Helicobacter pylori Eradication Can Reverse the Methylation-Associated Regulation of miR-200a/b in Gastric Carcinogenesis

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See editorial on page 533.

Background/Aims: Epigenetic change is one of the mechanisms that regulates the expression of microRNAs (miRNAs) and is known to play a role in Helicobacter pylori-associated gastric carcinogenesis. We aimed to evaluate the epigenetic changes of miR-200a/b in H. pylori-associated gastric carcinogenesis and restoration after eradication. Methods: The expression and methylation levels of miR-200a/b were evaluated in gastric cancer (GC) cell lines, human gastric mucosa of H. pylori-negative and -positive controls, and H. pylori-positive GC patients. Next, the changes in the expression and methylation levels of miR-200a/b were compared between H. pylori-eradication and H. pylori-persistence groups at 6 months. Real-time reverse transcription-polymerase chain reaction was conducted to investigate the miRNA expression levels, and MethyLight was performed to assess the methylation levels. Results: In the GC cell lines, the level of miR-200a/b methylation decreased and the level of expression increased after demethylation. In the human gastric mucosa, the miR-200a/b methylation levels increased in the following group order: H. pylori-negative control group, H. pylori-positive control group, and H. pylori-positive GC group. Conversely, the miR-200a/b expression levels decreased in the same order. In the H. pylori-persistence group, no significant changes were observed in the methylation and expression levels of miR-200a/b after 6 months, whereas the level of methylation decreased and the level of expression of miR-200a/b increased significantly 6 months in the H. pylori-eradication group. Conclusions: Epigenetic alterations of miR-200a/b may be implicated in H. pylori-induced gastric carcinogenesis. This field defect for cancerization is suggested to be improved by H. pylori eradication. (Gut Liver 2020;14:571-580)

Key Words: Helicobacter pylori; MicroRNAs; Methylation; Epigenetic alteration; Stomach neoplasm

INTRODUCTION

Gastric cancer (GC) is the fifth most common malignancy and the third leading cause of global cancer mortality.1 Although the incidence is declining globally, East Asia, including Korea and Japan, remains a region of high incidence of GC.2 It has been widely accepted that GC, especially intestinal-type GC, develops through progressive changes from chronic gastritis to gastric atrophy, intestinal metaplasia, dysplasia, and invasive carcinoma. Helicobacter pylori is thought to be crucial in the initiation of this sequence of changes in the Correa pathway.3 Despite several studies on the mechanism by which H. pylori leads to GC, this is not yet fully explained.

Recently, epigenetic changes have been attracting attention as one of the mechanisms of gastric carcinogenesis. One of the most consistent epigenetic changes in human cancer is aberrant DNA methylation, which has also been linked to gastric carcinogenesis.4 Importantly, previous reports have shown that methylation alterations of multiple genes occurs in both H. pylori infection and H. pylori-related gastric carcinogenesis.5,6 The accumulation of aberrant methylation through long-
The miR-200 family is a group of miRNAs consisting of miR-200a, -200b, -200c, -141, and -429. Among them, miR-200b/a/429 are encoded by the gene on chromosome 1 and miR-200a, -200b, -200c, -141, and -429. This family is closely linked to the expression of ZEB1 and ZEB2, key regulators of epithelial–mesenchymal transition, and regulates crucial processes in carcinogenesis, such as tumor initiation, progression, invasion, and metastasis of various types of cancer. The expression profiling of miR-200 family members is cancer-specific; downregulated in invasive bladder cancer and renal cell carcinoma, but overexpressed in lung, colorectal, and ovarian cancer. Several studies have demonstrated that miR-200 family was downregulated in GC, suggesting its role as a tumor suppressor in GC. Lower expression of miR-200 were known to be related to poor prognosis of GC: histological grade, size of tumor, invasion depth, lymphatic invasion, lymph node metastasis, intravascular cancer embolus, and disease-free survival. Although previous studies have shown that hypermethylation of the promoter CpG island was one of the mechanisms of miR-200c/141 downregulation, the exact mechanism of dysregulation of the remaining miR-200 members in GC has not been fully elucidated. To our knowledge, the methylation status and subsequent dysregulation of miR-200 family in the non-cancerous gastric mucosa of GC patients have not been studied. Furthermore, it has not yet been fully elucidated whether epigenetic alterations in the miR-200 family are affected by H. pylori infection and eradication.

Here, we examined whether epigenetic fields related to miR-200a/b were formed during H. pylori infection and gastric carcinogenesis, and recovered after the eradication of H. pylori.

MATERIALS AND METHODS

1. GC cell lines

Three GC cell lines, AGS, MKN-1, and MKN-45 were obtained from the Korean Cell Line Bank (Seoul, Korea) (Supplementary Table 1). Cells were incubated in RPMI-1640 medium containing 10% fetal bovine serum, L-glutamine (300 mg/L), 25 mM HEPES, and 25 mM NaHCO₃, and plated on day 0. On day 1, cells were treated with 2 μM 5-Aza-2’-deoxycytidine (Sigma-Aldrich and Merck KGaA, Darmstadt, Germany), demethylating agent, and replenished daily with the demethylating agent and medium. Cells were harvested on day 4.

2. Gastric mucosa specimens

We included 40 patients with H. pylori-positive GC, 20 H. pylori-positive and 20 H. pylori-negative controls. The H. pylori-positive GC group consisted of patients who underwent endoscopic submucosal dissection for GC, and the control group consisted of those diagnosed as normal or gastritis by upper gastrointestinal endoscopy. All subjects included in the present study were older than 18 years of age, had no other malignancy, were not taking antibiotics nor proton pump inhibitors within the last 4 weeks, and did not report history of H. pylori eradication during the last 4 weeks. The patients with H. pylori-positive GC were randomly allocated to the eradication or persistence group. The patients in the eradication group underwent 1-week of therapy (omeprazole 20 mg, amoxicillin 1 g, and clarithromycin 500 mg twice daily) 2 weeks after endoscopic resection. To determine the effect of H. pylori eradication on the epigenetic regulation of miRNA expression, we also collected gastric mucosal tissues at 6 months after endoscopic submucosal dissection. This study was approved by the Institutional Review Board of Seoul National University Hospital (IRB number: H-1507-112-690) and was performed in accordance with the Helsinki Declaration. All participants signed written informed consent before participation.

3. RNA extraction and quantitative reverse transcription–polymerase chain reaction

miRNAs were isolated from cell lines and gastric tissues preserved at –80°C using mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer’s instructions. Complementary DNA was synthesized from reverse transcription of the miRNAs using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). Quantitative reverse transcription–polymerase chain reaction was carried out using 2 μL of complementary DNA in a total mix of 20 μL containing 10 μL of TaqMan Universal Master Mix II (Applied Biosystems) and analyzed on an ABI PRISM 7000 Sequence De-
tection System (Applied Biosystems, Foster City, CA, USA). Human GAPDH gene served as an internal control and the relative expression levels of miRNAs were calculated using the comparative \(2^{-\Delta\DeltaCt}\) method. All samples were tested in triplicate.

4. DNA extraction, bisulfite conversion, and methylation analysis

DNA was isolated from the cell lines and tissues using the LaboPass\textsuperscript{TM} Blood Mini kit (Cosmogenetech, Seoul, Korea) following the manufacturer’s instructions. Bisulfite conversion was performed on 1 µg of genomic DNA using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA), which convert unmethylated cytosine to uracil.

Methylation status of the bisulfite-modified miRNA promoters was analyzed using real-time PCR-based MethyLight assay. Pairs of primers and probes were designed by the software, Beacon Designer (PREMIER Biosoft International, Palo Alto, CA, USA) (Supplementary Table 2). In both miR-200a and miR-200b, six CpG sites in the promoter region were included in the methylation analysis (Supplementary Fig. 1). Levels of DNA methylation were reported as a percentage of methylated reference (PMR), which was calculated as PMR=100\times[\text{[methylated reaction/ALU]}_{\text{sample}}]/[\text{[methylated reactoin/ALU]}_{\text{M.SssI}}]. The MethyLight assay was also performed in triplicate.

5. Statistical analysis

The Kruskal-Wallis test was recruited for the overall comparison of continuous variables of the three groups (H. pylori-negative controls, H. pylori-positive controls, and H. pylori-positive GC) since the data were not normally distributed. The Mann–Whitney U test was then used for pairwise group comparisons. The chi-square or Fisher exact tests were recruited for the analysis of the categorical variables. The ranked analysis of covariance model was applied to examine differences in the level of methylation and miRNA expression after correcting for baseline imbalances. The Wilcoxon signed-rank test was used for comparison between before and after eradication for H. pylori-eradication and H. pylori-persistence groups. Differences were considered statistically significant at \(p<0.05\). Statistical analyses were performed using IBM SPSS version 22.0 (IBM

![Fig. 1. Changes in promoter DNA methylation and microRNA (miRNA) expression following demethylating treatment in gastric cancer (GC) cell lines. (A) Promoter DNA methylation levels. The methylation levels of the promoter region of miR-200a and miR-200b was decreased in three GC cell lines after 72 hours of treatment with the demethylating agent 5-Aza-2’-deoxycytidine. (B) Relative expression levels. After 72 hours of treatment with the demethylating agent, the expression levels of both miR-200a and miR-200b increased in three GC cell lines. PMR, percentage of methylated reference.](image-url)
Corporation, Armonk, NY, USA).

RESULTS

1. miR-200a/b methylation and expression in GC cell lines

We measured the levels of miRNA expression and DNA methylation in the GC cell lines, AGS, MKN-1, and MKN-45. To assess the role of methylation in the expression of miRNAs, we examined their expression in three GC cell lines before and after the treatment with the demethylating agent, 5-Aza-2’-deoxycytidine. The levels of miR-200a methylation decreased by 25% in AGS, 63% in MKN-1, and 53% in MKN-45 after 72 hours of demethylation treatment (Fig. 1A). The expression of miR-200a increased by 1.7-fold in AGS, 12.7-fold in MKN-1, and 1.4-fold in MKN-45 after 72 hours of demethylation treatment (Fig. 1B).

The promoter methylation levels of miR-200b also decreased by 29% in AGS, 55% in MKN-1, and 33% in MKN-45, and the expression of miR-200b increased by 1.7-fold in AGS and MKN-1, and 1.8-fold in MKN-45 after demethylation treatment (Fig. 1).

2. Clinicopathological characteristics of subjects

In clinicopathological characteristics, the patients with H. pylori-positive GC were significantly older than H. pylori-positive and -negative controls (median, 66.5 vs 57.0 and 56.5, both p<0.001). The proportion of males was significantly higher in the H. pylori-positive GC group than that in the H. pylori-positive and -negative control groups (65.0% vs 20.0% and 35.0%, p=0.002 and p=0.028, respectively). In pathological characteristics, the degree of mucosal atrophy was more severe in the H. pylori-positive GC group than that in the H. pylori-negative control group (p=0.019), whereas no significant difference was seen between the H. pylori-positive GC and H. pylori-positive control groups. The degree of intestinal metaplasia was significantly more severe in the H. pylori-positive and -negative control groups (p=0.002 and p=0.007, respectively) (Table 1).

3. miR-200a/b methylation and expression in gastric mucosa according to H. pylori infection and disease state

We measured the level of methylation and corresponding miRNA expression in the gastric mucosa of the three groups. In the MethyLight assay, the promoter DNA methylation level of miR-200a was lowest in the H. pylori-negative control group, followed by the H. pylori-positive control group, and then the H. pylori-positive GC group (median, 11.5 vs 28.4 vs 40.3, all p<0.001) (Fig. 2). On the other hand, the expression level of miR-200a was highest in the H. pylori-negative control group, and significantly decreased in the H. pylori-positive control (0.6-fold that of the H. pylori-negative control) and H. pylori-positive GC groups (0.1-fold that of the H. pylori-negative control). miR-200b also followed the same pattern of promoter methylation and expression as miR-200a. The promoter methylation level of miR-200b increased gradually in the H. pylori-negative control, H. pylori-positive control, and H. pylori-positive GC groups (median, 9.9 vs 15.3 vs 22.6, all p<0.001) (Fig. 2B). The expression levels of miR-200b decreased significantly in the H. pylori-negative control, H. pylori-positive control (0.7-fold that of the H. pylori-negative control), and H. pylori-positive GC groups (0.4-

| Table 1. Clinicopathological Characteristics of the Subjects |
|-----------------------------------------------|
| Variable                      | H. pylori-positive GCs (n=40) | H. pylori-positive controls (n=20) | p-value* | H. pylori-negative controls (n=20) | p-value† |
|-----------------------------------------------|
| Age, yr                                      | 66.5 (59.3–72.0)              | 57.0 (48.0–61.0)                  | <0.001   | 56.5 (47.3–61.8)                  | <0.001   |
| Male sex                                    | 26 (65.0)                     | 4 (20.0)                         | 0.002    | 7 (35.0)                         | 0.028    |
| Mucosal atrophy                             | 0.077                         | 0.002                            | 0.019    |
| Absent to mild                              | 24 (60.0)                     | 17 (85.0)                        | 18 (90.0) |
| Moderate to severe                          | 16 (40.0)                     | 3 (15.0)                         | 2 (10.0)  |
| Intestinal metaplasia                       | 0.002                         | 0.007                            |
| Absent to mild                              | 17 (42.5)                     | 17 (85.0)                        | 16 (80.0) |
| Moderate to severe                          | 23 (57.5)                     | 3 (15.0)                         | 4 (20.0)  |
| Neutrophil                                  | 1.000                         | <0.001                           |
| Absent to mild                              | 1 (2.5)                       | 1 (5.0)                          | 19 (95.0) |
| Moderate to severe                          | 39 (97.5)                     | 19 (95.0)                        | 1 (5.0)   |
| Monocyte                                    | 0.595                         | <0.001                           |
| Absent to mild                              | 2 (5.0)                       | 2 (10.0)                         | 18 (90.0) |
| Moderate to severe                          | 38 (95.0)                     | 18 (90.0)                        | 2 (10.0)  |

Data are presented as the median (interquartile range) or number (%). H. pylori, Helicobacter pylori; GC, gastric cancer.

*Comparison between the H. pylori-positive GCs group and the H. pylori-positive controls; †Comparison between the H. pylori-positive GCs group and the H. pylori-negative controls.
fold that of the H. pylori-negative control. We compared miR-200a/b promoter methylation and expression levels according to the subtypes of GC in the H. pylori-positive GC group, but failed to find any significant correlations (Supplementary Table 3).

4. miR-200a/b methylation and expression in gastric mucosa with adjustment of baseline imbalance

Because of the significant difference in age and sex in the baseline characteristics of each group, further analysis was needed with adjustment of these variables. Promoter methylation and miRNA expression of miRNAs were analyzed using

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**Table 2. Differences in the Promoter Methylation and Expression Levels of miRNAs among the Groups after Adjustment for Covariates**

| Variable | miR-200a | miR-200b |
|----------|----------|----------|
|          | Promoter methylation | miRNA expression | Promoter methylation | miRNA expression |
|          | df  | F    | p-value | df  | F    | p-value | df  | F    | p-value |
| Age      | 1   | 0.482 | 0.490   | 1   | 0.016 | 0.900   | 1   | 0.004 | 0.952 |
| Sex      | 1   | 0.709 | 0.402   | 1   | 1.175 | 0.282   | 1   | 0.329 | 0.568 |
| Group    | 2   | 8.104 | 0.001   | 2   | 6.468 | 0.003   | 2   | 8.977 | <0.001 |

miRNA, microRNA; df, degree of freedom; F, variance ratio.
a non-parametric ranked analysis of covariance model with group as a factor, and age and sex as covariates. In this model, the levels of promoter methylation \( p=0.001 \) and expression of miR-200a \( p=0.003 \) were shown to be significantly different among groups after adjustment of age and sex (Table 2). Age and sex were not significantly associated with the promoter methylation levels and miR-200a expression. Similarly, the levels of promoter methylation \( p<0.001 \) and expression of miR-200b \( p=0.001 \) in each group were significantly different after adjustment of age and sex. Regarding miR-200b, age and sex were not significantly associated with the promoter methylation levels and miRNA expression.

5. Effect of \( H. pylori \) eradication on the methylation and expression of miR-200a/b

In 40 patients with \( H. pylori \)-positive GC, half of them received \( H. pylori \) eradication therapy (\( H. pylori \)-eradication group), and the other half did not receive eradication therapy (\( H. pylori \)-persistance group). In all 20 patients in the \( H. pylori \)-eradication group, \( H. pylori \) was confirmed to have been successfully eradicated. No significant difference was observed in baseline clinicopathological characteristics between the two groups (Table 3). Promoter methylation and expression levels in the non-cancerous gastric mucosa of \( H. pylori \)-positive GC patients were compared before and after 6 months of \( H. pylori \) eradication. In the \( H. pylori \)-eradication group, the promoter methylation level of miR-200a decreased significantly, and the expression level of miR-200a increased significantly (7.9-fold of baseline) at 6 months after \( H. pylori \) eradication, whereas the methylation and expression levels of miR-200a in the \( H. pylori \)-persistance group were not significantly different during follow-up at 6 months (Fig. 3A). In the \( H. pylori \)-eradication group, there was a significant decrease in the promoter methylation level and a significant increase in the expression level (4.4-fold of baseline) of miR-200b after 6 months compared with baseline. In the \( H. pylori \)-persistance group, there were no significant changes in the promoter methylation and expression levels of miR-200b between baseline and 6 months (Fig. 3B).

**DISCUSSION**

In this study, the level of DNA methylation might influence the level of miR-200a/b expression in GC cell lines. In addition, aberrant DNA methylation of miR-200a/b was observed in \( H. pylori \)-infected gastric tissue, which was also found in non-cancerous gastric tissue of patients with \( H. pylori \)-positive GC. The results indicate that \( H. pylori \) infection might affect the promoter methylation of miR-200a/b, alter the level of expression of miRNAs, and eventually form an epigenetic field for cancerization. \( H. pylori \) eradication has been shown to have the potential to improve these epigenetic changes and recover the expression of miR-200a/b. To our best knowledge, this research is the first to show that the regulation of miR-200a/b expression via promoter methylation may be an important mechanism for \( H. pylori \) to form the epigenetic field of GC, which can be recovered after eradication therapy.

Recently, epigenetic changes in cancer-related genes, such as tumor suppressor genes, have been proposed to be one of the

### Table 3. Clinicopathological Characteristics of the Helicobacter pylori-Eradication and -Persistence Groups at Baseline and the 6-Month Follow-up

| Characteristic          | Baseline          | 6-Month follow-up |
|-------------------------|-------------------|-------------------|
|                         | \( H. pylori \)-eradication \( n=20 \) | \( H. pylori \)-persistance \( n=20 \) | \( H. pylori \)-eradication \( n=20 \) | \( H. pylori \)-persistance \( n=20 \) | p-value* |
| Age, yr                 | 70.0 (56.5–72.8)  | 65.0 (60.3–70.8)  | -                     | -                     | 0.327    |
| Male sex                | 12 (60.0)         | 14 (70.0)         | -                     | -                     | 0.001    |
| Mucosal atrophy         |                   |                   |                       |                       |         |
| Absent to mild          | 10 (50.0)         | 14 (70.0)         | 14 (70.0)             | 11 (55.0)             |         |
| Moderate to severe      | 10 (50.0)         | 6 (30.0)          | 6 (30.0)              | 9 (45.0)              |         |
| Intestinal metaplasia   |                   |                   |                       |                       | 0.519    |
| Absent to mild          | 6 (30.0)          | 11 (55.0)         | 7 (35.0)              | 9 (45.0)              |         |
| Moderate to severe      | 14 (70.0)         | 9 (45.0)          | 13 (65.0)             | 11 (55.0)             |         |
| Neutrophil              |                   |                   |                       |                       | <0.001   |
| Absent to mild          | 1 (5.0)           | 0                 | 17 (85.0)             | 2 (10.0)              |         |
| Moderate to severe      | 19 (95.0)         | 20 (100.0)        | 3 (15.0)              | 18 (90.0)             |         |
| Monocyte                |                   |                   |                       |                       | 0.001    |
| Absent to mild          | 1 (5.0)           | 1 (5.0)           | 9 (45.0)              | 0                     |         |
| Moderate to severe      | 19 (95.0)         | 19 (95.0)         | 11 (55.0)             | 20 (100.0)            |         |

Data are presented as the median (interquartile range) or number (%).

*p-Value for comparison between the 6-month follow-up results in the \( H. pylori \)-eradication and \( H. pylori \)-persistance groups.*
important processes in *H. pylori*-associated gastric carcinogenesis. DNA methylation is one of the epigenetic mechanisms that has been known to occur not only in the promoters of protein-coding genes but also non-coding genes, such as miRNAs. When aberrant DNA methylation accumulates in normal-appearing gastric mucosal tissues due to prolonged *H. pylori* infection, these epigenetic alterations form an epigenetic field for cancerization that is susceptible to GC. In previous studies, *miR-133a* and Wnt antagonist genes were epigenetically silenced by promoter methylation in patients with *H. pylori*-associated GC. Considering that endoscopic resection has been a standard treatment for early type of GC, the importance of epigenetic field formed in residual stomach has been highlighted. To date, most of the studies on the expression of miRNA in GC patients have investigated the expression levels of miRNA in GC tissues, compared to the matched non-cancerous tissues of the same patient. There have been a few studies comparing the epigenetic status of non-cancerous gastric tissues of GC patients and normal gastric tissues of non-predisposed subjects, which might explore the risk of second primary GC after endoscopic resection of the primary one.

The *miR-200* family is one of the most studied miRNA family in many carcinomas. The *miR-200* family expression has been well known to be dysregulated in various cancers, including renal cell carcinoma, bladder cancer, colorectal cancer, and ovarian cancer. In GC, *miR-200* family acts as tumor suppressor and lower expression of these members has been reported to be related to poor prognosis of GC. Although the mechanisms controlling the expression of the *miR-200* family have been partially explained by DNA methylation and histone changes, they have not yet been fully understood.

The present study revealed that *miR-200a/b* expression might be epigenetically regulated both in vitro and in vivo. The level of promoter methylation of *miR-200a/b* decreased and the expression of *miR-200a/b* increased after demethylating treatment in three GC cell lines. In human gastric mucosa, promoter meth-

![Fig. 3. Change in promoter DNA methylation and corresponding microRNA (miRNA) expression according to the Helicobacter pylori eradication therapy. (A) *miR-200a* and (B) *miR-200b*. In the *H. pylori*-eradication group, there was a significant difference in the promoter methylation and expression levels of *miR-200a/b* at 6 months after eradication. FU, follow-up.](image-url)
ylation increased gradually in the order of H. pylori-negative control, H. pylori-positive control, and H. pylori-positive GC group, while miR-200a/b expression was gradually downregulated in the same order. These changes were significant even after adjustment of the covariates, such as age and sex. The present study highlights the role of miR-200a/b as a potential tumor suppressor in the initiation of H. pylori-related GC and suggests that methylation-dependent regulation may be an important mechanism to control its expression.

In this study, the eradication of H. pylori resulted in a decrease in the promoter methylation levels of miR-200a/b and an increase in the expression of these miRNAs in the H. pylori-positive GC group. Also, H. pylori eradication could prevent further progression of an epigenetic field for cancerization and restore the epigenetic changes that have already occurred during chronic infection. These findings might include one of the molecular mechanisms that explain the results of previous studies that the eradication of H. pylori reduced the incidence of metachronous GC and GC-related death.\[36,37\] It is remarkable that the promoter methylation levels of miR-200a/b in the H. pylori-eradication group were still higher than those in the H. pylori-negative control group at 6 months follow-up, which might be explained by the fact that aberrant methylation in H. pylori-infected gastric epithelial cells decreases via cell turnover after the successful eradication, while that in H. pylori-infected gastric stem cells might persist even after eradicating H. pylori.\[38\]

We demonstrated that the degree of neutrophil and monocyte infiltration decreased significantly at 6 months after eradication in the H. pylori-eradication group, but remained at a similar level in the H. pylori-persistence group. Previous studies that analyzed the mechanism of aberrant DNA methylation caused by the infection of H. pylori have suggested that infection-associated inflammatory response was a critical factor. The infection of H. pylori has been known to influence the release of many pro-inflammatory cytokines, including tumor necrosis factor-\(\alpha\), interleukin-1, 6, and 8, and nuclear factor-\(\kappa\)B, and also trigger a T1 helper cell inflammatory response.\[39\] These inflammatory dysregulations are considered to have a critical role in gastric inflammation and carcinogenesis.\[40\] In this study, the eradication of H. pylori led to a decrease in inflammatory response in gastric epithelial cells and further might reduce aberrant DNA methylation. Further researches are required to confirm the direct effect of H. pylori eradication on the cellular level, i.e., expression and methylation changes of miR-200a/b in the gastric epithelial cell itself.

In this study, the methylation levels of miR-200a/b were highly variable in the H. pylori-positive GC group, whereas those in the H. pylori-negative and -positive control groups were limited to a relatively narrow range (Supplementary Table 4). Similar results were obtained in previous studies that quantitatively analyzed the level of DNA methylation.\[5,38\] It has been suggested that aberrant promoter methylation may occur only in a fraction of cells in non-cancerous tissues.\[3\] Additional studies are needed to determine whether the levels of methylation vary depending on the location within the same individual.

This study has several strengths. To date, this is the first to demonstrate promoter methylation and subsequent dysregulation of miR-200a/b in non-cancerous gastric mucosa of patients with H. pylori-positive GC. In addition, we showed that the epigenetic alteration of gastric tissues could be recovered through H. pylori eradication even after the development of GC, which might suggest the need for re-discussion of the concept of “point of no return” in H. pylori eradication. Second, we used reverse transcription-polymerase chain reaction and MethyLight techniques to analyze miRNA expression and promoter methylation, which enabled sensitive and accurate quantitative analysis.

This study also has some limitations. First, a small number of patients were included for analysis. Nevertheless, the differences between the groups were large enough to reach statistical significance. In subgroup analysis of H. pylori-positive GC group, no significant association was found between characteristics of GC and levels of miR-200a/b expression and methylation. Further studies with large numbers of patients may be warranted. Second, there were differences in baseline characteristics, such as age and sex among the groups. We overcome the mismatches by performing further analysis with correction of these variables. Third, we did not include GC tissues in this study, which did not allow to compare between paired GC tissues and non-cancerous gastric mucosal tissues. Lastly, we did not include a study of target genes or functional analysis of miR-200a/b. ZEB1 and ZEB2 are one of potential target genes of miR-200a/b, which are known to be closely related to tumor growth and metastasis by engaging in epithelial-mesenchymal transition processes.\[15,16\] Subsequent studies are needed on the association between methylation changes in miR-200a/b and the expression and function changes in target genes, and, further, significance in the gastric carcinogenesis process.

In conclusion, aberrant DNA methylation of miR-200a/b might be associated with the infection of H. pylori and contributed to the formation of an epigenetic field for GC, which could be recovered by H. pylori eradication. Therefore, the eradication of H. pylori should be emphasized to prevent the development of metachronous tumor in non-cancerous gastric mucosa even in patients with GC.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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AUTHOR CONTRIBUTIONS

Study conception and design, data acquisition/analysis/interpretation, manuscript drafting: J.M.C., S.G.K., N.Y.C. Statistical analysis: J.M.C., H.J.Y. Critical revision of the manuscript: H.J.Y., J.H.L., N.Y.C., W.H.K., J.S.K., H.C.J. Obtained funding, study supervision: S.G.K. All authors read and approved the final manuscript.

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