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 3 groups: 25 patients in whom metachronous gastric cancers 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 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 3 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.

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 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.

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 (2018). *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 (Kuipers et al. 1995; Uemura et al. 2001). 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 (Schistosomes 1994; IARC Working Group Reports 2014). The Kyoto global consensus report in 2015 stated that all *H. pylori*-positive individuals worldwide should receive eradication therapy (Sugano et al. 2015). Successful eradication of *H. pylori* prevents gastric cancer development (Fukase et al. 2008).

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)) (Ford et al. 2014; Yoon et al. 2014; Chen et al. 2016; Lee et al. 2016; Terasawa et al. 2019; Fan et al. 2020). 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 (Correa 1992; Ito et al. 2002; Kamada et al. 2005; Toyokawa et al. 2010; Park and Kim 2015). On the other hand, there have been some reports on conflicting results, and whether *H. pylori* eradication improves atrophy and IM is controversial (Lahner et al. 2005; Wang et al. 2011; Kang et al. 2012).

DNA methylation, which accumulates in both gastric cancers and noncancerous mucosa, is an important epigenetic change associated with gastric carcinogenesis (Ushijima and Sasako 2004; Oka et al. 2006; Yamashita et al. 2011; Yousefi et al. 2019). *H. pylori* infection accumulates DNA methylation in the gastric mucosa (Maekita et al. 2006), and *H. pylori* eradication decreases aberrant methylation in some gene promoters, which also decreases the risk of gastric cancers (Chan et al. 2006; Leung et al. 2006; Nakajima et al. 2010). Asada et al. 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 (Asada et al. 2015). A persistently high methylation level in gastric mucosa after *H. pylori* eradication is presumed to be a risk factor for MGC (Ushijima et al. 2006; Maeda et al. 2017a). Although a positive correlation between histological IM and methylation levels in gastric mucosa has been reported (Shin et al. 2013), 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 (Kodama et al. 2012).

Methods

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 (Moertel et al. 1957). 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 less than 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 (Fig. 1, Online Resource 1).

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).

Determination of *H. pylori* infection status

H. pylori infection status was investigated by using the urea breath test (cutoff value < 2.5‰, Ubit®, Otsuka Pharmaceutical, Tokyo, Japan), and serum anti-*H. pylori* antibody test (cutoff value < 10 U/ml, E-Plate Eiken *H. pylori* antibody, Eiken Chemical Co., Ltd., Tokyo, Japan) 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.

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 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.

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* tests (Dixon et al. 1996). 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 (Kodama et al. 2012).

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 (Asada et al. 2015; Maeda et al. 2017).

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, Valencia, CA). Bisulfite treatment of DNA was performed using an EpiTect bisulfite kit (QIAGEN, Valencia, CA). Biotinate polymerase chain reaction was performed using primers that specifically amplify the sequences of the

three genes (Online Resource 2). All PCR assays included a denaturation step at 95°C for 30 seconds, followed by an annealing step at various temperatures for 30 seconds and an extension step at 72°C for 30 seconds. Pyrosequencing was performed using PSQ HS 96 Gold single-nucleotide polymorphism reagents on a Pyromark Q24 pyrosequencing machine (Biotage, Uppsala, Sweden). The protocol for pyrosequencing has been described in detail previously (Colella et al. 2003; Watanabe et al. 2009; Yamamoto et al. 2016). 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.

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. Z-score analysis was used to normalize the methylation level of the three genes in each sample. The Z-score for each gene was calculated as follows: $Z\text{-score} = (\text{methylation level of each sample} - \text{mean value of methylation level}) / \text{standard deviation of methylation level}$. A *P* value of < .05 in each analysis was considered statistically significant.

Results

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 than in the MGC group. For the IM score, there were significant differences between the HP group and MGC group before and long term after eradication therapy. However, there was no significant improvement in the histological score after eradication in each group.

Table1. Characteristics of patients and used samples.

	HP group N = 29	Non-MGC group N = 17	MGC group N = 25
Sex, M: F	22: 7	14: 3	21: 4
Age at the initial ER [†] , mean ± SD [‡] , y	–	66.2 ± 8.8	69.3 ± 6.9
Age at eradication, mean ± SD, y	67.5 ± 5.5	66.8 ± 8.9	70.8 ± 7.0
Follow-up periods after eradication, mean ± SD, m	83.2 ± 18	102.5 ± 29	97.4 ± 37
Periods from the initial ER to the sampling after eradication, mean ± SD, m	–	67.9 ± 10.2	83.8 ± 44.3
Periods from eradication to the sampling after eradication, mean ± SD, m	61.1 ± 2.7	62.2 ± 3.6	67.0 ± 37.2

[†]ER: endoscopic resection, [‡]SD: standard deviation.

In all the items, there were no significant differences among the three groups.

Table 2
Changes in histological atrophy and intestinal metaplasia.

	HP group N = 29	Non-MGC group N = 17	MGC-group N = 25
Atrophy score before eradication	0.95 ± 0.67	1.45 ± 0.82	1.66 ± 0.98*
Atrophy score after eradication	0.89 ± 0.70	1.00 ± 1.00	1.50 ± 1.17
Changes in atrophy score	-0.07 ± 1.21	-0.83 ± 0.98	-0.71 ± 1.38
IM [†] score before eradication	0.54 ± 0.88	0.94 ± 0.97	1.21 ± 1.18**
IM score after eradication	0.45 ± 0.78	0.82 ± 1.13	1.42 ± 1.21***
Changes in IM score	-0.07 ± 1.12	-0.11 ± 0.86	0.22 ± 1.28
[†] IM: intestinal metaplasia, *HP group vs MGC group, <i>P</i> = 0.01, **HP group vs MGC group, <i>P</i> = 0.03, ***HP group vs MGC group, <i>P</i> = 0.002			

Aberrant DNA methylation

Figure 2 shows changes in methylation levels before and after eradication in each group. There were no significant differences in the methylation level, including 3 genes and Z-score before eradication, among the 3 groups. In the HP group, the mean methylation levels of miR-124a-3 and EMX1 significantly

decreased after eradication (miR-124a-3: $P < 0.01$, EMX1: $P = 0.03$). In the non-MGC group, only the mean methylation level in miR-124a-3 significantly decreased ($P < 0.01$). In the MGC group, the mean methylation level in NKX6-1 was increased after eradication ($P = 0.04$), and the Z-score was significantly increased ($P = 0.03$). The mean Z-score decreased after eradication in the HP group (before: 0.13 ± 0.59 , after: -0.31 ± 0.56 , $P < 0.01$) but increased in the MGC group (before: -0.12 ± 0.71 , after: 0.30 ± 0.78 , $P = 0.03$).

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 ($P < 0.01$). Individual data is shown in Online Resource 3.

In the MGC group, 6 patients had repeated metachronous cancers more than twice after eradication, and methylation was measured using samples at the occurrence of MGC. In 4 patients who repeated MGC for more than 5 years after eradication, the methylation level showed an increase during follow-up (Fig. 3).

Correlations between histological atrophy/IM and DNA methylation

Correlations between histological atrophy/IM and DNA methylation are shown in Fig. 4. There was a good positive correlation between the methylation level of miR-124a-3 and the IM score after eradication ($P < 0.01$, $r = 0.82$). Additionally, the EMX1 gene promoter methylation level also had a moderate correlation with atrophy and IM after eradication ($P < 0.001$, $r = 0.59$; $P < 0.001$, $r = 0.64$).

Discussion

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 (Ushijima et al. 2006; Asada et al. 2015). High methylation levels after *H. pylori* eradication are one of the risk factors for MGC (Maekita et al. 2006; Shin et al. 2013; Suzuki et al. 2014; Asada et al. 2015; Maeda et al. 2017). *H. pylori* eradication reduces methylation and prevents carcinogenesis (Nakajima et al. 2010; Leung et al. 2006; Chan et al. 2006). 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 with 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 (Asada et al. 2015; Maeda et al. 2017). In other previous studies, individual methylation data had not been followed-up, and the observation periods were short (Shin et al. 2013; Michigami et al. 2018). 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 (Ushijima et al. 2006; Maeda et al. 2017). 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 (Shimazu et al. 2015; Yamashita et al. 2019; Kim et al. 2021).

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 (Wong et al. 2004). Kiriya 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* (Kirishima et al. 2016). Methylation levels, especially miR-124a-3 in gastric mucosa, were positively associated with IM after eradication. These results support that atrophy and IM after eradication are associated with carcinogenesis depending on epigenetic changes. Our results also show that mucosal damage in cases with MGC has accumulated to the point of no histological and genetic improvement.

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 didn't evaluate short-term changes in this study (Kamada et al. 2005; Toyokawa et al. 2010; Ito et al. 2002). 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 (Take et al. 2011; Choi et al. 2018), 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 good DNA quality (Ohomomo et al. 2021; Ueda et al. 2021). Actually, the EMX1 and NKX6-1 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.

Declarations

Funding

There is no funding for the study.

Conflict of Interest

The authors declare that they have no conflict of interest.

Author contributions

Conceptualization: Shoko Ono and Naoya Sakamoto, Methodology: Ikko Tanaka, Shoko Ono and Yoshiyuki Watanabe, Formal analysis and investigation: Ikko Tanaka, Yoshoyuki Watanabe, Hiroyuki Yamamoto, Ritsuko Oikawa, Shogo Matsumoto, Marina Kubo, Yusuke Nishimura, Yoshihiko Shimoda, Masayoshi Ono, and Keiko Yamamoto, Writing - original draft preparation: Ikko Tanaka, Writing - review and editing: Shoko Ono and Yoshiyuki Watanabe, Supervision: Hiroyuki Yamamoto and Naoya Sakamoto.

Ethics approval

This study was performed in accordance with the ethical standards detailed in the Declaration of Helsinki. The study was reviewed and approved by the Hokkaido University Hospital Review Board 019-015.

Consent to participate

All patients provided written informed consent.

Consent for publication

Not applicable.

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Figures

Figure 1

Outline of the subjects.

The metachronous gastric cancer (MGC) group included 25 patients who received *H. pylori* eradication after initial endoscopic resection (ER) and in whom MGC occurred after successful eradication. The non-MGC group included 17 patients in whom MGC did not occur for more than 5 years after the initial ER and successful eradication. The HP group included 29 patients without a history of gastric cancer for more than 5 years after eradication.

Figure 2

Changes in methylation levels in gastric mucosa before and after eradication.

Methylation levels before and after *H. pylori* eradication were compared among the 3 groups. The Z-score shows the mean normalized methylation level of 3 genes (miR-124a-3, EMX1, NKX6-1).

The methylation levels of miR-124a-3 and EMX1 and the Z-score significantly decreased after eradication in the HP group. That in miR-124a-3 significantly decreased after eradication in the non-MGC group. In the MGC group, the methylation levels of miR-124a-3 and EMX1 did not improve after eradication, and the NKX6-1 and Z-scores significantly increased.

Figure 3

Changes in DNA methylation levels in patients with repeated metachronous cancer.

In the MGC group, 6 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 6 patients. Aberrant methylation in all 3 genes seemed to accumulate over time after eradication.

a) Correlation between atrophy and methylation

Before eradication

After eradication

b) Correlation between IM and methylation

Before eradication

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 ($P < 0.001$, $r = 0.50$). After eradication, there was also a moderate correlation between EXM1 and atrophy ($P < 0.001$, $r = 0.59$).

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 ($P < 0.001$, $r = 0.82$). Additionally, the EMX1 gene promoter methylation level also had a significant correlation with IM after eradication ($P < 0.001$, $r = 0.64$).