**Helicobacter pylori** Is Associated with miR-133a Expression through Promoter Methylation in Gastric Carcinogenesis

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**Background/Aims:** To investigate whether *Helicobacter pylori* eradication can reverse epigenetic silencing of microRNAs (miRNAs) which are associated with *H. pylori*-induced gastric carcinogenesis. **Methods:** We examined expression and promoter methylation of miR-34b/c, miR-133a, let-7a, and let-7i in gastric cancer cell line, before/after demethylation. Among them, epigenetically controlled miRNAs were identified. Their expression and promoter methylation was examined in human tissues of *H. pylori*-positive gastric cancer (T), *H. pylori*-positive gastritis (H), and *H. pylori*-negative controls (C). We also compared changes of miRNA expression and promoter methylation in *H. pylori*-positive patients who were endoscopically treated for early gastric cancer, between baseline and 1 year later according to eradication status. **Results:** In gastric cancer cell line, miR-34b/c and miR-133a showed epigenetic silencing. In human tissues, miR-34b/c and miR-133a showed serial increase of promoter methylation in order of C, H, and T (all, p<0.01), and the miR-133a expression showed serial decrease (C vs H, p=0.02; H vs T, p=0.01; C vs T, p<0.01) while miR-34b and miR-34c expressions did not. *H. pylori* eradication induced decrease of methylation (p<0.01) and increase of miR-133a expression (p=0.03), compared with noneradication group. **Conclusions:** This result suggests *H. pylori* eradication could reverse methylation-silencing of miR-133a which is involved in *H. pylori*-induced gastric carcinogenesis. *(Gut Liver 2018;12:58-66)*

**Key Words:** Helicobacter pylori; Methylation; MicroRNAs; Stomach neoplasms

**INTRODUCTION**

MicroRNAs (miRNAs) are short noncoding RNAs consisting of about 22 nucleotides, which are known to function in post-transcriptional modulation in the way of epigenetic changes such as translational repression or messenger RNA cleavage. Recently, many studies have revealed that various miRNAs are involved in human carcinogenesis. For example, miR-21 has been shown to be associated with adenoma-carcinoma sequence of colon cancer. Also, miR-21 and miR-27a were reported to be related to gastric carcinogenesis. DNA methylation is another well-known epigenetic phenomenon which induces transcriptional regulation, and it is already established that a great deal of tumor suppressor genes are controlled by promoter methylation. In the meanwhile, miRNA expression is also influenced by epigenetic alterations. Several studies have reported that expression of certain miRNAs are epigenetically regulated by promoter CpG island methylation of the miRNA genes in gastric cancer. miR-34b/c was previously found to be silenced by promoter CpG island hypermethylation in colorectal cancer and gastric cancer. Also, a recent study has shown that aberrant methylation of miR-34b/c is associated with metachronous gastric cancer. This study also showed that the promoter methylation of miR-34b/c is related with *Helicobacter pylori* infection. *Let-7* family is an essential developmental regulator, which is one of the first known miRNAs. *Let-7* genes are thought to be related with tumor suppression, as the expression of which is considerably low in many cancers. In a previous study, *let-7* expression was shown to be downregulated by cytotoxin-associated gene A of *H. pylori* by histone modification and DNA methyla-
tion of its promoter.\textsuperscript{3} miR-133 is another miRNA which was reported to be associated with \textit{H. pylori} infection. A previous study has revealed that miR-133 was downregulated by long-term infection with \textit{H. pylori} in mice.\textsuperscript{15} In recent studies, miR-133 was demonstrated to play tumor suppressive role in gastric carcinogenesis.\textsuperscript{16-19} However, whether \textit{H. pylori} eradication therapy could reverse the modulation of these miRNAs has not been clarified yet. Therefore, we aimed to clarify whether the miRNAs which are associated with gastric carcinogenesis could be reversed by \textit{H. pylori} eradication therapy.

**MATERIALS AND METHODS**

1. **miRNA and promoter CpG island selection**

   Based on literature review, miR-34b/c, let-7, and miR-133a were selected as miRNAs which are possibly associated with \textit{H. pylori}-induced gastric carcinogenesis. miR-34b and miR-34c are a pair of miRNAs, whose genes are closely located within 418 bp distance in 11q23.1, and they are encoded by common promoter sequence. Therefore we measured expression levels of miR-34b and miR-34c each, and promoter methylation levels of miR-34b/c. Among let-7 series, let-7a-3 located in 22q13.31 and let-7i in 12q14.1 were selected, as they were the only miRNAs whose promoters contain CpG islands. miR-133a is a miRNA encoded by two genes, miR-133a-1 and miR-133a-2. Among them, we measured promoter methylation of miR-133a-2 gene for the same reason as in let-7.

2. **Cell line and demethylation**

   Gastric cancer cell lines AGS and KATO III (Table 1) were obtained from Korean Cell Line Bank and cultured in RPMI 1640 with L-glutamine (300 mg/L), 25 mM HEPES and 25 mM NaHCO\textsubscript{3}, 90%; heat inactivated fetal bovine serum, 10%. On day 0, cells were seeded, and the media was added with 2 μM 5-Aza-2’-deoxycytidine (Sigma-Aldrich and Merck KGaA, Darmstadt, Germany), the demethylating agent, on the next day. Cells were treated with 5-Aza-2’-deoxycytidine for 72 hours, while daily replacing the demethylating agent and medium. On day 4, cells were harvested.

3. **Tissue samples**

   Gastric mucosal tissue samples were obtained from cancerous mucosa of 24 patients with gastric cancer (T, tumor group), antral mucosa of 24 patients with \textit{H. pylori}-positive gastritis (H, \textit{H. pylori}-gastritis group), and antral mucosa of 24 \textit{H. pylori}-negative healthy volunteers (C, control group). All the T, H, and C groups were enrolled between October 2013 and September 2014. \textit{H. pylori} status was considered positive when either the rapid urease test or histologic examination showed a positive result. To assess the effect of \textit{H. pylori} eradication, noncancerous mucosal tissues of 24 \textit{H. pylori}-positive early gastric cancer
patients who were endoscopically treated were also obtained between January 2012 and December 2012. Then they were randomly assigned to H. pylori eradication group (E) and noneradication group (NE). E group received 20 mg of omeprazole, 1 g of amoxicillin, and 500 mg of clarithromycin, twice a day for 7 days. Twelve months later, gastric antral mucosal tissues were taken from the E and NE groups during the follow-up endoscopy and rapid urease tests were performed as well. Final H. pylori status was also evaluated with rapid urease test and histologic examination. When either of those studies showed positive, it was considered as positive. From all tissue samples, degrees of atrophic gastritis and intestinal metaplasia were measured by the updated Sydney system. And then the remaining tissue samples were restored at –80°C. Also, degrees of neutrophilic infiltration and monocytic infiltration were measured. All the patients enrolled in this study were ≥18 years old, had no other cancer, and were not taking nonsteroidal anti-inflammatory drugs nor proton pump inhibitors. This study was approved by Seoul National University Hospital Institutional Review Board (IRB number: H-1309-017-518) and complied with the Declaration of Helsinki. From all the participants, informed consent was obtained before tissue retrieval.

4. Real-time reverse transcription polymerase chain reaction of miRNA

The miRNAs were isolated from tissues and cells stored at −80°C using mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA). Reverse transcription of the miRNAs into the single-stranded cDNAs were performed using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). Quantitative polymerase chain reaction (PCR) was conducted using TaqMan Universal Master Mix II (Applied Biosystems). The relative expression levels of the miRNAs were calculated using the relative quantification ($2^{-\Delta\Delta Ct}$) method with duplicate measurements for tissue samples. However, cell line experiments were performed in triplicate to minimize potential errors induced by misidentification or contamination.

5. DNA isolation and bisulfite modification

DNA was isolated from the tissues and cells using phenol-chloroform extraction method. Bisulfite modification which converts unmethylated cytosine into uracil was performed using EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) (Table 2).

6. Methylation analysis

For methylation analysis, we used MethyLight technique based on quantitative PCR method with duplicate measurements for tissue samples and triplicate for cell lines. Pairs of primers and probes to bind bisulfite-converted DNA were designed using the software, Beacon Designer (Premier Biosoft, Palo Alto, CA, USA). To report the levels of DNA methylation, percentage of methylated reference (PMR) was calculated as follows: PMR=100×(methylated reaction/ALU)sample/(methylated reaction/ALU)M.Sssl.

7. Statistical analysis

To analyze continuous variables with normal distribution, t-test or analysis of variance was applied between two or more than two groups, respectively. To analyze continuous variables which are not normally distributed, Wilcoxon rank-sum test and Wilcoxon signed-rank test were applied for independent and paired samples, respectively. For nominal variables, chi-square test or Fisher exact test was applied. When >20% of expected frequencies were ≤5, Fisher exact test was used. Otherwise, chi-square test was used. For repeated measures, generalized linear mixed model was applied. p-values less than 0.05 were considered significant. p-values were presented without multiple test-

| Table 2. Primers and Probes Used in MethyLight Assay |
|-------------------------------|-----------------------------|----------------|-----------|
| **Gene** | **Primer/probe** | **Sequence (5’→3’)** | **Length, bp** | **T_m, °C** |
| miR-34b/c | Forward primer | TTCGCCGGGGTTTAAGGACG | 20 | 59.0 |
| | Reverse primer | CAAACCTAAAACACTCTCTCGAC | 26 | 58.9 |
| | Probe | CGCCCGCTCTAAACGACCAATAACTAT | 28 | 66.3 |
| let-7a-3 | Forward primer | GGAGTTGAGAGTTGATATGTC | 25 | 59.2 |
| | Reverse primer | CGCCCTACCTCCAAAATATCT | 22 | 59.1 |
| | Probe | AACATAAACCTACCTCCGACCCGACCTACTTC | 30 | 67.7 |
| let-7i | Forward primer | TTTCGAGGTTGTTGGGAAC | 21 | 59.0 |
| | Reverse primer | CGCAGCAAACTCCCCGACCAACA | 20 | 59.6 |
| | Probe | TACCCGCCGACTCCGACCCAACCACCA | 25 | 68.1 |
| miR-133a-2 | Forward primer | GTCAGATCTATTCACAACCTACG | 25 | 58.8 |
| | Reverse primer | CGGGAGGTTATGCGGT | 20 | 58.7 |
| | Probe | CACCACCGTAACGCACACCGCC | 26 | 66.6 |

T_m, melting temperature.
RESULTS

1. Identification of miRNAs silenced by promoter methylation in gastric cancer cell line

In gastric cancer cell lines, AGS and KATO III, the levels of expression of miRNAs and promoter DNA methylation were measured before and after demethylation with 5-Aza-2’-deoxycytidine. After demethylation, the promoter methylation levels of miR-34b/c and miR-133a-2 were decreased by about 30% in AGS and about 50% in KATO III, and the levels of expression of miR-34b, miR-34c, and miR-133a were increased by about 10-fold in AGS and more than 3-fold in KATO III. On the other hand, the promoter methylation levels of let-7i were decreased by less than 10% in AGS and 60% in KATO III, and its expression levels were increased by only about 2-fold in AGS and KATO III after demethylation. The promoter methylation levels of let-7a were decreased by about 30% in AGS and 50% in KATO III, and the expression levels of which were only increased by 70% in AGS and 40% in KATO III after demethylation (Fig. 1).

2. Clinicopathological characteristics of enrolled patients

Overall 96 patients were enrolled in this study. Their clinicopathological characteristics are presented in Table 3. Mean age was the highest in T, then H and C group, respectively (p<0.01). Also, the degrees of atrophic gastritis, intestinal metaplasia, neutrophilic infiltration, and monocytic infiltration showed the same pattern (all, p<0.01). However, there was no significant difference in gender proportion among the three groups (p=0.25). Also, there was no significant difference in clinicopathological characteristics between the eradication and noneradication group, at the time of enrollment.

3. Promoter methylation and expression levels of miRNAs according to disease status

In human gastric mucosal tissues, we measured promoter methylation levels of miR-34b/c and miR-133a-2, which were found to be epigenetically silenced by promoter methylation in above mentioned gastric cancer cell line experiment. In promoters of both miRNAs, the methylation levels were the highest in T, then H and C group, respectively, and the difference between each pair of groups was statistically significant (all, p<0.01) (Fig. 2). We then examined the expression levels of miR-34b, 34c,
Table 3. Baseline Clinicopathological Characteristics

| Characteristic                | Tumor (n=24) | H. pylori-gastritis (n=24) | Control (n=24) | p-value* | Near tumor eradication (n=12) | Near tumor noneradication (n=12) | p-value† |
|------------------------------|--------------|---------------------------|---------------|----------|------------------------------|---------------------------------|----------|
| Age, yr                      | 63.5 (53.5–69.8) | 59.0 (42.3–64.0) | 42.5 (33.3–52.0) | <0.01    | 53.5 (50.5–60.8) | 57.0 (49.0–67.3) | 0.50    |
| Sex                          | Male         | 14 (58.3) | 9 (37.5) | 9 (37.5) | 0.25 | 11 (91.7) | 8 (66.7) | 0.32    |
|                             | Female       | 10 (41.7) | 15 (62.5) | 15 (62.5) | 0.25 | 1 (8.3) | 4 (33.3) | 0.32    |
| Atrophic gastritis‡          | Absent       | 5 (29.4) | 9 (39.1) | 23 (95.8) | <0.01 | 2 (16.7) | 4 (33.3) | NA      |
|                             | Mild         | 3 (17.6) | 10 (33.3) | 1 (4.2) | 5 (41.7) | 3 (25.0) | 0.32    |
|                             | Moderate     | 7 (41.2) | 4 (17.4) | 0 | 5 (41.7) | 1 (8.3) | 0.32    |
|                             | Marked       | 2 (11.8) | 0 | 0 | 0 | 1 (8.3) | 0.32    |
| Intestinal metaplasia        | Absent       | 4 (16.7) | 10 (41.7) | 24 (100) | <0.01 | 2 (16.7) | 1 (8.3) | 0.83    |
|                             | Mild         | 7 (29.2) | 9 (37.5) | 0 | 4 (33.3) | 5 (41.7) | 0.32    |
|                             | Moderate     | 7 (29.2) | 4 (16.7) | 0 | 4 (33.3) | 4 (33.3) | 0.32    |
|                             | Marked       | 6 (25.0) | 1 (4.2) | 0 | 2 (16.7) | 2 (16.7) | 0.32    |
| Neutrophilic infiltration    | Absent       | 1 (4.2) | 0 | 21 (87.5) | <0.01 | 0 | 0 | 0.65    |
|                             | Mild         | 0 | 0 | 3 (12.5) | 1 (8.3) | 0 | 0 | 0.65    |
|                             | Moderate     | 16 (66.7) | 18 (75.0) | 0 | 9 (75.0) | 10 (83.3) | 0.32    |
|                             | Marked       | 7 (29.2) | 6 (25.0) | 0 | 2 (16.7) | 2 (16.7) | 0.32    |
| Monocytic infiltration       | Absent       | 0 | 0 | 0 | <0.01 | 0 | 0 | 1.00    |
|                             | Mild         | 2 (8.3) | 0 | 22 (91.7) | 0 | 0 | 0 | 1.00    |
|                             | Moderate     | 16 (66.7) | 15 (62.5) | 2 (8.3) | 10 (83.3) | 9 (75.0) | 0.32    |
|                             | Marked       | 6 (25.0) | 9 (37.5) | 0 | 2 (16.7) | 3 (25.0) | 0.32    |

Data are presented as median (interquartile range) or number (%).
H. pylori, Helicobacter pylori; NA, not available.
*Comparison was performed among tumor, H. pylori-gastritis, and control group; †Comparison was performed between near tumor eradication and noneradication group. Results are showing their initial status, not the status after treatment; ‡Because of missing data, the sum of each does not match the total.

Fig. 2. Promoter methylation levels, reported as percentage of methylated reference (PMR), of miR-34b/c (A) and miR-133a-2 (B) in gastric mucosal tissues of Helicobacter pylori negative control, H. pylori positive gastritis, and H. pylori positive gastric cancer. Hp+, H. pylori positive. *p<0.01.
and 133a. Among them, only miR-133a showed serial decrease of expression level in the order of C, H, and then T group. The difference between every two groups was also significant (p=0.02 in C vs H, p=0.01 in H vs T, and p<0.01 in C vs T) (Fig. 3C). In the meanwhile, although miR-34b and 34c showed lower levels of expression in T compared with C and H, respectively (both miR-34b and 34c; p<0.01 in T vs C, p<0.01 in T vs H), they showed no significant difference between C and H group [miR-34b, p=0.63; miR-34c, p=0.90] (Fig. 3A and B).

4. Promoter methylation and expression level of miR-133a under age adjustment

As the age showed significant difference among the three groups, we further performed adjustment analysis. We performed analysis of covariance for age adjustment in promoter methylation and expression levels of miR-133a, which was the only miRNA showing serial increase of methylation and decrease of miRNA expression in the progress of H. pylori-related gastric cancer development. In this analysis, the promoter methylation was found to be significantly different among C, H, and T group, even after the age adjustment (p<0.01). Besides, age was not a predictor for the promoter methylation (p=0.70). Also, the miR-133a expression level showed significant difference among the three groups under the age adjustment (p=0.01), and age was not associated with the miR-133a expression level (p=0.71) (Table 4).

5. Changes of promoter methylation and expression of miR-133a according to H. pylori eradication status

Among the E and NE groups which all had current H. pylori infection, half eradicated H. pylori (E group) and half did not (NE group). In E group, the eradication was successful in every patient. One year later, the promoter methylation level tended to decrease in the E group, while it increased in the NE group (Fig. 4A). On the other hand, miR-133a expression showed increasing tendency in the E group, while it did not show significant change among the NE group (Fig. 4B). The difference in changing patterns of promoter methylation among the E and NE groups was analyzed by using generalized linear mixed
miR-133a, Bcl-xl, growth factor 1 receptor, and proliferation, such as transcription factor Sp1, have been suggested to be involved in gastric carcinogenesis in gastric cancer was also confirmed in The Cancer Genome role in gastric cancer in several studies. has been revealed that cellu-
lar carcinoma, cervical cancer, prostate cancer, colorectal cancer, -small cell lung cancer, cervical cancer, pancreatic cancer, breast cancer, hepatocellular carcinoma, and gallbladder cancer. In addition, it has been revealed that miR-133a also plays tumor suppressive role in gastric cancer in several studies. Its downregulation in gastric cancer was also confirmed in The Cancer Genome Atlas dataset. So far, various target molecules of miR-133a have been suggested to be involved in gastric carcinogenesis and proliferation, such as transcription factor Sp1, insulin-like growth factor 1 receptor, antiapoptotic molecules, Mcl-1 and Bcl-xl, and ERBB2. However, the underlying mechanism of miR-133a downregulation has not been well discovered yet.

**DISCUSSION**

This study demonstrated that suppression of miR-133a by its promoter methylation is associated with H. pylori-related gastritis and gastric cancer, and it could be reversed by H. pylori eradication. As far as we know, this is the first study to verify the reversibility of methylation-induced miR-133a downregulation caused by H. pylori infection through H. pylori eradication in relation to gastric carcinogenesis.

Although miR-133a was previously known as a muscle-specific miRNA, it was recently established to function as a tumor suppressor in various types of cancer, such as bladder cancer, head and neck cancer, lung squamous cell carcinoma, prostate cancer, colorectal cancer, non-small cell lung cancer, cervical cancer, pancreatic cancer, breast cancer, hepatocellular carcinoma, and gallbladder cancer. In addition, it has been revealed that miR-133a also plays tumor suppressive role in gastric cancer in several studies. Its downregulation in gastric cancer was also confirmed in The Cancer Genome Atlas dataset. So far, various target molecules of miR-133a have been suggested to be involved in gastric carcinogenesis and proliferation, such as transcription factor Sp1, insulin-like growth factor 1 receptor, antiapoptotic molecules, Mcl-1 and Bcl-xl, and ERBB2. However, the underlying mechanism of miR-133a downregulation has not been well discovered yet.

A previous study, miR-133a downregulation was found to be related with H. pylori infection in mice. Herein, we assumed that miR-133a would be regulated by promoter methylation induced by H. pylori infection. We have demonstrated that epigenetic modification, especially promoter methylation downregulates miR-133a by showing the decrease of methylation and increase of miR-133a expression by treatment with demethylating agent in gastric cancer cell culture. However, in a previous study, treatment with demethylating agent could not upregulate miR-133a expression in gastric cancer cell line unlike histone methylation inhibitor and deacetylation inhibitor. Although the reason for this discrepancy is unclear, the different time interval before and after the treatment may have influenced. In the previous study, the intervals were 12 hours and 24 hours, whereas ours was 72 hours. Probably, sufficient recovery of miR-133a expression by demethylation may take longer than 24 hours. Similar to our results, there have been a study which revealed silencing of miR-133a by DNA methylation, although it was in colorectal cancer. Also another study have shown miR-133a downregulation by DNA methylation in relation to the development of cardiac hypertrophy induced by air-pollution. However, they showed methylation of only two CpG sites located at the transcription start site of miR-133a rather than a CpG island located in the promoter site.

In current study, the promoter methylation showed consistent increase and the miR-133a expression showed consistent decrease according to the H. pylori-related disease severity: from H. pylori-negative control to H. pylori-gastritis, and then H. pylori-positive gastric cancer. This implies the possibility that the epigenetic silencing of miR-133a may be initiated by chronic H. pylori infection. As this study only examined H. pylori-infected gastric cancer patients, the methylation-silencing of miR-133a may not be involved in every gastric carcinogenesis. H. pylori-unrelated gastric carcinogenesis may be free from this mecha-

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**Fig. 4.** Changes of promoter methylation levels (A) and expression levels (B) of miR-133a before and 1 year after Helicobacter pylori eradication therapy in normal mucosa of H. pylori positive gastric cancer patient. PMR, percentage of methylated reference.
nism. Also, most of the patients had intestinal type gastric cancer except for one diffuse type and one mixed type cancer in the noneradication group. Therefore we could not know if it would be the same in diffuse type gastric cancer. Nevertheless, in those with *H. pylori* infection, miR-133a downregulation may play important role in *H. pylori*-related intestinal type gastric cancer development.

Our study result also showed that the downregulation of miR-133a by *H. pylori* infection could be reversed by *H. pylori* eradication, which was also through the decrease of promoter methylation. This finding implies that some epigenetic modification involved in gastric carcinogenesis could be easily treated at certain time after once developed. Moreover, the gastric mucosal tissues used in this eradication experiment were normal-appearing mucosa from those with gastric cancer. Considering the epigenetic field cancerization,\(^6,7\) this result suggests that the reversibility of miR-133a expression remains after gastric cancer development. As miR-133a downregulation is already proven to promote tumor proliferation and migration,\(^4\) treating the modification might be also effective in gastric cancer treatment, not only prevention.

Although there were age differences among the three groups, the different methylation and miR-133a expression levels were the same even after the age adjustment. Also, the small number of patients enrolled in this study is another limitation. Nonetheless, the fact that we showed statistical significance even with this small number further supports our results. Another limitation is that the comparison between cancer and noncancerous mucosa of cancer patients could not be made in this study because we did not perform paired sampling. This comparison should be made in further researches. Also the fact that this study did not evaluate the mechanism how *H. pylori* modulates methylation or how miR-133a regulates tumorigenesis is another limitation. It was over the scheme of this study. We believe this should be further studied in later researches to better understand this phenomenon.

In conclusion, downregulation of miR-133a caused by promoter methylation might be associated with *H. pylori*-related gastric carcinogenesis, and *H. pylori* eradication showed capacity to reverse its expression. Therefore, treating *H. pylori* infection even after gastric cancer development may have some portion of therapeutic role. Also this could be used as a biomarker for early diagnosis and a therapeutic target in gastric cancer. For such clinical use, further large scale studies are warranted.

**CONFLICTS OF INTEREST**

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

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