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<td><strong>Author(s)</strong></td>
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Eradication of Helicobacter pylori infection reverses E-cadherin promoter hypermethylation

A O O Chan, J Z Peng, S K Lam, K C Lai, M F Yuen, H K L Cheung, Y L Kwong, A Rashid, C K Chan and B C-Y Wong

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Gastric carcinogenesis is a multistep process involving multiple genetic and epigenetic events, with postulated intestinal metaplasia-dysplasia-invasive carcinoma sequence during morphologic progressions. 

Helicobacter pylori infection is an important aetiological risk factor in gastric cancer, and has been classified as a group I or definite carcinogen by the World Health Organisation’s International Agency for Research on Cancer. 

E-cadherin is an adhesion molecule that is expressed on all epithelial cells. It is an important tumour suppressor and invasion suppressor gene. In gastric carcinogenesis, the critical role of E-cadherin is underlined by the observation that familial gastric cancer is related to germline mutations of the E-cadherin gene. Furthermore, somatic mutations of E-cadherin were found in approximately 50% of gastric carcinomas of the diffuse histological type. Finally, in two kindreds with familial gastric cancer and germline E-cadherin mutation, promoter CpG hypermethylation was found to be the second “genetic hit” in abrogating E-cadherin expression. 

We have previously shown that expression of E-cadherin protein was decreased early in precancerous lesions of gastric cancer, suggesting E-cadherin may play an early role in gastric cancer. Methylation of E-cadherin was identified early in precancerous lesions of gastric cancer, being found in 31% of gastric mucosa with chronic gastritis. More importantly, H pylori was an independent risk factor associated with methylation of E-cadherin in non-lesional gastric mucosa from patients with dyspepsia.

Targeting epigenetic changes that occur before the development of frank malignancy as chemopreventive intervention offers the maximal impact. Hence in gastric cancer, early reversal of promoter methylation at precancerous lesions before the development of frank gastric cancer might halt gastric carcinogenesis. We hypothesised that H pylori infection may be an important aetiology in causing methylation at E-cadherin and that eradication of H pylori might reverse methylation at E-cadherin. We tested this hypothesis by examining methylation of the E-cadherin gene in gastric mucosa with chronic gastritis from patients without gastric cancer before and after eradication of H pylori, and also in intestinal metaplasia from patients with and without H pylori infection.

**MATERIALS AND METHODS**

**Patients and specimens**

Two cohorts of patients were studied. The first cohort, which constituted the majority of patients in this study, was derived from a prospective randomised controlled study. Ninety patients from the Department of Medicine, Queen Mary Hospital, Hong Kong, who had upper endoscopy for investigation of dyspepsia and were confirmed to be H pylori positive were recruited to the study. The presence of H pylori infection was confirmed by rapid urease test and subsequent histological analysis using both haematoxylin-eosin and the modified Giemsa stains. Equivocal cases were excluded from study.

**Abbreviations:** PCR, polymerase chain reaction; MSP, methylation specific polymerase chain reaction
the analysis. This approach has been validated previously in our centre. Antral gastric mucosal biopsies were taken for histological analysis, E-cadherin immunohistochemical staining, and methylation study (week 0). Haematoylin-eosin stained slides were evaluated for the presence of chronic gastritis, intestinal metaplasia, or dysplasia. Histological assessment was categorised according to the Sydney classification.

Patients were included in the study only if the gastric mucosal histology showed chronic active gastritis after confirmation of H pylori infection. Patients were then randomised into two groups: group 1 subjects received H pylori eradication therapy while group 2 received no treatment. H pylori eradication therapy was performed in the case of one week of amoxicillin 1000 mg twice/day, clarithromycin 500 mg twice/day, and omeprazole 20 mg twice/day. All patients were followed up prospectively for six weeks. Upper endoscopy was repeated again at the end of six weeks (week 6). The status of H pylori infection was reassessed using the same method. Antral gastric mucosal biopsies were again taken for histology, immunostaining, and methylation analysis.

In order to assess the degree of concordant methylation at the antrum, five additional random antral mucosa biopsies were taken from each of the 10 patients at week 0. Concordant methylation was defined as the same methylation status (either negative or positive) at the E-cadherin gene in at least four biopsy samples in each patient. Informed consent for tissue procurement was obtained from all patients.

The second cohort comprised archival antral biopsy specimens with documented intestinal metaplasia from patients who were H pylori positive (n = 22) and from those who were H pylori negative (n = 19) as controls. The study was approved by our institutional review board.

### Table 1 Primers and polymerase chain reaction conditions for E-cadherin, DAPK, MGMT, p16, and OR beta

<table>
<thead>
<tr>
<th>Locus</th>
<th>Allele</th>
<th>Sense primers</th>
<th>Antisense primers</th>
<th>Annealing temp (°C)</th>
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<tr>
<td>E-cadherin</td>
<td>Methylated</td>
<td>5'-TTAGTTAGAGGTTAGCTCGG-3'</td>
<td>5'-TAACAAAATATCCTACCTACGAC-3'</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>Unmethylated</td>
<td>5'-TAATTAGTTAGAGGTTAGCTCGG-3'</td>
<td>5'-CAACACCAAATCAAAGAC-3'</td>
<td>53</td>
</tr>
<tr>
<td>DAPK</td>
<td>Methylated</td>
<td>5'-GGGATATCGGGCTGAGTATGTTTCTCGT-3'</td>
<td>5'-GGGGATATCGGGCTGAGTATGTTTCTCGT-3'</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Unmethylated</td>
<td>5'-GGGATATCGGGCTGAGTATGTTTCTCGT-3'</td>
<td>5'-CCTCCCCAAACCGCA-3'</td>
<td>60</td>
</tr>
<tr>
<td>MGMT</td>
<td>Methylated</td>
<td>5'-TTTCTAGGCTTCTGGATCTGTTTCTCGT-3'</td>
<td>5'-TTTCTAGGCTTCTGGATCTGTTTCTCGT-3'</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>Unmethylated</td>
<td>5'-TTTCTAGGCTTCTGGATCTGTTTCTCGT-3'</td>
<td>5'-GACCTCTCTCGGAAAGGAAAGGC-3'</td>
<td>59</td>
</tr>
<tr>
<td>hMLH1</td>
<td>Methylated</td>
<td>5'-TTAGTCTACTTCCTGCAGTCTGTTTCTCGT-3'</td>
<td>5'-CGGAAAAGAACCCCAACCTAA-3'</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Unmethylated</td>
<td>5'-TTAGTCTACTTCCTGCAGTCTGTTTCTCGT-3'</td>
<td>5'-GACCTCTCTCGGAAAGGAAAGGC-3'</td>
<td>60</td>
</tr>
<tr>
<td>p16</td>
<td>Methylated</td>
<td>5'-TTAGTATTAGAGGGTGGGGTGGATTGT-3'</td>
<td>5'-CCCTCCCAAACACCAACCTAA-3'</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>Unmethylated</td>
<td>5'-TTAGTATTAGAGGGTGGGGTGGATTGT-3'</td>
<td>5'-GACCTCTCTCGGAAAGGAAAGGC-3'</td>
<td>65</td>
</tr>
<tr>
<td>OR beta</td>
<td>Methylated</td>
<td>5'-TTTGGAGGTTGGGTTGTTGTC-3'</td>
<td>5'-CCACATGAAATAATATAACGAC-3'</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>Unmethylated</td>
<td>5'-TTTGGAGGTTGGGTTGTTGTC-3'</td>
<td>5'-CCACATGAAATAATATAACGAC-3'</td>
<td>49</td>
</tr>
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DAPK, DAP kinase; MGMT, O6-methylguanine methyltransferase; hMLH1, human Mut L homologue; OR beta, oestrogen receptor beta.

### Immunohistochemical staining for E-cadherin

E-cadherin expression was examined by immunostaining using the avidin-biotin complex immunoperoxidase method, as described previously. Briefly, 4 μm thick tissue slides were deparaffinised in xylene and rehydrated serially with sodium citrate buffer, pH 6.0. Slides were then incubated with an avidin conjugated monoclonal anti-E-cadherin antibody (HECD-1, 1:500 dilution in phosphate buffered saline; Zymed Laboratories Inc., South San Francisco, USA) in a moist chamber at 37 °C for one hour. Bound antibody was detected by a biotinylated secondary antibody and the avidin-biotin complex immunoperoxidase method (Dako Corp., Carpinteria, California, USA). Slides were finally counterstained with Mayer’s haematoxylin. As a negative control, the primary antibody was replaced with mouse IgG. Slides with normal colonic mucosa were used as positive controls. E-cadherin staining was classified as altered when cytoplasmic and/or membranous staining was reduced or absent.

### Statistical analysis

The χ2 test was used to compare categorical associations and the Student’s t test for continuous associations. Two sided tests were used to calculate p values.

### RESULTS

Demographic data from patients who presented with dyspepsia

Two patients from the first cohort were excluded from the study because the initial gastric mucosal biopsies showed evidence of intestinal metaplasia. The remaining 88 patients were randomised into groups 1 and 2. Three patients from group 1 and four patients from group 2 defaulted for the second upper endoscopy and were excluded from the final
analysis. There were nine men and 32 women in group 1 and 11 men and 29 women in group 2 (p = 0.6). Mean age of group 1 patients was 51 (11) years and 47 (16) years in group 2 (p = 0.2).

**Promoter methylation at E-cadherin at week 0 and week 6 in group 1 patients**

At week 0, chronic active gastritis was present in all specimens. Promoter methylation at E-cadherin was present in 46% (19/41) of the gastric mucosa specimens (examples in fig 1A). There was no difference in mean age between patients who had methylation in their gastric mucosa (51 (9) years) and those who had not (51 (11) years) (p = 0.8).

At week 6 after *H pylori* eradication therapy, none of the patients showed persistent *H pylori* infection. Normal histology was observed in the antral gastric biopsy specimens from five patients, while 35 biopsy specimens showed chronic inactive gastritis, and one specimen showed intestinal metaplasia (fig 2). Promoter methylation at E-cadherin was only present in 17% (7/41) of the gastric mucosa specimens, which was significantly different from that in the pre-eradication specimens (p = 0.004) (table 2; examples in fig 1B)—that is, among the 19 specimens which were positively methylated before *H pylori* eradication, 15 (78.9%) became unmethylated after eradication therapy (fig 2). The disappearance of promoter methylation at E-cadherin was associated with reversal of chronic active gastritis to inactive gastritis in 14 patients, and to normal mucosa in one patient. There were three unmethylated specimens which became methylated after eradication therapy (fig 2). One showed a histological change from chronic active gastritis to intestinal metaplasia (fig 2). Specificity of E-cadherin methylation was confirmed by DNA sequencing (fig 1C, 1D). Patients with or without E-cadherin methylation after *H pylori* eradication did not differ in mean age (50 (8) v 52 (11) years; p = 0.5).

**Promoter methylation at E-cadherin at week 0 and week 6 in group 2 patients**

At week 0, chronic active gastritis was present in all specimens. Promoter methylation at E-cadherin was present in 47.5% (19/40) of the gastric mucosa specimens (examples in fig 3A). Mean age of patients with positive and negative methylation in their gastric mucosa was 53 (10) years and 43 (14) years, respectively (p = 0.05). At week 6, none of the specimens showed inactive gastritis or normal gastric epithelium. Promoter methylation at E-cadherin was observed
in 52.5% (21/40) of specimens (examples in fig 3B) which showed no difference in methylation frequency compared with week 0 (p = 0.5). The methylation status at E-cadherin at week 0 and week 6 was confirmed by sequencing (fig 3C, 3D). Three specimens with negative methylation at week 0 became positive at week 6. One specimen with positive methylation at week 0 became negative at week 6.

**Promoter methylation at other genes at week 0 and week 6 in 10 of the group 1 patients**
The methylation pattern at week 0 and week 6 at these genes are summarised in table 3. Disappearance of methylation was not observed in these genes.

**Analysis for the presence of concordant methylation**
Each of the 10 patients had five additional antral biopsies taken. Eight showed concordant positive E-cadherin methylation (at least 4/5 biopsies were positive for E-cadherin methylation from each of these patients) and four showed concordant negative methylation (all five biopsies were negative for E-cadherin methylation from each of these patients). Each of the 10 patients had five additional antral biopsies taken. Eight showed concordant positive E-cadherin methylation (at least 4/5 biopsies were positive for E-cadherin methylation from each of these patients) and four showed concordant negative methylation (all five biopsies were negative for E-cadherin methylation from each of these patients).

**Immunostaining of E-cadherin**
Immunostaining of gastric mucosae from group 1 and group 2 patients showed strong membranous staining in the gastric epithelium, and there was no difference in staining between pre- and post-*H pylori* eradication in group 1 patients.

**Promoter methylation at E-cadherin in the intestinal metaplasia specimens**
In the second cohort of patients, among 19 *H pylori* negative specimens, 63% (12) showed E-cadherin methylation. Among the 22 *H pylori* positive specimens, 72.7% (16) of specimens were methylated (p = 0.5).

**DISCUSSION**
Our study showed that by eradicating *H pylori* infection early in the stage of chronic gastritis in patients without gastric cancer, promoter methylation at E-cadherin disappeared in a high proportion of patients.

The importance of the E-cadherin gene in gastric carcinogenesis has been well demonstrated, and hence the methylation pattern at E-cadherin before and after eradication of *H pylori* was of particular interest in our study. We have also studied other genes which have been reported to be methylated in patients with chronic gastritis. The genes that we chose are involved in cell cycle regulation (p16), DNA repair or protection (hMLH1, MGMT), and apoptosis (DAP kinase).

In addition to the important role E-cadherin plays in gastric cancer, we have also demonstrated in our previous work that methylation of the E-cadherin promoter is associated with gastric cancer and have generated a hypothesis linking E-cadherin methylation, *H pylori* infection, intestinal metaplasia, and gastric carcinogenesis. The current study again showed that *H pylori* infection was associated with E-cadherin methylation at the gastric mucosa in dyspeptic subjects without gastric cancer. Despite the fact that the role of E-cadherin methylation in the gastric epithelium in these dyspeptic subjects in the future development of gastric cancer remains uncertain, targeting epigenetic changes that occur before the development of frank malignancy as chemopreventive intervention may offer the maximal impact. Hence the disappearance of E-cadherin methylation in these dyspeptic patients may be important for preventing future development of gastric cancer.

In the current study we observed that the disappearance of E-cadherin methylation after *H pylori* eradication was associated with a decrease in activity of chronic gastritis, according to the Sydney classification. The underlying mechanism of the reversal of the methylation process is still uncertain. We postulate that this may relate to the decrease in inflammation after *H pylori* eradication. It is well known that promoter methylation is associated with chronic inflammatory conditions, such as inflammatory bowel disease, oesophageal mucosa in patients with Barrett’s oesophagitis, and in liver tissues in chronic hepatitis.

We also observed in the current study that, in the presence of...
intestinal metaplasia, E-cadherin methylation did not associate with the presence of \textit{H pylori} infection. This supports the fact that the presence of E-cadherin methylation relates to the underlying chronic inflammatory condition. In contrast, it has been reported that methylation dependent gene silencing can be induced by interleukin 1\textbeta via the action of nitric oxide.\textsuperscript{27} It has also been recently reported that \textit{H pylori} upregulates mRNA expression, promoter activity, and enzyme activity of inducible spermine oxidase in human gastric epithelial cells, resulting in DNA damage and apoptosis.\textsuperscript{28} Thus the disappearance of methylation at the stage of chronic gastritis may also be directly related to abolishment of oxidative stress caused by \textit{H pylori}.

The current observation is intriguing and will require further extensive studies to confirm the underlying mechanism. In addition, we also assessed the presence of concordant methylation within the antrum from 10 patients. We found 80\% concordant methylation status. This implies that the possibility that the difference in E-cadherin methylation at week 0 and week 6 was due to random biopsy errors is less likely.

Despite the fact that we observed differences in E-cadherin methylation pre- and post-\textit{H pylori} eradication in gastric mucosa, we did not observe any difference in immunostaining at E-cadherin in these specimens. We postulate that this may be due to the fact that MSP is a very sensitive method for detecting methylation. The sensitivity of MSP was $10^{-3}$ (results not shown). On the other hand,
imunohistochemical staining is a qualitative method, and
is not as sensitive as PCR in detecting subpopulations of
cells with gene methylation and hence downregulation of
E-cadherin. A large number of cells may be needed to be
methylated before this is reflected in expression by immu-
nostaining.

Methylation analysis at other genes was also performed in
10 pairs of pre- and post- H pylori eradicated specimens.
However, no difference in methylation at these genes was
observed. This could be due to the small sample size. On the
other hand, methylation frequencies in gastric mucosa at
DAPK, MGMT, hMLH1, and p16 genes were 41%, 18.7%,
10.9%, and 4.1%, respectively, according to Kang and
colleagues,20 which was not as high as that observed in
E-cadherin, and this may also account for the difference.

The current study corroborates the observation in a
prospective randomised controlled study from our group
showing that eradicating H pylori prevented gastric cancer in
patients with chronic gastritis but not in those with existing
precursor lesions, such as intestinal metaplasia.20 The current
study may partially explain the clinical observation. Whether
disappearance of E-cadherin methylation at the gastric
mucosa in dyspeptic patients can eventually prevent gastric
cancer development still requires further investigation.

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