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Blood leukocyte DNA hypomethylation and gastric cancer risk in a high-risk Polish population

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Abstract

Global hypomethylation has been shown to increase genome instability potentially leading to increased cancer risk. We determined whether global methylation in blood leukocyte DNA was associated with gastric cancer in a population-based study on 302 gastric cancer cases and 421 age- and sex-matched controls in Warsaw, Poland, between 1994 and 1996. Using PCR-pyrosequencing, we analyzed methylation levels of Alu and LINE-1, 2 CG-rich repetitive elements, to measure global methylation levels. Gastric cancer risk was highest among those with lowest level of methylation in either Alu (OR = 1.3, 95% CI = 0.9–1.9) or LINE-1 (OR = 1.4, 95% CI = 0.9–2.0) relative to those with the highest levels, although the trends were not statistically significant. For Alu, the association was stronger among those aged 70 or older (OR = 2.6, 95% CI = 1.3–5.5, *p* for interaction = 0.02). We did not observe meaningful differences in the associations by other risk factors and polymorphisms examined. For LINE-1, the association tended to be stronger among individuals with a family history of cancer (OR = 3.1, 95% CI = 1.4–7.0, *p* for interaction = 0.01), current alcohol drinkers (OR = 1.9, 95% CI = 1.0–3.6, *p* for interaction = 0.05), current smokers (OR = 2.3, 95% CI = 1.1–4.6, *p* for interaction = 0.02), those who rarely or never consumed fruit (OR = 3.1, 95% CI = 1.2–8.1, *p* for interaction = 0.03), CC carriers for the *MTRR* Ex5+123C>T polymorphism (OR = 2.3, 95% CI = 1.2–4.4, *p* for interaction = 0.01) and TT carriers for the *MTRR* Ex15+572T>C polymorphism (OR = 1.7, 95% CI = 1.0–2.8, *p* for interaction = 0.06). The association was not different by sex, *Helicobacter pylori* infection, intake of folate, vitamin B6 and total protein and the remaining polymorphisms examined. Our results indicate that interactions between blood leukocyte DNA hypomethylation and host characteristics may determine gastric cancer risk.

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Keywords

gastric cancer; methylation; global hypomethylation; gastric cancer risk

Gastric cancer is one of the most common malignancies worldwide and remains a leading cause of cancer death in Asia and some European countries.[1] A reduced level of genomic methylation content (also referred to as global DNA methylation) has been shown to increase genome instability and mutation rates, thus potentially leading to an increase in cancer risk. Mice with global hypomethylation showed a tendency to develop multiple malignancies.[2] Many types of cancer cells and premalignant adenomas have been found to exhibit a reduced level of CpG sequence methylation globally.[3–5] It is estimated that more than one-third of DNA methylation occurs in repetitive elements in the human genome. There are ~1.4 million Alu repetitive elements and a half-million long interspersed nucleotide elements (LINE-1 elements) that are normally methylated in the human genome.[6] Each Alu element is ~300 base pairs long. LINE-1 elements are remnants of reverse transcription inserted into genomic DNA at new locations and can be up to 6 kb long, accounting for up to a third of the genome.[7] Thus, methylation of repetitive elements throughout the human genome has been shown to be a major contributor to total genomic DNA methylation.[6] In cancer cells, chromosomal instability is thought to be initiated by global hypomethylation of the genome with subsequent hypermethylation of CpG sequences in the promoter region of tumor suppressor genes.[8]

Unlike tumor DNA, leukocyte DNA can be obtained non-invasively and relatively inexpensively. Recently, measures of DNA methylation in surrogate tissues, such as leukocyte DNA, have been used as a marker of potential cancer risk. For example, one study reported that head and neck squamous cell carcinoma cases had significant lower levels of global methylation in whole blood DNA relative to cancer-free controls.[9] Another recent study examining peripheral leukocyte DNA hypomethylation in a large hospital-based case–control study of bladder cancer also demonstrated a significant association.[10] In addition, a number of gene promoter methylation patterns detected in genomic DNA were found to be highly correlated with patterns detected in tumor tissues, suggesting the important cancer predictive value of DNA methylation markers.[11–18]

In this study, we determined whether levels of genomic DNA methylation in peripheral leukocyte DNA were associated with gastric cancer risk in a population-based case–control study in Poland, which has one of the highest gastric cancer incidences in the world.[1] We also examined potential effect modification and confounding by factors that are important either to DNA methylation, such as intake of folate and protein, and/or to gastric cancer etiology, such as smoking and *Helicobacter pylori* (*H. pylori*) carriage.

Material and Methods

Study population

Cases and controls were part of a population-based study of gastric cancer conducted in Warsaw, Poland; the methods of this study have been described in detail.[19] Gastric cancer cases were Caucasian residents of Warsaw, ages 21–79 years, who were newly diagnosed with gastric cancer (ICD-9 151) during 1994–1996 and identified by physicians in each of the 22 hospitals in the study area. All cases were confirmed by study pathologists. Controls were randomly selected from a computerized registry of Warsaw residents and frequency-matched to cases by gender and 5-year age groups. The study was approved by the Institutional Review Boards of the U.S. National Cancer Institute and the M. Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Warsaw, Poland. Information on

adjuvant treatment was collected by asking if the patients had undergone radiation or chemotherapy before the blood draw. Written informed consent was obtained from all participants. An ever-smoker was defined as a smoker of at least 1 cigarette per day for 6 months or longer. An ever-drinker was defined as a person drinking at least 1 serving of beer (12 oz), wine (4 oz) or liquor (1.5 oz) per month for 6 months or longer. Among smokers and drinkers, information was collected on the age when exposure to each product started and stopped, and total years and frequency of use. Pack-years of smoking were calculated as the product of packs of cigarettes smoked per day and total years of smoking. Total drink-years were calculated as the product of yearly frequency and total years of alcohol use.

Information on dietary intake was collected as previously described.[20] Briefly, usual frequency of intake of 118 food and beverage items was obtained. Nutrient intake was estimated from the weekly consumption of food items, the average portion size and the nutrient composition of each food item. Then, total intake of each nutrient was summed across all food items.[20] The following tertile cutoff points were used ≤0.6, >0.6–0.7 and >0.7 μg/day for dietary vitamin B6 intake and ≤91.8, 91.9–119.6 and >119.6 g/day for total protein intake. The tertile cutoff points for folate intake were 93.2–268.1, 268.2–342.4 and 342.5–678.5 μg/day.

Of 464 cases and 480 controls who provided questionnaire information through in-person or proxy-interviews, a 30-mL blood sample was obtained from 345 (74.4%) cases and 442 (92.1%) controls. Tumor samples were collected, but the quality and quantity of the DNA were inadequate for molecular studies as previously described.[21] Genomic DNA was obtained from peripheral blood leukocytes as previously described.[22] Serum levels of IgG antibodies to *H. pylori* and to the cagA protein were determined by antigen-specific ELISA as described previously.[23]

DNA methylation measurement

We used highly quantitative pyrosequencing assays to test the DNA methylation levels of Alu and LINE-1 throughout the genome. First, 1 μg DNA (concentration 50 ng/μl) was treated using EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA) according to the manufacturer's protocol. Final elution was performed with 30 μl of M-Elution Buffer. Bisulfite-treated DNA was stored at −20°C and used shortly after treatment. DNA methylation was quantitated using bisulfite-PCR and Pyrosequencing.[6] The PCR primers were designed toward a consensus Alu or LINE-1 sequence and allowed the amplification of a representative pool of repetitive elements to serve as a surrogate for global DNA methylation changes, as previously described.[24] Analysis of DNA methylation in Alu and LINE-1 repetitive elements was performed using previously published methods.[6,25,26] Briefly, for each reaction, a 50-μl PCR was carried out in 50 μl of GoTaq Green Master mix (Promega, Madison, WI), 1 pmol of the forward primer, 1 pmol of the reverse primer, 50 ng of bisulfite-treated genomic DNA and water. One of the primers was biotin-labeled and used to purify the final PCR product using Sepharose beads. The PCR product was bound to Streptavidin Sepharose HP (Amersham Biosciences, Uppsala, Sweden), and the Sepharose beads containing the immobilized PCR product were purified, washed, denatured using a 0.2 M NaOH solution and washed again using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing, Westborough, MA), as recommended by the manufacturer. Then, 0.3-μM pyrosequencing primer was annealed to the purified single-stranded PCR product, and pyrosequencing was performed using the PSQ HS 96 Pyrosequencing System (Pyrosequencing). Methylation quantification was performed using the provided software. The degree of methylation was expressed as % 5-methylated cytosines (%5 mC) over the sum of methylated and unmethylated cytosines. We used built-in controls to verify bisulfite conversion efficiency. Each Pyrosequencing plate included controls containing water, annealing solution, binding solution or PCR product. In addition, a standard oligonucleotide

was used for assessing sequencing accuracy. All assays were run in duplicate to measure interrun variability and reduce measurement error. The average of the two duplicates was used in the statistical analysis. In addition, we also interspersed 5% blind duplicate samples within the study samples to evaluate intrasample variability. Analytical variability [coefficient of variation (CV) calculated in duplicate runs] was 0.7% for the LINE-1 methylation assay and 1.6% for the Alu methylation assay.

Statistical Analysis

Linear regression models were used to evaluate differences in methylation level (continuous dependent variable in the models) among controls in relation to age at blood draw, gender, *H. pylori* infection status, family history of cancer and other oxidative stress-related factors, including smoking, alcohol drinking and fruit and vegetable consumption, and folate, vitamin B6 and total protein intakes. We categorized DNA methylation variables in tertiles, using cut-points of methylation distribution among controls. The highest tertile of methylation was used as the reference in estimating odds ratios. We evaluated the association of gastric cancer with Alu or LINE-1 methylation categories using unconditional logistic regression models adjusted for age, sex, educational levels, smoking (never, former and current, or pack-year) and alcohol (non drinker, former drinker or current drinker). In addition, we also ran a second set of models in which Alu or LINE-1 methylation was fitted as continuous variables. To allow for comparison of the size effects of Alu and LINE-1 methylation, the results from this second set of models were reported as odds ratios, estimating the relative odds of gastric cancer associated with a decrease from the 90th to the 10th percentile of Alu or LINE-1 methylation. This variable was also used in the effect-modification analyses. Further adjustment by other potential confounding variables, including body mass index, intake of fruits, vegetables, sausages, red meats or preserved vegetables; intake of folate, vitamin B6 and total protein; family history of gastric cancer or other cancers and *H. pylori* infection status, did not alter the risk estimates. Therefore, these variables were not included in the final models. We tested for modification effects of several gastric cancer risk factors on the associations of Alu or LINE-1 methylation levels with gastric cancer risk using likelihood-ratio tests. We also tested for effect modification of 10 genetic polymorphisms in 3 key folate-metabolizing genes (*MTHFR*, *MTR* and *MTRR*) previously studied in this same population.[27] These polymorphisms included *MTHFR* Ex5+79C>T (C677T; rs1801133) and Ex8−62A>C (A1298C; rs1801132), *MTR* Ex26−20A>G (A2756G; rs1805087), *MTRR* Ex2−64A>G (A66G; rs1801394), Ex5+123C>T (rs1532268), Ex15+572C>T (rs9282787), Ex15−405A>T (rs8659), Ex9−85C>T (rs2287780), Ex15−526G>A (rs9332) and Ex14+14C>T (rs10380) and were genotyped at the Core Genotyping Facility (CGF) at the National Cancer Institute.[28] In addition, we assessed the relationship of Alu or LINE-1 methylation with pathological features. We conducted sensitivity analysis by excluding patients who received chemotherapy before the blood draw. All tests were 2-sided, and an alpha level of less than 0.05 was considered significant. All statistical analyses were performed by Stata 10.1 software (StataCorp. Stata Statistical Software: Release 10.0. College Station, TX: StataCorp LP).

Results

The characteristics of the 302 gastric cancer cases and 421 age- and gender-matched controls are shown in Table 1. Cases had lower educational levels than controls, with 47.3% of cases and 37.1% of controls had high school or less education. A family history of gastric cancer was present in 12.4% of cases compared to 4.3% of controls. Compared to the controls, cases were more likely to be current smokers (41.0 *vs*. 28.0%) but less likely to be current alcohol drinkers (31.5 *vs*. 52.5%) and less frequently consumed vegetables (49.3 *vs*.

60.2%). There were not significant differences between cases and controls in dietary intake of folate, vitamin B6 or total protein. Among controls, age was inversely associated with Alu methylation levels ($p = 0.02$) but not with LINE1 methylation. We did not observe meaningful associations between other risk factors examined and DNA methylation level in either Alu or LINE1 among controls (data not shown). There was no significant correlation between Alu and LINE-1 methylation among controls (Pearson's coefficient = 0.06, $p = 0.11$).

Gastric cancer risk increased with decreasing levels of methylation in Alu (p-trend = 0.19) or LINE-1 (p-trend = 0.12) (Table 2). An elevated risk of borderline significance was observed among those with the lowest tertile of methylation in either Alu (OR = 1.3, 95% CI = 0.9–1.9) or LINE-1 (OR = 1.4, 95% CI = 0.9–2.0) relative to those with the highest tertile. Alternatively, using DNA methylation measures as continuous variables, we estimated an OR = 1.1 (95% CI 0.8–1.7) for decreasing levels of Alu methylation, and an OR = 1.0 (95% CI 0.7–1.5) for decreasing levels of LINE-1 methylation (OR estimates are scaled to express the risk associated with a decrease in methylation from the 90th to the 10th centile). In addition, the use of other quantiles (*i.e.*, median, quartiles and quintiles), arbitrary cutoffs (top 20%, top 10%, top 5%, bottom 20%, bottom 10% and bottom 5%) or transformations (log or square) did not yield any results that suggested a relation between DNA methylation and relative odds of cancer different from the linear relation.

We further evaluated the effect modification of potential and known risk factors using methylation as a continuous variable (Table 3). For the association with Alu methylation, we did not observe a consistent pattern of effect modification by other gastric cancer risk factors, except for a significantly elevated risk among those aged 70 or older (OR = 2.6, 95% CI = 1.3–5.5, p for interaction = 0.02). For LINE-1, the magnitude of gastric cancer risk associated with methylation level tended to be stronger among individuals with other cancer family history (OR = 3.1, 95% CI = 1.4–7.0, p for interaction = 0.01), current alcohol drinkers (OR = 1.9, 95% CI = 1.0–3.6, p for interaction = 0.05), current smokers (OR = 2.3, 95% CI = 1.1–4.6, p for interactions = 0.02), smokers who started smoking younger than age 19 (OR = 2.3, 95% CI = 1.1–4.5, p for interaction = 0.1) or persons with lower intake of fruits (OR = 3.1, 95% CI = 1.2–8.1, p for interaction = 0.03) or vegetables (OR = 3.7, 95% CI = 1.0–12.9, p for interaction = 0.09).

Among 10 polymorphisms in folate metabolizing genes examined, we found no consistent pattern of interaction with Alu methylation in gastric cancer risk (data not shown). For the association with LINE-1 methylation, we observed a significant interaction with 2 polymorphisms in the *MTRR* gene. Gastric cancer risk was significantly higher among carriers of the CC allele in polymorphism Ex5+123C>T (OR = 2.3, 95% CI = 1.2–4.4) *versus* CT and TT carriers (OR = 0.8, 95% CI = 0.5–1.4, p for interaction = 0.01) and higher with borderline significance (OR = 1.7, 95% CI = 1.0–2.8) among carriers of the TT allele in polymorphism Ex15+572T>C *versus* TC and CC carriers (OR = 0.7, 95% CI = 0.4–1.4, p for interaction = 0.06) (data not shown).

DNA methylation did not vary significantly by Lauren pathologic classification ($p = 0.82$ for Alu and $p = 0.54$ for LINE-1) or tumor localization ($p = 0.34$ for Alu and $p = 0.33$ for LINE-1) (Table 4), although the number of patients with diffuse Lauren classification ($n = 50$) and cardia localization ($n = 35$) were small. In addition, excluding patients who received chemotherapy before the blood draw ($n = 42$) produced no meaningful difference in the results, with no changes in statistical significance.

Discussion

This population-based case–control study showed inverse associations between gastric cancer risk and levels of global methylation in both Alu and LINE-1 repetitive elements. These associations were modified by certain gastric cancer risk factors including age, tobacco and alcohol use and low intake of fruits and vegetables. Two polymorphic variants in a folate metabolism gene, *MTRR*, also appeared to modify the association with LINE-1 global methylation level.

Global hypomethylation has been found in tumor tissues and considered one of the molecular features of malignancies.[29–31] Several etiologies have been proposed, including abnormal cellular proliferation and deficiency of methyl donors due to uncontrolled cell growth in tumor tissues. However, loss of genomic DNA methylation content in the surrogate nontumor tissues has not been well studied. Recently, 2 studies examined genomic hypomethylation of peripheral blood leukocyte DNA. Hypomethylation of LRE1, a LINE-1 repeat that has been used as an indicator of global hypomethylation, was associated with an increased risk of head and neck squamous cell cancer.[9] Similarly, Moore *et al*. demonstrated a significant inverse association between total cytosine methylation content of peripheral leukocyte DNA and bladder cancer risk.[10] Both of these studies provide evidence that methylation of leukocyte DNA may be a potential biomarker of select cancer risk.

A number of hypotheses have been put forward about how global hypomethylation may increase cancer risk.[29–31] Direct reactivation of transposable repetitive elements, such as LINE-1 and Alu, has been shown to be activated by hypomethylation and commonly has adverse effects such as functional repression through insertion into transcribed sequences. Hypomethylation may also represent a loss of imprinting in the genome that causes activation of a wide spectrum of genes that convey various growth advantages, possibly including oncogenes. Loss of methylation also affects gene expression and genome stability that favors proliferation and differentiation, leading to neoplastic transformation. Alternatively, hypomethylation may be an epigenetic phenomenon associated with genomic alterations common to neoplasia.[32]

Deficiency of methyl donors has been thought to be one of the major causes for loss of DNA methylation. In both experimental animals and human studies, deficiencies in methyl group availability have been linked to cancers.[33] Cellular S-adenosyl-L-methionine (SAM) is the universal methyl donor to cytosine. SAM levels are dependent on a sufficient supply of methyl groups from both dietary methionine-rich food (protein-rich and low fat food) and methionine recycled from homocysteine. This recycling requires several vitamins, particularly folic acid, B6 and B12, which are rich in fruits and vegetables.[34,35] Smoking and alcohol use have also been previously shown to cause global genomic hypomethylation through their effects on vitamin B12 pathway.[36,37]

In this study, the association between gastric cancer and hypomethylation of LINE-1 was more pronounced among current smokers or smokers who started smoking when they were young (between age 11 and 19), current alcohol drinkers and those who had the least fruits, vegetables or vitamin B6 intakes.

For Alu repeats, a more pronounced increased risk was observed among those who were 70 years old or older. The modifying effect of age is interesting from an epigenetic perspective. A recent study showed a significant change in methylation over time in an Icelandic and Utah cohort over a span of 11–16 years.[32] The Utah cohort, in particular, showed statistically significant familial clustering of decreased methylation, indicating these changes occur with age and are likely inheritable. Taken together, our results suggest that individuals

older than 70, smokers, alcohol drinkers and/or subjects on high-risk diets might have lower genetic instability due to repetitive element hypomethylation.

Genetic variations in folate metabolism genes may affect DNA methylation and therefore determine the susceptibility of gastric cancer. In our previous study, we examined several polymorphisms in *MTHFR* and *MTRR* and gastric cancer risk and did not find significant associations or interactions between polymorphisms and dietary folate and alcohol consumption.[27] In this study, we examined 10 polymorphisms in *MTHFR*, *MTRR* and *MTR* in this investigation and observed a significant or borderline significant modifying effect of *MTRR* Ex5+123C>T or Ex15+572T>C polymorphisms on the risk by LINE-1 hypomethylation, suggesting a possible role of folate metabolism genes in the association between global hypomethylation and gastric cancer risk.

The effect modifications by the risk factors and polymorphisms on the associations of gastric cancer risk with Alu and LINE-1 methylation are different, and Alu and LINE-1 methylation levels were not significantly correlated (Pearson's coefficient = 0.06, $p = 0.11$) in this investigation. Differences between LINE-1 and Alu elements in methylation regulation mechanisms and their responses to cellular stressors and environmental exposures may account for the differences.[25,38–42] Although studies using tumor DNA have shown that the LINE-1 and Alu methylation in tumor samples were correlated with each other,[43,44] no significant correlations have been reported, to the best of our knowledge, between LINE-1 and Alu methylation levels in surrounding normal samples as well as in nontarget tissues, such as blood leukocytes.[45] In addition, growing evidence has also shown that Alu and LINE-1 have distinct functional roles that may participate in cancer development and progression.[25,40,42] Therefore, our results provide further indirect evidence that LINE-1 and Alu are independent of each other and might have distinct functional roles in nonmalignant tissues.

There were no statistical differences in the hypomethylation levels in pathological features. Therefore, our findings suggest that the loss of LINE-1 and Alu methylation may occur more significantly at the early onset of carcinogenesis and play a critical role in tumor initiation, but not in the disease progression. It is also possible that the small number in the subgroups hindered us from detecting the associations.

This is the first study evaluating peripheral leukocyte DNA global hypomethylation and gastric cancer risk. Our study has the advantage of relatively high participation rates and being population-based. Misclassification in DNA methylation was minimal owing to using a highly quantitative method, *i.e*., pyrosequencing, to measure methylation levels and the high reproducibility and accuracy of DNA methylation measurements.

There are also limitations in our study. Because the blood samples were collected after cancer diagnosis, we cannot rule out the possibility that our observations in cases are the results of gastric cancer development *per se*. Other concomitant chronic conditions, such as hypertension, diabetes or other chronic diseases, could also potentially impact DNA methylation. Another potential limitation is the differential recall between cases and controls, as patients may selectively recall past exposure to potential risk factors.[46] Our previous investigation showed that use of proxy data, which are more prone to reporting bias, yielded negligible changes in gastric cancer risk or in the precision of the estimates for the 13 nutrients and micronutrients that were examined.[20] As blood samples and laboratory analysis were conducted using standardized protocols for both cases and controls and laboratory personnel was blinded to the case status, the main variables of the interest, *i.e*., LINE-1 and Alu methylation levels, could not be affected by differential misclassification.

There is evidence that circulating whole blood DNA is increased in cancer patients, although estimates vary widely.[47] It is thought that both tumor cells and surrounding necrotic stromal and inflammatory cells contribute to this DNA.[47] Because global hypomethylation is a molecular feature of tumor tissues and several studies that have reported the reduced levels of global DNA methylation in gastric cancer tissues,[48–50] it is possible that circulating tumor DNA might have contributed to our results. However, it is worth noting that circulating tumor DNA is found as free DNA in the plasma component of blood. In our study, DNA was extracted from peripheral blood leukocytes, and therefore tumor DNA in our samples can be assumed to be minimal. In addition, we could not measure global methylation levels in gastric tumor tissue DNA because of, as previously mentioned, the poor quality and quantity.[21] In addition, caution needs to be exercised in interpreting these results because the study sample size is limited for interaction tests and stratified analyses.

Our results suggest that blood leukocyte DNA hypomethylation may influence gastric cancer risk. More studies, particularly, longitudinal studies of high-risk population, are warranted for a better understanding of the pathological role of global hypomethylation and its predictive value in cancer prevention.

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Table 1

Characteristics of study subjects

	Cases		Controls	
	No.	**%**	**No.**	**%**
Age group				
≤61	113	37.4	152	36.1
62–69	99	32.8	139	33.0
≥70	90	29.8	130	30.9
Gender				
Male	200	66.2	272	64.6
Female	102	33.8	149	35.4
Educational levels				
≤High school	143	47.3	156	37.1
Some college	102	33.8	145	34.4
≥College	57	18.9	120	28.5
Family history of gastric cancer				
No	177	61.0	276	66.7
Stomach	36	12.4	18	4.3
Other	77	26.6	120	29.0
H. pylori **infection status**[1]				
Negative	51	16.9	64	15.2
Positive	251	83.1	356	84.8
Current alcohol drinking				
No	202	68.5	200	47.5
Yes	93	31.5	221	52.5
Current smoking				
No	177	59.0	303	72.0
Yes	123	41.0	118	28.0
Age smoking started				
Never	87	29.2	169	40.2

	Cases		Controls	
	No.	%	No.	%
20–73	86	28.9	129	30.6
11–19	125	41.9	123	29.2
Pack-years of smoking				
0	87	29.1	169	40.3
0.1–30	90	30.1	132	31.4
>30–162	122	40.8	119	28.3
Fruit intake				
Daily/weekly	131	46.0	203	48.3
Several times/month	102	35.8	140	33.3
Rarely/never	52	18.2	77	18.4
Vegetable intake				
Daily/weekly	141	49.3	251	60.2
Several times/month	110	38.5	126	30.2
Rarely/never	35	12.2	40	9.6
Dietary folate intake (tertile) (μg/day)				
93.2–268.1	67	26.3	134	32.9
268.2–342.4	86	33.9	135	33.2
342.5–678.5	101	39.8	138	33.9
Dietary vitamin B6 intake (tertile) (μg/day)				
≤0.6	96	37.8	134	32.9
>0.6–0.7	87	34.3	135	33.2
>0.7	71	27.9	138	33.9
Total protein intake (tertile) (g/day)				
≤91.8	74	29.1	134	32.9
91.9–119.6	94	37.0	135	33.2
>119.6	86	33.9	138	33.9

[1] Negative, tested negative for both IgG antibodies to *H. pylori* and cagA; positive, tested positive for either IgG antibodies to *H. pylori* or cagA antibody, or both.

Table 2

Gastric cancer risk and global hypomethylation in blood leukocytes

	Cases		Controls				
Methylation	**No.**	**%**	**No.**	**%**	**OR[1]**	**95% CI**	***p*-trend**
Alu (tertile)							
25.7–31.6	96	31.8	153	36.3	1.0	(Reference)	0.19
24.5–<25.7	101	33.4	135	32.1	1.2	0.8–1.8	
20.6–<24.5	105	34.8	133	31.6	1.3	0.9–1.9	
Alu (continuous)[2]	302		421		1.1	0.8–1.7	0.52
LINE-1 (tertile)							
81.7–90.4	96	32.0	145	34.6	1.0	(Reference)	0.12
78.6–<81.7	97	32.3	143	34.1	1.2	0.8–1.8	
67.6–<78.6	107	35.7	131	31.3	1.4	0.9–2.0	
LINE-1 (continuous)[2]	300		419		1.0	0.7–1.5	0.81

[1] Adjusted for age, sex, educational level, smoking (never, former and current, or pack-years) and alcohol (nondrinker, former drinker or current drinker).

[2] Odds ratio and 95% CI estimated from models including Alu or LINE-1 methylation as a continuous variable. The ORs and 95% CIs are scaled to express the risk increase associated with a decrease from the 90th to the 10th centile in Alu or LINE-1 DNA methylation.

Table 3

Gastric cancer risk and global hypomethylation by risk factors

	Cases		Controls		Alu		LINE-1	
	No.	%	No.	%	OR[1]	95% CI	OR[1]	95% CI
Age group[2]								
≤61	113	37.4	152	36.1	0.7	0.3–.3	1.2	0.6–2.4
62–69	99	32.8	139	33.0	1.1	0.5–2.1	1.8	0.9–3.7
≥70	90	29.8	130	30.9	2.6	1.3–5.5	0.8	0.4–1.7
Gender								
Male	200	66.2	272	64.6	1.1	0.7–1.8	1.3	0.8–2.1
Female	102	33.8	149	35.4	1.3	0.7–2.5	1.1	0.5–2.2
Education levels								
≤High school	143	47.3	156	37.1	1.5	0.8–2.8	0.9	0.5–1.7
Some college	102	33.8	145	34.4	1.3	0.7–2.6	1.7	0.8–3.33
≥College	57	18.9	120	28.5	0.6	0.3–1.5	1.2	0.6–2.8
Family history of gastric cancer[3]								
No	177	61.0	276	66.7	1.3	0.8–2.2	0.9	0.5–1.4
Gastric cancer	36	12.4	18	4.3	0.4	0.8–2.1	0.4	0.1–2.3
Other	77	26.6	120	29.0	1.2	0.6–2.4	3.1	1.4–7.0
H. pylori **infection status**[4]								
Negative	51	16.9	64	15.2	2.5	0.8–7.8	1.0	0.4–2.5
Positive	251	83.1	356	84.8	1.0	0.7–1.6	1.3	0.8–2.0

	Cases		Controls		Alu		LINE-1	
	No.	%	No.	%	OR[1]	95% CI	OR[1]	95% CI
Current alcohol drinking[5]								
No	202	68.5	200	47.5	1.3	0.8–2.1	0.9	0.5–1.4
Current drinker	93	31.5	221	52.5	1.0	0.6–2.0	1.9	1.0–3.6
Current smoking[6]								
No	177	59.0	303	72.0	1.1	0.7–1.8	0.9	0.5–1.4
Yes	123	41.0	118	28.0	1.3	0.7–2.6	2.3	1.1–4.6
Age smoking started								
Never	87	29.1	169	40.2	0.9	0.5–1.9	1.0	0.5–2.1
20–73	86	28.9	129	30.6	1.8	0.8–3.8	0.9	0.4–1.8
11–19	125	42.0	123	29.2	1.0	0.6–1.9	2.3	1.1–4.5
Pack-years of smoking								
0	87	29.1	169	40.3	1.0	0.5–1.9	1.0	0.5–2.1
0.1–30	90	30.1	132	31.4	0.9	0.4–1.9	1.1	0.5–2.2
>30–162	122	40.8	119	28.3	1.7	0.9–3.4	1.7	0.9–3.4
Fruit intake[7]								
Daily/weekly	131	46.0	203	48.4	1.2	0.7–2.2	0.7	0.4–1.3
Several times/month	102	35.8	140	33.3	1.0	0.5–2.0	1.3	0.7–2.5
Rarely/never	52	18.2	77	18.3	1.4	0.5–3.8	3.1	1.2–8.1
Vegetables intake								
Daily/weekly	141	49.3	251	60.2	1.2	0.7–2.0	0.9	0.5–1.5

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	Cases		Controls		Alu		LINE-1	
	No.	%	No.	%	OR[1]	95% CI	OR[1]	95% CI
Several times/month	110	38.5	126	30.2	1.1	0.5–2.3	1.4	0.7–2.7
Rarely/never	35	12.2	40	9.6	1.9	0.6–6.1	3.7	1.0–12.9
Dietary folate intake (tertile) (μg/day)								
93.2–268.1	67	26.3	134	32.9	1.5	0.7–3.2	1.2	0.6–2.7
268.2–342.4	86	33.9	135	33.2	1.1	0.5–2.2	1.6	0.8–3.2
342.5–678.5	101	39.8	138	33.9	1.0	0.5–2.0	1.3	0.6–2.6
Dietary vitamin B_6 intake (tertile) (μg/day)								
≤0.6	96	37.8	134	32.9	1.4	0.6–2.8	1.1	0.5–2.4
>0.6–0.7	87	34.2	135	33.2	0.8	0.4–1.7	1.0	0.5–2.1
>0.7	71	28.0	138	33.9	1.3	0.7–2.6	2.1	1.0–4.3
Total protein intake (tertile) (g/day)								
≤91.8	74	29.1	134	32.9	0.8	0.4–1.8	1.4	0.6–3.0
91.9–119.6	94	37.0	135	33.2	1.9	1.0–3.8	1.4	0.7–2.7
>119.6	86	33.9	138	33.9	0.7	0.3–1.5	1.2	0.6–2.6

[1]Odds ratio and 95% CI estimated from models including Alu or LINE-1 methylation as a continuous variable, adjusted for age, sex, educational level, smoking (never, former and current, or pack-year) and alcohol (nondrinker, former drinker or current drinker). The ORs and 95% CIs are scaled to express the risk increase associated with a decrease from the 90th to the 10th centile in Alu or LINE-1 DNA methylation.

[2]*p* for interaction for Alu = 0.02.

[3]*p* for interaction for LINE-1 = 0.01.

[4]Negative, tested negative for both IgG antibodies to *H. pylori* and cagA; positive, tested positive for either IgG antibodies to *H. pylori* or cagA antibody, or both.

[5]*p* for interaction for LINE-1 = 0.05.

[6]*p* for interaction for LINE-1 = 0.02.

[7] p for interaction for LINE-1 = 0.03.

Table 4

Global hypomethylation stratified by Lauren classification and tumor localization among cases

	No.	Mean Alu methylation (SD)	No.	Mean LINE-1 methylation (SD)
Lauren classification				
Intestinal	203	25.0 (1.4)	202	80.1 (3.7)
Diffuse	50	24.9 (1.4)	50	79.7 (3.8)
Indeterminate	32	25.0 (1.5)	31	80.6 (2.9)
p-value		0.82		0.54
Tumor localization				
Cardia only	35	24.7 (1.4)	35	79.4 (4.1)
Distal stomach	221	25.0 (1.4)	219	80.1 (3.6)
Combined cardia/distal	35	25.1 (1.3)	35	80.7 (2.9)
p-value		0.34		0.33