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Role of *Helicobacter pylori* infection in aberrant DNA methylation along multistep gastric carcinogenesis

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CpG island hypermethylation is frequently found during gastric carcinogenesis. We investigated methylation profiles of *p16*, *LOX*, *HAND1*, *THBD*, *p41ARC*, and *APC* along multistep gastric carcinogenesis and determined their association with *Helicobacter pylori* infection. Methylation levels in these six genes were evaluated in noncancerous gastric biopsy specimens using quantitative methylation-specific PCR in 459 patients with gastric cancer (GC), 137 with dysplasia, and 248 controls. Controls were divided into four subgroups sorted by current *H. pylori* infection status (active *vs* past or negative infection) and the presence of intestinal metaplasia (IM). In controls, active *H. pylori* infection significantly increased methylation levels in *THBD*, *LOX*, and *HAND1* (all $P < 0.001$), and hypermethylation of *THBD*, *HAND1*, and *APC* was associated with IM. Aberrant DNA hypermethylation was correlated well with activity of *H. pylori*-associated gastritis. However, methylation levels in *LOX*, *HAND1*, *THBD*, and *p41ARC* remained increased in cases with past *H. pylori* infection compared to those that were *H. pylori* negative (all $P < 0.05$). Hypermethylation of *THBD*, and possibly *p16*, was significantly associated with GC, regardless of the status of current *H. pylori* infection (all $P < 0.05$). These results suggest that aberrant DNA hypermethylation caused by *H. pylori*-associated gastritis occurs in a gene-specific manner along gastric carcinogenesis, which can be persistent even after the disappearance of *H. pylori*. Aberrant methylation of *THBD* might provide a link between *H. pylori* infection and development of GC. (*Cancer Sci* 2010; 101: 1337–1346)

Promoter CpG island (CGI) hypermethylation is an important mechanism for the silencing of tumor suppressor genes, and has been identified among the earliest and most common alterations in human cancers.[1] Gastric cancer (GC) is known to be associated with tumor-suppressor or tumor-related genes that are inactivated more frequently by CGI hypermethylation than by mutations.[2] CGI hypermethylation has also been found in the adjacent noncancerous tissues of patients with GC and in non-neoplastic gastric mucosa of subjects without GC.[3]

It is now generally accepted that *Helicobacter pylori* infection is an important etiological risk factor for GC. Gastric carcinogenesis is regarded as a multistep process with a postulated intestinal metaplasia (IM)-dysplasia-invasive carcinoma sequence that occurs during morphologic progression;[4] this ''Correa cascade'' is promoted by *H. pylori* infection which causes a chronic active inflammation with severe oxidative damage of the gastric mucosa.[5] Several reports have suggested association of *H. pylori* infection with specific gene promoter methylation,[6,7] and *H. pylori*-associated active inflammation seems to be responsible for the promoter CGI hypermethylation.[2,8,9] However, it is still unclear whether aberrant DNA hypermethylation remains persistent even after active *H. pylori* infection discontinues.

In this study, we used quantitative profiling of DNA methylation states in a panel of six GC-associated genes (*p16*, *LOX*, *HAND1*, *THBD*, *p41ARC*, and *APC*) in a large case–control study. Among them, *p16*, *LOX*, and *APC* are tumor-suppressor genes.[2] Although the *p16* core region is resistant to methylation,[10] hypermethylation in this region was reported to be critical for *p16* silencing and is associated with *H. pylori* infection in gastric precancerous lesions.[11] In addition, *LOX*, *HAND1*, and *THBD* genes have been reported to be frequently methylated in GC, which were silenced by CGI hypermethylation.[12] *p41ARC* and *APC* have been also reported frequently methylated in GC.[13,14] This study was performed to investigate the methylation profiles of *p16*, *LOX*, *HAND1*, *THBD*, *p41ARC*, and *APC* along multistep gastric carcinogenesis in the condition of current *H. pylori* infection (positive for endoscopy-based tests) or a past one (*H. pylori* serology-positive only), to find a link between *H. pylori* infection and development of GC.

Materials and Methods

Subjects. Enrollment of 459 patients with primary GC, 137 patients with dysplasia, and 248 controls was carried out at Seoul National University Bundang Hospital from 2003 to 2008. All subjects were of Korean origin. Most of them had undergone a standard gastroscopy as part of a screening program for premalignant gastric mucosal lesions or GC. All GC or dysplasia patients were histologically confirmed to have gastric adenocarcinoma or dysplasia, respectively. When the endoscopy did not show any evidence of GC, dysplasia, mucosa-associated lymphoid tissue lymphoma, esophageal cancer, or peptic ulcer, the subjects were enrolled as controls. Next, the controls were divided into four subgroups sorted by the status of current *H. pylori* infection (active *vs* past or negative infection) and the presence of IM for further analysis. If there was histological evidence of IM in either the antral or body biopsy specimens, the subject was defined as IM positive. The study protocol was approved by the Ethical Committee at Seoul National University Bundang Hospital. All subjects, who provided informed consent, were asked to complete a questionnaire, which included questions regarding demographic information (age and gender) and socioeconomic habits (smoking and drinking). Patients with a history of *H. pylori* eradication were excluded from the study.

***Helicobacter pylori* testing and histology.** To determine the presence of a current *H. pylori* infection, 10 biopsy specimens were obtained for three types of *H. pylori* testing (histology, the *Campylobacter*-like organism [CLO] test, and culture). Among them, two from the greater curvature side of the antrum and two

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from the body were fixed in formalin to assess the presence of *H. pylori* (by modified Giemsa staining) and the degree of inflammatory cell infiltration, atrophy, and IM (all determined by hematoxylin–eosin staining). These histologic features of the gastric mucosa were recorded using the updated Sydney scoring system (i.e. 0 = none, 1 = slight, 2 = moderate, and 3 = marked).[15] One specimen from each, the lesser curvature of the antrum and the body, was used for rapid urease testing (CLO-test; Delta West, Bentley, Australia), and two specimens from the antrum and from the body, respectively, were used for culture, and the organisms present were identified as *H. pylori* by Gram staining, colony morphology, and positive oxidase, catalase, and urease reactions. In addition, anti-*H. pylori* immunoglobulin G was determined qualitatively using an enzyme-linked immunosorbent assay (Genedia *H. pylori* ELISA; Green Cross Medical Science, Eumseong, Korea). If the *H. pylori* serology was positive but no bacteria were found on the histology or CLO test or culture, the diagnosis was a past *H. pylori* infection without evidence of current ongoing infection. The remaining biopsy specimens were immediately frozen at −70°C until DNA extraction.

DNA preparation. Genomic DNA was extracted directly from noncancerous antral biopsy specimens. Briefly, specimens were homogenized in proteinase K solution (20 mmol/L Tris–HCl [pH 8.0], 10 mmol/L ethylenediaminetetraacetic acid, 0.5% sodium dodecyl sulfate, and 10 mg/mL proteinase K) using a sterile micropestle, and then incubated for 3 h at 52°C. DNA was isolated from homogenates by phenol/chloroform extraction and ethanol precipitation.

Sodium bisulfite modification of DNA and quantitative methylation-specific PCR. DNA was subjected to sodium bisulfite modification.[16] In summary, 1 μg of DNA was denatured with 1 M NaOH, followed by treatment with 550 μL bisulfite/hydroquinone solutions for 16–18 h at 55°C. Following purification using a JETSORB gel extraction kit (Genomed, Bad Oeynhausen, Germany), the DNA was treated with 3 M NaOH and precipitated with three volumes of 100% ethanol and a one-third volume of 7.5 M ammonium acetate at −20°C. The precipitated DNA was washed with 70% ethanol and was dissolved in distilled water. The six genes (*p16*, *LOX*, *HAND1*, *THBD*, *p41ARC*, and *APC*) were analyzed for their methylation status. For the *p16*, *LOX*, *HAND1*, and *APC* genes, core regions of their promoter CGIs were measured. For *THBD*, the noncore region of its promoter CGI was evaluated because the core region was not methylated.[13] For *p41ARC*, we analyzed its exon CGI (exon 8), which was frequently methylated in GC.[12] For the *APC* gene, among two promoters identified (1A and 1B), we analyzed the methylation of the *APC* 1A promoter,[17] because no methylation of the *APC* 1B promoter has ever been detected in the stomach.[14] The primer sequences and PCR conditions are shown in Table 1. An aliquot of 2 μL was used for real-time PCR with a primer set specific to methylated or unmethylated sequences, with a specific annealing temperature of 53–66°C. Real-time PCR was performed using 2× SYBR Premix Ex Taq (Takara Bio, Shiga, Japan) and the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster, CA, USA). Standard DNA was prepared by cloning PCR products into the pGEM-T Easy vector (Promega, Madison, WI, USA), or was kindly provided by Professor T. Ushijima's laboratory (National Cancer Center Research Institute, Tokyo, Japan). The number of molecules in a test sample was determined by comparing the amplification with those of standard samples containing a known number of molecules ($10^6 - 10^1$). The number of methylated and unmethylated molecules was measured separately, and the methylation level was calculated as following: (Methylation level) = (Number of methylated molecules)/{Total number of DNA molecules (methylated + unmethylated molecules)}.

The reproducibility of the methylation proportions obtained by quantitative methylation-specific PCR was analyzed for the *HAND1* and *p16* CGIs, following a Japanese study.[18] Briefly, test DNA samples were prepared by mixing the DNA that was completely methylated by *SssI* methylase with that of samples where no methylation was detected with methylation ratios (60%, 6%, 4.5%, 3%, 1.5%, and 0.8%). The same DNA was modified by bisulfite on two different dates, and each group was analyzed in triplicates. The methylation levels were fairly reliable (Supporting information, Fig. S1).

Statistical analysis. Initially, univariate analysis was performed to evaluate whether any significant difference was present in demographic or clinical features among the study groups. Then, the methylation levels of the genes under study were compared between the study groups. As most of the data on methylation profiles were skewed and in some cases heterogeneity of variances was observed, rank-transformed ANOVA was used to compare the four subgroups in controls sorted by the absence or presence of current *H. pylori* infection and IM; it was also used to compare the methylation levels in the six genes with regard to the three categorized *H. pylori* infection statuses (negative, current, and past). The Bonferroni method was applied for multiple comparisons. In addition, unconditional multivariate logistic regression analysis stratified for the status of current *H. pylori* infection was performed to compare the methylation levels in the six genes between controls and patients with dysplasia or GC, adjusted for age, gender, and smoking and drinking habits. For this analysis, the methylation levels in each gene were binomially categorized. For the analysis of the match-paired

Table 1. Primer sequences and PCR conditions for real-time methylation-specific PCR

Genes		Primer sequence		Size (bp)	AT (°C)
		Forward (5′–3′)	Reverse (5′–3′)		
p16	M	5′-TTG GTA GTT AGG AAG GTT GTA TCG C-3′	5′-TCC CTA CTC CCA ACC GCG-3′	126	66
	UM	5′-GGT AGT TAG GAA GGT TGT ATT GT-3′	5′-TCC CTA CTC CCA ACC ACA-3′	124	60
LOX	M	5′-ATA AAT AGT TGA GGG GCG GTC-3′	5′-CGA CAA TCC CGA AAA ACG-3′	120	61
	UM	5′-ATA AAT AGT TGA GGG GTG GTT-3′	5′-ACA ACA ATC CCA AAA AAC A-3′	121	58.5
HAND1	M	5′-ATA GTT TAG GGC GTT GGT C-3′	5′-CTA CTC TAC GAA CTT AAA AAA ACG-3′	100	57
	UM	5′-AAT AGT TTA GGG TGT TGG TT-3′	5′-CTA CTC TAC AAA CTT AAA AAA ACA-3′	101	55
THBD	M	5′-CGT TCG TTT TTA TTC GGC GTC-3′	5′-GCC AAA CCC CAT CTC ATC G-3′	118	60
	UM	5′-ATG TGT TTG TTT TTA TTT GGT GTT-3′	5′-CAA ACC CCA TCT CAT CAA A-3′	119	56
p41ARC	M	5′-CGT GTT TTT AAT TCG TTG CGT C-3′	5′-CGA CCT CTA CCG ACC TCC G-3′	133	64
	UM	5′-GGT GTG TTT TTA ATT TGT TGT GTT-3′	5′-CAA CCT CTA CCA ACC TCC A-3′	135	60
APC	M	5′-TAT TGC GGA GTG CGG GTC-3′	5′-TCA ACG AAC TCC CGA CGA-3′	98	62
	UM	5′-GTG TTT TAT TGT GGA GTG TGG GTT-3′	5′-CCA ATC AAC AAA CTC CCA ACA A-3′	108	62

AT, annealing temperature; bp, base pair; M, methylated sequence; UM, unmethylated sequence.

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samples, we used the Wilcoxon's sign paired test. Differences were considered significant when the *P*-values were <0.05. All analyses were performed using the statistical software package SAS (version 9.1; SAS Institute, Cary, NC, USA) or SPSS (version 12.0; SPSS, Chicago, IL, USA).

Results

Subject characteristics. The distribution of selected demographic variables in controls, patients with dysplasia, and GC patients are shown in Table 2. When controls were categorized based on the presence of IM and *H. pylori* infection, there was no significant difference in demographic and clinical variables among the four groups, except for age ($P < 0.001$). That is, subjects with IM were significantly older than those without IM. On the other hand, there were marked demographic and clinical differences between controls and the patients with GC. Therefore, controls were randomly sampled to match the GC cases by age (within ± 5 years) and gender for the comparison between the two groups, stratified for the status of current *H. pylori* infection. There were 56 *H. pylori*-negative and 140 *H. pylori*-positive case–control pairs matched to investigate the role of methylation in the gastric carcinogenesis.

Aberrant methylation in gastric mucosa with regard to *Helicobacter pylori* infection and intestinal metaplasia. In controls, the methylation levels in the six genes were analyzed in the four subgroups sorted by the status of *H. pylori* infection (active *vs* negative or past infection) and the presence of IM (Fig. 1). For *LOX* (Fig. 1b), *HAND1* (Fig. 1c), and *THBD* (Fig. 1d), methylation levels in *H. pylori*-positive cases were significantly increased compared to the levels in *H. pylori*-negatives ($P < 0.001$). IM significantly increased the methylation levels in *HAND1* and *THBD* among *H. pylori*-negative cases (Group A *vs* Group C, defined in Fig. 1, $P < 0.05$). On the other hand, methylation levels in *APC* (Fig. 1f) were significantly decreased with *H. pylori* among IM-negative cases (Group A *vs* Group B, $P = 0.016$), but were increased with IM, especially in the presence of *H. pylori* (Group B *vs* Group D, $P = 0.004$). For *p16* (Fig. 1a) and *p41ARC* (Fig. 1e), there was no significant association of their methylation levels with *H. pylori* infection and/or IM.

Persistent DNA hypermethylation caused by *Helicobacter pylori* after the *Helicobacter pylori*-associated gastritis discontinues. In the overall study population, we evaluated the association between histologic grades of *H. pylori*-associated gastritis and aberrant DNA hypermethylation in the six genes under study (Table 3). All genes except for *APC* were positively correlated with *H. pylori* density, polymorphous nuclear cell (PMN), and mononuclear cell infiltration in histology. Intriguingly, hypermethylation of the *APC* gene was negatively correlated with *H. pylori* density, as well as PMN and mononuclear cell infiltration. In addition, *HAND1*, *THBD*, and *APC* genes were positively correlated with the grade of IM.

Table 2. Characteristics of subjects

	No. of subjects (%)						
	Controls (n = 248)						
	IM negative*		IM positive*				
	Hp negative†	*Hp* positive†	*Hp* negative†	*Hp* positive†	*P*-values‡	Patients with dysplasia	GC patients
	Group A	Group B	Group C	Group D			
	(n = 76)	(n = 65)	(n = 30)	(n = 77)		(n = 137)	(n = 459)
Females	57 (75.0)	41 (63.1)	19 (63.3)	49 (63.6)	0.358	36 (26.3)	154 (33.6)
Age (years, mean ± SD)	48.8 ± 12.8	50.4 ± 12.1	57.6 ± 9.5§	55.9 ± 11.2¶	< 0.001	63.1 ± 9.0	59.5 ± 11.7
Hp status						123 (89.8)	398 (86.7)
Hp negative	58 (76.3)	0 (0.0)	19 (63.3)	0 (0.0)		23 (16.8)	59 (12.9)
Current active infection	0 (0.0)	65 (100.0)	0 (0.0)	77 (100.0)		92 (67.2)	327 (71.2)
Past infection	18 (23.7)	0 (0.0)	11 (36.7)	0 (0.0)		22 (16.1)	73 (15.9)
Diffuse type	–	–	–	–		–	197 (42.9)
Smoking	(n = 236)					(n = 131)	(n = 434)
Nonsmoker	54 (75.0)	44 (68.8)	18 (66.7)	50 (68.5)	0.772	44 (33.6)	155 (35.7)
Current/ex-smoker	18 (25.0)	20 (31.3)	9 (33.3)	23 (31.5)		87 (66.4)	279 (64.3)
Drinking	(n = 228)					(n = 130)	(n = 434)
Never/rare drinker	14 (20.0)	16 (26.2)	5 (18.5)	15 (21.4)	0.800	69 (53.1)	223 (51.4)
Current/ex-drinker	56 (80.0)	45 (73.8)	22 (81.5)	55 (78.6)		61 (46.9)	211 (48.6)
Intestinal metaplasia							
Antrum	(n = 248)					(n = 135)	(n = 451)
None	76 (100.0)	65 (100.0)	9 (30.0)	15 (19.5)	–	29 (21.5)	140 (31.0)
Mild	0 (0.0)	0 (0.0)	9 (30.0)	29 (37.7)		40 (29.6)	123 (27.3)
Moderate/severe	0 (0.0)	0 (0.0)	12 (40.0)	33 (42.9)		66 (48.9)	188 (41.7)
Corpus	(n = 248)					(n = 135)	(n = 449)
None	76 (100.0)	65 (100.0)	12 (40.0)	32 (41.6)	–	46 (34.1)	229 (51.0)
Mild	0 (0.0)	0 (0.0)	10 (33.3)	23 (29.9)		36 (26.7)	96 (21.4)
Moderate/severe	0 (0.0)	0 (0.0)	8 (26.7)	22 (28.6)		53 (39.3)	124 (27.6)

Counts within categories may not be the sum of the total shown at the top of the table owing to missing data. *IM positive denotes any histological evidence of intestinal metaplasia in either the antrum or body. †*Hp* positive refers to a current active *Helicobacter pylori* infection, and *Hp* negative includes those with a past *H. pylori* infection (*H. pylori* serology-positive only, as well as those negative to all *H. pylori* tests (CLOtest, histology, culture, and serology). ‡*P*-values for chi-squared test or one-way ANOVA analysis for the comparison of variables across groups. Post-hoc HSD-Turkey test for pair-wise comparisons between groups; §$P = 0.003$ for Group A *versus* Group C; ¶$P = 0.030$ for Group B *versus* Group D. Current infection refers to positivity for any one of the endoscopy-based *H. pylori* tests, and past infection positive for *H. pylori* serology only. *Hp*, *H. pylori*; IM, intestinal metaplasia.

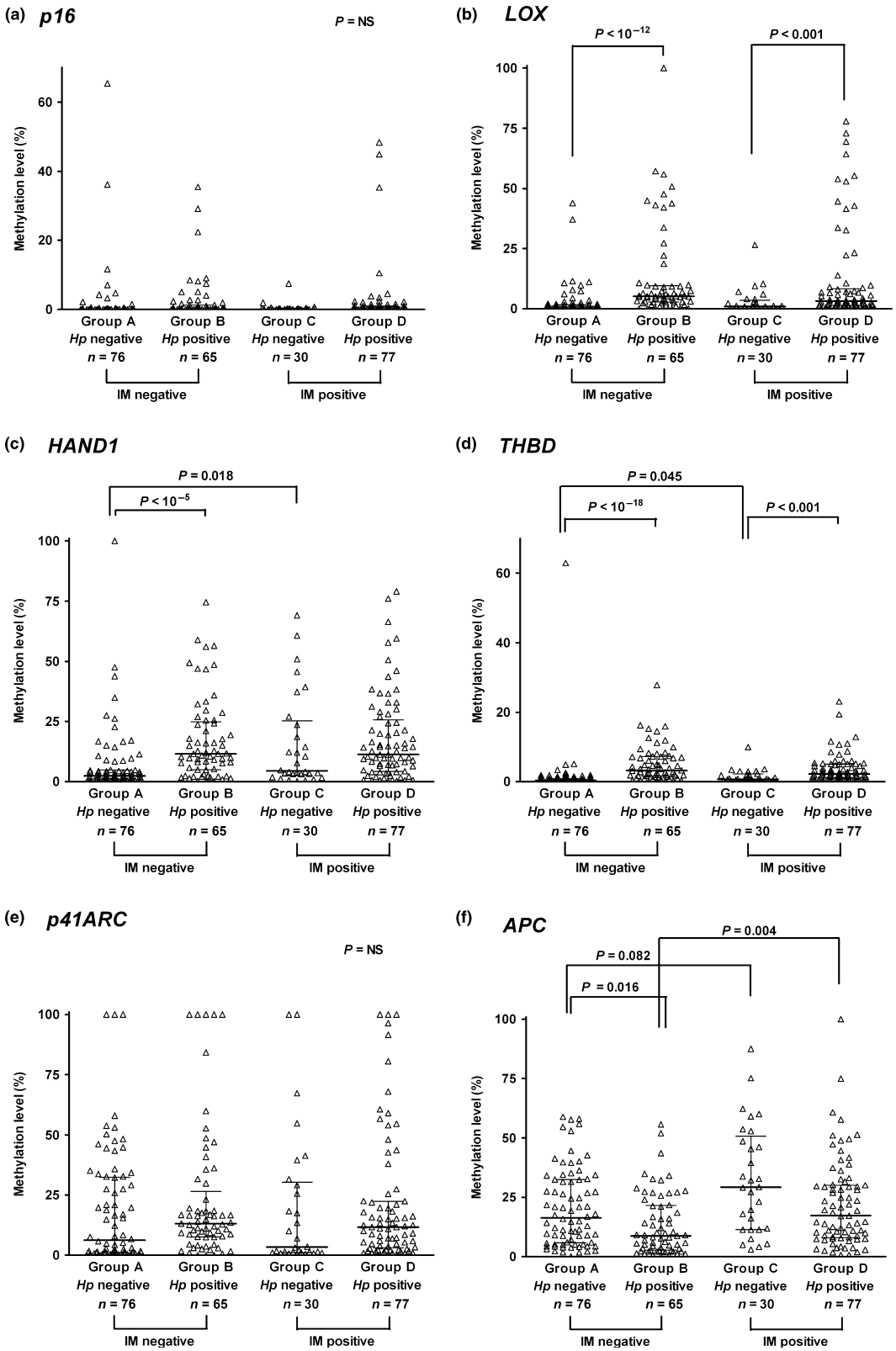

Fig. 1. Methylation levels in the six genes: *p16* (a), *LOX* (b), *HAND1* (c), *THBD* (d), *p41ARC* (e), and *APC* (f) according to the presence of current active *Helicobacter pylori* (*Hp*) infection and intestinal metaplasia (IM) in controls (n = 248). ''*Hp* positive'' refers to a current active *H. pylori* infection, and ''*Hp* negative'' includes those with a past *H. pylori* infection (serology-positive only), as well as negatives to all *H. pylori* tests (CLOtest, histology, culture, and serology). ''IM positive'' denotes any histological evidence of IM in either the antrum or body. Results are given as median and interquartile range. *Helicobacter pylori* significantly increased methylation in *LOX*, *HAND1*, and *THBD*, and IM was associated with hypermethylation in *HAND1*, *THBD* (in the absence of current *H. pylori* infection, $P < 0.05$), and *APC* (especially in the presence of *H. pylori*, $P = 0.003$).

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Table 3. Correlations between methylation levels in *p16*, *LOX*, *HAND1*, *THBD*, *p41ARC*, and *APC* genes, and histologic grades of *Helicobacter pylori*-associated gastritis and/or intestinal metaplasia

Histologic findings	*n*	Methylation level (median, %)					
		p16	*LOX*	*HAND1*	*THBD*	*p41ARC*	*APC*
Density of *H. pylori* (*n* = 839)							
0	471	0.07	1.09	5.50	1.81	10.41	21.83
1	141	0.31	2.35	12.94	7.44	16.55	18.53
2	162	0.19	4.04	11.92	5.54	14.75	15.33
3	65	0.10	4.35	11.59	7.16	16.94	13.25
ρ†		**0.087***	**0.265****	**0.168****	**0.292****	**0.169****	**−0.160****
Polymorphous nuclear cell infiltration (*n* = 837)							
0	283	0.00	0.91	4.37	1.31	8.35	20.56
1	212	0.16	1.54	8.18	3.15	13.20	23.24
2	307	0.21	3.00	11.75	6.10	15.50	14.83
3	35	0.21	5.57	10.76	7.42	23.42	10.56
ρ†		**0.108****	**0.231****	**0.196****	**0.309****	**0.220****	**−0.123*****
Mononuclear leukocyte infiltration (*n* = 836)							
0 or 1‡	218	0.00	0.56	3.00	1.21	9.41	20.95
2	559	0.18	2.40	10.58	4.76	13.61	18.48
3	59	0.25	5.52	11.75	5.55	16.40	17.49
ρ†		**0.115*****	**0.265****	**0.233****	**0.281****	**0.151****	**−0.071***
Grade of IM (*n* = 834)							
0	334	0.07	1.98	5.71	2.13	13.89	16.33
1	201	0.19	1.97	9.75	4.54	14.68	19.73
2	224	0.13	1.92	11.02	4.09	12.48	21.84
3	75	0.08	1.61	10.04	4.81	10.41	24.89
ρ†		0.019	−0.007	**0.125*****	**0.091****	−0.027	**0.143****

Sum of counts within categories may not be same as the number of overall study subjects (*n* = 844) owing to missing data. The histologic scores of the gastric mucosa were recorded following the updated Sydney scoring system (0 = none, 1 = slight, 2 = moderate, and 3 = marked). †Spearman correlation coefficient. ‡Only two cases had no mononuclear cell infiltration (0 score) in histology. Bold style indicates statistical significance. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.00001$. IM, intestinal metaplasia.

In the next, we investigated methylation levels in the six genes under study with regard to the three categorized *H. pylori* infection statuses (*H. pylori* negative, current and past infection, Fig. 2). Methylation levels in all genes but *APC* showed a decrease after active *H. pylori* infection discontinued; in particular, methylation levels in *THBD* were significantly decreased ($P < 0.001$). However, compared to *H. pylori* negatives, aberrant DNA hypermethylation in *LOX* (Fig. 2b), *HAND1* (Fig. 2c), *THBD* (Fig. 2d), and *p41ARC* (Fig. 2e) remained significantly increased in past *H. pylori*-infected cases (all $P < 0.05$).

Association of high methylation levels with the precancerous gastric lesions or cancer. The methylation levels in the six genes were compared between controls and patients with dysplasia or GC with reference to the status of *H. pylori* infection, adjusted for age, gender, and smoking and drinking habits (Table 4). When controls and patients with dysplasia were compared, the methylation profiles did not significantly differ in all six genes. However, methylation levels in specific genes were significantly elevated in GC patients. Among *H. pylori*-negative subjects ($n = 263$), the samples with higher levels of methylation (% of methylation, ≥1% for *p16* and ≥5% for *LOX*, *HAND1*, and *THBD*) had an increased risk for GC compared to those with lower levels of methylation (% of methylation, <1% for *p16*, <5% for *LOX*, *HAND1*, and *THBD*): 4.66-fold (*p16*), 3.32-fold (*LOX*), 2.66-fold (*HAND1*), and 24.59-fold (*THBD*). However, among *H. pylori*-positive subjects ($n = 525$), the methylation levels in *p16*, *LOX*, and *HAND1* were not different between controls and GC cases.

The similar tendency was observed when the analysis was carried out between the cases and their age and gender-matched controls (Fig. 3). The methylation levels in *p16* (Fig. 3a) and *THBD* (Fig. 3d) were significantly elevated in GC patients, regardless of *H. pylori* infection (all $P < 0.05$). Among active *H. pylori*-infected cases, the methylation levels in *APC* (Fig. 3f) were significantly elevated with GC ($P = 0.001$). For *LOX* (Fig. 3b), its methylation profile was quite different depending on the status of *H. pylori* infection. That is, among *H. pylori*-negative subjects, the methylation levels in *LOX* were significantly increased with GC (Fig. 3b, $P = 0.021$); however, they were decreased with GC among *H. pylori*-positive cases ($P = 0.002$). In addition, methylation levels in *HAND1* (Fig. 3c) and *p41ARC* (Fig. 3e) did not differ significantly between controls and GC patients. Furthermore, when methylation levels of the six genes under study were compared according to the histologic type of GC (intestinal *vs* diffuse type), the difference was not statistically different (data not shown).

Discussion

In the present study, the methylation levels in the six genes (*p16*, *LOX*, *HAND1*, *THBD*, *p41ARC*, and *APC*) were evaluated from non-neoplastic gastric mucosae using quantitative real-time PCR after bisulfite modification. Previously, a Japanese group reported that hypermethylation of eight selected genes (*p16* core, *p16* noncore, *HRASLS*, *LOX*, *HAND1*, *THBD*, *p41ARC*, and *FLNc*) was associated with both *H. pylori* infection and GC.[18] In addition, we investigated the methylation profiles of the six genes at variable stages of gastric carcinogenesis including IM and dysplasia in relation to *H. pylori* infection. Furthermore, a systematic analysis was performed taking into consideration three statuses of *H. pylori* infection (negative, current active, and past) using both invasive tests (culture, histology, and CLOtest) and serology testing; a past *H. pylori* infection could be distinguished, at least in part, from a current active one. The association of *APC* methylation with *H. pylori* infection and precancerous gastric lesions was also evaluated,

Table 4. Methylation level of *p16*, *LOX*, *HAND1*, *THBD*, *p41ARC*, and *APC* genes in normal controls, and patients with dysplasia and gastric cancer patients with respect to *Helicobacter pylori* infection status

Gene (methylation level, %)	No. of subjects (%)			OR† (95% CI)	*P*-values†	OR‡ (95% CI)	*P*-values‡
	Controls	DYS	GC				
Helicobacter pylori negative or past infection (*n* = 263)	(*n* = 97)	(*n* = 42)	(*n* = 124)				
P16 (≥1%)	11 (11.3)	4 (9.5)	37 (29.8)	2.03 (0.42–9.88)	0.379	**4.66 (1.83–11.85)**	**0.001**
LOX (≥5%)	13 (13.4)	8 (19.0)	31 (25.0)	1.24 (0.35–4.42)	0.738	**3.32 (1.31–8.39)**	**0.011**
HAND1 (≥5%)	30 (30.9)	16 (38.1)	71 (57.3)	0.88 (0.33–2.35)	0.793	**2.66 (1.30–5.44)**	**0.007**
THBD (≥5%)	3 (3.1)	8 (19.0)	69 (55.6)	2.55 (0.50–12.95)	0.260	**24.59 (6.78–89.26)**	**<0.001**
p41ARC (≥10%)	44 (45.4)	20 (47.6)	68 (54.8)	1.63 (0.63–4.20)	0.313	1.93 (0.97–3.82)	0.061
APC (≥10%)	66 (68.0)	24 (57.1)	96 (77.4)	0.48 (0.18–1.25)	0.131	1.42 (0.67–3.01)	0.368
Current active *H. pylori* infection (*n* = 525)	(*n* = 132)	(*n* = 87)	(*n* = 306)				
P16 (≥1%)	31 (23.5)	16 (18.4)	100 (32.7)	0.84 (0.39–1.78)	0.641	1.56 (0.95–2.55)	0.080
LOX (≥5%)	58 (43.9)	27 (31.0)	103 (33.7)	0.64 (0.33–1.25)	0.195	0.72 (0.46–1.13)	0.152
HAND1 (≥5%)	96 (72.7)	50 (57.5)	199 (65.0)	0.60 (0.31–1.19)	0.145	0.75 (0.47–1.20)	0.231
THBD (≥5%)	41 (31.1)	19 (21.8)	202 (66.0)	0.82 (0.39–1.72)	0.604	**5.37 (3.31–8.70)**	**<0.001**
p41ARC (≥10%)	76 (57.6)	45 (51.7)	213 (69.6)	0.83 (0.44–1.57)	0.562	**1.90 (1.20–3.00)**	**0.006**
APC (≥10%)	75 (56.8)	57 (65.5)	225 (73.5)	1.24 (0.64–2.41)	0.526	**2.02 (1.27–3.19)**	**0.003**

Analyses were performed in 788 subjects because of the missing data for some variables. All ORs and *P*-values were calculated using logistic regression model adjusted for age, gender and smoking and drinking habits. †ORs and *P*-values of normal controls *versus* patients with dysplasia. ‡ORs and *P*-values of normal controls *versus* gastric cancer patients. Bold style indicates statistical significance. DYS, patients with dysplasia; GC, patients with gastric cancer; OR, odds ratio.

and it showed a distinct pattern from the other five genes under study.

In controls, the univariate analysis showed that methylation levels in *THBD* and *APC*, but not the other four genes evaluated in this study, were significantly correlated with aging (Spearman's rank correlation test; $P = 0.039$ and 0.001 for *THBD* and *APC* respectively); this finding favors the notion that susceptibility to age-related CGI methylation differs among genes and tissues.[19] On the other hand, gender, alcohol drinking, and smoking history did not affect the methylation level in any of the six genes significantly (data not shown), which implies that these factors play a minor, if any, role in inducing epigenetic changes in noncancerous gastric mucosae.[6] Methylation levels in *LOX*, *HAND1*, and *THBD* were significantly higher in the cases with active *H. pylori* infection than those with *H. pylori*-negative or past infection (Fig. 2). Of note was that *APC* gene promoter CGI methylation increased with IM, but decreased with *H. pylori* infection (Fig. 1f). A few studies have reported that promoter methylation of *APC* is often found in biopsies with histologically normal mucosa, and that such alterations were found in normal mucosa adjacent to IM.[14,20] In addition, a recent study has shown that *APC* 1A methylation was observed in similar levels (20–40%) in both normal gastric mucosae of healthy volunteers and noncancerous gastric mucosae of GC patients and that *H. pylori* infection did not affect its methylation.[21] Negative correlation between *APC* methylation and *H. pylori* infection appears to be plausible because the histologic grade of IM was negatively correlated with *H. pylori* density in our study population (Spearman's rank correlation test; $\rho = -0.182$, $P < 0.001$). Overall, hypermethylation of *APC* appears to be associated with IM, but *H. pylori* infection does not induce its methylation.

Two types of aberrant DNA methylation have been postulated; one a temporary methylation induced in progenitor or differentiated cells and the other a permanent methylation induced in stem cells.[22] The former fluctuates during the course of *H. pylori* infection and decreases after active inflammation resolves and new epithelial cells are supplied from unmethylated stem cells. On the other hand, the latter gradually increases during active infection and persists even after *H. pylori* is no longer present.[2] *Helicobacter pylori* is thought to induce aberrant DNA methylation in both temporary and permanent components.[23] Our results demonstrated that methylation levels in all genes but *APC* were positively correlated with both density of *H. pylori* and histologic grade of gastritis (Table 3), which is consistent with previous studies.[11,24] In addition, methylation level was decreased in those who had undergone *H. pylori* infection before (past infection or serology-positive only) compared to those with current active infection; this probably originates from a decrease in temporary methylation (Fig. 2). However, we found the methylation levels in such genes as *LOX*, *HAND1*, *THBD*, and *p41ARC* were significantly increased in the subjects with past *H. pylori* infection compared to those without evidence of *H. pylori* infection (Fig. 2); this supports the notion that methylation profiles induced by *H. pylori* infection can be persistent even after discontinuation of active infection.[2,23,24]

Hypermethylation of *HAND1*, *APC*, and *THBD* were positively correlated with the grade of IM (Table 3). Among the cases without evidence of active *H. pylori* infection, the methylation profiles of the six genes under study showed an increasing tendency with the progression of gastric carcinogenesis (Supporting information Fig. S2). However, among cases with active *H. pylori* infection, their methylation profiles were different depending on the susceptibility to *H. pylori*-induced CGI hypermethylation (Table 4 and Supporting information Fig. S3). CGI hypermethylation of *THBD*, and possibly *p16*, was significantly associated with GC, regardless of active *H. pylori* infection (Table 4 and Figs 3, S2, S3). However, the association of the methylation levels in *LOX* and *HAND1* with GC appears to be more dependent on active *H. pylori* infection. Methylation profiles obtained from noncancerous gastric mucosae could be affected by the activity of *H. pylori*-associated gastritis. Thus, methylation levels during current active *H. pylori* infection do not necessarily reflect the future risk for GC.[23] We postulated that CGI hypermethylation in such genes as *LOX* and *HAND1* is closely related to the activity of *H. pylori*-induced gastritis; thus, their methylation profiles are more affected by *H. pylori* infection itself than by the stage in multistep gastric carcinogenesis.

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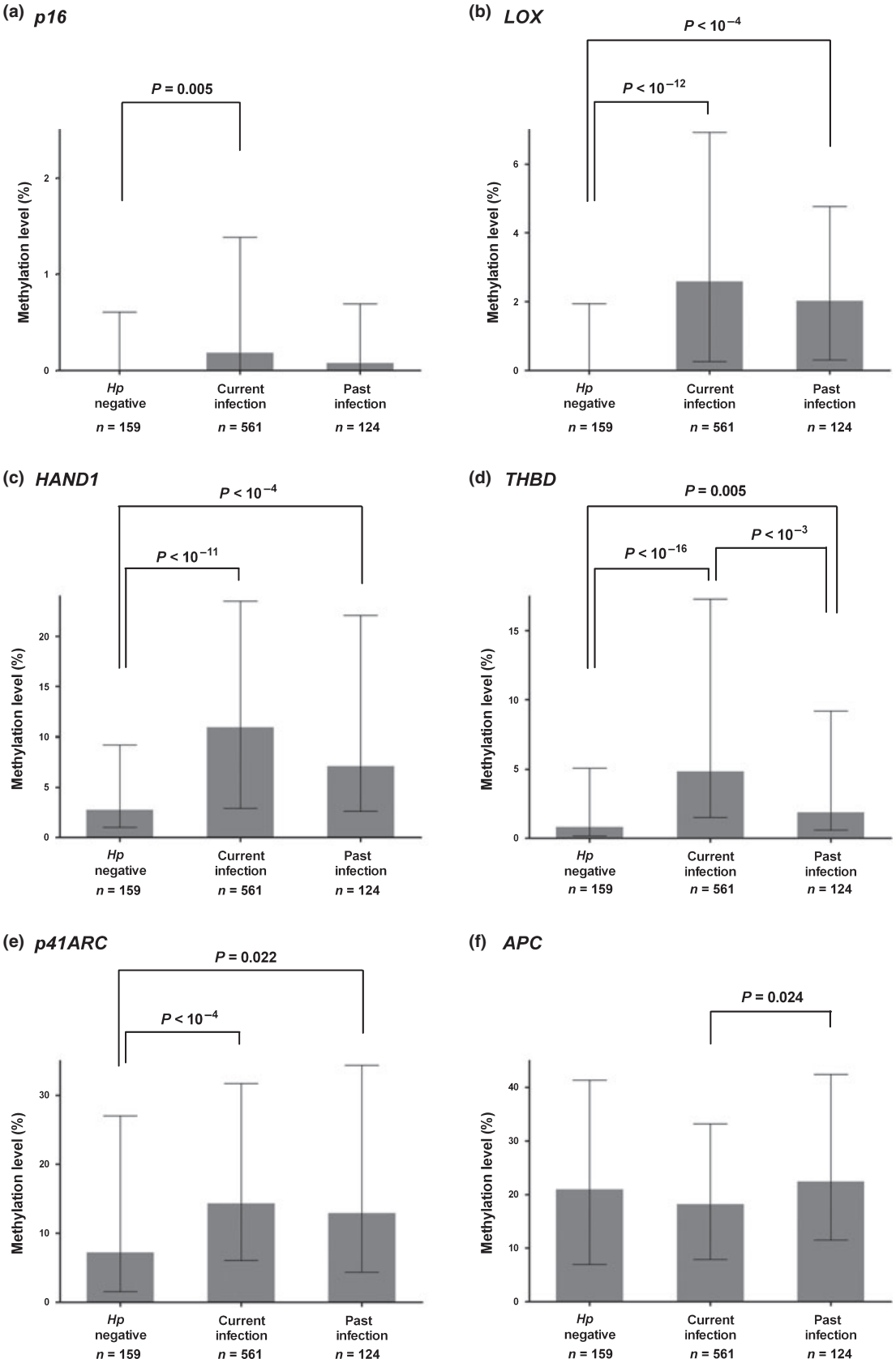

Fig. 2. Methylation levels in the six genes: *p16* (a), *LOX* (b), *HAND1* (c), *THBD* (d), *p41ARC* (e), and *APC* (f) with regard to the *Helicobacter pylori* infection status. ''Current infection'' refers to positivity for any one of the endoscopy-based active *H. pylori* tests, and ''past infection'' positive for *H. pylori* serology only. Results are given as median and interquartile range. Aberrant DNA hypermethylation decreased as *H. pylori*-associated active infection resolved. However, methylation levels in *LOX*, *HAND1*, *THBD*, and *p41ARC* remained significantly increased in the subjects with past infection compared to those with *H. pylori* negativity (all $P < 0.05$).

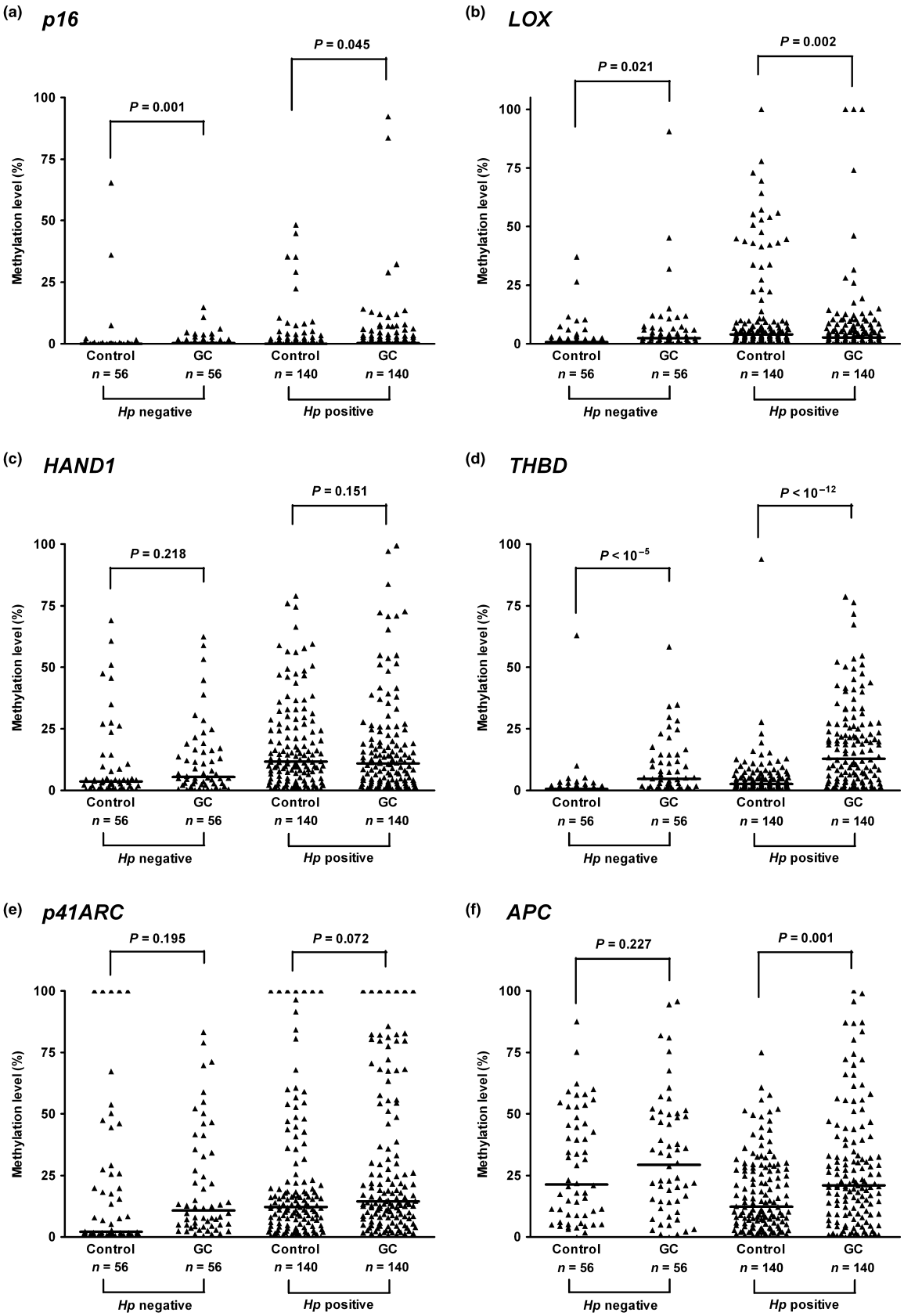

Fig. 3. Methylation levels of the six genes: *p16* (a), *LOX* (b), *HAND1* (c), *THBD* (d), *p41ARC* (e), and *APC* (f) in the gastric cancer (GC) cases and their age- and gender-matched controls according to the presence of current active *Helicobacter pylori* infection (*H. pylori* negative, $n = 56$; *H. pylori* positive, $n = 140$). Bar represents median. Methylation levels in *p16* and *THBD* significantly increased in patients of GC (all *P*-values <0.05), whereas the methylation levels in *HAND1* and *p41ARC* did not. Hp, *Helicobacter pylori*; GC, patients with gastric cancer.

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We could not identify any significant difference in the methylation levels in the six genes in the comparisons between controls and patients with dysplasia (Table 4), which is in part due to relatively small sample size in dysplasia cases. Overall, all of these findings are consistent with the concept that aberrant DNA hypermethylation induced by *H. pylori* occurs in a gene-specific manner, with different methylation susceptibility, during the multistep carcinogenesis.

It has been suggested that gene promoter hypermethylation could be used as a marker to predict the future risk for GC.[22,23,25] In this study, aberrant DNA hypermethylation in *THBD* was not only closely linked to *H. pylori* infection, but also accumulated with the progression of gastric carcinogenesis. Thrombomodulin, which is important for anticoagulant activity, is also known to be involved in various physiologic and pathologic processes including thrombosis, arteriosclerosis, inflammation, and carcinogenesis.[26] Hypermethylation and silencing of *THBD* have been frequently observed in ovarian cancer,[27] pancreatic cancer,[28] and melanoma,[29] as well as in GC.[12,18] The duration of *H. pylori* exposure and the epigenetic alterations induced by *H. pylori*, not the *H. pylori* infection status itself, are known to be closely associated with the future risk for GC;[18,30] the methylation level of specific genes such as *THBD* might be a useful marker associated with the methylation fingerprint of *H. pylori* infection and the risk for GC.[31]

The limitations of this study include the following. In this study, the profiles of aberrant CGI hypermethylation were obtained from antral biopsy specimens, although different sites of gastric mucosae (antrum or corpus) may have different methylation profiles.[6,23] However, one study has reported no significant difference in methylation levels between the corpus and antrum;[18] therefore, biopsy site(s) may not have affected our results substantially. Second, some of those who were classified as *H. pylori* negative possibly had a remote *H. pylori* infection; one study has shown the seroconversion rate was 45% at 18 months after *H. pylori* eradication in Koreans.[32] This may attenuate the differences in methylation levels between the two groups categorized as *H. pylori* negative and past *H. pylori* infection, and may result in the reduction of the statistical power.

In conclusion, aberrant DNA methylation was found to occur in a gene-specific manner during the multistep gastric carcinogenesis, and *H. pylori* infection status was closely associated with promoter methylation of specific genes. Furthermore, the aberrant hypermethylation induced by *H. pylori* was persistent even after *H. pylori* infection disappeared. Hypermethylation of specific genes such as *THBD* has been found to be associated with both *H. pylori* infection and the development of GC, and thus might provide a link between *H. pylori* infection and development of GC.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Reproducibility of methylation levels for *HAND1* (a) and *p16* (b) CGIs. Two independent bisulfite modifications, with the methylation ratios of 60%, 6%, 4.5%, 3%, 1.5%, and 0.8%, were performed on two different dates (Groups 1 and 2; Groups 3 and 4). Each group was analyzed by quantitative real-time PCRs in triplicate (a, b, c). The resultant methylation levels were highly reliable.

Fig. S2. Methylation profiles of the six genes from non-neoplastic gastric mucoase at various stages of gastric carcinogenesis among the cases with *Helicobacter pylori* negativity or past infection ($n = 283$). The methylation levels of the six genes showed an increasing tendency along gastric carcinogenesis. Specifically, hypermethylation of *HAND1* (c) and *THBD* (d) was associated with IM ($P < 0.05$) and dysplasia ($P < 0.01$), respectively. Results are given as median and interquartile range. All *P*-values were calculated using rank ANOVA analysis, and the Bonferroni method for multiple comparisons was applied. CG, subjects with normal gastric mucosa or chronic gastritis only; DYS, patients with dysplasia; GC, patients with gastric cancer; IM, subjects with intestinal metaplasia. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Fig. S3. Methylation profiles of the six genes from non-neoplastic gastric mucoase at various stages of gastric carcinogenesis in active *Helicobacter pylori*-infected cases ($n = 559$). The methylation profiles showed a difference with genes. For *p16* (a), *THBD* (d), and *APC* (f), their methylation profiles showed an increase with the progression of gastric carcinogenesis. For *LOX* (b), however, its methylation levels showed a decrease with the progression of the gastric carcinogenesis. Results are given as median and interquartile range. All *P*-values were calculated using rank ANOVA analysis, and the Bonferroni method for multiple comparisons was applied. CG, subjects with normal gastric mucosa or chronic gastritis only; DYS, patients with dysplasia; GC, patients with gastric cancer; IM, subjects with intestinal metaplasia. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

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