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## HELIOBACTER PYLORI

Eradication of *Helicobacter pylori* infection reverses E-cadherin promoter hypermethylation

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**Background:** E-cadherin methylation is important in gastric carcinogenesis. Reversing hypermethylation may halt the carcinogenic process. We have previously reported that *Helicobacter pylori* infection is associated with E-cadherin methylation in chronic gastritis patients.

**Aim:** To examine if eradication of *H pylori* could reverse E-cadherin methylation.

**Methods:** Patients with dyspepsia and positive for *H pylori* infection, with a mucosal biopsy showing chronic active gastritis, were randomised to receive *H pylori* eradication therapy (group 1, n=41) or no treatment (group 2, n=40), and were followed up prospectively. Gastric mucosae were taken for methylation assay at week 0 (before treatment) and week 6 (after treatment). Archived specimens of intestinal metaplasia with *H pylori* infection (n=22) and without (n=19) were retrieved for methylation analysis. Methylation was assessed using methylation specific polymerase chain reaction and sequencing.

**Results:** Methylation at E-cadherin was detected in 46% (19/41) and 17% (7/41) of patients at weeks 0 and 6, respectively, in group 1 (p=0.004); 78.9% (15/19) of specimens were unmethylated after eradication of *H pylori*. Mucosal biopsy showed chronic inactive gastritis in 35 patients, intestinal metaplasia in one, and normal mucosa in five at week 6. Methylation was detected in 47.5% (19/40) and 52.5% (21/40) of patients at weeks 0 and 6, respectively, in group 2 (P=0.5). Gastric mucosal biopsy showed persistent chronic active gastritis in all cases. Methylation frequency did not differ in *H pylori* positive or negative intestinal metaplastic specimens (72.7% v 63%; p=0.5).

**Conclusion:** *H pylori* eradication therapy could reverse methylation in patients with chronic gastritis. This demonstrates an environmental effect on methylation.

Gastric carcinogenesis is a multistep process involving multiple genetic and epigenetic events, with a postulated intestinal metaplasia-dysplasia-invasive carcinoma sequence during morphologic progression.<sup>1</sup> *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.<sup>2</sup>

E-cadherin is an adhesion molecule that is expressed on all epithelial cells.<sup>3-4</sup> 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.<sup>5-6</sup> Furthermore, somatic mutations of E-cadherin were found in approximately 50% of gastric carcinomas of the diffuse histological type.<sup>7</sup> 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.<sup>8</sup> It is now increasingly recognised that epigenetic silencing of gene expression by promoter CpG hypermethylation is an important alternative mechanism in inactivating tumour suppressor genes and tumour associated genes in cancers,<sup>9-10</sup> and in gastric cancer and its precursors.<sup>11-12</sup>

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.<sup>13</sup> Methylation of E-cadherin was identified early in precancerous lesions of gastric cancer, being found in 31% of gastric mucosa with chronic gastritis.<sup>14</sup> More importantly, *H pylori* was an independent risk factor

associated with methylation of E-cadherin in non-lesional gastric mucosa from patients with dyspepsia.<sup>14</sup>

Targeting epigenetic changes that occur before the development of frank malignancy as chemopreventive intervention offers the maximal impact.<sup>10-15</sup> 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

**Abbreviations:** PCR, polymerase chain reaction; MSP, methylation specific polymerase chain reaction

**Table 1** Primers and polymerase chain reaction conditions for E-cadherin, DAPK, MGMT, hMLH1, p16, and OR beta

Locus	Allele	Sense primers	Antisense primers	Annealing temp (°C)
E-cadherin	Methylated	5'- TTAGGTTAGAGGGTTATCGCGT -3'	5'- TAACTAAAAATTCACCTACCGAC-3'	57
	Unmethylated	5'- TAATTTAGGTTAGAGGGTATTGT-3'	5'- CACAACCAATCAACAACACA-3'	53
DAPK	Methylated	5'- GGATAGTCGGATCGAGTTAACGTC -3'	5'- CCCTCCCAAACGCCGA -3'	60
	Unmethylated	5'- GGAGGATAGTTGGATTGAGTTAATGTT -3'	5'- CAAATCCCTCCCAAACACCAA -3'	60
MGMT	Methylated	5'- TTCGACGTTCTAGTTCGTCG -3'	5'- GCACTCTCCGAAAACGAAACG -3'	59
	Unmethylated	5'- TTTGTGTTTGTATGTTTGTAGGTTTTGT-3'	5'- AACTCCACACTCTCCAAAAACAAAACA-3'	59
hMLH1	Methylated	5'- TATATCGTTCGTAGTATTCGTGT -3'	5'- TCCGACCCGAATAAACCCAA-3'	60
	Unmethylated	5'- TTTGATGTAGATGTTTATTAGGGTGT -3'	5'- ACCACCTCATCATACTACCACA -3'	60
p16	Methylated	5'- TTATTAGAGGGTGGGGCGGATCGC-3'	5'- GACCCCGAACCCGCGACCGTAA-3'	65
	Unmethylated	5'- TTATTAGAGGGTGGGGTGGATTGT-3'	5'- CAACCCCAAACCAACCAACATAA-3'	60
OR beta	Methylated	5'- TTTGGAAGGTGGGTTTGGTC-3'	5'- CGCATACAAATATAATAACTAACG-3'	49
	Unmethylated	5'- TTTGGAAGGTGGGTTTGGTT-3'	5'- CACATACAAATATAATAACTAACCA-3'	45

DAPK, DAP kinase; MGMT, O<sup>6</sup>-methyl-guanine methyltransferase; hMLH1, human Mut L homologue; OR beta, oestrogen receptor beta.

the analysis. This approach has been validated previously in our centre.<sup>16</sup> Antral gastric mucosal biopsies were taken for histological analysis, E-cadherin immunohistochemical staining, and methylation study (week 0). Haematoxylin-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 consisted 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.

### Methylation specific polymerase chain reaction (MSP)

Gastric mucosal tissue was obtained by microdissection from 5 µm thick haematoxylin-eosin stained, paraffin embedded tissue sections without a coverslip, followed by DNA extraction, as described previously.<sup>17</sup> The methylation status of the *E-cadherin* promoter was determined by bisulfite treatment of DNA followed by MSP, as described previously.<sup>18</sup> MSP was performed by an operator unaware of the clinical data of the specimens. Briefly, 2 µg of DNA were denatured with 2 M NaOH at 37°C for 10 minutes, followed by incubation with 3 M sodium bisulfite, pH 5.0, at 50°C for 16 hours. Bisulfite 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 µm) was then amplified for

the E-cadherin gene by polymerase chain reaction (PCR) with the primers described by Herman and colleagues (table 1).<sup>18</sup> CpGenome Universal Methylated DNA (Intergen, Purchase, New York, USA) 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 the MSP, PCR products from the methylated and unmethylated primers were gel purified and sequenced, as previously described.<sup>19</sup>

Pre- and post-*H pylori* eradication methylation pattern was also assessed in 10 additional pairs of biopsy specimens from group 1 at DAP kinase (DAPK), O<sup>6</sup>-methyl-guanine methyltransferase (MGMT), human Mut L homologue (hMLH1), p16 genes,<sup>20</sup> and oestrogen receptor beta (table 1).<sup>21</sup>

### Immunohistochemical staining for E-cadherin

E-cadherin expression was examined by immunostaining using the avidin-biotin complex immunoperoxidase method, as described previously.<sup>13</sup> 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. 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.

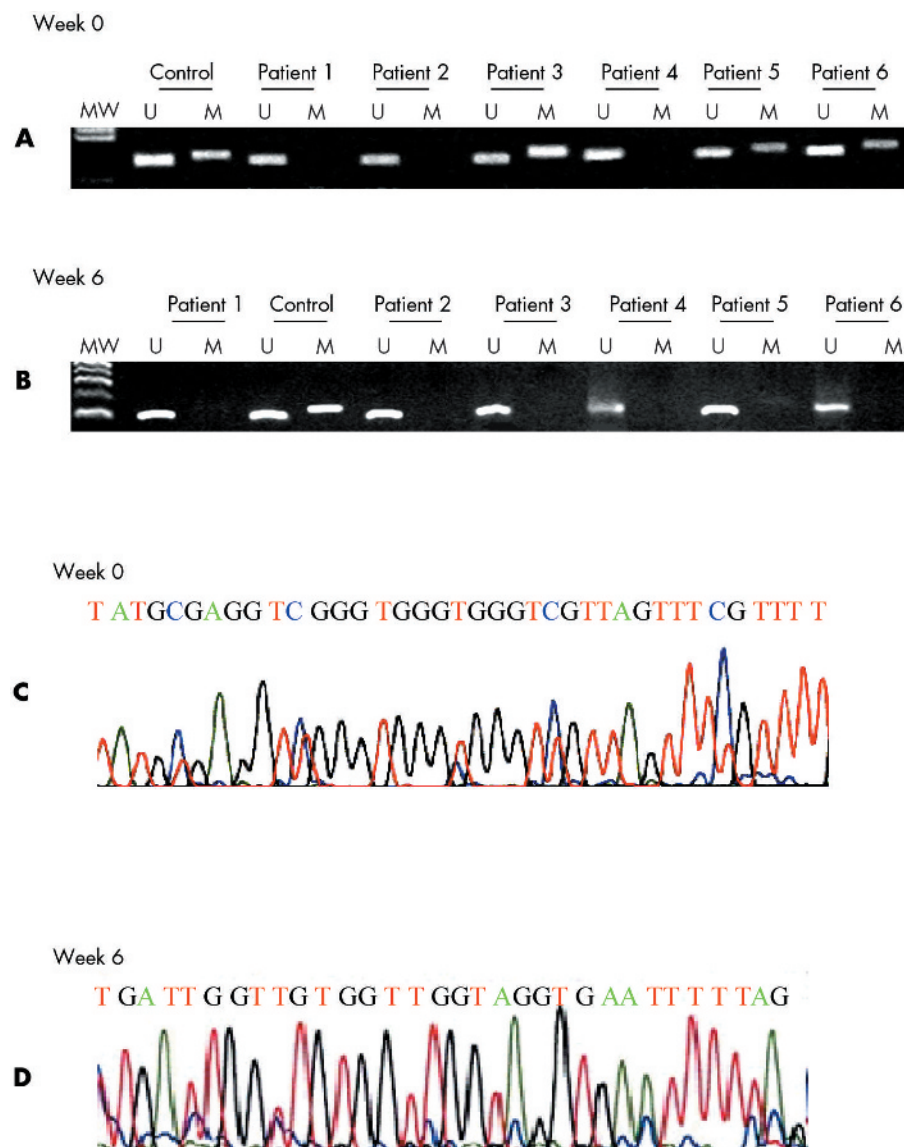
### Statistical analysis

The  $\chi^2$  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



**Figure 1** CpG island methylation pattern at the E-cadherin gene in gastric mucosa from patients with dyspepsia. MW, molecular weight marker; U, unmethylated band; M, methylated band. (A) At week 0 before eradication of *Helicobacter pylori*, methylation was present in patient Nos 3, 5, and 6. (B) At week 6 after eradication of *H pylori*, methylation was not present in any patient. (C) At week 0 before eradication of *H pylori*, the methylated product was confirmed by sequencing using the same methylated primer. The unmethylated cytosines were converted to thymidine (red colour) while the methylated cytosines remained unchanged (blue colour). (D) At week 6 after eradication of *H pylori*, the methylated product was again confirmed by sequencing using the same methylated primer. No methylated cytosine was seen.

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 ( $p = 0.2$ ).

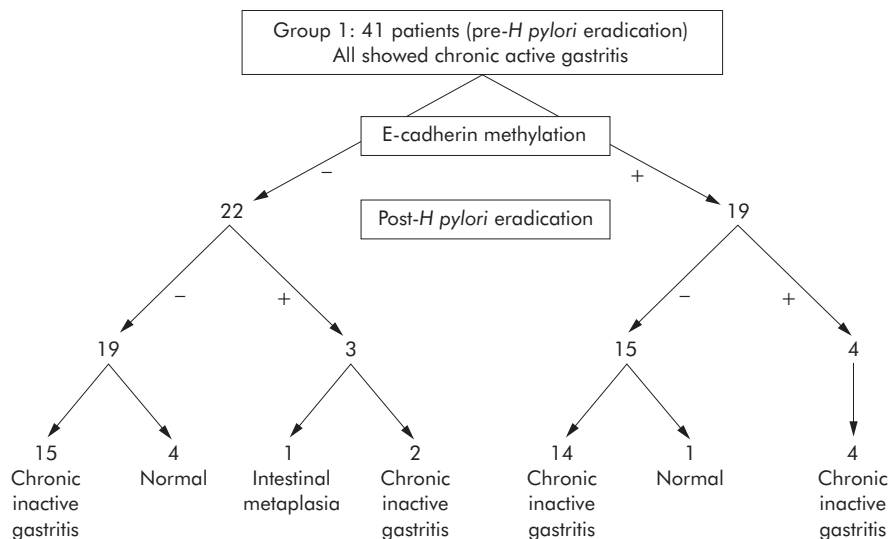
#### Promoter methylation at *E-cadherin* at week 0 and week 6 in group 1 patients

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



**Figure 2** Summary of the methylation and histology status of the 41 patients in group 1, before and after *Helicobacter pylori* eradication.

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 methylation: four 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,<sup>5-7</sup> 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.<sup>20</sup> The genes that we chose are involved in cell cycle regulation (p16), DNA repair or protection (hMLH1, MGMT), and apoptosis (DAP kinase).

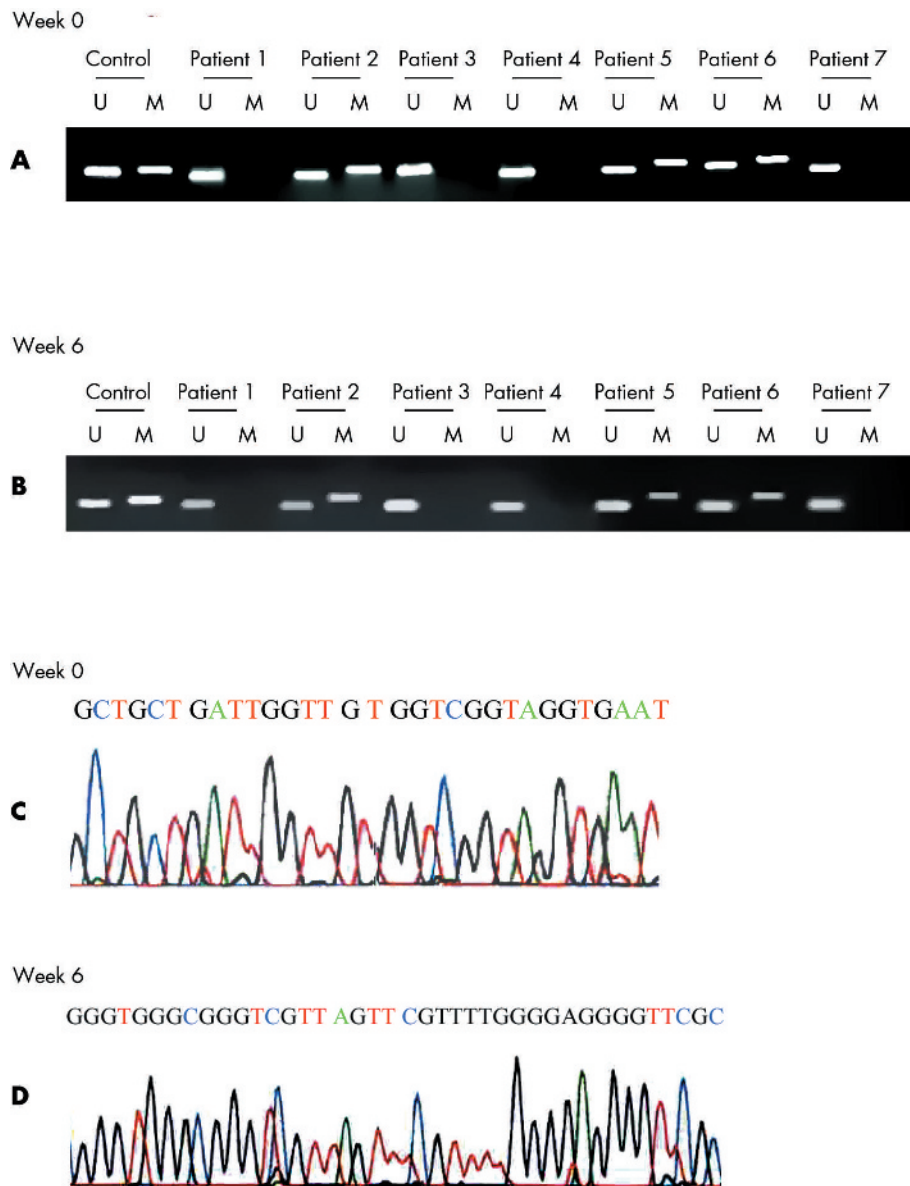
In addition to the important role *E-cadherin* plays in gastric cancer, we have also demonstrated in our previous work that methylation of the *E-cadherin* promoter is associated with gastric cancer and have generated a hypothesis linking *E-cadherin* methylation, *H pylori* infection, intestinal metaplasia, and gastric carcinogenesis.<sup>13,14</sup> 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,<sup>22,23</sup> oesophageal mucosa in patients with Barrett's oesophagitis,<sup>24,25</sup> and in liver tissues in chronic hepatitis.<sup>26</sup> We also observed in the current study that, in the presence of

**Table 2** *E-cadherin* methylation in gastric mucosal specimens before and after *Helicobacter pylori* eradication

	E-cadherin methylated	E-cadherin unmethylated
Before <i>H pylori</i> eradication	19	22
After <i>H pylori</i> eradication	7*	34

\**E-cadherin* was previously methylated in four patients but unmethylated in three patients.



**Figure 3** CpG island methylation at the E-cadherin gene in gastric mucosa from patients with dyspepsia without receiving *Helicobacter pylori* eradication therapy. MW, molecular weight marker; U, unmethylated band; M, methylated band. (A) At week 0, methylation was present in patient Nos 2, 5, and 6. (B) At week 6, methylation was still present in patient Nos 2, 5, and 6. (C) At week 0, methylated products were confirmed by sequencing using the same methylated primer. Methylated cytosines were seen. (D) At week 6, methylated cytosines were still observed after sequencing using the same methylated primer.

intestinal metaplasia, E-cadherin methylation did not associate with the presence of *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 $\beta$  via the action of

nitric oxide.<sup>27</sup> It has also been recently reported that *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.<sup>28</sup> Thus the disappearance of methylation at the stage of chronic gastritis may also be directly related to abolishment of oxidative stress caused by *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-*H pylori* eradication in gastric mucosae, 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<sup>-3</sup> (results not shown). On the other hand,

**Table 3** Promoter methylation at DAPK, MGMT, hMLH1, OR beta, and p16 at week 0 and week 6 in 10 of the group 1 patients

	Week 0 (n = 10) (No (%))	Week 6 (n = 10) (No (%))
DAPK	2 (20)	1 (10)
MGMT	0	2 (20)
hMLH1	1 (10)	1 (10)
OR beta	6 (60)	5 (50)
p16	0	0
E-cadherin	5 (50)	2 (20)

DAPK, DAP kinase; MGMT, O<sup>6</sup>-methyl-guanine methyltransferase; hMLH1, human Mut L homologue; OR beta, oestrogen receptor beta.

immunohistochemical 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 immunostaining.

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,<sup>20</sup> 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.<sup>29</sup> 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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Conflict of interest: None declared.

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