High Levels of Aberrant DNA Methylation in *Helicobacter pylori* – Infected Gastric Mucosae and its Possible Association with Gastric Cancer Risk

Takao Maekita,[1,3] Kazuyuki Nakazawa,[3] Mami Mihara,[1] Takeshi Nakajima,[1,2] Kimihiko Yanaoka,[3] Mikitaka Iguchi,[3] Kenji Arii,[3] Atsushi Kaneda,[1] Tetsuya Tsukamoto,[4] Masae Tatematsu,[4] Gen Tamura,[5] Daizo Saito,[2] Takashi Sugimura,[1] Masao Ichinose,[3] and Toshikazu Ushijima[1]

Abstract

Introduction: Risk prediction of gastric cancers is important to implement appropriate screening procedures. Although aberrant DNA methylation is deeply involved in gastric carcinogenesis, its induction by *Helicobacter pylori*, a strong gastric carcinogen, is unclear. Here, we analyzed the effect of *H. pylori* infection on the quantity of methylated DNA molecules in noncancerous gastric mucosae and examined its association with gastric cancer risk.

Experimental Design: Gastric mucosae were collected from 154 healthy volunteers (56 *H. pylori* negative and 98 *H. pylori* positive) and 72 cases with differentiated-type gastric cancers (29 *H. pylori* negative and 43 *H. pylori* positive) by endoscopy. The numbers of DNA molecules methylated and unmethylated for eight regions of seven CpG islands (CGI) were quantified by quantitative PCR after bisulfite modification, and fractions of methylated molecules (methylation levels) were calculated.

Results: Among healthy volunteers, methylation levels of all the eight regions were 5.4- to 303-fold higher in *H. pylori* positives than in *H. pylori* negatives ($P < 0.0001$). Methylation levels of the *LOX, HAND1*, and *THBD* promoter CGIs and *p41ARC* exonic CGI were as high as 7.4% or more in *H. pylori* – positive individuals. Among *H. pylori* – negative individuals, methylation levels of all the eight regions were 2.2- to 32-fold higher in gastric cancer cases than in age-matched healthy volunteers ($P \leq 0.01$). Among *H. pylori* – positive individuals, methylation levels were highly variable, and that of only *HAND1* was significantly increased in gastric cancer cases (1.4-fold, $P = 0.02$).

Conclusions: It was indicated that *H. pylori* infection potently induces methylation of CGIs to various degrees. Methylation levels of specific CGIs seemed to reflect gastric cancer risk in *H. pylori* – negative individuals.

Gastric cancer is one of the most common malignancies worldwide and remains a leading cause of cancer death in Asia and some European countries (1). To reduce its mortality, early detection by endoscopy and curative resection are important (2). However, considering the potential risk and costs of early detection by endoscopic examination, implementation reflecting an individual's risk for developing a gastric cancer would be ideal. Also, endoscopic mucosal resection, which conserves the noncancerous gastric mucosae, is becoming popular, and the problem of metachronous gastric cancer recurrence is being recognized (3). Again, if the future risk of developing metachronous cancers in a specific case can be estimated, the information will be useful in the decision on either surgical resection or endoscopic mucosal resection for the case.

The major etiologic risk factor for gastric cancers is *Helicobacter pylori* infection, which increases gastric cancer risk 2.2- to 21-fold (4–6). In an animal model with Mongolian gerbil chronic infection with *H. pylori* rarely induces gastric cancers by itself, but markedly enhances their incidences after initiation with a mutagen, such as *N*-methyl-*N*-nitrosourea (7). This promoting effect of *H. pylori* has been attributed to the induction of chronic inflammation and cell proliferation (8). Cell proliferation increases a chance for initiated cells to escape growth suppression and undergo further mutations. Other risk factors for gastric cancers include high salt intake and smoking (9), and a cancer risk marker incorporating these factors is awaited.

Authors' Affiliation: [1]Carcinogenesis Division, National Cancer Center Research Institute; [2]Endoscopy Division, National Cancer Center Hospital, Tokyo, Japan; [3]Second Department of Internal Medicine, Wakayama Medical University, Wakayama, Japan; [4]Division of Oncological Pathology, Aichi Cancer Center Research Institute, Nagoya, Japan; and [5]Department of Pathology, Yamagata University School of Medicine, Yamagata, Japan

Received 9/26/05; revised 11/11/05; accepted 11/21/05.

Grant support: Research Resident Fellowships from the Foundation for Promotion of Cancer Research (T. Maekita and K. Nakazawa); Grant-in-Aid for Cancer Research from the Ministry of Health, Labour, and Welfare; and a Special Coordination Fund for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Requests for reprints: Toshikazu Ushijima, Carcinogenesis Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, 104-0045 Tokyo, Japan. Phone: 81-3-3547-5240; Fax: 81-3-5565-1753; E-mail: tushijim@ncc.go.jp.

doi:10.1158/1078-0432.CCR-05-2096

As an additional mechanism of gastric cancer induction by *H. pylori* infection, induction of aberrant methylation was suggested by Chan et al. (10). They observed that *E-cadherin* methylation was more frequent in the gastric mucosae of dyspepsia cases with *H. pylori* infection than those without. In contrast, Kang et al. (11) did not observe a difference in the number of methylated genes in gastric mucosae with and without *H. pylori* infection. The discrepancy could be due to the lack of quantification of aberrantly methylated DNA molecules. Aberrant methylation in noncancerous tissues occurs only in a fraction of cells, which is expected to be highly variable, and qualitative analysis of methylation does not seem suitable. Also, different CpG islands (CGI) and, even within a CGI, different regions show different susceptibility to aberrant DNA methylation (12), and analysis considering the different susceptibility has not been done. Most importantly, there is no former study regarding the relationship between the level of aberrant methylation in gastric mucosae and risks of gastric cancer development. It seems possible that levels of aberrant methylation could reflect past exposure to *H. pylori* and other carcinogens, and that the methylation levels could be used as a cancer risk marker.

In this study, we aimed to clarify the effects of *H. pylori* infection on the induction of aberrant methylation by quantifying methylation levels of multiple CGIs and regions in healthy volunteers with and without *H. pylori* infection. Then, to clarify whether accumulated levels of aberrant DNA methylation are associated with a risk of gastric cancer development, we quantified methylation levels in gastric mucosae of healthy volunteers and noncancerous gastric mucosae of gastric cancer cases, which are known to have an elevated risk of gastric cancers (13, 14).

Materials and Methods

Cases, tissue samples, and DNA extraction. Healthy volunteers (82 males and 72 females) with an average age of 54.2 (range, 23-98) were recruited with informed consents on the occasion of a gastric cancer screening program under the approval of institutional review boards. Cases with well-differentiated gastric cancers (60 males and 12 females) with an average age of 67.2 (range, 37-85) were recruited with informed consents and under the approval of institutional review boards. To obtain a group of healthy volunteers whose average age was matched to the cancer cases, the same number of volunteers as cancer cases was randomly selected from each age group. The age-matched group (35 males and 37 females) had an average age of 64.4 (range 39-91; Table 1). *H. pylori* infection status was analyzed by a serum anti–*H. pylori* IgG antibody test (SBS, Kanagawa, Japan), rapid urease test (Otsuka, Tokushima, Japan), or culture test (Eiken, Tokyo, Japan). The sensitivities of the serum anti–*H. pylori* IgG antibody test and rapid urease test are ≥90% of the culture test (15, 16).

Gastric mucosae were obtained by endoscopic biopsy of two standard sites, the upper corpus and antral regions in the lessor curvature, with sterilized biopsy forceps (Olympus, Tokyo, Japan). Gastric cancer cases that had cancers in either of the two standard sites were excluded from the analysis. Histologic analysis of selected biopsied materials showed that these samples contain 40% to 80% of epithelial tissues. The samples were frozen and stored at −80°C. High molecular weight DNA was extracted by the phenol/chloroform method.

Sodium bisulfite modification and quantitative methylation-specific PCR. Bisulfite treatment was done using 500 ng genomic DNA, digested with *Bam*HI, as previously described (17), and the treated DNA was suspended in 40 μL of TE buffer. An aliquot of 2 μL was used for real-time PCR with a primer set specific to methylated or unmethylated sequences. Using DNA from gastric mucosae from a young individual without *H. pylori* infection and DNA methylated with *Sss*I methylase (New England Biolabs, Beverly, MA), an annealing temperature specific for a primer set was determined. Real-time PCR was done using SYBR Green PCR Core Reagents (PE Biosystems, Warrington, United Kingdom) and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). Standard DNA was prepared by cloning PCR products into the pGEM-T Easy vector (Promega, Madison, WI). The number of molecules in a test sample was determined by comparing its amplification with those of samples containing a known number of molecules (10-10^5 molecules). The number of molecules methylated and unmethylated for a genomic region in a sample was measured separately, and the methylation level was calculated as the fraction of methylated molecules in the total number of DNA molecules (number of methylated molecules + number of unmethylated molecules). The primer sequences and PCR conditions are shown in Supplementary Table S1, and standard DNA for real-time methylation-specific PCR is available upon request.

Statistical analysis. The differences of mean methylation levels were analyzed by using the t test Welch method (both sided). Association between the age and methylation level was analyzed by calculating correlation coefficients and t_0 values.

Results

Quantification of methylation levels and reproducibility. We analyzed methylation levels of two regions of the *p16* promoter CGI and one region of six genes (*LOX*, *FLNc*, *HRASLS*, *HAND1*, *THBD*, and *p41ARC*; Fig. 1A), which could be methylated in human gastric cancers (18, 19). The two regions of *p16* promoter CGIs were selected because one region (core region)

Table 1. Characteristics of the individuals analyzed

	All cases		Age matched	
	n **(male:female)**	**Mean age (range)**	***n*** **(male:female)**	**Mean age (range)**
Healthy volunteers				
HP (−)	56 (30:26)	51 (25-91)	29 (14:15)	63 (48-91)
HP (+)	98 (52:46)	56 (23-98)	43 (21:22)	64 (39-86)
Gastric cancer cases				
HP (−)	29 (24:5)	69 (52-85)	n/a	n/a
HP (+)	43 (36:7)	67 (37-85)	n/a	n/a

Abbreviations: *HP* (−), *H. pylori* negative; *HP* (+), *H. pylori* positive; n/a, not applicable.

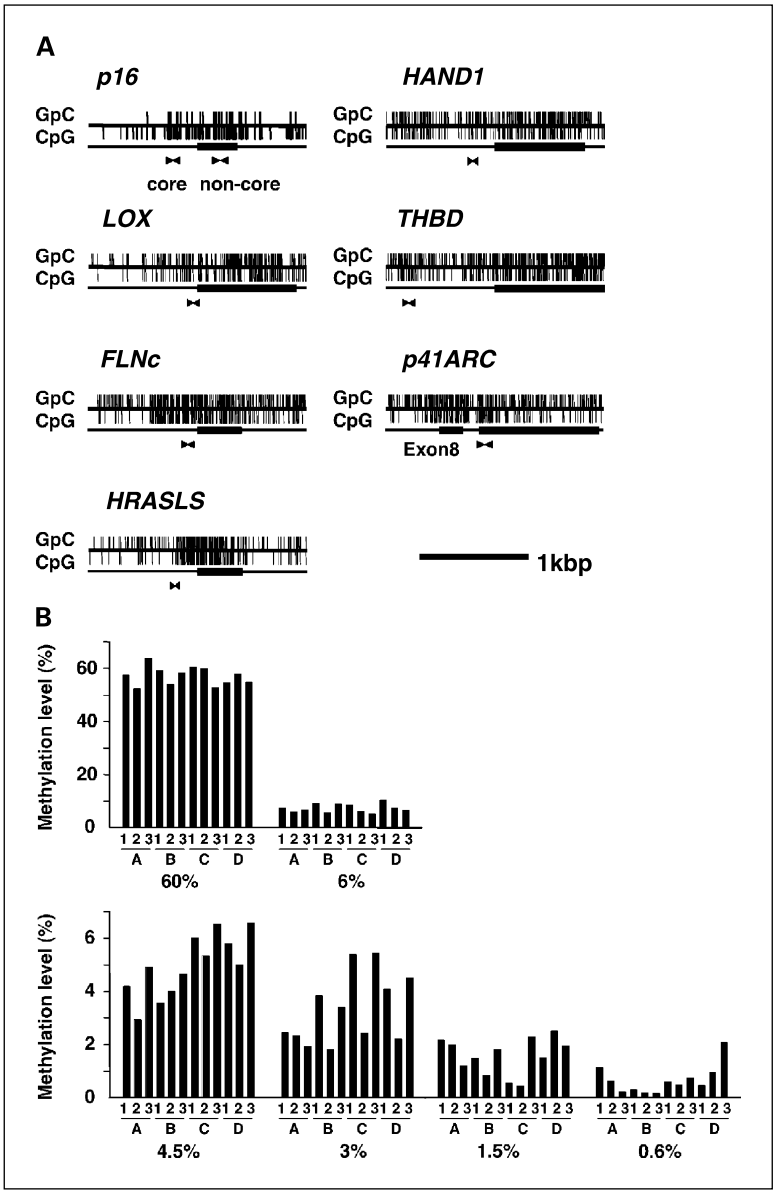

Fig. 1. Locations of regions analyzed and reproducibility of methylation levels. *A,* CGIs and regions analyzed. *B,* reproducibility of methylation levels. Two independent bisulfite modifications (*A* and *B*; *C* and *D*) were done and four independent quantitative PCRs were done in triplicate. The methylation levels obtained were highly reproducible.

was known to be critical for *p16* silencing but resistant to methylation and the other region (noncore region) was known to be susceptible to methylation but does not suppress gene expression (12). For *LOX*, *FLNc*, *HRASLS*, and *HAND1*, core regions of their promoter CGIs were analyzed. For *THBD*, noncore region of its promoter CGI was analyzed because the core region was not methylated at all (data not shown). For *p41ARC*, we analyzed its exonic CGI, which was frequently methylated in gastric cancers (19).

The reproducibility of the values obtained by quantitative methylation-specific PCR was analyzed for the *HAND1* CGI. Test DNA samples were prepared by mixing DNA completely methylated by *Sss*I methylase and that in which no methylation was detected at the ratios of 60%, 6%, 4.5%, 3%, 1.5%, and 0.7% of methylation. The same DNA was modified by bisulfite on two different dates (groups A and B on the same date, and groups C and D on the other date). Each group was analyzed by quantitative PCR on different dates in triplicate. The resultant 12 values (mean ± SD) for the three samples were as follows: 57.1 ± 3.5%, 7.3 ± 1.3%, 5.0 ± 1.2%, 3.3 ± 1.3%, 1.6 ± 0.7%, and 0.7 ± 0.5% (Fig. 1B). Therefore, methylation levels obtained here were highly reliable when they were larger than 1.5% to 3%, and were also reliable but more number of measures were necessary when smaller than 1.5% to 3%.

***High levels of aberrant methylation in gastric mucosae with* H. pylori.** Methylation levels of the eight regions were analyzed by quantitative methylation-specific PCR in 56 *H. pylori* – negative volunteers and 98 *H. pylori* – positive volunteers (Fig. 2; Table 2). For all the eight CGIs analyzed, methylation levels in the *H. pylori* – positive healthy volunteers were significantly and markedly elevated compared with those in the *H. pylori* – negative healthy volunteers. In the corpus, the mean methylation levels were elevated 303-fold (*p16* core region), 20-fold (*p16* noncore region), 14-fold (*LOX*), 11-fold (*THBD*), 49-fold (*FLNc*), 13-fold (*HRASLS*), 9.3-fold (*HAND1*), and 5.4-fold (*p41ARC*). In the antrum, they were

elevated 54-fold (*p16* core region), 22-fold (*p16* noncore region), 16-fold (*LOX*), 17-fold (*THBD*), 30-fold (*FLNc*), 18-fold (*HRASLS*), 7.8-fold (*HAND1*), and 5.7-fold (*p41ARC*). Especially, methylation levels of *LOX*, *HAND1*, *THBD*, and *p41ARC* were higher than 7.4% in *H. pylori* – positive individuals, and this unequivocal effect of *H. pylori* infection supported the increases in methylation levels of the other CGIs in smaller ranges. This strongly indicated that *H. pylori* infection potently induced aberrant methylation in multiple CGIs. Mean methylation levels were in the same range in the corpus and antrum.

Effect of age and sex on methylation levels. Because age-dependent methylation was reported for various CGIs (20, 21), association between age and methylation levels of antral mucosa was analyzed by calculating correlation coefficients. In the 56 *H. pylori* – negative healthy volunteers, correlation coefficients (*P* values) for *p16* core region, *p16* noncore region, *LOX*, *THBD*, *FLNc*, *HRASLS*, *HAND1*, and *p41ARC* were 0.09 (0.51), 0.23 (0.08), 0.18 (0.18), 0.26 (0.05), 0.17 (0.21), 0.34 (0.01), 0.32 (0.02), and 0.29 (0.03), respectively. In the 98 *H. pylori* – positive healthy volunteers, they were 0.18 (0.08), 0.13 (0.20), −0.02 (0.85), 0.08 (0.43), −0.07 (0.49), 0.05 (0.62), 0.18 (0.08), and 0.13 (0.20), respectively. Only *HRASLS*, *HAND1*, and *p41ARC* showed very weak correlation ($0.01 < P < 0.05$) with age in *H. pylori* – negative healthy volunteers.

Because males have a twice as high incidence of gastric cancers as females (2), we also examined the sex differences of methylation levels. However, no significant differences were observed between the 30 males and 26 females among the 56 *H. pylori* – negative healthy volunteers, or between the 52 males and 46 females among the 98 *H. pylori* – positive healthy volunteers.

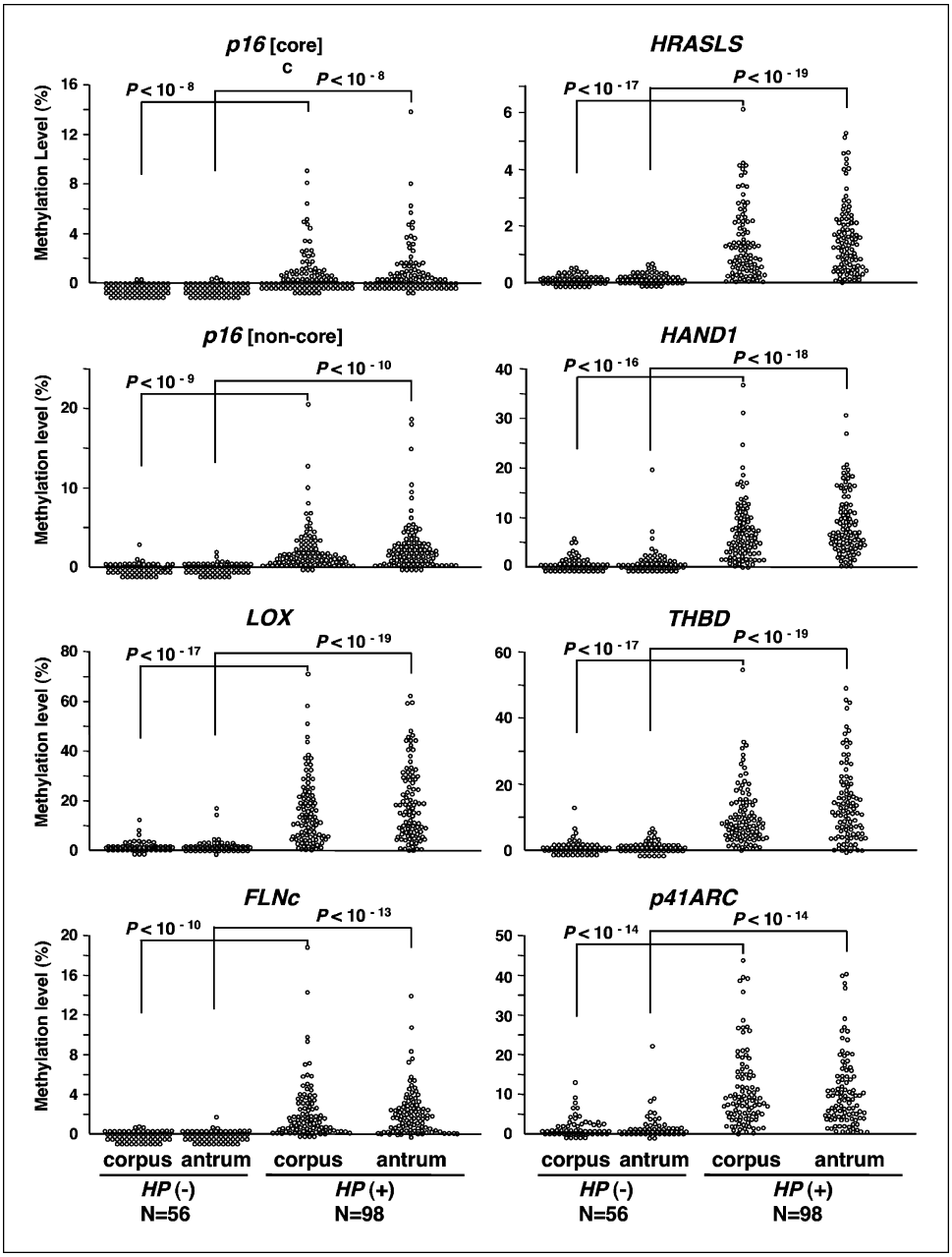

Fig. 2. Higher levels of methylation in gastric mucosae of *H. pylori* – positive volunteers than in those of *H. pylori* – negative volunteers. Methylation levels were measured in the corpus and antrum of 56 *H. pylori* – negative volunteers and 98 *H. pylori* – positive volunteers. All the eight CGIs (core region of *p16*, noncore regions of *p16* and *THBD*; core regions of *LOX*, *HRASLS*, *FLNc*, and *HAND1*; and *p41ARC* exonic CGI) showed significantly elevated methylation levels (5.4- to 303-fold) in the *H. pylori* – positive volunteers. Methylation levels in the corpus were at the same levels as those in the antrum.

Table 2. Methylation levels of the seven CGIs in *H. pylori* – negative and *H. pylori* – positive individuals and in healthy volunteers and gastric cancer cases

	n	Mean age	Corpus								Antrum							
			p16 (core)	*p16* (non)	*LOX*	*FLNc*	*HRASLS*	*HAND1*	*THBD*	*p41ARC*	*p16* (core)	*p16* (non)	*LOX*	*FLNc*	*HRASLS*	*HAND1*	*THBD*	*p41ARC*
HV																		
HP(−)	56	51	0.0%	0.1%	1.2%	0.0%	0.1%	0.8%	1.0%	2.1%	0.0%	0.1%	1.2%	0.1%	0.1%	1.1%	0.8%	1.9%
HP(+)	98	57	0.9%	2.2%	16.0%	2.3%	1.3%	7.4%	10.7%	11.2%	1.1%	2.6%	18.8%	2.2%	1.5%	8.8%	14.2%	10.7%
P			$<10^{-8}$	$<10^{-9}$	$<10^{-17}$	$<10^{-10}$	$<10^{-17}$	$<10^{-16}$	$<10^{-17}$	$<10^{-14}$	$<10^{-8}$	$<10^{-10}$	$<10^{-19}$	$<10^{-13}$	$<10^{-19}$	$<10^{-18}$	$<10^{-19}$	$<10^{-14}$
GCC																		
HP(−)	29	69	0.2%	0.6%	8.2%	0.6%	0.6%	4.1%	7.1%	6.6%	0.2%	0.5%	4.6%	1.3%	0.4%	7.5%	5.5%	4.9%
HP(+)	43	67	0.4%	2.0%	12.7%	0.8%	1.2%	8.1%	12.3%	11.5%	0.3%	2.5%	11.2%	1.0%	0.8%	11.3%	10.2%	7.8%
P			0.07	<0.01	0.02	0.17	<0.01	<0.01	0.01	<0.01	0.28	0.04	<0.01	0.35	0.02	0.05	0.02	0.02
HP(−)																		
AM-HV	29	63	0.0%	0.2%	1.7%	0.1%	0.1%	1.0%	1.3%	3.1%	0.0%	0.2%	1.4%	0.1%	0.1%	1.7%	1.1%	2.2%
GCC	29	69	0.2%	0.6%	8.2%	0.6%	0.6%	4.1%	7.1%	6.6%	0.2%	0.5%	4.6%	1.3%	0.4%	7.5%	5.5%	4.9%
P			<0.01	0.01	<0.01	<0.01	<0.01	0.01	<0.01	<0.01	0.05	0.02	0.02	0.03	<0.01	<0.01	<0.01	0.02
HP(+)																		
AM-HV	43	64	1.0%	1.9%	12.8%	1.4%	1.2%	6.7%	9.4%	9.4%	0.8%	2.1%	13.9%	1.5%	1.2%	7.8%	10.0%	9.8%
GCC	43	67	0.4%	2.0%	12.7%	0.8%	1.2%	8.1%	12.3%	11.5%	0.3%	2.5%	11.2%	1.0%	0.8%	11.3%	10.3%	7.8%
P			0.03	0.44	0.48	0.02	0.48	0.13	0.07	0.11	0.07	0.38	0.17	0.09	0.05	0.02	0.45	0.12

Abbreviations: HV, healthy volunteers; GCC, gastric cancer cases (noncancerous mucosae); AM-HV, age-matched healthy volunteers; non, noncore.

Association between high methylation levels and gastric cancer development. Finally, we examined whether accumulated levels of aberrant DNA methylation in gastric mucosae are associated with a risk of gastric cancer. Methylation levels in noncancerous gastric mucosae of 72 gastric cancer cases (29 *H. pylori* – negative and 43 *H. pylori* – positive cases) were compared with those of 72 healthy volunteers (29 *H. pylori* – negative and 43 *H. pylori* – positive individuals) that were randomly selected to match the average age and *H. pylori* infection status of gastric cancer cases (Table 2).

When *H. pylori* – negative healthy volunteers and *H. pylori* – negative gastric cancer cases were compared, mean methylation levels of antral mucosae in gastric cancer cases (Fig. 3) were significantly elevated at 4.9-fold (*p16* core region), 2.3-fold (*p16* noncore region), 3.3-fold (*LOX*), 5.1-fold (*THBD*), 10-fold (*FLNc*), 3.9-fold (*HRASLS*), 4.4-fold (*HAND1*), and 2.2-fold (*p41ARC*). The same tendency was observed in the corpus. When *H. pylori* – positive healthy volunteers and *H. pylori* – positive gastric cancer cases were compared, variations within both groups were very large. A possibly significant increase ($P = 0.02$) was observed only for *HAND1* at 1.4-fold.

Discussion

It was shown here that significantly higher levels of aberrant methylation (5.4- to 303-fold) were present in multiple CGIs in the gastric mucosae of healthy volunteers with *H. pylori* infection. This finding strongly indicated that *H. pylori* infection potently induces aberrant methylation in multiple CGIs, although there has been controversy (10, 11). The induction of aberrant methylation by the strong gastric carcinogen *H. pylori* is in a good agreement with the fact that tumor suppressor genes, like *p16*, *E-cadherin*, and *hMLH1*, are inactivated more frequently by aberrant DNA methylation than by mutations in gastric cancers (2). Aberrant DNA methylation was shown to be present in noncancerous mucosae of ulcerative colitis by pioneering studies (21, 22), and a role of chronic inflammation in methylation induction has been proposed. Because *H. pylori* infection also causes strong chronic inflammation (23), the role of chronic inflammation in induction of aberrant methylation seems very clear.

Methylation levels of all of the eight CGIs were associated with gastric cancer risk in *H. pylori* – negative individuals. It must be noted that clinical tests for *H. pylori* infection detect only current (culture and urease tests) or recent (serum antibody test) status of *H. pylori* infection and cannot detect past exposure to *H. pylori* (4, 16). On the other hand, epidemiologic studies showed that past exposure to *H. pylori*, rather than current exposure, is more closely associated with a risk of gastric cancer development and that a majority of *H. pylori* – negative gastric cancer cases had past exposure to *H. pylori* (5, 24).

However, methylation levels in *H. pylori* – positive individuals were higher than *H. pylori* – negative gastric cancer cases. The high methylation levels in the *H. pylori* – positive individuals are considered to drop down to various degrees when active *H. pylori* infection discontinues as observed in the *H. pylori* – negative gastric cancer cases. This suggests that *H. pylori* infection induces DNA methylation in both stem cells, which will persist, and nonstem cells, which will drop off the gastric mucosae in a few days (25). If the methylation status of stem cells is copied into the cells in the entire gland without active induction, the methylation levels in the gastric mucosae will reflect the fraction of methylated stem cells among the entire

stem cell population and thus a fraction of stem cells with increased cancer risk. It seems important to examine whether eradication of *H. pylori* leads to decrease in methylation levels.

Mechanistic analysis of how *H. pylori* infection induces aberrant DNA methylation is necessary. *H. pylori* infection almost always induces chronic inflammation and cell proliferation (8). Cell proliferation itself has been suggested as a promoting factor for *de novo* DNA methylation (21, 26). In addition, expression of many genes is repressed during the inflammatory processes and decreased gene expression is known to promote *de novo* methylation (27–29). Further, it was recently reported that stimulation of myeloma cells by interleukin-6 increased expression of DNA methyltransferase 1 (*DNMT1*) mRNA expression (30).

The methylation level of the *p16* core region was consistently much lower than methylation levels of other CGIs. No methylation was detected in 46 (47%) of 98 *H. pylori* – positive healthy volunteers, whereas it was only 3 (3%) when the *p16* noncore region was analyzed. Also, absolute levels of methylation were much higher in *LOX*, *HAND1*, *THBD*, and *p41ARC* of *H. pylori* – positive individuals and *H. pylori* – negative gastric cancer cases. This suggested that extensive methylation of multiple, and possibly preferential, CGIs precedes infrequent occurrence of methylation of a core region of a promoter CGI of critical tumor suppressor gene(s).

As for the effect of locations within the stomach, no significant difference in methylation levels was observed between the corpus and antrum, regardless of *H. pylori*

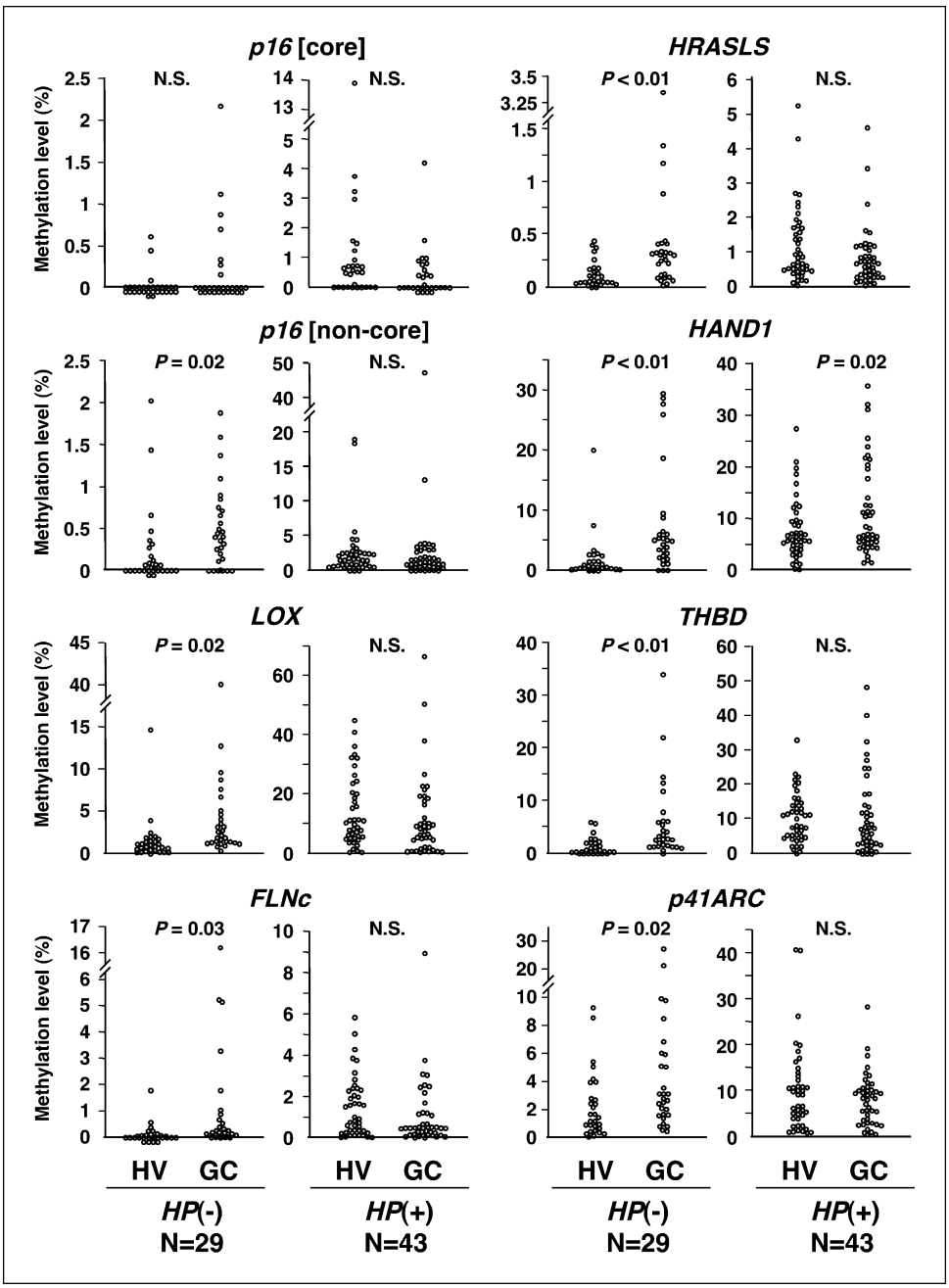

Fig. 3. Association between high methylation levels and a risk of gastric cancer development. Methylation levels of antral mucosae were measured in 29 *H. pylori* – negative and 43 *H. pylori* – positive cases with differentiated-type gastric cancers (*GC*), and the levels were compared with those in 29 *H. pylori* (*HP*) – negative and 43 *H. pylori* – positive age-matched healthy volunteers (*HV*). Among the *H. pylori* – negative individuals, methylation levels of gastric cancer cases were significantly higher (2.2- to 10-fold) than those in healthy volunteers, which showed that methylation levels in noncancerous gastric mucosae are associated with a risk of gastric cancer development. Among the *H. pylori* – positive individuals, methylation levels were highly variable within each group, and a significant increase was observed only for *HAND1* at 1.4-fold. N.S., not significant.

infection status. As for the effect of histologic changes, analysis of limited number of samples showed methylation levels of the eight CGIs were not associated with mucosal atrophy, intestinal metaplasia, or degree of inflammation (data not shown). It seems important to search for specific CGIs whose methylation levels are associated with *H. pylori* infection, with a gastric cancer risk, and with histologic changes, respectively, because various CGIs and regions within one CGI show different susceptibility to methylation (12).

In conclusion, it was indicated that *H. pylori* infection potently and temporarily induces methylation of multiple CGIs. Methylation levels of specific CGIs in noncancerous gastric mucosae may be associated with gastric cancer risk in *H. pylori* – negative individuals.

References

1. Jemal A, Tiwari RC, Murray T, et al. Cancer statistics, 2004. CA Cancer J Clin 2004;54:8–29.
2. Ushijima T, Sasako M. Focus on gastric cancer. Cancer Cell 2004;5:121–5.
3. Ono H, Kondo H, Gotoda T, et al. Endoscopic mucosal resection for treatment of early gastric cancer. Gut 2001;48:225–9.
4. Ekstrom AM, Held M, Hansson LE, Engstrand L, Nyren O. *Helicobacter pylori* in gastric cancer established by CagA immunoblot as a marker of past infection. Gastroenterology 2001;121:784–91.
5. Forman D, Webb P, Parsonnet J. *H. pylori* and gastric cancer. Lancet 1994;343:243–4.
6. Uemura N, Okamoto S, Yamamoto S, et al. *Helicobacter pylori* infection and the development of gastric cancer. N Engl J Med 2001;345:784–9.
7. Sugiyama A, Maruta F, Ikeno T, et al. *Helicobacter pylori* infection enhances *N*-methyl-*N*-nitrosourea-induced stomach carcinogenesis in the Mongolian gerbil. Cancer Res 1998;58:2067–9.
8. Szaleczky E, Pronai L, Molnar B, Berczi L, Feher J, Tulassay Z. Increased cell proliferation in chronic *Helicobacter pylori* positive gastritis and gastric carcinoma-correlation between immuno-histochemistry and Tv image cytometry. Anal Cell Pathol 2000;20:131–9.
9. Koizumi Y, Tsubono Y, Nakaya N, et al. Cigarette smoking and the risk of gastric cancer: a pooled analysis of two prospective studies in Japan. Int J Cancer 2004;112:1049–55.
10. Chan AO, Lam SK, Wong BC, et al. Promoter methylation of E-cadherin gene in gastric mucosa associated with *Helicobacter pylori* infection and in gastric cancer. Gut 2003;52:502–6.
11. Kang GH, Lee HJ, Hwang KS, Lee S, Kim JH, Kim JS. Aberrant CpG island hypermethylation of chronic gastritis, in relation to aging, gender, intestinal metaplasia, and chronic inflammation. Am J Pathol 2003;163:1551–6.
12. Ushijima T. Detection and interpretation of altered methylation patterns in cancer cells. Nat Rev Cancer 2005;5:223–31.
13. Arima N, Adachi K, Katsube T, et al. Predictive factors for metachronous recurrence of early gastric cancer after endoscopic treatment. J Clin Gastroenterol 1999;29:44–7.
14. Takeda J, Toyonaga A, Koufuji K, et al. Early gastric cancer in the remnant stomach. Hepatogastroenterology 1998;45:1907–11.
15. Nishikawa K, Sugiyama T, Kato M, et al. A prospective evaluation of new rapid urease tests before and after eradication treatment of *Helicobacter pylori*, in comparison with histology, culture and ^{13}C-urea breath test. Gastrointest Endosc 2000;51:164–8.
16. Schembri MA, Lin SK, Lambert JR. Comparison of commercial diagnostic tests for *Helicobacter pylori* antibodies. J Clin Microbiol 1993;31:2621–4.
17. Kaneda A, Kaminishi M, Sugimura T, Ushijima T. Decreased expression of the seven ARP2/3 complex genes in human gastric cancers. Cancer Lett 2004;212:203–10.
18. Kaneda A, Kaminishi M, Yanagihara K, Sugimura T, Ushijima T. Identification of silencing of nine genes in human gastric cancers. Cancer Res 2002;62:6645–50.
19. Kaneda A, Kaminishi M, Nakanishi Y, Sugimura T, Ushijima T. Reduced expression of the insulin-induced protein 1 and p41 Arp2/3 complex genes in human gastric cancers. Int J Cancer 2002;100:57–62.
20. Issa JP, Ottaviano YL, Celano P, Hamilton SR, Davidson NE, Baylin SB. Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. Nat Genet 1994;7:536–40.
21. Issa JP, Ahuja N, Toyota M, Bronner MP, Brentnall TA. Accelerated age-related CpG island methylation in ulcerative colitis. Cancer Res 2001;61:3573–7.
22. Hsieh CJ, Klump B, Holzmann K, Borchard F, Gregor M, Porschen R. Hypermethylation of the p16INK4a promoter in colectomy specimens of patients with long-standing and extensive ulcerative colitis. Cancer Res 1998;58:3942–5.
23. Tatematsu M, Tsukamoto T, Mizoshita T. Role of *Helicobacter pylori* in gastric carcinogenesis: the origin of gastric cancers and heterotopic proliferative glands in Mongolian gerbils. Helicobacter 2005;10:97–106.
24. Forman D, Newell DG, Fullerton F, et al. Association between infection with *Helicobacter pylori* and risk of gastric cancer: evidence from a prospective investigation. BMJ 1991;302:1302–5.
25. Tatematsu M, Tsukamoto T, Inada K. Stem cells and gastric cancer: role of gastric and intestinal mixed intestinal metaplasia. Cancer Sci 2003;94:135–41.
26. Velicescu M, Weisenberger DJ, Gonzales FA, Tsai YC, Nguyen CT, Jones PA. Cell division is required for *de novo* methylation of CpG islands in bladder cancer cells. Cancer Res 2002;62:2378–84.
27. Song JZ, Stirzaker C, Harrison J, Melki JR, Clark SJ. Hypermethylation trigger of the glutathione-*S*-transferase gene (GSTP1) in prostate cancer cells. Oncogene 2002;21:1048–61.
28. De Smet C, Loriot A, Boon T. Promoter-dependent mechanism leading to selective hypomethylation within the 5′ region of gene MAGE-A1 in tumor cells. Mol Cell Biol 2004;24:4781–90.
29. Ushijima T, Okochi-Takada E. Aberrant methylations in cancer cells: where do they come from? Cancer Sci 2005;96:206–11.
30. Hodge DR, Peng B, Cherry JC, et al. Interleukin 6 supports the maintenance of p53 tumor suppressor gene promoter methylation. Cancer Res 2005;65:4673–82.

Clinical Cancer Research

High Levels of Aberrant DNA Methylation in *Helicobacter pylori*–Infected Gastric Mucosae and its Possible Association with Gastric Cancer Risk

Takao Maekita, Kazuyuki Nakazawa, Mami Mihara, et al.

Clin Cancer Res 2006;12:989-995.