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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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A O-O Chan, S-K Lam, B C-Y Wong, W-M Wong, M-F Yuen, Y-H Yeung, W-M Hui, A Rashid and Y-L Kwong

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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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Background: E-cadherin is an adhesion molecule involved in tumour invasion/metastasis. Silencing of E-cadherin by promoter CpG methylation has been shown in both familial and sporadic gastric cancers. Helicobacter pylori is a class I carcinogen in gastric cancer.

Aims: This study was undertaken to investigate the association between methylation of E-cadherin and H pylori in gastric mucosa from dyspeptic patients, and in intestinal metaplasia and primary and metastatic adenocarcinoma from surgical specimens of patients with gastric cancer.

Methods: E-cadherin methylation was studied using methylation specific polymerase chain reaction in microdissected tissue from biopsies or surgical resection specimens. E-cadherin expression was studied by immunohistochemistry.

Results: E-cadherin methylation was present in 31% (11/35) of gastric mucosa from dyspeptic patients, and was associated with H pylori infection (p=0.002), but was independent of the age of the patient or presence or absence of gastritis. E-cadherin methylation was present in 0% (0/8) of normal mucosa, 57% (12/21) of intestinal metaplasias, and 58% (15/26) of primary and 65% (21/32) of metastatic cancers. E-cadherin methylation status was concordant in 92% (11/12) of intestinal metaplasias and primary cancers, and in 85% (17/20) of primary and metastatic cancers from the same resected specimen. E-cadherin methylation in gastric cancer was associated with depth of tumour invasion (p=0.02) and regional nodal metastasis (p=0.05).

Conclusion: E-cadherin methylation is an early event in gastric carcinogenesis, and is initiated by H pylori infection.

Helicobacter pylori infection is an important aetiologial 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.

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 mucosae, 21 intestinal metaplasias, and 26 primary adenocarcinomas and 32 metastatic lymph nodes from patients undergoing surgery for gastric cancer were tested.

DNA microdissection

DNA was extracted from microdissected mucosa from 35 patients undergoing upper endoscopy for dyspepsia. H pylori infection was confirmed by histology and urease breath test. DNA methylation analysis was performed on 11 dyspeptic patients, 21 intestinal metaplasias, 26 primary gastric cancers, and 32 metastatic lymph nodes from patients undergoing surgery for gastric cancer.

Methylation-specific polymerase chain reaction

Methylation specific polymerase chain reaction was performed as described previously. Primer sequences specific for the promoter region of E-cadherin were kindly provided by Dr H Lee (National University of Singapore).

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 Methylation 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: 1:50 dilution in phosphate buffered saline; ZymedLaboratories 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 heterogeneous.

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 mucosa 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 mucosa, 12 of 21 (57%) intestinal metaplasia, 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 metaplasia 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 mucosa 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).24 25 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,26 or in metaplasia, dysplasia, and carcinoma developing due to chronic reflux injury of the oesophagus and inflammatory bowel disease in the colorectal region.27 28 The former is supported by a previous study which showed that H

| Table 1 Association of E-cadherin promoter methylation with age, gastritis, and Helicobacter pylori infection status in 35 patients with dyspepsia |
|---------------------------|---------------------------|---------------------------|
|                        | Methylated (No (%))       | Unmethylated (No (%))     | p Value  |
| Age (y)*                | 53 (13)                   | 43 (15)                   | 0.048    |
| Chronic gastritis       | Present: 11 (35)          | 20 (65)                   | N5       |
|                         | Absent: 0 (0)             | 4 (100)                   |          |
| H pylori                | Present: 10 (53)          | 9 (47)                    | 0.002    |
|                         | Absent: 1 (6)             | 15 (94)                   |          |

*Mean (SD).

Figure 2 (A) Analysis of methylation of E-cadherin in gastric mucosa from patients with dyspepsia. Samples N3–N6 are methylated, and samples N1, N2, and N7 are unmethylated. Methylation specific polymerase chain reaction (MSP) was performed with the first primer set, resulting in a methylated polymerase chain reaction (PCR) product of 116 bp and an unmethylated PCR product of 97 bp. (B) Analysis of methylation of E-cadherin in gastric mucosa from patients with dyspepsia, as shown in (A), but with MSP performed with the second primer set. Samples N3–N6 are methylated, and samples N1, N2, and N7 are unmethylated. (C) Analysis of methylation of E-cadherin in intestinal metaplasia (IM), gastric adenocarcinomas (T), and metastatic lymph nodes (LN) from patients with gastric cancer. Samples IM2, T1, T2, LN1, and LN2 are methylated, and sample IM1 is unmethylated. MSP was performed with the first primer set.
*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. 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>E-cadherin Methylated (%)</th>
<th>E-cadherin Unmethylated (%)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)*</td>
<td>65 (14)</td>
<td>58 (13)</td>
<td>NS</td>
</tr>
<tr>
<td><em>H pylori</em> Present</td>
<td>6 (67%)</td>
<td>3 (33%)</td>
<td>NS</td>
</tr>
<tr>
<td>Histological subtype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucinous/signet ring</td>
<td>8 (80%)</td>
<td>2 (20%)</td>
<td>NS</td>
</tr>
<tr>
<td>Tubular</td>
<td>7 (44%)</td>
<td>9 (56%)</td>
<td></td>
</tr>
<tr>
<td>Regional nodal metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N&lt;sub&gt;o&lt;/sub&gt;</td>
<td>1 (20%)</td>
<td>4 (80%)</td>
<td>0.05</td>
</tr>
<tr>
<td>N&lt;sub&gt;o&lt;/sub&gt;/N&lt;sub&gt;i&lt;/sub&gt;</td>
<td>14 (67%)</td>
<td>7 (33%)</td>
<td></td>
</tr>
<tr>
<td>Depth of invasion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>0 (0%)</td>
<td>3 (100%)</td>
<td>0.02</td>
</tr>
<tr>
<td>T2/T3/4</td>
<td>15 (65%)</td>
<td>8 (35%)</td>
<td></td>
</tr>
<tr>
<td>Distant metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>2 (50%)</td>
<td>2 (50%)</td>
<td>NS</td>
</tr>
<tr>
<td>Absent</td>
<td>13 (59%)</td>
<td>9 (41%)</td>
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*Mean (SD).
immunostaining. However, 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.15 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 studies16 17 and serum enzyme linked immunosorbant assays.18 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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