Research Article

Slow Overmethylation of Housekeeping Genes in the Body Mucosa Is Associated with the Risk for Gastric Cancer

Jung-Hwan Oh1, Mun-Gan Rhyu2, Sung-Hoon Jung1, Sang-Wook Choi1, Suk-Il Kim3, and Seung-Jin Hong2

Abstract

Helicobacter pylori infection increases age-related diverse overmethylation in gene-control regions, which increases the risk of gastric cancer. The H. pylori-associated overmethylation changes subsequently disappear when gastric atrophy and cancer develop. To identify cancer-risk epigenotypes, we traced dynamic methylation changes in the background mucosa of the stomach depending on the extent of gastric atrophy. Paired biopsy specimens were obtained from the noncancerous antrum and body mucosa of 102 patients with cancer and 114 H. pylori–positive and 112 H. pylori–negative controls. The grade of gastric atrophy was evaluated using the endoscopic atrophic border score. The methylation-variable sites at the CpG-island margins and near the transcriptional start sites lacking CpG islands were semiquantitatively analyzed by radioisotope-labeling methylation-specific PCR. We selected eight housekeeping genes adjacent to Alu (CDH1, ARRDC4, PPARG, and TRAPPC2L) or LTR retroelements (MMP2, CDKN2A, RUNX2, and RUNX3) and eight stomach-specific genes (TFF2, PGC, ATP4B, TFF1, TFF3, GHRL, PGA, and ATP4A). Analysis of age-related methylation in the H. pylori–positive controls revealed slow overmethylation in the body and in the LTR-adjacent genes. A high-frequency overmethylation defined based on the slowly overmethylated genes was frequently observed in the body of patients with gastric cancer with open-type atrophy (OR, 12.7; 95% confidence interval, 3.2–49.8). The rapidly changing methylation of Alu-adjacent genes was barely increased in the antrum of patients with gastric cancer. Among diverse methylation changes associated with H. pylori infection, an increase in slowly changing methylation could serve as a cancer-risk marker. Cancer Prev Res; 7(6); 1–11. ©2014 AACR.

Introduction

Gastric cancer is the second most common cause of cancer-related deaths worldwide (1). Early cancer detection can greatly reduce morbidity and mortality (2). One strategy for early detection is to identify and screen individuals at high risk of developing cancers (3). Endoscopy plays a leading role in the early detection of gastric cancer (4). Biannual endoscopy screening was recently reported to reduce gastric cancer mortality (5, 6). However, mass screening for gastric cancer by endoscopy may be an unfeasible approach because of medical expenses and a very small number of cancer cases (3, 6). In addition, interobserver variation exists between expert and beginner operators.

Therefore, cancer markers may present a more economical screening option to identify high-risk individuals who would benefit from undergoing further invasive screening. Helicobacter pylori is well known to be a carcinogen that promotes progression of gastritis to atrophy, metaplasia, and dysplasia (7, 8). H. pylori infection occurs mostly during childhood and persists for years (9). In an aging population, the infection tends to disappear in the presence of advanced atrophy, which creates an unfavorable environment for H. pylori colonization (8). However, the oncogenic potential of H. pylori infection persists after loss of the infection (10). Interestingly, H. pylori infection is closely associated with frequent overmethylation of housekeeping genes containing CpG islands (11–13). The H. pylori-associated methylation changes have been proposed as one of the major causes of gastric cancer (11, 14). High methylation levels of housekeeping genes in the noncancerous mucosa of the stomach may be useful for detecting gastric cancer–risk events.

Although the elderly typically show advanced atrophic changes caused by long-standing H. pylori infection, the infection has usually disappeared by the time cancer is detected (15). It is likely that current H. pylori infection potently induces the overmethylation of housekeeping genes, and a high level of methylation decreases to a low level after active H. pylori infection discontinues. In fact, H. pylori eradication significantly reduces the overmethylation

Note:

Supplementary data for this article are available at Cancer Prevention Research Online (http://cancerprevention.aacrjournals.org/).

J.-H. Oh and M.-G. Rhyu contributed equally to this work.

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changes in housekeeping genes (16, 17). Postinfection maintenance of methylation status in some of the densely methylated genes could be related to the risk of gastric cancer (18, 19). The sustained methylation may show promise as an epigenetic cancer-risk marker, even in individuals with past infection. For this reason, the \textit{H. pylori}-associated dynamic methylation changes need to be elucidated to understand how they appear and subsequently disappear in the background mucosa of patients with gastric cancer.

Methylation changes occur gradually during subsequent cell divisions in an age-dependent manner (20, 21). It is noteworthy that the speed of dynamic methylation changes is high in the antrum, which harbors fast self-renewal stem cells, and low in the body, which contains slow self-renewal stem cells (13). A temporal difference in the dynamic methylation changes between the antrum and body is likely to be informative for understanding epigenetic cancer-risk patterns. High-speed methylation changes may appear during the cancer-risk period of \textit{H. pylori} infection, with most of these changes not present at the time of gastric cancer detection. Low-speed methylation changes may remain for a long time in the background mucosa of gastric cancer. The aim of this study is to identify cancer-related methylation patterns in the antral and body mucosa of gastric cancer. The transitional-CpG sites between unmethylated promoters and nearby methylated retroelements, including the CpG-island margins and the non-island CpG sites of the genes lacking CpG islands, were methylated to various degrees in an age-dependent manner (13). The CpG-island margins, termed CpG-island shores, were concurrently methylated during stem cell differentiation (21–25). Using a transitional-CpG marker set, we investigated the dynamic methylation changes through small biopsy samples obtained from the stomach antrum and body.

\textbf{Materials and Methods}

\textbf{Collection of stomach biopsy specimens}

Noncancerous mucosal tissues were collected from healthy subjects and patients with gastric cancer who underwent gastric endoscopy from March 2008 to April 2012 at St. Paul’s Hospital. During an endoscopic examination, paired gastric mucosa specimens were obtained from the lesser curvatures of the proximal antrum (1–2 cm distal from the angle) and the greater curvatures of the middle body of the stomach by endoscopic biopsy using sterile forceps (Olympus FB 24-K-1, Olympus Optical Co; Supplementary Fig. S1A). Normal-appearing area adjacent to cancerous lesion might be composed of epigenetically heterogeneous cells when analyzing methylation patterns (26). To ensure the epigenetic purity of noncancerous cells, biopsy tissues were taken from normal-appearing mucosa more than 2 cm away from the cancerous lesion. The biopsy specimens were frozen immediately and stored at –70°C. Additional biopsy specimens for histologic examination were collected adjacent to the first site. A pathologist confirmed the presence of more than 80% normal epithelial cells and no cancer cells in the biopsy specimens (Supplementary Fig. S1B). \textit{H. pylori} infection was examined by the Warthin–Starry silver impregnation method. Gastric cancer was further classified as early or advanced gastric cancer according to the endoscopic findings evaluated by 2 endoscopists (27, 28). The extent of gastric atrophy was evaluated using the endoscopic atrophic border score proposed by Kimura and Takemoto (29, 30), which correlated with the results of the histologic evaluations (Supplementary Methods). This study was approved by St. Paul’s hospital Institutional Review Board, the Catholic University of Korea, Catholic Medical Center. Written informed consent was obtained from each participating subject before the study.

\textbf{Methylation analysis of small amounts of DNA}

Detailed methodology for the DNA preparation and radioisotope methylation-specific PCR (MSP) has been described elsewhere (Supplementary Methods; refs. 12, 31, and 32). We have analyzed the methylation-variable transitional-CpG sites of 19 genes for a limited amount of DNA extracted from endoscopic biopsy specimens (Supplementary Table S1; ref. 13). MSP primer sets for the 19 transitional-CpG sites were designed to amplify 6 \textit{Alu}-adjacent housekeeping genes (CDH1, ARRDCA4, PPARG, TRAPPC2L, MLH1, and SHH). 4 \textit{LTR}-adjacent housekeeping genes (MMP2, CDKN2A, RUNX2, and RUNX3). 8 stomach-specific genes (TFF2, PGC, ATP4B, TFF1, TFF3, GHRL, PGA, and ATP4A), and 1 inactive gene (APC). The transitional-CpG sites of housekeeping genes were chosen from the CpG-island margins. However, the transitional-CpG sites of the genes lacking CpG islands were selected from the non-island CpG sites near the transcriptional start sites.

A hot start MSP using primer isotope was necessary for specific amplification of the transitional-CpG sites (12, 22, 31, 32). To ensure similar PCR efficiency for unmethylated versus methylated amplicons, PCR amplification of each primer set was adjusted to reach a subplateau level at 32 PCR cycles (13). A low GC content and repetitive sequence in the transitional-CpG sites limited the template-primer specificity when using nonradioisotope methods (12, 22, 26, 31, 32). The PCR specificity of the transitional-CpG sites of 4 genes (PPARG, CDKN2A, CDH1, and TFF2) was compared between the radioisotope MSP and the pyrosequencing (Supplementary Fig. S2A). The radioisotope MSP generated specific amplification products (Supplementary Fig. S2B). Although, the PPARG and CDKN2A genes could not be analyzed by pyrosequencing because of nonspecific PCR bands (Supplementary Fig. S2C). When common PCR for pyrosequencing generated a specific band, such as CDH1 and TFF2 genes, the methylation values from pyrosequencing and radioisotope MSP were similar.

A stringent MSP condition using radioisotope has been found to provide reproducible results for repeated bisulfite modification and paired experiments (12, 13). The biased MSP results tended to be attributed to dissimilar biopsy sizes. Thirty pairs of 1-cm-adjacent tissues were obtained by size-matched biopsies from the \textit{H. pylori}–negative stomach.
(Supplementary Fig. S3). The length, width, and height of each biopsy specimen were measured in the largest diameter. The biopsy size was calculated using the ellipsoid formula: volume of tissue size = \(1/6 \times \pi \times \text{length} \times \text{width} \times \text{height}\). The size of biopsy tissues was categorized into 3 groups: 0.1 to <0.2 mm\(^3\), 0.2 to <0.3 mm\(^3\), and 0.3 to <0.4 mm\(^3\). Nineteen transitional-CpG sites per sample were amplified under stringent MSP conditions. The 10-level classification of the paired samples reproduced the similarly methylated cases within 2 levels in 79% of small-size pairs, 77% of middle-size pairs, and 65% of large-size pairs. The small-sized biopsy was performed to obtain uniformed mucosal tissues.

**Statistical analysis**

See Supplementary Methods.

**Results**

**Baseline characteristics of patients with gastric cancer and noncancer controls**

The baseline characteristics of the study population were summarized in Table 1. Of 226 controls, 114 were *H. pylori*–positive and 112 were *H. pylori*–negative. The distributions of age, gender, and atrophic border score were similar between *H. pylori*–negative and –positive controls. Of 102 patients with cancer, 36 were *H. pylori*–positive and 66 were *H. pylori*–negative. The incidence of gastric cancer was significantly high in elderly individuals (≥60 years, \(P < 0.0001\)), males (\(P < 0.01\)), *H. pylori*–negative (\(P = 0.011\)) and open-type gastric atrophy (\(P < 0.01\)) as compared with the noncancer controls. Of the gastric cancer cases, 65 (64%) cases were endoscopically categorized into early gastric cancer and 37 (36%) cases into advanced gastric cancer.

**Increased overmethylation in the body of patients with gastric cancer**

We evaluated the overmethylation frequencies of 19 genes examined in the background gastric mucosa of noncancer controls and patients with cancer (Fig. 1). Because patients with gastric cancer revealed few differences in the number of overmethylated genes between the *H. pylori*–positive and –negative cases (Supplementary Table S2), the overmethylated genes in the patients with cancer were counted irrespective of *H. pylori* infection. The mean number of overmethylated genes was calculated for comparison of the overall overmethylation degree between the antrum and body as well as between closed- and open-types of gastric atrophy (Fig. 1A). The number of overmethylated genes estimated in the body tended to be higher in the patients with cancer than in the *H. pylori*–positive controls. In the antrum, the overmethylated genes were similarly observed in the patients with cancer and the *H. pylori*–positive controls. When considering the extent of gastric atrophy, the number of overmethylated genes estimated in the body was higher in the patients with cancer with open-type atrophy than in the *H. pylori*–positive controls (8.4 vs. 6.0, \(P < 0.0001\)), but not in the patients with cancer with close-type atrophy (6.5 vs. 6.0, \(P = 0.313\)). The antrum made no significant difference in the mean number of overmethylated genes between the *H. pylori*–positive controls and the patients with cancer irrespective of the gastric atrophy.

The overmethylation frequencies of individual genes were shown in Fig. 1B. In comparison of *H. pylori*–positive controls and patients with cancer, 4 LTR-adjacent genes containing CpG islands (MMP2, CDKN2A, RUNX2, and RUNX3) were more frequently overmethylated in the patients with cancer when analyzing the antrum (MMP2, \(P < 0.0001\)) and the body (MMP2, \(P < 0.0001\); CDKN2A, \(P = 0.001\); RUNX2, \(P = 0.041\); RUNX3, \(P = 0.027\)). In contrast, 4 Alu-adjacent genes containing CpG islands (CDH1, ARRDC4, PPARG, and TRAPPCL2) were more frequently overmethylated in the *H. pylori*–positive controls when analyzing the antrum (CDH1, \(P < 0.0001\); ARRDC4, \(P = 0.04\); PPARG, \(P = 0.019\)) and the body (TRAPPCL2, \(P = 0.023\)).

Overall, the stomach-specific genes lacking CpG islands tended to be frequently overmethylated in the body of patients with cancer compared with the body of *H. pylori*–positive controls, even though only 2 genes showed statistical significances (TFF2, \(P = 0.029\); GHRL, \(P = 0.002\); Fig. 1B). The GHRL gene and the inactive APC gene were frequently overmethylated in both the antrum and body of patients with cancer (\(P < 0.01\)). The ATP4A gene

### Table 1. Descriptive characteristics of study participants

<table>
<thead>
<tr>
<th>Variables</th>
<th>Controls (n = 226)</th>
<th>Patients with gastric cancer (n = 102)</th>
<th>P value</th>
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<tbody>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
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<tr>
<td>≤49</td>
<td>56 (25)</td>
<td>14 (14)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>50–59</td>
<td>95 (42)</td>
<td>19 (18)</td>
<td></td>
</tr>
<tr>
<td>≥60</td>
<td>75 (33)</td>
<td>69 (68)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>120 (53)</td>
<td>79 (78)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Female</td>
<td>106 (47)</td>
<td>23 (22)</td>
<td></td>
</tr>
<tr>
<td>Helicobacter pylori</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>112 (49)</td>
<td>66 (65)</td>
<td>0.011</td>
</tr>
<tr>
<td>Positive</td>
<td>114 (51)</td>
<td>36 (35)</td>
<td></td>
</tr>
<tr>
<td>Grade of gastric atrophy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No atrophic change</td>
<td>19 (8)</td>
<td>0 (0)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>C1</td>
<td>24 (10)</td>
<td>5 (5)</td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>88 (36)</td>
<td>24 (23)</td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>70 (31)</td>
<td>28 (28)</td>
<td></td>
</tr>
<tr>
<td>O1</td>
<td>23 (10)</td>
<td>30 (29)</td>
<td></td>
</tr>
<tr>
<td>O2</td>
<td>7 (3)</td>
<td>12 (12)</td>
<td></td>
</tr>
<tr>
<td>O3</td>
<td>1 (1)</td>
<td>3 (3)</td>
<td></td>
</tr>
</tbody>
</table>

Note: Percentages are indicated in parenthesis.
producing gastric juice was frequently overmethylated in the antrum of patients with cancer \((P < 0.0001)\). Of the 6 \(Alu\)-adjacent genes, the \(MLH1\) and \(SHH\) genes, both containing a long transitional-CpG segment, were found to be similarly methylated among \(H. pylori\)-negative and -positive controls and patients with cancer.

The influence of gastric atrophy on transitional-CpG methylation distinct between the \(Alu\) and \(LTR\)-adjacent genes

The concurrent methylation of the 19 genes was evaluated by analyzing \(R_5\) values between each gene (Supplementary Fig. S4). Four \(Alu\)-adjacent genes with a short

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**Figure 1.** Comparison of genes overmethylated in the antrum and body among \(H. pylori\)-negative and -positive controls and patients with gastric cancer. A, the mean number of overmethylated genes was calculated for all cases and for closed-type and open-type atrophic cases. Error bars indicate the SEM. Statistical analysis was performed by Student t test. B, the overmethylation frequencies of individual genes were statistically analyzed by \(\chi^2\) test. \(*\), \(P < 0.05\); \(**\), \(P < 0.01\).
transitional-CpG segment (CDH1, ARRDC4, PPARG, and TRAPPC2L) and 4 LTR-adjacent genes (MMP2, CDKN2A, RUNX2, and RUNX3) tended to be concurrently methylated in the H. pylori-positive controls and the patients with cancer. The mean number of overmethylated genes of the 2 gene groups was analyzed according to the grade of gastric atrophy (Fig. 2). The body with open-1 atrophy showed significant increases in the number of overmethylated Alu- and LTR-adjacent genes in the patients with cancer compared with the H. pylori-positive controls \((P = 0.001\) and \(P < 0.001\)). Meanwhile, the 4 Alu-adjacent genes tended to be less methylated in the patients with cancer than in the H. pylori-positive controls \((1.5 \text{ vs. 2.5, } P = 0.006)\) when analyzing the antrum with closed-3 atrophy.

**Analysis of age-related diverse methylation patterns**

The methylation of inactive APC gene was positively correlated with age in both the H. pylori-positive controls \((R_S = 0.464 \text{ in the antrum, } P < 0.001; R_S = 0.461 \text{ in the body, } P < 0.001)\) and the H. pylori-negative controls \((R_S = 0.398 \text{ in the antrum, } P < 0.001; R_S = 0.373 \text{ in the body, } P < 0.001; \text{Supplementary Fig. S5})\). The methylation level of the APC gene was used as a standard age-related methylation for the delineation of heterogeneously age-related methylation changes observed in other active genes (Fig. 3). The H. pylori-positive controls with closed-type atrophy revealed that the peak overmethylation of Alu-adjacent genes was rapid in the antrum (level-4 APC-methylation; mean age, 50 years) compared with the body (level-5 APC-methylation; mean age, 55 years). In contrast, the 4 LTR-adjacent genes slowly increased to peak overmethylation in level-5 APC-methylation controls in the antrum (mean age, 54 years) and the body (mean age, 55 years). The H. pylori-positive controls showed peak methylation values of the Alu-adjacent genes higher than those of the LTR-adjacent genes.

The methylation of the APC gene was greater than level 3 (mean age, \(\geq 51\) years) in the open-type-atrophy controls and the patients with cancer, and most of them showed post-peak methylation curves. The methylation peaks of both the Alu- and LTR-adjacent genes were high in the body of the patients with cancer with open-type atrophy compared with that of H. pylori-positive controls. In the antrum with closed-type atrophy, the peak methylation value of the Alu-adjacent genes was lower than that of the LTR-adjacent genes in the patients with cancer.

**High-frequency overmethylation defined on the basis of slowly overmethylated genes**

The housekeeping genes adjacent to the Alu retroelements (CDH1, ARRDC4, PPARG, and TRAPPC2L) and the LTR retroelements (MMP2, CDKN2A, RUNX2, and RUNX3) as well as the stomach-specific genes tended to be frequently overmethylated in the body of patients with cancer (Figs. 1–3). Given that low-speed methylation changes could remain in the body, we evaluated whether the slowly changing overmethylation was associated with an increased risk of gastric cancer (Table 2). High-frequency overmethylation was scored if the overmethylation of transitional-CpG sites involved 2 or more Alu- and LTR-adjacent genes higher than those of the

\[\text{Mean number of overmethylated genes compared among } H.\text{ pylori-positive and -negative controls and gastric cancer. The housekeeping genes were subgrouped into Alu-adjacent genes (CDH1, ARRDC4, PPARG, and TRAPPC2L) and LTR-adjacent genes (MMP2, CDKN2A, RUNX2, and RUNX3). Error bars indicate the SEM. Statistical analysis was performed by Student t test. ‘*’, } P < 0.05; ‘**, P < 0.01.\]

Figure 2. The mean number of overmethylated genes compared among H. pylori-negative and –positive controls and gastric cancer.

The housekeeping genes were subgrouped into Alu-adjacent genes (CDH1, ARRDC4, PPARG, and TRAPPC2L) and LTR-adjacent genes (MMP2, CDKN2A, RUNX2, and RUNX3) was subdivided into 2 or more LTR-adjacent genes showing slow methylation changes and at least 1 gene in each of the Alu-adjacent gene group and the stomach-specific gene group. When analyzing the body, high-frequency overmethylation defined based on the slowly overmethylated genes was significantly associated with the risk of gastric cancer with open-type atrophy \([OR, 12.7; 95\% \text{ confidence interval (CI), 3.2–49.8}]\), but not with closed-type atrophy \([OR, 1.7; 95\% \text{ CI, 0.8–3.5}]\) as compared with H. pylori-positive controls. The OR value was 2 times higher in early gastric cancer \([OR, 17.7; 95\% \text{ CI, 3.1–101.7}]\) compared with advanced gastric cancer \([OR, 7.9; 95\% \text{ CI, 1.6–37.9}]\) when analyzing the body of open-type atrophic cases. However, in analysis of the antrum, there was no significant difference in high-frequency overmethylation...
between the patients with gastric cancer (62%) and the *H. pylori*-positive controls (54%, OR, 1.8; 95% CI, 0.9–3.2).

Overmethylation of non–*Alu*-adjacent genes frequent in the antrum

In the closed-type atrophic cases, the number of overmethylated genes was similar between *H. pylori*-positive controls and patients with cancer (Fig. 1A). When subgrouped into the *Alu* and LTR-adjacent housekeeping genes, the overmethylation of *Alu*-adjacent genes was more common in the *H. pylori*-positive controls than in the patients with cancer (Figs. 2 and 3). Both the LTR-adjacent genes and the stomach-specific genes tended to be similarly overmethylated in the antrum of patients with cancer and *H. pylori*-positive controls (Figs. 1B and 2). This indicated that genes other than the *Alu*-adjacent genes were frequently overmethylated in the antrum of patients with gastric cancer. Non-*Alu*-gene overmethylation was therefore defined when (i) 1 or more overmethylated genes were found in each of the LTR-adjacent gene group and the stomach-specific gene group; and (ii) the number of overmethylated genes was lower in the *Alu*-adjacent gene group than in the LTR-adjacent gene group (Table 3). In comparison of the patients with cancer and the *H. pylori*-positive controls, non-*Alu*-gene overmethylation was significantly associated with the risk of gastric cancer when analyzing the antrum (OR, 4.7; 95% CI, 2.5–8.8). The closed-type atrophic cases with non-*Alu*-gene overmethylation showed an elevated risk of gastric cancer (81%) as compared with the *H. pylori*-positive controls (34%; OR, 7.2; 95% CI, 3.1–16.8). The OR value was 5.7 (95% CI, 0.3–109.3) for the cases with closed-1 atrophy, 9.7 (95% CI, 2.4–39.7) for the cases with closed-2 atrophy, and 6.3 (95% CI, 1.9–21.2) for the cases with closed-3 atrophy. There was no significant difference in non-*Alu*-gene overmethylation between the patients with cancer with open-type atrophy (76%) and the *H. pylori*-
Discussion

A long time lag between H. pylori infection and cancer detection makes it difficult to identify H. pylori-associated overmethylation marks that predict the risk of gastric cancer (33). In fact, H. pylori--positive cases were less common in the patients with gastric cancer than in the noncancer controls (Table 1). This study analyzed age-related diverse overmethylation changes using a subset of transitional-CpG sites. The overmethylated genes were most frequent in the antrum of H. pylori--positive controls and were reduced to low levels in the antrum with open-type atrophy (Figs. 1 and 3; ref. 13). This reflected dynamic methylation changes corresponding to the natural history of H. pylori infection, because the infection disappeared in the antrum when gastric atrophy extended to the body in the older population (Fig. 4A; ref. 8 and 34). In the patients with gastric cancer, high-frequency overmethylation was commonly found in the body with open-type atrophy, but not in the antrum (Table 2). It is likely that the overmethylated genes associated with the risk of gastric cancer are preserved for a long time in the body background mucosa.

Diverse dynamic methylation changes of the transitional-CpG sites could be categorized into rapidly and slowly changing patterns based on a standard age-dependant pattern of an inactive gene (Supplementary Fig. S5; ref. 13). The age-related overmethylation of transitional-CpG sites rapidly increased to a peak value in the antrum, whereas the body showed slowly peaking overmethylation curves (Fig. 3). In addition, the peak of the overmethylation curve was found to be rapid for the Adjacent-LTR-genes with a short transitional-CpG segment but slow for the LTR-adjacent genes. The open-type-atrophy controls showed post-peak methylation curves and a decrease in methylation of rapidly changing Adjacent-LTR genes (Fig. 3).

The frequency of overmethylated LTR- and Adjacent-LTR genes tended to be high in the body of patients with cancer compared with the control body when analyzing the open-type atrophic cases (Figs. 2 and 3). This suggested that both the slowly and rapidly changing overmethylation increased in an extensive gastric mucosa of patients with cancer, which remained in the post-peak stage. We used high-frequency overmethylation, involving Adjacent-LTR-adjacent genes as well as stomach-specific genes, as a criterion for accessing cancer-associated methylation. The criterion of Adjacent-LTR overmethylation was defined with a relatively high number of overmethylated genes in order to reflect the slow overmethylation of LTR-adjacent genes. The high-frequency overmethylation cases were found to be significantly frequent in the body of patients with cancer compared with the body of both H. pylori--positive and --negative controls when analyzing the open-type atrophic cases (Table 2).

### Table 2. Association of high-frequency overmethylation with the risk for gastric cancer stratified by the extent of gastric atrophy

<table>
<thead>
<tr>
<th></th>
<th>Gastric cancer</th>
<th>All noncancer controls</th>
<th>OR (95% CI)</th>
<th>H. pylori-positive controls</th>
<th>OR (95% CI)</th>
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<td><strong>Antrum</strong></td>
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<tr>
<td>Total cases</td>
<td>63/102 (62)</td>
<td>95/226 (42)</td>
<td>2.5 (1.5–4.2)</td>
<td>62/114 (54)</td>
<td>1.8 (0.9–3.2)</td>
</tr>
<tr>
<td>No atrophic change</td>
<td>0/0</td>
<td>3/19 (16)</td>
<td>–</td>
<td>1/4 (25)</td>
<td>–</td>
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<tr>
<td>Closed type</td>
<td>33/57 (58)</td>
<td>77/176 (44)</td>
<td>2.0 (1.0–3.7)</td>
<td>51/91 (57)</td>
<td>1.5 (0.7–3.2)</td>
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<tr>
<td>Open type</td>
<td>30/45 (67)</td>
<td>15/31 (48)</td>
<td>2.3 (0.9–6.1)</td>
<td>10/19 (53)</td>
<td>2.0 (0.6–6.0)</td>
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<tr>
<td>EGC</td>
<td>17/26 (65)</td>
<td>15/31 (48)</td>
<td>2.2 (0.7–6.7)</td>
<td>10/19 (53)</td>
<td>0.5 (0.2–1.8)</td>
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<tr>
<td>AGC</td>
<td>13/19 (68)</td>
<td>15/31 (48)</td>
<td>2.3 (0.7–7.9)</td>
<td>10/19 (53)</td>
<td>0.5 (0.1–1.9)</td>
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</tr>
<tr>
<td>Total cases</td>
<td>66/102 (65)</td>
<td>62/226 (27)</td>
<td>4.5 (2.7–7.7)</td>
<td>40/114 (35)</td>
<td>3.4 (1.8–6.1)</td>
</tr>
<tr>
<td>No atrophic change</td>
<td>0/0</td>
<td>0/19 (0)</td>
<td>–</td>
<td>0/4 (0)</td>
<td>–</td>
</tr>
<tr>
<td>Closed type</td>
<td>26/57 (46)</td>
<td>49/176 (28)</td>
<td>2.2 (1.2–4.2)</td>
<td>32/91 (35)</td>
<td>1.7 (0.8–3.5)</td>
</tr>
<tr>
<td>Open type</td>
<td>40/45 (89)</td>
<td>13/31 (42)</td>
<td>13.4 (3.8–46.8)</td>
<td>8/19 (42)</td>
<td>12.7 (3.2–49.8)</td>
</tr>
<tr>
<td>EGC</td>
<td>24/26 (92)</td>
<td>13/31 (42)</td>
<td>18.8 (3.5–99.6)</td>
<td>8/19 (42)</td>
<td>17.7 (3.1–101.7)</td>
</tr>
<tr>
<td>AGC</td>
<td>16/19 (84)</td>
<td>13/31 (42)</td>
<td>8.3 (1.9–36.5)</td>
<td>8/19 (42)</td>
<td>7.9 (1.6–37.9)</td>
</tr>
</tbody>
</table>

**NOTE:** Data are n/N (%) unless otherwise stated.

**Abbreviations:** AGC, advanced gastric cancer; EGC, early gastric cancer.

*Two or more overmethylated genes among the LTR-adjacent genes, and one or more overmethylated genes in both the Adjacent-LTR-adjacent gene group and the stomach-specific gene group.

*Adjusted for age and sex.
Therefore, a high level of age-related concurrent overmethylation seemed to represent cancer-associated methylation patterns in the body mucosa of the stomach.

Methylation studies on the *H. pylori*-infected stomach have targeted 2 distinct CpG sites. The CpG-island centers are methylated within a narrow range of variation (11, 25). Meanwhile, the CpG-island margins, termed transitional-CpG sites and CpG-island shores, are methylated to various degrees in a tissue-type-dependent manner (13, 35). Most previous studies concentrated on the overmethylation of CpG-island centers, which could lead to complete gene inactivation. However, it is unclear whether the overmethylated genes are suppressed during the aging process, old stem cells seem to be replaced with new stem cells when losing the self-renewal abilities of native stem cells (20, 39). The stabilization of newly expanding stem cells in the gastric mucosa can be inferred by analyzing the concurrent methylation changes in the CpG-island margins (21). Inflammatory condition such as *H. pylori* infection may facilitate the recruitment of new stem cells and the overmethylation changes for the replacement of old stem cells (40, 41). High-frequency overmethylation in the body is likely to be associated with an extensive field of enrichment of new stem cells.

This study showed no difference in the mean number of overmethylated genes between the *H. pylori*-positive controls and the patients with gastric cancer when analyzing the closed-type atrophic cases (Fig. 1A). When categorizing the *Alu*- and *LTR*-adjacent genes, the frequencies of overmethylated *Alu*-adjacent genes were low in the antrum of patients with gastric cancer with closed-type atrophy (Figs. 2 and 3). In previous CpG-island center studies, the level of methylation in the antrum tended to be lower in patients with gastric cancer than in *H. pylori*-positive noncancer controls (11, 19). We analyzed the type of retroelements adjacent to the previously examined genes (11, 19), and found that most CpG-island centers capable of predicting cancer-risk individuals in a population with atrophic lesions and *H. pylori* infection.

Here, we used a transitional-CpG marker set to identify cancer-risk methylation patterns. A series of studies on the methylation-variable sites at the CpG-island margins have found concurrent methylation changes that have a stabilizing effect on stem cell phenotypes (21, 24, 32, 37, 38). During the aging process, old stem cells seem to be replaced with new stem cells when losing the self-renewal abilities of native stem cells (20, 39). The stabilization of newly expanding stem cells in the gastric mucosa can be inferred by analyzing the concurrent methylation changes in the CpG-island margins (21). Inflammatory condition such as *H. pylori* infection may facilitate the recruitment of new stem cells and the overmethylation changes for the replacement of old stem cells (40, 41). High-frequency overmethylation in the body is likely to be associated with an extensive field of enrichment of new stem cells.

The limited dose of transcription components in a nuclear space can lead to a transcription-dose counterbalance between the stomach-specific genes lacking CpG islands and the housekeeping genes containing CpG islands (21, 43). Thus, housekeeping genes are likely to be down-regulated when new stem cells induce extremely high

### Table 3. Association of non-*Alu*-gene overmethylation with the risk of gastric cancer stratified by the extent of gastric atrophy

<table>
<thead>
<tr>
<th></th>
<th>Gastric cancer</th>
<th>All noncancer controls</th>
<th>OR (95% CI)b</th>
<th>H. pylori-positive controls</th>
<th>OR (95% CI)b</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antrum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cases</td>
<td>80/102 (78)</td>
<td>78/226 (35)</td>
<td>5.5 (3.1–9.6)</td>
<td>43/114 (38)</td>
<td>4.7 (2.5–8.8)</td>
</tr>
<tr>
<td>No atrophic change</td>
<td>0/0</td>
<td>0/4 (0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Closed type</td>
<td>46/57 (81)</td>
<td>60/176 (34)</td>
<td>7.3 (3.4–15.5)</td>
<td>31/91 (34)</td>
<td>7.2 (3.1–16.8)</td>
</tr>
<tr>
<td>C1</td>
<td>4/5 (80)</td>
<td>7/24 (29)</td>
<td>5.7 (0.3–94.0)</td>
<td>2/10 (20)</td>
<td>5.7 (0.3–109.3)</td>
</tr>
<tr>
<td>C2</td>
<td>19/24 (79)</td>
<td>24/82 (29)</td>
<td>10.2 (3.1–33.7)</td>
<td>13/44 (30)</td>
<td>9.7 (2.4–39.7)</td>
</tr>
<tr>
<td>C3</td>
<td>23/28 (82)</td>
<td>29/70 (41)</td>
<td>6.2 (2.1–18.5)</td>
<td>16/37 (43)</td>
<td>6.3 (1.9–21.2)</td>
</tr>
<tr>
<td>Open type</td>
<td>34/45 (76)</td>
<td>15/31 (48)</td>
<td>3.4 (1.2–9.3)</td>
<td>12/19 (63)</td>
<td>1.8 (0.5–5.9)</td>
</tr>
<tr>
<td><strong>Body</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cases</td>
<td>61/102 (50)</td>
<td>89/226 (39)</td>
<td>1.9 (1.1–3.1)</td>
<td>38/114 (33)</td>
<td>2.5 (1.4–4.5)</td>
</tr>
<tr>
<td>No atrophic change</td>
<td>0/0</td>
<td>0/4 (0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Closed type</td>
<td>32/57 (56)</td>
<td>64/176 (36)</td>
<td>2.0 (1.3–3.8)</td>
<td>29/91 (32)</td>
<td>2.4 (1.2–5.1)</td>
</tr>
<tr>
<td>Open type</td>
<td>29/45 (64)</td>
<td>19/31 (61)</td>
<td>1.1 (0.4–2.9)</td>
<td>9/19 (47)</td>
<td>2.0 (0.6–5.9)</td>
</tr>
</tbody>
</table>

**NOTE:** Data are n/N (%) unless otherwise stated.

*a*Non-*Alu*-gene overmethylation was scored (i) if at least 1 *LTR*-adjacent gene and at least 1 stomach-specific gene were overmethylated and (ii) if the number of overmethylated *Alu*-adjacent genes was fewer than that of overmethylated *LTR*-adjacent genes.

*b*Adjusted for age and sex.
expression of stomach-specific genes in the gastric mucosa (13). The methylation spreading of gene-adjacent retroelement was reported to be enhanced by transcriptional repression (44). The transitional-CpG sites of housekeeping genes were concurrently overmethylated under the influence of the type and proximity of adjacent retroelements. CpG-island margins are enriched with the Alu retroelements and their methylation changes are rapid. The overmethylated genes are subsequently demethylated in the stabilized cells. B, the risk for gastric cancer is increased with early severe *H. pylori* infection and extensive atrophic changes, which increase the overmethylation of Alu- and LTR-adjacent genes in the body. The antrum with gastric atrophy induces non-Alu-gene overmethylation that is not sufficient to stabilize cell phenotypes.

As the antral biopsy specimens were taken inside the atrophic lesion, in contrast to the body biopsy specimens, the Alu-adjacent genes might not reach a high level of methylation under the influence of atrophic changes. It has been suggested that *H. pylori* infection and atrophic change early in life are closely associated with the development of gastric cancer (Fig. 4B; ref. 34). Even though the atrophic changes were similarly observed in the antrum of noncancer controls and patients with cancer, the early atrophic change seemed to be associated with the non–Alu-gene overmethylation in patients with gastric cancer. Once the housekeeping genes are sufficiently overmethylated to stabilize stem cells, stabilized cell phenotypes are likely to be resistant to the effect of the subsequent atrophic changes. The timing of the stem cell replacement following atrophic change may be crucial for increasing the unstable methylation pattern. There were few differences in the overmethylation frequencies of transitional-CpG sites between the *H. pylori*-positive and –negative cases of gastric cancers. This may be because (i) the overmethylation of transitional-CpG sites starting...
after the atrophic changes can remain for a long time after an external infectious origin has disappeared, and (ii) a variety of inflammatory gastric lesions other than H. pylori infection also can promote the overmethylation changes.

Previous in vitro experiments showed effective induction of gastric epithelial differentiation of stem cells by stomach tissue extracts (40, 45). This suggests that the intact glandular structures of the stomach with no atrophy are important for the induction of stem cell phenotypes as well as the downregulation of housekeeping genes. The antrum with gastric atrophy can give rise to aberrant changes in glandular structures. If the replacement of stem cells initiates late in the poor tissue environment, the new stem cells are likely to fail to increase the overmethylated Alu-adjacent genes (Fig. 4B). Alternatively, non–Alu-gene overmethylation may be associated with noncancer controls in the poststabilization stage, because the overmethylated Alu-adjacent genes are rapidly demethylated after the stabilization of cell phenotype. Therefore, the detailed criteria for the cancer-risk pattern of non–Alu-gene overmethylation need to be established with longitudinal study on high-risk individuals.

In summary, the concurrent methylation of CpG-island margins has a stabilizing effect on the downregulated housekeeping genes and new stem cell phenotypes (21). This provides important information about the repopulation of invisible stem cells in the gastric mucosa. The methylation of CpG-island margins slowly changed in the gastric body, which was sustained for a long time. The slowly overmethylated genes were frequent in the body of patients with gastric cancer with open-type atrophy. The methylation of Alu-adjacent genes rapidly changed in the antrum, which was barely increased in the antrum of patients with gastric cancer. Therefore, the age-related methylation patterns of the transitional-CpG sites are expected to serve as useful surrogate markers for evaluating an extent of field carcinization in the stomach.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J.-H. Oh, M.-G. Rhyu, S.-J. Hong
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.-H. Oh, S.-H. Jung, S.-W. Choi
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.-G. Rhyu, S.-I. Kim, S.-J. Hong
Writing, review, and/or revision of the manuscript: J.-H. Oh, M.-G. Rhyu, S.-H. Jung, S.-J. Hong
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.-H. Oh, M.-G. Rhyu, S.-I. Kim, S.-J. Hong
Study supervision: M.-G. Rhyu, S.-J. Hong

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References
Frequent Methylation in the Body Mucosa of Gastric Cancer


Slow Overmethylation of Housekeeping Genes in the Body Mucosa Is Associated with the Risk for Gastric Cancer

Jung-Hwan Oh, Mun-Gan Rhyu, Sung-Hoon Jung, et al.

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