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Somatic DNA Hypomethylation in *H. pylori*-Associated High-Risk Gastritis and Gastric Cancer: Enhanced Somatic Hypomethylation Associates with Advanced Stage Cancer

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OBJECTIVES: *Helicobacter pylori*-related high-risk gastritis (HRG) is a severe risk factor for gastric cancer (GC). The link between HRG and long-term risk for GC may involve genetic and epigenetic alterations underlying a field defect, i.e. a region of the mucosa prone to cancer development. Global DNA hypomethylation is a pervasive alteration in GC that associates with chromosomal instability and poor prognosis. The aim of this study was to determine the chronology of this alteration along the progression of HRG to GC, to test the hypothesis that it occurs early in the chronology of this pathway and plays a mechanistic role in the long-term cancer risk.

METHODS: We comparatively measured the genomic methylation level in gastric biopsies from 94 GC patients and 16 of their cancer-free relatives, 38 HRG patients, and 17 GERD patients, using a quantitative enzymatic method.

RESULTS: GC biopsies were hypomethylated compared to their matching non-tumor mucosa ($P = 9.4 \times 10^{-12}$), irrespective of the tumor location or patients' country of origin. Genome-wide hypomethylation was also found in gastric mucosa of GC ($P = 1.5 \times 10^{-5}$) and HRG ($P = 0.004$) patients compared with healthy donors and GC relatives, regardless of the biopsy location within the stomach or previous *H. pylori* eradication therapy. An enhanced hypomethylation, distinguished by a bi-slope distribution of the differences in methylation between tumor and normal tissues, associated with a more invasive ($P = 0.005$) and advanced stage ($P = 0.017$) type of GC.

CONCLUSIONS: Universal DNA demethylation in normal gastric mucosa in GC patients appears sporadic rather than familial. Genomic hypomethylation in HRG possibly contributes to a field defect for cancerization that is not reversed by bacterial eradication. Enhanced somatic hypomethylation may stratify GC for prognostic purposes.

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INTRODUCTION

Gastric cancer (GC) is the leading cause of cancer-related deaths in many parts of the world.[1,2] GC is a multistep and multifactorial process involving genetic, epigenetic and environmental factors. Up to 70% of all GC cases are associated with previous *H. pylori* infection.[3] The GC risk increases about 6-fold in *H. pylori* infected individuals,[4] particularly in the presence of corpus-predominant gastritis as well as pangastritis.[5] In the current model of gastric carcinogenesis of the intestinal type, the oncogenic process starts with *H. pylori*-related chronic active gastritis, followed by glandular atrophy, intestinal metaplasia, dysplasia, and finally gastric adenocarcinoma.[6] Despite the well-established association between *H. pylori* and GC, the oncogenic mechanism of this bacterium and why the GC-risk remains elevated even after successful eradication therapy are not yet well understood.[7] This long-term 'memory' effect is thought to involve genetic and epigenetic alterations of the gastric mucosa contributing to the subsequent development of GC.

The human genome is nearly fully methylated at birth, with values typically around 80.0–95.0% depending on the tissue and the analytical technique employed.[8,9] Methylation occurs almost exclusively at CpG dinucleotides in a post-replication event catalyzed by DNA (cytosine-5)-methyltransferases (DNMTs).[10] After birth, methylation relentlessly decrease during aging in essentially all tissues, at different rates depending on their proliferative potential.[11] Cancer cells generally present an aberrant DNA methylation profile, comprising both loss of methylation (hypomethylation) and *de novo*

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gain of methylation (hypermethylation).[12] Whereas hypermethylation occurs typically in gene promoters and associates with transcriptional silencing, hypomethylation is genome-wide and associates with genetic instability.[13–16] Genome-wide DNA hypomethylation of human cancer cells was described in the early eighties,[17] and has been found in almost all types of cancers, including gastric carcinomas.[18–20] Paradoxically, both germ line and somatic mutations in DNMTs are rare in cancer and thus some factors - endogenous or exogenous - must underlie the abnormal methylation patterns.

In a previous study, we found that global DNA hypomethylation accumulates with patient age, associates with copy number alterations and is an independent predictor of poor prognosis in gastrointestinal cancers.[21] Other studies have explored the association of *H. pylori* with hypermethylation of specific genes,[22–26] as well as hypomethylation of genes,[27,28] and repetitive elements Alu, long interspersed nuclear element -1 (LINE-1) and Satellite α (Satα).[20,29–31] These studies provided several biomarkers for gastric cancer risk, and shed light on the link between epigenetics and *H. pylori*-associated gastric carcinogenesis. There is still no consensus, however, on the chronology of these alterations, nor a clear explanation for the discrepancies found between Alu and LINE-1 hypomethylation in both their timing and prognostic value.[20,29]

In this study, we applied a biochemical quantitative assay to estimate the percentage of methylated CpG sites (^{m}CpG%) in normal and cancer gastric tissues from patients with GC and patients with high-risk gastritis (HRG) caused by *H. pylori* infection. We investigated the incidence and extent of somatic genome-wide hypomethylation to elucidate its chronology in the progression of gastric carcinogenesis.

PATIENTS AND METHODS

Patients. Ninety-four primary gastric cancers and matched normal tissue from patients who had undergone surgery were collected as freshly frozen tissues from the Cooperative Human Tissue Network ($n=61$; USA) and the Institute of Pathology, University of Magdeburg ($n=33$; Germany). Thirty-eight patients with previously diagnosed *H. pylori* infection and pangastritis or corpus-predominant gastritis and/or intestinal metaplasia were identified in the endoscopy database of the University of Magdeburg. All patients had no gross pathology, and only chronic erosion, or erythema, were described by the gastroenterologist in the initial upper GI-endoscopy. In 24 patients, *H. pylori* eradication therapy had been performed consciously according to the documentation, at least 2 years before the current investigation. In addition, 16 cancer-free first-degree relatives of GC patients were included in the study. In all of them an upper GI-endoscopy was performed and specimens were taken form the antrum and the corpus for further investigations. Seventeen patients suffering from gastro-esophageal reflux disease without acid-related medication, normal endoscopic appearance, and normal histopathology of the stomach, were recruited as a control group (GERD patients).

H. pylori infection was diagnosed by the rapid urease test, culture and histological examination (greater and lesser curvature both antrum and corpus) with Giemsa staining. The patients were classified as *H. pylori* positive when two out of three detection methods were positive. Gastritis was evaluated in accordance with the updated Sydney classification.[32] The definition of pangastritis and corpus-predominant gastritis (grade of neutrophil infiltration in the corpus equal or higher than in the antrum) was based on the parameters described in Uemura *et al*.[5] The distribution of the gastritis parameters is shown in Table 1.

Approval from the Sanford-Burnham Institutional Review Board and the Ethical Committee of the University of Magdeburg was obtained for this work, complying with the ethical guidelines of the Declaration of Helsinki.[33]

Sample dissection and DNA extraction. Genomic DNA from gastric biopsies was prepared by standard proteinase-K digestion, phenol-chloroform extraction and ethanol precipitation.[34] Ten of the American GC cases were randomly selected for a more detailed analysis of the methylation levels in the different layers of the transmural resection of the gastric non-tumor tissues. In these ten cases a gastroenterologist dissected the tissue samples in 3 parts, corresponding to mucosa, muscularis, and serosa, respectively. DNA was prepared and analyzed separately for each layer.

Analysis of global genome methyl-CpG content (^{m}CpG%). Methyl-CpG content was measured using a modified M.SssI protocol.[21,35] The method estimates the global genome content of methylated cytosines at CpG sites by *in vitro* incorporation of tritiated S-adenosyl-L-[methyl-^{3}H] methionine (SAM) into non-methylated CpG sites, catalized by the M.SssI methylase. The originally methylated cytosines in the genomic DNA are protected from incorporation. The reaction was conducted as follows: 100 ng of DNA were

Table 1 Cases and controls

	GERD patients ($n=17$)	High risk gastritis ($n=38$)	Cancer-free relatives ($n=16$)	Gastric cancer ($n=94$)
Median age (range)	57 (33–73)	63 (34–77)	53 (32–74)	68 (25–89)
Gender (male:female)	9:8	20:18	11:5	63:29
H. pylori positive[a]	0 (0%)	14 (37%)	9 (56%)	n/a
Distribution of gastritis[b]				
Pangastritis	0 (0%)	29 (76%)	5 (31%)	n/a
Corpus predominant	0 (0%)	9 (24%)	2 (13%)	n/a
Intestinal metaplasia	0 (0%)	19 (50%)	6 (38%)	n/a
Atrophy[c]	0 (0%)	5 (13%)	5 (31%)	n/a

[a]At the time of the determination of global DNA methylation. [b]At the time of the initial investigation (index endoscopy). [c]Mild degree.

treated with 2 U of M.SssI methylase (CpG methylase, New England BioLabs, USA) at 37 °C for 4 h in 20 µl of buffer containing 0.75 µM S-adenosyl-L-[methyl-^{3}H]-methionine (80.0 Ci/mmol, Amersham Biosciences, USA), 0.75 µM unlabeled S-adenosylmethionine, 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol. Reactions were inactivated by 20 min incubation at 75 °C. DNA was purified using a silica-based purification kit (Geneclean kit, Q.BIOgene, USA), which provided a faster, easily scalable and equally efficient method compared to the previously described precipitation with trichloroacetic acid and ethanol and subsequent filtration through GF/C filters.[35] Purified DNA was dissolved in 200 µl of water and transferred to a scintillation vial with 2 ml of scintillation solution (EcoLume, ICN, USA). Incorporation of the radioactive methyl group was measured using a Beckman liquid scintillation counter. To control the quantity and quality of the DNA, a parallel assay was performed using the *dam* methylase, which methylates the deoxyadenosine within the sequence GATC (virtually never methylated in humans). Conditions were identical to the M.SssI reaction, except that 6 U of *dam* methylase were used in 10 mM EDTA, 50 mM Tris-HCl, 5 mM 2-mercaptoethanol buffer.

The percentage of methylated CpG sites (^{m}CpG%) was estimated from the M.SssI and *dam* methylase ^{3}H incorporation values as follows: $^m\text{CpG}\% = (1 - 0.2323 \times \text{M.SssI}/\textit{dam}) \times 100$, were 0.2323 is the proportion between GATC sites and CpG sites in the human genome (GRh37). All experiments were performed in triplicate. The standard error of the technique was $3.1 \pm 1.8\%$, CI 95% = [0.4–5.9%]. No statistically significant difference in CpG methylation level between antrum and corpus was found (mean difference = 0.5%, $P = 0.24$, paired t Student test). Therefore, for some of the analyses presented in this paper, methylcytosine content of antrum and corpus was averaged per patient. For comparison with previously published work conducted with different techniques that report the relative methyl-cytosine content (^{m}C% = methyl-cytosine divided by total cytosine content), the values were transformed as follows: ^{m}CpG % = ^{m}C% x (number of cytosines/cytosines in CpG sites in the human genome) = $^m\text{C}\% \times 20.7506$.

Statistics. Statistical analyses were performed with R environment for statistical computing.[36] Methylation in non-tumor vs. tumor tissue was compared by Student's *t*-test for paired samples. Differences between two groups were analyzed by Student's *t*-test for unpaired samples, or by repeated measures ANOVA (rANOVA) when two independent biopsies (antrum and corpus) were available per individual. For comparison of three or more groups, we performed ANOVA with Tukey's Honest Significant Differences method (Tukey's HSD), or one-tailed Cochran-Armitage test for ordinal categorical variables.[37] The threshold for abnormal global hypomethylation (AGH) was calculated as the mean percentage of global methylation in controls minus two times the standard deviation. The threshold for enhanced somatic hypomethylation (ESH) was calculated as the mean

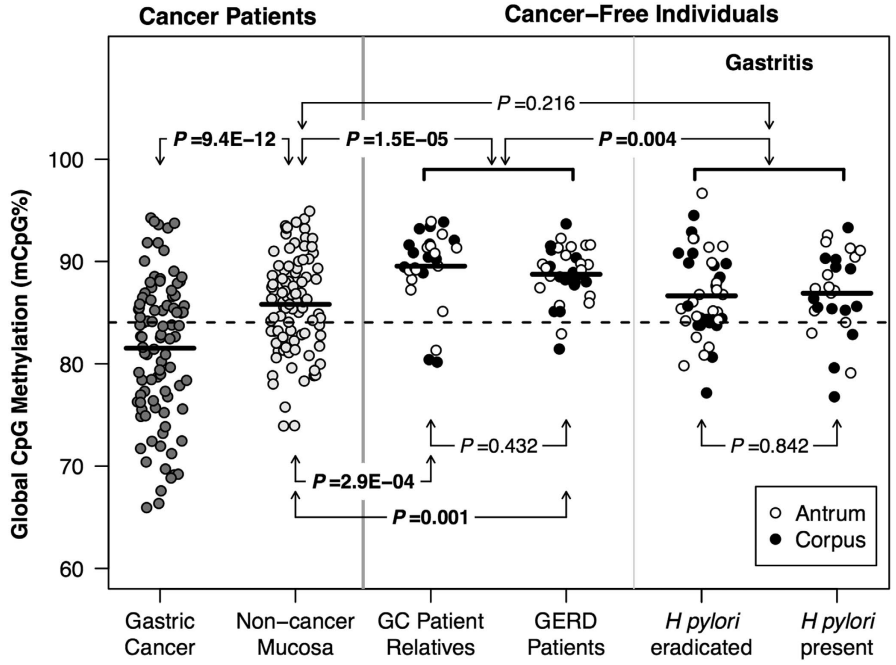

Figure 1 Global CpG methylation levels in gastric biopsies of healthy donors, gastric-cancer patients' relatives, gastric biopsies from risk gastritis patients, and from gastric mucosa and cancer tissues from gastric cancer patients. The difference in ^{m}CpG% between tumor and non-tumor samples remained statistically significant in a multifactorial regression analysis ($P = 1.7 \times 10^{-6}$, Supplementary Table 1). No association was found between any of the parameters analyzed (distribution of gastritis, grade of neutrophil/lymphocytic infiltration, presence of intestinal metaplasia, or atrophy) and the degree of hypomethylation in the gastric mucosa of HRG patients (Supplementary Table S2). The dashed horizontal line indicates the abnormal genome-wide hypomethylation threshold (AGH, 84.04%, mean of controls minus 2 times their standard deviation). *P* values of paired *t*-test for Non-cancer mucosa vs. cancer tissues in cancer patients or repeated measures ANOVA considering type and, where available, location (antrum vs. corpus) for all other comparisons, are shown. In bold type, *P* values < 0.05. In the majority of cancer-free cases, each individual donated two biposies, one from antrum and one from corpus.

^{m}CpG% difference between the tumor and the combined value of mucosa, muscularis and serosa, minus two times the standard deviation. Unless otherwise specified, *P*-values were two-sided. Statistical significance threshold was set at $P<0.05$.

RESULTS

Primary gastric cancers exhibit abnormally low levels of CpG methylation. Our study design compared the methylation levels in biopsies from GC and matching normal tissues ($n=94$, median age 68, male:female ratio 63:29), between normal mucosa of GC patients and the mucosa of GERD patients ($n=17$, median age 57, male:female ratio 9:8) or cancer free relatives ($n=16$, median age 53, male:female ratio 11:5), and finally, between all of these and gastric mucosa biopsies of HRG ($n=38$, median age, 63, male: female ratio, 20:18) before and after *H. pylori* eradication. The results are condensed in Figure 1 that is described stepwise below.

In GC patients, primary tumors had significantly lower methylation than non-tumor tissues (Figure 1 left) regardless of the gender, age of the patients, or the anatomical location (Table 2). The cancer-specific demethylation of German and American GC patients was nearly identical (Supplementary Figure S1). The degree of hypomethylation in primary tumors associated with stage ($P=0.024$), especially with the extent of invasion through the stomach layers (T-stage, $P=5\times10^{-4}$), but not with lymph node invasion (N-stage, $P=0.11$) or distant metastasis spread (M-stage, $P=0.31$).

Table 2 Demographics of gastric cancer patients and ^{m}CpG% content

	n	Normal	Tumor	*P*-value[a]
Patient Origin				
Informative cases	94	85.8 ± 4.7	81.5 ± 6.8	9.4×10^{-12}
Germany	33	86.5 ± 4.7	81.0 ± 7.4	3.3×10^{-8}
US	61	85.5 ± 4.6	81.8 ± 6.6	6.1×10^{-6}
P-value[b]		0.294	0.592	
Patient Gender				
Informative cases	92	85.8 ± 4.7	81.5 ± 6.9	8.4×10^{-12}
Female	29	85.3 ± 4.7	80.9 ± 7.5	3.7×10^{-4}
Male	63	86.0 ± 4.7	81.7 ± 6.6	6.7×10^{-9}
P-value[b]		0.524	0.627	
Patient Age				
Informative cases	91	85.7 ± 4.6	81.3 ± 6.8	7.7×10^{-12}
<68 years	41	85.7 ± 4.4	81.9 ± 6.5	6.4×10^{-5}
>68 years	50	85.7 ± 4.8	80.8 ± 7.1	2.9×10^{-8}
P-value[b]		0.966	0.442	
Tumor Location				
Informative cases	45	85.6 ± 4.9	81.8 ± 6.9	8.8×10^{-5}
Cardias	14	85.4 ± 5.2	80.5 ± 7.6	0.015
Fundus	3	85.5 ± 6.4	82.3 ± 5.0	0.334
Corpus	6	82.0 ± 4.9	75.6 ± 7.1	0.104
Antrum	22	86.7 ± 4.3	84.1 ± 5.6	0.030
P-value[b]		0.234	0.043[c]	

Normal and Tumor columns show the mean value of mCpG% ± standard deviation, in each group.
[a]*P*-value of the comparison of ^{m}CpG% in normal tissues vs. tumors, calculated by paired *t*-test.
[b]*P*-values of one-way ANOVA analyses, separately conducted on normal and tumor samples.
[c]Statistically significant difference in the ^{m}CpG% in tumors was observed between tumors from the antrum (84.1 ± 5.6%) and from the corpus (75.6 ± 7.1%) ($P=0.032$, Tukey's HSD test).

Normal gastric tissue of GC patients also undergoes global demethylation compared with GERD patients and cancer-free first-degree relatives of GC patients. Figure 1 also shows that DNA hypomethylation accumulated in the normal tissue of GC patients compared with that of cancer-free first-degree relatives and GERD patients (Figure 1, center). GERD patients and cancer-free relatives of GC patients exhibited similar methylation levels as previously reported for healthy individuals.[20] In contrast, the mean methylation level in non-tumor tissue of GC patients was significantly lower ($P=1.5\times10^{-5}$).

Hypomethylation in *H. pylori*–related gastritis patients persists after eradication. Biopsies from patients with *H. pylori*-related high-risk gastritis (HRG) also exhibited lower levels of ^{m}CpG compared to the control groups of GERD patients and cancer-free first-degree relatives of GC patients ($P=0.004$, Figure 1, right). According to the results of histology, culture and rapid urease test, 14 of the HRG patients were *H. pylori* positive, whereas 24 patients were *H. pylori* negative after having being successfully treated to eradicate the bacteria. No difference was found between the ^{m}CpG levels in HRG patients with *H. pylori* present and absent after eradication ($P=0.842$, Figure 1 right).

GC and *H. pylori*–related gastritis patients show abnormal global hypomethylation (AGH). We determined a threshold to distinguish an abnormal global hypomethylation (AGH) level (dashed horizontal line in Figure 1) from the background level of methylation shared by adult GERD patients, who show no difference with the healthy relatives of GC patients. The AGH was estimated as 84.04% methylated genomic CpG sites based on the mean percentage of global methylation in GERD patients minus two times the standard deviation (see methods). Fifty-four of the 94 primary GCs (57.4%) and 32 of the corresponding matching non-tumor specimens (34.0%) exhibited levels below the AGH threshold. Notably, similar incidence rates of AGH were found in patients with untreated *H. pylori* associated high-risk gastritis (5/14, 35.7%), as well as with gastritis patients whereby *H. pylori* was eradicated by antibiotic treatment (8/24, 33.3%). Only one of the 17 GERD patients (two data-points in Figure 1 represent two biopsies from the same individual), and 2 of the 16 GC relatives (three biopsies from two individuals) showed methylation below the AGH threshold (Figure 1).

Hypomethylation preferentially affects the mucosa layer. Normal tissue from GC patients consists of a heterogeneous mixture of different cell types derived from the mucosa, muscularis and serosa layers of the stomach. We investigated the hypomethylation in the mucosa and the other layers. We randomly selected 10 GC cases to study the methylation levels separately in the three layers of the non-tumor biopsy, as well as in the tumor sample (Figure 2). We found no difference between the levels of DNA methylation in

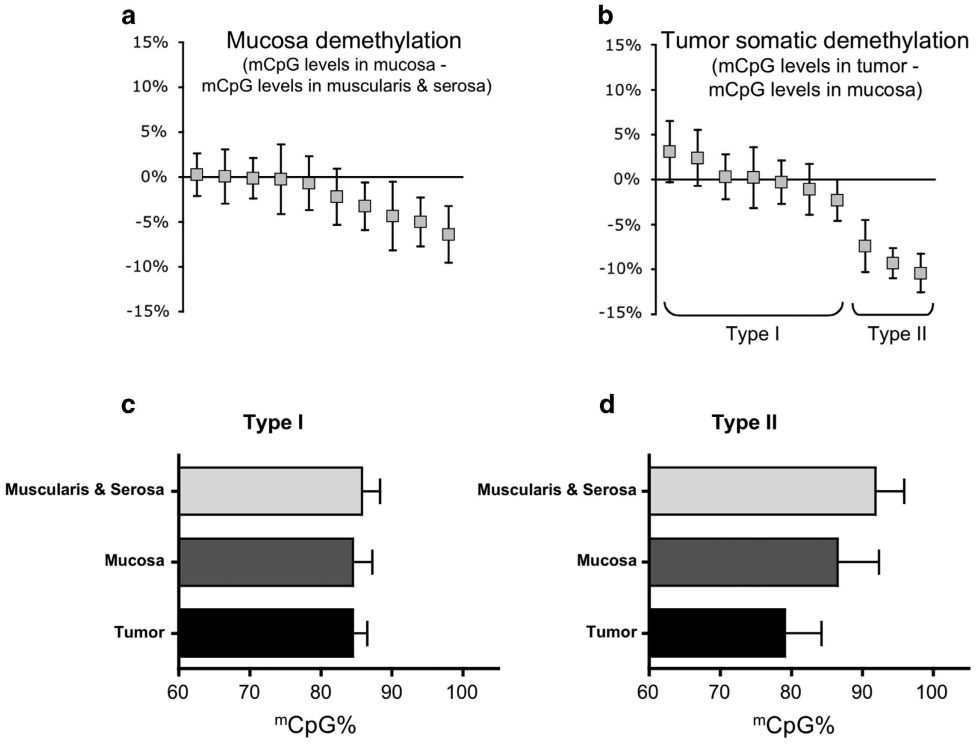

Figure 2 (**a**) Global CpG methylation differences between the mucosa cells and the serosa and muscularis cells from non-tumor gastric tissues (mucosa demethylation) and (**b**) between tumor cells and gastric mucosa cells (tumor somatic demethylation) in ten samples from gastric cancer patients. Error bars indicate the standard errors of the difference. Mucosa demethylation occurs gradually but tumor somatic demethylation dramatically increases in a subset of tumors (type II). (**c** and **d**) Average global CpG methylation levels in the transmural layers of the non-tumor tissues and primary tumors from patients without (**c**, Type I, $n=7$) and with enhanced somatic hypomethylation (**d**, Type II, $n=3$). Error bars represent the standard deviation.

the muscularis and serosa layers in any of the cases (not shown). Therefore, the data from these two layers was averaged as a single value of non-mucosa methylation. Methylation was frequently lower in the mucosa than in the other two layers. This difference might reflect the higher number of mitoses undergone by these cells.[11] The distribution of the differences in methylation between mucosa and non-mucosa layers was gradual, with no evident discontinuity (Figure 2a).

A subset of gastric cancers exhibit enhanced somatic hypomethylation (ESH). The relative differences of ^{m}CpG% between tumor and mucosa cells in the ten cases selected for methylation analysis showed a non-gradual distribution. Thus, 3 out of 10 cases seemed to have an increased tumor-specific hypomethylation (Figure 2b). Despite the limited number of samples, this suggested the existence of two groups of tumors according to their somatic hypomethylation level. One group (type I), with slightly lower ^{m}CpG% than the surrounding mucosa (Figure 2c), and another group (type II), that exhibited a more pronounced demethylation (Figure 2d). We termed this enhanced somatic hypomethylation (ESH). Based on these ten cases, we tentatively defined an ESH threshold of 9.4% less methylation in CpG sites in tumor relative to the matched normal tissue (see Materials and Methods).

The comparison of ^{m}CpG% between normal and tumor tissues from the whole set of 94 GC cases revealed a significant correlation ($R^2=0.401$, $P=7.4\times10^{-12}$, Figure 3). The majority of cancers (83.0%) exhibited a ^{m}CpG content only slightly lower (2.5 ± 3.7% difference) than their matched normal tissue samples. This indicates that, in most cases, the methylation drop of the tumor was superposed to the preexisting hypomethylation of the correponding non-tumor tissue. However, there was a subgroup of 16 tumors (17%) that exhibited methylation levels below the proposed ESH threshold (Figure 3).

Ranking the cases according to the difference of ^{m}CpG% between non-tumor and matched tumor tissue revealed the existence of two groups of tumors with different slopes (Figure 4). Notably, the apparent inflexion point of the distribution graph was almost coincident with the proposed ESH threshold. This suggests that demethylation in tumors was gradual, mainly reflecting that of the mucosa, but in some cases the demethylation process accelerated before and/or during tumor progression.

Gastric cancers with ESH present a more aggressive tumor phenotype. Based on these observations, we classified GC into types I and II according to the degree of somatic hypomethylation. Both type I and II cancers exhibited AGH, but only type II cases exhibited ESH (Figure 4). Type II tumors displayed a more aggressive phenotype than type I tumors, i.e. more advanced stage, and invasion through the gastric layers and into the adjacent lymph nodes (Table 3).

DISCUSSION

Genome-wide hypomethylation is a frequent somatic epigenetic alteration in cancer cells.[12] In a previous study we analyzed methylation alterations in gastrointestinal cancers, and found that hypomethylation preceded diploidy loss in a significant subset of gastrointestinal cancers, and had a stronger association with genetic damage and poorer prognosis than hypermethylation.[21] In the present work, we focused on genome-wide hypomethylation in GC and HRG to explore the occurrence of epigenetic alterations in the early stages of GC development.

DNA hypomethylation in pre-cancerous gastric lesions, in particular chronic gastritis patients, have been already explored.[25,38,39] Yoshida *et al.*,[20] described gradual hypomethylation of Alu and Sat-α repetitive sequences in the antral region of the gastric mucosa, correlating with the levels of

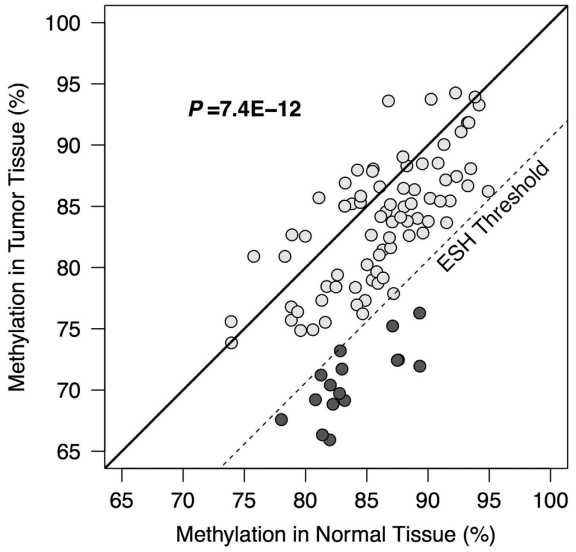

Figure 3 ^{m}CpG% in tumors (y-axis) vs. matching non-tumor tissues (x-axis). The diagonal solid line represents the no-change line, i.e. same mCpG% for tumor and non-tumor tissue. The dashed diagonal line indicates the enhanced somatic hypomethylation threshold (ESH). In dark grey, cases with somatic demethylation below this threshold (Type II tumors).

Table 3 Association of enhanced somatic hypomethylation (ESH) with advanced GC

TNM	Type I	Type II	*P*-value[a]
T2	8	0	**0.0047 (0.0029)**
T3	17	0	
T4 (a or b)	26	10	
N0	15	1	**0.025 (0.034)**
N1	10	1	
N2	16	2	
N3	10	5	
M0	48	8	0.07 (0.18)
M1	3	2	
Stage			
I	5	0	**0.017 (0.024)**
II	16	1	
III	27	7	
IV	3	2	

[a]One-sided *P*-value of Cochran-Armitage test. Since data in some cells were low for Chi-square test, *P*-values of Monte Carlo simulations with 10,000 iterations were also calculated (in parenthesis). In bold type, *P*-values < 0.05.

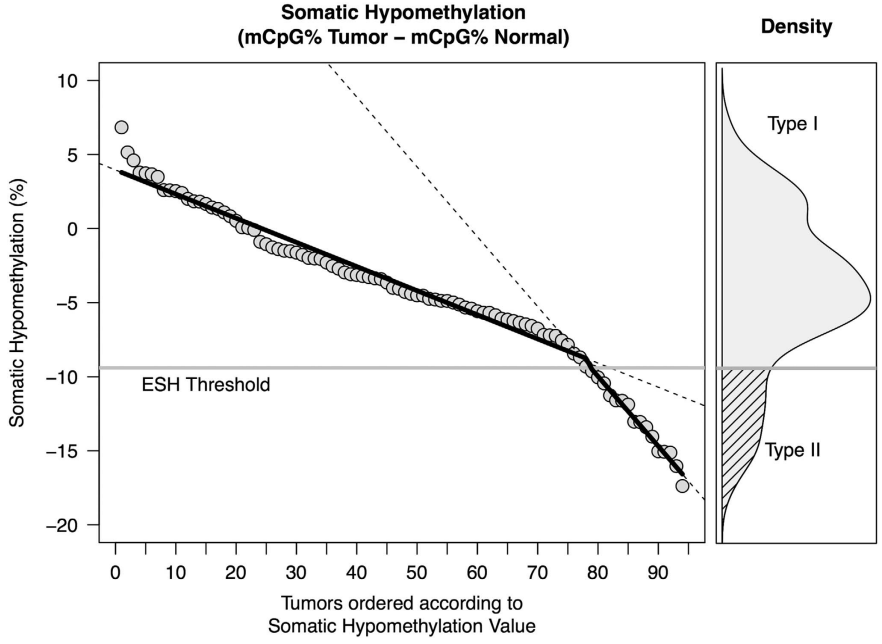

Figure 4 Left: Global CpG methylation difference between tumor and non-tumor samples from 94 gastric cancer patients, ordered according to the magnitude of the difference. The horizontal grey line indicates the proposed severe somatic hypomethylation threshold (−9.4%). The slopes of the tumors above and below the ESH threshold are shown with dashed lines. The solid black line indicates the predicted values of somatic hypomethylation using lineal regression modeling. Right: density of the somatic demethylation, with the ESH threshold indicated by the horizontal grey line, and the proportion of Type II tumors shaded.

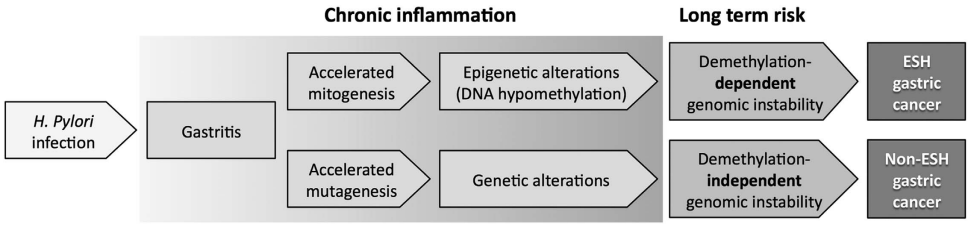

Figure 5 Schematic model of the proposed *H. pylori*-associated DNA demethylation gastric carcinogenic pathway. In this model, *H. pylori* infection causes chronic inflammation. During the inflammation process, epithelial cells are subjected to an increased mitotic rate and the mutagenic effect of the reactive oxygen and nitrogen species, leading to the accumulation of epigenetic and genetic lesions, respectively. Antibiotic treatment effectively eradicates the bacterium alleviating the chronic inflammation, but does not revert the lesions. Genome-wide demethylation impairs proper mitosis, causing the cells to increasingly accumulate genomic damage. In the long term, this genomic instability can contribute to tumorigenesis. (Pre)neoplastic lesions (i.e. intestinal metaplasia) may develop into non-ESH cancers mainly due to the somatic genetic alterations (i.e. oncogenes and tumor suppressors). The pre-existing background demethylation undergone by the precursor cells (AGH) may or may not contribute to their genomic instability. In addition, some cancers exhibit an enhanced demethylation (ESH tumors), due to the accelerated demethylation undergone by the precursor tumor cells during inflammation. This may associate to higher levels of genomic instability, and may be reflected in a more aggressive phenotype. The diagram is simplified and does not depict the overlapping between the two pathways.

H. pylori antibodies in serum.[25] Compare *et al.* analyzed changes in total methylcytosine content by immunostaining, concluding that demethylation is an early event in the gastritis-cancer pathway. In 10 patients with preneoplastic lesions that they analyzed, the levels of DNA methylation decreased over time despite the eradication treatment.[39] Differences in methylation of several tumor-related genes and repetitive elements, in non-neoplastic tissue of GC patients and patients with advanced stages of gastritis compared with healthy controls have been also recently described.[40,41]

In contrast with these previous studies that restricted the data to individual loci or a subset of CpG sites, such as CpG islands or repetitive sequences, we employed an analytical method that measures methylation changes in all genomic CpG sites. This approach provides an estimation of the absolute levels of methylation in comparative analyses of intra- and inter-individual experimental data points. We found significant genome-wide somatic DNA hypomethylation in primary gastric cancers from American and German patients. This highlights one of the original findings of our work because it generalizes the phenomenon to Caucasians from two Continents.

Our results differ from those published by Yoshida *et al.*,[20] who found no difference in the global 5-methylcytosine content in gastric mucosa regardless of *H. pylori* infection and presence or absence of GC. Yoshida *et al.* used another technique (liquid chromatography coupled to mass spectrometry) to estimate global genome methylation. However, our approach is clearly superior in its sensitivity to detect drops of methylation levels because it measures *demethylation* events (unmethylated CpG sites) instead of methylation events (Supplementary Figure 2).

The proportion of patients with hypomethylation below the proposed AGH was very similar in patients with high-risk gastritis (13/38, 34.2%) and in patients with gastric cancer (32/94, 34.0%) (Figure 1). Moreover, there was no detectable differences in methylation between HRG patients with and without active *H. pylori* infection, which suggests that demethylation is irreversible once it has taken place. This hypothesis is in agreement with evidence from a previous clinical trial that concluded that *H. pylori* eradication in patients with advanced changes in gastric mucosa was not able to prevent the development of GC.[42] These data together suggest a putative mechanistic link between *H. pylori* infection and long-term risk for GC after bacterial eradication, through global and irreversible DNA hypomethylation.

Another original observation in this study is that first-degree relatives of GC patients have a low incidence of AGH (Figure 1). This suggests that inherited genetic factors have a weak effect in the demethylation process, although this is open for further investigation. In contrast, at least one third of the patients with corpus-predominant or pangastritis, with or without intestinal metaplasia, exhibit AGH (Figure 1). The hypomethylation in patients with active gastritis and therefore with inflammatory infiltrate was independent of the activity, grade or pattern of gastritis (Supplementary Table 2), in accordance with previous observations.[43] Hence, it is improbable that the differences in methylation reflect differences in the proportion of inflammatory cells within the sample. In support of this conclusion is the novel data on the levels of methylation in the different tissue layers of the stomach.

In this study, we recruited gastric mucosa samples from healthy donors, relatives and HRG patients by gastroscopy, whereas the non-tumor samples from GC patients were whole transmural surgical resections including mucosa, muscularis and serosa layers. We investigated the possible bias due to the presence of non-epithelial cells by comparing the methylation levels of these layers in a subset of 10 gastric cancer patients. Most cases exhibited lower levels in the mucosa layer (Figure 2a). This experiment indicated that (a) hypomethylation preferentially occurs in the mucosa cells of the stomach and accumulates in a gradual manner and (b) the estimated ^{m}CpG% value of non-tumor samples is not significantly affected by the presence of the muscularis and serosa derived cells. And in case of gross contamination with non-mucosa cells, it would result in underestimation, not in overestimation, of the extent of hypomethylation.

Based on these observations, we propose that DNA hypomethylation may contribute to the initial stages of the carcinogenesis process at least in some gastric cancers (Figure 5). Several additional facts support this hypothesis: (i) the association between DNA hypomethylation and aneuploidy has been confirmed in other reports;[12,13,15] (ii) it has been demonstrated that hypomethylation, associated with

chromosomal alterations and instability, is sufficient to induce tumors in animal models;[16,44] and (iii) we have shown that global DNA hypomethylation precedes and correlates with genomic damage in gastrointestinal cancer.[21]

Our results also provide further support to the concept of an epigenetic field defect for cancerization in gastric cancer, associated with *H. pylori* infection (reviewed in Ushijima *et al.*).[45] The exact mechanism linking *H. pylori* infection and aberrant DNA methylation is still mainly unknown. In addition to the suggested role of inflammatory damage in signaling induced by interleukin 1ß,[46] and the link between E-cadherin methylation and *H. pylori* infection,[47] another possible explanation for the link between inflammation due to *H. pylori* infection and cancer may be through the increased mitotic activity leading to an accelerated hypomethylation rate[48] (Figure 5).

We found a strong correlation between the methylation in tumors and matching non-tumors samples, where most of the tumors analyzed (72%) exhibited slightly lower levels than their normal tissue counterparts ($-2.37 \pm 3.7\%$, Figure 3). The simplest interpretation is that in these cases the tumor methylation reflects that of the surrounding mucosa cells, and that the increased number of cell replications during the tumor clonal expansion underlies the small increase in demethylation.[48,49] Interestingly, a subset of tumors (18%) had significant lower levels of global genomic methylcytosine compared to their matching normal mucosa (ESH, Figures 3 and 4). This is one of the novel findings of our study and the main implication is that gastric cancer of the intestinal type can follow at least two pathways, one of which is more influenced by hypomethylation than the other. We do not have data on genetic differences between these two types of tumors, but we found important phenotypic differences, i.e. higher invasiveness and more advanced stage (Table 3), suggesting underlying differences in genotype. ESH may be explained by an augmented demethylation frequency during tumor progression, or it might reflect a particularly low level of methylation already present in the precursor cell that originated the tumor, or a combination of both phenomena. In any case, the results may be useful for clinical classification since ESH tumors associated with a more aggressive phenotype, i.e. more advanced stage, invasion through the gastric wall and into adjacent lymph nodes (Table 3). This sets the stage for potential diagnostic and prognostic tests for gastric cancer based on the determination of global DNA demethylation by second-generation less or non-invasive procedures, for instance by using liquid biopsies.

In summary, the occurrence of genome-wide hypomethylation in the non-neoplastic tissue of patients with gastritis and gastric cancer further exposes the fundamental role of epigenetic alterations in the early steps of carcinogenesis. Our data provide an additional mechanistic insight over the series of events involved in the process of *H. pylori*-associated GC, through the predicted subsequent genome damage and aneuploidy triggered by DNA global hypomethylation, whether gradual (AGH) or enhanced (ESH). Whether the demethylation may be stochastic or linked to some predisposition by some individuals in the population, that may eventually yield predictive biomarkers, is the subject for further investigation.

CONFLICT OF INTEREST

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Specific author contributions: Study design: A. Leodoloter, S. Alonso, and M. Perucho; Patient recruitment: A. Leodolter, M. PA. Ebert, M. Vieth, C. Roecken, T. Wex, U. Peitz, P. Malfertheiner, and M. Perucho; Sample preparation: A. Leodolter, M. PA. Ebert, M. Vieth, and S. Alonso; Data acquisition: A. Leodoloter and S. Alonso.; Data analysis and interpretation: A. Leodolter, S. Alonso, B. González, and M. Perucho; Manuscript preparation: A. Leodolter, S. Alonso, B. González, and M. Perucho. All the above-mentioned authors have approved the final draft submitted.
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Potential competing interests: None.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ *Helicobacter pylori* (*H. pylori*) infection-related gastritis constitutes one of the major risk factors for the development of gastric cancer.
- ✓ Genetic and epigenetic alterations are germane to gastric cancer etiology and progression.
- ✓ Epigenetic alterations affect not only the tumor but also the non-tumor gastric mucosa of gastric cancer patients, suggesting the existence of an epigenetic field defect affecting the mucosa cells and contributing to the long-term risk to develop GC.
- ✓ Studies primarily conducted on Asian populations showed that *H. pylori* infection associates with DNA methylation alterations in gastric mucosa. Several genes have been found to undergo hypermethylation, and a number of repetitive elements have been found to undergo hypomethylation, upon *H. pylori* infection.

WHAT IS NEW HERE

- ✓ Global DNA demethylation was studied with a biochemical method that provides absolute measurements of the drop in methylated CpG sites, more sensitive for estimation of demethylation than previous approaches.
- ✓ DNA demethylation of cancer and non-cancerous gastric mucosa occurs in GC patients from American and European populations with no distinguishable differences in incidence or degree.
- ✓ Genome-wide hypomethylation occurs in the mucosa, with less impact in the serosa or muscularis layers, possibly due to the mucosa higher mitotic activity.
- ✓ The demethylation present in the mucosa of gastric cancer patients is significantly greater than that of GERD patients (already reported) and also cancer-free first-degree relatives of GC patients (our original data). This suggests that the underlying defect has no clear familial component.
- ✓ A subset of tumors (18%) exhibits a more pronounced reduction of somatic DNA methylation that associates with a more aggressive phenotype. These tumors might possess a distinct cancer etiology, with different response to treatment.

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Supplementary Information accompanies this paper on the Clinical and Translational Gastroenterology website (http://www.nature.com/ctg)