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

Ji Min Choi[1], Sang Gyun Kim[2], Hyo-Joon Yang[3], Joo Hyun Lim[1], Nam-Yun Cho[4], Woo Ho Kim[5], Joo Sung Kim[1,2], and Hyun Chae Jung[2]

[1]Department of Internal Medicine, Healthcare Research Institute, Seoul National University Hospital Healthcare System Gangnam Center, [2]Department of Internal Medicine and Liver Research Institute, Seoul National University College of Medicine, [3]Department of Internal Medicine, Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, [4]Laboratory of Epigenetics, Cancer Research Institute, and [5]Department of Pathology, Seoul National University College of Medicine, Seoul, Korea

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-

Correspondence to: Sang Gyun Kim
Division of Gastroenterology, Department of Internal Medicine and Liver Research Institute, Seoul National University College of Medicine, 103 Daehak-ro, Jongno-gu, Seoul 03080, Korea
Tel: +82-2-740-8112, Fax: +82-2-743-6701, E-mail: harley1333@hanmail.net
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term *H. pylori* infection forms an epigenetic field defect that is susceptible to GC.[7] Genes that are hypermethylated during *H. pylori* infection are not only tumor suppressor genes, but also those of non-coding RNAs, such as microRNAs (miRNAs).[8] To date, more than 1,000 miRNAs have been identified in humans, and various miRNAs are implicated in the development of cancer.[9] A growing number of studies point out that abnormal expression of miRNA has a crucial role in the initiation and progression of GC. For example, the expression of *miR-1*, *-9*, *-34b*, and *-129*, which have a tumor suppressor function, was downregulated in GC by hypermethylation of their promoter CpG islands.[10,11] In contrast, *miR-196a/b*, an oncogenic miRNA, was upregulated in GC by hypomethylation of the promoter region.[12,13]

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-200c/141* are encoded by the gene on chromosome 12.[14] 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.[14-16] 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.[17-21] Several studies have demonstrated that *miR-200* family were downregulated in GC, suggesting its role as a tumor suppressor in GC.[16,22,23] 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.[16,22,23] 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.[23,24] 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 $NaHCO_3$, 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 endoscopy, gastric mucosa specimens were harvested from two sites in the antrum and two sites in the corpus (in case of GC patients, non-cancerous tissues were collected) for histological evaluation according to the updated Sydney System: glandular atrophy, intestinal metaplasia, neutrophils and mononuclear cells infiltration, and *H. pylori*.[25] Additional two samples of gastric mucosa were harvested from the antrum for miRNA-related analysis and restored at –80°C. *H. pylori* infection was considered positive if the histological (hematoxylin-eosin and modified Giemsa staining) or rapid urease test (Delta West Ltd., Bentley, Australia) was positive. 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 Detection 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 $2^{-\Delta\Delta Ct}$ method.[26] 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™ 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.[27,28] 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×[(methylated reaction/ALU)$_{sample}$/(methylated reactoin/ALU)$_{M.SssI}$]. 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

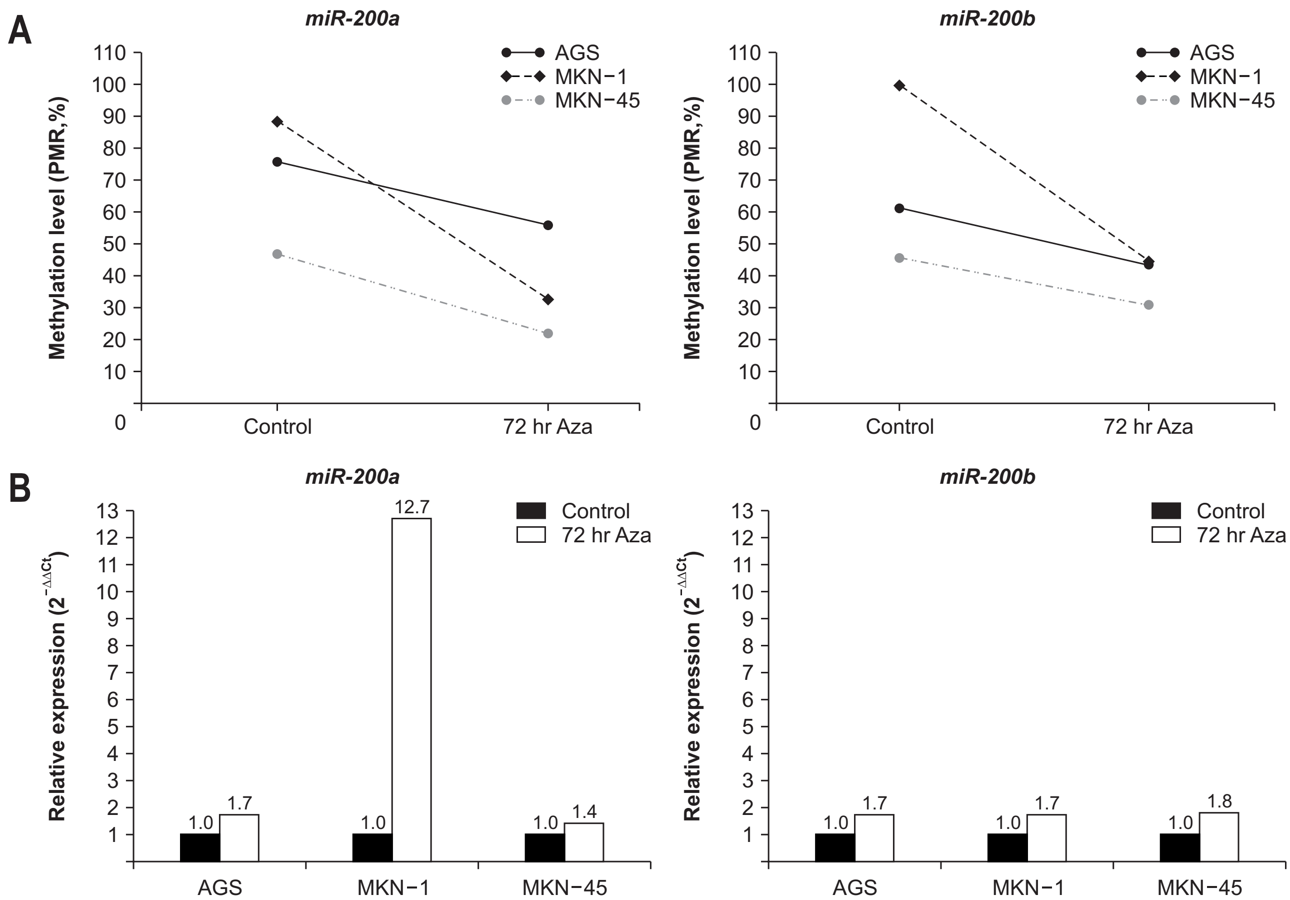

Fig. 1. Changes in promoter DNA methylation and microRNA (miRNA) expression following demethylating treatment in gastric cancer (GC) cell lines. (A) Promoter DNA methylation levels. The methylation levels of the promoter region of *miR-200a* and *miR-200b* was decreased in three GC cell lines after 72 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.

Corp., 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 MNK-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 GC group than that 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		0.019
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.

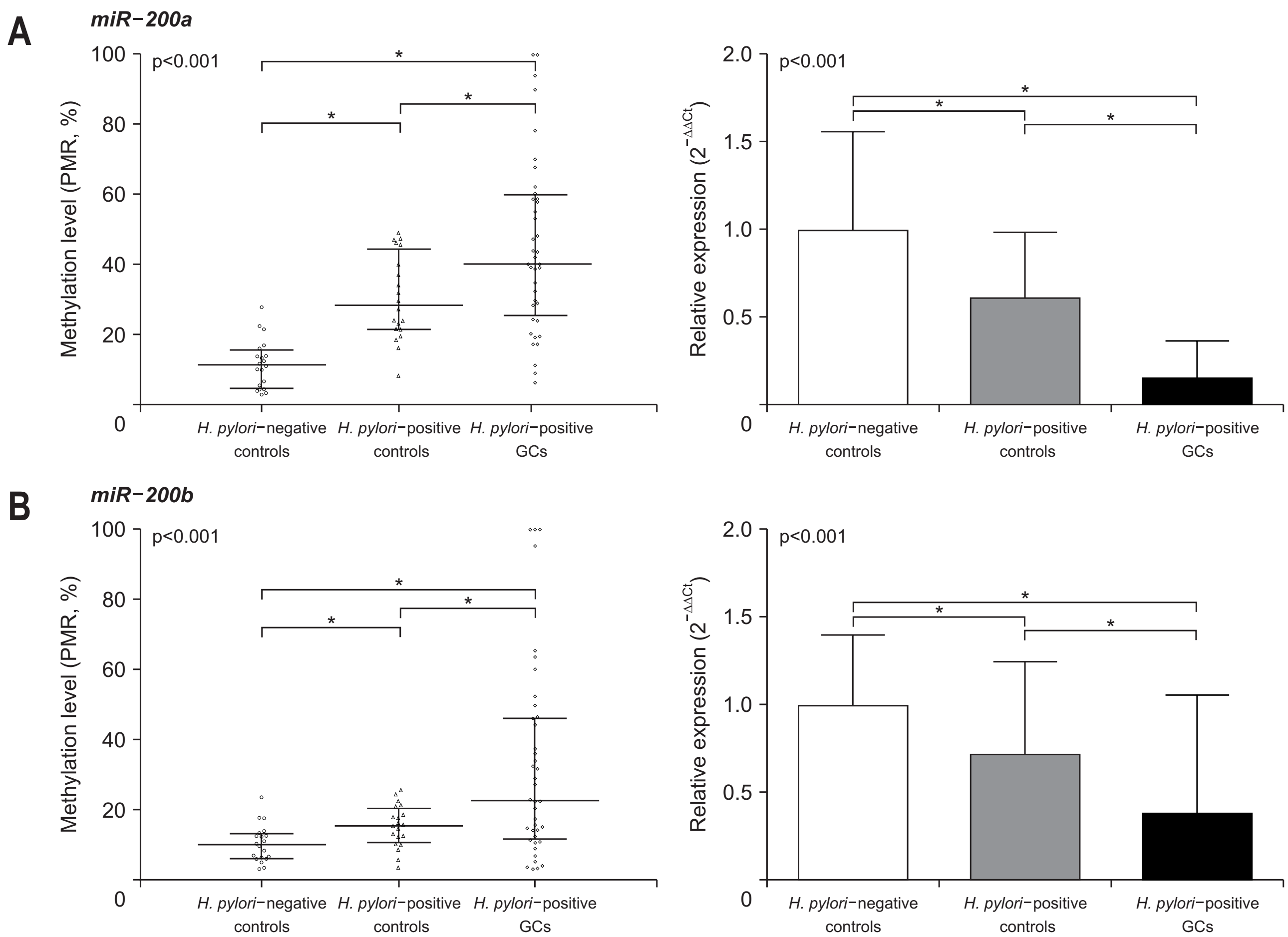

Fig. 2. The levels of promoter DNA methylation and corresponding miRNA expression in the study groups. The levels of *miR-200a/b* methylation are presented as medians and interquartile ranges. The levels of *miR-200a/b* expression are shown as fold changes relative to the *Helicobacter pylori*-negative controls and standard deviations. (A) *miR-200a*. The promoter levels of *miR-200a* (left) methylation were lowest in the *H. pylori*-negative controls, followed by the *H. pylori*-positive controls and then the *H. pylori*-positive GCs (all p<0.001). The *miR-200a* expression (right) level was highest in the *H. pylori*-negative controls, followed by the *H. pylori*-positive controls and then the *H. pylori*-positive GCs (all p<0.001). (B) *miR-200b*. *miR-200b* showed the same pattern as *miR-200a* for promoter DNA methylation and expression.
miRNA, microRNA; PMR, percentage of methylated reference; GC, gastric cancer. *p<0.001.

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	df	F	p-value
Age	1	0.482	0.490	1	0.016	0.900	1	0.004	0.952	1	0.565	0.455
Sex	1	0.709	0.402	1	1.175	0.282	1	0.329	0.568	1	1.343	0.250
Group	2	8.104	0.001	2	6.468	0.003	2	8.977	<0.001	2	8.415	0.001

miRNA, microRNA; df, degree of freedom; F, variance ratio.

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 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*-persistence 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*-persistence 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*-persistence 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*-Eradiation and -Persistence Groups at Baseline and the 6-Month Follow-up

Characteristic	Baseline		6-Month follow-up		p-value*
	H. pylori-eradication (n=20)	*H. pylori*-persistence (n=20)	*H. pylori*-eradication (n=20)	*H. pylori*-persistence (n=20)	
Age, yr	70.0 (56.5–72.8)	65.0 (60.3–70.8)	-	-	
Male sex	12 (60.0)	14 (70.0)	-	-	
Mucosal atrophy					0.327
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 at the median (interquartile range) or number (%).
*Comparison between the 6-month follow-up results in the *H. pylori*-eradication and *H. pylori*-persistence groups.

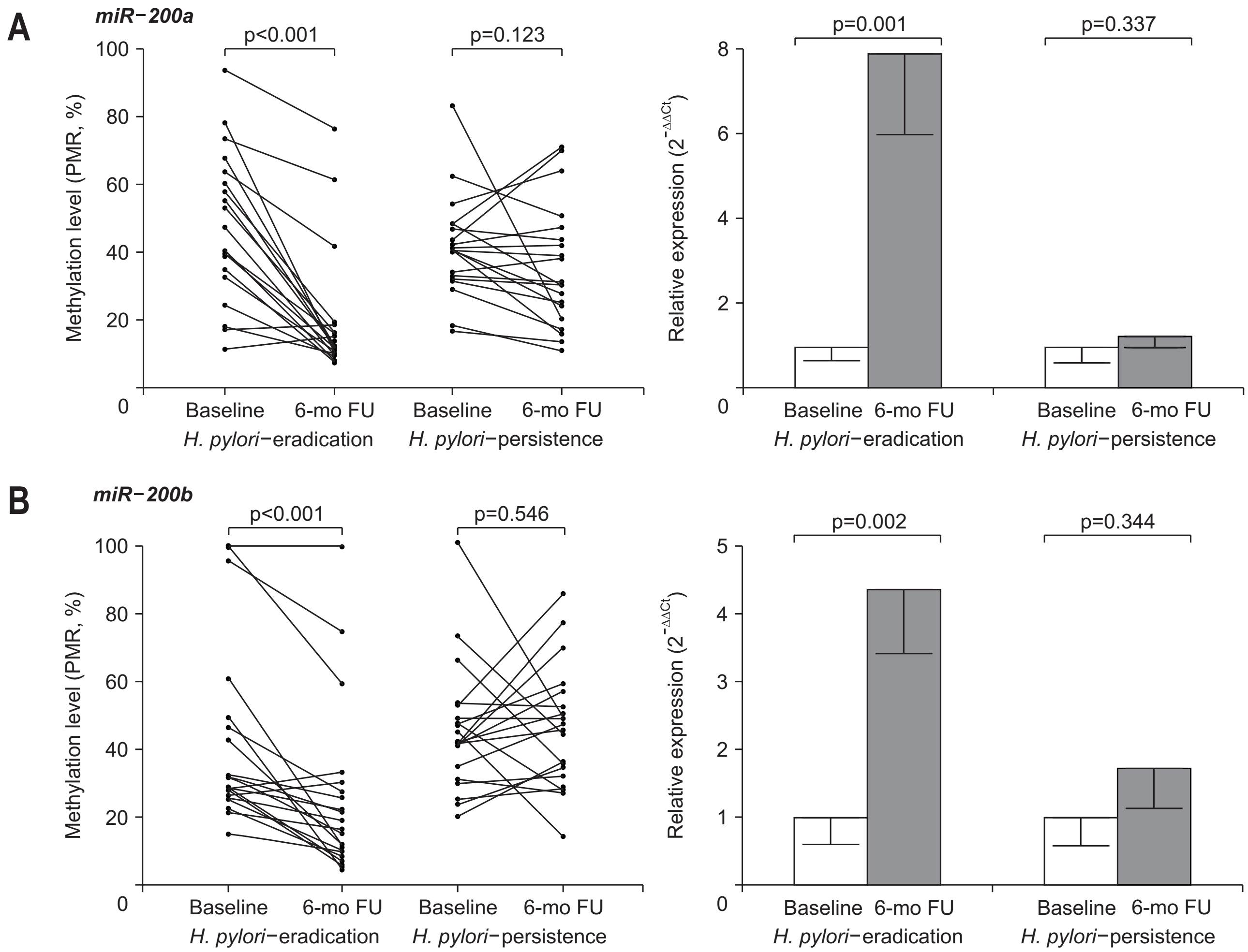

Fig. 3. Change in promoter DNA methylation and corresponding microRNA (miRNA) expression according to the *Helicobacter pylori* eradication therapy. (A) *miR-200a* and (B) *miR-200b*. In the *H. pylori*-eradication group, there was a significant difference in the promoter methylation and expression levels of *miR-200a/b* at 6 months after eradication.
FU, follow-up.

important processes in *H. pylori*-associated gastric carcinogenesis.[5,6] 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.[10-13] 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.[7,29] In previous studies, *miR-133a* and Wnt antagonist genes were epigenetically silenced by promoter methylation in patients with *H. pylori*-associated GC.[30] 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.[31] 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.[32,33] 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.[17,18,20,21] 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.[16,22,23] 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.[23,34,35]

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 methylation 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 deaths.[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 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-α, interleukin-1, 6, and 8, and nuclear factor κB, 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.[38] 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.[5,30] It has been suggested that aberrant promoter methylation may occur only in a fraction of cells in non-cancerous tissues.[5] 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 overcame 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.

ORCID

Ji Min Choi	https://orcid.org/0000-0001-8611-4647
Sang Gyun Kim	https://orcid.org/0000-0003-1799-9028
Hyo-Joon Yang	https://orcid.org/0000-0002-0265-672X
Joo Hyun Lim	https://orcid.org/0000-0002-8437-096X
Nam-Yun Cho	https://orcid.org/0000-0002-5736-2956
Woo Ho Kim	https://orcid.org/0000-0003-0557-1016
Joo Sung Kim	https://orcid.org/0000-0001-6835-4735
Hyun Chae Jung	https://orcid.org/0000-0001-5442-4341

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