Effects of *Helicobacter pylori* Eradication on Methylation Status of *E-Cadherin* Gene in Noncancerous Stomach

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Abstract

**Purpose:** Promoter hypermethylation of *E-cadherin* plays an important role on gastric cancer development. Whereas *E-cadherin* methylation was frequently detected in the stomach of *Helicobacter pylori*–infected individuals, we tested whether eradication of *H. pylori* alters the methylation status of the noncancerous gastric epithelium.

**Experimental Design:** Endoscopic biopsies were taken from the antrum and corpus of *H. pylori*–infected subjects without gastric cancer. Presence of methylated *E-cadherin* sequences in the gastric specimens was detected by methylation-specific PCR. Bisulfite DNA sequencing was done to determine the topographical distribution and changes in methylation profiles with *H. pylori* eradication.

**Results:** Among the 28 *H. pylori*–infected subjects (median age, 44.5 years), 15 (53.6%) had *E-cadherin* methylation detected in stomach at baseline. Discordant methylation patterns between the antrum and corpus were noted in six patients. One year after successful *H. pylori* eradication, there was a significant reduction in the methylation density of the promoter region and exon 1 of the *E-cadherin* gene as detected by bisulfite DNA sequencing (*P* < 0.001).

**Conclusion:** Promoter methylation in *E-cadherin* was frequently detected in the stomach of *H. pylori*–infected individuals. Eradication of *H. pylori* might possibly reduce the methylation density in *E-cadherin* gene and the chance of subsequent neoplastic transformation.

Epigenetic inactivation of tumor-related genes by promoter hypermethylation is increasingly recognized to play a pivotal role on tumorigenesis (1, 2). Promoter hypermethylation in tumor-related genes is frequently detected in gastric cancer (3, 4) as well as in premalignant gastric lesions (5, 6). Moreover, it is shown that the nonneoplastic gastric mucosa of normal individuals may harbor *E-cadherin* methylation (7–9).

The *E-cadherin* gene (CDH1) is located on chromosome 16q22.1, which contains 16 exons. The mature protein product belongs to the family of cell-cell adhesion molecules and plays a fundamental role in the maintenance of cell differentiation and the normal architecture of epithelial tissues. Abrogation of the *E-cadherin* function induces loss of adherens junctions and impairment of cell adhesiveness and cell proliferation signaling pathways (10). In gastric cancer, loss of function of the *E-cadherin* gene has been linked to diffuse-type gastric cancer (11). *CDH1* mutations are considered to be the commonest somatic alterations in diffuse gastric cancer and germ-line inactivating mutations of *CDH1* gene leads to the rare hereditary diffuse gastric cancer syndrome (12). In addition to mutation, epigenetic inactivation of *E-cadherin* by promoter hypermethylation was frequently reported in sporadic diffuse gastric cancer (13, 14). It is also recently suggested that promoter methylation of *E-cadherin* in noncancerous gastric epithelium is associated *Helicobacter pylori* infection (9). It is reported that up to 52.6% of *H. pylori*–infected noncancer patients have methylated *E-cadherin* detected in stomach. In contrast, only one of the 15 *H. pylori* negative patients had methylation in *E-cadherin* detected.

Although *H. pylori* is considered to be the most important etiologic agent for gastric carcinoma, the exact mechanism underlying the gastric carcinogenesis remains elusive. It is therefore tempting to test whether eradication of *H. pylori* could reverse epigenetic changes in the noncancerous gastric epithelium. In this study, we determined the comprehensive methylation profiles of *E-cadherin* gene in the antrum and corpus of *H. pylori*–infected individuals without gastric cancer and examined the effects of *H. pylori* eradication on methylation.

Materials and Methods

Patients and gastric biopsy. Dyspeptic patients referred for upper gastrointestinal endoscopy were recruited. None of these patients took aspirin or nonsteroidal anti-inflammatory drugs. All patients were confirmed to have *H. pylori* infection by rapid urease test and histology. None of these patients had peptic ulcer or gastric cancer on endoscopy.
Gastric mucosal biopsies were obtained during endoscopy according to a standard protocol. Briefly, two biopsies were taken from the lesser curve and greater curve of the antrum and corpus. Gastric biopsies were fixed in buffered formalin and embedded on paraffin for histologic examination and DNA analysis. All patients were given a 1-week course of anti-Helicobacter therapy consisting of a proton pump inhibitor and two antibiotics after the baseline endoscopy. Clearance of H. pylori infection was confirmed by a negative urea breath test 2 months after the course of antimicrobial therapy. Patients with confirmed H. pylori eradication underwent a follow-up endoscopy at 1 year with biopsy taken as described in baseline endoscopy. All patients were negative for H. pylori by histology on follow-up. The study protocol was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong and all patients gave informed written consent for participation in this study.

Histology. Histologic sections of the antrum and the corpus were graded separately for the severity of acute polymorphonuclear infiltrates, chronic mononuclear cell infiltrates, and intestinal metaplasia as stipulated by the updated Sydney system (15). A global score of 0 (none), 1 (mild), 2 (moderate), or 3 (marked) was given for the inflammatory scores.

H. pylori and cell culture. To determine the in vitro effects of H. pylori infection on E-cadherin methylation in gastric epithelial cells, the AGS cell line was cocultured with H. pylori strain TN2GF4, which is positive for cag pathogenicity island and vacA s1 m1 (16). AGS cells were cultured with H. pylori in a six-well plate (multiplicity of infection, 100). Cells were harvested at 12, 24, and 48 hours post H. pylori infection. DNA was extracted for determination of methylation status in E-cadherin gene.

Methylation-specific PCR. Antral and corpus biopsies from the same individual were processed separately. All samples were randomly coded before processing. Genomic DNA was extracted from paraffin-embedded gastric biopsy sections by QIAamp DNA Mini kit (Qiagen, Hilden, Germany) as previously described (17). Presence of methylated E-cadherin DNA in gastric mucosal biopsies was detected by methylation-specific PCR as previously described (18). Briefly, purified DNA samples were chemically modified by sodium bisulfite with the EZ DNA Methylation kit (Zymo Research, Orange, CA) to convert all unmethylated cytosines to uracils while leaving methylcytosines unaltered. The bisulfite-modified DNA was amplified by using primer pairs that specifically amplify either methylated or unmethylated sequences of the E-cadherin genes (17, 18). PCR was done in 25-μL reaction volumes containing 1× PCR buffer [10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl], 2 mmol/L MgCl2, 0.25 mmol/L each of the deoxynucleoside triphosphates (MBI Fermentas), 5 pmol of primer, and 1 unit of AmpliTaq Gold Taq polymerase (Applied Biosystems, Foster City, CA). The condition for amplification was 10 minutes at 95°C followed by 40 cycles of denaturing at 95°C for 10 minutes at 95°C.

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Abbreviations: Al, acute inflammation; Cl, chronic inflammation; MSP, methylation-specific PCR.
45 seconds, 45 seconds of annealing at 57°C, and 1 minute of extension at 72°C. CpGenome Universal methylated DNA (Chemicon International, Inc., CA, USA) was used as positive control and water was included as negative control during amplification. As a further internal control, three gastric cancer cells (AGS, MKN45, and NCI-N87) with different methylation status in the E-cadherin gene were included.

**Bisulfite DNA sequencing.** To determine the comprehensive CpG island methylation status of the E-cadherin gene in H. pylori–infected gastric epithelium and the effects of H. pylori eradication, bisulfite DNA sequencing was done in paired gastric biopsies taken from patients before and after H. pylori eradication. PCR primers were designed to amplify the CpG-rich promoter region and exon 1 of the E-cadherin gene (−187 to +177) by using the Methprimer freeware (available at http://www.ucsf.edu/urogene/methprimer/index1.html). The sequences of the primers were forward, 5′-TAGTAATTTTAGGTTAGAGGGTTAA-3′, and reverse, 5′-AAATACCTACAACAAACACCA-3′. The 387-bp PCR product, which covered 32 CpG sites, was then cloned into the pCR 2.1-TOPO vector using the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). At least 10 colonies were selected and grown in LB overnight at 37°C. Plasmid DNA was isolated by QIAprep Miniprep Spin Miniprep Kit (Qiagen) and 2 μL were sequenced using M13 primers and the ABI Prism Dye Terminator Cycle Sequencing Kit (Applied Biosystems) in ABI Prism 3100 DNA Sequencer (Applied Biosystems).

**Immunohistochemistry.** Expression of E-cadherin in gastric biopsy specimens was examined by immunohistochemistry. Paraffin-embedded sections were dewaxed in xylene and rehydrated in a graded series of ethanol. Microwave antigen retrieval was done in citrate buffer (pH 6.0) for 10 minutes before peroxidase quenching with 3% H2O2 ethanol. Microwave antigen retrieval was done in citrate buffer (pH 6.0) for 10 minutes before peroxidase quenching with 3% H2O2 ethanol. Primary antibody against E-cadherin (Zymed Laboratories, San Francisco, CA) was applied overnight in LB at 4°C. Biotinylated secondary antibody and enzyme conjugate (Histostain-Plus Kits; Zymed Laboratories) were incubated for 45 minutes each. 3,3′-Diaminobenzidine was then added and incubated until chromogen developed. Percentage of gastric epithelial cells with loss of E-cadherin expression was computed by counting >500 representative cells in at least 10 different fields.

**Statistical analysis.** Statistical analysis was done with SPSS software (ver. 11.0; SPSS, Chicago, IL). χ2 test or Fisher’s exact test was used for analysis of categorical data. Numerical data were analyzed by Student’s t test. Wilcoxon signed-rank test was used for paired comparison of methylation frequency before and after H. pylori eradication. Two-sided P < 0.05 was considered to be statistically significant.

## Results

**Patients.** Gastric biopsies from 28 H. pylori–infected patients (male 42.9%; median age, 44.5 years; range, 34-55 years) were available for analysis. Ten (35.7%) patients had history of gastric cancer in first-degree relatives. The severity of gastric inflammation was listed in Table 1. Moreover, 18 of them had intestinal metaplasia on microscopic examination (16 in antrum only, 1 in corpus only, and 1 in both antrum and corpus). Among the 28 patients, 25 had serial gastric biopsies taken before and 1 year after H. pylori eradication. Intestinal metaplasia was still detectable in 15 patients 1 year after H. pylori eradication (12 in antrum only, 1 in corpus only, and 2 in both antrum and corpus).

**Promoter methylation of E-cadherin gene in normal gastric epithelium.** At baseline, gastric biopsies from 15 (53.6%) H. pylori–infected patients had methylation detected. Specifically, 14 (50%) antral and 10 (35.7%) corpus biopsies were positive for methylated E-cadherin sequences. Six (21.4%) patients had discordant methylation results in the antrum and corpus at baseline (Table 1).

Serial gastric biopsies were available from 25 patients after H. pylori eradication. There was a considerable dynamic change in the methylation-specific PCR results after H. pylori eradication (Table 1). As shown in Fig. 1, there was a mild and nonsignificant reduction in the number of methylation-specific PCR positive gastric samples after H. pylori eradication.

**Bisulfite DNA sequencing.** In addition to methylation-specific PCR, we did bisulfite DNA sequencing in a subset of randomly selected samples to determine the comprehensive methylation pattern of individual CpG islands in the E-cadherin gene. We randomly selected seven sets of paired antral and corpus biopsies taken from patients before and after H. pylori eradication (Fig. 2). All methylation-specific PCR positive samples had methylated CpG islands detected on bisulfite DNA sequencing.

Three patterns of methylation changes were noted after H. pylori eradication: no change, decrease, and increase in methylation density. The most common pattern was seen in sample no. 2, the antrum of sample no. 19, and the corpus of sample nos. 9, 17, and 18, in which both the extent and density of methylation decrease with H. pylori eradication. On the other hand, the extent and density of methylation were mildly increased in sample no. 1, the antrum of sample nos. 17 and 18, and the corpus of sample no. 19. There was no change in methylation pattern and density of sample no. 20. Overall, H. pylori eradication resulted in a significantly reduction in mean methylation density of the E-cadherin gene in both the promoter region and exon 1 (P < 0.0001; Fig. 3).

**Methylated E-cadherin and gastric inflammation.** We also determined the correlation between gastric inflammation and E-cadherin methylation. Both acute and chronic inflammatory scores were significantly higher in gastric biopsies with methylation in E-cadherin gene. The mean acute and chronic...
Inflammatory scores were 0.88 and 1.48 in gastric biopsies with methylated E-cadherin whereas the corresponding scores were 0.42 (\( P = 0.013 \)) and 0.98 (\( P = 0.003 \)) in biopsies without methylation. Promoter hypermethylation was detected in 43.2% of gastric biopsies with intestinal metaplasia and 37.5% of gastric biopsies without intestinal metaplasia (\( P = 0.67 \)). There was no association between promoter hypermethylation of E-cadherin gene and age of patients (antrum, \( P = 0.35 \); corpus, \( P = 0.90 \)). There is also no association between E-cadherin methylation and family history of gastric cancer.

**E-cadherin expression and methylation.** The localization of E-cadherin expression was detected by immunohistochemistry (Fig. 4). Membranous expression of E-cadherin was detected at the basolateral border of normal glandular and foveolar epithelial cells. Despite the presence of E-cadherin methylation, there was no complete loss of E-cadherin expression in these noncancerous gastric mucosal specimens. There was, however, a tendency towards a higher percentage of partial loss of E-cadherin expression in gastric biopsies containing intestinal metaplasia. The percentage loss of E-cadherin expression was significantly higher in the glandular epithelium of gastric biopsies containing intestinal metaplasia than those without intestinal metaplasia (median, 10% versus 5%; \( P = 0.034 \)). Similar tendency was observed in the foveolar epithelium (\( P = 0.05 \)).

**Effect of transient H. pylori infection on E-cadherin methylation.** To further test the effect of H. pylori infection on gastric cells, we determined the methylation changes in AGS gastric cells after coculture with H. pylori for up to 48 hours. Methylated E-cadherin DNA was, however, not detected in AGS cells by both methylation-specific PCR and bisulfite DNA sequencing after coculture with H. pylori (Fig. 2).

### Discussion

In this study, we focused on H. pylori – associated gastritis, the early stage of the gastric carcinogenesis cascade. Although...
promoter hypermethylation of E-cadherin gene in the noncancerous gastric epithelium has previously been reported (5–9), the topographical methylation pattern in different parts of the stomach has not been properly evaluated. Among the 28 paired gastric biopsies taken from the antrum and corpus of H. pylori–infected individuals, we found that 53.6% of patients had methylation in E-cadherin gene as detected by methylation-specific PCR. This frequency was comparable to previous reports in noncancerous biopsies (6, 9). Interestingly, discordant methylation patterns were noted in the antrum and corpus of patients with H. pylori–associated gastritis. The frequency of methylation tended to be higher in the antrum than in the corpus (50% versus 35.7%) although the difference did not reach statistical significance. The bisulfite DNA sequencing results further confirmed the discrepant methylation patterns between the antrum and corpus.

We further determined the role of H. pylori eradication on the methylation pattern of E-cadherin gene in the noncancerous gastric epithelium by both methylation-specific PCR and bisulfite DNA sequencing. One year after H. pylori eradication, there was a marked reduction in the methylation density as detected by the comprehensive bisulfite DNA sequencing. The methylation density dropped significantly in the promoter region and exon 1 of the E-cadherin gene (Fig. 3). Our findings are therefore in support of the potential beneficial effects of H. pylori eradication on the reversal of E-cadherin promoter hypermethylation. On the other hand, there was no significant difference on the numbers of methylated gastric samples after H. pylori eradication by the extremely sensitive methylation-specific PCR. In this regard, bisulfite DNA sequencing may provide a better delineation of subtle methylation changes after clearance of H. pylori, which could not be provided by qualitative methylation-specific PCR alone.

Whereas the triggering event leading to promoter hypermethylation of E-cadherin in the noncancerous stomach remains elusive, it is strongly believed that environmental factors, particularly chronic H. pylori infection, play an important role (19). It is highly plausible that the direct effects of H. pylori or the adaptive response of the host to the chronic inflammation triggers the development of these epigenetic changes in noncancerous stomach, which eventually lead to neoplastic transformation. However, we failed to show any increase in methylation of the CpG islands of the E-cadherin gene in AGS gastric cells after short-term coculture of H. pylori. It may be necessary to determine the effects of H. pylori on methylation changes in longer-term experiments. However, our findings are in favor of the potential beneficial effects of H. pylori eradication by eliminating the possible drive to methylation changes. In keeping with this finding, we have previously shown that eradication of H. pylori in AGS cells reverts the aberrant expression of cyclin D2 and p27 in intestinal metaplasia (20).

Epigenetic inactivation in the stomach is likely to be multifocal in nature, which may be related to the severity of inflammation. In this study, we have shown a significant correlation between acute and chronic inflammation scores and the presence of methylated E-cadherin genes. This may also partly explain the disparate methylation pattern between the antrum and corpus of the stomach. Despite the presence of methylated E-cadherin sequences, there was no apparent loss of E-cadherin expression in most noncancerous gastric epithelium as detected by immunohistochemistry (Fig. 4). This could be
explained by the extremely sensitive methylation-specific PCR which can detect a small subpopulation of cells showing these abnormalities. In contrast, a partial loss of E-cadherin expression was more commonly detected in gastric intestinal metaplasia, a presumed preneoplastic gastric lesion. Similar observation was made in past studies which reported loss of E-cadherin expression in intestinal metaplasia as well as in early gastric cancer (21, 22).

Although previous studies have suggested the detection of E-cadherin methylation in normal gastric and bladder epithelium of aged patients (9, 23), we failed to show any significant association between promoter hypermethylation and age in this study. This may be related to the recruitment of relatively young patients (median age, 44.5 years) in this study. However, it may be more difficult to show any potential effects of H. pylori eradication in elderly patients with advanced histologic and possibly epigenetic changes. In addition, further studies may be necessary to characterize the methylation changes in patients with early gastric cancer, particularly the role of H. pylori eradication in those who received endoscopic treatment. However, early gastric cancer is an uncommon disease in this locality and most of our patients are treated with surgical resection, which made these specimens unavailable for analysis in the present study.

In conclusion, we have shown the comprehensive methylation pattern in noncancerous stomach of H. pylori–infected individuals. In addition to the demonstration of a discordant methylation pattern between the antrum and corpus, we have shown that eradication of H. pylori resulted in a significant reduction in methylation density of the noncancerous stomach. The result is therefore in favor of the potential beneficial effect of H. pylori eradication on epigenetic changes in the noncancerous stomach and possibly on chemoprevention of cancer.

References
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