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ORIGINAL ARTICLE

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헬리코박터 파일로리 제균 치료가 위암 관련 유전자의 CpG Island 과염기화에 미치는 영향

최정민, 김상균[1], 김병관[2], 고성준[2], 김지원[2], 이국래[2]

인제대학교 의과대학 상계백병원 내과학교실, 서울대학교 의과대학 내과학교실 및 간연구소[1], 서울대학교병원운영 서울특별시보라매병원 내과[2]

Helicobacter pylori Eradication Modulates Aberrant CpG Island Hypermethylation in Gastric Carcinogenesis

Jeongmin Choi, Sang Gyun Kim[1], Byeong Gwan Kim[2], Seong-Joon Koh[2], Ji Won Kim[2], and Kook Lae Lee[2]

Department of Internal Medicine, Inje University Sanggye Paik Hospital, Inje University College of Medicine, Department of Internal Medicine and Liver Research Institute, Seoul National University College of Medicine[1], Department of Internal Medicine, SMG-SNU Boramae Medical Center[2], Seoul, Korea

Background/Aims: *Helicobacter pylori* infection induces aberrant DNA methylation in gastric mucosa. We evaluated the long-term effect of *H. pylori* eradication on promotor CpG island hypermethylation in gastric carcinogenesis.
Methods: *H. pylori*-positive patients with gastric adenoma or early gastric cancer who underwent endoscopic resection were enrolled. According to *H. pylori* eradication after endoscopic resection, the participants were randomly assigned to *H. pylori* eradication or non-eradication group. *H. pylori*-negative gastric mucosa from normal participants provided the normal control. CpG island hypermethylation of tumor-related genes (p16, CDH1, and RUNX-3) was evaluated by quantitative MethyLight assay in non-tumorous gastric mucosa. The gene methylation rate and median values of hypermethylation were compared after one year by *H. pylori* status.
Results: In *H. pylori*-positive patients, hypermethylation of p16 was found in 80.6%, of CDH1 in 80.6%, and of RUNX-3 in 48.4%. This is significantly higher than normal control (p16, 10%; CDH1, 44%; RUNX-3, 16%) ($p<0.05$). In the *H. pylori* eradication group, methylation rates of p16 and CDH1 decreased in 58.1% and 61.3% of the patients, and the median values of hypermethylation were significantly lower at one year compared with the non-eradication group. However, RUNX-3 hypermethylation did not differ significantly at one year after *H. pylori* eradication. The non-eradication group hypermethylation did not change after one year.
Conclusions: *H. pylori* infection was associated with promotor hypermethylation of genes in gastric carcinogenesis, and *H. pylori* eradication might reverse p16 and CDH1 hypermethylation. **(Korean J Gastroenterol 2016;68:253-259)**

Key Words: *Helicobacter pylori*; CpG hypermethylation; p16; CDH1; Carcinogenesis

INTRODUCTION

Helicobacter pylori infection is one of the most prevalent infectious diseases worldwide and 40-50% of the global human population is estimated to be infected. *H. pylori* has been identified as group I carcinogen by the World Health Organization International Agency for Research on Cancer and is associated with the development of gastric cancer.[1]

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교신저자: 김상균, 03080, 서울시 종로구 대학로 101, 서울대학교 의과대학 내과학교실 및 간연구소
Correspondence to: Sang Gyun Kim, Department of Internal Medicine and Liver Research Institute, Seoul National University College of Medicine, 101 Daehak-ro, Jongno-gu, Seoul 03080, Korea. Tel: +82-2-740-8112, Fax: +82-2-743-6701, E-mail: harley@snu.ac.kr

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Aberrant DNA methylation is one of the most frequent epigenetic changes, usually at the 5'position of the cytosine ring within CpG dinucleotides, and its influence is gene silencing and noncoding genomic regions.[2] Promotor CpG island hypermethylation is a crucial mechanism to silence tumor suppressor genes.[3] Aberrant CpG island hypermethylation occurs early in the multi-stage carcinogenesis. Gastric cancer is linked to tumor suppressor-related genes that are inactivated with by CpG island hypermethylation.[4] CpG island hypermethylation has been found in the adjacent noncancerous tissues of gastric cancer patients as well as normal gastric mucosa.[5]

H. pylori infection induces aberrant DNA methylation in gastric mucosa, which raises gastric cancer risk.[6] Aberrant DNA methylation could be suppressed by *H. pylori* eradication.[7] However, whether suppression of aberrant DNA methylation lasts over the long term is unknown. We evaluated long-term effect of *H. pylori* eradication on promotor CpG island hypermethylation in gastric carcinogenesis.

In this study, we postulated that *H. pylori* infection might cause aberrant DNA hypermethylation of three gastric cancer-related genes (p16, CDH1, and runt-related transcription factor 3 [RUNX-3]), which are all tumor suppressor genes.[7-9] Eradication of *H. pylori* might reverse methylation of these genes over the long term. We investigated methylation of the p16, CDH1, and RUNX-3 genes in gastric mucosa from patients with gastric adenoma or early gastric cancer (EGC) before and after eradication of *H. pylori* after one year.

SUBJECTS AND METHODS

1. Patients and study design

In this study, gastric tissues were obtained from samples that were collected for another study.[10] *H. pylori*-positive patients with gastric adenoma or EGC who underwent endoscopic resection were enrolled. According to *H. pylori* eradication after endoscopic resection, the participants were randomly assigned to *H. pylori* eradication or non-eradication group. Patients in the eradication group received omeprazole, 20 mg; amoxicillin, 1 g; and clarithromycin, 500 mg, twice daily for one week. Patients in the non-eradication group received no antibiotics.[10] All patients underwent endoscopic examination regularly at one year intervals. Successful eradication for *H. pylori* was confirmed in the eradication group by both histologic examination and rapid urease test. *H. pylori* status was considered positive if the result of one or both tests (histology or rapid urease test) was positive. Negative in both histology and rapid urease test deemed *H. pylori*-negative. We used samples from that study to evaluate the effect of *H. pylori* eradication on hypermethylation of genes before and one year after endoscopic resection. *H. pylori*-negative dyspepsia patients without adenoma or EGC were enrolled as normal control. All patients gave informed consent and the institutional review board of Seoul National University Hospital approved this study (H-1008-115-329).

2. Tissue collection

Biopsy samples were taken from the lesser curvature of the antrum and the lesser curvature of the body for evaluation of *H. pylori*, rapid urease test, gastric atrophy, intestinal metaplasia (IM), and DNA methylation study. The degree of atrophic gastritis (AG) and IM in the gastric mucosa was classified according to the updated Sydney system.[11] Negative AG/IM was considered no evidence of AG/IM in both antrum and body. AG/IM was considered positive if the antrum or body result was positive. *H. pylori* density, neutrophilic inflammation activity, and mononuclear inflammation were also evaluated according to updated Sydney system.

3. DNA extraction and Bisulfite modification

Biopsy specimens obtained from non-tumorous mucosa were stored at −80°C. DNA was extracted and verified. Specimens were homogenized in proteinase K solution (20 mmol/L Tris-hydrochloride [pH 8.0], 10 mmol/L EDTA, 0.5% sodium lauryl sulfate, and 10 mg/mL proteinase K), then maintained for over 3 hours at 50°C. DNA was separated from homogenates by phenol/chloroform extraction and ethanol precipitation.[12] Genomic DNA was modified by sodium bisulfite to convert unmethylated cytosines to uracil using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol.

4. MethyLight assay

The methylation assay of the p16, CDH1, and RUNX-3 genes from bisulfite modified DNA samples was performed with real-time PCR-based quantitative MethyLight technology.[13-15] Primers and probe for sequencing have been described.[16] Two sets of primers and probes designed to bond to bisulfite-con-

verted DNA were used: One set of primers and a probe were used for methylated reaction, and another set was utilized as the reference locus. The DNA methylation of each examined marker was quantified and reported as a percent of methylated reference (PMR; degree of methylation). PMR was calculated as 100×([methylated reaction/ALU] sample/[methylated reaction/ALU] M.SssI-reference).[17] We considered a CpG island locus methylated if the PMR value was >4.[15]

Table 1. Clinicopathological Characteristics among Groups

Characteristic	Group			
	Helicobacer pylori eradication	Non-eradication	p-value[a]	Normal control
Gender			1.00	
Male	21 (67.7)	22 (64.7)		11 (61.1)
Female	10 (32.3)	12 (35.3)		7 (38.9)
Age (yr)	60.0 (50.0-66.0)	59.5 (54.7-66.0)	0.68	58.5 (50.0-67.5)
Gastric atrophy			0.42	
Absent	5 (16.1)	2 (5.9)		7 (38.9)
Mild	6 (19.4)	6 (17.6)		9 (50.0)
Moderate	11 (35.5)	10 (29.4)		2 (11.1)
Marked	3 (9.7)	6 (17.6)		0 (0)
NA	6 (19.4)	10 (29.4)		0 (0)
Intestinal metaplasia			1.00	
Absent	1 (3.2)	2 (5.9)		11 (61.1)
Mild	9 (29.0)	8 (23.5)		3 (16.7)
Moderate	13 (41.9)	19 (55.9)		4 (22.2)
Marked	8 (25.8)	5 (14.7)		0 (0)
Gastric atrophy or IM			0.97	
Absent	1 (3.2)	1 (2.9)		7 (38.9)
Mild	9 (29.0)	9 (26.5)		9 (50.0)
Moderate	12 (38.7)	15 (44.1)		2 (11.1)
Marked	9 (29.0)	9 (26.5)		0 (0)
H. pylori density			0.75	0 (0)
Mild	10 (32.3)	14 (41.2)		
Moderate	14 (45.2)	13 (38.2)		
Marked	7 (22.6)	7 (20.6)		
Neutrophilic inflammation			0.25	
Absent	1 (3.2)	0 (0)		
Mild	0 (0)	3 (8.8)		
Moderate	25 (80.6)	27 (79.4)		
Marked	5 (16.1)	4 (11.8)		
Mononuclear inflammation			0.43	
Absent	0 (0)	0 (0)		
Mild	0 (0)	0 (0)		
Moderate	20 (64.5)	25 (73.5)		
Marked	11 (35.5)	9 (26.5)		
Histology			0.55	
Low-grade dysplasia	12 (38.7)	10 (29.4)		0 (0)
High-grade dysplasia	4 (12.9)	3 (8.8)		0 (0)
Adenocarcinoma	15 (48.4)	21 (61.8)		0 (0)
Well differentiated	5 (16.1)	11 (32.4)		
Moderately differentiated	7 (22.6)	6 (17.6)		
Poorly differentiated	2 (6.5)	1 (2.9)		
Signet ring cell	1 (3.2)	3 (8.8)		
Total	31 (100)	34 (100)		18 (100)

Values are presented as n (%) or median (interquartile range).
NA, not available; IM, intestinal metaplasia.
[a]Comparison between *H. pylori* eradication and non-eradication group.

5. Statistical analysis

Because the data were not normally distributed, gene hypermethylation rate before eradication and at one year after was compared using the McNemar test. The median methylation value (PMR) at baseline and at one year after was compared with a Wilcoxon signed-rank test. Other comparisons used the Mann-Whitney U test and Fischer's exact test. Null hypotheses of no difference were rejected if p-values were less than 0.05. Statistical calculations were done using R version 2.15.3 (R foundation for Statistical Computing, Vienna, Austria).

RESULTS

1. Subject characteristics

Eighty-three patients were enrolled in the study: 31 patients in *H. pylori* eradication group, 34 patients in non-eradication group, or 18 patients in normal control. There were no significant differences in baseline clinicopathological variables among the groups (Table 1). The median age was 60 years old (interquartile range, 50-66 years) and male was 67.7%. Most patients had AG/ IM while only two patients (3.0%) had no AG or IM. Half of patients had EGC and remaining half had gastric adenoma. Most of the normal control group had no AG/IM or mild AG/IM.

2. MethyLight assay

In the *H. pylori* eradication group, rate of hypermethylation of p16, CDH1, and RUNX-3 genes at baseline was 80.6% (25/31), 80.6% (25/31), and 48.4% (15/31), respectively. At one year after eradication, methylation was 58.1% (18/31), 61.3% (19/31), and 67.7% (21/31), respectively (Table 2). There was no statistical difference in hypermethylation rates between baseline and one year after eradication. We also analyzed quantitative methylation value of individual genes, which was possible owing to MethyLight assay. We found that median methylation value (PMR) of p16 and CDH1 was significantly lower at one year after eradication. For the p16 gene, median baseline PMR decreased significantly from 11.7 to 5.7 (p=0.004). For CDH1, median PMR decreased significantly from 47.9 to 7.0 (p=0.001). Conversely, RUNX-3 did not show any difference in methylation value (Fig. 1).

In the non-eradication group, rates of methylated the three genes were CDH1, 71.1%; PMR, 97.4%; and RUNX-3, 55.3%. There were no significant changes in both hypermethylation rate and median methylation value at one-year follow-up.

Baseline p16, CDH1, and RUNX-3 genes had significantly higher methylation levels in *H. pylori* positive patients than in *H. pylori*-negative normal control (10%, 44%, 16%, respectively; $p < 0.05$) (Table 2).

We observed significant decrease in both neutrophilic and mononuclear inflammation in the gastric mucosa one year after *H. pylori* eradication (p=0.01). Conversely, there was no

Table 2. Promotor Gene Methylation Rates and Median Methylation Values at Baseline and One Year among Groups

Variable	Group							
	Helicobacter pylori eradication			Non-eradication			Normal control	
	Baseline	1 yr	p-value[a]	Baseline	1 yr	p-value[b]	Baseline	p-value[c]
Methylated gene (%)								
P16	80.6	58.1	1.00	71.1	84.2	0.33	10.0	0.01
RUNX-3	48.4	67.7	0.25	55.3	65.8	0.05	16.0	0.03
CDH1	80.6	61.3	1.00	97.4	100	1.00	44.0	0.01
Median methylation value (PMR)								
P16	11.7	5.7	0.01	12.9	17.8	0.25	3.4	0.01
RUNX-3	2.2	9.8	0.41	30.5	50.4	0.18	13.9	0.01
CDH1	47.9	7.0	0.01	67.7	76.6	0.24	5.3	0.01
Patient (total, n)	31			34			18	

PMR, percentage of methylated reference.
[a]Comparison of methylation between baseline and one year after in the *H. pylori* eradication group.
[b]Comparison of methylation between baseline and one year after in the non-eradication group.
[c]Comparison of baseline methylation in the eradication group with normal control.

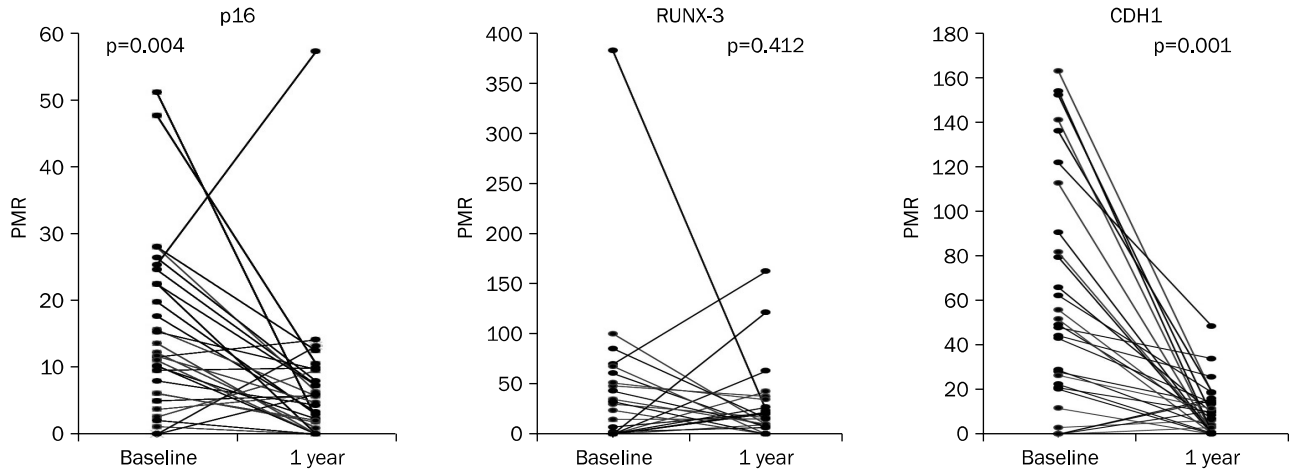

Fig. 1. Changes in quantitative value of MethyLight assay (percentage of methylated reference, PMR) of p16, RUNX-3 and CDH1 at baseline and after one year in the *Helicobacter pylori* eradication group. PMR value in p16 and CDH1 were significantly reduced one year after eradication.

Table 3. Promotor Gene Methylation Rates and Median Methylation Values at Baseline and One Year according to Gastric Adenoma or Early Gastric Cancer

Variable	*Helicobacter pylori* eradication						Non-eradication					
	Adenoma			Cancer			Adenoma			Cancer		
	Baseline	1 yr	p-value	Baseline	1 yr	p-value	Baseline	1 yr	p-value	Baseline	1 yr	p-value
Methylated gene (%)												
P16	75.0	68.8	1.00	86.7	46.7	0.07	61.5	69.2	1.00	76.2	95.2	0.13
RUNX-3	62.5	75.0	0.73	33.3	60.0	0.13	61.5	69.2	1.00	52.4	71.4	0.29
CDH1	81.3	56.3	0.29	80.0	66.7	0.69	100	100	1.00	95.2	100	1.00
Median methylation value (PMR)												
P16	9.7	5.7	0.20	15.3	3.2	0.02	5.24	10.4	0.55	6.92	14.4	0.06
RUNX-3	19.1	15.9	0.61	0.01	9.6	0.96	11.3	22.2	0.02	9.1	25.4	0.59
CDH1	49.7	2.4	0.01	28.9	9.7	0.04	44.0	99.7	0.04	78.1	78.3	0.59
Patient (n)	16			15			13			21		

PMR, percentage of methylated reference.

change of the inflammation in the non-eradication group (data not shown). We tried to explain why some of eradicated patients still showed CDH1 and/or p16 hypermethylation even after the eradication. We compared patients who showed decreased methylation level of CDH1/p16 after the eradication with those did not in terms of initial *H. pylori* density, initial neutrophilic/mononuclear inflammation activity, or inflammation activity one year after eradication. We found no significant difference of inflammation activity or *H. pylori* density between groups.

We also analyzed methylation rates and median methylation level according to gastric adenoma or EGC type. In the eradication group, significant change in median methylation level of p16 was predominant in the EGC (PMR, 15.3 [baseline], 3.2 [1 year]; p=0.02) rather than gastric adenoma (PMR, 9.7 [baseline], 5.7 [1 year]; p=0.20) (Table 3). We evaluated median methylation level of p16 in the EGC patients by differentiation type (well, moderately, poorly differentiated adenocarcinoma, or signet ring cell carcinoma). Initial methylation level of p16 was not different between well/moderately differentiated EGC and poorly differentiated/signet ring cell EGC. However, only well or moderately differentiated EGC decreased significantly in the methylation level of p16 after eradication (p=0.01). In terms of CDH1, significant change in PMR was found in EGC as well as adenoma. However, in the non-eradication group, there was no significant difference in methylation rate according to gastric adenoma or EGC.

DISCUSSION

In this study, the methylation levels in three genes (p16, CDH1, and RUNX-3) were evaluated from non-neoplastic gastric mucosae using quantitative real-time PCR, MethyLight assay. Baseline p16, CDH1, and RUNX-3 genes showed significantly higher methylation levels in *H. pylori*-positive patients than in *H. pylori*-negative normal control group. Some articles reported that patients with *H. pylori* had hypermethylation of p16 and CDH1 in 46-80%, while normal participants without *H. pylori* had no/little hypermethylation,[8,18] which was in line with our study. However, we should consider that not all patients with *H. pylori* infection had hypermethylation of p16 and CDH1 although hypermethylation of these genes are associated with *H. pylori* infection. Rates of hypermethylation were variable, ranging from 30% to 82%.[8,19] In addition, some studies reported that methylation of MLH1 and MGMT did not decrease significantly after *H. pylori* eradication.[8,20] Conversely, a recent study showed that MGMT methylation was significantly reduced after *H. pylori* eradication in patients with *H. pylori* gastritis (from 70% to 48%).[21] These contradictory results suggest that the exact mechanism needs to be described, although researchers found epigenetic alteration associated with *H. pylori* in gastric carcinogenesis.

MethyLight is a sodium-bisulfite-dependent, quantitative, real-time PCR method to sensitively detect and quantify DNA methylation in genomic DNA. Methylation specific polymerase chain reaction (MSP) after sodium bisulfite conversion is used to determine DNA methylation status. However, due to its qualitative nature of assay, MSP cannot distinguish level of methylation.[15] The high sensitivity and specificity of MethyLight assay allows detection of low-frequency DNA methylation markers. The advantages of MethyLight technology include its quantitative and high-throughput nature and relatively simple assay.[14] Therefore, MethyLight allows better detection of DNA and less normalization errors caused by copy number changes.[15]

Research of the effect of *H. pylori* eradication on CpG hypermethylation of genes in gastric mucosa[8,18,21,22] typically analyzed hypermethylation of genes at six to eight weeks after *H. pylori* eradication. We postulated that some genes need longer than eight weeks, for reversal of CpG hypermethylation after *H. pylori* eradication. We also assumed some genes were more likely to remain methylated once their CpG island was methylated, so eradication might have little impact. We aimed to determine whether *H. pylori* eradication affects methylation of relevant genes over the long term.

In this study, p16 and CDH1 hypermethylation decreased in 58.1% and 61.3% of the patients, and the median values of methylation reduced significantly at one year after *H. pylori* eradication compared with the non-eradication group.

Hypermethylation of these genes is associated with *H. pylori* and suppressed by *H. pylori* eradication.[8] Specifically, CDH1 and p16 methylation significantly decreased after *H. pylori* eradication,[8,18] inconsistent with our study. This result might stem from patient differences. In this study, patients had gastric adenoma/EGC and most patients had AG/IM, while other studies had no gastric adenoma or EGC.[8,18] However, not all patients experienced reduction of hypermethylation of relevant genes by *H. pylori* eradication.[8,18] Approximately 22-76% of patients who underwent eradication still had hypermethylation of the CDH1 gene and 18% of patients still had p16 hypermethylation.[8,18]

Hypermethylation of RUNX-3 increased one year after *H. pylori* eradication in this study, which suggests RUNX-3 may not be crucial in gastric carcinogenesis. However, some methylation profiles induced by *H. pylori* infection can persist even after eradication.[12] Several studies suggested RUNX-3 methylation as a risk factor for the gastric carcinogenesis in patients with *H. pylori* infection.[23,24]

One study suggested that epigenetic events and gene methylation were not evenly distributed throughout the gastric mucosa, so multiple biopsies in different parts of stomach should be performed to determine methylation status.[8] In this study, one biopsy was used for methylation status, which might be a limitation. Another limitation was the small number of participants.

In conclusion, *H. pylori* infection is associated with promotor methylation of genes in gastric carcinogenesis. *H. pylori* eradication might reverse CDH1 and p16 methylation levels. Further studies are warranted to determine long-term effect of *H. pylori* eradication on DNA methylation.

REFERENCES

1. Schistosomes, liver flukes and *Helicobacter pylori*. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Lyon,

7-14 June 1994. IARC Monogr Eval Carcinog Risks Hum 1994;61:1-241.
2. Na HK, Woo JH. *Helicobacter pylori* induces hypermethylation of CpG islands through upregulation of DNA methyltransferase: possible involvement of reactive oxygen/nitrogen species. J Cancer Prev 2014;19:259-264.
3. Jones PA, Baylin SB. The epigenomics of cancer. Cell 2007;128: 683-692.
4. Ushijima T. Epigenetic field for cancerization. J Biochem Mol Biol 2007;40:142-150.
5. Kang GH, Shim YH, Jung HY, Kim WH, Ro JY, Rhyu MG. CpG island methylation in premalignant stages of gastric carcinoma. Cancer Res 2001;61:2847-2851.
6. Maekita T, Nakazawa K, Mihara M, et al. High levels of aberrant DNA methylation in *Helicobacter pylori*-infected gastric mucosae and its possible association with gastric cancer risk. Clin Cancer Res 2006;12:989-995.
7. Ito K, Liu Q, Salto-Tellez M, et al. RUNX3, a novel tumor suppressor, is frequently inactivated in gastric cancer by protein mislocalization. Cancer Res 2005;65:7743-7750.
8. Perri F, Cotugno R, Piepoli A, et al. Aberrant DNA methylation in non-neoplastic gastric mucosa of *H. pylori* infected patients and effect of eradication. Am J Gastroenterol 2007;102:1361-1371.
9. Zou XP, Zhang B, Zhang XQ, Chen M, Cao J, Liu WJ. Promoter hypermethylation of multiple genes in early gastric adenocarcinoma and precancerous lesions. Hum Pathol 2009;40:1534-1542.
10. Choi J, Kim SG, Yoon H, et al. Eradication of *Helicobacter pylori* after endoscopic resection of gastric tumors does not reduce incidence of metachronous gastric carcinoma. Clin Gastroenterol Hepatol 2014;12:793-800.e1.
11. Dixon MF, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. Am J Surg Pathol 1996;20:1161-1181.
12. Shin CM, Kim N, Jung Y, et al. Role of *Helicobacter pylori* infection in aberrant DNA methylation along multistep gastric carcinogenesis. Cancer Sci 2010;101:1337-1346.
13. Park SY, Yoo EJ, Cho NY, Kim N, Kang GH. Comparison of CpG island hypermethylation and repetitive DNA hypomethylation in premalignant stages of gastric cancer, stratified for *Helicobacter pylori* infection. J Pathol 2009;219:410-416.
14. Eads CA, Danenberg KD, Kawakami K, et al. MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res 2000;28:E32.
15. Ogino S, Kawasaki T, Brahmandam M, et al. Precision and performance characteristics of bisulfite conversion and real-time PCR (MethyLight) for quantitative DNA methylation analysis. J Mol Diagn 2006;8:209-217.
16. Shin SH, Park SY, Ko JS, Kim N, Kang GH. Aberrant CpG island hypermethylation in pediatric gastric mucosa in association with *Helicobacter pylori* infection. Arch Pathol Lab Med 2011;135: 759-765.
17. Kim JH, Rhee YY, Bae JM, et al. Subsets of microsatellite-unstable colorectal cancers exhibit discordance between the CpG island methylator phenotype and MLH1 methylation status. Mod Pathol 2013;26:1013-1022.
18. Chan AO, Peng JZ, Lam SK, et al. Eradication of *Helicobacter pylori* infection reverses E-cadherin promoter hypermethylation. Gut 2006;55:463-468.
19. Tahara T, Arisawa T, Shibata T, et al. Increased number of methylated CpG islands correlates with *Helicobacter pylori* infection, histological and serological severity of chronic gastritis. Eur J Gastroenterol Hepatol 2009;21:613-619.
20. Bartchewsky W Jr, Martini MR, Squassoni AC, et al. Influence of *Helicobacter pylori* infection on the expression of MLH1 and MGMT in patients with chronic gastritis and gastric cancer. Eur J Clin Microbiol Infect Dis 2009;28:591-597.
21. Sepulveda AR, Yao Y, Yan W, et al. CpG methylation and reduced expression of O6-methylguanine DNA methyltransferase is associated with *Helicobacter pylori* infection. Gastroenterology 2010;138:1836-1844.
22. Leung WK, Man EP, Yu J, et al. Effects of *Helicobacter pylori* eradication on methylation status of E-cadherin gene in noncancerous stomach. Clin Cancer Res 2006;12:3216-3221.
23. Kitajima Y, Ohtaka K, Mitsuno M, et al. *Helicobacter pylori* infection is an independent risk factor for Runx3 methylation in gastric cancer. Oncol Rep 2008;19:197-202.
24. Lu XX, Yu JL, Ying LS, et al. Stepwise cumulation of RUNX3 methylation mediated by *Helicobacter pylori* infection contributes to gastric carcinoma progression. Cancer 2012;118:5507-5517.