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

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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

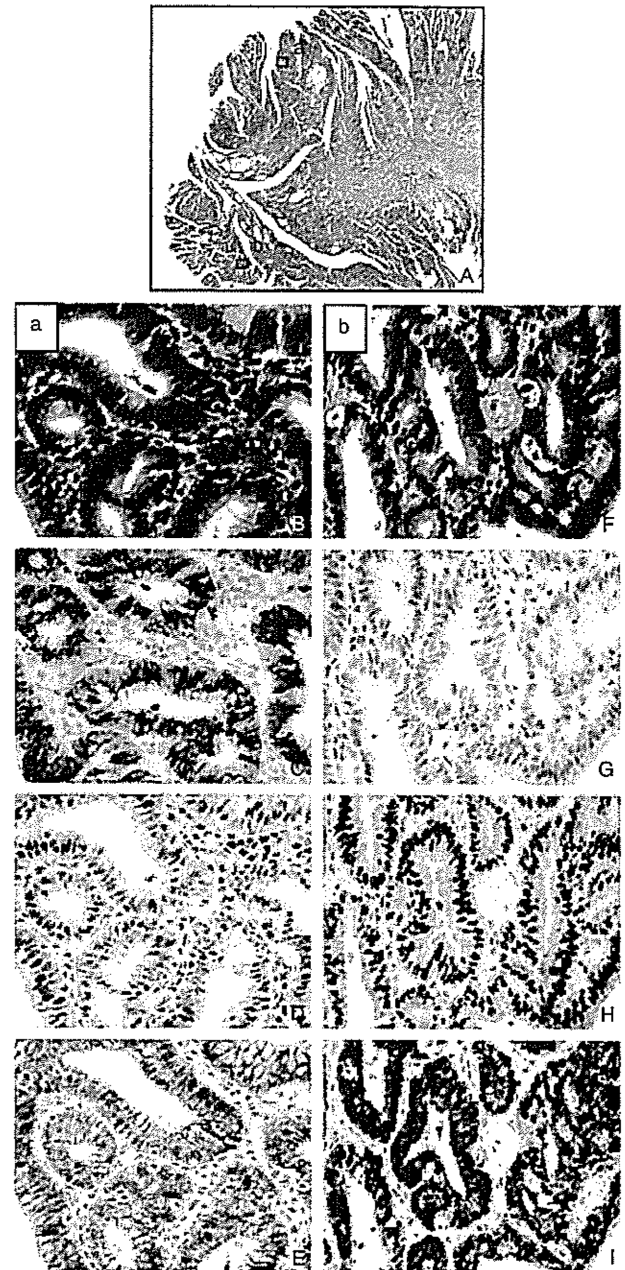

Figure 1. A case of human gastric adenocarcinoma showing intratumour heterogeneity in terms of phenotypic expression and β-catenin localization. A, An overview of adenocarcinoma showing two areas. H&E staining. Area a, B–E, Cancer cells forming tubular structure (B, H&E staining) are immunoreactive for 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).

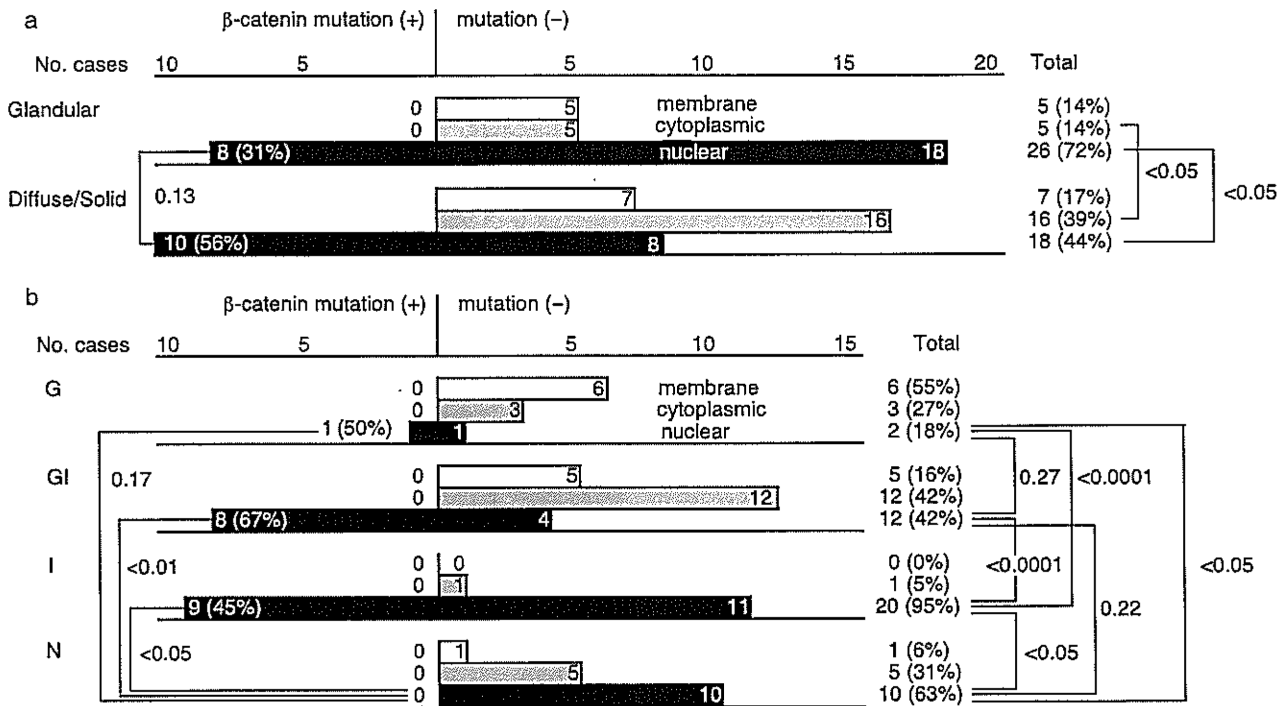

Figure 2. The relation between β-catenin localization/mutation and histological/phenotypical classification. a, Classified histologically. b, Classified according to phenotypes.

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).

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

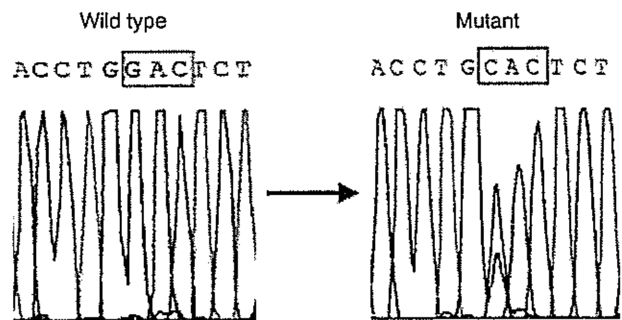

Figure 3. Representative sequencing analysis of *β-catenin* exon 3. A wild type and a mutant sequence with a point mutation (G→C) at codon 32 resulting in amino acid substitution from aspartic acid (D) to histidine (H).

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

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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

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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

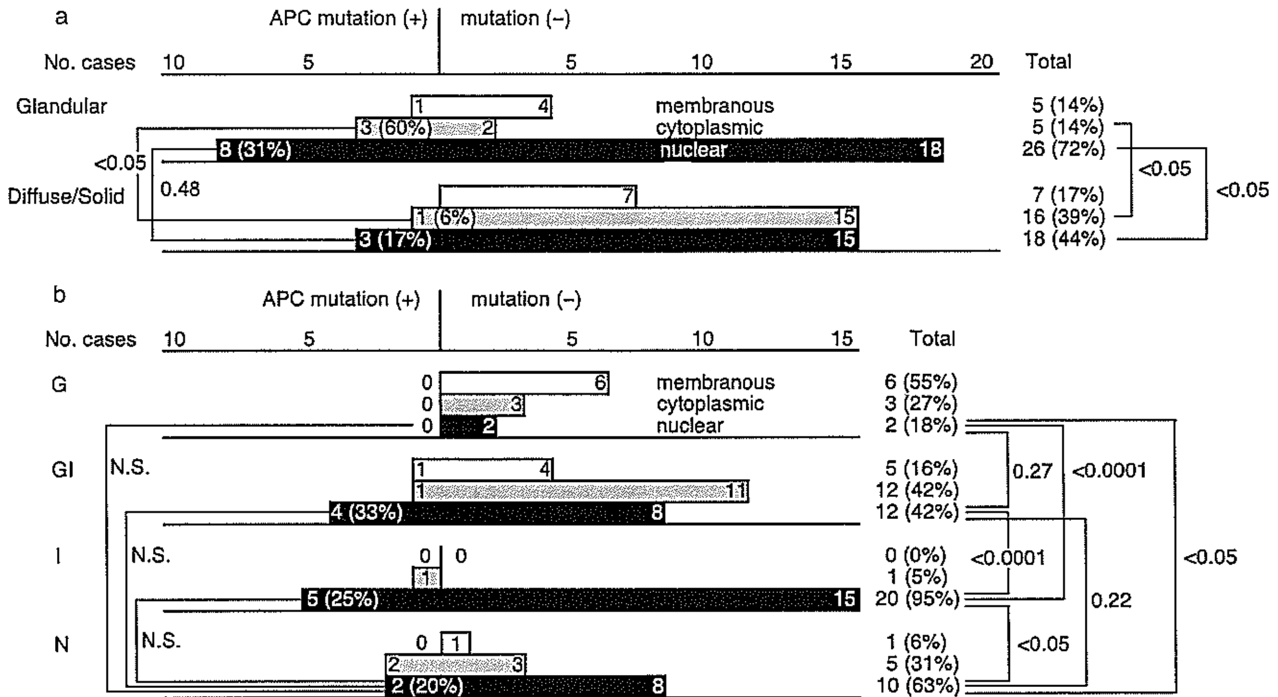

Figure 4. The relation between the β-catenin localization/*APC* mutation and histological/phenotypic classification. a, Classified histologically. b, Classified according to phenotypes.

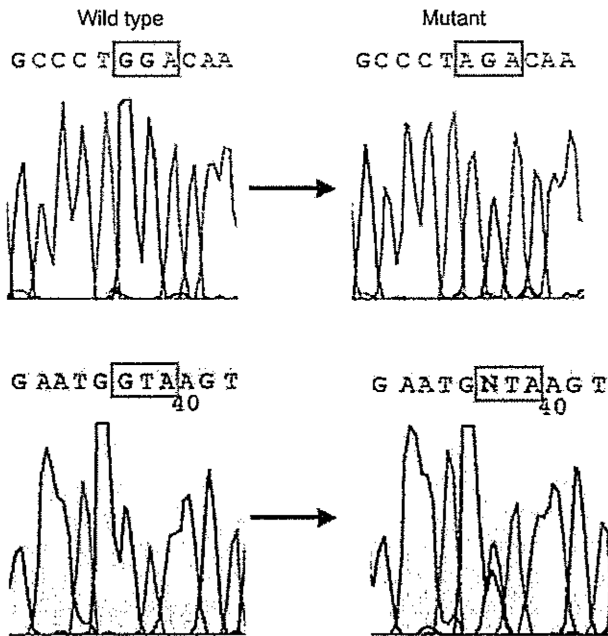

Figure 5. Representative sequencing analysis of *APC*. (Top panel) A wild-type and a mutant sequence with a point mutation (G→A) at codon 1428 resulting in amino acid substitution from glycine (G) to arginine (R). No wild-type sequence shows complete loss of the wild-type allele. (Bottom panel) Point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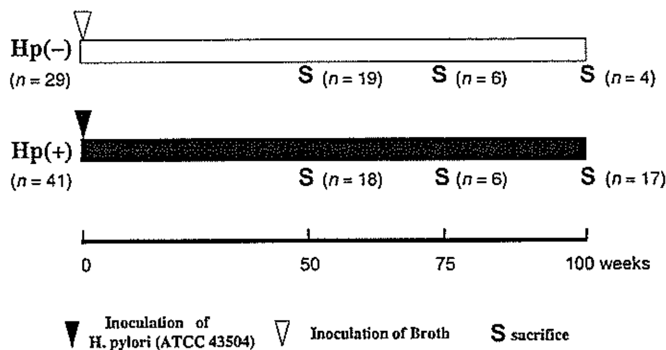

Fig. 1. Experimental design. Hp, *Helicobacter pylori.*

glands was carried out for the pyloric region and jejunum. After death, successful H. pylori infection was confirmed by remarkable elevation of serum IgG titers and/or histological inflammatory change in all H. pylori-infected MG (data not shown).

Each tissue sample was fixed in 95% ethanol plus 1% acetic acid, sectioned at 4 μM, and stained with hematoxylin and eosin for histochemical examination.[18,19]

Immunohistochemistry. Immunohistochemical staining was carried out with the polyclonal antibodies listed in Table 1.[14] The precise procedures for immunohistochemical techniques were as described previously.[14,15,18–22] Briefly, 4 μm-thick consecutive sections were deparaffinized and hydrated through a graded series of ethanol. After inhibition of endogenous peroxidase activity by immersion in 3% H_2O_2/methanol solution, sections were incubated with primary antibodies, washed thoroughly in phosphate-buffered saline (PBS), then incubated with biotinylated secondary antibody followed by the avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA). Finally, immune complexes were visualized by incubation with 0.01% H_2O_2 and 0.05% 3.3′-diaminobenzidine tetrachloride (DAB). Nuclear counterstaining was accomplished with Mayer's hematoxylin. Two independent investigators (YT and TT) judged the histology and immunohistochemical staining of each marker.

Classification of glandular ducts in the stomachs of MG. The endocrine cells in each glandular duct were identified as cells with chromogranin A (CgA) cytoplasmic expression. Gastrin is a marker of gastric endocrine cells, whereas GIP is a typical intestinal endocrine cell marker (Table 1).[14]

Regarding the phenotypes of glandular ducts with reference to mucous cell differentiation, we used Alcian blue–periodic acid–Schiff staining (AB-PAS) for identifying gastric surface mucous cells with mucin stained red and goblet cells stained blue (Table 1).[21,23] Non-neoplastic glandular ducts in the stomach were divided histologically and phenotypically into three types: gastric phenotypic glandular (G-type gland), gastric-and-intestinal-mixed phenotypic glandular (GI-type gland), and intestinal phenotypic glandular (I-type gland) ducts.[15,20]

Multiple staining with AB-PAS, gastrin and GIP. Multiple staining with AB-PAS, gastrin and GIP was achieved as follows. After staining with AB-PAS, sections were incubated with anti-gastrin antibody, followed by application of the biotin-labeled goat antirabbit IgG and the peroxidase-labeled avidin–biotin complex method. Binding was visualized with DAB. After thorough washing with Tris-buffered saline, incubation was with the anti-GIP antibody, followed by application of alkaline phosphatase-labeled antirabbit IgG, and development with nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (BCIP/NBT substrate system for immunohistochemistry and in situ hybridization; Dako, Glostrup, Denmark) using the indirect immuno-alkaline phosphatase method. With this multiple staining, gastrin-positive and GIP-positive cells were stained brown and dark purple, respectively. Glands were classified by AB-PAS staining into G-type, GI-type and I-type glands.

Gland isolation. Gland isolation was carried out as described previously.[20,24] Fresh gastrointestinal tissues were obtained from MG, washed thoroughly in calcium- and magnesium-free PBS to remove the luminal contents and cut into 2–4-cm squares. These were then incubated in calcium- and magnesium-free Hanks' balanced salt solution containing 30 mM ethylenediamine-tetraacetate (pH 7.0) and shaken for 15–20 min at 37°C. The gastrointestinal epithelium was then shaven off using the back of a knife, and harvested. The isolated glands were fixed in 70% ethanol and stored at –20°C until RNA extraction.

Sequencing of CgA, gastrin and GIP cDNA in MG. Total RNA from MG stomach and jejunum mucosae were extracted using ISOGEN (Nippon Gene, Toyama, Japan).[20] First strand cDNA was synthesized basically as described previously[25] using random primers with the Thermoscript RT-PCR System (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. To obtain partial cDNA sequences for CgA in MG, the most homologous regions were selected by comparison of the rat (DNA Bank of Japan [DDBJ]/European Molecular Biology Laboratory [EMBL]/GenBank accession number, NM_021655) and mouse (NM_007693) sequences (Table 2). To determine gastrin cDNA sequences, those of rat (NM_012849) and mouse (NM_010257) were compared for selection of one primer pair (Table 2). Regarding GIP, the most homologous regions were selected by comparison with the rat (X66724) and mouse (NM_008119) sequences (Table 2). After successful RT-PCR amplification using cDNA from gerbil stomach and jejunum as a template, sequencing was carried out using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster, CA, USA) utilizing ABI Prism 3100 (Applied Biosystems) according to the manufacturer's instructions.

Real-time RT-PCR. The oligo(dT)-primed cDNA was synthesized as described previously, using the Thermoscript RT-PCR System.[20,25] Relative quantitative real-time RT-PCR of CgA, gastrin and GIP was carried out using the LightCycler system (Roche Diagnostics, Mannheim, Germany) with a SYBR Green PCR Kit (Qiagen, Hilden, Germany).[20] The primer sequences of each marker are shown in Table 2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; DDBJ/EMBL/GenBank accession number AB040445) was used as an internal control. Relative quantification was carried out as established earlier using an internal control, without the necessity for external standards.[25] Values are expressed as a percentage of the CgA-positive and gastrin-positive cells in the pyloric region, and of GIP-positive

Table 1. Phenotypic markers for gastrointestinal endocrine and mucous cells

Cell types	Tissue specificity	Marker	Source of antibody
Endocrine	Ubiquitous	Chromogranin A	Dako (Glostrup, Denmark)
	Gastric	Gastrin	Yanaihara Institute (Fujinomiya, Japan)
	Intestinal	Gastric inhibitory polypeptide	Yanaihara
Mucous	Gastric	Periodic acid–Schiff† staining (mucin stained red)	
	Intestinal	Alcian blue‡ staining (mucin stained blue)	

†Periodic acid-schiff (Nakalai Tesque, Kyoto, Japan), ‡Alcian blue (Wako Pure Chemical Industries Ltd, Osaka, Japan)

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Table 2. Primer sequences of chromogranin A (CgA), gastrin and gastric inhibitory polypeptide (GIP) in Mongolian gerbils

Gene	Direction	Sequence	Product length (bp)
CgA	Sense	5′-CAAAAGGGGACACCAAGGTGATGAAGTG-3′	153
	Antisense	5′-TCAGCAGATTCTGGTGTCGCAGGATAGA-3′	
Gastrin	Sense	5′-GGAAGCCCCGCTCCCAGCTACAGGATG-3′	171
	Antisense	5′-TCCGTGGCCTCTGCTTCTTGGACAGGTC-3′	
GIP	Sense	5′-AGTGATTACAGCATCGCCATGGACAA-3′	243
	Antisense	5′-CCAGGCCAGTAGCTCTTGAATCAGAAGG-3′	
GAPDH	Sense	5′-AACGGCACAGTCAAGGCTGAGAACG-3′	118
	Antisense	5′-CAACATACTCGGCACCGGCATCG-3′	

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

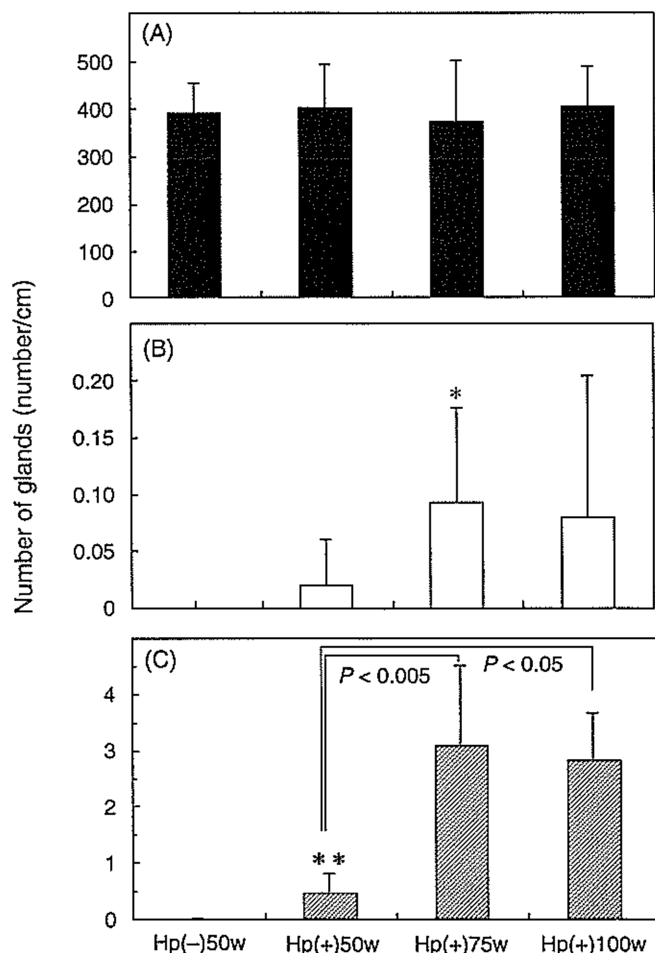

Fig. 2. The average numbers (mean ± SD) of (A) glandular (G)-type, (B) gastric and intestinal mixed (GI)-type and (C) intestinal (I)-type glandular ducts in Hp(–)-50-week ($n = 8$), Hp(+)-50-week ($n = 10$), Hp(+)-75-week ($n = 6$), and Hp(+)-100-week ($n = 9$) cases. *$P < 0.05$, **$P < 0.005$ vs the average Hp(–)-50-week number.

cells in the duodenum (both set as 100%). Specificity of the PCR reaction was confirmed using the melting program provided with the LightCycler software. To further confirm that there was no obvious primer dimer formation or amplification of any extra bands, the samples were electrophoresed in 2.5% agarose gels and visualized with ethidium bromide after the LightCycler reaction. Total RNA samples without RT provided a control for PCR amplification (data not shown).

Statistical analysis. The data were analyzed between groups using the Mann–Whitney U-test. The Kruskal–Wallis test was applied to establish the significance of differences among G-, GI- and I-type glands with reference to gastrin and GIP expression among the Hp(+)-50-week, Hp(+)-75-week and Hp(+)-100-week groups. P-values < 0.05 were considered statistically significant.

Results

Progression of intestinal metaplasia: average numbers of G-, GI- and I-type glands in *H. pylori*-infected MG. Fig. 2 shows the average numbers of G-, GI- and I-type glands per 1 cm of the glandular stomach mucosa (number/cm) in the Hp(–)-50-week (n = 8), Hp(+)-50-week (n = 10), Hp(+)-75-week (n = 6) and Hp(+)-100-week (n = 9) cases. The average numbers of G-type glands were 387.8 ± 62.8 (mean ± SD), 398.5 ± 91.3, 368.8 ± 128.9 and 401.2 ± 83.9, respectively. The average numbers of GI-type glands were 0 ± 0, 0.018 ± 0.039, 0.092 ± 0.083 and 0.079 ± 0.125, respectively, whereas those of I-type glands were 0 ± 0, 0.47 ± 0.32, 3.09 ± 1.40 and 2.83 ± 0.82, respectively. Thus, no GI- or I-type glands were observed in Hp(–)-50-week animals as controls.

The number of I-type glands in Hp(+)-50-week, Hp(+)-75-week and Hp(+)-100-week stomachs was increased significantly compared with the Hp(–)-50-week case (P < 0.005). In the Hp(+) groups, the average number of I-type glands increased significantly from week 50 to week 75, and then did not change at week 100. Regarding the GI-type glands, there was a significant difference between Hp(–)-50-week and Hp(+)-75-week (P < 0.05).

Immunolocalization of CgA, gastrin and GIP in the normal alimentary tract of MG. Expression of CgA, gastrin and GIP in the normal gastrointestinal tract was estimated by immunohistochemistry in Hp(–)-50-week animals (n = 5) (Fig. 3). The average numbers of CgA-positive cells per 1 cm of the mucosa (number/cm) in fundic regions, pyloric regions, duodenum, small intestines and large intestines were 124.1 ± 19.3 (mean ± SD), 106.7 ± 38.6, 30.7 ± 14.9, 16.9 ± 5.2 and 44.3 ± 25.2, respectively. The expression of CgA decreased gradually from the fundic region to the small intestine and increased from the small to large intestine (Fig. 4A). The average numbers of gastrin-positive cells in fundic regions, pyloric regions, duodenum, small intestines and large intestines were 1.1 ± 1.3, 99.8 ± 39.9, 2.6 ± 3.4, 0 ± 0 and 0 ± 0, respectively. No gastrin expression was detected in the small and large intestines. The average numbers of GIP-positive cells in fundic regions, pyloric regions, duodenum, small intestines and large intestines were 0.6 ± 0.6, 1.1 ± 0.5, 25.2 ± 12.1, 22.3 ± 7.5 and 0.9 ± 0.2, respectively. GIP expression was observed frequently in the small but not the large intestine.

Immunohistochemical analysis of CgA, gastrin and GIP in the pyloric regions of *H. pylori*-infected MG stomachs. For immunohistochemical analyses of CgA, gastrin and GIP, stomach samples of eight Hp(–)-50-week, six Hp(–)-75-week, four Hp(–)-100-week, ten Hp(+)-50-week, six Hp(+)-75-week and nine Hp(+)-100-week

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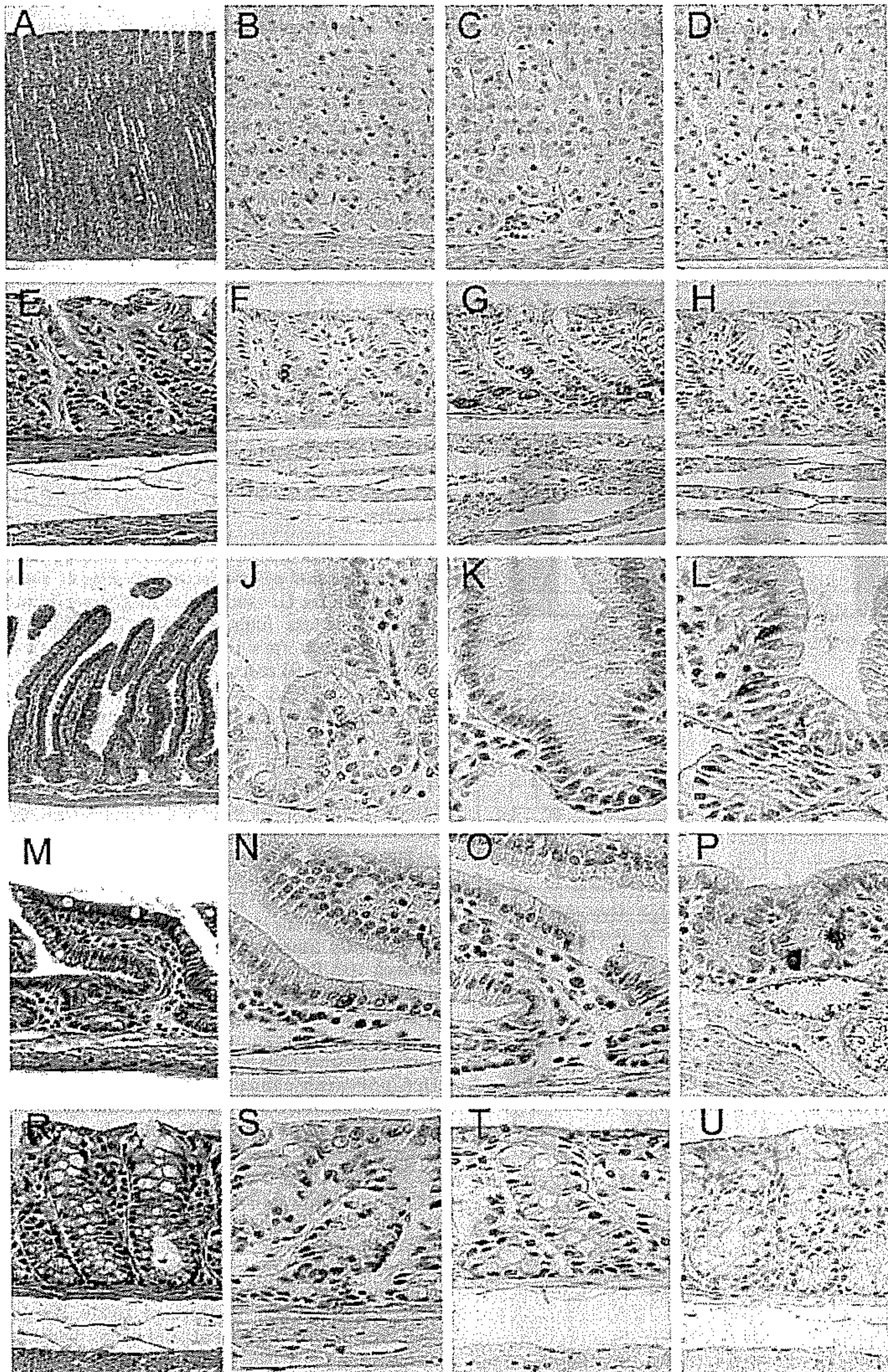

Fig. 3. Immunohistochemical staining of chromogranin A (CgA), gastrin and gastric inhibitory polypeptide (GIP) in (A–D) normal fundic and (E–H) pyloric regions, (I–L) duodenum, (M–P) small intestines and (R–U) large intestines of Mongolian gerbils. Expression of CgA was observed in the bottom of (B) normal fundic, (F) pyloric, (J) duodenal, (N) small intestinal and (S) colonic glandular ducts. (C,D) No gastrin or GIP was observed in the fundic glands. In the pyloric glands, (G) expression of gastrin was detected clearly, but (H) no GIP expression was observed. In the duodenum and small intestine, (L,P) GIP expression was detected, but (K,O) no gastrin expression was observed. Neither (T) gastrin nor (U) GIP were detected in the large intestine. Original magnification: (A) ×100; (B–U) ×400.

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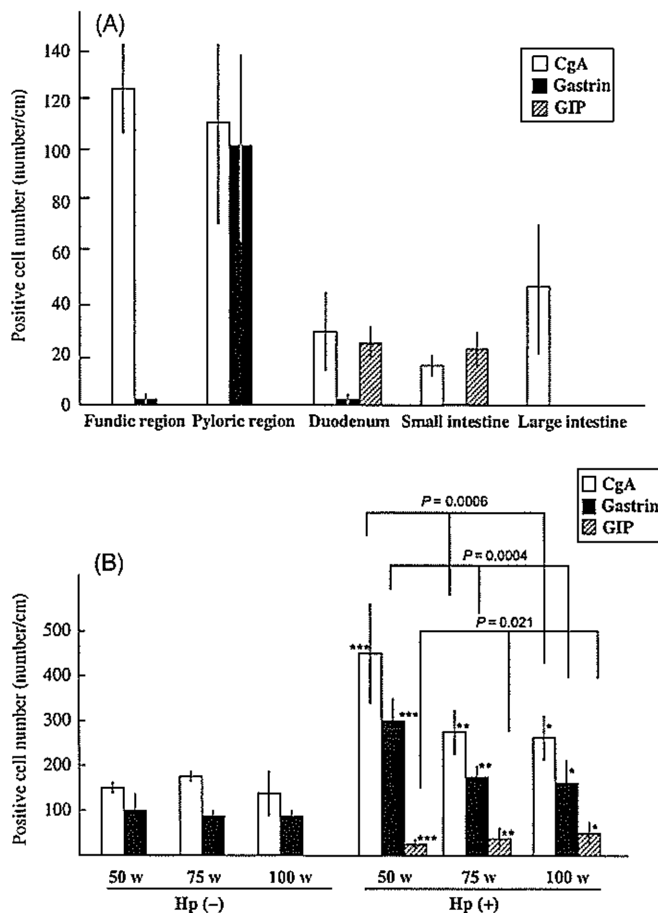

Fig. 4. Immunohistochemical analysis of chromogranin A (CgA)-, gastrin- and gastric inhibitory polypeptide (GIP)-positive cells. (A) Normal gastrointestinal tract. (B) *Helicobacter pylori*-infected Mongolian gerbils. $^{*}P < 0.01$, $^{**}P < 0.005$, $^{***}P < 0.0005$ compared with each control group.

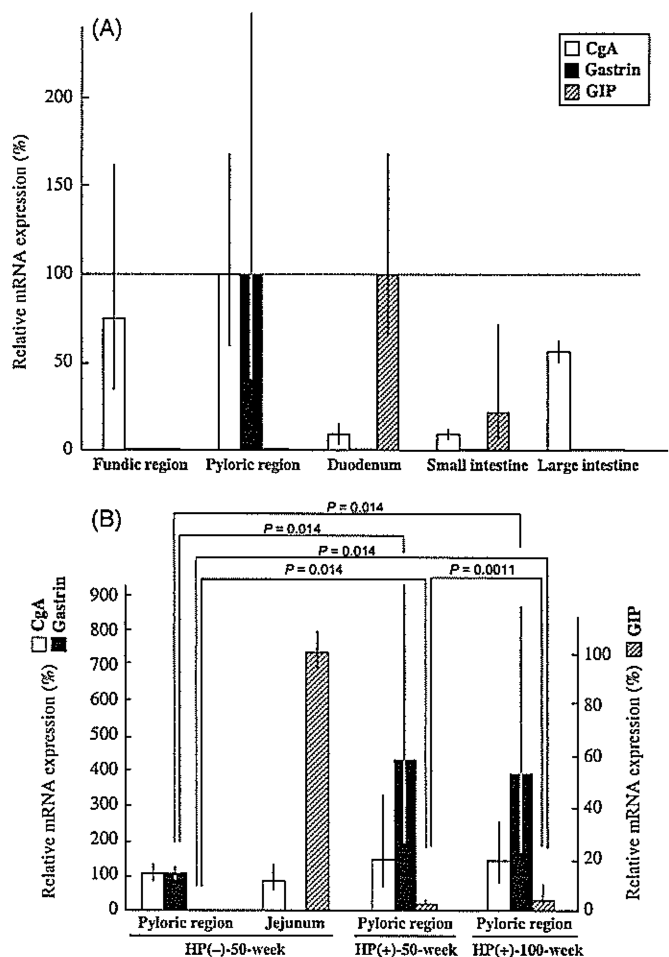

Fig. 5. Real-time reverse transcription-polymerase chain reaction analysis of chromogranin A (CgA), gastrin and gastric inhibitory polypeptide (GIP) in the pyloric region of Mongolian gerbils. (A) Normal alimentary tract. (B) Isolated pyloric glands from *Helicobacter pylori*-infected stomachs.

animals were used (Fig. 4B). The average numbers of CgA-positive cells per 1 cm of the pyloric region (number/cm) were 156.7 ± 6.4, 175.7 ± 2.5, 136.3 ± 60.4, 446.1 ± 104.2, 271.9 ± 175.4 and 250.0 ± 60.0, respectively. There was no significant variation in the number of CgA-positive cells in the controls at the three time points. Values were elevated in each H. pylori-infected group, but decreased gradually from Hp(+)-50-week through Hp(+)-75-week to Hp(+)-100-week (P = 0.0006). The average number of gastrin-positive cells in Hp(–)-50-week, Hp(–)-75-week, Hp(–)-100-week, Hp(+)-50-week, Hp(+)-75-week and Hp(+)-100-week were 96.1 ± 46.7, 83.7 ± 8.6, 92.7 ± 11.5, 295.1 ± 54.1, 175.4 ± 24.5 and 162.7 ± 44.7, respectively. The average number of GIP-positive cells was 1.9 ± 1.4, 2.1 ± 0.2, 2.8 ± 1.8, 20.0 ± 5.0, 35.5 ± 15.0 and 40.4 ± 24.3, respectively.

Determination of partial nucleotide sequences of CgA, gastrin and GIP. Partial nucleotide sequences of neuroendocrine markers in the MG were determined and deposited at the DNA Data Bank of Japan (DDBJ) (http://www.ddbj.nig.ac.jp/Welcome-e.html). DDBJ/EMBL/GenBank The accession numbers for CgA, gastrin and GIP are AB253527, AB253528 and AB253529, respectively.

Expression of CgA, gastrin and GIP mRNA in the normal alimentary tract. The expression of CgA, gastrin and GIP mRNA in the normal gastrointestinal tract was also estimated by real-time RT-PCR in Hp(–)-50-week animals (n = 3) (Fig. 5A), values being expressed as a percentage of those in the pyloric mucosa of stomach for CgA and gastrin, and relative to that in the duodenum for GIP. The average relative expression levels of CgA in fundic regions, pyloric regions, duodenum, small intestines and large intestines were 74.9% (35.05% [mean – SD]–160.11% [mean + SD]), 100% (59.87–167.02%), 10.25% (6.29–16.69%), 9.39% (7.62–11.56%) and 57.04% (51.50–63.17%), respectively. The corresponding figures for gastrin were 1.69% (0.45–6.34%), 100% (39.36–254.08%), 1.12% (0.15–8.34%), 0.0036% (0.00032–0.041%) and 0.015% (0.000024–8.76%), and for GIP were 0.00019% (0.000059–0.00064%), 1.30% (0.46–3.67%), 100% (61.69–162.11%), 22.85% (7.00–74.60%) and 0.0088% (0.0014–0.057%). In line with the immunohistochemical and real-time RT-PCR findings, gastrin and GIP were used as gastric and intestinal endocrine cell markers, respectively, in MG.

Alteration of CgA, gastrin and GIP mRNA expression in isolated pyloric glands from *H. pylori*-infected MG. We used gland isolation to avoid contamination of epithelial cell elements with stromal or inflammatory cells. Isolated glands were obtained from the pyloric regions of Hp(–)-50-week (n = 3), Hp(+)-50-week (n = 8) and Hp(+)-100-week (n = 8) animals. Examples were also collected as controls of GIP mRNA expression from Hp(–)-50-week jejunums (n = 3). Values are expressed as the percentage of those in isolated glands obtained from the pyloric region for

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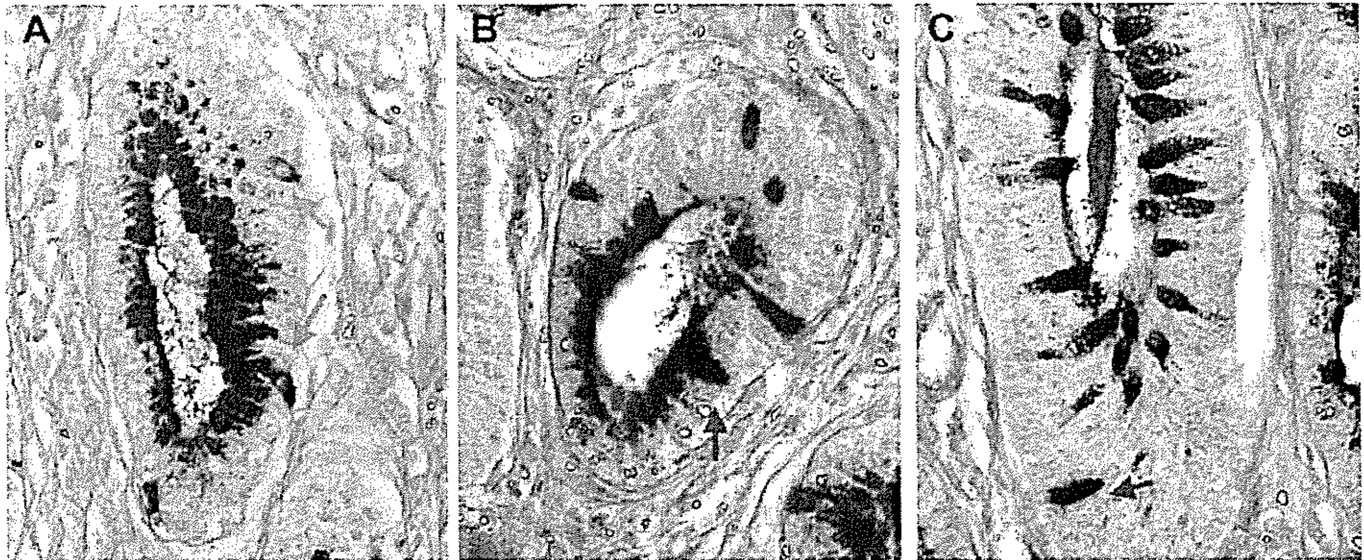

Fig. 6. Expression of gastrin and gastric inhibitory polypeptide (GIP) in (A) glandular (G)-type, (B) gastric and intestinal mixed (GI)-type and (C) intestinal (I)-type glands. (A) In the G-type glands, gastrin-positive cells were identified by brown cytoplasmic staining (red arrow), but no GIP expression was observed. (B) In the GI-type glands, gastrin- and GIP-positive cells were identified by brown (red arrow) and dark purple (blue arrow) cytoplasmic staining. (C) In the I-type glands, GIP expression was detected by dark purple staining (blue arrow), but no gastrin expression was apparent. Original magnification: (A–C) ×500.

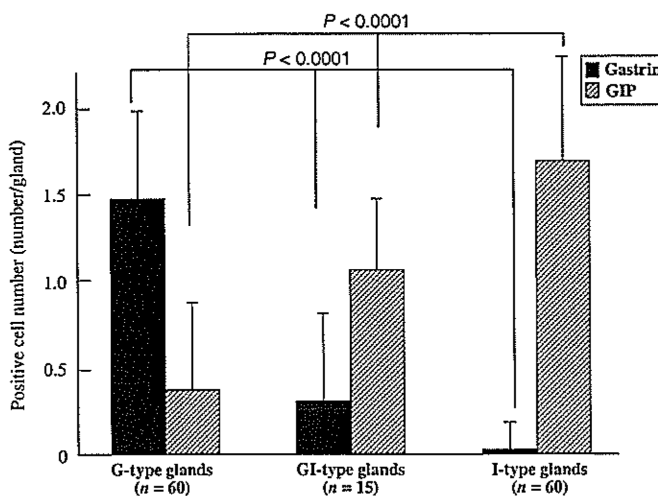

Fig. 7. Expression of gastrin and gastric inhibitory polypeptide (GIP) in glandular (G)-type, gastric and intestinal mixed (GI)-type and intestinal (I)-type glands. The number of gastrin-positive cells decreased gradually from G-type through GI-type to I-type glands ($P < 0.0001$), correlating inversely with the number of GIP-positive cells ($P < 0.0001$).

CgA and gastrin and in the jejunum for GIP (Fig. 5B). Average relative expression levels for CgA mRNA were 100% (81.50–122.70%), 80.11% (54.32–118.14%), 136.60% (55.78–334.55%) and 147.55% (82.65–263.43%) in the normal Hp(–)-50-week pyloric region and the jejunum, and in the H. pylori-infected pyloric regions of Hp(+)-50-week and Hp(+)-100-week animals, respectively. Those for gastrin mRNA were 100% (89.95–111.17%), 0.21% (0.10–0.44%), 436.46% (205.35–927.65%) and 399.88% (182.48–876.31%), respectively, and for GIP mRNA were 0.00057% (0.000037–0.0086%), 100% (92.68–107.90%), 0.29% (0.14–0.57%) and 3.38% (1.40–8.17%), respectively.

The expression of gastrin mRNA in H. pylori-infected groups (Hp[+]-50-week and Hp[+]-100-week) was increased significantly compared with the Hp(–)-50-week case. However, there was no significant difference in gastrin mRNA expression between Hp(+)-50-week and Hp(+)-100-week groups. The expression of GIP mRNA in H. pylori-infected groups (Hp[+]-50-week and Hp[+]-100-week) was also increased significantly compared with Hp(–)-50-week. In addition, there was a significant difference in GIP between Hp(+)-50-week and Hp(+)-100-week (Fig. 5B). Regarding CgA mRNA expression, there was no statistically significant variation among the groups.

Colocalization of gastrin- and GIP-positive endocrine cells of GI-type glands. The presence of gastrin- and GIP-positive cells was double-immunohistochemically evaluated with AB-PAS staining (Fig. 6) to directly compare the localization of endocrine and mucous G-type and I-type markers. The numbers of gastrin-positive and GIP-positive cells per gland (number/gland) were analyzed in 60 G-type, 15 GI-type and 60 I-type glands of H. pylori-infected groups. The average numbers of gastrin-positive cells were 1.48 ± 0.57, 0.53 ± 0.51 and 0.03 ± 0.18 in G-, GI- and I-type glands, respectively. Those for GIP-positive cells were 0.15 ± 0.36, 1.00 ± 0.53 and 1.68 ± 0.65, respectively (Fig. 7). Gastrin-positive cells decreased gradually from G- through GI-, to I-type glands ($P < 0.0001$), whereas GIP-positive cells were correlated inversely ($P < 0.0001$) (Fig. 7). Coexistence of gastrin- and GIP-positive cells was detected in the same gland in GI-type glands (Fig. 6).

Discussion

To our knowledge, this is the first report of expression of endocrine and mucous cell markers observed periodically in the glandular stomach of H. pylori-infected MG, although several studies have shown that long-term H. pylori colonization produces hyperplasia of gastrin-producing antral G-cells and carcinoid tumors in MG.[7,9,26] In the present study, the immunohistochemical data demonstrated that the numbers of CgA- and gastrin-positive cells in H. pylori-infected groups was increased significantly compared with the non-infected condition, but both demonstrated a gradual decrease over time, despite the lack of any significant variation in CgA or gastrin mRNA expression between Hp(+)-50-week and Hp(+)-100-week. In humans, there have been several

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reports of no significant differences in the number of G-cells and G-cell density in the stomach mucosa between H. pylori-infected and -uninfected healthy volunteers,[27–29] although the number of G-cells was significantly less in patients with both H. pylori infection and duodenal ulcer than in either infected or uninfected controls.[27–30] Kamada et al. reported that G-cell number in H. pylori-associated gastritis mucosa was decreased in comparison with an uninfected case.[30] Diamaline et al. suggested that CgA production in enterochromaffin-like cells of the rat stomach was part of the functional response of these cells to circulating gastrin.[31] We consider that the expression of some factors, including CgA and gastrin, is influenced by the time after H. pylori infection, and further analyses in the MG model may explain the discrepancy with human reports. Regarding the relationship between IM and the change in endocrine cells, several reports demonstrated that G-cells disappeared and I-type endocrine cells conversely appeared in human IM mucosa,[4–6] and that many Glicentin-positive, intestinal phenotype cells were found at the IM gland level,[6,32] in line with our gland isolation findings. However, few GIP-positive cells were observed in non-infected groups. H. pylori infection may trigger intestinalization of the stomach mucosa.[21] We have showed previously that the phenotypes of endocrine cells are associated strongly with those of mucous cells in human IM,[14] and the GI-type glands in the present study had both gastrin- and GIP-positive cells in the H. pylori-infected MG glandular stomach. This evidence supports the concept that all of the different types of mucous and endocrine cells may be generated from a single stem cell.[15]

Regarding intestinalization of the stomach mucosa, changes in the expression of various genes, especially homeobox examples determining cell structures and functions, may be involved. The fetal stomach, which develops from the foregut, displays areas of I-type mucosa with goblet cells and epithelial cells with striated borders in the antrum and cardia.[33] It is important to consider the correlation between expression of phenotypes and organ-specific genes. We earlier showed that Sox2 and Cdx1/2 are gastric- and intestinal-specific transcription factors, respectively.[15,20] In isolated pyloric and intestinal metaplastic glands, the phenotypes of mucous cells were found to be associated strongly with these specific transcription factors. In isolated GI-type glands, Sox2 and Cdx1/2 were both observed, as well as gastric and intestinal mucous cell markers such as MUC5AC, MUC6, MUC2 and villin. Recently, La Rosa et al. demonstrated that Cdx2 may be a sensitive and specific marker of midgut endocrine cells and endocrine tumors.[34] We think that Cdx2 might be important in the regulation of intestinal phenotype endocrine cell markers such as GIP, glicentin and glucagon-like polypeptide-1 because of its localization. Jenny et al. reported previously that neurogenin3, a basic helix-loop-helix transcription factor, is required for endocrine cell fate specification in multipotent intestinal progenitor cells, whereas gastric endocrine development is both neurogenin3 dependent and independent.[35] Thus, specific transcription factors, including Cdx2, might play an important role in the intestinalization of endocrine cells as well as mucous cells, because a phenotypic link was here observed sequentially between mucous and endocrine cells. Moreover, recently, several reports have demonstrated that impaired expression of the stomach morphogenic factor Sonic hedgehog (Shh) by parietal cells and increased expression of the transcriptional activators of intestinal and pancreatic differentiation, namely CDX2 and PDX1, are crucial for the development of stomach atrophy and for intestinal, endocrine and pancreatic transdifferentiation processes.[36,37] Suzuki et al. described that prolonged colonization by H. pylori led to extension of inflammation from the antrum to the corpus of the stomach with downregulation of Shh in gastric epithelial differentiation in the MG model.[38] Again, evaluation of the expression of the specific transcription factors detailed above should be carried out periodically in the H. pylori-infected MG model.

Our present data demonstrate that most glands in the stomach of H. pylori-infected MG were G-type. The numbers of GI- and I-type glands were extremely low in comparison. In the non-infected MG, no GI- or I-type glands were detected. We have demonstrated previously that most stomach cancers present with gastric phenotypic expression in the glandular stomach of H. pylori-infected MG treated with carcinogens.[21] In the rat, Tatematsu et al. reported that pepsinogen 1-altered pyloric glands, which are low in pepsinogen 1, are putative preneoplastic lesions in the glandular stomach.[39–41] In humans, most early-stage gastric cancers consist mainly of G-type cancer cells, irrespective of histological type.[15,42–44] Thus, it is important to note the possibility that precancerous lesions exist in G-type as well as IM glands.[21]

In conclusion, the phenotype of endocrine cells is in line with that of their mucous cell counterparts in the glands of the H. pylori-infected MG stomach, supporting the concept that the development of IM is due to abnormal differentiation of stem cells. For elucidation of stomach carcinogenesis, it is very important to evaluate factors related to H. pylori infection periodically in the MG model.

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Review article

Gastric-and-intestinal mixed-type intestinal metaplasia: aberrant expression of transcription factors and stem cell intestinalization

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Abstract
Helicobacter pylori **plays a causative role in the development of chronic atrophic gastritis, intestinal metaplasia (IM), and stomach cancer. Although IM has long attracted attention as a putative preneoplastic lesion for stomach cancers, its clinicopathologic significance has yet to be clarified in detail. Using gastric and intestinal epithelial cell markers, IM was here divided into two major types: a gastric-and-intestinal (GI) mixed type and a solely intestinal (I) type. In the former, gastric and intestinal phenotypic markers appeared not only at the glandular but also at the cellular level. Furthermore, neuroendocrine cells also showed intestinalization along with their exocrine counterparts. In animal models, GI-type IM was found to appear first, followed by the solely I type. Summarizing these data, it was suggested that IM might be caused by the gradual intestinalization of stem cells from the GI to the I type. The molecular mechanisms of IM include the ectopic expression of CDX1, CDX2, OCT-1, and members of the Erk pathway. Suppression of the expression of gastric transcription factors such as SOX2, genes that are involved in the Sonic hedgehog pathway, and RUNX3, a tumor suppressor gene, could be additional relevant alterations. The expression of PDX1 may also be associated with pseudopyloric gland metaplasia and IM. Detailed analysis of gene regulation may shed light on the molecular bases of gastric lesions, leading to strategies for chemoprevention.**

Key words Gastric-and-intestinal mixed-type intestinal metaplasia · Stem cell · Transcription factor

Introduction

Since the discovery of *Helicobacter pylori* by Warren and Marshal [1] in Australia, it has been well established that this microorganism plays important roles in the development of chronic gastritis, intestinal metaplasia (IM), and stomach cancers, including malignant lymphomas [2–7]. In 1994, the World Health Organization (WHO)/International Agency for Research on Cancer (IARC) categorized *H. pylori* as a group 1 "definite carcinogen" [8]. IM has been extensively studied as a putative preneoplastic lesion in the human stomach, due to its strong association with stomach cancer development [9–18]. Although controversy exists as to its real significance [19,20], IM is considered by some to be a precancerous lesion for so-called intestinal adenocarcinomas of Lauren's classification [21]. This type comprises well- and moderately differentiated adenocarcinomas, irrespective of the presence of the intestinal properties. Clearly, the pathogenesis of IM, as well as its molecular background, needs to be detailed for the elucidation of the actual relation between IM and stomach cancer [22].

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Stem cells in the gastrointestinal mucosa

To investigate the cellular origin of tissues, mosaicism of cellular genetic markers is often used. One approach is to use chimeric animals, produced experimentally by the amalgamation of cells from allelically different strains. Recently, numerous histological markers have also been applied for the analysis of mosaicism in chimeric mice. Antibodies strictly recognizing C3H strain-specific antigens (CSAs) [23] enable the immunohistochemical discrimination of C3H cells in histological sections of chimeric mouse tissues. In normal gastric and intestinal mucosa of chimeric mice, each gland is composed entirely of CSA-positive or -negative cells, and no mixed glands are found, indicating that each individual gland in the adult mouse is derived from a single progenitor cell. Surface mucous cells (foveolar epithelial cells), mucous neck cells, parietal cells, and chief cells in the fundic glands thus all arise from the same cell. Similarly, surface mucous cells and pyloric gland cells arise from a single progenitor cell [24–26].