Research Article

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DNA Methylation Predicts Progression of Human Gastric Lesions

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Abstract

Background: Development of the intestinal subtype of gastric adenocarcinoma is marked by a progression of histopathologic lesions. Residents of the Andean regions of Colombia are at high risk for gastric cancer.

Methods: A cohort of 976 Colombian subjects was followed over 16 years examining effects of *Helicobacter pylori* eradication and treatment with antioxidants on progression of lesions. We performed methylation analysis of DNA from baseline antral biopsies from 104 subjects for whom follow-up data were available for at least 12 years. Methylation was quantitated for *AMPH*, *CDKN2A*, *CDH1*, *EN1*, *EMX1*, *NKX6-1*, *PCDH10*, *RPRM*, *RSPO2*, *SORCS3*, *ZIC1*, and *ZNF610* genes, using Pyrosequencing.

Results: Levels of DNA methylation were associated with baseline diagnosis for *AMPH*, *EMX1*, *RPRM*, *RSPO2*, *SORCS3*, and *ZNF610*. After adjusting for baseline diagnosis and *H. pylori* infection, methylation levels of *AMPH*, *PCDH10*, *RSPO2*, and *ZNF610* had progression coefficients that increased and *P* values that decreased over 6, 12, and 16 years. Methylation for *SORCS3* was associated with progression at all 3 time points but without the continual strengthening of the effect. Scores for mononuclear leukocytes, polymorphonuclear leukocytes, or intraepithelial lymphocytes were unrelated to progression.

Conclusions: Methylation levels of *AMPH*, *PCDH10*, *RSPO2*, *SORCS3*, and *ZNF610* predict progression of gastric lesions independent of the effect of duration of *H. pylori* infection, baseline diagnosis, gender of the patient, or scores for mononuclear leukocytes, polymorphonuclear leukocytes, or intraepithelial lymphocytes.

Impact: DNA methylation levels in *AMPH*, *PCDH10*, *RSPO2*, *SORCS3*, and *ZNF610* may contribute to identification of persons with gastric lesions likely to progress. *Cancer Epidemiol Biomarkers Prev; 24(10); 1607–13. ©2015 AACR.*

Introduction

Gastric cancer was responsible for an estimated 723,000 deaths in 2012 worldwide and more than 70% of these are in the developing world (1). Gastric infection with *Helicobacter pylori*, designated a class I carcinogen (2), is the major risk factor for gastric cancer. The most common type of gastric cancer, adenocarcinoma of the intestinal subtype (3), develops in association with a series of precancerous lesions, called Correa's cascade (4, 5): non-atrophic gastritis (NAG), multifocal atrophic gastritis (MAG), intestinal metaplasia (IM), dysplasia, and adenocarcinoma (6, 7). The extent of atrophy is correlated with gastric cancer risk (8), a finding that is consistent with this pattern of progression. We developed a histopathologic score that uses an increasing ordinal scale to categorize lesions by severity and extent, to estimate progression along the cascade. Characterization of lesions may be useful in predicting progression toward tumor development, yet in developing countries, precancerous lesions such as intestinal metaplasia are common. Therefore, identification of additional markers of risk would be beneficial to select persons who should be closely monitored for progression of disease.

Aberrant DNA methylation is a well-documented feature of cancers, and gastrointestinal cancers show the highest frequency of DNA methylation alterations among the tumor types reported to date in The Cancer Genome Atlas project (9). Methylation changes occur in premalignant lesions and in normal-appearing gastric mucosae adjacent to gastric cancers, but the mechanism by which such changes occur is not understood. Infection with *H. pylori* is associated with alterations in DNA methylation in gastric epithelial cells, in humans, and in animal models (10, 11), and this methylation may partially reverse if the infection is eradicated (12–14).

In 1991, we began a chemoprevention trial among residents of an Andean region of Colombia with a high incidence of gastric cancer. Subjects were randomized to receive either placebo only, anti–*H. pylori* therapy (amoxicillin, metronidazole, and bismuth subsalicylate), β-carotene, ascorbic acid, both antioxidants, either antioxidant with anti–*H. pylori* therapy, or all 3 treatments combined (15). At the end of 6 years, subjects who had not received

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anti–*H. pylori* therapy were offered this. Subsequently, individuals who continued to participate were endoscoped again at 6, 12, and 16 years to monitor progression of lesions and infection status. Gastric biopsies were classified as indicating progression, regression, or no change from baseline diagnosis. At 6 years, all interventions were associated with reduced risk of progression of lesions, but the effects of different treatments were not additive (15). At 12 years, 51% of the total set of subjects were *H. pylori*–positive and 9 participants had developed gastric cancer. Regression of lesions was found to be related to the square of time without *H. pylori* infection (16). On average, the uninfected subjects had 14.8% more regression and 13.7% less progression, compared with infected subjects. For the current study, baseline biopsy DNA was available for analysis of DNA methylation as a potential predictor of progression of lesions at years 6, 12, and 16. From this baseline DNA, we sought to examine DNA methylation in a set of candidate genes identified from prior studies in gastric precancerous lesions or tumors for relevance to prediction of outcome in later years.

Materials and Methods

Human tissues

Volunteers for the double-blinded chemoprevention trial were recruited from Pasto and Túquerres. From 1,219 screened participants, 976 subjects were randomized to receive 1 of the 8 treatments listed above, as previously described (15). All participants were *H. pylori*–positive at baseline by C^{13} urea breath (UBT) testing and had NAG or more advanced gastric histopathology. Treatments or placebos were given over 6 years. The study was approved by the Institutional Review Boards of Louisiana State University Health Sciences Center (New Orleans, LA) and Vanderbilt University (Nashville, TN) and Ethics committees of Universidad del Valle and Hospital Departamental de Nariño in Colombia. All participants provided informed consent. Results were reported at 6 (15) and 12 (16) years.

Histopathology

Endoscopies were performed at 6, 12, and 16 years, and at each visit, 4 gastric mucosal biopsies (antrum at lesser curvature, antrum at greater curvature—both within 3 cm from the pylorus—incisura angularis and corpus anterior wall) were harvested, formalin-fixed, and paraffin-embedded for histopathologic diagnosis. All tissues were stained with modified Steiner stain to detect *H. pylori* organisms (17). When needed to classify the type of intestinal metaplasia, Alcian blue/periodic acid Schiff, and high-iron diamine/Alcian blue stains were performed. Tissues were diagnosed by 2 experienced pathologists (M.B. Piazuelo and J.C. Bravo), who were blinded to the treatment assignment and prior diagnoses. Global diagnoses were assigned, considering the most advanced lesion for each subject. In addition to the global diagnoses, a histopathology score was assigned that assessed MAG (mild, moderate, or marked MAG), intestinal metaplasia (assessed for extent and type), and dysplasia (indefinite, low, or high grade), as published previously (16, 18). Any cases with discordant diagnoses between the 2 pathologists were reviewed by a third pathologist (P. Correa) until a consensus was reached. Scores for infiltration by polymorphonuclear, mononuclear cells, or intraepithelial lymphocytes (IEL) were assigned on the basis of a 0 to 3 scale (normal, mild, moderate, marked) according to published criteria (19). Scores were averaged over all 4 biopsies from all sites in each individual.

Quantitative methylation analysis by Pyrosequencing

DNA from whole gastric biopsies (from antrum, one biopsy per person) was isolated with DNAzol (Life Technologies) following the manufacturer's protocol. DNA samples (n = 104) were randomly selected from those of 620 participants for whom follow-up information for at least 12 years was available. The person performing the analyses was blinded to the status of the subjects. DNA was bisulfite-modified using a Zymo EZ Methylation Direct kit (Zymo Research Corp.). Modified DNA (30–60 ng) was amplified by PCR, using Amplitaq Gold DNA polymerase, 2 units per reaction (Applied Biosystems, Life Technologies). Specific assays included the following: Pyromark assays (Qiagen) for *EN1* (Hs_EN1_02_PM, PM00101752), *PCDH10* (Hs_PCDH10_06_PM, PM00111783), *RSPO2* (Hs_RSPO2_01_PM, PM00036267), *ZIC1* (Hs_ZIC1_03_PM, PM00015099), and *ZNF610* (Hs_ZNF610_01_PM, PM00191331), as described (20). The Pyrosequencing assay for *RPRM* was performed as previously described (21). Pyrosequencing primers for *AMPH and SORCS3* were as described by Matsusaka and colleagues (22). Other primer sequences were as follows: for *CDH1*: (F) GGTTGTGGTAGGTAGGTGAAT, (R) biotin-AACTTCCCCAAACTCACAAATACTTTAC, (Seq) GTAGGTGAATTTTTAGTTAATTAG, described by Oh and colleagues (23); for *CDKN2A*: (F) TTAGAGGATTTGAGGGATAGGG, (R) biotin-CCCTACCTACTCTCCCCCTCTC, (Seq) GTTGGTTGGTTATTAG; for *EMX1*: (F) GTGGGGTTTAGTTTGTTGTATTAAGA, (R) biotin-ATCCCCACCAAACCTCTAAACT, (Seq) TTGAGATTAGGTTTTTTAGAA; for *NKX6-1*: (F) GGTTTGGGTGAGTTTATTGAAGATAGT, (R) biotin-CAACCTATACCAACCCCCAAAAT, (Seq) TAGTTTAGAGTTTTTAGGGTAG. Sites of noncommercial assays are shown in Table 1, and maps of CpG locations are shown in Supplementary Fig. S1. Sites of sequences analyzed by the Pyromark assays may be found at www.qiagen.com. All sites queried are within CpG islands. Sites queried for *AMPH*, *RPRM*, *SORCS3*, *PCDH10*, *RSPO2*, and *ZIC1* overlap regions with bivalent histone marks in human embryonic stem cells. Assay sites for *EMX1* and *NKX6-1* overlapped those queried in Asada and colleagues (24), and the assay site for *CDKN2A* overlapped the region queried in the gastric adenocarcinoma study for The Cancer Genome Atlas

Table 1. Locations of noncommercial assays

Gene	Position queried[a]	Citation/source
AMPH	chr7:38,670,985-38,671,002	Matsusaka and colleagues (22)
CDH1	chr16:68,771,138-68,771,155	Oh and colleagues (23)
CDKN2A	chr9:21,974,879-21,974,899	This study
EMX1	chr2:73,147,660-73,147,675	This study, based on Nanjo (34)
NKX6-1	chr4:85,418,054-85,418,072	This study, based on Nanjo (34)
RPRM	chr2:154,335,079-154,335,098	Schneider and colleagues (21)
SORCS3	chr10:106,400,876-106,400,893	Matsusaka and colleagues (22)

[a]Position indicates location in February 2009 (hg19) Assembly.

project (9). For all genes except *RPRM*, the amplification program was 95°C for 15 minutes and then 45 cycles of 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds, followed by a 10-minute incubation at 72°C. Biotinylated single strands were purified on Sepharose beads and annealed to the appropriate sequencing primer. Methylation was quantitated using a Pyromark MD Pyrosequencing instrument (Qiagen) according to the manufacturer's instructions.

H. pylori virulence

Virulence of *H. pylori* was evaluated by characterizing the s region of the vacuolating cytotoxin, *vacA*, as s1 or s2 (25). Biopsy DNA was used as template, using conditions for PCR as previously described (26). Given the relatively low amount of *H. pylori* DNA in the biopsy DNA, to minimize false negatives, we used *vacA* s1 as a proxy for *cagA*. The s1 allele is highly correlated with the presence of *cagA* in strains from this high-risk population (Spearman's ρ, 0.8243, $P < 0.0001$; ref. 21).

Statistical analysis

Histopathologic scores and their changes over time were assessed in relation to the levels of methylation at the gene promoters, using multivariate models (a separate model for each gene) that included gender, age, *H. pylori* status over time, and virulence genes. Sample size was selected to have greater than 80% power to detect a significant difference in histopathologic scores over time with a 5% methylation change. Correlation and rank correlation coefficients were used to contrast different factors, as appropriate. To assess the contribution of the different variables to differences in histopathology scores between baseline and 6, 12, or 16 years and to account for confounders, multivariate generalized linear models with a Gaussian or logistic link were used, as appropriate. These models were used to evaluate the relationship of baseline methylation of each gene to outcome, accounting for covariates, including gender, age, and *H. pylori* presence and virulence. Data were analyzed using Stata MP v13 (Stata Corporation).

Results

Demographics, baseline diagnoses, and *H. pylori* status of the group for the methylation study are shown in Table 2. The *H. pylori* virulence factor *vacA* s region was detectable in 101 of the 104 baseline biopsy DNA samples (97%), and 99 (95% of the 104) were identified as carrying *vacA* s1 alleles. Within this limited range of diversity, histopathologic scores were not significantly related to the presence of the *vacA* s1 allele but were significantly associated with *H. pylori* infection at each point in time. The score difference at 16 years was significantly associated with cumulative time with *H. pylori* infection ($P = 0.028$, or after adjusting for baseline diagnosis, $P = 0.016$). IEL scores at baseline were associated with baseline histologic score ($P = 0.004$).

Table 2. Characteristics of participants

Age (mean, SD), y	50.7 ± 8.3
Median	50
Interquartile range	44.5–56
Gender, *n* (%)	
Male	54 (51.9%)
Female	50 (48.1%)
Baseline diagnosis, *n* (%)	
NAG	12 (11.5%)
MAG	18 (17.3%)
IM	64(61.5%)
Dysplasia	10 (9.6%)
H. pylori (%, *n* pos/*n* total)[a]	
Baseline	100% (104/104)
6 y	83.9% (68/81)
12 y	56.7% (59/104)
16 y	50% (39/78)
Progression (%, *n* prog/*n* total)	
6 y	49.4% (40/81)
12 y	43.3% (45/104)
16 y	55.1% (43/78)

[a]Established by Steiner staining.

Univariate analysis: variables related to methylation at baseline

Methylation levels of *AMPH*, *PCDH10*, *RPRM*, *RSPO2*, *ZIC1*, *and ZNF610* were associated with gender, being elevated in males ($P = 0.005$–0.048, Table 3). A similar trend was noted for *SORCS3* ($P = 0.078$). Baseline methylation was associated with baseline diagnosis for *AMPH*, *EMX1*, *RPRM*, *RSPO2*, *SORCS3*, and *ZNF610* ($P = 0.001$–0.048). Methylation levels of all genes except *CDH1*, *CDKN2A*, and *NKX6-1* were significantly correlated with each other (Supplementary Table S1, univariate correlation coefficients, after adjusting for multiple comparisons), with Pearson correlation coefficients varying from 0.7646 (*ZIC1* and *ZNF610*) to 0.9129 (*SORCS3* and *AMPH*). Within the age range of the participants, levels of methylation were not significantly associated with age, comparing subjects 50 and younger to all others.

Multivariate analysis: variables related to changes in diagnosis over time

The genes differed in their utility of predicting progression by their methylation levels (Table 4). Four genes (*AMPH*, *PCDH10*, *RSPO2*, and *ZNF610)* showed a strengthening effect over time, having increasing progression coefficients (a coefficient represents the predicted change in histopathology units associated with 1% change in methylation, when all other variables are held constant) and decreasing *P* values at 6, 12, and 16 years, after adjusting for baseline diagnosis and *H. pylori* status (Table 4). For example, after those adjustments, *AMPH* methylation was related to progression with *P* equal to 0.087, 0.029, and 0.011 at 6, 12, and 16 years, respectively. If instead of *H. pylori* status at the time point, cumulative *H. pylori* status over the entire study period was included in the model for each gene, the effect of methylation in predicting progression was even stronger for those 4 genes (e.g., $P = 0.006$ for *AMPH*). Methylation of the *SORCS3* gene predicted progression at 6, 12, and 16 years ($P = 0.034$, 0.013, and 0.029), but without an increasing effect. In contrast to these 5 genes, *ZIC1* showed no significant associations of methylation with progression at the 3 time points, and *EN1* had marginal significance ($P = 0.046$) of methylation with progression at 6 years, but the associations became nonsignificant at 12 and 16 years. Methylation at *RPRM* was associated with progression at 6 years ($P = 0.013$) but lost significance thereafter. Methylation of *EMX1* was significantly associated with disease progression at 6 years ($P = 0.025$) and 12 years ($P = 0.005$) but not at 16 years. Methylation at *NKX6-1* was significantly related to progression at 12 years ($P = 0.015$) but not at other time points. Scores for polymorphonuclear, morphonuclear, and IEL were not associated with progression and did not affect the association of methylation with progression.

Table 3. Average methylation related to baseline variables

Baseline variables ($n = 104$)	*AMPH*	*CDH1*	*CDKN2A*	*EMX1*	*EN1*	*NKX6-1*	*PCDH10*	*RPRM*	*RSPO2*	*SORCS3*	*ZIC1*	*ZNF610*
Sex (*P*)	0.029	0.651	0.579	0.113	0.179	0.266	0.007	0.010	0.030	0.078	0.048	0.005
Female	21.1 ± 1.3[a]	19.2 ± 1.1	3.4 ± 0.3	8.2 ± 0.8	22.5 ± 1.0	47.8 ± 1.5	26.9 ± 1.3	17.1 ± 1.5	17.9 ± 1.1	21.1 ± 1.5	13.0 ± 1.0	17.1 ± 1.3
Male	25.0 ± 1.3	19.8 ± 1.2	3.7 ± 0.3	10.3 ± 0.8	24.4 ± 1.1	45.8 ± 1.6	31.9 ± 1.4	22.9 ± 1.6	21.3 ± 1.1	24.9 ± 1.6	15.9 ± 1.0	22.6 ± 1.3
Age (*P*), y	0.735	0.934	0.065	0.498	0.227	0.307	0.742	0.792	0.254	0.684	0.374	0.852
≤50	23.2 ± 1.3	19.3 ± 1.2	3.2 ± 0.3	9.7 ± 0.8	24.2 ± 1.0	45.9 ± 1.5	29.6 ± 1.3	20.2 ± 1.6	20.4 ± 1.1	23.3 ± 1.5	15.1 ± 1.0	20.1 ± 1.3
>50	22.7 ± 1.3	19.7 ± 1.2	3.9 ± 0.3	8.7 ± 0.8	22.5 ± 1.1	47.9 ± 1.6	29.1 ± 1.4	19.6 ± 1.6	18.6 ± 1.2	22.5 ± 1.6	13.8 ± 1.0	19.4 ± 1.4
Baseline diagnosis (*P*)	0.032	0.800	0.812	0.008	0.092	0.379	0.128	0.048	0.009	0.039	0.224	0.001
NAG	18.3 ± 2.7	17.4 ± 2.4	3.4 ± 0.6	6.6 ± 1.7	20.1 ± 2.2	45.5 ± 3.2	25.1 ± 2.8	14.7 ± 3.3	15.2 ± 2.3	16.6 ± 3.2	12.2 ± 2.1	15.2 ± 2.7
MAG	21.6 ± 2.2	20.7 ± 2.0	3.7 ± 0.5	6.2 ± 1.4	22.9 ± 1.8	42.3 ± 2.7	29.3 ± 2.3	17.7 ± 2.7	17.5 ± 1.9	21.3 ± 2.6	13.5 ± 1.7	15.0 ± 2.3
IM	23.8 ± 1.2	19.7 ± 1.1	3.6 ± 0.3	10.4 ± 0.7	23.9 ± 0.9	48.8 ± 1.4	30.0 ± 1.2	21.3 ± 1.4	20.3 ± 1.0	24.4 ± 1.4	15.0 ± 0.9	20.8 ± 1.2
Dysplasia	25.6 ± 2.9	18.4 ± 2.7	3.0 ± 0.6	10.5 ± 1.8	24.9 ± 2.4	43.7 ± 3.5	30.7 ± 3.0	21.8 ± 3.6	23.1 ± 2.6	23.9 ± 3.5	14.9 ± 2.3	27.5 ± 3.0

NOTE: From a multivariate linear model where methylation values for each gene are evaluated in conjunction with age, sex, and baseline diagnosis of the subject.
Abbreviation: IM, intestinal metaplasia.
[a]Values are means ± SE.

We asked how the effect of *H. pylori* compared in the models to the effect of methylation as a predictive factor for disease progression. We examined the effect of *H. pylori* for its status at a single point in time and also for its cumulative effects over the total period of the study. For the *ZNF610* model for the 12-year interval, using a single-time-point measurement for *H. pylori* as present or absent, the bacterial effect corresponded to 0.392 histopathology units, compared with 0.589 units for the difference between the 10th and 90th percentiles of methylation levels for the *ZNF610* gene. Similarly, for the *AMPH* gene, its model predicts 0.391 units for the effect of *H. pylori* at 12 years, compared with 0.494 histopathology units for the difference between 10th and 90th percentiles of methylation levels for *AMPH*. Results for models using *H. pylori* status at the 16-year time point were similar, with the effect of *H. pylor*i at the single time point being smaller than the effect of methylation of either gene. If instead, we examined the cumulative effect of the presence of *H. pylori* over the total 16-year interval, the corresponding differences in histopathology units were 0.82 for the cumulative effect of *H. pylori* and 0.55 units for the effect of the middle 80-percentile difference for methylation levels of *ZNF610*. For the *AMPH* gene, the corresponding score changes were 0.766 units for the cumulative *H. pylori* effect and 0.66 units for 10th to 90th percentile of methylation levels.

Discussion

Our findings support the hypothesis that methylation alterations can predict progression toward gastric disease, even 16 years prior to such progression. Our study was specifically focused on the histopathologic lesions associated with development of the intestinal type of gastric adenocarcinoma, and the DNA we examined was from gastric biopsies containing the epithelial cells from which such tumors arise.

The genes we chose to examine for methylation changes included 6 genes (*EN1*, *PCDH10*, *RPRM*, *RSPO2*, *ZIC1*, and *ZNF610)* that were already established in cross-sectional studies to show changes in methylation in premalignant lesions, and those changes were quantitatively related to risk determinants such as *H. pylori* virulence factors (20, 21). *EN1* encodes engrailed homeobox 1, which is involved in control of pattern formation during development of the central nervous system. *PCDH10* encodes protocadherin 10, a cadherin-related neuronal receptor that is a possible tumor suppressor in gastric cancer (27, 28). *RPRM*, encoding a cell-cycle checkpoint mediator, was identified from a screening study as a possibly useful biomarker for gastric cancer (29). *RSPO2* encodes R-spondin 2, which regulates β-catenin signaling and is involved in development of limbs, lungs, and hair follicles (30). *ZIC1* encodes a zinc finger protein involved in regulating organogenesis in the central nervous system but also modulates cell-cycle distributions through the PI3K and MAPK signaling pathways in gastric cancer (31). *ZNF610* encodes zinc finger protein 610, a member of the Krüppel C2H2-type zinc finger protein family and a likely transcription regulator. In the Cancer Genome Atlas study for gastric adenocarcinoma (ref. 9; which did not address methylation in premalignant lesions), *ZNF610* was the 9th most frequently epigenetically silenced gene

Table 4. Associations of methylation with progression of gastric lesions

Baseline variables ($n = 104$)	*AMPH*	*CDH1*	*CDKN2A*	*EMX1*	*EN1*	*NKX6-1*	*PCDH10*	*RPRM*	*RSPO2*	*SORCS3*	*ZIC1*	*ZNF610*
Progression at year 6 (P^a)	0.087	ns[b]	ns	0.025	0.046	ns	0.082	0.013	0.091	0.034	ns	0.045
Coefficient[c]	0.018			0.039	0.026		0.018	0.021	0.021	0.019		0.020
(SE)	(0.010)			(0.017)	(0.013)		(0.010)	(0.008)	(0.012)	(0.009)		(0.010)
Progression at year 12 (P^a)	0.029	ns	ns	0.005	ns	0.015	0.039	0.061	0.051	0.013	ns	0.012
Coefficient	0.023			0.047		0.021	0.0212	0.017	0.024	0.022		0.025
(SE)	(0.011)			(0.017)		(0.009)	(0.010)	(0.008)	(0.012)	(0.009)		(0.010)
Progression at year 16 (P^a)	0.011	ns	ns	ns	ns	0.074	0.013	0.087	0.033	0.029	ns	0.012
Coefficient	0.028					0.019	0.025	0.015	0.027	0.019		0.025
(SE)	(0.011)					(0.011)	(0.01)	(0.009)	(0.012)	(0.009)		(0.010)
Progression at year 16 + cumulative *H. pylori* status (P^a)	0.006	ns	ns	ns	ns	ns	0.008	0.071	0.025	0.026	ns	0.009
Coefficient	0.029						0.027	0.015	0.028	0.020		0.026
(SE)	(0.010)						(0.01)	(0.008)	(0.012)	(0.009)		(0.010)

[a]*P* values after adjusting for baseline diagnosis and time-dependent *H. pylori* status or cumulative *H. pylori* status.
[b]Nonsignificant (ns) indicates a *P* value of ≥ 0.1.
[c]Coefficients may be interpreted in the model as the predicted change in difference in histopathology units between time points as methylation increases by 1%.

(of 769 silenced genes) in gastric cancers tested using the Illumina HM450 platform. *ZNF610* was both methylated and decreased in expression in 71% of 220 gastric cancers.

In addition to these 6 genes already tested in premalignant lesions, we added methylation assays for *CDKN2A* and *CDH1*, as possible tumor suppressor genes in gastric cancer. Germline inactivation of *CDH1*, which encodes E-cadherin, is associated with high risk for hereditary diffuse gastric cancer. The site we examined in the *CDH1* promoter was previously reported in a cross-sectional study to show methylation changes in the nonmalignant antrum adjacent to gastric cancers (23). We also added *AMPH* and *SORCS3*, which had been identified from a screen of 14,495 genes, as hypermethylated in both Epstein Barr virus–associated gastric cancers and uninfected tumors (22). *AMPH* encodes amphiphysin, a protein associated with the cytoplasmic surface of synaptic vesicles. *AMPH* is involved in endocytosis and is expressed in gastric enterochromaffin–like cells (32). *SORCS3* encodes sortilin-related receptor 3, a modulator of neuronal viability and memory extinction (33). We added *NKX6-1* and *EMX1* identified as genes that have higher DNA methylation in gastric cancer patients than in healthy volunteers with former *H. pylori* infection (34) and that may be useful in prediction of risk for gastric cancer (24). *NKX6-1* encodes a transcription factor involved in regulation of pancreatic beta cells and modulates transcription of insulin. *EMX1* encodes a homeobox protein called empty spiracles homolog 1, involved in regulating development in the nervous system.

H. pylori infection increases methylation of specific genes in humans and animal models (10, 11). In cross-sectional studies, we have found elevated levels of methylation of *EN1*, *PCDH10*, *RPRM*, *RSPO2*, *ZIC1*, and *ZNF610* in gastric biopsy DNA of *H. pylori*–infected persons compared with that of uninfected ones (20, 21). Of those genes, *PCDH10*, *RSPO2*, and *ZNF610* showed increases in methylation that predicted progression in the current study. We found no significant interaction between *H. pylori* presence (either single point or cumulative) and baseline methylation of any of the genes we examined, indicating the independence of these variables in our models.

A popular concept states that it is inflammation, rather than infection *per se*, that is responsible for methylation changes associated with *H. pylori* infection (35). Niwa and colleagues found in a gerbil model that suppression of inflammation with cyclosporin A reduced aberrant DNA hypermethylation even without eradication of the *H. pylori* infection (13). Therefore, we asked whether measures of infiltration with polymorphonuclear, morphonuclear cells, or IEL predicted progression of disease and found that they did not. However, in univariate analysis, IEL scores at baseline were associated with elevated histopathologic scores. Because our progression models adjusted for baseline scores, the effect of IEL on such scores was not independently detectable in those models.

Because of the choice of the high-risk population, our study has some limitations. We found little variation in virulence of infecting *H. pylori* strains, by the means we used to measure it, as 95% of the subjects had highly virulent strains at baseline. Therefore, it is not surprising that we were unable to detect a significant effect of *H. pylori* virulence on methylation. Previously, in cross-sectional studies with a more diverse cohort, we found that *H. pylori* virulence was related to methylation of *EN1*, *PCDH10*, *RSPO2*, *ZIC1*, *ZNF610* (20), and *RPRM* (21). Adjusting the multivariate model for cumulative exposure to *H. pylori* did not eliminate the significant effect of methylation, indicating a predictive value for baseline methylation, independent of the influence of *H. pylori*, whether virulent or not.

Another limitation of the study is that we had a relatively narrow age range of participants. The median age at baseline was relatively young (50 years) and the interquartile range was 44.5 to 56 years. This narrow range may be the reason that we did not find age of the participants at baseline to be a significant variable associated with baseline methylation. However, over the total duration of the study, *P* values for the association of methylation at *AMPH*, *PCDH10*, *RSPO2*, and *ZNF610* with histopathologic scores decreased over time and coefficients increased, indicating a strengthening effect as the cohort aged.

The tissues used in our study were not microdissected. We had available to us DNA only from whole biopsies, which contain precancerous lesions, but which also contain normal gastric cells and immune cells. Therefore, we were unable to ascertain whether elevated DNA methylation that we measured came from precancerous lesions alone, from some field effect, or from some combination of cell types.

DNA methylation in an individual is known to change over lifespan (36, 37). Fraga and colleagues noted that monozygotic twins at age 3 had similar DNA methylation profiles in blood, but these profiles were different in adults, and the adult twins became more variable from each other, especially if they had different lifestyles (38). The increasing variation in DNA methylation with age is called epigenetic drift and has been characterized at the genomic level as overall loss of CpG methylation, increased variation in neighboring CpG sites, and increasing methylation at CpG island promoters (39) with bivalent chromatin domains (40). Of the 5 significant genes, all but *ZNF610* have bivalent histone marks in human embryonic stem cells. Several investigators have devised models to predict age from DNA methylation microarray data (40–44). Both tissue-specific and tissue-independent changes have been identified (43, 45, 46). Promoters of some of the same genes that become hypermethylated in normal aging are similarly affected in cancers (47). Cancer cells may undergo a process similar to accelerated aging; Horvath calculated from 20 tissue types an average age acceleration of 36.2 years in patients with cancer (43). Men show acceleration compared with women (48), and environmental influences such as obesity may accelerate epigenetic drift (49). Methylation changes in blood and tissues have been associated with risk for liver disease (41, 43, 49), for diabetes (50), and for all-cause mortality (48). It is possible that DNA methylation of the significant genes in our models may reflect the activity of a similar epigenetic clock examined at baseline of our study. We speculate that the baseline methylation we detected may reflect the influence of other unknown variables that may advance the epigenetic clock, so as to predispose a person to develop disease, even 12 to 16 years later. Our method of analysis allows us to see how variables affecting both the epigenetic clock and an environmental factor (*H. pylori* infection) are associated with progression of lesions, with the *H. pylori* effect increasing in strength when analyzed cumulatively.

Our results demonstrate the multifactorial nature of associations with histopathologic progression. The cumulative effect of *H. pylori* infection during the entire study had a major influence in the models, consistent with findings of its association with progression in previous analyses in this chemoprevention cohort (16). However, our results predict that in the case of a study that would be restricted to samples taken at a single time point, when

the duration of infection cannot be known, methylation of the significant genes may have greater benefits for prediction of progression than current *H. pylori* status. Studies are in progress to examine the utility of these methylation assays in additional populations.

In summary, we found that methylation levels of *AMPH*, *PCDH10*, *RSPO2*, *SORCS3*, and *ZNF610* predicted progression of gastric disease in a high-risk prospective cohort, over a period of 16 years. Methylation levels, in conjunction with baseline histopathology scores and *H. pylori* status or cumulative exposure, have potential as markers of risk for gastric disease in high-incidence populations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

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.

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