Slow overmethylation of housekeeping genes in the body mucosa is associated with the risk for gastric cancer

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Abstract

*Helicobacter pylori* (*H. 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 non-cancerous antrum and body mucosa of 102 cancer patients 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 gastric cancer patients with open-type atrophy (odds ratio, 12.7; 95% confidence interval, 3.2-49.8). The rapidly changing methylation of Alu-adjacent genes was barely increased in the antrum of gastric cancer patients. Among diverse methylation changes associated with *H. pylori* infection, an increase in slowly changing methylation could serve as a cancer-risk marker.
**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, inter-observer 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* (*H. 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 non-cancerous 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 changes in housekeeping genes (16, 17). Post-infection 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 H. pylori-associated dynamic methylation changes need to be elucidated in order to understand how they appear and subsequently disappear in the background mucosa of gastric cancer patients.

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 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.

Materials and Methods

Collection of stomach biopsy specimens

Non-cancerous mucosal tissues were collected from healthy subjects and gastric cancer patients 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., Tokyo, Japan) (Supplementary Fig. S1A). Normal-appearing area adjacent to cancerous lesion might be composed of epigenetically heterogeneous cells when analyzing methylation patterns (26). In order 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 histological examination were collected adjacent to the first site. A pathologist confirmed the presence of more than 80 percent normal epithelial cells and no cancer cells in the biopsy specimens (Supplementary Fig. S1B). 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 two 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 histological
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 prior to the study.

**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) (12, 31, 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) (13). MSP primer sets for the 19 transitional-CpG sites were designed to amplify six Alu-adjacent housekeeping genes (*CDH1, ARRDC4, PPARG TRAPPC2L, MLH1*, and *SHH*), four LTR-adjacent housekeeping genes (*MMP2, CDKN2A, RUNX2*, and *RUNX3*), eight stomach-specific genes (*TFF2, PGC, ATP4B, TFF1, TFF3, GHRL, PGA*, and *ATP4A*), and one inactive gene (*APC*). The transitional-CpG sites of housekeeping genes were chosen from the CpG-island margins. While, 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 dTTP isotope was necessary for specific amplification of the transitional-CpG sites (12, 22, 31, 32). To ensure similar PCR efficiency for unmethylated vs. methylated amplicons, PCR amplification of each primer set was adjusted to reach a sub-plateau 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 non-radioisotope methods (12, 22, 26, 31, 32). The PCR specificity of the transitional-CpG sites of four 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). While, the \textit{PPARG} and \textit{CDKN2A} genes could not be analyzed by pyrosequencing due to non-specific PCR bands (Supplementary Fig. S2C). When common PCR for pyrosequencing generated a specific band, such as \textit{CDH1} and \textit{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 = \frac{1}{6} \pi \times \text{length} \times \text{width} \times \text{height}. The size of biopsy tissues was categorized into three 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 two 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.

\textbf{Statistical analysis}

See Supplementary Methods.
Results

Baseline characteristics of gastric cancer patients and non-cancer 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 cancer patients, 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 to the non-cancer 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 gastric cancer patients

We evaluated the overmethylation frequencies of 19 genes examined in the background gastric mucosa of non-cancer controls and cancer patients (Fig. 1). Because gastric cancer patients 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 cancer patients 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 cancer patients than in the *H. pylori*-positive controls. In the antrum, the overmethylated genes were similarly observed in the cancer patients 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 cancer patients with open-type atrophy than in the H. pylori-positive controls (8.4 vs. 6.0, \( P < 0.0001 \)), but not in the cancer patients 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 cancer patients irrespective of the gastric atrophy.

The overmethylation frequencies of individual genes were shown in Fig. 1B. In comparison of H. pylori-positive controls and cancer patients, four LTR-adjacent genes containing CpG islands (\( \text{MMP2} \), \( \text{CDKN2A} \), \( \text{RUNX2} \) and \( \text{RUNX3} \)) were more frequently overmethylated in the cancer patients when analyzing the antrum (\( \text{MMP2}, P < 0.0001 \)) and the body (\( \text{MMP2}, P < 0.0001; \text{CDKN2A}, P = 0.001; \text{RUNX2}, P = 0.041; \text{RUNX3}, P = 0.027 \)). In contrast, four Alu-adjacent genes containing CpG islands (\( \text{CDH1} \), \( \text{ARRDC4} \), \( \text{PPARG} \), and \( \text{TRAPPC2L} \)) were more frequently overmethylated in the H. pylori-positive controls when analyzing the antrum (\( \text{CDH1}, P < 0.0001; \text{ARRDC4}, P = 0.04; \text{PPARG}, P = 0.019 \)) and the body (\( \text{TRAPPC2L}, P = 0.023 \)).

Overall, the stomach-specific genes lacking CpG islands tended to be frequently overmethylated in the body of cancer patients compared to the body of H. pylori-positive controls, even though only two genes showed statistical significances (\( \text{TFF2}, P = 0.029; \text{GHRL}, P = 0.002 \)) (Fig. 1B). The \( \text{GHRL} \) gene and the inactive \( \text{APC} \) gene were frequently overmethylated in both the antrum and body of cancer patients (\( P < 0.01 \)). The \( \text{ATP4A} \) gene producing gastric juice was frequently overmethylated in the antrum of cancer patients (\( P < 0.0001 \)). Of the six Alu-adjacent genes, the \( \text{MLH1} \) and \( \text{SHH} \) genes both containing a long transitional-CpG segment were found to be similarly methylated among H. pylori-negative and -positive controls and cancer patients.
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 Rs values between each gene (Supplementary Fig. S4). Four Alu-adjacent genes with a short transitional-CpG segment (CDH1, ARRDC4, PPARG, and TRAPPC2L) and four LTR-adjacent genes (MMP2, CDKN2A, RUNX2, and RUNX3) tended to be concurrently methylated in the H. pylori-positive controls and the cancer patients. The mean number of overmethylated genes of the two 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 cancer patients compared to the H. pylori-positive controls (P = 0.001 and P < 0.001). Meanwhile, the four Alu-adjacent genes tended to be less methylated in the cancer patients than in the H. pylori-positive controls (1.5 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 (Rs = 0.464 in the antrum, P < 0.001; Rs = 0.461 in the body, P < 0.001) and the H. pylori-negative controls (Rs = 0.398 in the antrum, P < 0.001; Rs = 0.373 in the body, P < 0.001) (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 to the body (level-5 APC-methylation; mean age, 55 years). In contrast, the four 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, ≥ 51 years) in the open-type-atrophy controls and the cancer patients, 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 cancer patients with open-type atrophy compared to 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 cancer patients.

**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 cancer patients (Figs. 1, 2 and 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 two or more LTR-adjacent genes showing slow methylation changes and at least one 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% CI, 3.2-49.8), but not with closed-type atrophy (OR, 1.7; 95% CI, 0.8-3.5) as compared to *H. pylori*-positive controls. The OR value was two times higher in early gastric cancer (OR, 17.7; 95% CI, 3.1-101.7) compared to advanced gastric cancer (OR, 7.9; 95% 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 gastric cancer patients (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 cancer patients (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 cancer patients (Figs. 2 and 3). Both the LTR-adjacent genes and the stomach-specific genes tended to be similarly overmethylated in the antrum of cancer patients 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 gastric cancer patients. Non-Alu-gene overmethylation was therefore defined when 1) one or more overmethylated genes were found in each of the LTR-adjacent gene group and the stomach-specific gene group and 2) 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 cancer patients 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 \textit{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 cancer patients with open-type atrophy (76%) and the \textit{H. pylori}-positive controls (63%, OR, 1.8; 95% CI, 0.5-5.9). The OR value for non-Alu-gene overmethylation was decreased when analyzing the body (OR, 2.5; 95% CI, 1.4-4.5).

\textbf{Discussion}

A long time lag between \textit{H. pylori} infection and cancer detection makes it difficult to identify \textit{H. pylori}-associated overmethylation marks that predict the risk of gastric cancer (33). In fact, \textit{H. pylori}-positive cases were less common in the gastric cancer patients than in the non-cancer 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 \textit{H. pylori}-positive controls and were reduced to low levels in the antrum with open-type atrophy (Figs. 1 and 3) (13). This reflected dynamic methylation changes corresponding to the natural history of \textit{H. pylori} infection, because the infection disappeared in the antrum when gastric atrophy extended to the body in the older population (Fig. 4A) (8, 34). In the gastric cancer patients, 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-dependent pattern of an inactive gene (Supplementary Fig. S5) (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 Alu-adjacent 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 Alu-adjacent genes (Fig. 3).

The frequency of overmethylated LTR- and Alu-adjacent genes tended to be high in the body of cancer patients 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 cancer patients, which remained in the post-peak stage. We used high-frequency overmethylation, involving Alu- and LTR-adjacent genes as well as stomach-specific genes, as a criterion for accessing cancer-associated methylation. The criterion of LTR-adjacent 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 cancer patients compared with the body of both \textit{H. pylori}-positive and -negative controls when analyzing the open-type atrophic cases (Table 2). Therefore, a high level of age-related concurrent overmethylation appeared to represent cancer-associated methylation patterns in the body mucosa of the stomach.

Methylation studies on the \textit{H. pylori}-infected stomach have targeted two 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 *H. pylori* infection (36). To our knowledge, there are few recognized 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 appear 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 gastric cancer patients 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 gastric cancer patients 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 gastric cancer patients than in *H. pylori*-positive non-cancer controls (11, 19). We analyzed the type of retroelements
adjacent to the previously examined genes (11, 19), and found that most CpG-island studies were done toward describing the Alu-adjacent genes. Meanwhile, some CpG-island genes that contained few Alu retroelements in the upstream regions were reported to be frequently methylated in the antrum of cancer-risk individuals (42). Therefore, the Alu-adjacent genes appeared to be insufficiently overmethylated in the antrum of cancer patients compared to the *H. pylori*-positive non-cancer antrum.

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 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 (Fig. 1, Supplementary Fig. S4). The concurrent overmethylation of transitional-CpG sites is suitable for the stabilization of the down-regulated genes, which is necessary for prompt stabilization of new stem cell phenotypes (21, 35, 38). Because the Alu retroelements are enriched at the CpG-island margins (21), the methylation spreading of Alu retroelements can be increased more rapidly than that of LTR retroelements (Fig. 3) (44). This suggests that the overmethylation of genes other than the Alu-adjacent genes is not sufficient to stabilize cell phenotypes, which may represent an unstable epigenetic pattern (Fig. 4B). The unstable epigenotype of non-Alu-gene overmethylation was considered to allow the dedifferentiation of new stem cells and to predispose stem cells to gastric cancer.

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) (34). Even though the atrophic changes were similarly observed in
the antrum of non-cancer controls and cancer patients, the early atrophic change appeared to
be associated with the non-Alu-gene overmethylation in gastric cancer patients. 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 1) 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 2) 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 down-regulation 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 non-cancer controls in the post-stabilization 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 down-regulated 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 gastric cancer patients with open-type atrophy. The methylation of Alu-adjacent genes rapidly changed in the antrum, which was barely increased in the antrum of gastric cancer patients. 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 cancerization in the stomach.
Authors' Contributions

Conception and design: J.H. Oh, M.G. Rhyu, S.J. Hong

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Study supervision: M.G. Rhyu, S.J. Hong
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45. Okumura T, Wang SS, Takaishi S, Tu SP, Ng V, Ericksen RE et al. Identification of a bone marrow-derived mesenchymal progenitor cell subset that can contribute to the
Table 1. Descriptive characteristics of study participants

<table>
<thead>
<tr>
<th>Variables</th>
<th>Controls (n = 226)</th>
<th>Gastric cancer patients (n = 102)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 49</td>
<td>56 (25)</td>
<td>14 (14)</td>
<td>&lt; 0.001</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; 0.001</td>
</tr>
<tr>
<td>Female</td>
<td>106 (47)</td>
<td>23 (22)</td>
<td></td>
</tr>
<tr>
<td><em>Helicobacter pylori</em></td>
<td></td>
<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; 0.001</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.
Table 2. Association of high-frequency 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 non-cancer 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>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>
</tr>
<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>
</tr>
<tr>
<td>Open type</td>
<td>30/45 (67)</td>
<td>15/31 (48)</td>
<td>2.3(1.0-6.1)</td>
<td>10/19 (53)</td>
<td>2.0(0.6-6.0)</td>
</tr>
<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>
</tr>
<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>
</tr>
<tr>
<td><strong>Body</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</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>

Abbreviations: OR, odds ratio; CI, confidence interval; EGC, early gastric cancer; AGC, advanced gastric cancer.

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

aTwo or more overmethylated genes among the LTR-adjacent genes, and one or more overmethylated genes in each of the Alu-adjacent gene group and the stomach-specific gene group.

bAdjusted for age and sex.
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 non-cancer 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>3/19 (16)</td>
<td>-</td>
<td>0/4 (0)</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 (60)</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>6/19 (32)</td>
<td>-</td>
<td>0/4 (0)</td>
<td>-</td>
</tr>
<tr>
<td>Closed type</td>
<td>32/57 (56)</td>
<td>64/176 (36)</td>
<td>2.0 (1.1-3.8)</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>

Abbreviations: OR, odds ratio; CI, confidence interval.

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

^Non-Alu-gene overmethylation was scored 1) if at least one LTR-adjacent gene and at least one stomach-specific gene were overmethylated and 2) if the number of overmethylated Alu-adjacent genes was fewer than that of overmethylated LTR-adjacent genes.

^Adjusted for age and sex.
Figure legend

Figure 1. Comparison of genes overmethylated in the antrum and body among *H. pylori*-negative and -positive controls and gastric cancer patients. (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 standard error of the mean. Statistical analysis was performed by Student *t*-test. (B) The overmethylation frequencies of individual genes were statistically analyzed by \( \chi^2 \) test. Asterisk indicates *P* value < 0.05. Double asterisk indicates *P* value < 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*). Error bars indicate the standard error of the mean. Statistical analysis was performed by Student *t*-test. Asterisk indicates *P* value < 0.05. Double asterisk indicates *P* value < 0.01.

Figure 3. Age-related methylation patterns of Alu- and LTR-adjacent genes under the influence of atrophic changes. Curves of the mean number of overmethylated genes are plotted as a function of the *APC*-methylation level. The mean number of overmethylated genes was calculated for housekeeping genes adjacent to Alu retroelements (*CDH1*, *ARRDC4*, *PPARG*, and *TRAPPC2L*) or LTR retroelements (*MMP2*, *CDKN2A*, *RUNX2*, and *RUNX3*).
Figure 4. Schematics of methylation changes in the background gastric mucosa of the stomach. (A) *H. pylori* infection leads to gastric atrophy and disappears when atrophy creates an unfavorable environment for *H. pylori*. Cell phenotypes are stabilized by the concurrent overmethylation of housekeeping genes, which are influenced by the methylation 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 of 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.
Figure 1.

A

Mean No. of overmethylation

B

CDH1

ARRDC4

PPARG

TRAPPC2L

MMP2

CDKN2A

RUNX2

RUNX3

TFF2

PGC

ATP4B

TFF1

TFF3

GHRL

PGA

ATP4A

MLH1

SHH

APC

H. pylori-negative control
H. pylori-positive control
Gastric cancer patient
Figure 2.

Mean No. of overmethylation

** H. pylori-negative

** H. pylori-positive

control

Gastric cancer

patient

Downloaded from cancerpreventionresea...
Figure 3.

**H. pylori-negative control**

- Alu-adjacent genes
- LTR-adjacent genes
- No. of cases

**H. pylori-positive control**

- Alu-adjacent genes
- LTR-adjacent genes
- No. of cases

**Gastric cancer patient**

- Alu-adjacent genes
- LTR-adjacent genes
- No. of cases

Mean No. of overmethylation

Closed-type atrophy

Open-type atrophy

Antrum

Body

No. cases

APC methylation level (Mean age, years)
Figure 4.

A. *H. pylori* infection

- Stably adapted cell
- Housekeeping genes containing CpG-island
- Stomach-specific genes lacking CpG-island
- Overmethylation
- Insufficient overmethylation
- Methylated CpG
- Unmethylated CpG
- Transcription start site

B. Early *H. pylori* infection

- Unsatisfactory cancer-risk cell
- Housekeeping genes containing CpG-island
- Stomach-specific genes lacking CpG-island
- Overmethylation
- Insufficient overmethylation
- Methylated CpG
- Unmethylated CpG
- Transcription start site
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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