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<td>Author(s)</td>
<td>Chan, AOO; Lam, SK; Wong, BCY; Wong, WM; Yuen, MF; Yeung, YH; Hui, WM; Rashid, A; Kwong, YL</td>
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Promoter methylation of E-cadherin gene in gastric mucosa associated with Helicobacter pylori infection and in gastric cancer

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HELCOBACTER PYLORI

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 Organization’s International Agency for Research on Cancer.17

In this study, we evaluated the role of E-cadherin methylation in dyspeptic patients without metaplasia or dysplasia, and in sporadic gastric carcinomas, by studying promoter hypermethylation of the gene in gastric mucosa in patients without cancer, in intestinal metaplasia, and in primary carcinoma and metastatic lymph nodes from patients undergoing surgery for gastric cancer. In particular, the association between E-cadherin methylation and H pylori infection was assessed.

MATERIALS AND METHODS

Patients and specimens
Thirty five gastric mucosal biopsies obtained from patients undergoing upper endoscopy for dyspepsia were studied. Haematoxylin and eosin stained slides were evaluated for the presence of gastritis, intestinal metaplasia, or dysplasia. H pylori infection was evaluated by histology and urease breath test. DNA from eight normal mucosa, 21 intestinal metaplasias, and 26 primary adenocarcinomas and 32 metastatic lymph nodes from gastrectomy specimens of patients undergoing resection for gastric adenocarcinoma were studied. The tumours were staged according to the Japanese Research Society for Gastric Cancer17 and classified according to the

Abbreviations: PCR, polymerase chain reaction; MSP, methylation specific polymerase chain reaction; COX-2, cyclooxygenase 2.
World Health Organization classification scheme. DNA was obtained by microdissection from 5 µm thick haematoxylin and eosin stained paraffin embedded tissue sections without coverslip, as described previously. 

H pylori infection status was evaluated in 18 primary adenocarcinoma specimens by histology. Informed consent for tissue procurement was obtained from all patients.

Methylation specific polymerase chain reaction (MSP) for E-cadherin promoter methylation

The methylation status of the E-cadherin promoter was determined by bisulphite treatment of DNA followed by MSP, as described previously.

Briefly, 2 µg of DNA were denatured with 2 M NaOH at 37°C for 10 minutes, followed by incubation with 3 M sodium bisulphite, pH 5.0, at 50°C for 16 hours. Bisulphite treated DNA was then purified (DNA Cleanup Kit; Promega, Madison, Wisconsin, USA), incubated with 3 M NaOH at room temperature for five minutes, precipitated with 10 M ammonium acetate and 100% ethanol, washed with 70% ethanol, and resuspended in 20 µl of distilled water. DNA (2 µl) was then amplified by polymerase chain reaction (PCR) with two sets of primers specific for the methylated and unmethylated alleles, as described by Herman and colleagues and Graff and colleagues (fig 1). CpGenome Universal Methylated DNA (Intergen, Purchase, New York, USA) in which E-cadherin was methylated and reagent blanks were used as positive and negative controls in each experiment. All tests were performed in duplicate. For confirmation of the specificity of MSP, PCR products from the methylated and unmethylated primers were gel purified and sequenced, as previously described.

Immunohistochemical staining for E-cadherin

E-cadherin expression was examined by immunostaining using the avidin-biotin complex immunoperoxidase method. Briefly, 4 µm thick tissue slides were deparaffinised in xylene and rehydrated serially with alcohol and water. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 minutes, followed by microwave antigen retrieval for nine minutes at 95°C in 10 mM sodium citrate buffer, pH 6.0. The 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, USA). The slides were finally counterstained with Mayer’s haematoxylin. For a negative control, the primary antibody was replaced with mouse IgG. Slides with normal gastric mucosa were used as a positive control. Furthermore, positive E-cadherin staining in the adjacent non-involved gastric mucosa served as an internal positive control. E-cadherin staining was classified as absent staining, cytoplasmic distribution, heterogeneous staining, or normal membranous staining. Because staining patterns often varied within the same tumour, particularly when the degree of differentiation varied, the classification was based on the dominant pattern. Tumours with more than 10% variation were scored as heterogenous.

Statistical analysis

Fisher’s exact test and χ² test were used to compare categorical associations and the Student’s t test for continuous associations. Two sided tests were used to calculate p values. Multivariate analysis using a forward conditional model was used to evaluate the contribution of age, gastritis, and H pylori infection to E-cadherin methylation in the gastric mucosae of patients with dyspepsia. The relationship between E-cadherin methylation status and clinicopathological features, including age, histological subtypes of the carcinoma, depth of tumour invasion, presence and extent of regional lymph node, and distant metastasis, were evaluated in the 26 primary cancers.

RESULTS

MSP for E-cadherin

MSP with both sets of primers for the methylated and unmethylated E-cadherin promoter gave expected results in...
the positive and negative controls, and concordant results in all of the tested specimens. Sequencing of the PCR products confirmed the expected nucleotide changes associated with bisulphite modification in the methylated and unmethylated alleles (fig 1A, 1B).

**E-cadherin methylation, expression, and clinicopathological associations in gastric mucosae from patients with dyspepsia**

The clinicopathological associations of 35 patients with dyspepsia are tabulated in table 1. Gastritis was present in 31 (89%) cases, and *H pylori* infection in 19 (54%) cases. Patients with *H pylori* infection were older compared with those without infection (53 (14) years v 38 (12) years; p=0.002). *H pylori* infection was also associated with gastritis (p=0.009). Intestinal metaplasia or dysplasia was not present in any biopsy on histological assessment. *E-cadherin* methylation in gastric mucosa was present in 11 (31%) patients (fig 2A, 2B). All 11 cases with *E-cadherin* methylation had gastritis (NS) (table 1). *E- cadherin* methylation was associated with *H pylori* infection: 53% (10/19) of patients with *H pylori* infection had *E-cadherin* methylation versus 94% (15/16) of patients without *H pylori* infection and no methylation (p=0.002) (table 1). Patients with *E-cadherin* methylation were older compared with those without methylation (53 (13) years v 43 (15) years; p=0.048). *H pylori* infection was the only factor associated with *E-cadherin* methylation by multivariate analysis (p=0.013 (95% confidence interval 0.007–0.55)). However, immunohistochemical staining showed normal membranous staining of *E-cadherin* in samples with or without *E- cadherin* methylation. This was probably due to the fact that immunohistochemical staining was not as sensitive as PCR in detecting subpopulations of cells with gene methylation and hence downregulation of *E-cadherin*.

**E-cadherin methylation, expression, and clinicopathological associations in resected specimens in patients with gastric cancer**

*E-cadherin* methylation was present in 0 of 8 (0%) normal mucosas, 12 of 21 (57%) intestinal metaplasias, 15 of 26 (58%) primary cancers, and 21 of 32 (65%) metastatic cancers (fig 2C). Methylation status was concordant between 11 of 12 (92%) intestinal metaplasias and cancers, and 17 of 20 (85%) primary and metastatic cancers from the same patients. One patient had methylation in intestinal metastasis but not primary cancer, another had unmethylated primary cancer but methylated metastatic cancer, and two patients had methylated primary cancer but unmethylated metastatic cancer. *E-cadherin* immunohistochemistry was performed on 13 primary and nine metastatic cancers. Eleven primary cancers had loss of membranous distribution of *E-cadherin* (fig 3A) and had *E- cadherin* methylation (100%). Of two primary cancers with retained *E-cadherin* expression, the gene was methylated in one case and unmethylated in the other. Seven metastatic cancers in the lymph node had diminished *E-cadherin* staining (fig 3B), and five (71%) had *E- cadherin* methylation. In the two remaining cancers with retained *E-cadherin* expression, the gene was methylated in one cancer and unmethylated in the other. *E-cadherin* methylation was associated with the depth of tumour invasion. All three tumours confined to the lamina propria (T1) were unmethylated but 15 of 23 (65%) tumours with invasion of the muscularis propria or beyond were methylated (p=0.02) (table 2). *E-cadherin* methylation was also associated with regional nodal metastasis. Four of five (80%) patients without nodal metastasis were unmethylated but 14 of 21 (67%) patients with regional metastasis were methylated (p=0.05) (table 2). However, methylation was not related to age, *H pylori* infection status, histological subtype, or distant metastasis status.

**Discussion**

In this study, we found *E-cadherin* methylation in patients with *H pylori* infection without metaplasia or dysplasia, in intestinal metaplasia, and in primary and metastatic cancers from patients undergoing gastrectomy for gastric cancer. *E-cadherin* methylation was present in one third of gastric mucosae from patients with dyspepsia, and was associated with age and *H pylori* infection. However, by multivariate analysis *H pylori* infection was the only significant contributing factor. *H pylori* can induce transcriptional activation by inflammatory mediators such as nuclear factor κB and cyclooxygenase 2 (COX-2). Methylation in gastric epithelial cells may occur as a consequence of or as an adaptive protective response due to chronic exposure to inflammatory mediators overproduced during infection, a mechanism that has been described in viral infection, or in metaplasia, dysplasia, and carcinoma developing due to chronic reflux injury of the oesophagus and inflammatory bowel disease in the colorectal region. The former is supported by a previous study which showed that *H pylori* infection, a mechanism that has been described in viral infection, or in metaplasia, dysplasia, and carcinoma developing due to chronic reflux injury of the oesophagus and inflammatory bowel disease in the colorectal region.

![Figure 2](image-url)
*H pylori* stimulated COX-2 expression in gastric cell lines without promoter methylation of the COX2 gene but not in cell lines with methylation of COX-2 promoter. However, COX-2 expression can be induced by demethylation treatment with 5-azacytidine followed by exposure to *H pylori* in cell lines with methylation of COX-2 promoter. It is therefore possible that *E-cadherin* methylation may be mediated through similar mechanisms in response to *H pylori* infection. Finally, it is intriguing to note that only about half of gastritis cases with *H pylori* infection showed *E-cadherin* methylation. This could be related to a sampling effect as *E-cadherin* methylation in relation to *H pylori* infection might be focal. Alternatively, other collaborative mechanisms together with *H pylori* infection may be involved in *E-cadherin* methylation.

Also, it is apparent that only a small proportion of *H pylori* related gastritis cases progress to carcinoma. According to Knudson’s “two hit” hypothesis, two genetic mutations are required for inactivation of a putative tumour suppressor gene. Promoter methylation with suppression of gene expression may collaborate with mutation or deletion to cause gene inactivation, thereby fulfilling Knudson’s hypothesis. Thus the occurrence of additional genetic events may be necessary for *E-cadherin* gene inactivation, leading to initiation of carcinogenesis.

We did not observe an association between *H pylori* infection and *E-cadherin* methylation in gastric cancers. This may be related to the fact that this was a retrospective analysis so that prior *H pylori* infection status was not known in all of the cases of cancer. However, it is also well known that *H pylori* infection declines with the development of gastric cancer and histological examination alone cannot reflect accurately previous exposure to *H pylori* infection.

*E-cadherin* methylation was present in 57% of intestinal metaplasias in patients with gastric cancers in our study. The timing of hypermethylation during tumour development may vary among different genes and tumour types. Kang and colleagues demonstrated that methylation at DAP-kinase, THBS1, and TIMP-3 was present in gastritis whereas p16 and hMLH1 were present in intestinal metaplasia and cancer. We demonstrated that the frequency of *E-cadherin* methylation was the highest compared with the methylation frequency at p16 (2.1%), hMLH1 (6.3%), THBS-1 (34.7%), DAP-kinase (36.7%), and TIMP-3 (36.7%) in intestinal metaplasia.

Methylation of *E-cadherin* was present in 58% of primary cancers and 66% of cancers metastatic to lymph nodes in our study, a frequency comparable with previous reports. Interestingly, in the metastatic nodes without *E-cadherin* methylation, there were two cases where the primary tumour showed *E-cadherin* methylation. Similar findings have been observed in immunohistochemical studies of *E-cadherin* expression. It has been postulated that expression of *E-cadherin* might be dynamic. Thus downregulation of *E-cadherin* in the primary tumour may allow cell dispersal into the circulation while re-expression of *E-cadherin* may allow cell deposition at distant sites to form metastases.

There was, in general, concordance between *E-cadherin* methylation and diminished *E-cadherin* expression by

### Table 2

Clinicopathological associations of *E-cadherin* methylation in patients with gastric cancer

<table>
<thead>
<tr>
<th>Clinicopathological parameter</th>
<th><em>E-cadherin</em></th>
<th>p Value</th>
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<tbody>
<tr>
<td></td>
<td>Methylated (%)</td>
<td>Unmethylated (%)</td>
</tr>
<tr>
<td>Age (y)*</td>
<td>65 (14)</td>
<td>58 (13)</td>
</tr>
<tr>
<td>H pylori</td>
<td>Present</td>
<td>6 (67%)</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>5 (56%)</td>
</tr>
<tr>
<td>Histological subtype</td>
<td>Mucinous/signet ring</td>
<td>8 (80%)</td>
</tr>
<tr>
<td>Tubular</td>
<td>7 (44%)</td>
<td>9 (56%)</td>
</tr>
<tr>
<td>Regional nodal metastasis</td>
<td>N0</td>
<td>1 (20%)</td>
</tr>
<tr>
<td></td>
<td>N1/N2</td>
<td>14 (67%)</td>
</tr>
<tr>
<td>Depth of invasion</td>
<td>T1</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>T2/T3/4</td>
<td>15 (65%)</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td>Present</td>
<td>2 (50%)</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>13 (59%)</td>
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*Mean (SD).*
E-cadherin in invasive cancer. Such a hypothesis will require vigorous immunostaining. There was one primary and one metastatic cancer in a lymph node where E-cadherin methylation was not associated with diminished E-cadherin staining. This may indicate that cancer cells with E-cadherin methylation were only a minor subclone of the tumour, which was picked up by the sensitive MSP.

Clinicopathological correlations showed that E-cadherin methylation was associated with depth of invasion and nodal metastasis. This pattern suggests that increasing acquisition of E-cadherin methylation may be related to progressive tumour development. If E-cadherin methylation is also an important initiating event, as occurs in intestinal metaplasia progressing to gastric cancer, a quantitative increase in E-cadherin methylation may be expected as the neoplastic clone progressively increases in size. To further validate these possibilities, analysis of E-cadherin methylation by a quantitative assay is required. These biological considerations notwithstanding, depth of invasion and nodal metastasis in relation to E-cadherin methylation were parameters that may be associated with poor prognosis. In fact, the role of E-cadherin as a prognostic marker in gastric cancer has been previously shown by immunohistochemical studies and serum enzyme linked immunosorbent assays. Furthermore, loss of E-cadherin expression is most pronounced in epithelial carcinomas with an infiltrative growth pattern associated with no intercellular cohesion, such as invasive lobular breast cancer and diffuse-type gastric adenocarcinoma. The increased invasiveness of tumours with E-cadherin methylation identified in our study might be consistent with these findings.

In conclusion, we have shown an association between E-cadherin methylation in the stomach and H. pylori infection in dyspeptic patients without cancer, and in intestinal metaplasia and cancer. Furthermore, E-cadherin methylation in gastric cancer was also associated with depth of tumour invasion and nodal metastasis in gastric adenocarcinoma. However, the results of this study do not exclude mechanisms other than H. pylori infection that may lead independently to E-cadherin methylation. A working hypothesis may be that gastric mucosal infection by H. pylori initiates E-cadherin methylation which may subsequently progress to intestinal metaplasia and invasive cancer. Such a hypothesis will require rigorous testing in future studies. Finally, the potential contribution of E-cadherin methylation to other H. pylori related pathologies may warrant further investigation.

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REFERENCES


