Virulence of infecting *Helicobacter pylori* strains and intensity of mononuclear cell infiltration are associated with levels of DNA hypermethylation in gastric mucosae


To cite this article: Barbara G Schneider, M Blanca Piazuelo, Liviu A Sicinschi, Robertino Mera, Dun-Fa Peng, Juan Carlos Roa, Judith Romero-Gallo, Alberto G Delgado, Thibaut de Sablet, Luis E Bravo, Keith T Wilson, Wael El-Rifai, Richard M Peek Jr & Pelayo Correa (2013) Virulence of infecting *Helicobacter pylori* strains and intensity of mononuclear cell infiltration are associated with levels of DNA hypermethylation in gastric mucosae, Epigenetics, 8:11, 1153-1161, DOI: 10.4161/epi.26072

To link to this article: https://doi.org/10.4161/epi.26072
Virulence of infecting *Helicobacter pylori* strains and intensity of mononuclear cell infiltration are associated with levels of DNA hypermethylation in gastric mucosae

Barbara G Schneider1,*, M Blanca Piazuelo1, Liviu A Sicinschi1,2, Robertino Mera1, Dun-Fa Peng1, Juan Carlos Roa4, Judith Romero-Gallo1, Alberto G Delgado1, Thibaut de Sabela1, Luis E Bravo5, Keith T Wilson1,6, Wael El-Rifai3,4, Richard M Peek Jr1, and Pelayo Correa1

1Division of Gastroenterology; Department of Medicine; Vanderbilt University Medical Center; Nashville, TN USA; 2Holmes Regional Medical Center; Melbourne, FL USA; 3Department of Surgery; Vanderbilt University Medical Center; Nashville, TN USA; 4Department of Pathology; School of Medicine; Pontificia Universidad Catolica de Chile; Santiago, Chile; 5Department of Pathology; School of Medicine; Universidad del Valle; Cali, Colombia; 6Veterans Affairs Tennessee Valley Healthcare System and Office of Medical Research; Department of Veterans Affairs; Nashville, TN USA

Keywords: methylation, chronic inflammation, gastric cancer, pyrosequencing, *Helicobacter*, biopsy

Abbreviations: EBV, Epstein Barr virus; LCM, laser capture microdissection; FFPE, formalin-fixed, paraffin-embedded; MLST, multi-locus sequence typing; HR, high risk; LR, low risk; H. pylori, *Helicobacter pylori*; NAG, non-atrophic gastritis; IM, intestinal metaplasia; MAG, multi-focal atrophic gastritis; DYS, dysplasia; PMN, polymorphonuclear cells; MN, mononuclear cells; CI, confidence interval; IRB, institutional review board

DNA methylation changes are known to occur in gastric cancers and in premalignant lesions of the gastric mucosa. In order to examine variables associated with methylation levels, we quantitatively evaluated DNA methylation in tumors, non-tumor gastric mucosae, and in gastric biopsies at promoters of 5 genes with methylation alterations that discriminate gastric cancers from non-tumor epithelia (*EN1, PCDH10, RSPO2, ZIC1*, and *ZNF610*). Among Colombian subjects at high and low risk for gastric cancer, biopsies from subjects from the high-risk region had significantly higher levels of methylation at these 5 genes than samples from subjects in the low risk region (*P* ≤ 0.003). When results were stratified by *Helicobacter pylori* infection status, infection with a *cagA* positive, *vacA* s1m1 strain was significantly associated with highest methylation levels, compared with other strains (*P* = 0.024 to 0.001). More severe gastric inflammation and more advanced precancerous lesions were also associated with higher levels of DNA methylation (*P* ≤ 0.001). In a multivariate model, location of residence of the subject and the presence of *cagA* and *vacA* s1m1 in the *H. pylori* strain were independent variables associated with higher methylation in all 5 genes. High levels of mononuclear cell infiltration were significantly related to methylation in *PCDH10, RSPO2*, and *ZIC1* genes. These results indicate that for these genes, levels of methylation in precancerous lesions are related to *H. pylori* virulence, geographic region and measures of chronic inflammation. These genes seem predisposed to sustain significant quantitative changes in DNA methylation at early stages of the gastric precancerous process.

Introduction

Gastric cancer was responsible for approximately 738,000 deaths worldwide in 2008, and is the 3rd most common lethal cancer in men and 5th in women.1 This disease is distinctive in its association with two infectious agents, the bacterium *Helicobacter pylori* (*H. pylori*) and Epstein-Barr virus (EBV). While EBV is associated with approximately 10% of gastric cancer cases, *H. pylori* is a risk factor for at least 80–90% of cases, especially for non-cardiac tumors.2 Furthermore, *H. pylori* has been classified as a human carcinogen3 and, as a bacterium, can be eradicated with antibiotics. Yet, half the world’s population harbors this infection. Even if cost were not an issue, treatment of large populations with antibiotics poses the risk of development of antibiotic-resistant strains of *H. pylori* and other pathogenic bacteria, as well as side effects from therapy. Identification of better biomarkers for gastric cancer risk would allow more efficient targeting of prevention efforts.

*H. pylori* isolates are genetically diverse, and virulence differs from strain to strain. A well-established virulence factor is encoded...
Hypermethylated promoter DNA is of additional interest as representing potential biomarkers for gastric cancer. Hypermethylated DNA for Reprimo (RPRM, a tumor suppressor gene in gastric cancer), CDKN2A, and other markers have been detected in serum or plasma of gastric cancer patients at levels significantly different from those found in control patients. These findings raise hope that biomarkers may eventually be developed, that will facilitate early detection and thus reduce mortality of this disease.

Gastric cancer of the intestinal subtype develops over decades, through a series of well-defined premalignant lesions. Infection with H. pylori induces gastritis, which may progress to multifocal atrophic gastritis (MAG), intestinal metaplasia (IM), dysplasia, and carcinoma. Although, in theory, the many years typically required for these stages to advance provide an opportunity for intervention, in practice, gastric cancers are typically diagnosed at late stages, especially in developing countries where screening is not common. To investigate DNA methylation with respect to this process of progression, we examined several variables, including severity of lesions and H. pylori virulence, to determine how these influenced levels of DNA methylation in 5 genes that become hypermethylated in gastric cancers.

### Results

#### DNA methylation in tumors

When gastric cancer samples were analyzed by pyrosequencing for methylation at EN1, PCDH10, RSPO2, ZIC1, and ZNF610, results for the set of diffuse tumors were not significantly different from that of the set of intestinal tumors, so results for both subtypes were combined. Mean methylation was 48% (38.1–57.8, 95% CI) for EN1, 67.9% (61–74.7, 95% CI) for PCDH10, 53.2% (43.4–63, 95% CI) for RSPO2, 51.5% (40.5–62.4, 95% CI) for ZIC1, and 61.8% (53.4–70.2, 95% CI) for ZNF610 (Fig. 1). Corresponding values for histologically normal mucosae were 22.1% (16.1–28.2, 95% CI), 21.8% (14.4–29.2, 95% CI), 11.2% (7.2–15.1, 95% CI), 9.6% (5.4–13.8, 95% CI), and 7.2% (3.3–11.1, 95% CI), respectively, all significantly different from the tumors (p < 0.0001 for all). As discriminators of tumor from non-tumor mucosae, the area under the curve (AUC) for ZIC1 was 0.95 (95% CI, 0.85–0.99); for ZNF610, 0.94 (95% CI, 0.85–0.99); for RSPO2, 0.89 (95% CI, 0.77–0.97); for PCDH10, 0.90 (95% CI, 0.77–0.96); and for EN1, 0.77 (95% CI, 0.62–0.89).

#### Univariate analysis of biopsy results

For the biopsy analysis, Table 1 provides information regarding the study population and infecting H. pylori strains. Subjects residing in the high-risk region showed more advanced mucosal lesions (p = 0.024). For each of the five genes (EN1, PCDH10, RSPO2, ZIC1, and ZNF610), we stratified DNA methylation results by geographic risk region, by histopathological diagnosis, and by characteristics of the infecting H. pylori strains, including cagA, vacA s and m alleles, and the number of EPIYA motifs (3 vs. more than 3) within the CagA of the infecting strains. For all five genes, methylation was significantly increased in DNA from subjects from the high-risk region compared with those from the low risk region (P values ranging from 0.003 to 0.0003; Fig. 2).
When results from all subjects were stratified into categories of uninfected, infected \((cagA\) negative) and infected \((cagA\) positive), samples associated with \(cagA\) positive strains showed significantly higher methylation than did those from uninfected persons. Results associated with persons harboring \(cagA\) negative \(H. pylori\) strains were intermediate in DNA methylation, although for \(EN1\) and \(ZNF610\), the difference in methylation between DNA from uninfected persons and those with \(cagA\) negative infections was not significant (Fig. 3). The \(vacA\ s1m1\) genotype is highly associated with \(cagA\) positivity and the \(vacA\ s2m2\) genotype with \(cagA\) negativity, so the relationship of \(vacA\) genotypes to methylation produced an almost identical pattern to that of the \(cagA\) analysis (data not shown). The level of methylation was not related to the number of EPIYA motifs or to strain ancestry (European vs. African) as characterized by MLST (data not shown).

When the relationship of diagnoses to methylation was evaluated, MAG and IM/dysplasia categories had higher methylation in all genes except \(ZNF610\), compared with samples from uninfected persons or persons with non-atrophic gastritis (NAG) (Fig. 4). Methylation levels measured in samples from subjects diagnosed with MAG vs IM / dysplasia were not different from each other. Results were similar, for evaluation by both categories, or by histopathology score in a multivariate model. Biopsies diagnosed with dysplasia were too few to analyze separately, so they were grouped with those diagnosed with IM. In the age range of our subjects (39 to 60 y), methylation was not significantly related to age for any of the five genes. Methylation in all 5 genes was highly correlated with methylation in the other genes, with correlation coefficients for each comparison ranging from 0.7882 for \(EN1\) with \(ZNF610\) to 0.9609 for \(ZIC1\) with \(RSPO2\). In univariate analysis, the mononuclear cell (MN) score was significantly related to methylation in all 5 genes \((P < 0.001\) for all); however, because the MN score is closely associated with \(H. pylori\) virulence, region and histology score, a multivariate approach was necessary. In univariate analysis, the PMN score was significantly related to methylation for \(PCDH10\) only \((P = 0.007)\).

**Multivariate analysis of biopsy results**

Table 2 shows multivariate analysis of methylation differences in gastric biopsies. Location of residence (HR vs. LR) and \(H. pylori\) virulence factors \(cagA\) and \(vacA\) s and m remained significant variables in the model. Histological diagnosis lost

<table>
<thead>
<tr>
<th>Variables</th>
<th>Low risk for gastric cancer (n = 45)</th>
<th>High risk for gastric cancer (n = 42)</th>
<th>Two-tailed P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years, mean (SD)</td>
<td>47.5 (6.0)</td>
<td>49.6 (4.5)</td>
<td>0.071</td>
</tr>
<tr>
<td>(cagA) assessment by PCR, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>6 (13.3)</td>
<td>2 (4.8)</td>
<td>0.361</td>
</tr>
<tr>
<td>(cagA) negative</td>
<td>7 (15.6)</td>
<td>6 (14.3)</td>
<td></td>
</tr>
<tr>
<td>(cagA) positive</td>
<td>32 (71.1)</td>
<td>34 (80.9)</td>
<td></td>
</tr>
<tr>
<td>(H. pylori) genotypes, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>6 (13.3)</td>
<td>2 (4.8)</td>
<td>0.192</td>
</tr>
<tr>
<td>(cagA) positive, (vacA\ s1m1)</td>
<td>29 (64.4)</td>
<td>34 (80.9)</td>
<td></td>
</tr>
<tr>
<td>Other genotype</td>
<td>10 (22.2)</td>
<td>6 (14.3)</td>
<td></td>
</tr>
<tr>
<td>Histopathological diagnosis, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal/NAG</td>
<td>28 (62.2)</td>
<td>14 (33.3)</td>
<td>0.024</td>
</tr>
<tr>
<td>MAG</td>
<td>6 (13.3)</td>
<td>8 (19.1)</td>
<td></td>
</tr>
<tr>
<td>IM/Dysplasia</td>
<td>11 (24.4)</td>
<td>20 (47.6)</td>
<td></td>
</tr>
<tr>
<td>% Methylation, mean (SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(EN1)</td>
<td>20.2 (4.7)</td>
<td>24.5 (6.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(PCDH10)</td>
<td>24.4 (6.6)</td>
<td>30.0 (7.7)</td>
<td>0.001</td>
</tr>
<tr>
<td>(RSPO2)</td>
<td>14.9 (4.7)</td>
<td>19.4 (6.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(ZIC1)</td>
<td>11.5 (3.9)</td>
<td>15.8 (6.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(ZNF610)</td>
<td>14.3 (5.2)</td>
<td>18.9 (8.5)</td>
<td>0.003</td>
</tr>
</tbody>
</table>

*P value from \(X^2\), Fisher’s exact, t-test or Wilcoxon/Mann-Whitney test as appropriate; †This category includes a subject with a strain of \(H. pylori\) that was \(cagA\) positive and \(vacA\ s1\), but which repeatedly failed PCR for \(vacA\ m\); SD, standard deviation; NAG, non-atrophic gastritis; IM, intestinal metaplasia.
significance in the model, once the *H. pylori* virulence factors were included. In a multivariate model for average methylation across the 5 genes, region and *H. pylori* virulence remained as significant variables. Chronic inflammation, as measured by the level of infiltration of MN cells, remained significant for methylation at PCDH10, RSPO2, and ZIC1 after adjusting for other variables. The PMN score, significantly related to methylation only for PCDH10 in univariate analysis, lost significance in the multivariate analysis.

**Discussion**

For analyses of DNA methylation, we selected genes with possible functionality in promoting tumor development, and which showed marked discrimination of tumor from non-tumor gastric mucosae in their levels of promoter methylation. Notably, these genes also showed variation in methylation in premalignant lesions, unlike some others we previously reported, for example, APC. EN1 (engrailed homeobox 1) encodes a homeodomain-containing protein involved in development of the central nervous system. EN1 lies within the middle portion of a region of long-range epigenetic silencing in colorectal cancer, located on chromosome 2q14.2. EN1 is hypermethylated in 70% of colorectal cancers and has been suggested as a potential biomarker for this tumor type. In addition, EN1 is hypermethylated in 65% of prostate cancers and in 80% of serous ovarian cancers.

PCDH10, a member of the protocadherin family, encodes a calcium-dependent cell-cell adhesion molecule. Although expressed at highest levels in the central nervous system, PCDH10 has tumor suppressor activity in gastric cancer, as well as in nasopharyngeal, esophageal, and colorectal cancers. PCDH10 is hypermethylated in 82% of nasopharyngeal carcinomas, 51% of esophageal carcinomas, 45% of breast cancers, 42% of hepatocellular carcinomas, and in 42% to over 80% of gastric cancers.

RSPO2 (R spondin family member 2) encodes a secreted protein that regulates β-catenin signaling and can modulate invasiveness of cultured mammary epithelial cells. RSPO2 is an important regulator of lung, limb, and tracheal development. Although reports mainly indicate a growth-promoting effect of RSPO2 expression, stable expression of transfected RSPO2 in mammary epithelial cells was associated with lowered rates of cell proliferation in monolayer culture. The RSPO2 protein may maintain, rather than increase Wnt pathway activation. In a genetic screen following insertion of a transposon, RSPO2 was identified as a gene that when dysregulated, may lead to colorectal cancer in a mouse model. Of interest, cagA-positive *H. pylori* strains can activate β-catenin.

ZIC1 (zinc finger protein of the cerebellum 1) encodes a zinc finger protein important in organogenesis in the central nervous system, especially for development of the dorsal spinal cord and cerebellum. The ZIC1 protein regulates Nodal and Sonic Hedgehog signaling, as well as retinoic acid signaling, and may serve as a tumor suppressor gene in colorectal cancers. In earlier studies, ZIC1 was reported to be hypermethylated in 95% of gastric cancers and in 85% of colorectal cancers. When the ZIC1 protein was ectopically expressed in gastric cancer cell lines, colony formation was reduced. Zhong et al. showed that when the ZIC1 protein was ectopically expressed in gastric cancer cell lines, cell cycle distributions were altered (more cells were found in G1 phase), expression of the cyclin-dependent kinase inhibitors p21 and p27 was increased, and expression of sonic hedgehog was reduced. While methylation of the ZIC1 promoter had a numerically higher AUC value in discriminating tumors from non-tumor mucosae, the difference from the other genes was not significant.

ZNF610 encodes zinc finger protein 610, which belongs to the Krüppel C2H2-type zinc finger protein family. ZNF610 encodes 9 C2H2-type zinc fingers and 1 Krüppel-associated box (KRAB) domain. KRAB domains may serve as transcriptional repressors by recruiting histone-modifying proteins and interacting with the co-repressor KAP1. Other members of the KRAB family of proteins act to inhibit transformation by Myc and affect p53-dependent apoptosis.

Our study detected quantitative alterations in methylation occurring in biopsies of subjects diagnosed with MAG, compared with biopsies from uninfected subjects or those with NAG. This finding is interesting, in light of the fact that MAG (or chronic atrophic gastritis) is the gastric lesion recognized as the earliest in the precancerous cascade. This finding is also consistent with the Epigenetic Progenitor model of cancer development, which proposes that the earliest disruptions in the development of a malignancy are epigenetic ones. Notably, we found no significant differences between MAG and IM lesions in the biopsies, but in the gastric cancers, levels of methylation were highly variable, with some approaching 100% and some having levels similar to those of the precancerous lesions.

Subjects infected with the more virulent strains of *H. pylori* had higher levels of methylation. Levels of hypermethylation increased progressively in DNA from uninfected subjects, to subjects with cagA negative infections, and to subjects with cagA

---

**Figure 2.** Area of residence and methylation. Geographic location of residence of the subjects was significantly associated with levels of methylation in the 5 genes, both in the univariate and multivariate models. Bars indicate means, with 95% CI. LR, low risk; HR, high risk.
positive infections. This effect of the *H. pylori* virulence had a stronger association than the histology variable in the multivariate model. We were unable to separate the effect of *cagA* from that of *vacA* s1m1, due to the strong association of these virulence factors with each other. Although the association of *H. pylori* infection with DNA methylation has been known for some years, the effect of virulence determinants has been less explored. Examination of gastric washings from gastric cancer patients and controls indicated no association with *cagA* positivity in the strains. However, Sepulveda et al. found a higher proportion of their set of gastric biopsies designated as positive for MGMT methylation to be associated with *cagA* positivity vs. *cagA* negativity in the infecting strain. In contrast to these prior studies, our study measured average levels of methylation in individual tissues, which may reflect the cell composition and expansion of cell compartments.

The finding of the association of mononuclear inflammatory cell infiltration with high levels of methylation in three of the genes is consistent with the idea that chronic inflammation promotes aberrant DNA methylation. In the gerbil model of *H. pylori*-induced gastric cancer, treatment of infected animals with cyclosporin A to repress inflammation suppressed methylation while not altering the presence of the *H. pylori* infection; *cagA* negative, methylation in biopsies from subjects with *H. pylori* strains lacking the *cagA* gene; *cagA* positive, methylation in biopsies from subjects with *H. pylori* infections bearing the *cagA* gene.

In conclusion, we have identified a panel of genes that sustain DNA hypermethylation in gastric cancers, and that also show significant methylation differences in gastric mucosae in early stages of the precancerous process. These differences are associated with *H. pylori* virulence, geographic area and (for *PCDH10*, *RSPO2*, *ZIC1*, and *ZNF610*), only *RPRM* showed an association of promoter hypermethylation with more virulent infecting *H. pylori* strains, more severe gastric lesions, and geographic location. The genes *EN1*, *PCDH10*, *RSPO2*, *ZIC1*, and *ZNF610*, with *RPRM*, form a list of genes that seem prone to sustain significant measurable changes in DNA methylation at early stages of disease.

In conclusion, we have identified a panel of genes that sustain DNA hypermethylation in gastric cancers, and that also show significant methylation differences in gastric mucosae in early stages of the precancerous process. These differences are associated with *H. pylori* virulence, geographic area and (for *PCDH10*, *RSPO2*, and *ZIC1*) differences in chronic inflammation, in a multivariate model. All these variables are known to be related to gastric cancer risk. The practical utilities of these genes as markers for disease progression in high-risk populations are under investigation.

**Methods**

**Human gastric cancer tissues**

A total of 53 gastric adenocarcinomas (9 frozen, 44 formalin-fixed, paraffin-embedded, or FFPE) were used in the study.
Of these, 43 were of the intestinal subtype and 10 were diffuse. De-identified frozen gastric adenocarcinomas (n = 9) plus matched non-tumor gastric mucosa from the same patients were obtained from Vanderbilt’s Translational Pathology Shared Resource and from the Cooperative Human Tissue Network. These tissues were collected with approval of Vanderbilt’s Institutional Review Board, in association with the Cooperative Human Tissue Network. Sections (5 microns) were harvested on slides, fixed in 70% ethanol and dehydrated in graded ethanol solutions and xylene. FFPE gastric adenocarcinomas were obtained from Temuco, Chile; Pasto, Colombia; New Orleans, LA; San Antonio, TX; and Nashville, TN. Chilean tumors (n = 22 FFPE) came from the Hospital Regional Temuco. Colombian tumors (n = 14 FFPE) were provided from the Hospital Fundacion San Pedro. Gastric adenocarcinoma tissues from North American patients (n = 8 FFPE) were obtained from University Hospital, Touro Infirmary, and Baptist Medical Center. The use of these tissues was approved by the Ethics Committee of the Universidad de la Frontera, the Ethics Committee of the Hospital Fundacion San Pedro, the Institutional Review Boards of the Louisiana State University Health Sciences Center, the Vanderbilt University Medical Center, and the University of Texas Health Sciences Center. Cancer cells or normal epithelia were harvested either by laser capture microdissection (LCM) with an Arcturus Pixcell IIe instrument (Life Technologies), or by manual microdissection when the desired cell type was at least 50% of total cells. Following LCM, harvested cells were digested overnight from the Capsure® using proteinase K, heated to denature the proteinase K, and subjected to bisulfite modification without additional purification.

**Table 2. Multivariate generalized linear model* analysis**

<table>
<thead>
<tr>
<th>Genes (% methylation)</th>
<th>EN1 (n = 87)</th>
<th>P</th>
<th>PCDH10 (n = 86)</th>
<th>P</th>
<th>RSPO2 (n = 86)</th>
<th>P</th>
<th>ZIC1 (n = 87)</th>
<th>P</th>
<th>ZNF610 (n = 86)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Area</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low risk</td>
<td>20.5 ± 0.7</td>
<td>Ref</td>
<td>25.2 ± 0.9</td>
<td>Ref</td>
<td>15.4 ± 0.7</td>
<td>Ref</td>
<td>11.8 ± 0.7</td>
<td>Ref</td>
<td>14.4 ± 1.0</td>
<td>Ref</td>
</tr>
<tr>
<td>High risk</td>
<td>24.1 ± 0.8</td>
<td>0.003</td>
<td>29.1 ± 0.9</td>
<td>0.003</td>
<td>18.9 ± 0.8</td>
<td>0.002</td>
<td>15.4 ± 0.8</td>
<td>0.002</td>
<td>18.8 ± 1.0</td>
<td>0.005</td>
</tr>
<tr>
<td><strong>Diagnosis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal/NAG</td>
<td>21.7 ± 0.8</td>
<td>Ref</td>
<td>26.5 ± 0.9</td>
<td>Ref</td>
<td>16.6 ± 0.8</td>
<td>Ref</td>
<td>13.3 ± 0.8</td>
<td>Ref</td>
<td>17.2 ± 1.1</td>
<td>Ref</td>
</tr>
<tr>
<td>MAG</td>
<td>22.8 ± 1.4</td>
<td>0.51</td>
<td>27.8 ± 1.6</td>
<td>0.51</td>
<td>17.8 ± 1.4</td>
<td>0.44</td>
<td>13.8 ± 1.3</td>
<td>0.79</td>
<td>16.3 ± 1.8</td>
<td>0.69</td>
</tr>
<tr>
<td>IM/dysplasia</td>
<td>22.7 ± 0.9</td>
<td>0.47</td>
<td>27.6 ± 1.1</td>
<td>0.47</td>
<td>17.4 ± 0.9</td>
<td>0.53</td>
<td>13.8 ± 0.9</td>
<td>0.71</td>
<td>15.6 ± 1.2</td>
<td>0.37</td>
</tr>
<tr>
<td><strong>H. pylori genotypes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>16.5 ± 1.8</td>
<td>Ref</td>
<td>15.2 ± 2.1</td>
<td>Ref</td>
<td>9.4 ± 1.8</td>
<td>Ref</td>
<td>6.8 ± 1.8</td>
<td>Ref</td>
<td>9.9 ± 2.5</td>
<td>Ref</td>
</tr>
<tr>
<td>cagA negative</td>
<td>19.4 ± 1.5</td>
<td>0.214</td>
<td>23.7 ± 1.7</td>
<td>0.002</td>
<td>14.5 ± 1.5</td>
<td>0.024</td>
<td>11.2 ± 1.4</td>
<td>0.05</td>
<td>11.8 ± 2.0</td>
<td>0.53</td>
</tr>
<tr>
<td>cagA positive</td>
<td>23.5 ± 0.6</td>
<td>0.001</td>
<td>29.3 ± 0.7</td>
<td>0.000</td>
<td>18.5 ± 0.6</td>
<td>0.000</td>
<td>14.9 ± 0.6</td>
<td>0.000</td>
<td>18.1 ± 0.8</td>
<td>0.003</td>
</tr>
<tr>
<td><strong>Chronic Inflammation†</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st Quartile (MN)</td>
<td>21.9 ± 0.69</td>
<td>Ref</td>
<td>25.9 ± 0.78</td>
<td>Ref</td>
<td>16.1 ± 0.66</td>
<td>Ref</td>
<td>12.5 ± 0.64</td>
<td>Ref</td>
<td>15.8 ± 0.92</td>
<td>Ref</td>
</tr>
<tr>
<td>3rd Quartile (MN)</td>
<td>22.7 ± 0.78</td>
<td>0.470</td>
<td>28.6 ± 0.88</td>
<td>0.020</td>
<td>18.2 ± 0.75</td>
<td>0.036</td>
<td>15.0 ± 0.75</td>
<td>0.003</td>
<td>17.3 ± 1.04</td>
<td>0.288</td>
</tr>
</tbody>
</table>

*Multivariate model adjusted for age and including risk area, diagnosis and H. pylori genotypes; †Multivariate model adjusted for risk area and H. pylori genotypes; interquartile range comparison; Ref, Referent category; SE, standard error; MN, mononuclear cells

©2013 Landes Bioscience. Do not distribute.
experienced pathologists (MBP and PC). Discordant diagnoses were reviewed until a consensus developed. Diagnosis was performed by established guidelines\textsuperscript{41,42} discriminating categories of normal, non-atrophic gastritis (NAG), multifocal atrophic gastritis without intestinal metaplasia (MAG), intestinal metaplasia (IM), or dysplasia. Infiltration by polymorphonuclear (PMN) and mononuclear (MN) cells was evaluated in biopsies from antrum and incisura angularis and classified with a 0–3 scale (normal, mild, moderate, marked) according to published criteria.\textsuperscript{41} Augmented histopathology scores were characterized as described previously.\textsuperscript{43} Average inflammation scores were calculated for all biopsies from antrum and incisura angularis for each subject. An individual was designated as uninfected with \textit{H. pylori} only if both these conditions were met: no \textit{H. pylori} isolates could be cultured, and no \textit{H. pylori} organisms were visible in Steiner stained preparations of any of 3 biopsies. This protocol identified 8 subjects who were designated as currently uninfected; of these, 4 had normal mucosa, 3 had NAG, and one had IM. Pathologists evaluated diagnoses and inflammation without knowledge of residence area of the subjects.

Quantitative analysis of DNA methylation by pyrosequencing

Up to 2 μg of DNA per sample was bisulfite modified using a Zymo EZ Methylation Direct kit (Zymo Research Corp.) or an Epitect Bisulfite kit (Qiagen). Laser captured templates were used undiluted from the 20 μl sample eluted from the column supplied with the bisulfite modification kit. Modified DNA (20 ng per reaction for DNA from frozen tissue) was amplified by PCR, using Pyromark assays for \textit{EN1} (Hs\_EN1\_02\_PM, PM00101752), \textit{PCDH10} (Hs\_PCDH10\_06\_PM, PM0011783), \textit{RSPO2} (Hs\_RSPO2\_01\_PM, PM00036267), \textit{ZIC1} (Hs\_ZIC1\_03\_PM, PM0005099), or \textit{ZNF610} (Hs\_ZNF610\_01\_PM, PM00191331), using AmpliTAQ Gold DNA polymerase (Applied Biosystems, Life Technologies). The amplification program was 95 °C for 15 min, then 45 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s, followed by a 10 min. incubation at 72 °C. PCR products were analyzed by gel electrophoresis in 2% agarose, to determine that a single band was obtained. The biotinylated strand of each PCR product was isolated from 10 μl of each PCR product according to the manufacturer’s protocol. Pyrosequencing\textsuperscript{5} reactions were performed using a PyroMark MD Pyrosequencing instrument (Qiagen), following the manufacturer’s recommendations. Bisulfite-modified Methylated HeLa DNA (New England Biolabs) was employed as a positive control with each assay; bisulfite-modified pooled normal human blood DNA (Promega Corp) and no-template buffer were used as negative controls, at least one of which was used with each assay. Methylation values used for analysis were the mean of the percent methylation of the first 2 to 4 potential methylation sites quantitated in the assay following the sequencing primer.

\textit{H. pylori} culture and genotyping

Culture of \textit{H. pylori} organisms (one colony each, from one antral biopsy), isolation of DNA, and genotyping for \textit{cagA} and \textit{vacA} s and m regions for this set of subjects was previously reported.\textsuperscript{15} Sanger sequencing of the polymorphic 3′ end of the \textit{cagA} gene was used to characterize EPIYA motifs.\textsuperscript{44} Previously, \textit{H. pylori} strains from these subjects were characterized for ancestry by Multilocus Sequence Typing (MLST).\textsuperscript{45}

Statistical analysis

Histological diagnoses, age, \textit{H. pylori} virulence genes, numbers of EPIYA motifs, and levels of methylation at the five gene promoters were compared by risk area using χ², Spearman, and the Student t tests, as appropriate. To assess the contribution of the different variables to levels of methylation and account for confounders, multivariate generalized linear models were employed. For binary and continuous variables, the coefficients represent the average difference in percentage of methylation for a one unit change in the predictor (i.e., for every year of age, or low vs. high risk area). For \textit{H. pylori} genotypes, the coefficient represents the average difference in percentage of methylation associated with membership in the specified category (\textit{cagA} and \textit{vacA} s1m1 or other genotypes), compared with the reference group (uninfected). Nonparametric estimation of receiver operating characteristic (ROC) curves was used to compute AUC values for percent methylation of all genes. Data were analyzed using Stata MP v13 (Stata Corporation).

To assess reproducibility of the Pyrosequencing results for the biopsies, Lin’s concordance correlation coefficient was calculated on 152 replicates. The coefficient’s value was 0.939 (95% CI 0.920–0.958). The average difference between the replicates was 0.031 with a standard deviation of 2.6 (95% limit of agreement, −5.201–5.263).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This study was supported by the National Center for Research Resources, grant UL1 RR024975-01, which is now at the National Center for Advancing Translational Sciences, Grant 2 UL2 TR000445-06; grants P01 CA28842, R01 CA095103, R01 CA77955, R01 CA93999, P01 CA116387 from the National Cancer Institute, P30 DK58404, R01 DK053620, and R01 DK58587. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute, NIH, Department of Veterans Affairs or Vanderbilt University. We wish to acknowledge assistance of Anthony Frazier in Vanderbilt’s Translational Pathology Shared Resource. Frozen gastric cancers and matched non-tumor epithelia were provided by the Vanderbilt Translational Pathology Shared Resource and the Cooperative Human Tissue Network.
References


