LINE-1 Hypomethylation Is Associated with Increased CpG Island Methylation in *Helicobacter pylori*–Related Enlarged-Fold Gastritis

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Abstract

Background: **The molecular mechanism by which *Helicobacter pylori* infection leads to gastric cancer is not fully understood. Similarly, patients with enlarged-fold (EF+) gastritis, one cause of which is *H. pylori* infection, have an increased risk for gastric cancer, although again molecular mechanism is unclear. In the present study, we analyzed the methylation status of long interspersed nucleotide elements (LINE-1) and three cancer-related genes in a panel of gastric mucosae, with or without EF+ gastritis.**

Methods: **We used bisulfite pyrosequencing to assess the levels of LINE-1, CDH1, CDH13, and PGP9.5 methylation in 78 gastric mucosa specimens from 48 patients.**

Results: **Levels of LINE-1 methylation were significantly reduced in mucosae from patients with EF+ gastritis. This hypomethylation of LINE-1 was associated with increased methylation of the 5′ CpG islands of the genes, which suggests that, in EF+ gastritis, the methylation of the promoter regions of certain genes is accompanied by global demethylation of repetitive sequences.**

Conclusions: **Our results indicate that genomewide hypomethylation and regional hypermethylation occur in EF+ gastritis and may contribute to the tumorigenesis of diffuse-type gastric cancers.** (Cancer Epidemiol Biomarkers Prev 2008;17(10):2555–64)

Introduction

Etiologic analysis has shown that infection by *Helicobacter pylori* plays a critical role in the development of gastric cancer, although the molecular mechanism is not fully understood (1). Moreover, most patients with *H. pylori*–related gastritis never develop a gastric malignancy. It would thus be highly desirable if one could identify molecular markers that were predictive of the risk for *H. pylori*–related gastric cancer. For instance, it is now widely accepted that intestinal-type gastric cancers arise from lesions containing intestinal metaplasia (2).

Enlarged gastric folds are associated with a variety of diseases, including hypertrophic gastritis, Ménétrier disease, Zollinger-Ellison syndrome, primary gastrin cell hyperplasia, gastric cancer, and lymphoma (3). *H. pylori* is a known cause of enlarged-fold (EF+) gastritis accompanied by foveolar hyperplasia, massive infiltration of inflammatory cells, and increased production of interleukin 1β and hepatocyte growth factor in the corpus mucosa (4-6). Notably, the mutagenicity of gastric juice in the body region of the stomach is significantly higher in the patients with *H. pylori*–related EF+ gastritis than in either *H. pylori*–negative (HP-) controls or *H. pylori*–positive (HP+) patients without enlarged fold (EF-; ref. 7). It has also been reported that the prevalence of diffuse-type gastric cancer in the gastric body region increases with increasing fold width (4). Taken together, these findings suggest that HP+/EF+ gastritis puts one at high risk of developing diffuse-type gastric cancer.

Epigenetic alterations play a key role in silencing genes in human tumors (8). In gastric cancer, for example, DNA methylation leads to the silencing of a variety of cancer-related genes involved in cell cycle regulation, apoptosis, immune function, cell signaling, and tumor invasion and metastasis (9-12). *H. pylori* infection reportedly induces methylation of various genes in the gastric mucosae, among which CDH1 is reportedly hypermethylated in HP+/EF+ gastritis (13). On the other hand, global levels of DNA methylation reportedly decline in various types of cancer (14-16). The role of global hypomethylation in cancer remains largely unexplored, although it is reportedly associated with

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Table 1. *H. pylori* infection, age, and sex of 48 patients

	H. pylori (−)	*H. pylori* (+), enlarged (−)	*H. pylori* (+), enlarged (+)
Age (y)	46.3 (23-78)	57.9 (22-81)	51.8 (38-63)
Age differences (SE) HP+/EF-	−12.7 (5.1), $P = 0.051$		
when compared with HP+/EF+	−5.5 (5.7), $P > 0.999$	7.2(4.8), $P = 0.431$	
Sex (male/female)	7/4	16/8	9/4

chromosomal instability. Moreover, global hypomethylation has been reported to correlate with hypermethylation of CpG islands (17), although other studies do not support this concept (16), and it remains unclear whether global hypomethylation and CpG island hypermethylation are two independent phenomena or are mechanistically linked.

Our aim in the present study was to begin to address these issues by using quantitative bisulfite pyrosequencing to examine the methylation of repetitive sequence long interspersed nucleotide elements (LINE-1) in a panel of gastric mucosae, with and without *H. pylori* infection. In addition, we analyzed the correlation between LINE-1 hypomethylation and the levels of CpG island methylation in patients with EF+ gastritis.

Materials and Methods

Patients and Specimens. In total, 78 specimens from 48 patients with gastritis were examined for methylation. These included 23 specimens of gastric mucosa from 13 patients who were HP+/EF+ (13), 37 specimens from 24 patients who were HP+/EF-, and 18 specimens from 11 patients who were HP- and served as controls. Average ages and sex ratios in the three groups are shown in Table 1. Written informed consent was obtained from every patient, and this study was approved by the institutional review board. In each case, the widths of the gastric body folds on double-contrast radiographs were measured using a computerized image analyzer. The diagnostic criteria for EF+ gastritis were *H. pylori* positivity and a maximum fold width in the gastric body >5 mm, which are consistent with earlier studies and the Sydney system (18). EF- patients were divided into two groups on the basis of the presence or absence of *H. pylori* infection, which was identified by histologic examination and by a rapid urease test (Pyloritek test, Serim Research Corp.). If either of these assays was positive, the patient was considered to be HP+. From each patient, gastric mucosa biopsy specimens were taken from the gastric body and antrum. DNA was prepared using a QIAamp DNA Mini Kit (Qiagen).

Bisulfite Treatment. Bisulfite treatment was carried out as described previously (19). Briefly, 2 μg of DNA was incubated with 10 mmol/L hydroquinone and 3 mol/L sodium bisulfite for 16 h at 37°C. The DNA was then purified using a PCR purification system (Promega). After precipitation with ethanol, the DNA was resuspended in 20 μL of distilled water and stored at -20°C until used.

Bisulfite Pyrosequencing. Pyrosequencing was carried out as described previously (14, 20). Bisulfite PCR primers were designed using PSQ Assay Design software (Biotage), and the primers and PCR conditions used for specific target genes are shown in Table 2. After PCR, the biotinylated PCR product was purified and made single stranded to act as a template in a pyrosequencing reaction run as recommended by the manufacturer (Biotage). The PCR products were bound to streptavidin-coated Sepharose beads, after which beads containing the immobilized PCR products were purified,

Table 2. Primer sequences used in this study

Pyrosequencing	Sequences	No. CpG sites analyzed
CDH13	F: 5′-GYGAGGTGTTTATTTYGTATTTGT-3′ R: 5′-AACCAACTTCCCAAATAAATCAAC-3′ Sequence: 5′-TGTTATGTAAAAYGAGGG-3′	2
CDH1		
Set1	F: 5′-TTTTTTGATTTTAGGTTTTAGTGAGTTAT-3′ R: 5′-TACCRACCACAACCAATCAACAAC-3′ Sequence: 5′-GATTTTAGGTTTTAGTGAGT-3′	3
Set2	F: 5′-GGAATTGTAAAGTATTTGTGAGTTTG-3′ R: 5′-RAAATACCTACAACAACAACAACAAC-3′ Sequence: 5′-GGAAGTTAGTTTAGATTTTA-3′	4
PGP9.5	F: 5′-AGTGAGATTGTAAGGTTTGGGGGTT-3′ R: 5′-ACCGCCCAAACTACAACTATAAAAC-3′ Sequence: 5′-GGGGTTYGTATTTATTTG-3′	4
LINE-1	F: 5′-TTTTGAGTTAGGTGTGGGATATA-3′ R: 5′-AAAATCAAAAAATTCCCTTTC-3′ Sequence: 5′-GGGTGGGAGTGAT-3′	3
Bisulfite sequencing		
CDH1	F: 5′-GGATTYGAATTTAGTGGAATTAGA-3′ R: 5′-CAAAACTAAAACRCRAAACTTAC-3′	38

NOTE: Y = C or T, R = A or G.

washed, and denatured using a 0.2 mol/L NaOH solution. Thereafter, 0.3 μmol/L pyrosequencing primers were annealed to the purified single-stranded PCR product, and pyrosequencing was carried out using a PSQ HS 96 Pyrosequencing System (Biotage), after which methylation was then quantified using PSQ Assay Design software (Biotage).

Bisulfite Sequencing. To amplify the CDH1 promoter region, PCR was carried out using primers that amplify the region around the transcription start site of the gene (Fig. 1B, Table 2). The resultant PCR products were then cloned into pCR4.0 vector using a TOPO-TA Cloning Kit (Invitrogen). The sequencing reaction was carried out using a BigDye terminator cycle sequencing kit (PE Biosystems), and sequencing was done using an ABI PRISM 3100 sequencer according to the manufacturer guidelines (Applied Biosystems). Alleles that showed methylation of >80% of their CpG sites were considered to be densely methylated.

Statistical Analysis. Proportions and mean values between two groups were compared using Fisher's exact test (two tailed) or Student's *t* test. Methylation levels and densities among three groups were compared using one-way ANOVA with Games-Howell post hoc test and age-adjusted analysis of covariance (ANCOVA) with Bonferroni Correction for multiple comparisons. Pearson's correlation coefficients were calculated between methylation levels of each gene and by each method. $P < 0.05$ was considered statistically significant. All statistical analyses were carried out using Statistical Package for the Social Sciences software 15.0 J (SPSS, Inc.).

Results

Hypomethylation of LINE-1 in EF+ Gastritis. We initially used pyrosequencing to assess the levels of LINE-1 methylation in a panel of gastric mucosae (Figs. 1 and 2, Table 3). Among 48 patients, 13 patients were

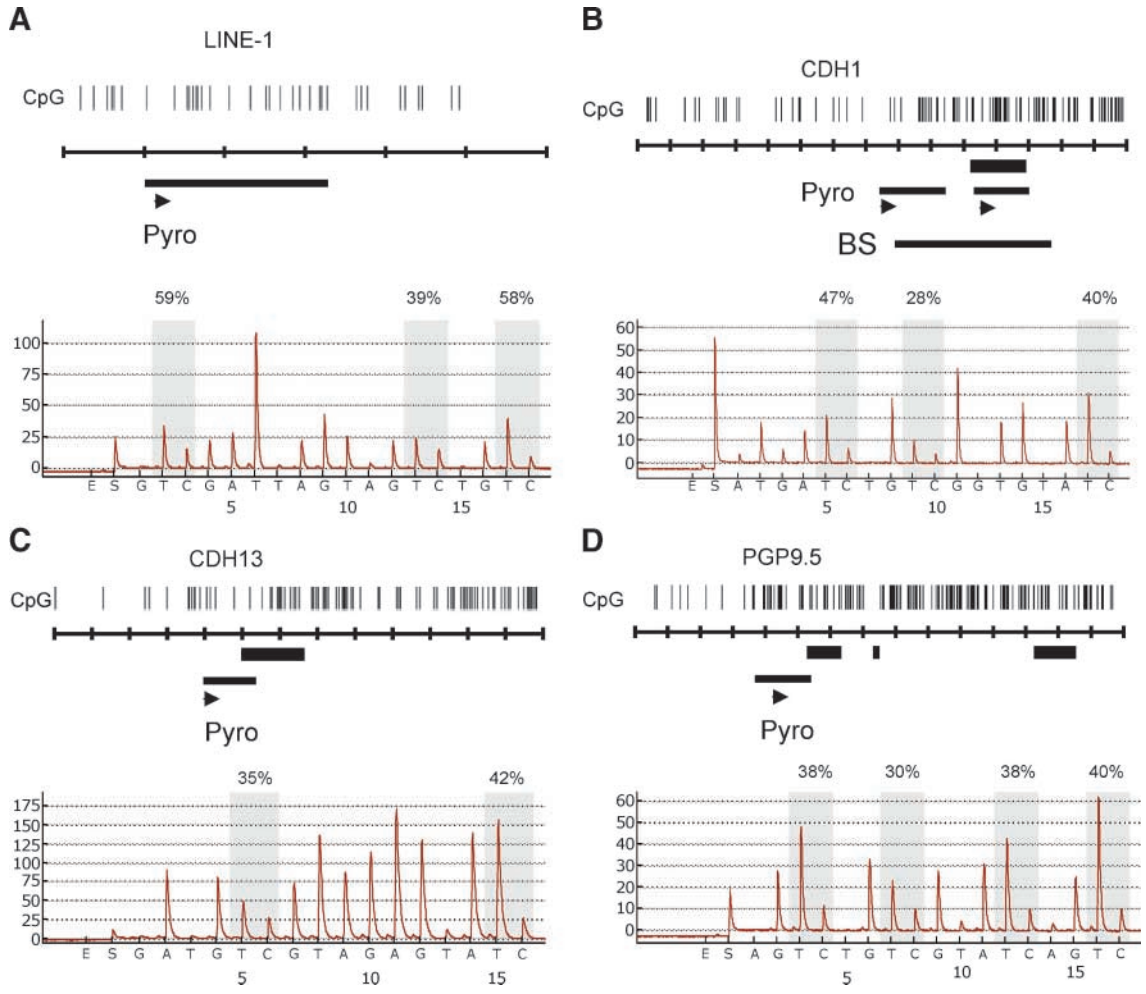

Figure 1. Quantitative pyrosequencing analysis of LINE-1 (**A**), CDH1 (**B**), CDH13 (**C**), and PGP9.5 (**D**) methylation. *Top,* schematic representation of the CpG islands analyzed; *horizontal bars,* regions analyzed by pyrosequencing (*Pyro*) and bisulfite sequencing (*BS*); *bent bars,* transcription start sites; *bottom,* representative pyrograms; *gray columns,* regions of C-to-T polymorphic sites. Percent methylation is shown above the peak. *Y-axis,* signal peaks reflecting to the number of nucleotides incorporated; *X-axis,* nucleotides incorporated.

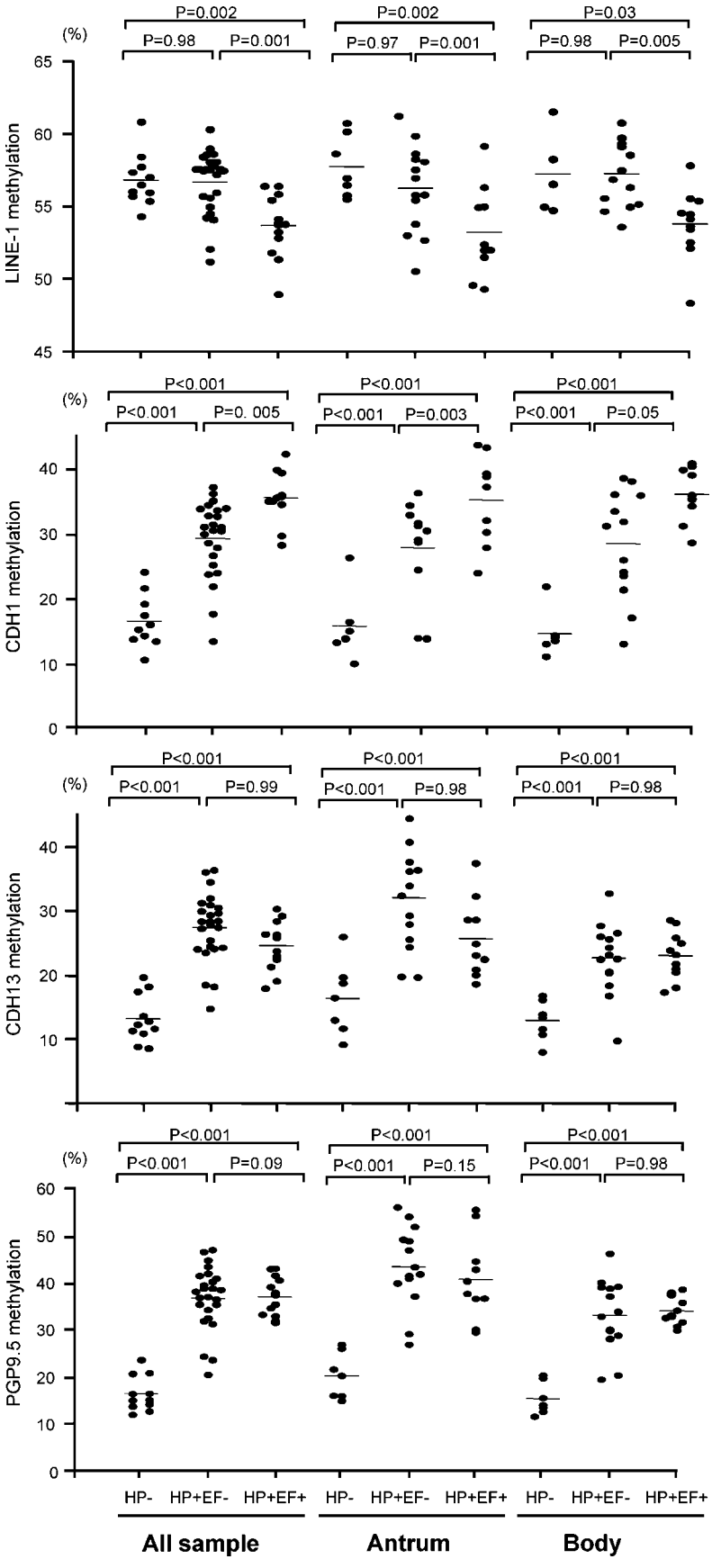

Figure 2. Methylation levels. Percent methylation of LINE-1, CDH1, CDH13, and PGP9.5 was compared among gastric mucosae from patients with HP+/EF+, HP+/EF-, and HP- gastritis. *Filled circles,* average methylation of multiple CpG sites. In 30 cases, specimens were obtained from the antrum and body, and the average methylation levels are shown. Genes analyzed are shown on the left. *Horizontal bars,* average methylation levels in total cases.

Table 3. Summary of pyrosequence results

All subjects				Antrum				Body			
Gene	HP/EF status	*n* (mean ± SD)	95% CI	Gene	HP/EF status	*n* (mean ± SD)	95% CI	Gene	HP/EF status	*n* (mean ± SD)	95% CI
LINE-1	HP−	11 (56.8 ± 1.8)	55.6-57.9	*LINE-1*	HP−	7 (57.7 ± 2.1)	55.7-59.6	*LINE-1*	HP−	7 (57.2 ± 2.3)	55.0-59.3
	HP+/EF−	24 (56.7 ± 2.2)	55.7-57.6		HP+/EF−	13 (56.2 ± 3.1)	54.2-58.0		HP+/EF−	13 (57.4 ± 2.3)	56.0-58.7
	HP+/EF+	13 (53.6 ± 2.1)	52.3-54.9		HP+/EF+	10 (53.2 ± 3.1)	50.9-55.4		HP+/EF+	10 (53.8 ± 2.5)	51.9-55.5
CDH1	HP−	10 (16.4 ± 4.1)	13.4-19.2	*CDH1*	HP−	6 (15.6 ± 5.6)	9.74-21.4	*CDH1*	HP−	6 (14.4 ± 3.7)	10.5-18.3
	HP+/EF−	23 (29.8 ± 4.9)	27.6-31.9		HP+/EF−	12 (28.9 ± 5.9)	25.1-32.6		HP+/EF−	12 (29.6 ± 7.1)	25.0-34.1
	HP+/EF+	12 (35.3 ± 4.2)	32.6-37.9		HP+/EF+	9 (35.0 ± 7.0)	29.6-40.3		HP+/EF+	9 (35.9 ± 4.3)	32.6-39.2
CDH13	HP−	11 (16.4 ± 3.7)	13.8-18.8	*CDH13*	HP−	7 (20.2 ± 4.9)	15.6-24.7	*CDH13*	HP−	7 (15.3 ± 3.4)	12.1-18.4
	HP+/EF−	24 (37.2 ± 6.3)	34.5-39.8		HP+/EF−	13 (44.5 ± 7.4)	40.0-49.0		HP+/EF−	13 (34.0 ± 6.9)	29.8-38.1
	HP+/EF+	13 (36.9 ± 4.1)	34.4-39.4		HP+/EF+	10 (40.7 ± 8.8)	34.3-46.9		HP+/EF+	10 (33.6 ± 2.9)	31.5-35.6
PGP9.5	HP−	11 (13.2 ± 3.7)	10.6-15.6	*PGP9.5*	HP−	7 (16.3 ± 5.7)	11.0-21.5	*PGP9.5*	HP−	7 (12.9 ± 3.1)	10.0-15.7
	HP+/EF−	24 (27.7 ± 5.0)	25.6-29.8		HP+/EF−	13 (32.9 ± 7.3)	28.5-37.3		HP+/EF−	13 (23.0 ± 5.4)	19.7-26.3
	HP+/EF+	13 (24.5 ± 3.8)	22.2-26.8		HP+/EF+	10 (25.6 ± 6.0)	21.3-29.9		HP+/EF+	10 (22.7 ± 3.8)	19.9-25.3

HP+/EF+, 24 patients were HP+/EF-, and 11 patients were HP-. We found that the average levels of LINE-1 methylation were similar in specimens from patients with HP+/EF- and HP- gastritis (56.7% and 56.8%; 95% CI, 55.7-57.6 and 55.6-57.9, respectively; $P = 0.98$). On the other hand, specimens from patients with HP+/EF+ gastritis showed significantly lower levels of LINE-1 methylation (53.6%; 95% CI, 52.3-54.9) than either of the other two groups (HP- versus HP+/EF+ 95% CI, 1.2-5.2; $P = 0.002$; HP+/EF- versus HP+/EF+ 95% CI, 1.2-4.9; $P = 0.001$). Because there were age differences between HP+/EF+ and other two groups, we did age-adjusted ANCOVA with Bonferroni Correction for multiple comparisons. We found the same tendency for the differences by age-adjusted model, indicating that age-related differences in methylation did not account for the results (Table 4).

In 30 patients, specimens were obtained from the gastric antrum and body, which enabled us to compare the levels of LINE-1 methylation in these two areas. We found that in HP+/EF+ cases, methylation of LINE-1 in the gastric body was significantly lower than that in either HP+/EF- (53.8% versus 57.4%; $P = 0.005$) or HP- cases (53.8% versus 57.2%; $P = 0.03$). Likewise, methylation of LINE-1 in the antrum in HP+/EF+ cases was significantly lower than that in HP+/EF- (53.2% versus 56.2%; $P = 0.001$) or HP- cases (53.2% versus 57.7%; $P = 0.002$). There was a significant correlation between the methylation levels of LINE-1 in the antrum and body ($R = 0.58$; $P = 0.001$; Table 5).

Table 4. The difference of mean age-adjusted levels of *LINE-1, CDH1, CDH13, and PGP9.5* methylation

Gene	HP/EF status	compared with	difference	SE	*P*	95% CI
LINE-1	HP−	HP+/EF−	0.3	0.8	>0.999	(−1.8 to 2.3)
		HP+/EF+	3.2	0.9	0.002	(1.0 to 5.4)
	HP+/EF−	HP+/EF+	3.0	0.7	0.001	(1.0 to 4.8)
CDH1	HP−	HP+/EF−	−13.4	1.8	<0.001	(−18.0 to −8.8)
		HP+/EF+	−19.5	2.1	<0.001	(−24.8 to −14.1)
	HP+/EF−	HP+/EF+	−6.0	1.8	0.006	(−10.5 to −1.4)
CDH13	HP−	HP+/EF−	−18.2	1.7	<0.001	(−22.4 to −13.9)
		HP+/EF+	−18.4	1.9	<0.001	(−23.0 to −13.7)
	HP+/EF−	HP+/EF+	−1.2	1.6	>0.999	(−5.2 to 2.7)
PGP9.5	HP−	HP+/EF−	−12.3	1.4	<0.001	(−15.8 to −8.7)
		HP+/EF+	−10.4	1.5	<0.001	(−14.1 to −6.5)
	HP+/EF−	HP+/EF+	1.9	1.3	0.442	(−1.3 to 5.1)

Table 5. Correlation of methylation levels in antrum and body

	Mean (SD)		Correlation coefficient (R =)	P
	Antrum	Body		
LINE-1	55.5 (3.3)	56.2 (2.9)	0.58	0.001
CDH1	28.0 (9.4)	28.3 (9.6)	0.82	<0.001
CDH13	37.6 (12.2)	29.5 (9.4)	0.71	<0.001
PGP9.5	26.6 (9.1)	20.5 (6.0)	0.67	<0.001

Hypermethylation of 5′ CpG Islands of Genes Is Associated with Hypomethylation of LINE-1 in EF+ Gastritis. To examine the methylation levels of the 5′ CpG islands of genes involved in HP+/EF+ gastritis, we used pyrosequencing to assess methylation of CDH1, CDH13, and PGP9.5 (Fig. 1). For all three genes, the level of methylation was significantly higher in specimens from patients with *H. pylori* infection (Fig. 2; Table 3). Furthermore, methylation of CDH1 was significantly higher in HP+/EF+ cases (35.3%) than in either HP+/EF- (29.8%) or HP- (16.4%) cases ($P < 0.001$; P = 0.005). Methylation of CDH13 and PGP9.5 was higher in HP+/EF+ cases (CDH13, 36.9%; PGP9.5, 24.5%) than in HP-/EF- cases (CDH13, 16.4%; $P < 0.001$; PGP9.5, 13.2%; $P < 0.001$) but was similar to that in HP+/EF- cases (CDH13, 37.2%; $P = 0.99$; PGP9.5, 27.7%; $P = 0.09$). Regression analysis of the levels of methylation of all three genes revealed significant correlations between the methylation of CDH1 and CDH13, CDH1 and PGP9.5, and CDH13 and PGP9.5 (Fig. 3A). Overall, there was a significant correlation between the levels of methylation in the antrum and body (CDH1, $R = 0.82$; $P < 0.001$; CDH13, $R = 0.71$; $P < 0.001$; PGP9.5, $R = 0.67$; $P < 0.001$; Table 5).

We next examined the correlation between levels of LINE-1 methylation and methylation of the CDH1, CDH13, and PGP9.5 CpG islands (Fig. 3B), and observed inverse relations between the level of LINE-1 methylation and methylation of CDH1 ($R = -0.40$; $P = 0.0003$), CDH13 ($R = -0.31$; $P = 0.005$), and PGP9.5 ($R = -0.23$; $P = 0.04$).

Because the median value of LINE-1 methylation was 56.0%, we used that as a cut point to separate specimens with hypomethylation of LINE-1 (<56%) from those without it (LINE-1>56%). We found that hypomethylation of LINE-1 was significantly associated with EF+ gastritis ($P = 0.008$) and methylation of CDH1 ($P = 0.05$; Table 1).

Dense Methylation of CDH1 in EF+ Gastritis. With pyrosequencing, we examined only seven CpG sites in the CDH1 promoter. For that reason, we also investigated the density of CDH1 methylation by bisulfite-sequencing PCR products amplified using primers that contained the region around the transcription start site of the gene (Fig. 4A and B). Gastric mucosae from HP+/EF- cases showed increased methylation of CDH1, but the density of CDH1 methylation was higher in HP+/EF+ cases ($n = 8$; average, 27.8%) than in either HP- ($n = 8$; average, 3.3%) or HP+/EF- cases ($n = 17$; average, 15.6%). When we then determined the percentages of clones that showed >80% methylation of CpG sites, we found that, in the antrum, the percentages were higher in HP+/EF+ cases (average, 11.6%) than in HP- (average, 0%) and HP+/EF- (average, 9.6%) cases, although the difference between the two HP+ groups was not significant (Fig. 1A). In the gastric body too, the percentages of clones showing methylation of >80% of CpG sites were higher in HP+/EF+ cases (average, 9.5%) than in the other two groups (average, 0% and 3.7%, respectively;

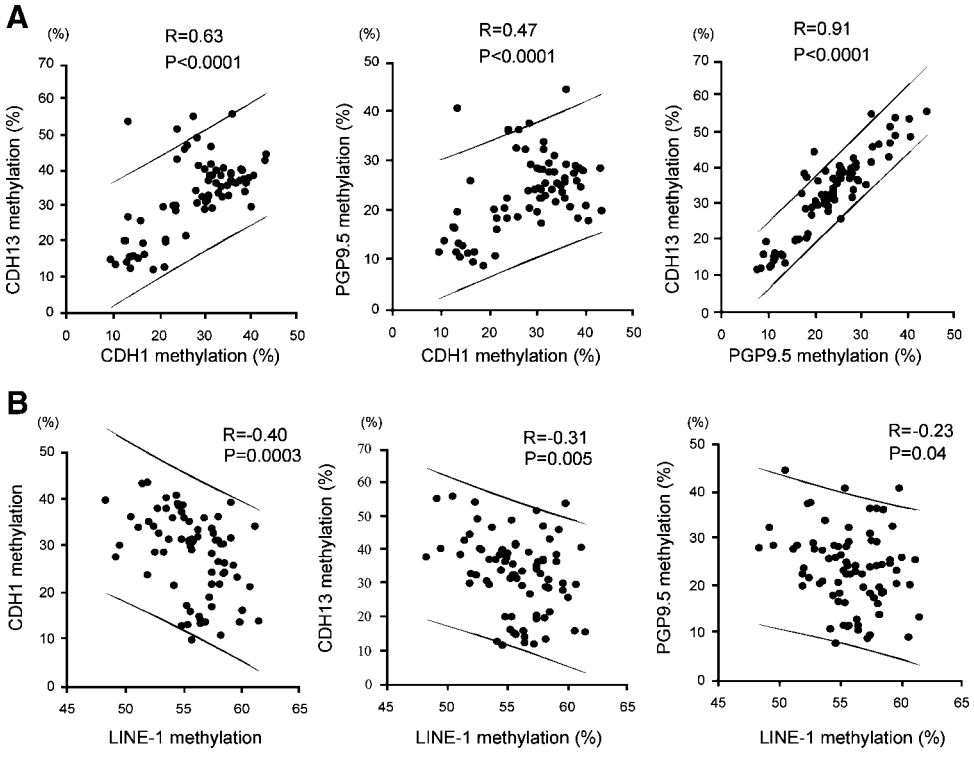

Figure 3. Correlation of methylation among genes. **A.** Regression analysis showing concordance of the methylation of CDH1, CDH13, and PGP9.5 in gastric mucosae. *Solid lines,* 95% CI. **B.** Inverse relation between LINE-1 methylation and CpG island methylation.

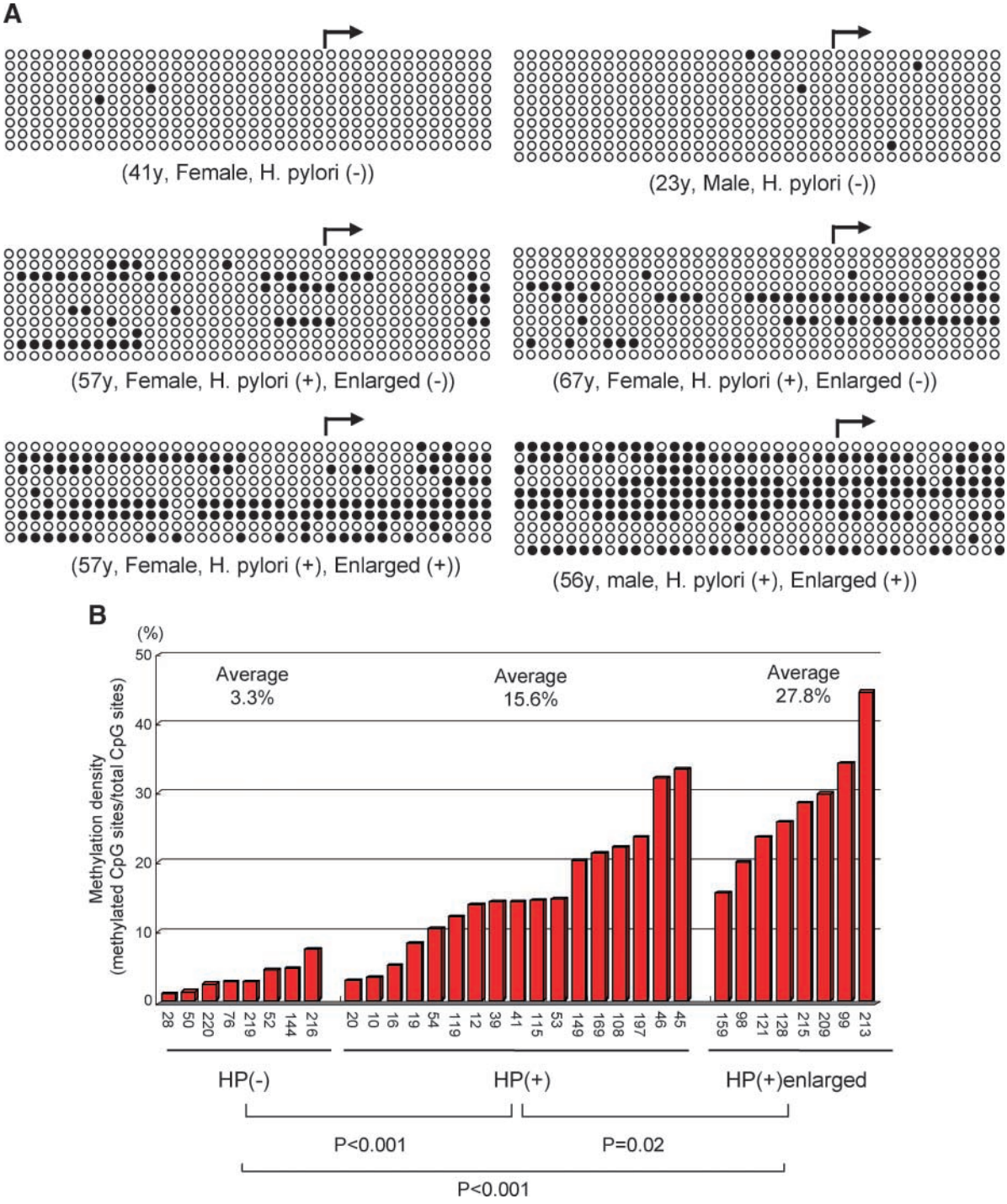

Figure 4. A. Bisulfite sequencing of CDH1 in HP+/EF+ gastritis. *Vertical columns,* CpG sites; *horizontal arrows,* transcription start sites; *open circles,* unmethylated CpG sites; *filled circles,* methylated CpG sites. The cases examined are below the columns. **B.** Density of CDH1 methylation in gastric mucosae; CpG site methylation is expressed as a percentage of the total (*Y-axis*). Average of density of methylation in HP+/EF+ gastritis is higher than in HP+/EF- and HP- gastritis.

Fig. 1B). Note that there was a good correlation between the methylation levels detected by pyrosequencing and those detected by bisulfite sequencing ($R = 0.59$; $P < 0.0001$; Fig. 5).

Discussion

DNA methylation plays a key role in the silencing of genes in cancer (8). Indeed, DNA methylation seems to

be an early event during tumorigenesis that is even detectable in premalignant regions (e.g., intestinal metaplasia; refs. 21, 22). However, little is known about genomewide methylation status in premalignant regions during gastric tumorigenesis. In the present study, we have shown that levels of LINE-1 methylation are significantly reduced in EF+ gastritis and that this hypomethylation was associated with increased methylation of 5′ CpG islands of genes, which suggests that methylation of the promoter regions of these genes is accompanied by global demethylation of repetitive sequences. Such global hypomethylation is known to occur during the development of colorectal cancer (15). Its functional consequences in tumorigenesis remain unclear, although it has been proposed that genomewide hypomethylation gives increase to chromosomal instability (23, 24). That levels of LINE-1 methylation strongly correlate with methyl cytosine content detected by liquid chromatography–mass spectrometry suggests LINE-1 methylation can be used as a surrogate for genomewide methylation (20, 25). Thus, the findings that LINE-1 hypomethylation inversely correlates with microsatellite instability in colorectal cancers (14) and is associated with alteration of chromosome 8 in prostate cancers (26) suggest genomewide hypomethylation is associated with genetic and epigenetic alterations in neoplasias.

Although genomewide hypomethylation and regional hypermethylation of 5′ CpG islands are common features of neoplasias, the link between the two is controversial (27). Recent studies using methylation of LINE-1 and/or Alu as a marker revealed that genomewide hypomethylation is tightly linked to CpG island hypermethylation in prostate cancers (17, 28) and neuroendocrine tumors (29). Our studies indicate that genomewide hypomethylation and CpG island hypermethylation are tightly linked in EF+ gastritis, and that these events are some of the earliest alterations occurring during gastric tumorigenesis. The reason for the discrepancies between the published results on genomewide hypomethylation and CpG island hypermethylation remains unclear, although they may be attributable, to some extent, to differences in the methods used to detect methylation. For example, several studies used methylation-specific PCR to detect hypermethylation of promoter regions (16), whereas others used quantitative methylation analyses such as MethyLight or Pyrosequencing (14, 30). Alternatively, methylation in cancer and noncancerous tissues may involve different molecular mechanisms. Consistent with that idea, Iacopetta et al. (30) reported that, whereas there is no correlation between LINE-1 hypomethylation and CpG island methylation in colorectal cancer, there is a significant correlation between the two events in normal colon. Further studies will be needed to clarify the molecular mechanisms responsible for aberrant methylation in noncancerous tissues.

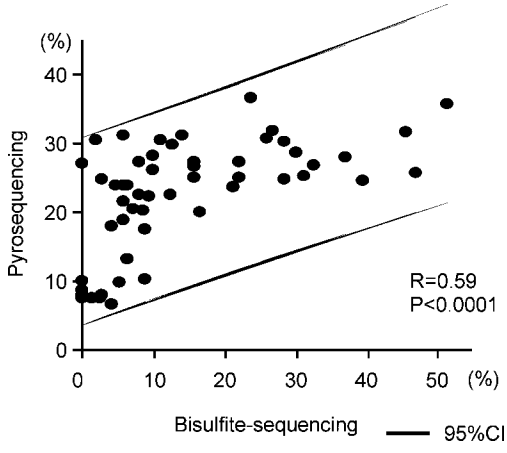

Figure 5. Regression analysis of methylation levels detected using pyrosequencing and bisulfite sequencing. Levels of CDH1 methylation detected by pyrosequencing are plotted against the density of CDH1 methylation detected by bisulfite sequencing.

Aberrant methylation of CpG islands is known to be one of the molecular mechanisms involved in *H. pylori*–associated carcinogenesis (31); moreover, CDH1 methylation is known to be increased in EF+ gastritis (13). In the present study, we observed that CDH13 and PGP9.5 are also methylated in gastric mucosae with *H. pylori* infection, which is consistent with an earlier study showing CDH13 to be aberrantly methylated in gastric cancer (32). CDH13 (also known as H-cadherin) encodes a protein related to the cadherin superfamily of cell adhesion molecules and is involved in suppressing cell growth, invasion, and metastasis (33, 34). Thus, methylation of CDH13 in the gastric mucosa may provide a growth advantage to cells during tumorigenesis. PGP9.5 was previously shown to be aberrantly methylated in diffuse-type gastric cancers (35). Introduction of PGP9.5 into cancer cells suppresses cell growth and induces apoptosis, suggesting PGP9.5 serves as a tumor suppressor in gastric cancer (35). In addition to methylation of CDH13 and PGP9.5, EF+ gastritis shows CDH1 hypermethylation and LINE-1 hypomethylation, which is consistent with the fact that, among patients with *H. pylori*–associated gastritis, those with EF+ gastritis have a much higher risk of developing cancer (7). There were no HP-/EF+ cases because diseases in which EF+ gastritis is present without *H. pylori* infection (e.g., Zollinger-Ellison syndrome and lymphoma) are rare and were not included in this study. Further study will be needed to determine whether EF+ gastritis in the absence of *H. pylori* infection also shows LINE-1 hypomethylation and high levels of CpG island methylation.

We applied bisulfite pyrosequencing to assess the methylation levels of LINE-1 and three cancer-related genes. In several earlier studies, methylation-specific PCR was used to detect changes in gene methylation in premalignant regions during gastric tumorigenesis (21, 22, 36). With methylation-specific PCR, however, only the frequency of methylation in each population can be analyzed. Bisulfite pyrosequencing offers a semiquantitative, high throughput, and reliable method that has an inbuilt internal control for adequacy of bisulfite treatment (37). This approach enabled us to determined that levels of CDH1, CDH13, and PGP9.5 methylation are positively correlated, and that there is an inverse relation between LINE-1 and CpG island methylation.

The molecular mechanism underlying genomewide hypomethylation in EF+ gastritis remains unknown; however, our finding that methylation levels in the

Table 6. Characteristics of gastritis with or without *LINE-1* hypomethylation

	LINE-1<56% (n = 24)	*LINE-1*>56% (n = 24)	*P*
Sex			
Male	12	19	0.07
Female	12	5	
Age			
Mean (SD)	52.7 (12.6)	54.5 (15.1)	0.85
H. pylori			
Positive	19	18	1
Negative	5	6	
Enlarged fold			
Positive	11	2	0.008
Negative	13	22	
Methylation			
CDH1	32.2 (7.7)	24.2 (8.2)	0.054
CDH13	36.0 (10.4)	29.4 (11.4)	0.312
PGP9.5	25.3 (7.8)	21.7 (8.3)	0.742

antrum and body correlate with one another suggests that it is the degree of inflammation that affects DNA methylation in both the pyloric and fundic glands from different parts of stomach. Recent studies suggest a role for proteins involved in the maintenance of heterochromatin in the silencing of repetitive elements (38). For example, Lsh deficiency leads to abnormal heterochromatin organization, with a genomewide loss of DNA methylation (39). Moreover, histone H3 lysine-27 demethylase Jmjd3 is induced by inflammation and is involved in altered histone modification in inflamed tissues (40). This suggests that altered chromatin structure and/or histone modification induced by inflammation may be involved in global demethylation, although that idea remains to be tested. Nevertheless, hypomethylation of LINE-1 may represent a much needed molecular marker with which to predict the risk for gastric cancer associated with EF+ gastritis.

In conclusion, we have shown that LINE-1 hypomethylation is significantly associated with CpG island hypermethylation in EF+ gastritis. Our results not only shed light on the pathogenesis of gastric cancer associated with *H. pylori* infection, but also suggest that hypermethylation of gene promoters is often associated with global demethylation of repetitive sequences during tumorigenesis Table 6.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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