Long-term changes in aberrant DNA methylation and gastritis after *Helicobacter pylori* eradication focused on metachronous gastric cancer

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**Abstract**

**Background:** A persistently high methylation level in gastric mucosa after *Helicobacter pylori* (*H. pylori*) eradication is presumed to be a risk for metachronous gastric cancer (MGC); however, long-term changes in aberrant DNA methylation and histological gastritis have been unclear. Our aim was to examine changes in DNA methylation and histological gastritis according to the occurrence of MGC.

**Methods:** Subjects were classified into three groups: 25 patients in whom MGCs occurred after the initial endoscopic resection (ER) for early gastric cancer and *H. pylori* eradication (MGC group), 17 patients in whom MGC did not occur for more than 5 years after the initial ER and *H. pylori* eradication (non-MGC group) and 29 patients without a history of gastric cancer who succeeded in eradication more than 5 years ago (HP group). Aberrance of DNA methylation in three genes (miR-124a-3, EMX1, NKX6-1) and histological score of atrophy and intestinal metaplasia (IM) were evaluated using biopsy samples before and more than a mean of 5 years after *H. pylori* eradication. Also, the mean Z-score was calculated using Z-score values of the three genes.

**Results:** The methylation level of miR-124a-3 in the HP group and non-MGC group and that of EMX1 in the HP group significantly decreased in the long term after eradication. In the MGC group, *H. pylori* eradication did not improve aberrant methylation, and the mean Z-score significantly increased. There were significant positive correlations between methylation levels in miR-124a-3 and EMX1 and histological findings after eradication.

**Conclusions:** A persistently high methylation level after *H. pylori* eradication reflected precancerous mucosal conditions and led to long-term MGC.

**Keywords**
DNA methylation, *Helicobacter pylori*, metachronous neoplasm
1 | INTRODUCTION

According to the GLOBOCAN 2018 report, gastric cancer is the third deadliest cancer, with an estimated 783,000 deaths and 1,000,000 new cases worldwide in 2018.\(^1\) *Helicobacter pylori* (*H. pylori*) infection induces histological inflammatory changes in the gastric mucosa, such as atrophic gastritis and intestinal metaplasia (IM), which is a risk factor for gastric cancer.\(^2,3\) The International Agency for Research on Cancer working group declared in 1994 that *H. pylori* was a human carcinogen and that screening and treatment programs for *H. pylori* were recommended in all countries.\(^4,5\) The Kyoto global consensus report in 2015 stated that all *H. pylori*-positive individuals worldwide should receive eradication therapy.\(^6\) Successful eradication of *H. pylori* prevents gastric cancer development.\(^7\) Several randomized controlled trials and meta-analyses showed the cancer-preventive effects of *H. pylori* eradication, but gastric cancer also occurred to some degree after *H. pylori* eradication (1.0–1.6% in primary gastric cancer, 2.7–6.1% in metachronous gastric cancer [MGC]).\(^8–13\) Risk factors for MGC after successful *H. pylori* eradication have been reported to be age, male sex, severe atrophy, and IM. Atrophy and IM are considered to be the most important factors in gastric carcinogenesis, and *H. pylori* eradication improves atrophy and IM, which leads to the prevention of gastric cancer.\(^14–18\) On the other hand, there have been some reports on conflicting results, and whether *H. pylori* eradication improves atrophy and IM is controversial.\(^19–21\)

DNA methylation, which accumulates in both gastric cancers and noncancerous mucosa, is an important epigenetic change associated with gastric carcinogenesis.\(^22–25\) *H. pylori* infection accumulates DNA methylation in the gastric mucosa,\(^26\) and *H. pylori* eradication decreases aberrant methylation in some gene promoters, which also decreases the risk of gastric cancers.\(^27–29\) Asada et al.\(^30\) reported that the occurrence of MGC after endoscopic resection (ER) of early gastric cancers in patients with *H. pylori*-negative or *H. pylori*-eradicated gastric cancer was associated with a high methylation level of miR-124a-3 at a median 3-year follow-up. A persistently high methylation level in gastric mucosa after *H. pylori* eradication is presumed to be a risk factor for MGC.\(^31,32\) Although a positive correlation between histological IM and methylation levels in gastric mucosa has been reported,\(^33\) there have been no reports on both of these changes for long-term surveillance of MGC. Based on the fact that recovery of atrophy and IM takes for at least several years after *H. pylori* eradication, herein, we examined the relationship between histological gastritis and methylation level focused on the long-term occurrence of MGC.\(^34\)

2 | METHODS

2.1 | Patients

Between January 2004 and December 2018, 184 patients were positive for *H. pylori* at the initial ER for early gastric cancer and were successfully eradicated after ER at our institution. Among these patients, patients who received annual endoscopy for more than 5 years and in whom biopsy specimens were properly obtained were selected, and they were divided into groups with and without MGC. MGC was defined as newly discovered cancer more than 1 year after eradication. The criteria of MGC were as follows: (1) each lesion was histopathologically malignant, (2) each lesion was separated from another, and (3) each lesion was not the result of a local extension or metastasis of other lesions.\(^35\) The following patients were excluded: (1) those who were *H. pylori*-negative at the initial ER (including those who had spontaneous or previous eradication), (2) those who failed or refused *H. pylori* eradication, (3) those whose biopsy specimens were not properly collected or too small, (4) those whose follow-up was <5 years after eradication, and (5) those in whom local recurrence was found, (6) those who received surgical gastrectomy before and after eradication. Finally, 25 patients in whom MGCs occurred were included in the MGC group, and 17 patients in whom MGC did not occur were included in the non-MGC group. As a control (HP group), 29 patients without gastric cancer for more than 5 years in annual endoscopy follow-up after successful eradication were analyzed (Figure 1, Figure S1).

This study was performed in accordance with the ethical standards detailed in the Declaration of Helsinki. The authors’ institutional ethics committee approved this study, and all patients provided written informed consent (Hokkaido University Hospital Review Board 019-015).

2.2 | Determination of *H. pylori* infection status

*Helicobacter pylori* infection status was investigated by using the urea breath test (cutoff value <2.5‰; Ubit®, Otsuka Pharmaceutical), and serum anti-*H. pylori* antibody test (cutoff value <10 U/ml, E-Plate Eiken *H. pylori* antibody, Eiken Chemical Co., Ltd.) and histopathology from the greater curvatures in the antrum and the corpus. *H. pylori*-positive status was determined when at least one test was positive. For judgment, at least two tests were performed more than 8 weeks after completion of eradication therapy.

2.3 | Biopsy samples

Endoscopic biopsy specimens obtained from noncancerous gastric mucosa of the antrum (2 cm from the pylorus at the greater curvature) were used for the measurement of methylation and histological assessment. For the MGC group, biopsy samples before the first ER (before eradication) and when MGCs were detected after eradication were used. In the HP group and non-MGC group, biopsy samples before and at 5 years after eradication were prepared.

2.4 | Histological assessment of atrophy and IM

All biopsy specimens were stained with hematoxylin and eosin and evaluated according to the updated Sydney system by pathologists who had no information about the results of endoscopy and *H. pylori* infection status.
tests. A visual analog scale according to the updated Sydney system was converted to a score in which 0 = absent, 1 = mild, 2 = moderate, and 3 = severe.

2.5 | Quantitative methylation analysis

The DNA methylation levels of three gene promoters (miR-124a-3, EMX1 and NKX6-1) related to gastric cancer after *H. pylori* eradication were analyzed. From formalin-fixed paraffin-embedded specimens, twenty 10-μm-thick tissue sections were cut for DNA extraction. DNA was extracted using the QIAamp DNA FFPE Tissue Kit (QIAGEN). Bisulfite treatment of DNA was performed using an EpiTect bisulfite kit (QIAGEN). Biotinate polymerase chain reaction was performed using primers that specifically amplify the sequences of the three genes (Figure S2). All PCR assays included a denaturation step at 95°C for 30 s, followed by an annealing step at various temperatures for 30 s and an extension step at 72°C for 30 s. Pyrosequencing was performed using PSQ HS 96 Gold single-nucleotide polymorphism reagents on a Pyromark Q24 pyrosequencing machine (Biotage). The protocol for pyrosequencing has been described in detail previously. Pyrosequencing quantitatively measures the methylation status of several CpG sites in a given promoter. These adjacent sites usually show highly concordant methylation. Therefore, the mean percentage of methylation of detected sites was used as a representative value for each gene promoter.

Also, Z-score was calculated for normalization of the methylation level in each three gene as follows; Z-score = (methylation level of each sample – mean value of methylation level)/standard deviation of methylation level. The mean Z-score was calculated using Z-score values of the three genes.

2.6 | Statistical analysis

Continuous variables such as methylation level, age, and period were compared using Student’s t-test or paired t-test. Histological atrophy and IM scores were compared using the Mann–Whitney U test or Wilcoxon’s rank-sum test. Correlations between methylation level and histological atrophy/IM score were analyzed using Spearman’s rank correlation coefficient. A p value of <.05 in each analysis was considered statistically significant.

3 | RESULTS

3.1 | Characteristics of patients and histological score

The characteristics of the patients and samples used are shown in Table 1. Table 2 shows histological score of atrophy and IM. The atrophy score before eradication was significantly lower in the HP group.
**Figure 2** shows changes in methylation levels before and after eradication in each group. There were no significant differences in the methylation level, including three genes and mean Z-score, before eradication among the three groups. In the HP group, the mean methylation levels of miR-124a-3 and EMX1 significantly decreased after eradication (miR-124a-3: \( p < .01, \) EMX1: \( p = .03 \)). In the non-MGC group, only the mean methylation level in miR-124a-3 significantly decreased (\( p < .01 \)). In the MGC group, the mean methylation level in NKX6-1 was increased after eradication (\( p = .04 \)), and mean Z-score was significantly increased (before: \( -0.12 \pm .71 \), after: \( 0.30 \pm .78, p = .03 \)). But mean Z-score decreased after eradication in the HP group (before: \( 0.13 \pm .59, \) after: \( -0.31 \pm .56, p < .01 \)).

There were significant differences in the mean methylation levels of miR-124a-3 and EMX1 after \( H.\ pylori \) eradication between the MGC group and HP group (\( p < .01 \)). Individual data are shown in Figure 3.

In the MGC group, six patients had repeated metachronous cancers more than twice after eradication, and methylation was measured using samples at the occurrence of MGC. In four patients who repeated MGC for more than 5 years after eradication, the methylation level and mean Z-score showed an increase during follow-up (Figure 3).

### 3.2 Aberrant DNA methylation

Correlations between histological atrophy/IM and DNA methylation

Correlations between histological atrophy/IM and DNA methylation are shown in Figure 4. There was a good positive correlation between the methylation level of miR-124a-3 and the IM score after eradication (\( p < .01, r = .82 \)). Additionally, the EMX1 gene promoter methylation level also had a moderate correlation with atrophy and IM after eradication (\( p < .001, r = .59; p < .001, r = .64 \)).

To identify risk factors for GC development, we performed multivariate logistic regression analysis including age at \( H.\ pylori \) eradication, sex, severity of IM after \( H.\ pylori \) eradication and changes in methylation levels. There were no significant predictors for GC development (data not shown).
Our data showed that methylation in gastric mucosa did not improve long term after *H. pylori* eradication in the MGC group, and a persistently high methylation level that was correlated with severe atrophy and IM after successful eradication would be a risk factor for MGC.

It has been reported that methylation before eradication reflects both temporary factors such as inflammation and permanent factors such as chronic gastritis, and methylation levels before eradication are not necessarily associated with gastric cancer risk. High methylation levels after *H. pylori* eradication are one of the risk factors for MGC. *H. pylori* eradication reduces methylation and prevents carcinogenesis. However, persistence of high methylation caused MGC, and patients with repeated MGC tended to have increased methylation. Asada et al reported that high methylation in gastric mucosa after
ESD for early gastric cancer related to high incidence of MGC, but in many cases the timing of eradication is unclear and long-term changes in methylation levels after eradication were not studied. In other previous studies, individual methylation data had not been followed up, and the observation periods were short. The strength of our study was that we were able to measure methylation levels and to examine changes in histology in the long term after successful eradication.

The mechanism by which methylation improves after eradication is postulated to be as follows. First, *H. pylori* infection induced methylation of differentiated cells. After *H. pylori* eradication, differentiated cells with methylation disappear over time and are replaced by normal differentiated cells derived from a stem cell without methylation. In contrast, if a stem cell is methylated, the methylation of differentiated cells in that gland duct will not improve after eradication because an entire gland reflects the methylation status of its stem cell and methylation status in stem cells is preserved and replicated. In cases with MGC after eradication, many stem cells had been methylated before eradication. Even if many methylated cells are shed after *H. pylori* eradication because cell differentiation from methylated stem cells continues, it is possible that the methylation level does not improve after eradication. Additionally, there may be other factors besides *H. pylori* that increase methylation levels in gastric mucosa, such as age, familial history, and smoking.

**FIGURE 3** Changes in DNA methylation levels in patients with repeated metachronous cancer. In the MGC group, six patients had repeated metachronous gastric cancer (MGC) more than twice after eradication. Methylation levels before eradication and when MGCs were detected after eradication were plotted for each of the six patients. Aberrant methylation in all three genes seemed to accumulate over time after eradication.

**FIGURE 4** Correlations between histological findings and DNA methylation level in gastric mucosa. Correlation coefficients between histological score and methylation level were calculated using Spearman's rank correlation coefficient. (A) Correlations between histological atrophy and methylation level. There were moderate correlations between miR-124a-3 and atrophy score before and after eradication (*p* < .001, *r* = .50). After eradication, there was also a moderate correlation between EXM1 and atrophy (*p* < .001, *r* = .59). (B) Correlations between histological intestinal metaplasia and methylation level. There was a strong correlation between the methylation level of miR-124a-3 and the intestinal metaplasia (IM) score after eradication (*p* < .001, *r* = .82). Additionally, the EMX1 gene promoter methylation level also had a significant correlation with IM after eradication (*p* < .001, *r* = .64).
(A) Correlation between atrophy and methylation

Before eradication

miR-124a-3

EMX1

NKKX6-1

After eradication

miR-124a-3

EMX1

NKKX6-1

(B) Correlation between IM and methylation

Before eradication

miR-124a-3

EMX1

NKKX6-1

After eradication

miR-124a-3

EMX1

NKKX6-1
Wong et al. suggested a “point of no return,” in which the benefit of *H. pylori* eradication diminished when *H. pylori*-associated gastritis reached a certain degree.46  Kiriyama et al. also stated that mucosal damage with IM may not recover to gastric-type mucosa for a "histological point of no return" with eradication of *H. pylori.*47  It has been reported that methylation of some genes, especially miR-124a-3p, was more frequently identified in IM.33,34 Therefore, we have added sub-analysis to investigate that DNA methylation is surrogate marker of severe atrophy and IM. As a result, changes in methylation levels were not significantly affected by changes in histological gastritis after *H. pylori* eradication (Table S1). We considered that irreversible epigenetic change in atrophy and IM was related to continuous carcinogenesis. Our results show that mucosal damage in cases with MGC has accumulated to the point of no histological and genetic improvement. However, a further investigation is required because of small sample size in our sub-analysis.

In addition, the mean methylation level in the NKKX6-1 gene increased after eradication without histological changes of atrophy/IM in the MGC group, and it would be a predictive marker of persistent cancer risk unaffected by inflammation and histological findings.

This study has some limitations. First, the study was a single-center retrospective study with a small sample size. Second, methylation after eradication in the other group except for the repeated MGC group was evaluated at only one point. Because it takes a long time to change chronic gastritis after successful eradication, we did not evaluate short-term changes in this study.16–18 Third, we defined the control group as those patients who did not develop cancer for more than 5 years after eradication. Although there have been some reports of cases with gastric cancer detected more than 5 years after eradication, our subjects continued annual endoscopy after successful eradication and were retroactively confirmed to have no occurrences of gastric cancer to date. Finally, the DNA methylation was measured using FFPE samples in this study. Generally, fresh frozen samples are more suitable for methylation analysis because of the good DNA quality.49,50 Actually, the EMX1 and NKKX6-1 genes were not amplified by PCR in some samples. We have to perform a further study using a large sample size from multiple centers.

In conclusion, the persistence of a high level of aberrant DNA methylation in gastric mucosa after *H. pylori* eradication reflected severe atrophy and IM and was associated with the occurrence of MGC in the long term.

**AUTHOR CONTRIBUTIONS**

Shoko Ono and Naoya Sakamoto conceptualized the study; Ikko Tanaka, Shoko Ono and Yoshiyuki Watanabe contributed to methodology; Ikko Tanaka, Yoshiyuki Watanabe, Hiroyuki Yamamoto, Ritsuko Oikawa, Shogo Matsumoto, Marina Kubo, Yusuke Nishimura, Yoshihiko Shimoda, Masayoshi Ono, and Keiko Yamamoto formally analyzed and investigated the study; Ikko Tanaka contributed to writing—original draft preparation; Shoko Ono and Yoshiyuki Watanabe contributed to writing—review and editing; Hiroyuki Yamamoto and Naoya Sakamoto supervised the study.

**CONFLICT OF INTEREST**

The authors declare that they have no conflict of interest.

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SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.

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