의학박사 학위논문

Helicobacter pylori eradication modulates the methylation-associated regulation of *miR-200a/b* in gastric carcinogenesis

헬리코박터 파일로리 제균치료가 위암 발병에서 메틸화 연관 *miR-200a/b* 조절에 미치는 영향

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Helicobacter pylori eradication modulates the methylation-associated regulation of *miR-200a/b* in gastric carcinogenesis

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ABSTRACT

Introduction: We aimed to evaluate the epigenetic changes of *miR-200a/b* in *H. pylori*-associated gastric carcinogenesis and restoration of these findings after eradication.

Methods: The levels of expression and promoter methylation of *miR-200a/b* were evaluated in gastric cancer (GC) cell lines and human gastric mucosa of *H. pylori*-negative and -positive controls, and *H. pylori*-positive GC patients. Next, the changes of expression and methylation levels of *miR-200a/b* were compared between *H. pylori*-eradication and *H. pylori*-persistence groups 6 months after endoscopic resection of GCs. Real-time reverse transcription-polymerase chain reaction was conducted to investigate the miRNA expression levels, and MethyLight assay was performed to assess methylation levels.

Results: In GC cell lines, the levels of *miR-200a/b* methylation decreased and the levels of expression increased after demethylation. In human gastric mucosa, the *miR-200a/b* methylation levels increased in the order of *H. pylori*-negative control, -positive control, and *H. pylori*-positive GC patients ($P < 0.001$). Conversely, the *miR-200a/b* expression levels decreased in the same order ($P < 0.001$). 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 ($P < 0.001$) and the level of expression of *miR-200a/b* increased ($P = 0.001$ and $P = 0.002$, respectively) significantly 6 months after *H. pylori* eradication.

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.

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Keywords: *Helicobacter pylori*; MicroRNAs; Methylation; Epigenetic Alteration; Stomach Neoplasm

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LIST OF ABBREVIATIONS

miRNAs: microRNAs

GC: gastric cancer

RT-PCR: reverse transcription-polymerase chain reaction

cDNA: complementary DNA

PMR: percentage of methylated reference

ANCOVA: analysis of covariance

Hp: *Helicobacter pylori*

IQR: interquartile range

IM: intestinal metaplasia

INTRODUCTION

Gastric cancer (GC) is the fifth most frequent cancer 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 studies have reported 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-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 1000 miRNAs have been found in humans, and various miRNAs are involved in the development of cancer.[9] A growing number of

studies point out that abnormal miRNA expression plays an essential 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 located on chromosome 1 and *miR-200c/141* are encoded by the gene located 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 members were downregulated in GC, suggesting its role as a tumor suppressor in GC.[16,22,23] Lower expression levels of *miR-200* were known to be associated with poor prognosis of GC: histological grade, tumor size, depth of invasion, 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* family members in GC has not been fully elucidated.[23,24] To our knowledge, the methylation status and subsequent dysregulation of *miR-200* family members 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.

In this study, we examined whether epigenetic fields related to *miR-200a/b* were formed during *H. pylori* infection and gastric carcinogenesis, and recovered after *H. pylori* eradication.

MATERIALS AND METHODS

GC cell lines

Three GC cell lines, AGS, MKN-1, and MKN-45 were obtained from the Korean Cell Line Bank (Seoul, Republic of Korea) (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. Cells were treated with demethylating agent, 2 μM 5-Aza-2'-deoxycytidine (Sigma-Aldrich and Merck KGaA, Darmstadt, Germany), on day 1 and replenished daily with the demethylating agent and medium. On day 4, cells were harvested.

Table 1. Short tandem repeat profiles of gastric cancer cell lines

Allele	**Profile**		
	AGS	**MKN-1**	**MKN-45**
D3S1358	15.2	15,17	15,16
vWA	16,17	16	19
FGA	16,17	20,23	19
Amelogenin	X	X	X
TH01	6,7	9	7
TPOX	11,12	8	8
CSF1P0	11,12	9,12	12
D5S818	9,12	11	10,11
D13S317	12	10,12	8,11
D7S820	10,11	10	10,11

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 this study were older than 18 years of age, had no other malignancy, were not taking antibiotics nor proton pump inhibitors within the last four weeks, and did not report history of *H. pylori* eradication. During endoscopy, gastric mucosa specimens were obtained 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 obtained 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 (CLO test; Delta West Ltd., Bentley, Australia) was positive. The patients with *H. pylori*-positive GC were randomly assigned to the eradication or persistence group. The patients in the eradication group received one week of treatment (20 mg omeprazole, 1 g amoxicillin, and 500 mg clarithromycin twice daily) two weeks after endoscopic resection. To evaluate the effect of *H. pylori* eradication on the epigenetic regulation of miRNA expression, we also collected gastric mucosal tissues at six

months after endoscopic submucosal dissection. This study was approved by the Institutional Review Board of Seoul National University Hospital (H-1507-112-690) and was conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent before participation.

RNA extraction and quantitative reverse transcription-polymerase chain reaction (RT-PCR)

miRNAs were isolated from cell lines and gastric tissues stored at –80°C using mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from reverse transcription of the miRNAs using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). Quantitative RT-PCR was carried out using 2 μL of cDNA 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 as described previously.[26] All samples were tested in triplicate.

DNA extraction, bisulfite conversion, and methylation analysis

DNA was isolated from the cell lines and tissues using the LaboPass™ Blood Mini kit (Cosmogentech, Seoul, Korea) according to 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) to convert unmethylated cytosine into uracil.

Methylation status of the bisulfite-modified miRNA promoters was analyzed using real-time PCR-based MethyLight assay as previously described.[27,28] Pairs of primers and probes were designed by the software, Beacon Designer (PREMIER Biosoft International, Palo Alto, CA, USA) (Table 2). In both *miR-200a* and *miR-200b*, six CpG sites in the promoter region were included in the methylation analysis (Figure 1). Levels of DNA methylation were reported as a percentage of methylated reference (PMR), which was calculated as $\mathrm{PMR} = 100 \times [(\text{methylated reaction/ALU})_{\text{sample}}/(\text{methylated reactoin/ALU})_{\text{M.SssI}}]$. The MethyLight assay was also performed in triplicate.

Table 2. The individual primers and probes for MethyLight assay

Gene	Primer/probe	Sequence (5'→3')	Size (bp)	Tm (℃)
miR-200a	Forward primer	CCCTATCCGATCCCGACACC	20	59.5
	Reverse primer	AGGGACGTGGGGTTTCGTTA	20	59.1
	Probe	CCTAACTACTCACCGCTCCGATTCTTCCC	29	67.5
mir-200b	Forward primer	AGTGTTTTAGGAGGACGAGGTTT	23	58.3
	Reverse primer	ATACTACCCAATAAAATAACCACGACTA	28	58.0
	Probe	CCGAACTAAATCCGAAAACCGCCCGA	26	66.7

Tm, melting temperature

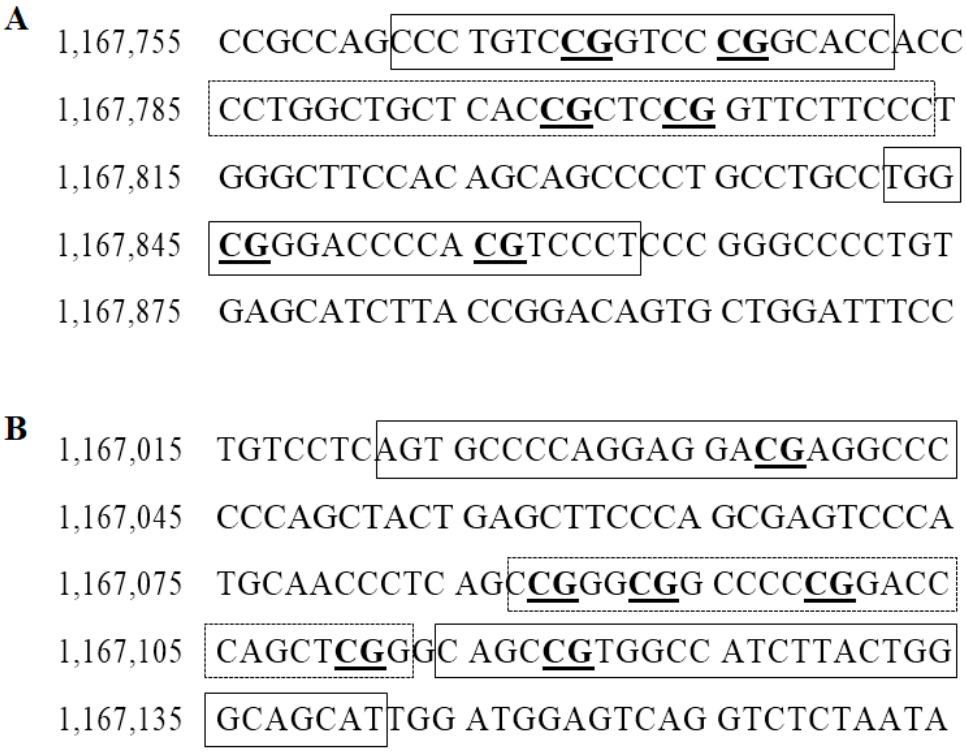

Figure 1. The sequence of the promoter CpG island region of *miR-200a/b*. (A) *miR-200a* and (B) *miR-200b*. The binding sites of the forward and reverse primers were boxed with solid line, and the binding sites of the probe were boxed with dotted line. In this study, methylation status of six CpG sites in the promoter region was analyzed for both *miR-200a* and *miR-200b*, which were highlighted as bold and underline.

Statistical analysis

Since the data were not normally distributed, the Kruskal-Wallis test was used for the overall comparison of continuous variables of the three groups (*H. pylori*-negative controls, *H. pylori*-positive controls, and *H. pylori*-positive GC). The Mann–Whitney U test was then used for pairwise group comparisons. Categorical variables were analyzed by chi-square or Fisher's exact tests. The ranked analysis of covariance (ANCOVA) 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 22.0 (IBM SPSS Statistics for Windows, Version 22.0, IBM Corp., Armonk, NY).

RESULT

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 h of demethylation treatment (Figure 2A). 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 h of demethylation treatment (Figure 2B). 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 (Figure 2A, B).

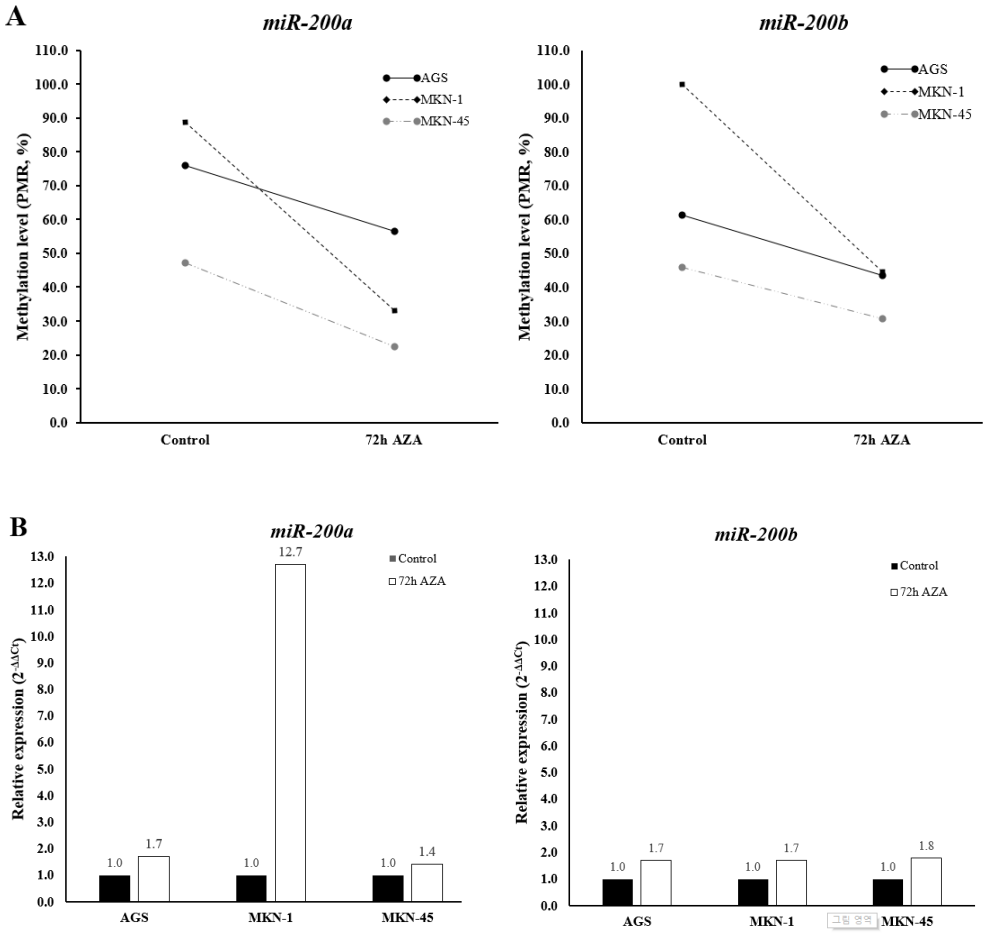

Figure 2. Changes in promoter DNA methylation and miRNA expression following demethylating treatment in gastric cancer (GC) cell lines. (A) Promoter DNA methylation levels. The methylation levels of promoter region of *miR-200a* and *-200b* was decreased in three GC cell lines after 72 hours treatment of demethylating agent, 5-Aza-2'-deoxycytidine. (B) Relative expression levels. After 72 hours of demethylating agent treatment, expression levels of both *miR-200a* and *-200b* increased in three GC cell lines.

PMR, percentage of methylated reference

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 3).

Table 3. Clinicopathological Characteristics of Subjects

	***Hp*-positive GCs (n=40)**	***Hp*-positive controls (n=20)**	***P* value***	***Hp*-negative controls (n=20)**	***P* value†**
Age, yrs	66.5 (59.3-72.0)	57.0 (48.0-61.0)	<0.001	56.5 (47.3-61.8)	<0.001
Sex, male	26 (65.0)	4 (20.0)	0.002	7 (35.0)	0.028
Atrophy			0.077		0.019
Absent/mild	24 (60.0)	17 (85.0)		18 (90.0)	
Moderate/severe	16 (40.0)	3 (15.0)		2 (10.0)	
IM			0.002		0.007
Absent/mild	17 (42.5)	17 (85.0)		16 (80.0)	
Moderate/severe	23 (57.5)	3 (15.0)		4 (20.0)	
Neutrophil			1.000		<0.001
Absent/mild	1 (2.5)	1 (5.0)		19 (95.0)	
Moderate/severe	39 (97.5)	19 (95.0)		1 (5.0)	
Monocyte			0.595		<0.001
Absent/mild	2 (5.0)	2 (10.0)		18 (90.0)	
Moderate/severe	38 (95.0)	18 (90.0)		2 (10.0)	

Data are n (%) or median (IQR).

Hp, H. pylori; GC, gastric cancer; IQR, interquartile range; IM, intestinal metaplasia

*Comparison between the *Hp*-positive GCs and the *Hp*-positive controls

†Comparison between the *Hp*-positive GCs and the *Hp*-negative controls

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 $P < 0.001$) (Figure 3A). 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 $P < 0.001$) (Figure 3B). 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-fold that of the *H. pylori*-negative control).

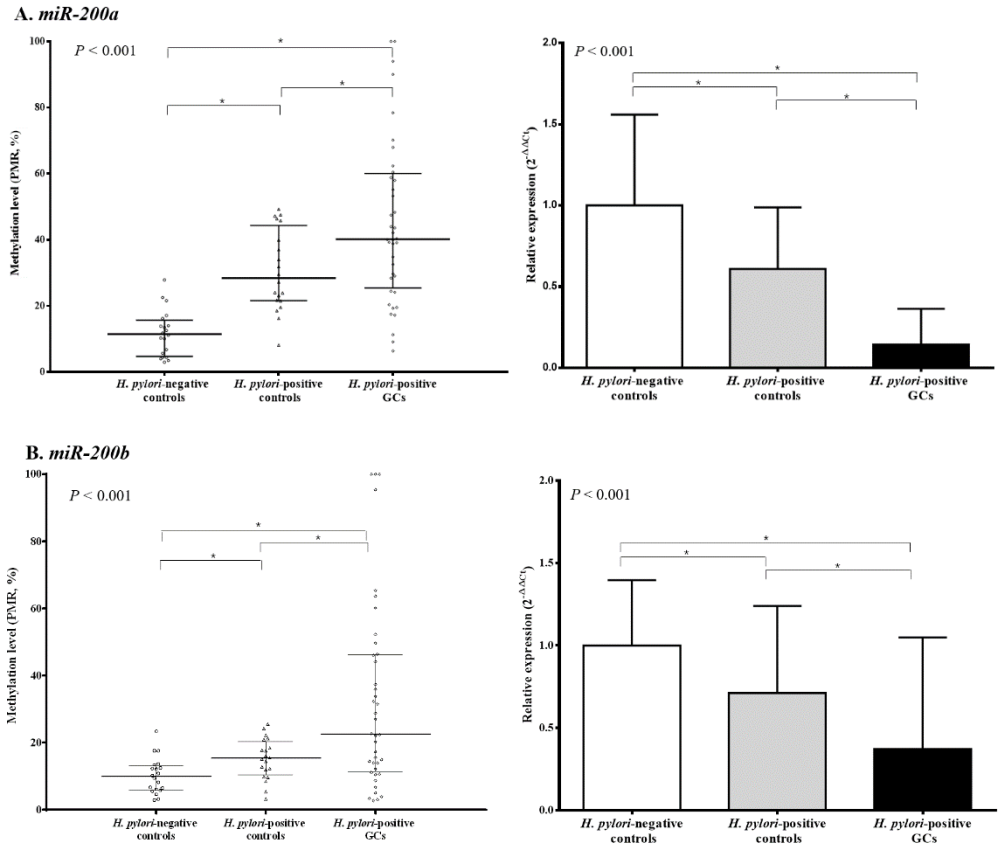

Figure 3. The levels of promoter DNA methylation and corresponding miRNA expression in the study groups. The levels of *miR-200a/b* methylation are presented as medians and interquartile ranges. The levels of *miR-200a/b* expression are shown as fold changes relative to *H. pylori*-negative controls and standard deviations. (A) *miR-200a.* The promoter methylation levels of *miR-200a* (left) were lowest in *H. pylori*-negative controls, followed by *H. pylori*-positive controls and then *H. pylori*-positive GCs (all $P < 0.05$). The *miR-200a* expression (right) were highest in *H. pylori*-negative controls, followed by *H. pylori*-positive controls and then *H. pylori*-

positive GCs (all $P < 0.05$). (B) *miR-200b*. *miR-200b* also showed the same pattern of promoter DNA methylation and expression as *miR-200a*.

miRNA, microRNA; PMR, percentage of methylated reference; *H. pylori*, *Helicobacter pylori;* GC, gastric cancer.

*P <0.05

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

Because there was a 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 ANCOVA 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 4). Age and sex were not significantly associated with the levels of promoter methylation and expression of *miR-200a*. 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 levels of promoter methylation and expression of miRNA.

Table 4. Differences in the Promoter Methylation and Expression Levels of miRNAs among the Groups after Adjustment for Covariates

	Promoter methylation			**miRNA expression**		
	df	F	*P* value	df	F	*P* value
miR-200a						
Age	1	0.482	0.490	1	0.016	0.900
Sex	1	0.709	0.402	1	1.175	0.282
Group	2	8.104	0.001	2	6.468	0.003
miR-200b						
Age	1	0.004	0.952	1	0.565	0.455
Sex	1	0.329	0.568	1	1.343	0.250
Group	2	8.977	<0.001	2	8.415	0.001

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

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. There were no significant differences in baseline clinicopathological characteristics between the two groups (Table 5). Promoter methylation and expression levels in the non-cancerous gastric mucosa of *H. pylori*-positive GC patients were compared before and after six 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 six 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 (Figure 4A). 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 six 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 six months (Figure 4B).

Table 5. Clinicopathological Characteristics of the *H. pylori*-Eradiation and Persistence Groups at Baseline and the 6-Month Follow-up

	Baseline		**6-months follow-up**		
	H. pylori-eradication (n=20)	*H. pylori*-persistence (n=20)	*H. pylori*-eradication (n=20)	*H. pylori*-persistence (n=20)	*P* value*
Age, yrs	70.0 (56.5-72.8)	65.0 (60.3-70.8)			
Sex, male	12 (60.0)	14 (70.0)			
Atrophy					0.327
Absent/mild	10 (50.0)	14 (70.0)	14 (70.0)	11 (55.0)	
Moderate/severe	10 (50.0)	6 (30.0)	6 (30.0)	9 (45.0)	
IM					0.519
Absent/mild	6 (30.0)	11 (55.0)	7 (35.0)	9 (45.0)	
Moderate/severe	14 (70.0)	9 (45.0)	13 (65.0)	11 (55.0)	
Neutrophil					<0.001
Absent/mild	1 (5.0)	0 (0.0)	17 (85.0)	2 (10.0)	
Moderate/severe	19 (95.0)	20 (100.0)	3 (15.0)	18 (90.0)	
Monocyte					0.001
Absent/mild	1 (5.0)	1 (5.0)	9 (45.0)	0 (0.0)	
Moderate/severe	19 (95.0)	19 (95.0)	11 (55.0)	20 (100.0)	

Data are n (%) or median (IQR).

IQR, interquartile range; IM, intestinal metaplasia

*Comparison between 6-months follow-up results of *H. pylori*-eradication and *H. pylori*-persistence group

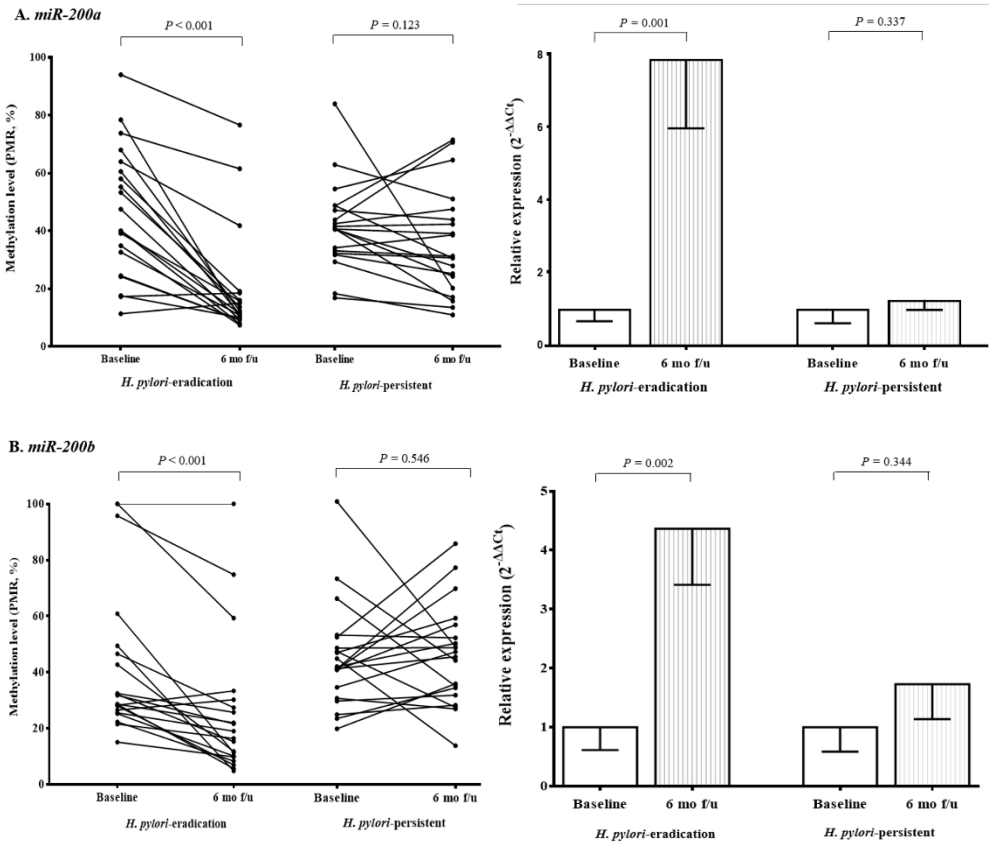

Figure 4. Change in promoter DNA methylation and corresponding miRNA expression according to the *H. pylori* eradication therapy. (A) *miR-200a.* (B) *miR-200b.* In *H. pylori*-eradication group, there was a significant difference in promoter methylation and expression level of *miR-200a/b* at 6 months after the eradication. miRNA, microRNA

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 gastric mucosa with *H. pylori* infection, which was also observed in non-cancerous gastric mucosa of patients with *H. pylori*-positive GC. These data suggest 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 knowledge, this is the first study 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 important mechanisms 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 patients with GC have investigated the expression levels of miRNA in GC tissues, compared with 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 patients with GC and normal gastric tissues of non-predisposed subjects, which might explore the risk of second primary GC after endoscopic treatment of the primary one.

The *miR-200* family is one of the most studied miRNA family in many carcinomas. The expression of *miR-200* family members has been 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 functions as tumor suppressor and lower expression of these members has been reported to be associated with poor prognosis of GC.[16,22,23] Although the mechanisms controlling the expression of the *miR-200* family members 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 5-Aza-2'-deoxycytidine 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 suggests the association of *miR-200a/b* in the *H. pylori*-related gastric carcinogenesis and suggests that methylation-dependent silencing may be an important mechanism to control its expression.

In this study, *H. pylori* eradication led to 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 *H. pylori* eradication 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 group at 6 months follow-up, which might be explained by the fact that aberrant methylation in *H. pylori*-infected gastric epithelial cells decreases through cell turnover after successful *H. pylori* eradication, while that in *H. pylori*-infected gastric stem cells

might persist even after *H. pylori* eradication.[38]

We demonstrated that the percentage of inflammatory cells dropped significantly at 6 months after *H. pylori* eradication. The degree of neutrophil and monocyte infiltration also significantly decreased 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 *H. pylori* infection have suggested that infection-associated inflammatory response was a critical factor. *H. pylori* infection has been known to induce the release of many pro-inflammatory cytokines, such as interleukin-1, 6, and 8, tumor necrosis factor-alpha, and nuclear factor κB, and also trigger a T1 helper cell-mediated inflammatory reaction.[39] These inflammatory dysregulations are considered to play a critical role in gastric inflammation and carcinogenesis.[38] In this study, *H. pylori* eradication led to a decrease in inflammatory response in gastric epithelial cells and further might reduce aberrant DNA methylation. Further studies are required to confirm the effect of *H. pylori* eradication on the cell-specific level, ie, 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 (Table 6). 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] Further research is needed to determine whether the levels of methylation vary depending on the location within the same individual.

Interestingly, mucosal atrophy and intestinal metaplasia did not show significant improvement after *H. pylori* eradication in this study (table 5). Recently, studies on the patients with advanced histologic changes have reported that *H. pylori* eradication can lead to histologic improvement of mucosal atrophy and intestinal metaplasia.[40,41] However, the time required to reach significant histologic improvement varies from study to study, ranging from 1 to 3 years for mucosal atrophy and 3 to over 5 years for intestinal metaplasia.[40,41] The follow-up period may have been too short to observe the reversibility of mucosal atrophy or intestinal metaplasia in this study.

This study has several strengths. To date, this is the first study 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 RT-PCR and MethyLight techniques to analyze miRNA expression and promoter methylation, which enabled sensitive and accurate quantitative analysis.

This study has also 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. 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-alteration of miR-200a/b and function changes in target genes, and, further, significance in the process of *H. pylori*-related gastric carcinogenesis.

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

Table 6. The promoter methylation levels of *miR-200a/b* in the *H. pylori*-negative, -positive controls, and *H. pylori*-positive gastric cancer (GC) patients

	***H. pylori*-negative controls (n=20)**	***H. pylori*-positive controls (n=20)**	***H. pylori*-positive GCs (n=40)**
miR-200a	11.5 (3.0, 27.9)	28.4 (8.2, 49.2)	40.3 (6.4, 100.0)
miR-200b	9.9 (3.0, 23.5)	15.3 (3.3, 25.5)	22.6 (2.8, 100.0)

Data are median (ranges) of percentage of methylated reference.

GC, gastric cancer

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국문 초록

서론: 본 연구에서는 헬리코박터 파일로리 연관 위암 발병과 관련된 *miR-200a/b*의 후성유전학적 변화를 확인하고, 제균치료를 통해 이러한 후성유전학적 변형을 역전시킬 수 있는지를 밝히고자 한다.

방법: 위암 세포주 및 헬리코박터 파일로리 음성 및 양성 대조군, 헬리코박터 파일로리 양성 위암 환자군의 위점막 조직에서 *miR-200a/b*의 발현 및 프로모터 메틸화 정도를 비교하였다. 또한, 위암에 대한 내시경적 절제술 후 헬리코박터 파일로리를 제균한 군과 감염이 지속된 군에서 6개월 후 *miR-200a/b*의 발현 및 프로모터 메틸화 정도의 변화를 비교하였다. miRNA 발현 수준은 real-time RT-PCR을 통하여, 프로모터 메틸화 정도는 MethyLight assay를 통하여 각각 측정하였다.

결과: 위암 세포주에서 탈메틸화 후 *miR-200a/b*의 발현이 증가함이 확인되었다. 인체 위점막 조직에서는 헬리코박터 파일로리 음성 대조군, 양성 대조군, 헬리코박터 파일로리 양성 위암 환자군의 순서로 *miR-200a/b*의 프로모터 메틸화 정도가 증가하였다 ($P < 0.001$). 반대로, *miR-200a/b*의 발현 수준은 같은 순서로 감소하였다 ($P < 0.001$). 6개월 후 헬리코박터 파일로리 감염 지속군에서는 *miR-200a/b*의 발현 및 프로모터 메틸화 정도에 유의한 차이가 없었으나, 제균치료 군에서는 프로모터 메틸화 정도가 유의하게 감소하고 ($P < 0.001$) *miR-200a* 및 *miR-200b*의

발현 수준이 유의하게 증가함이 관찰되었다 ($P = 0.001$ 및 $P = 0.002$).

결론: *miR-200a/b*의 후성유전학적 변화가 헬리코박터 파일로리 연관 위암 발병에 역할을 할 가능성이 있다. 이를 통해 형성된 필드 종양화는 헬리코박터 파일로리 제균치료를 통해 호전될 수 있는 것으로 나타났다.

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주요어: 헬리코박터 파일로리, 마이크로 RNA, 메틸화, 후성유전학적 변화, 위암

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