IM. (D) MUC2 is evident in the cytoplasm of IM, but not in the normal gastric mucosa. (E) Villin is positive at the luminal surface of IM, but not the normal gastric mucosa. (F) Cdx2 nuclear staining is apparent in the IM, not in the normal gastric mucosa (original magnification, 80).

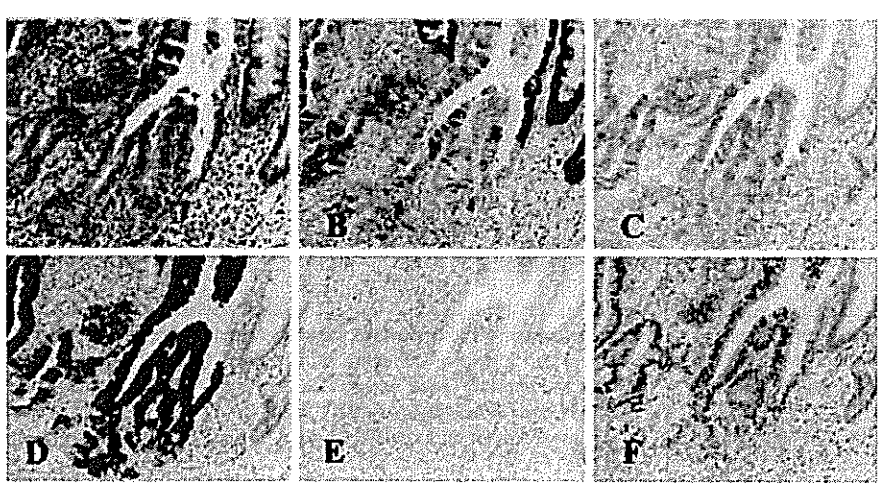

Figure 3 An early moderately-differentiated adenocarcinoma with both gastric and intestinal phenotypic expression with adjacent normal gastric mucosa (right). (A) HE staining; (B) MUC5AC is positive in the cytoplasm of cancer cells and the normal gastric foveolar epithelia; (C) MUC6 is present in the cytoplasm of cancer cells, but not in the normal gastric foveolar epithelia. (D) MUC2 is evident in the cytoplasm of cancer cells, but not in the normal gastric mucosa. (E) Villin is not detected in the luminal surface of either cancer cells, or the normal gastric mucosa. (F) Cdx2 nuclear staining is apparent in the cancer cells, not in the normal gastric mucosa (original magnification, 160).

markers. GI-IM has staining for at least one gastric epithelial cell, and at least one intestinal epithelial cell marker, while I-IM is positive for at least one intestinal epithelial cell marker, with no staining for gastric epithelial cell markers. Three glandular ducts on each side of the microcarcinoma were examined as non-neoplastic surrounding mucosa.

Statistical analysis

The data were analyzed by Fisher's exact test or ^{2} test between the groups. *P*-values <0.05 were considered as statistically significant.

RESULTS

Expression of gastric and intestinal epithelial cell markers in intramucosal gastric cancers

Typical findings for mucin and brush border staining in early GC are shown in Figs 3 and 4. Expression of MUC5AC, MUC6, MUC2, and villin was demonstrated in 41 (58.6%), 17 (24.3%), 30 (42.9%), and 30 (42.9%), respectively, of 70 intramucosal GC. Taking into account the combinations of expression of these four markers, the lesions were divided phenotypically into 22 G, 23 GI, 17 I, and eight N types, independent of the histological classification (Table 1). As compared with the undifferentiated type, the differentiated type statistically had significantly more intestinal phenotypic expression, while the undifferentiated type had more gastric phenotypic expression ($P = 0.0015$).

Expression of gastric and intestinal epithelial cell markers in gastric cancers with submucosal invasion

Expression of MUC5AC, MUC6, MUC2, and villin was demonstrated in 36 (60.0%), 20 (33.3%), 22 (36.7%), and 16 (26.7%), respectively, of 60 GC with submucosal invasion. Taking into account the combinations of expression of these four markers, the lesions were divided phenotypically into 22 G, 19 GI, 13 I, and six N types, independent of the histological classification (Table 2). As compared with the undifferentiated type, differentiated lesions statistically had significantly more intestinal phenotypic expression, while the undifferentiated type had more gastric phenotypic expression ($P = 0.0013$).

Immunohistochemical analysis of Cdx2 in intramucosal gastric cancers

Cdx2 nuclear staining was detected in IM mucosa, but not normal gastric epithelium (Figs 1,2). Of the 70 intramucosal GC, 54 and 16 cases were judged to be Cdx2-positive and -negative, independent of the histological types (Table 1). In the GI- and I-type GC, the areas of the Cdx2-positive nuclear staining were in perfect accord with those demonstrating intestinal phenotypic expression (Figs 3,4). The results of each antibody staining were evaluated using the percentage of positively stained cancer cells, and G- or N-type GC occasionally had <10% of the cancerous areas with intestinal phenotypic expression. However, these cases also exhibited nuclear staining of Cdx2 in the minor area of intestinal phenotypic cancer cells, without exception. Of 22 G-type intramucosal cancers, three differentiated and seven undifferentiated cancers were judged Cdx2-positive. In these cases, Cdx2 nuclear staining was detected in >10% of cancerous areas, not only where intestinal phenotypic expression was apparent but also in tissue exhibiting only gastric markers. Similarly, one differentiated and three undifferentiated cancers were judged to be Cdx2-positive in eight N-type intramucosal cancers, in which Cdx2 nuclear staining was also detected outside the cancerous area with intestinal phenotypic expression (Fig. 5). The rates for Cdx2-positive cases in the differentiated and undifferentiated types were 91.7% and 61.8%, respectively, the difference being significant ($P = 0.0029$).

Table 1 Histological and phenotypic classification, and Cdx2 expression in 70 intramucosal gastric carcinomas

Histological classification‡	Phenotypic classification† G	GI	I	N	Total
Differentiated	6 (3)	15 (15)	14 (14)	1 (1)	36 (33)§
Undifferentiated	16 (7)	8 (8)	3 (3)	7 (3)	34 (21)
Total	22 (10)	23 (23)	17 (17)	8 (4)	70 (54)

†The number of Cdx2 positive cases are given in the parentheses.

‡Classified based on structure of elements. 'Differentiated' includes tubular and papillary types, while 'undifferentiated' consists of signet-ring cell and poorly differentiated types.

§Differentiated versus undifferentiated, $P = 0.0029$ with Fisher's exact test.

Table 2 Histological and phenotypic classification, and Cdx2 expression in 60 gastric carcinomas with submucosal invasion

Histological classification‡	Phenotypic classification† G	GI	I	N	Total
Differentiated	5 (4)	11 (11)	11 (11)	3 (1)	30 (27)§
Undifferentiated	17 (9)	8 (8)	2 (2)	3 (2)	30 (21)
Total	22 (13)	19 (19)	13 (13)	6 (3)	60 (48)

†The number of Cdx2 positive cases are given in the parentheses.

‡Classified based on structure of elements. 'Differentiated' includes tubular and papillary types, while 'undifferentiated' consists of signet-ring cell and poorly differentiated types.

§Differentiated versus undifferentiated, $P = 0.053$ with Fisher's exact test.

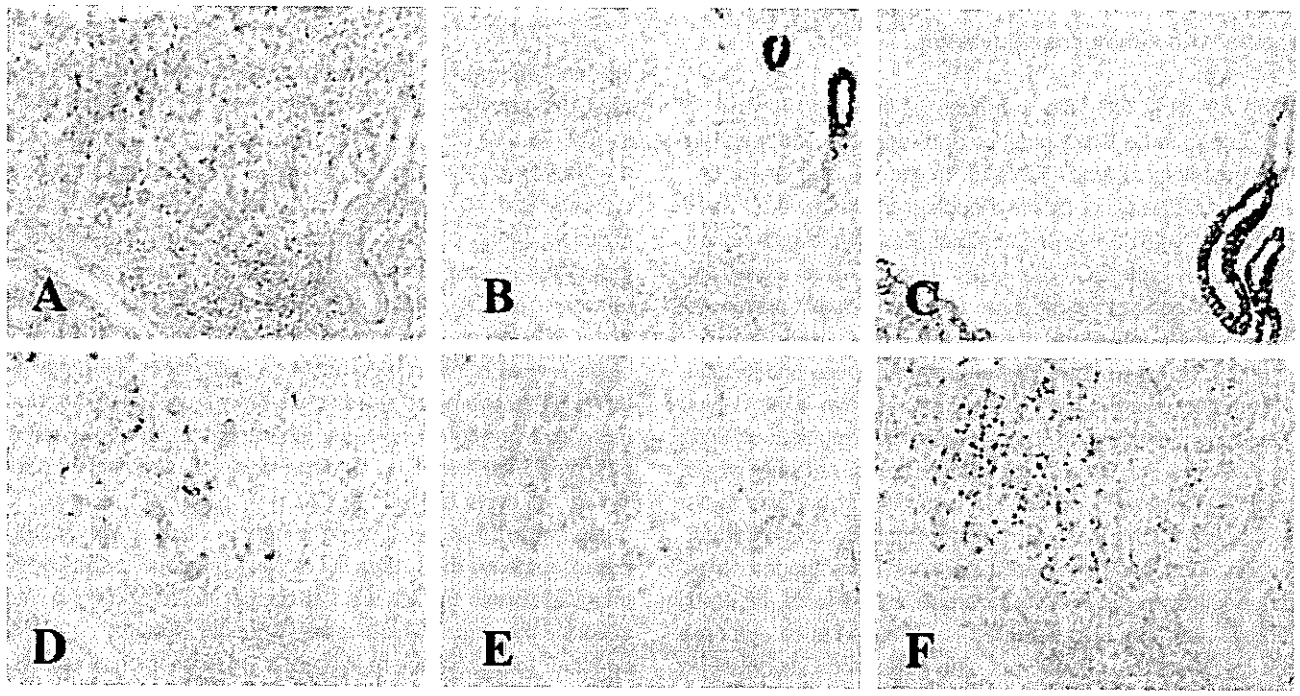

Figure 4 A signet-ring cell carcinoma with intestinal phenotypic expression and adjacent gastric foveolar epithelium. (A) HE staining. (B) MUC5AC is positive in the cytoplasm of the normal gastric foveolar cells, but not cancer cells. (C) MUC6 is present in the cytoplasm of the normal pyloric glands, but not cancer cells. (D) MUC2 is evident in the cytoplasm of cancer cells, but not in the normal gastric mucosa. (E) Villin is partially positive in the cytoplasm as well as the luminal surface of cancer cells, but not the normal gastric mucosa. (F) Cdx2 nuclear staining is apparent in the cancer cells, but not in the normal gastric mucosa (original magnification, 160).

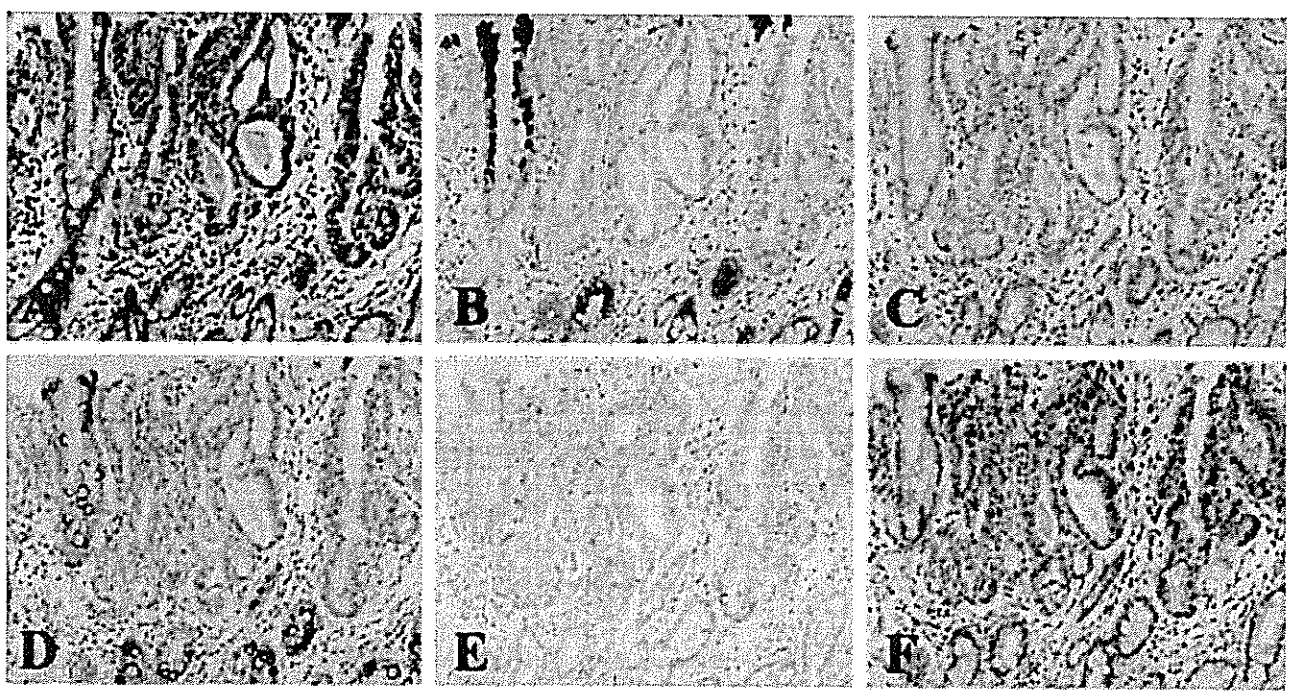

Figure 5 A well-differentiated adenocarcinoma adjacent to gastric and intestinal mixed phenotype intestinal metaplasia (left). (A) HE staining. (B) MUC5AC is positive in the cytoplasm of intestinal metaplasia (IM), but not in cancer cells. (C) MUC6 is not present in the cytoplasm of either cancer cells or IM. (D) MUC2 is evident in the cytoplasm of the IM, but not in the cancer cells. (E) Villin is not detected at the luminal surface of either cancer cells, or IM. (F) Cdx2 nuclear staining is apparent in both the cancer cells and IM (original magnification, 160).