Published in final edited form as:
Int J Cancer. 2010 December 1; 127(11): 2588–2597. doi:10.1002/ijc.25274.

Promoter DNA Hypermethylation in Gastric Biopsies from Subjects at High and Low Risk for Gastric Cancer

Barbara G. Schneider[1,*], **Dun-Fa Peng**[2], **M. Constanza Camargo**[3], **M. Blanca Piazuelo**[1], **Liviu A. Sicinschi**[1], **Robertino Mera**[1], **Judith Romero-Gallo**[1], **Alberto G. Delgado**[1], **Luis E. Bravo**[4], **Keith T. Wilson**[1,5], **Richard M. Peek Jr.**[1], **Pelayo Correa**[1], and **Wael El-Rifai**[2]

[1]Division of Gastroenterology, Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

[2]Department of Surgery, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

[3]Division of Epidemiology and Biostatistics, University of Illinois at Chicago, Chicago, IL, U.S.A.

[4]Department of Pathology, [3]School of Medicine, Universidad del Valle, Cali, Colombia

[5]Veterans Affairs Tennessee Valley Healthcare System and Office of Medical Research, Department of Veterans Affairs, Nashville, TN

Abstract

Gene promoter CpG island hypermethylation is associated with *Helicobacter pylori (H. pylori*) infection and may be an important initiator of gastric carcinogenesis. To examine factors influencing methylation, we utilized bisulfite Pyrosequencing® technology for quantitative analysis of promoter DNA methylation in *RPRM*, *APC*, *MGMT* and *TWIST1* genes using DNA from 86 gastric biopsies from Colombian residents of areas with high and low incidence of gastric cancer. *H. pylori* colonies were cultured from the same subjects, and gastric pathology was evaluated. Virulence factors *cagA* (including EPIYA polymorphisms of the 3' end) and *vacA s* and *m* regions were characterized in the *H. pylori* strains. Using univariate analysis, we found significantly elevated levels of *RPRM* and *TWIST1* promoter DNA methylation in biopsies from residents of the high risk region compared to those from residents of the low risk region. The presence of *cagA* and *vacA s1m1* alleles were independently associated with elevated levels of promoter DNA methylation of *RPRM* and *MGMT*. Using multivariate analysis, DNA methylation of *RPRM* was associated with location of residence, *cagA* and *vacA s1m1* status, and methylation of *TWIST1*. We conclude that *cagA* and *vacA* virulence determinants are significantly associated with quantitative differences in promoter DNA methylation in these populations, but that other as yet undefined factors that differ between the populations may also contribute to variation in methylation status.

Introduction

DNA methylation of promoter CpG islands is an important epigenetic regulatory mechanism for transcription. Methylated cytosines provide binding sites for various methyl-binding proteins, which interact with chromatin remodeling enzymes to stably repress transcription. In normal cells, repetitive elements are inactivated by DNA methylation, while the CpG islands of many housekeeping genes are protected from methylation. Conversely, in cancer cells, repetitive elements lose their methylation, and CpG islands in many promoters become

[*]Corresponding author: Barbara G. Schneider, Ph.D., 2215 Garland Ave., Room 1030C, MRB IV, Nashville, TN 37232-0252; USA, Phones: (615) 343-3959 (office), Fax: (615) 343-6229, barbara.schneider@vanderbilt.edu.

aberrantly methylated. Inactivation of tumor suppressor genes by promoter hypermethylation is considered to be an important contributor to carcinogenesis[1].

Gastric cancer is responsible for approximately 800,000 deaths annually worldwide[2], and the majority of these occur in the developing world. Infection with *Helicobacter pylori (H. pylori*) is the major environmental risk factor for gastric cancer[3]. However, identification of *H. pylori* infection alone as a risk factor does not simplify the problem of gastric cancer prevention, because approximately half of the world's population is infected. Most of these persons will have only gastritis, and less than 1% will develop cancer. Global eradication of the infection by antibiotic use is impractical, due to cost and to the problem of generation of antibiotic-resistant strains of *H. pylori* and other pathogenic bacteria. *Homo sapiens* and *H. pylori* have co-evolved over millennia, and infection may produce some benefits for the host, such as reduced rates of asthma and lower risk of esophageal diseases[4–6]. Therefore identification of more accurate biomarkers for increased risk of gastric cancer could be beneficial in reducing the burden of mortality from this disease.

H. pylori strains vary in their ability to induce and promote gastric cancer. Among the well-established virulence factors is *cagA*, a marker for a pathogenicity island carried by some strains[7, 8]. This gene encodes the CagA protein, which is injected into gastric epithelial cells by the type IV Cag secretion system of *H. pylori*. Once within the cell, CagA becomes tyrosine phosphorylated by Abl and Src family kinases, and then is able to bind to Src Homology 2-containing tyrosine phosphatase (SHP-2) and disrupt cell signaling9. Numerous studies have found an association between the presence of CagA in infecting strains and gastric cancer risk10. Among CagA positive strains, some variants are associated with an even greater risk for gastric cancer. CagA is polymorphic, containing 3 or more EPIYA (Glu-Pro-Ile-Tyr-Ala) motifs within its C-terminus. Among Western *H. pylori* strains, those with more than 3 EPIYA motifs have greater affinity for SHP-2 and disrupt epithelial cell morphology *in vitro*[11]. Notably, persons infected with strains bearing CagA proteins with more than 3 EPIYA motifs are at greater risk for gastric cancer than those harboring strains bearing only 3 motifs[12]. We previously reported in Colombian populations that persons infected with CagA positive strains with more than 3 EPIYA motifs have more severe gastric precancerous lesions[13].

Another important *H. pylori* virulence factor is *vacA*, which encodes the vacuolating cytotoxin. This gene is also polymorphic: the sequence encoding the signal peptide is either *s1* (with *s1a*, *s1b* and *s1c* subtypes), or s2; the middle portion of the gene is found as *m1* or *m2* alleles. Strains bearing *vacA s1m1* alleles produce more toxin and are associated with more severe pathology than those bearing *s2m2* alleles[14].

Recent reports indicate that infection with *H. pylori* is associated with elevated levels of aberrant DNA methylation. Chan et al. found the presence of aberrant methylation of the *CDH1* promoter to be associated with the presence of *H. pylori* in gastric mucosae of dyspeptic patients[15]. Maekita et al. quantitatively examined methylation levels within promoters of *LOX*, *HAND1*, *THBD*, *HRASLS*, *FLNC*, *ARC* and *CDKN2A* (p16), and found increased methylation in DNAs from *H. pylori*-infected subjects compared to uninfected individuals[16]. Additional affected gene promoters have been identified, including *TWIST1*[17–19]. Leung et al. found a lower methylation density at the *CDH1* promoter after *H. pylori* eradication[20], and an independent study examining *H. pylori*-associated promoter methylation in 5 genes (*CDH1*, p16, *MLH1*, *APC*, and *COX2*) similarly reported that aberrant methylation was significantly reduced or completely eliminated one year after *H. pylori* eradication[21]. Elevated methylation levels of the *FLNC* and *CDKN2A* (p16) genes in gastric mucosae of *H. pylori*-infected subjects have also been associated with elevated risk for gastric cancer[22, 23].

We aimed to determine whether DNA methylation differences could be detected within the context of *H. pylori* infection in two populations in Colombia. Though situated only 143 miles apart, these two populations differ in gastric cancer incidence by approximately 25-fold (150 vs 6 per 100,000)[24]. The high risk region is in the rural Andes mountain villages, where the population is supported by agriculture. This population is of mixed Amerindian and Spanish extraction. The low risk region is along the Pacific coast, where the economy is based on fishing. This population is of mixed African and Spanish ancestry. By 5 years of age, 80% of the children in both populations are infected with *H. pylori*, and infection is acquired at a rate that does not differ significantly between the two populations[25]. The high risk population has an elevated proportion of subjects infected with more virulent *H. pylori* strains (CagA positive, *vacA s1m1*), but this difference is estimated at 24% or less[26, 27]. These populations provide a useful natural laboratory in which to examine risk factors and potential biomarkers for gastric cancer.

Methods

Human Subjects

The study population consisted of 86 men with dyspeptic symptoms, aged 31 to 60 years old, who underwent upper endoscopy in gastroenterology clinics in two public hospitals between June and September, 2006. Ethics committees of the participating hospitals and of the Universidad del Valle in Cali, Colombia, approved the protocol for this study, and all patients provided informed consent. The subjects were residents of Tuquerres in the Andes Mountains and Tumaco on the Pacific Coast, both in the State of Nariño, Colombia. Exclusion criteria for the study were serious chronic diseases, previous gastrectomy, or ingestion of proton pump inhibitors, H_2-receptor antagonists, or antimicrobials in the month prior to the endoscopy. Biopsy samples from the antrum (greater curvature, within 5 cm of the pylorus), incisura angularis (lesser curvature), and corpus (middle anterior wall) were obtained by a single experienced gastroenterologist. One biopsy from each site was fixed in buffered formalin and embedded in paraffin for histopathology. One biopsy from the gastric antrum was frozen in glycerol and thioglycolate for culture of *H. pylori* organisms. Another biopsy, from the antrum, was frozen without any added solution, for analysis of DNA methylation. Frozen biopsies were shipped on dry ice to Vanderbilt University in Nashville, Tennessee, U.S.A., and stored at −80° C until thawed for culture or methylation analysis.

Histopathology

Four-micron sections were stained with hematoxylin and eosin for diagnosis performed independently by two experienced pathologists (MBP and PC). Discordant diagnoses were reviewed until a consensus was reached. Diagnosis was performed by established guidelines as normal, non-atrophic gastritis (NAG), multifocal atrophic gastritis without intestinal metaplasia (MAG), intestinal metaplasia (IM), or dysplasia (DYS) [28, 29]. Pathologists evaluated diagnoses blinded to residence of subjects.

H. pylori culture and genotyping

One antral and one corpus biopsy per person were homogenized under sterile conditions and cultured on selective Trypticase soy agar with 5% sheep blood and vancomycin (20 μg/ml), bacitracin (200 μg/ml), nalidixic acid (10 μg/ml) and amphotericin B (2 μg/ml), (all from Sigma, St Louis, MO), as previously described[13]. Small gray translucent colonies characteristic of *H. pylori* appeared after 4 to 8 days. Identity of *H. pylori* was confirmed by morphology, Gram stains and assays for oxidase and urease. Pellets from three single colonies per biopsy were frozen for analysis. DNA was isolated with either a Puregene kit (Qiagen) or by proteinase K digestion overnight followed by phenol / chloroform extraction. In preliminary experiments, 2 to 5 colonies per person were analyzed from 28 subjects by DNA fingerprinting analysis to

evaluate the frequency of mixed infections in the colonies harvested from the antral and corpus biopsies[30]. In all 28 subjects, all colonies from the same person produced identical DNA amplification patterns, indicating that there were no mixed infections in the strains harvested from a single individual. Subsequently, all analyses for *H. pylori* virulence markers were performed on DNA from a single colony per subject, isolated from the antral biopsy.

DNA from one colony per participant was genotyped for *cagA* and *vacA s* and *m* regions. Analysis of *cagA*, including the polymorphic 3' end, was previously described from *H. pylori* strains from this study population[13]. The *vacA s* region of each strain was amplified by primers VA1F[14] and Vac785 (R: AATACGCTCCCACGTATTGC), using an annealing temperature of 52°C. PCR products were treated with ExoSAP-IT (USB, Cleveland, OH) and sequenced using BigDye 3.1 terminator chemistry with an ABI 3130xl Genetic Analyzer. Two strains were genotyped for the *vacA s* region by PCR alone, using primers VA1F and VA1R[14] followed by electrophoresis in a 1.5% agarose gel, discriminating a 259 bp fragment for s1 or a 286 bp fragment for s2. The *vacA m* region was defined by multiplex PCR using primers M1F2, M1R2, M2F1, and M2R1[31]. Primer sequences and details of amplifications are described in Sicinschi et al[27].

Methylation Analysis

We used a Pyrosequencing® technique, which allows independent quantitative determination of methylation at selected CpG sites in the gene promoter. To obtain material for the analyses, gastric antral biopsies, one intact biopsy per person, were homogenized using disposable plastic pestles and digested overnight with proteinase K. DNA was isolated by phenol / chloroform extraction and quantitated with a Nanodrop instrument (Thermo Scientific, Wilmington, DE); 0.5 to 2 ug of DNA per biopsy was bisulfite modified, using a Zymo EZ Methylation Direct kit (Zymo Research Corp., Orange, CA). Modified DNA (20 ng per reaction) was amplified by PCR, using 0.2 uM of each primer, 2 Units of hot start *Taq* DNA polymerase, and 0.2 mM of each dNTP per reaction. Primer sequences and their location are listed in Table 1. Cycling programs were 95°C for 15 min., then 48 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72° C for 30 sec, followed by a 5 min. incubation at 72°C. PCR products were examined following gel electrophoresis in 1.5% agarose, in order to confirm that a single band was obtained. In preparation for Pyrosequencing, the biotinylated strand of each PCR product was isolated from 10 to 20 ul of each PCR product using a Vacuum Prep Tool (Qiagen Inc., Valencia, CA.), according to the manufacturer's protocol. Pyrosequencing reactions were performed in a PyroMark MD Pyrosequencing instrument (Qiagen), in the presence of 500 nM sequencing primer, following the manufacturer's recommendations. The positive control for methylation was methylated HeLa DNA (New England Biolabs, Ipswich, MA); the negative control was normal human blood DNA (Promega Corp., Madison, WI). Positive and negative controls were run with each experiment. Pyrosequencing assays contained a control for incomplete bisulfite modification. Methylation values used for analysis were the mean of the percent methylation measured for the first 3 potential methylation sites following the sequencing primer.

Statistical Analysis

Age, histological diagnoses, levels of methylation at the 4 gene promoters, *H. pylori* virulence genes, and number of EPIYA motifs were compared in a univariate fashion by risk area using $\chi 2$, Fisher's exact, Wilcoxon/Mann Whitney, Spearman, and Student's t tests, as appropriate. Mathematical transformations of the 4 levels of methylation were evaluated to reduce data skewness. *H. pylori* status was analyzed several ways combining *cagA* and *vacA* genotyping. In order to assess the degree to which various factors explained levels of methylation, multivariate generalized linear models were used. For continuous and binary 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 versus high risk area). In the case of *H.*

pylori genotypes, the coefficient represents the average difference in percentage of methylation associated with being a part of the specified category (*cagA* and *vacA s1m1* or other genotypes) as compared with the reference group (uninfected). Diagnosis was considered as an ordinal variable (0=Normal/NAG, 1=MAG, and 2=IM/DYS), for purposes of estimating the p-values in the test of linear trend (p-trend).

Regression analyses were individually applied to original and mathematically transformed variables. Since the results were similar, only those results based on the original variables are presented. Data were analyzed using the software package Stata 10.0 (Stata Corporation, College Station, TX). Prism 5.0b (GraphPad Software, La Jolla, CA) was used to generate Figure 1.

A kappa coefficient of reliability was run on 64 replicates to assess reproducibility of the Pyrosequencing results. This analysis produced a kappa statistic of 0.29 (95% CI=0.27 – 0.37, bias corrected), with complete agreement of 31% of samples, a standard error of 0.023, and a correlation coefficient of 0.983 ($p<0.0001$).

Results

Subjects and their Infecting *H. pylori* Strains

Table 2 shows the description of the subjects from the high and low risk populations and their infecting *H. pylori* strains. Age distributions and prevalence of *H. pylori* infection were similar in the two groups, but the high risk population had more advanced gastric precancerous lesions ($p=0.021$). Among the 64 strains that were analyzed for *vacA s* region by sequencing, all were *s1b*. Among the cagA positive strains, most had EPIYA patterns of ABC (66% of the cagA positive strains from the low risk region, and 57% of those from the high risk region). This difference was not significant. Of the remaining cagA positive strains, most were ABCC, but some variants were found: an AC strain in the low risk region, and one each of ABCCC, ABBCC, ABBC, ABCBC and BCC in the high risk region[32].

Unadjusted Analysis

In univariate analysis, levels of methylation (either medians or means) in the *RPRM* and *TWIST1* promoters were significantly associated with the area of residence of the subject, with more methylation being found in the DNA from subjects in the high risk area. No significant difference was detected for the *APC* or *MGMT* promoters.

Considering results from the entire set of DNA samples, generalized unadjusted linear regression models showed that levels of methylation in the *RPRM* and *MGMT* promoters were positively and significantly associated with histological diagnoses (Coefficient: 2.1; p-trend =0.018 for *RPRM*; Coefficient: 3.0; p-trend=0.003 for *MGMT*). Coefficients indicate that as lesions progressed, higher levels of methylation were observed. In the small number of uninfected patients, median per cent methylation was 8% for *RPRM* (n=4), 38% for *APC* (n=3), 39% for *MGMT* (n=4) and 6.5% for *TWIST1* (n=4). Regarding *H. pylori* infection, the presence of *cagA* and *vacA s1m1* alleles was associated with elevated levels of methylation of *RPRM* and *MGMT*. Interestingly, the association with *APC* was inverse, such that more virulent *H. pylori* strains were associated with less methylation of this gene. Age was not associated with levels of methylation.

Multivariate Analysis

Multivariate regression models incorporated effects of geographic area, diagnosis, age, and genotypes of the infecting *H. pylori* strain (classified as uninfected, *cagA* positive, *vacA s1m1* and all other genotypes; Table 3). Because alternative grouping of variables of *H.*

pylori status, including numbers of EPIYA motifs, or analysis by the presence of *cagA* alone, did not improve the explanatory capacity of the models, and led to similar results, parsimonious models with the highest adjusted R^2 (coefficient of determination) are presented. In these models, the observed mean differences between area and *RPRM* or *TWIST1* remained statistically significant, as did the differences between *H. pylori* virulence genes and *RPRM*, *MGMT*, *or APC*. Adjustment for covariates significantly attenuated the differences between histological diagnoses and levels of methylation of *RPRM* and *MGMT*. Age did not have any significant effect on the levels of methylation.

Because significant correlations exist between *TWIST1* (correlation coefficient rho=0.58; p<0.001) and *MGMT* (rho=0.39; p=0.001) with *RPRM*, the combined effect of methylation in these genes was simultaneously evaluated in a regression model using methylation of *RPRM* as outcome and including the covariates from the multivariate model. When *TWIST1* and *MGMT* were included as covariates, *TWIST1* was significantly associated with *RPRM* (coefficient, 1.04; p<0.0001) and *MGMT* showed a trend toward significance (Coefficient, 0.14; p=0.078). All effects were independent, as there was no significant interaction or effect modification between any of the covariates in the multivariate model.

An Interesting Outlier

One biopsy from a resident of the high risk region was a notable outlier. DNA from this biopsy showed elevated methylation at all four regions examined, for *RPRM* (55% methylation, compared to a median of 21%, 95% CI: 19–23%), for *TWIST1* (38% methylation, compared to a median of 10%, 95% CI: 8–11%), for *APC* (37%, compared to a median of 23%, 95% CI: 22–27%) and *MGMT* (73%, compared to a median of 58%, 95% CI: 55–61%; Figure 1). Histopathological analysis of paraffin-embedded biopsies of the same subject showed extensive intestinal metaplasia in all three sites examined (antrum, corpus and incisura angularis) and focal areas of indefinite dysplasia in antrum and corpus.

Discussion

In an attempt to understand mechanisms that regulate progression towards gastric cancer, we compared two populations that differ greatly in the incidence of this disease, although they have equally high prevalence of *H. pylori* infection[26, 27]. As expected, the two populations differed in diagnosis, with more severe lesions occurring more frequently in the high risk region. We detected a trend for the presence of the more virulent *cagA* positive, *vacA s1m1* strains more often in the high risk region, consistent with our findings in prior studies with different subjects from these populations[26, 27]. The quantitative method used to measure DNA methylation allowed us to detect differences in levels of methylation previously missed by earlier methods, such as methylation-specific PCR, which reveals only that methylation is present, without quantitating it. In our study, low levels of methylation present in uninfected patients could be measured; this methylation may occur due to environmental stressors not examined in our analysis. Univariate analysis comparing methylation levels of tissues representing the two populations showed differences in methylation of two genes: *RPRM* and *TWIST1*. When we examined several possible covariates of methylation, using multivariate analysis, geographic area remained as an independent effect, with more methylation in *RPRM* and *TWIST1* in the high risk region.

Because many epidemiological studies have shown an association of *cagA* and *vacA s1m1* with gastric cancer risk, we sought to determine if these virulence factors were associated with promoter methylation in the four genes examined. For *RPRM* and *MGMT*, an independent effect of *cagA* and *vacA s1m1* on methylation levels was detected. In contrast, for *APC*, decreased levels of promoter methylation were associated with more virulent *H. pylori* genotypes. Unadjusted analyses showed that levels of methylation in the *RPRM* and *MGMT*

promoters were significantly associated with the histological diagnoses, but adjustment for genotypes of the infecting *H. pylori* strains significantly attenuated the associations. This attenuation is likely due to the association between *H. pylori* infection and diagnosis of more severe lesions. Our study also demonstrated differential methylation of *RPRM* and *TWIST1* promoters by geographic area, even after adjustment for histological diagnosis and virulence determinants. These results indicate that other virulence determinants of the infecting strains are likely to be responsible for those differences, and/or that additional features distinguishing the two populations, such as diet or genetic features of the host, are important in differentiating risk.

In a prior examination of promoter methylation status and *H. pylori* virulence determinants, Watanabe et al. examined methylation of *MINT25*, *RORA*, *GDNF*, *ADAM23*, *PRDM5*, and *MLF1* in gastric washings from Korean subjects with and without gastric cancer[33]. These investigators tested for the presence of *cagA* in infecting strains and found no association with methylation. A difference from our study, besides the ethnicity of the subjects, is that Watanabe et al. assayed *cagA* by PCR on DNA from gastric washings, in contrast to our method of PCR using DNA from cultured bacteria. Culture is likely to be a more sensitive method for detection of *H. pylori* and its virulence determinants. In addition, as gastric lesions become more advanced, *H. pylori* infection is often lost, so that evaluation of *H. pylori* and its virulence determinants in gastric cancers may underrepresent the infection originally present[34]. Thus our study on premalignant lesions may have been more likely to detect infections originally present.

Park et al. examined methylation levels in a set of genes by quantitative methods, comparing premalignant gastric tissues and gastric cancers, with and without *H. pylori* infection[35], and found significant differences between infected and uninfected subjects, in the DNA samples from gastritis, but not from intestinal metaplasia, adenomas, or gastric cancers. While some studies have found *H. pylori*-related methylation to be associated with age[19, 36], others[15, 37], including the current study, have found no significant associations. However, the age range of our subjects was 29 years, and it is possible that with a broader age range, a significant association might be detected.

The genes we chose to study have been previously noted to be associated with development of gastric cancer. One such gene was *RPRM*, a *TP53*-dependent mediator of the G2/M cell cycle checkpoint[38]. *RPRM* is a tumor suppressor, which is inactivated by promoter methylation in a variety of human tumors, including gastric adenocarcinoma[39]. Notably, *RPRM* promoter methylation has been reported in premalignant conditions such as Barrett's esophagus[40] and non-malignant gastric epithelia[39]. Aberrant methylation of *RPRM* may be detected by methylation-specific PCR (MSP) in plasma of gastric cancer patients, in contrast to plasma of control subjects, suggesting the potential of *RPRM* as a biomarker for early gastric cancer[41]. Our results also support the identification of this gene as one that may be altered early in the progression towards gastric cancer.

Another gene we examined was the *APC* (adenomatous polyposis coli) gene, which encodes a component of a multiprotein complex that inactivates β-catenin in the Wnt signaling pathway. A critical tumor suppressor in the gastrointestinal tract, *APC* is expressed in the stomach as two isoforms originating from promoters 1A and 1B[42]. Methylation in gastric tissue occurs predominantly in promoter 1A, and for this reason, this promoter is frequently examined in studies of hypermethylation in gastric cancer[43, 44]. However, in contrast to the colon, methylation at promoter 1A in gastric mucosae is not predominantly tumor related, but is frequently found in non-malignant gastric mucosae[45], as we also observed. Promoter 1B is not methylated, and gene expression studies have shown that transcription of *APC* in the stomach is regulated primarily from this promoter[45, 46]. Consequently methylation at promoter 1A in gastric mucosae is likely to be a passenger, rather than a driver of carcinogenesis. Among the

four genes we examined, after adjustment for diagnosis, it is notable that only the *APC* promoter showed a decrease in methylation in DNAs from subjects from the high risk region.

MGMT encodes O-6-methylguanine-DNA methyltransferase (also called O^6-alkylguanine-DNA alkyltransferase), a DNA repair enzyme that removes alkylation adducts from guanine. MGMT must be constantly renewed, as part of defense against mutagens. *MGMT* promoter hypermethylation is associated with a wide variety of tumors, including gastric cancers[47]. Our assays detected a relatively high level of *MGMT* promoter methylation in most of the gastric biopsies. Additional studies will be necessary to determine how these elevated levels are related to *MGMT* gene expression.

TWIST1 encodes a basic-helix-loop-helix transcription factor called Twist1 that regulates metastasis[48] and epithelial-to-mesenchymal transition[49]. *TWIST1* represses transcription of E-cadherin and *TIMP1* (an inhibitor of matrix metalloproteinases)[50] and promotes migration in neoplastic cells51, 52. Although the Twist1 protein may have tumor- or metastasis-promoting effects, paradoxically, hypermethylated *TWIST1* is reported in cancers of the stomach and other tumor types[49, 53]. All of our Colombian subjects, with the exception of one outlier, had relatively low levels of *TWIST1* hypermethylation.

Limitations of our study include the fact that we examined DNA from biopsies containing premalignant lesions, instead of DNA from gastric cancers. Our goal was to investigate early events in gastric carcinogenesis, and these events may not all lead to cancer. Another limitation is the use of entire biopsies for our DNA preparation, so that mixed cell types are included. Although most of the cells contributing DNA were gastric epithelial cells, other cells including stromal and inflammatory cells may have contributed to the differences in methylation levels that we measured. Additional studies employing microdissection will be useful to confirm identity of the cell type demonstrating alterations in methylation levels.

A topic of intense investigation is the mechanism(s) by which promoter methylation becomes dysregulated during the development of cancer, and how inflammation may promote this process. In our study, we found that two of the four genes we examined (*RPRM* and *TWIST1*) increased together in levels of methylation, and a third (*MGMT*) trended toward such an increase, consistent with a common or related mechanism affecting most of the genes we studied. Results from an outlier subject, whose biopsy showed high or remarkably elevated promoter methylation in all four genes examined, are consistent with the existence of a global methylation defect that can occur early in the stages of carcinogenesis. Although this widespread hypermethylation was observed in only one subject, we expect progression of *H. pylori*-related disease to occur in only a small minority of infected persons, and therefore events occurring within the gastric mucosae of these rare individuals provoke great interest. Analysis of DNA methylation of additional genes and the effect of that methylation on gene transcription will be necessary to clarify early events in *H. pylori*-related gastric cancer development.

The mechanisms by which virulent *H. pylori* infection may influence promoter hypermethylation remain obscure, but several hypotheses which involve enhancement of DNA methyltransferase activity have been proposed. *H. pylori* infection promotes gastric epithelial cell proliferation and increased production of epidermal growth factor (EGF) and its receptor[54]; the EGF receptor has greater activity with the more virulent cagA positive strains55. EGF administered to gastric epithelial cells in culture increases DNA methyltransferase activity56.

Another possible mechanism by which *H. pylori* can promote hypermethylation involves the effect of nitric oxide on DNA methyltransferase. Katayama et al. noted that the *RUNX3* gene promoter in the human gastric cancer cell line MKN45 can become methylated by co-culture with *H. pylori* and macrophages, and this effect can be replicated by substituting nitric oxide

(NO) for the bacteria and macrophages[57]. Inhibition of NO synthesis in the co-culture reversed the methylation. Hmadcha et al reported that treatment of an insulinoma cell line by IL-1β or NO could inactivate the *FMR1* and *HPRT* genes by hypermethylation[58]. This effect was apparently mediated by enhancement of DNA methyltransferase activity, without an increase in transcription of the methyltransferase gene.

In conclusion, *RPRM* and *TWIST1* promoter DNA methylation were found to be elevated in gastric biopsies from residents of the high risk region compared to those from residents of the low risk region in Colombia. Virulence genes *cagA* and *vacA s1m1* in the infecting *H. pylori* strains were independently associated with elevated levels of methylation of *RPRM* and *MGMT*. Alterations in DNA methylation are early changes in the progression of premalignant lesions and have the potential to serve as biomarkers for gastric cancer risk.

Acknowledgments

Acknowledgement of Financial Support: This study was supported by 1 UL1 RR024975 from NCRR/NIH (CTSA), grants P01 CA028842, P50 CA095103, R01 CA77955, R01 CA93999 and P01 CA116087 from the National Cancer Institute, and P30DK058404, R01 DK053620, and DK058587. The content presented here is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health.

We wish to acknowledge Ms. Jacki Huckins (Qiagen) for assistance in assay design for the *RPRM* Pyrosequencing assay.

Bibliography

1. Vogiatzi P, Vindigni C, Roviello F, Renieri A, Giordano A. Deciphering the underlying genetic and epigenetic events leading to gastric carcinogenesis. J Cell Physiol 2007;211:287–295. [PubMed: 17238139]
2. Garcia M, Jemal A, Ward EM, Center MM, Hao Y, Siegel RL, Thun MJ. Global Cancer Facts & Figures 2007. American Cancer Society. 2007
3. Webb PM, Law M, Varghese C, Forman D, Yuan JM, Yu M, Ross R, Limberg PJ, Mark SD, Taylor PR, Dawsey SM, Qiao YL, et al. Gastric cancer and *Helicobacter pylori*: a combined analysis of 12 case control studies nested within prospective cohorts. Gut 2001;49:347–353. [PubMed: 11511555]
4. Chen Y, Blaser MJ. Inverse associations of *Helicobacter pylori* with asthma and allergy. Arch Intern Med 2007;167:821–827. [PubMed: 17452546]
5. Vaezi MF, Falk GW, Peek RM, Vicari JJ, Goldblum JR, Perez-Perez GI, Rice TW, Blaser MJ, Richter JE. *CagA*-positive strains of *Helicobacter pylori* may protect against Barrett's esophagus. Am.J.Gastroenterol 2000;95:2206–2211. [PubMed: 11007219]
6. Loffeld RJLF, Werdmuller BFM, Kuster JG, Pérez-Pérez GI, Blaser MJ, Kuipers EJ. Colonization with *cagA*-positive *Helicobacter pylori* strains inversely associated with reflux esophagitis and Barrett's esophagus. Digestion 2000;62:95–99. [PubMed: 11025356]
7. Tummuru MKR, Cover TL, Blaser MJ. Cloning and expression of a high-molecular mass major antigen of *Helicobacter pylori*: Evidence of linkage to cytotoxin production. Inf Immun 1993;61:1799–1809.
8. Covacci A, Censini S, Bugnoli M, Petracca R, Burroni D, Macchia G, Massone A, Papini E, Ziang Z, Figura N, Rappuoli R. Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. Proc Nat Acad Sci USA 1993;90:5791–5795. [PubMed: 8516329]
9. Hatakeyama M. SagA of CagA in Helicobacter pylori pathogenesis. Curr Opin Microbiol 2008;11:30–37. [PubMed: 18243773]
10. Huang JQ, Zheng GF, Sumanac K, Irvine EJ, Hunt RH. Meta-analysis of the relationship between cagA seropositivity and gastric cancer. Gastroenterology 2003;125:1636–1644. [PubMed: 14724815]

11. Naito M, Yamazaki T, Tsutsumi R, Higashi H, Onoe K, Yamazaki S, Azuma T, Hatakeyama M. Influence of EPIYA-repeat polymorphism on the phosphorylation-dependent biological activity of Helicobacter pylori CagA. Gastroenterology 2006;130:1181–1190. [PubMed: 16618412]
12. Yamaoka Y, Kodama T, Kashima K, Graham DY, Sepulveda AR. Variants of the 3' region of the *cagA* gene in *Helicobacter pylori* isolates from patients with different *H. pylori*-associated diseases. J Clin Microbiol 1998;36:2258–2263. [PubMed: 9666002]
13. Sicinschi LA, Correa P, Peek RM Jr, Camargo CM, Piazuelo MB, Romero-Gallo J, Hobbs SS, Krishna U, Mera R, Delgado AG, Bravo LE, Schneider BG. CagA C-terminal variations in *Helicobacter pylori* strains from Colombian patients with gastric precancerous lesions. Clin Microbiol Infect. 2009 in press.
14. Atherton JC, Cao P, Peek RM. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific vacA types with cytotoxin production and peptic ulceration. J Biol Chem 1995;270:17771–17777. [PubMed: 7629077]
15. Chan AO, Lam SK, Wong BC, Wong WM, Yuen MF, Yeung YH, Hui WM, Rashid A, Kwong YL. Promoter methylation of E-cadherin gene in gastric mucosa associated with *Helicobacter pylori* infection and in gastric cancer. Gut 2003;52:502–506. [PubMed: 12631658]
16. Maekita T, Nakazawa K, Mihara M, Nakajima T, Yanaoka K, Iguchi M, Arii K, Kaneda A, Tsukamoto T, Tatematsu M, Tamura G, Saito D, et al. High levels of aberrant DNA methylation in *Helicobacter pylori*-infected gastric mucosae and its possible association with gastric cancer risk. Clin Cancer Res 2006;12:989–995. [PubMed: 16467114]
17. Kang GH, Lee S, Cho NY, Gandamihardja T, Long TI, Weisenberger DJ, Campan M, Laird PW. DNA methylation profiles of gastric carcinoma characterized by quantitative DNA methylation analysis. Lab Invest 2008;88:161–170. [PubMed: 18158559]
18. Nakajima T, Yamashita S, Maekita T, Niwa T, Nakazawa K, Ushijima T. The presence of a methylation fingerprint of Helicobacter pylori infection in human gastric mucosae. Int J Cancer 2009;124:905–910. [PubMed: 19035455]
19. Kitajima Y, Ohtaka K, Mitsuno M, Tanaka M, Sato S, Nakafusa Y, Miyazaki K. Helicobacter pylori infection is an independent risk factor for Runx3 methylation in gastric cancer. Oncol Rep 2008;19:197–202. [PubMed: 18097595]
20. Leung WK, Man EP, Yu J, Go MY, To KF, Yamaoka Y, Cheng VY, Ng EK, Sung JJ. Effects of *Helicobacter pylori* eradication on methylation status of E-cadherin gene in noncancerous stomach. Clin Cancer Res 2006;12:3216–3221. [PubMed: 16707623]
21. Perri F, Cotugno R, Piepoli A, Merla A, Quitadamo M, Gentile A, Pilotto A, Annese V, Andriulli A. Aberrant DNA methylation in non-neoplastic gastric mucosa of *H. pylori* infected patients and effect of eradication. Am J Gastroenterol 2007;102:1361–1371. [PubMed: 17509026]
22. Nakajima T, Maekita T, Oda I, Gotoda T, Yamamoto S, Umemura S, Ichinose M, Sugimura T, Ushijima T, Saito D. Higher methylation levels in gastric mucosae significantly correlate with higher risk of gastric cancers. Cancer Epidemiol Biomarkers Prev 2006;15:2317–2321. [PubMed: 17119066]
23. Tahara T, Arisawa T, Shibata T, Wang FY, Nakamura M, Sakata M, Nagasaka M, Takagi T, Kamiya Y, Fujita H, Nakamura M, Hasegawa S, et al. Risk prediction of gastric cancer by analysis of aberrant DNA methylation in non-neoplastic gastric epithelium. Digestion 2007;75:54–61. [PubMed: 17438355]
24. Correa P, Cuello C, Duque E, Burbano LC, Garcia FT, Bolanos O, Brown C, Haenszel W. Gastric cancer in Colombia. III. Natural history of precursor lesions. J Natl Cancer Inst 1976;57:1027–1035. [PubMed: 1003539]
25. Camargo MC, Yepez MC, Ceron C, Guerrero N, Bravo LE, Correa P, Fontham ETH. Age at acquisition of *Helicobacter pylori* infection: Comparison of two areas with contrasting risk of gastric cancer. Helicobacter 2004;9:262–270. [PubMed: 15165263]
26. Bravo LE, van Doorn LJ, Realpe JL, Correa P. Virulence-Associated Genotypes of *Helicobacter pylori*: Do they Explain the African Enigma? Am J Gastroenterol 2002;97:2839–2842. [PubMed: 12425557]
27. Sicinschi LA, Correa P, Peek RMJ, Camargo MC, Delgado A, Piazuelo MB, Romero-Gallo J, Bravo LE, Schneider BG. *Helicobacter pylori* genotyping and sequencing using paraffin-embedded

biopsies from residents of Colombian areas with contrasting gastric cancer risks. Helicobacter 2008;13:135–145. [PubMed: 18321303]

28. Dixon MF, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. Am J Surg Pathol 1996;20:1161–1181. [PubMed: 8827022]
29. Rugge M, Correa P, Dixon MF, Hattori T, Leandro G, Lewin K, Riddell RH, Sipponen P, Watanabe H. Gastric dysplasia - The Padova international classification. Am J Surg Pathol 2000;24:167–176. [PubMed: 10680883]
30. Taylor DE, Eaton M, Chang N, Salama SM. Construction of a *Helicobacter pylori* genome map and demonstration of diversity at the genome level. J Bacteriol 1992;174:6800–6806. [PubMed: 1400229]
31. Koehler CI, Mues MB, Dienes HP, Kriegsmann J, Schirmacher P, Odenthal M. Helicobacter pylori genotyping in gastric adenocarcinoma and MALT lymphoma by multiplex PCR analyses of paraffin wax embedded tissues. Mol Pathol 2003;56:36–42. [PubMed: 12560462]
32. Sicinschi LA, Correa P, Peek RM Jr, Camargo CM, Piazuelo MB, Romero-Gallo J, Hobbs SS, Krishna U, Mera R, Delgado AG, Bravo LE, Schneider BG. CagA C-terminal variations in *Helicobacter pylori* strains from Colombian patients with gastric precancerous lesions. Clin Microbiol Infect. 2009 in press.
33. Watanabe Y, Kim HS, Castoro RJ, Chung W, Estecio MR, Kondo K, Guo Y, Ahmed SS, Toyota M, Itoh F, Suk KT, Cho MY, et al. Sensitive and specific detection of early gastric cancer with DNA methylation analysis of gastric washes. Gastroenterology 2009;136:2149–2158. [PubMed: 19375421]
34. Fukuda H, Saito D, Hayashi S, Hisai H, Ono H, Yoshida S, Oguro Y, Noda T, Sato T, Katoh M, et al. Helicobacter pylori infection, serum pepsinogen level and gastric cancer: a case-control study in Japan. Jpn J Cancer Res 1995;86:64–71. [PubMed: 7737912]
35. Park SY, Yoo EJ, Cho NY, Kim N, Kang GH. Comparison of CpG island hypermethylation and repetitive DNA hypomethylation in premalignant stages of gastric cancer, stratified for Helicobacter pylori infection. J Pathol. 2009
36. Kang GH, Lee HJ, Hwang KS, Lee S, Kim JH, Kim JS. Aberrant CpG island hypermethylation of chronic gastritis, in relation to aging, gender, intestinal metaplasia, and chronic inflammation. Am J Pathol 2003;163:1551–1556. [PubMed: 14507661]
37. Kaise M, Yamasaki T, Yonezawa J, Miwa J, Ohta Y, Tajiri H. CpG island hypermethylation of tumor-suppressor genes in H. pylori-infected non-neoplastic gastric mucosa is linked with gastric cancer risk. Helicobacter 2008;13:35–41. [PubMed: 18205664]
38. Ohki R, Nemoto J, Murasawa H, Oda E, Inazawa J, Tanaka N, Taniguchi T. Reprimo, a new candidate mediator of the p53-mediated cell cycle arrest at the G2 phase. J Biol Chem 2000;275:22627–22630. [PubMed: 10930422]
39. Takahashi T, Suzuki M, Shigematsu H, Shivapurkar N, Echebiri C, Nomura M, Stastny V, Augustus M, Wu CW, Wistuba II, Meltzer SJ, Gazdar AF. Aberrant methylation of Reprimo in human malignancies. Int J Cancer 2005;115:503–510. [PubMed: 15700311]
40. Hamilton JP, Sato F, Jin Z, Greenwald BD, Ito T, Mori Y, Paun BC, Kan T, Cheng Y, Wang S, Yang J, Abraham JM, et al. Reprimo methylation is a potential biomarker of Barrett's-Associated esophageal neoplastic progression. Clin Cancer Res 2006;12:6637–6642. [PubMed: 17121882]
41. Bernal C, Aguayo F, Villarroel C, Vargas M, Diaz I, Ossandon FJ, Santibanez E, Palma M, Aravena E, Barrientos C, Corvalan AH. Reprimo as a potential biomarker for early detection in gastric cancer. Clin Cancer Res 2008;14:6264–6269. [PubMed: 18829507]
42. Horii A, Nakatsuru S, Ichii S, Nagase H, Nakamura Y. Multiple forms of the APC gene transcripts and their tissue-specific expression. Hum Mol Genet 1993;2:283–287. [PubMed: 8388766]
43. Kang GH, Lee S, Kim JS, Jung HY. Profile of aberrant CpG island methylation along multistep gastric carcinogenesis. Laboratory Investigation 2003;83:519–526. [PubMed: 12695555]
44. Esteller M, Sparks A, Toyota M, Sanchez-Cespedes M, Capella G, Peinado MA, Gonzalez S, Tarafa G, Sidransky D, Meltzer SJ, Baylin SB, Herman JG. Analysis of *Adenomatous Polyposis Coli* promoter hypermethylation in human cancer. Cancer Res 2000;60:4366–4371. [PubMed: 10969779]

45. Tsuchiya T, Tamura G, Sato K, Endoh Y, Sakata K, Jin Z, Motoyama T, Usuba O, Kimura W, Nishizuka S, Wilson KT, James SP, et al. Distinct methylation patterns of two APC gene promoters in normal and cancerous gastric epithelia. Oncogene 2000;19:3642–3646. [PubMed: 10951570]
46. Hosoya K, Yamashita S, Ando T, Nakajima T, Itoh F, Ushijima T. Adenomatous polyposis coli 1A is likely to be methylated as a passenger in human gastric carcinogenesis. Cancer Lett. 2009
47. Soejima H, Zhao W, Mukai T. Epigenetic silencing of the *MGMT* gene in cancer. Biochem Cell Biol 2005;83:429–437. [PubMed: 16094446]
48. Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, Savagner P, Gitelman I, Richardson A, Weinberg RA. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell 2004;117:927–939. [PubMed: 15210113]
49. Puisieux A, Valsesia-Wittmann S, Ansieau S. A twist for survival and cancer progression. Br J Cancer 2006;94:13–17. [PubMed: 16306876]
50. Okamura H, Yoshida K, Haneji T. Negative regulation of TIMP1 is mediated by transcription factor TWIST1. Int J Oncol 2009;35:181–186. [PubMed: 19513566]
51. Matsuo N, Shiraha H, Fujikawa T, Takaoka N, Ueda N, Tanaka S, Nishina S, Nakanishi Y, Uemura M, Takaki A, Nakamura S, Kobayashi Y, et al. Twist expression promotes migration and invasion in hepatocellular carcinoma. BMC Cancer 2009;9:240. [PubMed: 19615090]
52. Hasselblatt M, Mertsch S, Koos B, Riesmeier B, Stegemann H, Jeibmann A, Tomm M, Schmitz N, Wrede B, Wolff JE, Zheng W, Paulus W. TWIST-1 is overexpressed in neoplastic choroid plexus epithelial cells and promotes proliferation and invasion. Cancer Res 2009;69:2219–2223. [PubMed: 19276370]
53. Rosivatz E, Becker I, Specht K, Fricke E, Luber B, Busch R, Hofler H, Becker KF. Differential expression of the epithelial-mesenchymal transition regulators snail, SIP1, and twist in gastric cancer. Am J Pathol 2002;161:1881–1891. [PubMed: 12414534]
54. Wallasch C, Crabtree JE, Bevec D, Robinson PA, Wagner H, Ulrich A. Helicobacter pylori-stimulated EGF receptor transactivation requires metalloprotease cleavage of HB-EBG. Biochem Biophys Res Comm 2002;295:695–701. [PubMed: 12099696]
55. Keates S, Sougioultzis S, Keates AC, D Z, Peek RM Jr, Shaw LM, Kelly CP. cag+ Helicobacter pylori induce transactivation of the epidermal growth factor receptor in AGS gastric epithelial cells. J Biol Chem 2001;276:48127–48134. [PubMed: 11604402]
56. Miyazaki T, Murayama Y, Shinomura Y, Yamamoto T, Watabe K, Tsutsui S, Kiyohara T, Tamura S, Hayashi N. E-cadherin gene promoter hypermethylation in H.pylori-induced enlarged fold gastritis. Helicobacter 2007;12:523–531. [PubMed: 17760721]
57. Katayama Y, Takahashi M, Kuwayama H. *Helicobacter pylori* causes *runx3* gene methylation and its loss of expression in gastric epithelial cells, which is mediated by nitric oxide produced by macrophages. Biochem Biophys Res Commun. 2009
58. Hmadcha A, Bedoya FJ, Sobrino F, Pintado E. Methylation-dependent gene silencing induced by interleukin 1beta via nitric oxide production. J Exp Med 1999;190:1595–1604. [PubMed: 10587350]

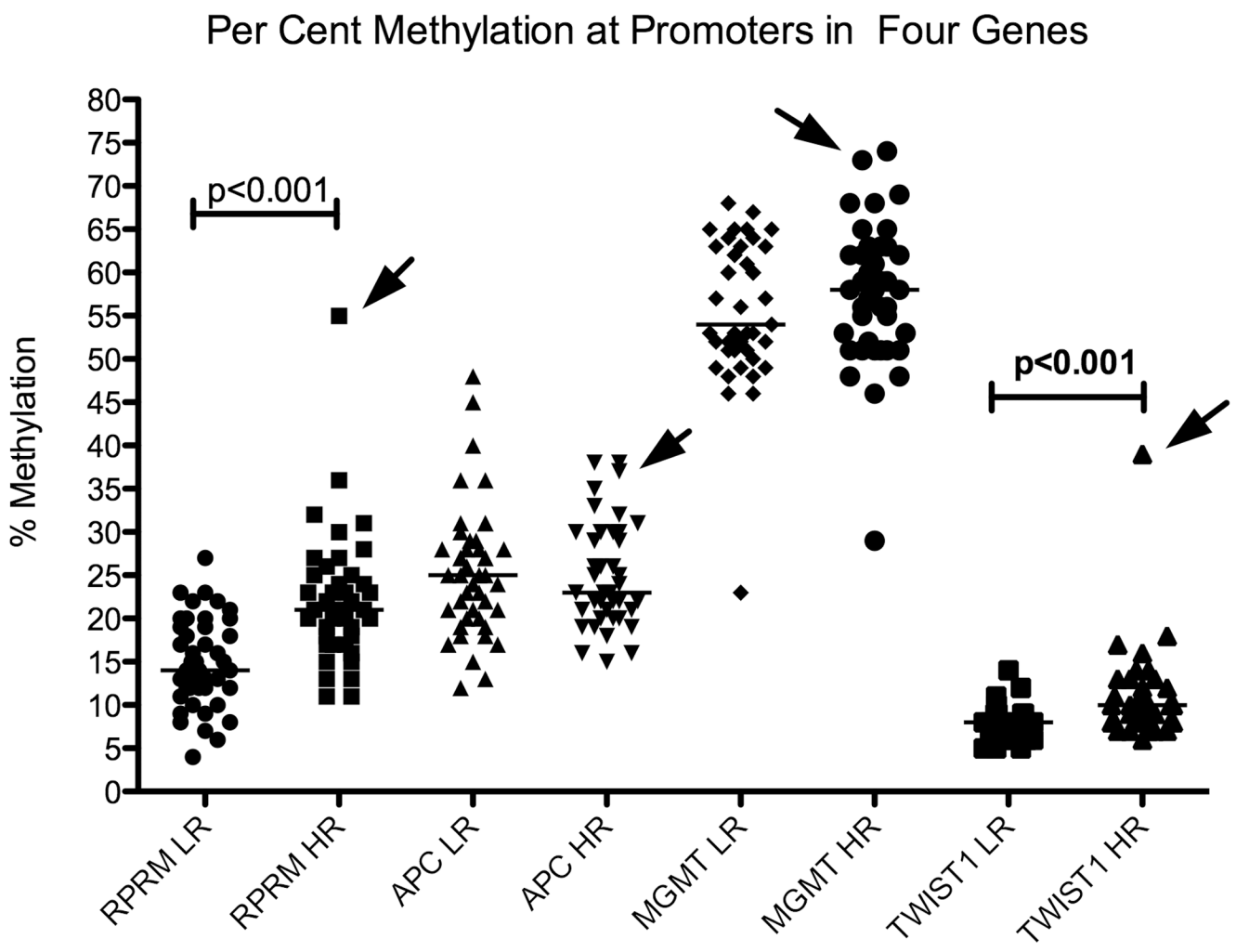

Figure 1.
Scatter plots indicate the percent methylation of each gene, measured from DNA from residents of low risk (LR) and high risk (HR) areas. Arrows indicate the points representing the outlier subject, whose gastric histology showed widespread intestinal metaplasia and focal areas of indefinite dysplasia.

Table 1

Primer sequences for Pyrosequencing

APC Promoter 1A	F: GGGGTTAGGGTTAGGTAGGTT R: biotin-ACTACACCAATACAACCACATATC Seq: GAGAGAAGTAGTTGTGTAAT	*chr 5: 112,101,273-112,101,293 antisense 112,101,444-112,101,472 112,101,348-112,101,367	** Industry source
MGMT	F: TGGTTTGGGGGTTTTTGA	chr 10, 131,154,890-131,154,907	This study
	R: biotin-CCTTTTCCTATCACAAAAATAATC	antisense 131,155,065-131,155,088	
	Seq: ATTAGGAGGGGAGAGAT	131,154,921-131,154,937	
RPRM	F: GGTTATTAAGGAAGTTTGGGTGTA	chr2:154,043,215-154,043,238	This study
	R: biotin-AACCCCACACCTATTCCTCC	antisense 154,043,387-154,043,406	
	Seq: TTGTTTAGGGTAGGATTTAT	154,043,306-154,043,325	
TWIST1	F: GTTAAGTGAGGTGGGAAGGTTGA	chr7:19,122,688-19,122,710	This study
	R: biotin-CCCACCCCCTCAACAAAAC	antisense 19,122,830-19,122,848	
	Seq: GGAGAGGGGAGGAAA	19,122,721- 19,122,735	

* Position indicates equivalent location of primer (in unmodified sequence) in the March 2006 human reference sequence (NCBI Build 36).

** assay designed by QIAGEN AB, formerly Biotage AB, as published in PyroMark™ Assay Database at www.pyrosequencing.com

Table 2

Study population and Characteristics of Infecting *H. pylori* Strains

Variables	Low risk for Gastric Cancer n=45	High risk for Gastric Cancer n=41	Two-tailed p-value *
Age in years, mean (SD)	47.5(6.0)	48.9(5.3)	0.257
cagA assessment by PCR, n(%)			
Uninfected	7(15.6)	2(4.9)	0.209
Negative	6(13.3)	4(9.7)	
Positive	32(71.1)	35(85.4)	
H. pylori genotypes, n(%)			
Uninfected	7(15.6)	2(4.9)	0.079
cagA positive, vacA s1m1**	29(64.4)	35(85.4)	
Other genotypes	9 (20.0)	4 (9.7)	
H. pylori genotypes, n(%)			
Uninfected	7(15.6)	2(4.9)	0.128
cagA negative	6(13.3)	4(9.7)	
cagA positive, 3 EPIYAs	25(55.6)	21(51.2)	
cagA positive, >3 EPIYAs	7(15.5)	14(34.2)	
H. pylori genotypes, n(%)			
uninfected	7(15.6)	2(4.9)	0.076
cagA positive, (3 EPIYAs), vacA s1m1*	7(15.6)	14(34.2)	
cagA positive (>3 EPIYAs) vacA s1m1*	22(48.8)	21(51.2)	
other genotypes	9(20.0)	4(9.7)	
Histopathological diagnosis§ , n(%)			
Normal/NAG	27(61.4)	13(31.7)	0.021
MAG	6(13.6)	8(19.5)	
IM/Dysplasia	11(25.0)	20(48.8)	
% methylation, median (interquartile range)			
RPRM	14(7)	21(7.5)	<0.001
APC	25(8)	23(9)	0.9158
MGMT	54(12)	58(11)	0.3788
TWIST1	8 (2)	10(4)	<0.001
Mathematically transformed values of % methylation, mean (SD)			
RPRM (logarithm)	2.6(0.4)	3.0(0.3)	<0.001
APC (logarithm)	3.2(0.3)	3.2(0.2)	0.9460
MGMT(square-root)	3160(848.4)	3369(906.6)	0.2869

Variables	Low risk for Gastric Cancer n=45	High risk for Gastric Cancer n=41	Two-tailed p-value *
TWIST1(inverse)	0.10 (0.03)	0.13 (0.03)	<0.001

Abbreviations: SD= standard deviation; NAG: non-atrophic gastritis; MAG: multifocal atrophic gastritis; IM: intestinal metaplasia.

* p-value from $\chi 2$, 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*.

§ A subject with gastritis in which we could not assess atrophy was excluded from this analysis.

Table 3

Multivariate Linear Regression Analysis.

Variables	Gene							
	***RPRM* (n=84)**		***APC* (n=84)**		***MGMT* (n=80)**		***TWIST1* (n=82)**	
	Coefficient (SE)	**p-value**	**Coefficient (SE)**	**p-value**	**Coefficient (SE)**	**p-value**	**Coefficient (SE)**	**p-value**
Area								
Low risk for Gastric Cancer	0		0		0		0	
High risk for Gastric Cancer	6.4 (1.4)	<0.001	1.0 (1.5)	0.511	−1.5 (1.8)	0.383	3.1 (0.9)	0.001
Diagnosis								
Normal/NAG	0		0		0		0	
MAG	1.1 (2.0)	0.583	−0.6 (2.1)	0.766	1.3 (2.5)	0.594	−0.4 (1.4)	0.746
IM/DYS	0.2 (1.7)	0.926	−1.4 (1.7)	0.431	3.3 (2.1)	0.124	0.4 (1.1)	0.718
H. pylori genotypes								
uninfected	0		0		0		0	
other genotypes	5.6 (2.6)	0.040	−9.3 (2.9)	0.002	5.3 (3.1)	0.104	0.6 (1.7)	0.746
cagA positive, *vacA s1m1*[*]	8.3 (2.4)	0.001	−9.9 (2.6)	<0.001	11.2 (2.9)	<0.001	1.8 (1.6)	0.244
Age in years	−0.1 (0.1)	0.271	0.1 (0.1)	0.403	−0.1 (0.1)	0.598	−0.1 (0.1)	0.462
Adjusted R-squared	0.3356		0.1267		0.2216		0.1281	

Abbreviations: SE= standard error; NAG: non-atrophic gastritis; MAG: multifocal atrophic gastritis; IM: intestinal metaplasia.

[*] 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*.