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Gastric and intestinal phenotypes and histogenesis of advanced glandular stomach cancers in carcinogen-treated, *Helicobacter pylori*-infected Mongolian gerbils

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The *Helicobacter pylori*-infected Mongolian gerbil (MG) has been established as an appropriate animal model for studies of stomach cancer development. However, there have hitherto been no data on the phenotypic classification of glandular stomach cancers in *H. pylori*-infected and non-infected MG. We therefore examined the phenotypes of 50 and six advanced glandular stomach cancers in *H. pylori*-infected and non-infected MG, respectively, as well as adjacent non-neoplastic mucosa, using several gastrointestinal epithelial phenotypic markers. The lesions were divided phenotypically into 21 gastric, 24 gastric-and-intestinal mixed, four intestinal and one null types, with 90.0% of the lesions harboring gastric elements and 56.0% demonstrating intestinal phenotypic expression in *H. pylori*-infected MG. All six lesions were classified as gastric type in non-infected MG. There was no clear correlation with the presence of intestinal metaplasia in surrounding mucosa. In conclusion, our data suggest that most advanced adenocarcinomas retain a gastric cellular phenotype in the glandular MG stomach. Thus, it might be proposed that intestinal metaplasia is a paracancerous phenomenon rather than a premalignant condition. *H. pylori* infection may trigger intestinalization of both stomach cancers and non-neoplastic mucosa. (*Cancer Sci* 2006; 97: 38–44)

Histologically, human gastric cancers present as two major groups, the 'intestinal' and 'diffuse' types of Lauren,[1] which correspond approximately to the 'differentiated' and 'undifferentiated' types, respectively, of Nakamura et al.[2] and Sugano et al.[3] It has also been suggested that intestinal type carcinomas arise in intestinalized mucosa, whereas the diffuse type develops from the gastric mucosa proper.[1,2,4,5] This hypothesis is based on morphological similarities between cancers and intestinal metaplasia (IM), and on the results of comparisons of carcinomas and surrounding mucosa.[6] However, previous studies on phenotypic expression and microsatellite instability (MSI) of each IM or stomach cancer cells have pointed to several contradictions to this hypothesis.[6–17] The phenotypic expression of stomach cancer cells of each histologic type can be classified clearly into gastric and intestinal epithelial cell types by immunohistochemistry and mucin histochemistry using gastrointestinal epithelial cell phenotypic markers such as human gastric mucin (HGM), intestinal type alkaline phosphatase (I-ALP), paradoxical concanavalin A staining (PCS), and Alcian blue–periodic acid Schiff staining (AB-PAS).[18,19]

The Helicobacter pylori-infected Mongolian gerbil (MG) has been established as an appropriate animal model for the study of stomach cancer development, with induction of adenocarcinomas by N-methyl-N-nitrosourea (MNU) and N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) as the carcinogens.[20–24] We previously demonstrated promoting effects on tumor development due to H. pylori infection in the MG model,[22–25] and several studies based on detailed histopathological assessment have shown no carcinomas in animals treated only with H. pylori infection, suggesting that H. pylori is a strong promoter of gastric carcinogenesis rather than an initiator.[21–23,26] Eradication of infection results in the curtailment of enhancing effects, particularly in the early stages of associated inflammation.[21,27,28] However, there have hitherto been no data on the phenotypic classification of glandular stomach cancers arising in H. pylori-infected and non-infected MG. As evaluation of the phenotype is very important with reference to histogenesis, the present study was conducted.

We here examine the phenotypes of advanced glandular stomach cancers, as well as adjacent non-neoplastic mucosa, using several gastrointestinal epithelial phenotypic markers in 50 H. pylori-infected and six non-infected MG treated with the carcinogen MNU. An especial focus was on the comparison of small tubular lesions less than 10 mm in diameter and the surrounding mucosa in H. pylori-infected and non-infected MG.

Materials and Methods

Samples and tissue collection

Advanced carcinomas of the glandular stomach in 50 H. pylori-infected and six non-infected MG treated with MNU[21,29] were classified histopathologically according to the Japanese Classification of Gastric Carcinomas.[30] Tumor

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tissues and adjacent non-neoplastic mucosa were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.2), Bouin's solution, 10% buffered formalin, or 95% ethanol containing 1% acetic acid,[25,28,31] sectioned at 5 μm, and stained with hematoxylin and eosin for histologic examination. All of the cancers analyzed demonstrated invasion into the muscularis propria, the subserosa, or the serosa and the peritoneal cavity, sometimes with involvement of adjacent organs.

Immunohistochemistry and mucin histochemistry

Immunohistochemical staining was carried out with antibodies against the following antigens: HGM (Novocastra Laboratories, Newcastle-upon-Tyne, UK); small intestinal mucinous antigen (SIMA) (Novocastra); I-ALP (kindly provided by Dr Kazuyuki Hirano, Department of Pharmaceutics, Gifu Pharmaceutical University, Gifu, Japan); and CD10 (Novocastra) (Table 1). The precise procedures for immunohistochemical analysis were as described previously.[15,18,19,32–34] With regard to gastric phenotypic markers, we used normal gastric mucosa and normal ileum as positive and negative controls, respectively, or vise versa for intestinal phenotypic controls. 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 by heating in 10 mM citrate buffer (pH 6.0) in a microwave oven for 10 min at 98°C. Sections were then incubated with primary antibodies. After thorough washing in PBS, they were next incubated with biotinylated secondary antibodies, and then with an avidin–biotin 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. Nuclear counterstaining was accomplished using Mayer's hematoxylin.

For mucin histochemistry, we used PCS for identifying class III mucins in mucous neck and pyloric gland cells.[35,36] We also carried out AB–PAS for identifying gastric surface mucous cells, with mucin staining red and goblet cells staining blue (Table 1).[26,33]

Two independent pathologists (TM and TT) judged the histology and the immunohistochemical and mucin histochemical stainings of the phenotypic markers. With regard to the phenotypic markers, the results of immunohistochemical and mucin histochemical stainings were evaluated in terms of the percentage of positively stained cancer cells, with 10% and above considered positive, as described previously.[15,18,19,32,34]

Classification of cancers. Tumors were classified phenotypically with reference to the expression patterns of the phenotypic markers (Table 1).[15,18,19,32,34] Glandular stomach 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 phenotype (G type) or intestinal phenotype (I type) cancers, respectively. Those that showed both gastric and intestinal phenotypes were classified as gastric-and-intestinal mixed phenotype (GI type) cancers, whereas those showing neither gastric nor intestinal phenotype expression were grouped as null (N type).

Table 1. Phenotypic markers for gastrointestinal epithelial cells

Tissue type	Cell type	Marker
Gastric	Foveolar	HGM, PAS (mucin stained red)
	Pyloric	PCS
Intestinal	Goblet	AB (mucin stained blue), SIMA
	Absorptive	I-ALP, CD10

AB, Alcian blue staining; HGM, human gastric mucin; I-ALP, intestinal type alkaline phosphatase; PAS, periodic acid–Schiff staining; PCS, paradoxical concanavalin A staining; SIMA, small intestinal mucinous antigen.

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

Non-neoplastic glandular ducts were divided histologically and phenotypically into three types: (i) gastric phenotypic glandular (G type gland), consisting of at least one gastric epithelial cell phenotype; (ii) gastric-and-intestinal mixed phenotypic glandular (GI type gland), showing both gastric and intestinal phenotypes; and (iii) intestinal phenotypic glandular (I type gland), harboring at least one intestinal counterpart.[15,18,33] Particular attention was paid to comparisons of adjacent normal glands with tubular adenocarcinomas less than 10 m in diameter in 13 H. pylori-infected and five non-infected MG.

Results

Phenotypic expression of non-neoplastic glandular ducts in the MG glandular stomach

Typical findings for immunohistochemical and mucin histochemical staining in the non-cancerous mucosa of glandular stomach are illustrated in Fig. 1. HGM was detected in the cytoplasm of foveolar epithelial cells of the pyloric (Fig. 1A,F) and fundic glandular ducts. It was also found in GI-IM. PCS was observed in the cytoplasm of normal pyloric glands (Fig. 1A,G) and mucous neck cells. In GI-IM, the cytoplasm of columnar and goblet cells was stained red and blue, respectively, with AB–PAS (Fig. 1B,H). Regarding I-IM, the cytoplasm of goblet cells was stained blue with AB, while that of columnar cells was not stained red with PAS (Fig. 1C,I). I-ALP and CD10 were apparent at the luminal surfaces of absorptive cells in the duodenum (Fig. 1D,E,J). SIMA was evident in the cytoplasm of goblet cells in the duodenum (Fig. 1D,K).

Phenotypic classification of advanced glandular stomach cancers in *H. pylori*-infected and non-infected MG treated with MNU

Typical findings for mucin and brush border staining in glandular stomach cancers are shown in Figs 2 and 3. We evaluated 44 differentiated and six undifferentiated glandular stomach cancers of H. pylori-infected MG phenotypically using several epithelial phenotypic markers. The lesions were divided phenotypically into 21 G, 24 GI, four I and one N type (Table 2). Of the 44 differentiated type cancers, 39 (88.6%) had gastric phenotypic expression, whereas 22 (50%) harbored intestinal elements. All six undifferentiated

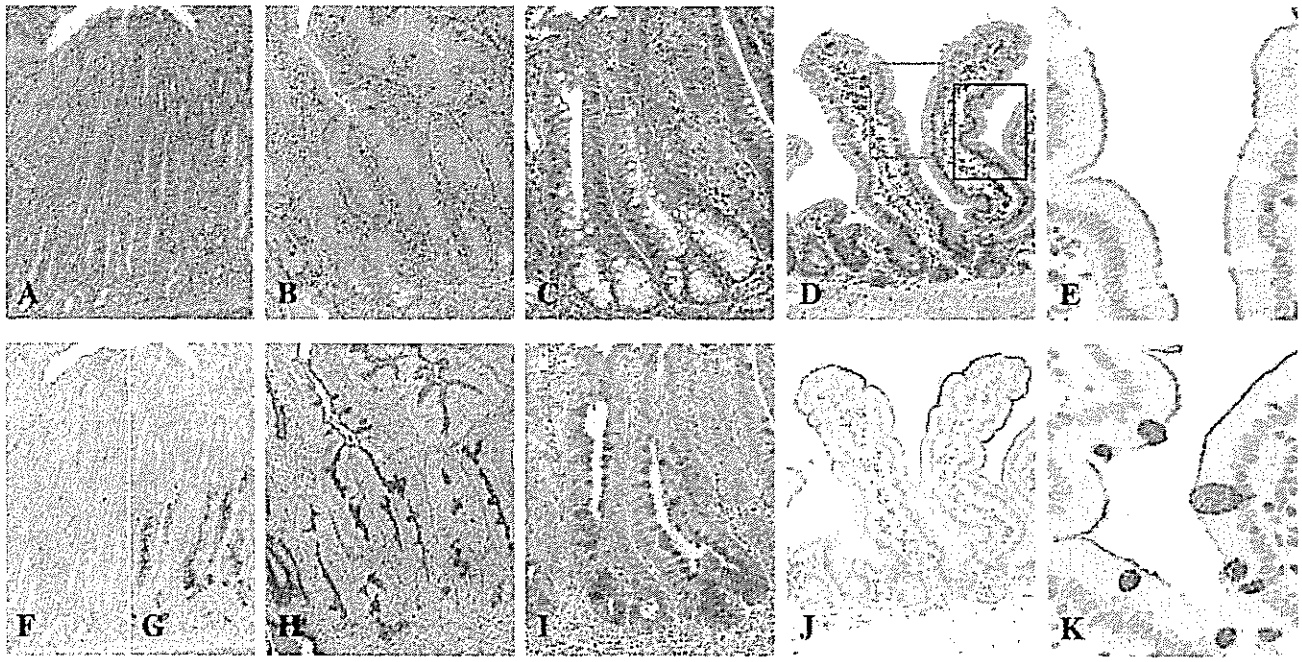

Fig. 1. Expression of gastrointestinal phenotypic markers in non-neoplastic mucosa. (A,F,G) gastric (G type) glands; (B,H) gastric-and-intestinal mixed (GI type) glands; (C,I) intestinal (I type) glands; and (D,E,J,K) normal duodenal glandular ducts. (A) H&E staining of pyloric glandular ducts. (B) H&E staining of GI-IM. (C) H&E staining of I-IM. (D) H&E staining of duodenal glandular ducts. (E) Higher magnification of the red square in part D. Note the presence of CD10 at the luminal surface of absorptive cells in the duodenum. (F) Human gastric mucin is evident in the cytoplasm of normal foveolar epithelial cells in the pyloric mucosa. (G) Paradoxical concanavalin A staining is present in the cytoplasm of normal pyloric gland cells. (H) The cytoplasm of columnar and goblet cells in the IM is stained red and blue, respectively, with AB–PAS. (I) The cytoplasm of goblet cells in IM is stained blue with AB, while that of columnar cells is not stained red with PAS. (J) Intestinal type alkaline phosphatase is present at the luminal surface of absorptive cells in duodenum. (K) Higher magnification of the blue square in part D. Small intestinal mucinous antigen is evident in the cytoplasm of goblet cells in the duodenum. Original magnification: A, F and G, ×100; B, D, H and J, ×160; C and I, ×200; E and K, ×640. AB–PAS, Alcian blue–periodic acid Schiff staining; GI-IM, gastric-and-intestinal mixed phenotype IM; IM, intestinal metaplasia; I-IM, solely intestinal phenotype IM.

Table 2. Histologic and phenotypic classification in advanced carcinomas of glandular stomach in 50 *Helicobacter pylori*-infected and six non-infected Mongolian gerbils treated with *N*-methyl-*N*-nitrosourea

H. pylori	Histologic classification†	Phenotypic classification‡				
		G	GI	I	N	Total
Infected	Differentiated	21	18	4	1	44
	Undifferentiated	0	6	0	0	6
	Subtotal	21	24	4	1	50
Non-infected	Differentiated	5	0	0	0	5
	Undifferentiated	1	0	0	0	1
	Subtotal	6	0	0	0	6

†Classification based on the structure of elements. 'Differentiated' includes tubular types, whereas 'undifferentiated' consists of signet-ring cell and poorly differentiated types. ‡The numbers of adenocarcinomas possessing intestinal phenotypic expression are 24 gastric-and-intestinal mixed (GI) and four intestinal (I) types in *H. pylori*-infected gerbils (57% among gastric [G], GI and I types), while corresponding cases do not exist in non-infected animals, the former was significantly higher (Fisher's exact test, $P < 0.05$). Null (N) type is excluded from this statistical analysis.

type cancers were judged as GI type. We also evaluated five differentiated and one undifferentiated glandular stomach cancers developing in non-infected MG, all six lesions being of G type (Table 2). The lesions of H. pylori-infected groups had more intestinal phenotypic expression compared with those of non-infected groups ($P < 0.05$). Tumor histology was mostly homogeneous in the gerbil stomach adenocarcinomas in the current study.

Relationships between carcinomas of tubular structure and non-neoplastic surrounding mucosa in the glandular stomach of *H. pylori*-infected and non-infected MG from the viewpoint of phenotypic expression

The relationship between adenocarcinomas of tubular structure, which measured less than 10.0 mm in largest dimension, and adjacent non-neoplastic surrounding mucosa is shown in Table 3. Of 13 lesions, 12 (92.3%) had non-neoplastic surrounding mucosa with G type glands. One G type lesion (case 7) had adjacent non-cancerous mucosa harboring GI and I type glands, and no G type glands. The non-neoplastic surrounding mucosa had only G type glands in seven lesions of G type (cases 1, 2, 3, 4, 5, 6 and 8) (Table 3). In the remaining six cases (cases 7, 9, 10, 11, 12 and 13), the phenotypes of the adenocarcinomas of tubular structure did not coincide with those of non-neoplastic surrounding mucosa.

Regarding the glandular stomach cancers in the non-infected MG, the non-neoplastic surrounding mucosa had only G type glands in all of the five G type tubular lesions.

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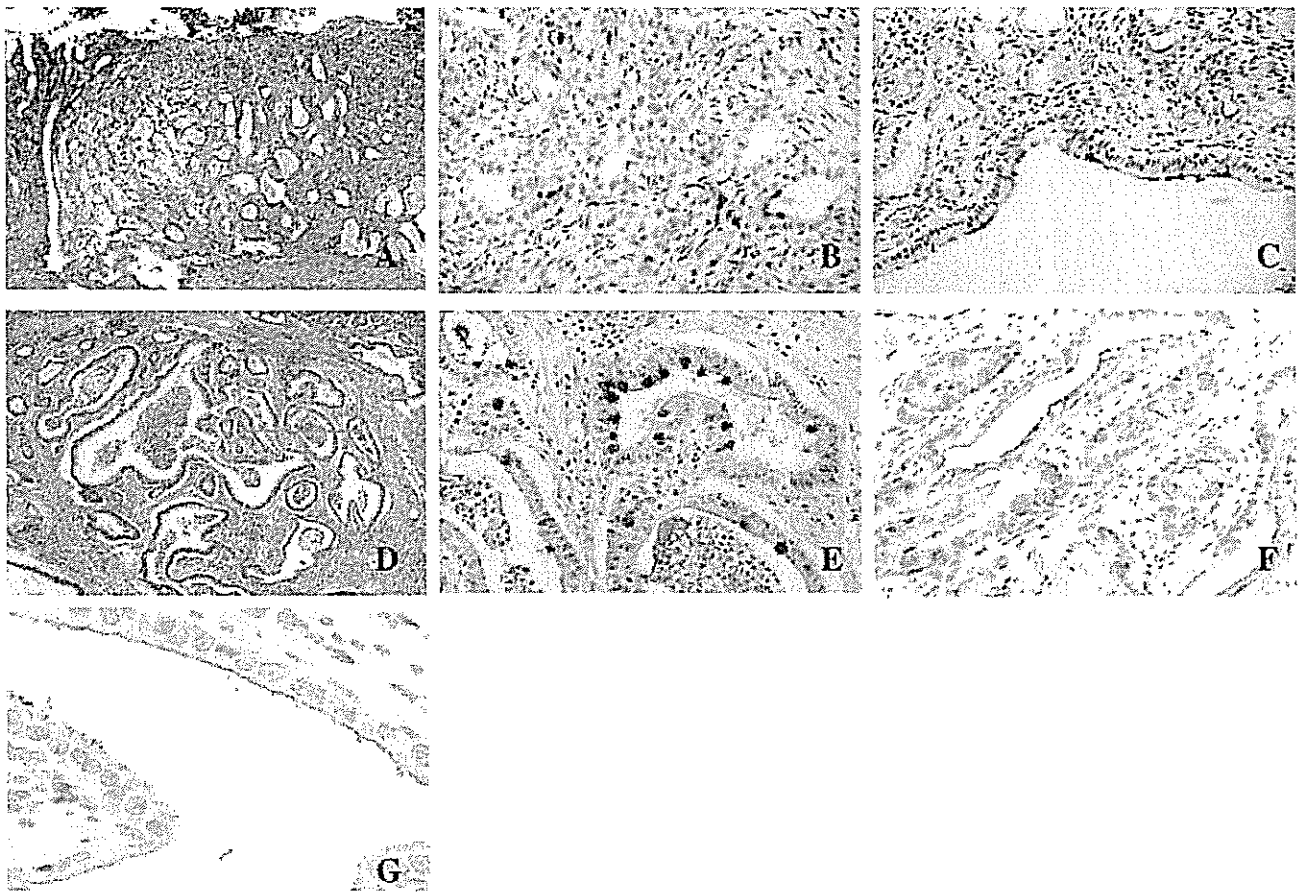

Fig. 2. Histology and phenotype of well-differentiated adenocarcinomas: (A–C) gastric (G type) and (D–G) intestinal (I type). (A) H&E staining of the G type tumor. (B) Human gastric mucin is present in the cytoplasm of tumor cells. (C) Paradoxical concanavalin A is present in the cytoplasm of cancer cells. (D) H&E staining of the I type tumor. (E) Higher magnification of the red square in part D. The cytoplasm of cancer cells is stained blue by Alcian blue–periodic acid Schiff staining. (F) Small intestinal mucinous antigen is evident in the cytoplasm of cancer cells. (G) CD10 is present at the luminal surface of cancer cells. Original magnification: A, ×40; D, ×100; B and C, ×200; E and F, ×320; G, ×640.

Table 3. Relationships between carcinomas and adjacent non-neoplastic mucosa in the glandular stomach of *Helicobacter pylori*-infected Mongolian gerbils treated with *N*-methyl-*N*-nitrosourea

Number	Clinicopathological findings of tumors			Phenotype of adjacent non-neoplastic mucosa		
	Histologic type	Depth	Phenotype	G type glands	GI type glands	I type glands
Case 1	well	mp	G	+	–	–
Case 2	well	mp	G	+	–	–
Case 3	well	mp	G	+	–	–
Case 4	mod	mp	G	+	–	–
Case 5	well	ss	G	+	–	–
Case 6	well	ss	G	+	–	–
Case 7	well	ss	G	–	+	+
Case 8	mod	ss	G	+	–	–
Case 9	mod	ss	G	+	+	–
Case 10	well	ss	GI	+	–	–
Case 11	well	ss	GI	+	–	–
Case 12	well	mp	I	+	–	–
Case 13	well	ss	I	+	+	–

mod, moderately differentiated adenocarcinoma; por, poorly differentiated adenocarcinoma; sig, signet-ring cell carcinoma; well, well differentiated adenocarcinoma. Tumor types are gastric (G), intestinal (I) and gastric-and-intestinal mixed (GI).

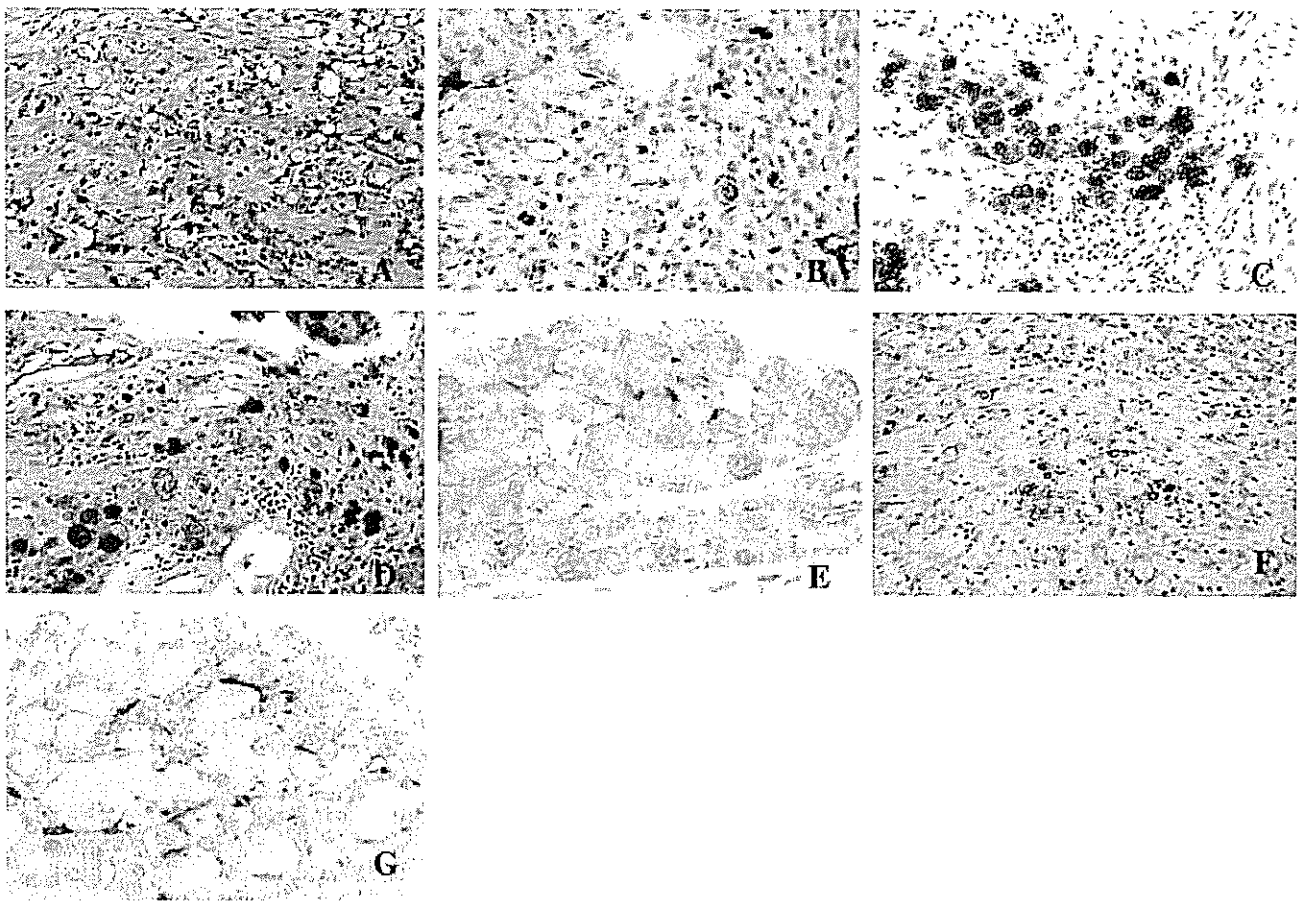

Fig. 3. Histology and phenotype of poorly differentiated adenocarcinomas: (A–C) gastric (G type) and (D–G) intestinal (I type). (A) H&E staining of the G type tumor. (B) Human gastric mucin is present in the cytoplasm of malignant cells. (C) Paradoxical concanavalin A is present in the cytoplasm of tumor cells. (D) The cytoplasm of tumor cells was stained blue by Alcian blue–periodic acid Schiff staining. (E) Small intestinal mucinous antigen is evident in the cytoplasm of cancer cells. (F) I-ALP is positive at the luminal surface of cancer cells. (G) CD10 is present at the luminal surface of cancer cells. Original magnification: A–D and F, ×200; E and G, ×640.

Discussion

Our present data provide clear evidence that most advanced adenocarcinomas of the glandular stomach in H. pylori-infected MG treated with MNU are characterized by gastric phenotypic expression. All of the six lesions were classified phenotypically as G type in non-infected MG treated with MNU. We have shown previously that experimentally induced adenocarcinomas in the rat glandular stomach consist mainly of gastric epithelial phenotypic cancer cells, with intestinal epithelial phenotypic cancer cells appearing later in larger tumors.[7,8,37,38] Similarly, in mice, experimentally induced adenocarcinomas in the glandular stomach[39,40] consist mainly of gastric epithelial phenotypic tumor cells.[41] We and others have also reported that the phenotypic shift from gastric to intestinal phenotypic expression occurs in accordance with increasing depth of invasion in human signet ring cell carcinomas and with progression in human differentiated gastric cancers.[42–44] Early stage papillary (papillary dominant) stomach cancers show significantly higher and more widespread high-frequency microsatellite instability (MSI-H) than other morphological types, and inactivation of human MutL homologue 1 (hMLH1) expression by promoter hypermethylation may be an early event in carcinogenesis of this type of stomach cancer.[45,46] MSI-positive differentiated gastric cancers with gastric foveolar phenotypic expression in the early stages sometimes demonstrate intestinal phenotypic expression in advanced stages.[47] Taking into account the previous reports and our present data, we consider that the same phenotypic shift occurs in both rodents and humans, and H. pylori infection may trigger intestinalization of stomach cancers and non-neoplastic mucosa.

It has been suggested that differentiated gastric carcinomas arise from mucosa with IM, but that undifferentiated gastric cancers originate from mucosa without IM in view of morphological similarities between each cancer and the surrounding mucosa.[1,2,4,5] However, this has not been supported by previous studies on phenotypic expression and MSI of individual intestinal metaplastic or stomach cancer cells.[6–17] We here found seven G type tubular lesions with non-neoplastic surrounding mucosa solely of G type, but in the remaining six cases the tumor phenotype did not match that of the immediately adjacent normal glands in H. pylori-infected MG (Table 3). Regarding the glandular stomach

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cancers in non-infected MG, the non-neoplastic surrounding mucosa had only G type glands in all five G type lesions. Heterotopic proliferative glands (HPG) frequently develop with H. pylori infection in the glandular stomach of infected MG, with slight dysplastic change of constituent cells.[26] They often resemble differentiated or mucinous adenocarcinomas, but do not appear to be malignant, disappearing with little evidence of persistence after eradication of bacteria. However, HPG also show a phenotypic shift from G type to GI or I type with the appearance of Paneth cells during the overall course of H. pylori infection.[26] Intestinalization progresses from G through GI to I type in non-cancerous and cancerous tissue independently in humans and MG.[18,48] Thus, it has been proposed that IM is important not as a precancerous lesion but as a paracancerous phenomenon.[11,12] Therefore, many questions remain regarding its pathogenesis as well as the actual relationship to gastric cancers.[18,48] In the present study, of 13 lesions, 12 (92.3%) had non-neoplastic surrounding mucosa with G type glands in H. pylori-infected MG treated with the carcinogens. In the rat, Tatematsu et al. have proposed pepsinogen 1-altered pyloric glands, which are low in pepsinogen 1 but appear normal in the pyloric mucosa after MNNG treatment, as putative preneoplastic lesions in the glandular stomach.[49–51] Thus, a kind of G type gland may be precancerous. We therefore consider that the origin of stomach cancers might be clarified by analysis of the genetic alteration in stomach cancers and non-neoplastic G type glands in H. pylori-infected MG treated with the carcinogens.

Stomach cancers frequently show variable morphology within individual tumors in humans and this may reflect different genetic alterations. A strong relationship has been observed between early stage papillary stomach cancer and MSI-H, and promoter hypermethylation of hMLH1 may be important for the development of this type of stomach cancer.[45] Mismatch repair deficiency in MSI-positive tumors causes multiple gene inactivations through frameshift mutations in short repetitive sequences in a heterogenous way within a histologically heterogenous tumor.[52] Song et al.[46] also suggested that a modest centromere numerical abnormality might be another characteristic of stomach cancer characterized by a papillary structure. Further studies of genetic alterations in stomach cancers of MG may be needed to clarify the different carcinogenetic pathways for various types of human stomach cancers.

In conclusion, our data suggest that most advanced adenocarcinomas retain a gastric cellular phenotype in the glandular MG stomach. Thus, it might be proposed that IM is a paracancerous phenomenon rather than a premalignant condition. H. pylori infection may trigger intestinalization of both stomach cancers and non-neoplastic mucosa.

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Mutations and nuclear accumulation of β-catenin correlate with intestinal phenotypic expression in human gastric cancer

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Mutations and nuclear accumulation of β-catenin correlate with intestinal phenotypic expression in human gastric cancer

Aims: Abnormal localization of β-catenin is frequently observed in human gastric cancers. The aim of the present study was to evaluate relationships among gastrointestinal differentiation phenotypes, β-catenin localization and mutations of *Wnt* signalling genes.

Methods and results: Seventy-seven regions in 39 gastric adenocarcinomas were classified according to β-catenin localization and gastric and intestinal phenotypes. Cases with membranous β-catenin localization showed a gradual decrease from gastric (G) (55% = 6/11) and gastric-and-intestinal-mixed (GI) (17% = 5/29) to intestinal (I) (0% = 0/21) phenotypes, while those with nuclear localization showed a concomitant increase: 18% (2/11), 41% (12/29), 95% (20/21) and 63% (10/16) for G, GI, I and null type (N), respectively ($P < 0.001$, membranous versus nuclear localization in G, GI through I). Mutations in exon 3 of the *β-catenin* gene were found in G (50% = 1/2), GI (67% = 8/12), I (45% = 9/20) and N (0% = 0/10) regions with nuclear β-catenin localization (GI versus N, $P < 0.01$; I versus N, $P < 0.05$). *Adenomatous polyposis coli* (*APC*) gene mutations were demonstrated only in GI, I and N types, irrespective of β-catenin localization. Molecular analysis of these genes revealed 10 tumours to be heterogeneous out of 16 informative cases (62.5%).

Conclusion: Intestinal phenotypic expression is accompanied by a shift from membranous to cytoplasmic/nuclear accumulation of β-catenin. In contrast, N-type regions may progress along a different pathway.

Keywords: β-catenin, *adenomatous polyposis coli* gene (*APC*), gastric cancer, intestinal phenotypic expression, nuclear accumulation

Abbreviations: APC, adenomatous polyposis coli; G, gastric type; GI, gastric-and-intestinal-mixed type; I, intestinal type; N, null type; PCR-SSCP, polymerase chain reaction-single-strand conformation polymorphism

Introduction

β-Catenin plays an important role in cell–cell adhesion and in wingless/Wnt signalling.[1] The oncogenic potential of β-catenin is derived from its nuclear pooling, which is associated with up-regulation of members of the T cell factor family of transcription factors. The resulting transcription complex activates genes such as those for c-myc and cyclin D1 involved in proliferation.[2,3] The frequency of β-catenin activation has been estimated to be 0–5% using gastric cancer tissue samples.[4,5] In contrast, colorectal adenocarcinomas show a higher mutation frequency ranging from 20% to 25%.[6,7] Further studies have revealed that nuclear localization of β-catenin occurs in 12–37% of cases of gastric cancer on the basis of immunohistochemistry.[8,9] Histologically, human gastric adenocarcinomas fall into

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two major groups, the 'intestinal' and 'diffuse' types of Lauren,[10] which almost correspond to the 'differentiated' and 'undifferentiated' types of Nakamura *et al.*[11] and Sugano *et al.*[12] Although these classifications have been widely used, they may not be appropriate for studies of the histogenesis of gastric adenocarcinoma and phenotypic expression at the cellular level, because they may engender confusion of the intestinal phenotype with a 'diffuse' structure and the gastric phenotype with the gland-forming 'intestinal' type of Lauren.[13] It is reported that gastric adenocarcinomas at an early stage, independent of histological type, consist mainly of gastric phenotype malignant cells expressing the gastric mucins, MUC5AC and/or MUC6. The intestinal phenotypes show emergence of MUC2 intestinal mucin, villin structural protein, or intestinal alkaline phosphatase expression, which then increases with progression.[14,15] However, the clinicopathological significance of this variation in phenotypic expression in gastric adenocarcinomas remains to be clarified. Several authors have demonstrated a correlation between prognosis and phenotypic markers in gastric adenocarcinoma,[16–18] but concrete conclusions have yet to be drawn. In addition, the relation between phenotypic expression and alteration of the Wnt signalling pathway also remains unclear.

In this study, therefore, we analysed the localization of β-catenin immunohistochemically and randomly selected small homogeneous areas with nuclear, cytoplasmic or membranous β-catenin localization. Then, we evaluated the relationship between phenotype and localization as well as mutations of *β-catenin* and *adenomatous polyposis coli* (*APC*) in these areas.

Materials and methods

SAMPLE AND TISSUE COLLECTION

This study was approved by the Ethical Review Board at the Aichi Cancer Centre and carried out after obtaining informed consent. We examined 39 primary gastric adenocarcinomas surgically resected at Aichi Cancer Centre Hospital between 1991 and 2002. The patients were 23 men with an average age of 68.4 ± 7.8 (SD) years (range 54–82 years) and 16 women aged 60.2 ± 14.8 years (range 43–78 years). All specimens were routinely processed and stained with haematoxylin and eosin (H&E) for histological examination.

LOCALIZATION OF β-CATENIN EXPRESSION

Gastric adenocarcinomas were analysed for immunoreactivity of β-catenin (clone14; BD Transduction Laboratories, Lexington, KY, USA), which was divided into three types: membranous, cytoplasmic and nuclear as previously reported.[19] Regions stained homogeneously with anti-β-catenin antibody were measured using a micrometer and were chosen, if > 2 mm in diameter, for further phenotypic analysis and microdissection. The regions were also classified into glandular (differentiated) and diffuse/solid (undifferentiated) with regard to their structure.

GASTRIC AND/OR INTESTINAL PHENOTYPIC CLASSIFICATION OF GASTRIC ADENOCARCINOMA REGIONS

MUC5AC (CLH2; Novocastra Laboratories, Newcastle upon Tyne, UK) and MUC6 (CLH5; Novocastra) are markers of gastric epithelial cells, whereas MUC2 (Ccp58; Novocastra), villin (12; BD Transduction Laboratories) and Cdx2 (CDX2-88; BioGenex, San Ramon, CA, USA) are typical of an intestinal epithelial cell phenotype. We first classified gastric cancers according to their gastric and intestinal phenotypic expression. Tumours in which > 10% of the section area consisted of a 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 having a gastric-and-intestinal-mixed (GI) phenotype, while those showing neither gastric nor intestinal phenotype expression were grouped as unclassified or null (N) type.[13] Then, small areas in which adenocarcinoma cells consisted of homogeneous β-catenin localization as described above were assessed for gastric and intestinal phenotypic expression and classified into the four phenotypes.

DNA EXTRACTION AND MUTATIONAL ANALYSIS OF *β-CATENIN* AND *APC* GENES

Microdissection, polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) analysis and sequencing were performed as previously reported.[19] PCR primer sequences to amplify exon 3 of the human *β-catenin* gene were 5′-AAC ATT TCC AAT CTA CTA ATG CTA AT-3′ and 5′-CCA GCT ACT TGT TCT TGA GTG A-3′(Gene Bank accession number X89579).[20] Since it has been reported that somatic mutations in upper gastrointestinal tumours cluster approximately between codons 1400 and 1580,[21] two sets of primers (5′-AGTTCACTTGATAGTTTTGA GAGTCG-3′ and 5′-AAATCCATCTGGAGTACTTTCT GTG-3′; 5′-GTCCAGGTTCTTCCAGATGCTGATAC-3′ and 5′-GACTTTGTTGGCATGGCAGAAATAA-3′) were

selected to amplify the mutation cluster legion of the *APC* gene.[22]

Results

RELATION BETWEEN GASTRIC ADENOCARCINOMA PHENOTYPE AND β-CATENIN

Gastric cancers were first classified into G, GI, I and N types using whole sections and then compared for patterns of β-catenin localization and mutations. However, there were no obvious correlations between gastrointestinal phenotype and β-catenin localization due to heterogeneity of the tumours. We further analysed exon 3 of the *β-catenin* gene using DNA isolated from whole tumour areas and found only three (7.7%) mutations among 39 cases, involving cases nos 8 [codon 32: GAC (D)→CAC (H)], 28 [codon 32: GAC (D)→CAC (H)] and 38 [codon 55: GAG (E)→GAA (E)], but failed to identify any relationship to their phenotype (data not shown).

CLASSIFICATION OF GASTRIC REGIONS WITH β-CATENIN LOCALIZATION

Since the tissue localization of β-catenin was heterogeneous in the same cancers, subregions were chosen for comparison with gastrointestinal phenotype. There were 77 homogeneously stained regions >2 mm in diameter (12 membranous, 21 cytoplasmic and 44 nuclear localization). These selected areas were then once again phenotypically evaluated for gastric and intestinal markers using antibodies against MUC5AC and MUC6 for the former and Cdx2, MUC2 and villin for the latter (Figure 1).

THE RELATIONSHIP BETWEEN THE LOCALIZATION OF β-CATENIN AND MORPHOLOGICAL CLASSIFICATION

Cytoplasmic localization of β-catenin was detected more frequently in diffuse/solid than in glandular regions ($P < 0.05$). On the other hand, nuclear localization of β-catenin was observed significantly more frequently in the glandular areas ($P < 0.05$) (Figure 2a).

THE RELATIONSHIP BETWEEN THE LOCALIZATION OF β-CATENIN AND PHENOTYPIC CLASSIFICATION

Data on the relationship between gastrointestinal phenotype and the localization of β-catenin are summarized in Figure 2b. Twelve cases of homogeneous cancerous areas with membranous β-catenin localization were divided phenotypically into six G, five GI, 0 I and one N types; note that no area with only intestinal phenotypic expression was observed in these cases. Of

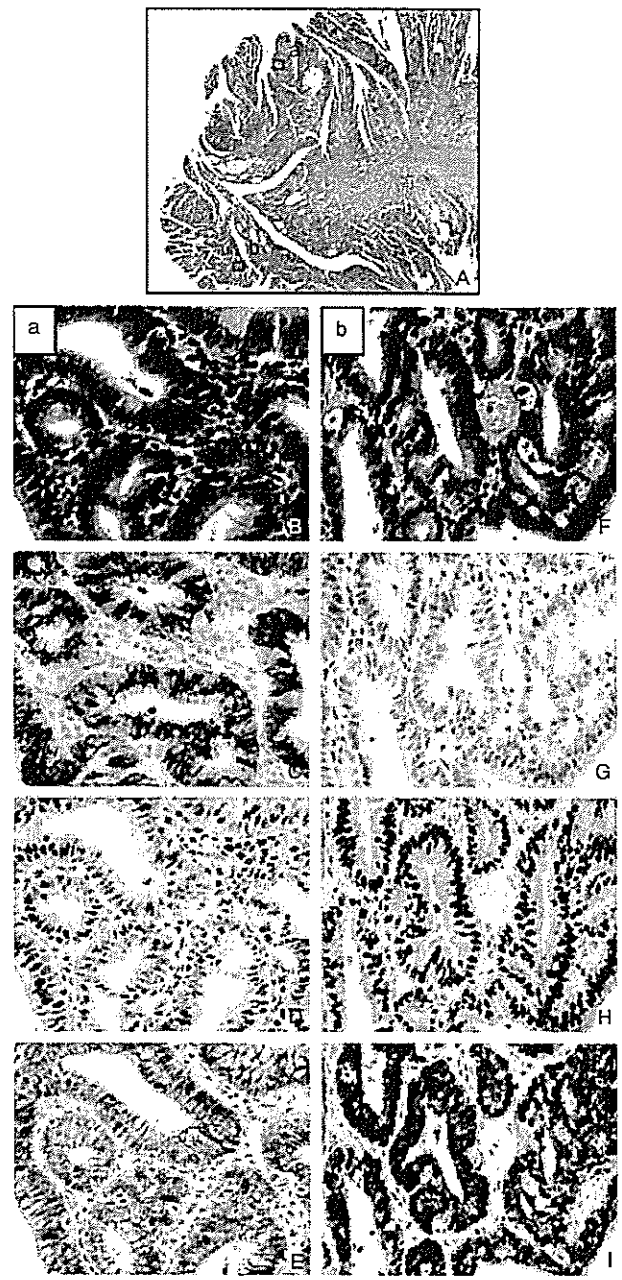

Figure 1. A case of human gastric adenocarcinoma showing intratumour heterogeneity in terms of phenotypic expression and β-catenin localization. A, An overview of adenocarcinoma showing two areas. H&E staining. Area a, B–E, Cancer cells forming tubular structure (B, H&E staining) are immunoreactive for MUC5AC (C) but lack Cdx2 nuclear protein (D), thus judged as being of gastric phenotype. β-Catenin is localized in membrane (E). Area b, F–I, Another area (F, H&E staining) harbours intestinal phenotype without MUC5AC (G) but with Cdx2 expression (H). β-Catenin nuclear accumulation is observed (I).

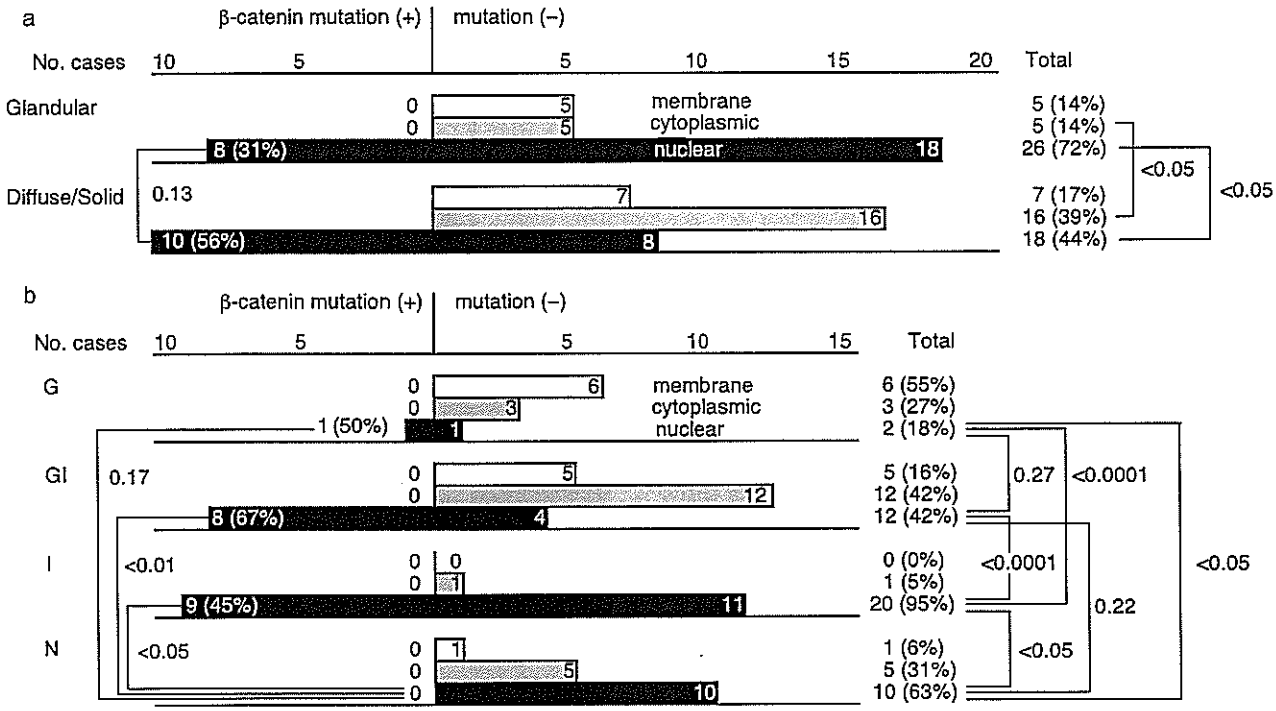

Figure 2. The relation between β-catenin localization/mutation and histological/phenotypical classification. a, Classified histologically. b, Classified according to phenotypes.

the 21 areas with cytoplasmic β-catenin localization, these could be divided phenotypically into three G, 12 GI, one I and five N types. Forty-four homogeneous cancerous areas with nuclear β-catenin localization were classified phenotypically as two G, 12 GI, 20 I and 10 N types. Areas with only gastric phenotypic expression were detected less frequently than other phenotypes in these areas. Cases with membranous β-catenin localization showed a gradual decrease from G, through GI, to I types, while those with nuclear β-catenin localization increased gradually ($P < 0.001$). In cases with cytoplasmic β-catenin localization, the GI type predominated (Figure 2b).

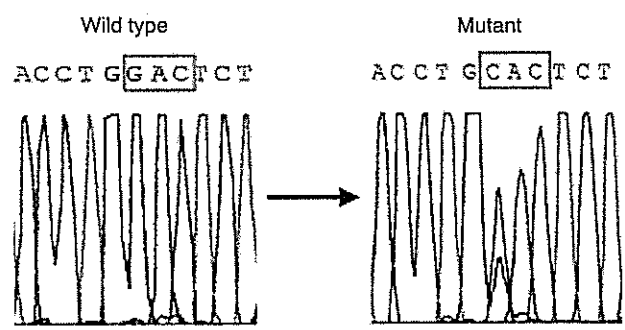

Figure 3. Representative sequencing analysis of *β-catenin* exon 3. A wild type and a mutant sequence with a point mutation (G→C) at codon 32 resulting in amino acid substitution from aspartic acid (D) to histidine (H).

MUTATIONAL ANALYSIS OF THE *β-CATENIN* GENE AND *APC* GENE

PCR-SSCP and direct sequencing of exon 3 of the *β-catenin* gene performed with the 77 microdissected samples revealed mutations in 18 (41%) of the 44 cases with nuclear β-catenin localization. None was found with other patterns of localization. Six patterns of mutation were detected (Figure 3, Table 1). All were missense mutations located in GSK-3β phosphorylation sites. Cases 8 and 28 had the same mutations as observed in whole section areas. However, case 38 harboured a different mutation. Regions with a diffuse/solid (10/18 = 56%) structure tended to be more frequently mutated than their glandular (8/26 = 31%) counterparts, but this did not reach statistical significance (Figure 2a, $P = 0.13$). There were no significant differences in phenotypic expression between G, GI and I types. Nevertheless, no mutations in N-type cases were observed, which showed significant differences from other phenotypes with regard to nuclear β-catenin localization (Figure 2b, N versus

Table 1. Relation of stomach cancer phenotypes and β-catenin localization and β-catenin and *Adenomatous polyposis coli* (*APC*) mutations

Case	Area	Phenotype*	β-catenin localization†	β-catenin mutation‡	APC mutation‡
1	1	G	C	WT	WT
2	1	N	M	WT	WT
3	1	GI	M	WT	WT
4	1	GI	C	WT	WT
5	1	G	C	WT	WT
6	1	GI	C	WT	WT
7	1	GI	C	WT	WT
	2	N	C	WT	WT
8	1	G	N	WT	WT
	2	GI	C	WT	WT
	3	I	N	codon32: GAC(D)→CAC(H)	WT
	4	G	C	WT	WT
	5	I	N	codon32: GAC(D)→CAC(H)	WT
	6	GI	N	codon32: GAC(D)→CAC(H)	WT
	7	N	N	WT	WT
	8	G	M	WT	WT
9	1	GI	N	codon32: GAC(D)→CAC(H)	WT
	2	GI	N	codon32: GAC(D)→CAC(H)	WT
	3	GI	N	codon32: GAC(D)→CAC(H)	WT
10	1	N	C	WT	codon1428: GGA(G)→AGA(R)
11	1	N	N	WT	WT
12	1	GI	C	WT	codon1414: GTA(V)→ATA(I)
13	1	I	N	WT	WT
14	1	N	N	WT	codon1459: ACT(T)→GCT(A)
15	1	I	N	codon37: TCT(S)→TTT(F)	codon1459: ACT(T)→GCT(A)
16	1	I	N	WT	codon1470: GCT(A)→ACC(T)
	2	I	N	WT	WT
	3	I	N	WT	WT
	4	N	C	WT	codon1452: GTA(V)→GCT(A)
17	1	I	N	WT	codon1470: GCT(A)→ACC(T)
	2	I	N	codon38: GGT(G)→CCC(P)	WT
	3	N	N	WT	WT

Table 1. (*Continued*)

Case	Area	Phenotype*	β-catenin localization†	β-catenin mutation‡	APC mutation‡
18	1	N	C	WT	WT
19	1	GI	N	WT	codon1452: GTA(V)→GCT(A)
	2	I	N	WT	WT
	3	GI	N	codon38: GGT(G)→CCC(P)	codon1452: GTA(V)→GCT(A)
	4	I	N	WT	WT
	5	GI	N	WT	WT
	6	N	N	WT	WT
20	1	GI	N	WT	WT
	2	GI	N	WT	codon1452: GTA(V)→GCT(A)
	3	GI	N	codon48: GGT(G)→GAT(D)	codon1452: GTA(V)→GCT(A)
	4	GI	N	codon32: GAC(D)→CAC(H)	WT
	5	N	N	WT	WT
21	1	GI	C	WT	WT
22	1	N	N	WT	WT
23	1	G	N	codon38: GGT(G)→CCC(P)	WT
	2	I	N	codon48: GGT(G)→GAT(D)	codon1459: ACT(T)→GCT(A)
	3	GI	N	codon36: CAT(H)→TAT(Y)	WT
	4	N	N	WT	codon1446: GCT(A)→ACT(T)
	5	N	N	WT	WT
24	1	N	N	WT	WT
25	1	GI	C	WT	WT
	2	I	N	codon38: GGT(G)→CCC(P)	WT
	3	N	C	WT	WT
26	1	GI	M	WT	WT
27	1	G	M	WT	WT
	2	GI	C	WT	WT
28	1	I	N	WT	WT
	2	I	N	codon32: GAC(D)→CAC(H)	WT
29	1	GI	C	WT	WT
30	1	G	M	WT	WT
	2	G	M	WT	WT
31	1	I	N	WT	WT
	2	I	N	WT	WT

Table 1. (*Continued*)

Case	Area	Phenotype*	β-catenin localization†	β-catenin mutation‡	APC mutation‡
32	1	GI	C	WT	WT
33	1	I	N	WT	codon1443: CCT(P)→CAT(H)
	2	GI	M	WT	codon1464: GAG(E)→AAG(K)
	3	G	M	WT	WT
34	1	GI	C	WT	WT
35	1	G	M	WT	WT
	2	GI	C	WT	WT
36	1	GI	M	WT	WT
37	1	I	N	codon36: CAT(H)→TAT(Y)	WT
38	1	I	N	codon32: GAC(D)→CAC(H)	WT
	2	GI	M	WT	WT
39	1	I	C	WT	codon1446: GCT(A)→GTG(V)

*G, Gastric type; GI, gastrointestinal type; I, intestinal type; N, null type.
†M, Membranous accumulatiuon; C, cytoplasmic accumulation; N, nuclear accumulation.
‡WT, Wild type.

G, $P = 0.17$; N versus GI, $P < 0.01$; N versus I, $P < 0.05$). PCR-SSCP and direct sequencing in exon 15 of the *APC* gene performed with the same 77 microdissected samples revealed mutations in 11 (25%) of the 44 cases with nuclear β-catenin localization, four (19%) of the 21 cases with cytoplasmic β-catenin localization and one (8%) of the 12 cases with membranous β-catenin localization (Figure 4). All were missense mutations located between 1414 and 1505 (Table 1). According to the sequencing analysis, 12 out of 16 mutation cases showed a mutated sequence (Figure 5, top panel) and four cases had a lower peak of the wild-type sequence (Figure 5, bottom panel), suggesting frequent loss of wild-type alleles, raising the possibility of stromal and/or inflammatory cell contamination. *APC* mutations were not found in G-type areas and were not associated with phenotypic expression among GI, I and N types.

HETEROGENEITY OF GASTRIC CANCERS

Sixteen tumours were analysed in multiple regions for *β-catenin* and *APC* gene mutations (Table 1). Among them, 10 (62.5%) tumours (cases 8, 16, 17, 19, 20, 23, 25, 28, 33 and 38) presented intratumour heterogeneity regarding these gene mutations, of which eight were from *β-catenin* mutations (cases 8, 17, 19, 20, 23, 25, 28 and 38) and six were from *APC* (cases 16, 17, 19, 20, 23 and 33) (Table 1). Four cases were revealed to have heterogeneity in both genes. Two areas in nos 19 (areas 1 and 3) and 20 (areas 2 and 3) harboured common *APC* mutations with or without *β-catenin* mutations, the former mutation considered to occur earlier. Tumour no. 23 showed five different patterns of heterogeneity (Figure 1, Table 1).

Discussion

The present study has shown, for the first time to our knowledge, a relationship between phenotype and mutations of the *β-catenin* and *APC* genes in human gastric cancers. Thus, the present data provide clear evidence that mutations in exon 3 of *β-catenin* are associated with phenotypic intestinal expression in cancerous areas with nuclear β-catenin localization. In contrast, no mutations were detected in cases with cytoplasmic or membranous localization, or in N-type regions with nuclear β-catenin localization. Clements *et al.*[8] have indicated that mutations of *β-catenin* may be strongly linked with β-catenin nuclear staining in gastric cancers, which is consistent with our present data. However, they did not evaluate the relationship

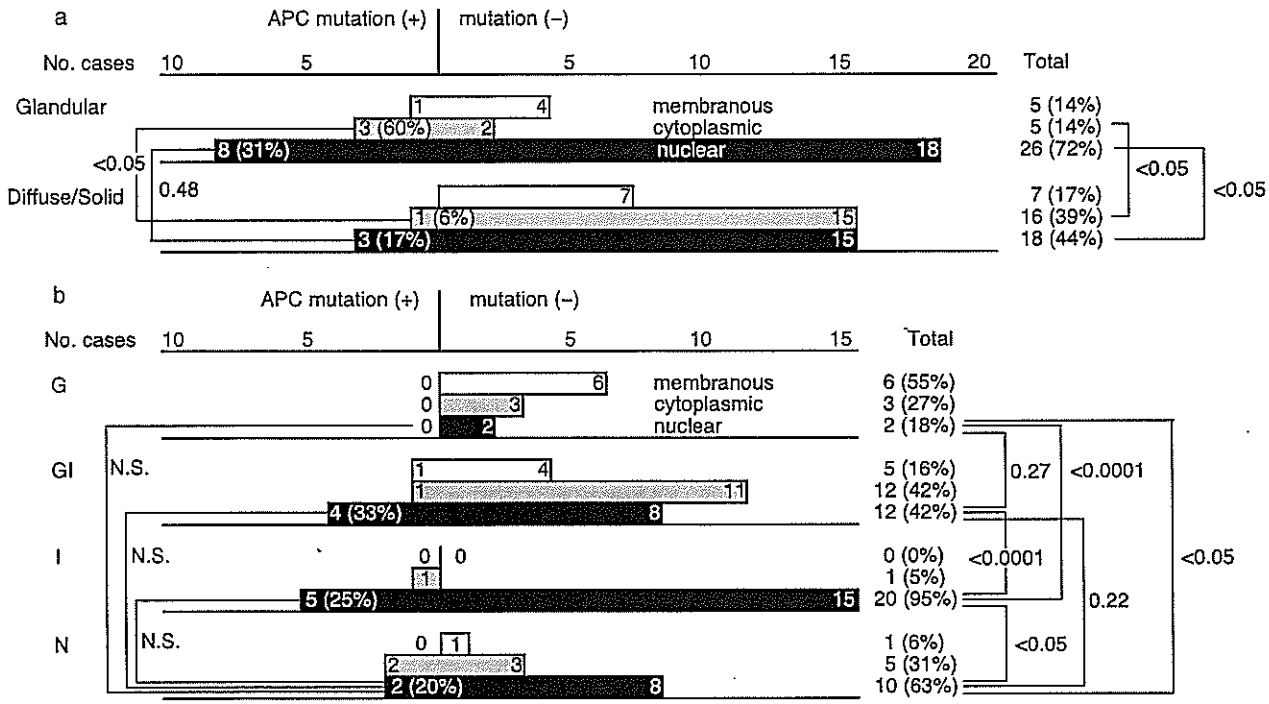

Figure 4. The relation between the β-catenin localization/*APC* mutation and histological/phenotypic classification. a, Classified histologically. b, Classified according to phenotypes.

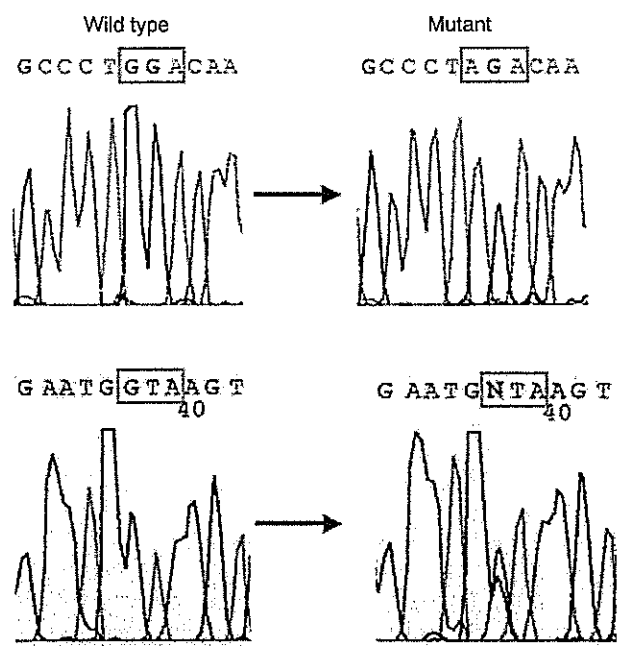

Figure 5. Representative sequencing analysis of *APC*. (Top panel) A wild-type and a mutant sequence with a point mutation (G→A) at codon 1428 resulting in amino acid substitution from glycine (G) to arginine (R). No wild-type sequence shows complete loss of the wild-type allele. (Bottom panel) Point mutation from G→A at codon 1414 causing substitution from valine (V) to isoleucine (I) with the presence of the remaining wild-type allele.

with phenotype. Human gastric cancers at an early stage, independent of histological type, mainly consist of gastric phenotype malignant cells,[18] whereas their advanced counterparts tend to have more malignant cells of the intestinal phenotype.[14,15,23] Taking into account the combination of our present data and previous reports, we consider that cytoplasmic and nuclear localization of β-catenin may be one of the important factors in the variation in gastric to intestinal expression in gastric cancers.

We performed sequencing of the mutational hot-spot of the *β-catenin* gene[19] and the mutation cluster region of the *APC* gene.[21] In contrast to *β-catenin* mutation, 26.7% (4/15) of N-type tumours with cytoplasmic or nuclear β-catenin localization harboured mutations in the *APC* gene. It suggests that Wnt activation in N-type cancers may be associated with occasional *APC* and infrequent *β-catenin* mutations. There was no significant difference among GI, I and N types in terms of *APC* mutations, unlike *β-catenin*. In cancerous areas of N type with nuclear β-catenin localization, another mechanism, other than *β-catenin* mutation, such as degradation of E-cadherin[9,24] or microsatellite instability,[25] might be responsible for the nuclear accumulation of β-catenin protein. Tumours with intestinal phenotypic expression have a significantly better

outcome than those without it.[17,18] Therefore, we consider that N-type cancers might be biologically different from G, GI and I-type tumours.

Mutations in exon 3 of the *β-catenin* gene were detected in codons 32, 33, 36, 37, 38 and 48 in the regions phosphorylated by GSK-3β. Earlier reports of gene alterations in GSK-3β phosphorylation sites in exon 3 involved codons 29, 37, 41 and 47.[8] Adjacent sites at codons 28, 32, 34, 39 and 48 also had mutations.[26] Our data are thus consistent with the literature. Tumours with cytoplasmic β-catenin localization did not have any mutations in exon 3 of the *β-catenin* gene in the present study. However, mutations of not only *β-catenin* but also *APC* cause activation of β-catenin–T cell factor (TCF) signalling in both human[27] and rat colorectal cancers.[28] Some of these tumours had mutations of the *APC* gene that might cause activation of β-catenin–TCF signalling. Such mutations of *APC* in our series might explain the abnormal β-catenin localization and accumulation in some cases.[29]

Direct sequencing of the tissue samples from topographically separate areas revealed that the tumour consisted of heterogeneous populations harbouring different mutations. Thus, progression may occur in several regions and in different directions from the original tumour and result in intratumour phenotypic heterogeneity as seen in the rat.[19] In the present series, mutations of the *β-catenin* gene were detected in 11 of 39 cases (28.2%) containing 18 (23.4%) mutated areas out of 77 regions. In contrast, only three mutations (7.7%) were found out of 39 cases when whole tumour areas were used. Thus, analysis of small regions was considered to be superior to that of whole tumour areas, especially in heterogeneous gastric cancers. *APC* gene mutations, in turn, were found in 16 areas (20.8%) in 11 cases (28.2%), being comparable to the frequency of *β-catenin* mutation. Among them, in nos 19 and 20 it was shown that the *APC* gene mutated earlier than *β-catenin* did. However, there is no obvious tendency for the order of mutations in *β-catenin* and *APC* genes in the current study.

In conclusion, our data suggest that the mutations of β-catenin are strongly associated with the intestinal phenotypic expression and that N type cancers with nuclear accumulation of β-catenin may not just have lost intestinal phenotypic markers but rather might be biologically different from G, GI and I tumors. Furthermore, shift of the β-catenin localization from the membrane to the cytoplasm and nuclei may be an important factor to determine the phenotypic variation including G, GI, I types in stomach cancers, at least partly associated with mutations of β-catenin and APC.

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Helicobacter pylori infection stimulates intestinalization of endocrine cells in glandular stomach of Mongolian gerbils

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Intestinal metaplasia has been investigated extensively as a possible premalignant condition for stomach cancer but its pathogenesis is still not fully understood. In the present study, we examined the relationship between endocrine and mucous cell marker expression periodically after *Helicobacter pylori* infection in the Mongolian gerbil model. The numbers of chromogranin A (CgA)-positive, gastrin-positive and gastric inhibitory polypeptide (GIP)-positive cells in *H. pylori*-infected groups was increased significantly compared with the non-infected case. However, CgA-positive and gastrin-positive cells then decreased from 50 through 100 experimental weeks after *H. pylori* infection, whereas GIP-positive cells increased. Coexistence of gastrin-positive and GIP-positive cells was detected in the same gastric and intestinal mixed phenotypic glandular-type glands. In conclusion, the endocrine cell phenotype is in line with that of the mucous counterpart in the glands of *H. pylori*-infected Mongolian gerbil stomach, supporting the concept that development of intestinal metaplasia is due to the abnormal differentiation of a stem cell. (*Cancer Sci* 2006; 97: 1015–1022)

The Mongolian gerbil (MG) is useful for examining the link between Helicobacter pylori infection and human gastric disorders, as the lesions induced by H. pylori in this experimental animal resemble those apparent in man.[1] In our animal model, we have previously demonstrated that eradication at early stages of inflammation is effective in preventing H. pylori-related stomach carcinogenesis.[2] Wong et al.[3] have demonstrated similar results in a human randomized-controlled trial of H. pylori eradication in China, and pointed out the importance of analyses of the factors that determine irreversibility – in other words, the point of no return. Thus, for the prevention of stomach cancer in MG, it is very important to estimate the histological and genetic alternations in the glandular stomach periodically and continuously after H. pylori infection, which is impossible in humans because of imprecise information on the time of infection with bacteria.

Several studies have demonstrated that changes in endocrine and mucous cells are observed in intestinal metaplasia (IM) in the human pyloric mucosa associated with H. pylori infection.[4–6] In the MG model, alterations in the endocrine cell population are also found during H. pylori infection.[7–9] Regarding the cellular differentiation of endocrine cells in the gastrointestinal tract, gastrin is detectable predominantly in the pyloric glands of the stomach, whereas gastric inhibitory polypeptide (GIP) is characteristic of the duodenum and small intestine.[10–14] Therefore, gastrin could be a gastric endocrine cell marker, in contrast to GIP as an intestinal example.[14] We have recently documented clear evidence that the phenotypes of endocrine cells are associated strongly with those of mucous cells in human IM as well as in normal gastric glands,[14] supporting the hypothesis that abnormal differentiation of stem cells underlies the development of IM in the human stomach.[15] With investigations of the histogenesis of H. pylori-related lesions, it is very interesting to focus on relationships between endocrine and mucous cells periodically in the MG model from the viewpoint of phenotypic expression.

In the present study, we therefore examined the expression of endocrine cell markers by immunohistochemistry and the quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR) using a gland isolation technique, and evaluated the relationship between endocrine and mucous cell marker expression at 50, 75 and 100 weeks after H. pylori infection in the MG model.

Materials and Methods

Samples. Seventy specific pathogen-free male MG (Seac Yoshitomi, Fukuoka, Japan), aged 7 weeks, and H. pylori (ATCC 43504; American Type Culture Collection, Rockville, MD, USA) were used for this study. The bacteria were grown from freezer stocks for 72 h and harvested in Brucella broth. Samples (0.8 mL) containing approximately 1.0×10^8 colony-forming units per mL were used as the inoculum, as described earlier.[16–19] Uninfected gerbils underwent sham inoculation using the same sterile Brucella broth.

The animals were divided into two major groups: H. pylori-infected (Hp[+]), and non-infected (Hp[–]) groups, and each group was subclassified with reference to time of death at 50, 75 and 100 weeks. Finally, the animals (n = 70) were divided into Hp(+)-50-week (n = 18), Hp(+)-75-week (n = 6), Hp(+)-100-week (n = 17), Hp(–)-50-week (n = 19), Hp(–)-75-week (n = 6) and Hp(–)-100-week (n = 4) groups (Fig. 1).

After 24 h fasting, all animals were subjected to deep ether anesthesia, laparotomized and exsanguinated from the inferior vena cava, with excision of their stomachs.[16] The numbers of stomach samples used for immunohistochemical analysis were 10 for Hp(+)-50-week, six for Hp(+)-75-week, nine for Hp(+)-100-week, eight for Hp(–)-50-week, six for Hp(–)-75-week and four for Hp(–)-100-week groups. The fundic and pyloric regions, duodenum, and small and large intestines of five Hp(–)-50-week gerbils were used as controls for immunohistochemical analyses. RNA extraction from the mucosa was also carried out for the fundic and pyloric regions, duodenum, and small and large intestines of three Hp(–)-50-week animals. For RNA extraction from isolated glands, pyloric regions were used from eight Hp(+)-50-week and eight Hp(+)-100-week gerbils. With the three Hp(–)-50-week cases, RNA extraction from isolated

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