# Global DNA Hypomethylation (LINE-1) in the Normal Colon and Lifestyle Characteristics and Dietary and Genetic Factors

**Jane C. Figueiredo,[1] Maria V. Grau,[2] Kristin Wallace,[2] A. Joan Levine,[1] Lanlan Shen,[3] Randala Hamdan,[3] Xinli Chen,[3] Robert S. Bresalier,[4] Gail McKeown-Eyssen,[5] Robert W. Haile,[1] John A. Baron,[2] and Jean-Pierre J. Issa[3]**

[1]Department of Preventive Medicine, Keck School of Medicine, University of Southern California, Los Angeles, California; [2]Departments of Community and Family Medicine, Dartmouth Medical School, Hanover, New Hampshire; [3]Department of Leukemia and Center for Cancer Epigenetics and [4]Department of Gastrointestinal Medicine and Nutrition, The University of Texas M. D. Anderson Cancer Center, Houston, Texas; and [5]Dala Lana School of Public Health and Department of Nutritional Sciences, University of Toronto, Toronto, Ontario, Canada

## Abstract

Background: **Global loss of methylated cytosines in DNA, thought to predispose to chromosomal instability and aneuploidy, has been associated with an increased risk of colorectal neoplasia. Little is known about the relationships between global hypomethylation and lifestyle, demographics, dietary measures, and genetic factors.**

Methods: **Our data were collected as part of a randomized clinical trial testing the efficacy of aspirin and folic acid for the prevention of colorectal adenomas. At a surveillance colonoscopy ~3 years after the qualifying exam, we obtained two biopsies of the normal-appearing mucosa from the right colon and two biopsies from the left colon. Specimens were assayed for global hypomethylation using a pyrosequencing assay for LINE-1 (long interspersed nucleotide elements) repeats.**

Results: **The analysis included data from 388 subjects. There was relatively little variability in LINE methylation overall. Mean LINE-1 methylation levels in normal mucosa from the right bowel were significantly lower than those on the left side ($P < 0.0001$). No significant associations were found between LINE-1 methylation and folate treatment, age, sex, body mass index, smoking status, alcohol use, dietary intake, or circulating levels of B vitamins, homocysteine, or selected genotypes. Race, dietary folic acid, and plasma $B_6$ showed associations with global methylation that differed between the right and the left bowel. The effect of folic acid on risk of adenomas did not differ according to extent of LINE-1 methylation, and we found no association between LINE-1 methylation and risk of adenomas.**

Conclusions: **LINE-1 methylation is not influenced by folic acid supplementation but differs by colon subsite.** (Cancer Epidemiol Biomarkers Prev 2009; 18(4):1041–9)

## Introduction

Growing evidence suggests that epigenetic alterations play a role in carcinogenesis (1). Both global loss of methylated cytosines (2, 3) and accumulation of abnormally hypermethylated sequences in CpG islands near promoter sites of specific genes (4) have been identified as potential mechanisms leading to the development of colorectal cancer. Alterations in methylation status within promoter regions may affect gene control by impairing transcription. Global loss of methylation may also affect chromatin structure, facilitate chromosomal instability and aneuploidy, and increase mutation rates (5-7).

Some evidence suggests that DNA methylation may be altered by dietary availability of methyl groups. Folate, in the form of 5-methyltetrahydrofolate, is involved in the remethylation of homocysteine to methionine, the precursor of *S*-adenosylmethionine, which is the primary methyl donor for most biological methylation reactions (8). Studies have inconsistently found that folate deficiency results in genomic DNA hypomethylation in hepatic and colonic tissues in rodent models (9-13) and *in vitro* systems (14). Folate depletion studies and intervention studies with folic acid supplementation in humans have also not been entirely consistent (15-20), suggesting that the role of folate in DNA methylation may be site, cell, and tissue specific and dependent on the dose and stage of cellular transformation (21).

Other dietary measures and demographic and lifestyle characteristics may also be associated with global DNA methylation. Some studies have reported on the potential associations between hypomethylation and age, gender, alcohol, dietary intake, and circulating levels of selected B-vitamin cofactors and homocysteine (15, 22-26), but results are inconsistent, and only a few of these investigations used human colorectal tissue (26-28). Additional evidence suggests that global DNA methylation may be lower in tumor or precursor lesions than in normal colonic tissue (24) and on the right compared with left side of the colon (29).

Received 10/1/08; revised 12/22/08; accepted 1/29/09; published OnlineFirst 3/31/09.

**Grant support:** National Cancer Institute, NIH grants R01-CA-105346, R01-CA-059005, and U54-CA-100971.

**Requests for reprints:** Jane C. Figueiredo, Department of Preventive Medicine, Keck School of Medicine, University of Southern California, 1450 Biggy Street, 1509B, Los Angeles, CA 90033. Phone: 323-442-7752; Fax: 323-442-7787. E-mail: janefigu@usc.edu



doi:10.1158/1055-9965.EPI-08-0926

In the present study, we investigate the relationships of lifestyle, demographic, dietary, and genetic factors with genomic methylation, using a LINE-1 (long interspersed nucleotide elements) pyrosequencing assay, in normal mucosal biopsies from individuals in a clinical trial of aspirin and folate for the prevention of large bowel adenomas. We also examine the relationship between LINE-1 methylation and risk of adenoma occurrence and whether methylation levels modify the association between folic acid treatment and risk.

## Materials and Methods

**Study Sample.** The Aspirin/Folate Polyp Prevention Study is a randomized, double-blind, placebo-controlled trial of the efficacy of oral aspirin, folic acid, or both to prevent colorectal adenomas in patients with a history of adenomas (30, 31). Recruitment at nine clinical centers in North America began on July 6, 1994 and ended on March 20, 1998. The study was originally designed to investigate the chemopreventive potential of aspirin. Shortly after enrollment began (after 100 subjects had been randomized), the study was extended to incorporate folic acid supplementation in a 3×2 factorial design, with 1 mg folic acid or placebo incorporated into each aspirin treatment arm.

Eligible individuals had at least one of the following: one or more histologically confirmed adenomas removed within 3 months before recruitment, one or more histologically confirmed adenomas removed within 16 months before recruitment and a lifetime history of two or more histologically confirmed adenomas, or a histologically confirmed adenoma at least 1 cm in diameter removed within 16 months before recruitment. Follow-up colonoscopy was obtained from 1,081 individuals ~3 years after the qualifying examination.

A total of 914 (84.6%) persons were approached for permission to obtain normal mucosal biopsy at the 3-year colonoscopy and 781 (85.4%) consented. Of the 167 individuals who were not approached, 92 (55%) were from one center that could not participate in the biopsy study, and the remaining individuals were randomized only to aspirin. Of the 781 individuals, we obtained two biopsies of normal mucosa from the rectum and two biopsies from the mid-sigmoid from 768 (98.3%) individuals (total samples = 3,072). Of the 13 individuals who consented but did not provide biopsies, the reasons were schedule conflicts ($n$ = 9), no institutional review board approval at hospital ($n$ = 3), and unknown ($n$ = 1). Our analysis includes data from 1,000 samples analyzed to date for the LINE-1 assay (499 from the left colon and 501 from the right colon) taken from 388 individuals. Of these, 1 subject had only one biopsy from the left colon, 274 subjects had two biopsies (273 from both sides of the colon and 1 from the right colon only), 1 subject had three biopsies (1 from the right colon and 2 from the left colon), and 112 subjects had four biopsies (111 had two biopsies from each side of the colon and 1 subject had three from the right colon and one from the left colon).

The samples were collected in 2 mL freezer tubes and immersed in liquid nitrogen. After freezing, tubes were transferred from liquid nitrogen to dry ice where they were stored in -70°C freezers at the center clinical center storage freezer until an annual shipment of all biopsy specimens during the previous year to M. D. Anderson Cancer Center for DNA extraction.

Written informed consent was obtained from each participant, and the institutional review board of every participating institution approved the studies.

**Follow-up.** Adenoma occurrence was determined by colonoscopy at the end of the 3-year follow-up and pathology review. All important medical events reported by participants were verified with medical record review. Records for all large bowel procedures (endoscopy or surgery) were obtained, and slides for all tissues removed from the bowel were retrieved and sent to a single study pathologist for uniform review. Lesions were classified as neoplastic (adenomatous, including sessile serrated adenomas) or nonneoplastic. Advanced lesions were defined as invasive carcinoma or adenomas with at least 25% villous component, high-grade dysplasia, or estimated size of ≥1 cm.

### Data Collection

*Questionnaires.* All participants completed a baseline questionnaire regarding personal characteristics, medical history, and lifestyle habits. Dietary information at baseline was collected using the Block food frequency questionnaire, whose validity and reliability have been described previously.[6] Questions assessed the average consumption of a food item during the past year. Brand and type of multivitamin supplement use were collected. Daily nutrient intakes were calculated by multiplying the frequency response by the nutrient content of the specified portion size using a comprehensive database.

*Measurement of Baseline Circulating Levels.* Blood samples from nonfasting participants were collected at baseline into 7 mL Vacutainer brand tubes containing EDTA. After collection, specimens were immediately put on ice and then centrifuged at 1,100 × $g$ for 10 min. Whole blood, plasma, and buffy coat fractions were stored at -20°C for 6 to 12 months and then transferred to Dartmouth Medical School where they were stored at -80°C until analysis.

Vitamin $B_2$ (riboflavin) and vitamin $B_6$ (pyridoxal 5′ phosphate, the main active form of vitamin $B_6$) were determined in plasma by liquid chromatography-tandem mass spectrometry and vitamin $B_{12}$ by microbiological assay using published methods (32, 33). Plasma folate was determined by microbiological assays using a colistin sulfate-resistant strain of *Lactobacillus leichmannii* (33) and RBC folate was determined by the ACS:180 folate assay, a competitive immunoassay using direct chemiluminescent technology (Bayer). Plasma homocysteine was analyzed by high-performance liquid chromatography with fluorescence detection (34). Biochemical analyses were conducted blinded to methylation features, randomized treatment assignment, and other subject characteristics.

*Genotyping Assay.* The following polymorphisms were considered in this study: cystathionine-β-synthase (*CBS*)-1080 C>T, A360A (rs1801181) and *CBS*-699 C>T, Y233Y (rs234706), methylenetetrahydrofolate reductase (*MTHFR*)-677 C>T, V222A (rs1801133) and *MTHFR*-1298

[6] http://www.nutritionquest.com/research/validation_study_ref.htm

C>T, A429E (rs1801131), methionine synthase (*MTR*)-2756 A>G, D919G (rs1805087), and methionine synthase reductase (*MTRR*)-66 A>G, I22M (rs1801394). These polymorphisms were genotyped using the 5′ nuclease TaqMan allelic discrimination assay on the ABI 7900HT (Applied Biosystems). Each assay contained quality-control DNA of the homozygous wild-type, heterozygous, and homozygous variant alleles for the respective polymorphisms in addition to the no-target controls. Specific experimental details are provided elsewhere (35).

*Bisulfite Pyrosequencing LINE-1 Analysis.* Specimens were assayed for global genomic methylation using LINE-1 bisulfite pyrosequencing. Each biopsy sample was assayed in duplicate; the correlation between replicates was $r = 0.7$. DNA was quantitated using spectrophotometry and quality was verified by gel electrophoresis on all samples; highly degraded samples were excluded from analysis (but were very rare). Sodium bisulfite modification of tumor DNA was done as reported previously (36). Methylation analysis of LINE-1 promoter (GenBank accession no. X58075) was investigated using a pyrosequencing-based methylation analysis. We carried out 50 μL PCR in 60 mmol/L Tris-HCl (pH 8.5), 15 mmol/L ammonium sulfate, 2 mmol/L $MgCl_2$, 10% DMSO, 1 mmol/L deoxyribonucleotide triphosphate mix, 1 unit Taq polymerase, 5 pmol of the forward primer (5-TTTTTTGAGTTAGGTGTGGG-3), 5 pmol of the reverse-biotinylated primer (5-BIO-TCTCACTAAAAAATACCAAACAA-3), and 50 ng bisulfite-treated genomic DNA. PCR cycling conditions were 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s for 50 cycles. If very low amounts of DNA are obtained after PCR, an internal quality check by the pyrosequencing software generates an error call and those values are excluded (and the PCR was repeated). The biotinylated PCR product was purified and made single-stranded to act as a template in a pyrosequencing reaction as recommended by the manufacturer using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing). In brief, the PCR product was bound to streptavidin Sepharose HP (Amersham Biosciences) and the Sepharose beads containing the immobilized PCR product were purified, washed, denatured using a 0.2 mol/L NaOH solution, and washed again. Then, 0.3 μmol/L pyrosequencing primer (5-GGGTGGGAGTGAT-3) was annealed to the purified single-stranded PCR product and pyrosequencing was done using the PSQ HS 96 Pyrosequencing System (Pyrosequencing).

**Statistical Analysis.** We compared individuals included in this study with those that we were unable to assay regarding selected characteristics using a $\chi^2$ test, *t* test, or Mann-Whitney two-sample test for categorical and continuous variables as appropriate. Spearman's rank correlation coefficient was used to compute the correlation between paired biopsies and between biopsies on the right and left colon. We used multilevel mixed-effects linear regression to assess the between-biopsy and between-person variances. To assess the association between LINE-1 methylation levels and selected variables, we used linear regression. Because some individuals had more than one sample, we used generalized estimating equations to account for the within-subject correlation of measurements.

We considered the following lifestyle variables: age, sex, baseline body mass index (slender/normal <25 $kg/m^2$, overweight 25-30 $kg/m^2$, and obese >30 $kg/m^2$), and race (White, Black, Hispanic, and Other). Alcohol intake at baseline was defined as the average number of drinks (beer, wine, and liquor) per day during the past year. Current smoking was defined at baseline as smoking at least one cigarette a day during the past 12 months. Former smoking was defined as individuals with a past history of smoking who have not smoked in the 12 months before interview. Individuals who regularly consumed multivitamins (at least once per week) were considered as multivitamin users. Dietary factors of interest included folate, $B_2$, and $B_6$. We were not able to examine dietary $B_{12}$ because it was not estimated by the food frequency questionnaire software. Circulating levels of plasma and RBC folate, plasma $B_2$, $B_6$, and $B_{12}$, and homocysteine were also considered. We examined the following genotypes: *MTHFR*-C677T, *MTHFR*-A1298C, *MTR*-A2756G, *MTRR*-A66G, *CBS*-C1080T, and *CBS*-C699T using a codominant model.

We present crude mean LINE-1 levels and 95% confidence intervals (95% CI). Adjustment for age, sex, race, smoking, alcohol, and folate did not significantly change these findings; therefore, the unadjusted models are presented. We also tested whether site within the colon (right versus left) modified the association between global methylation and selected characteristics by inclusion of an interaction term in model.

Overdispersed generalized linear models for the Poisson family as an approximation to the binomial family were used to compute crude and adjusted risk ratios to assess the association between LINE-1 methylation and the risk of at least one new adenoma. These models were adjusted for age and sex. Inclusion of length of follow-up, aspirin treatment group, and multivitamin use resulted in no significant change in the estimates of risk and the more parsimonious models are presented.

Additionally, to mitigate the influence of possible outlying values on levels of methylation, we performed an analysis excluding 10 samples (5 individuals) with very high values of LINE-1 methylation (>74.5%). Exclusion of these individuals did not affect any of the findings and we present results for all samples.

## Results

Table 1 compares selected characteristics between the subset of individuals included in this analysis and the remaining individuals involved in the trial. There were no significant differences, except for measures of baseline dietary and circulating (plasma and RBC) folate. Individuals in this analysis had lower dietary folate intake and circulating levels of folate compared with those not included ($P$ values < 0.04). The correlation between paired biopsies was only $r = 0.09$ ($P = 0.37$) on the right side and $r = 0.17$ ($P = 0.076$) on the left side of the colon. The between-biopsy variance (SD, 2.15%) was larger than the between-person variance (SD, 0.89%).

Mean LINE-1 methylation levels in normal mucosa among individuals with and without adenomas at 3-year follow-up colonoscopy were 64.37% (95% CI, 63.95-64.79) and 64.37% (95% CI, 64.03-64.71), respectively ($P$ for the difference = 0.99). For advanced lesions detected at the

**Table 1. Comparison of individuals in the Aspirin/Folate Polyp Prevention Trial who participated and did not participate in this study**

| Characteristics | Individuals excluded in this study | Individuals included in this study | *P** |
|---|---|---|---|
| No. participants | 733 | 388 | |
| Age at baseline, mean ± SD (y) | 57.3 ± 9.9 | 57.8 ± 9.1 | 0.83† |
| Male, *n* (%) | 466 (63.6) | 246 (63.4) | 0.96* |
| Body mass index >30 kg/m$^2$, *n* (%) | 176 (24.0) | 77 (19.9) | 0.12* |
| Current cigarette smoker, *n* (%) | 106 (14.5) | 58 (15.0) | 0.97* |
| Colorectal cancer in first-degree relative, *n* (%) | 224 (36.9) | 117 (37.9) | 0.78* |
| Self-identified as White, *n* (%) | 633 (86.4) | 325 (83.8) | 0.24* |
| Aspirin Treatment Group‡, *n* (%) | 489 (66.7) | 260 (67.0) | 0.92* |
| Folate Treatment Group, *n* (%) | 318 (50.2) | 198 (51.0) | 0.81* |
| Baseline RBC folate, mean ± SD (ng/mL) | 414.9 ± 6.3 | 396.4 ± 7.2 | 0.04§ |
| Baseline plasma folate, mean ± SD (nmol/L) | 24.8 ± 0.75 | 20.9 ± 0.80 | <0.01§ |
| Baseline plasma $B_2$, mean ± SD (nmol/L) | 30.9 ± 2.1 | 25.7 ± 2.3 | 0.38§ |
| Baseline plasma $B_6$, mean ± SD (nmol/L) | 80.7 ± 3.5 | 76.5 ± 4.6 | 0.44§ |
| Baseline plasma $B_{12}$, mean ± SD (pmol/L) | 319.3 ± 6.2 | 338.6 ± 9.1 | 0.09§ |
| Baseline plasma homocysteine, mean ± SD (μmol/L) | 10.1 ± 4.2 | 9.9 ± 2.95 | 0.80§ |
| Multivitamin use, *n* (%) | 265 (36.3) | 130 (33.5) | 0.36§ |
| Dietary intake | | | |
| Folate intake, mean ± SD (μg/d) | 327.9 ± 6.1 | 305.0 ± 7.7 | 0.01† |
| Vitamin $B_2$, mean ± SD (mg/d) | 1.79 ± 0.03 | 1.72 ± 0.43 | 0.11† |
| Vitamin $B_6$, mean ± SD (mg/d) | 1.67 ± 0.03 | 1.64 ± 0.04 | 0.26† |
| Vitamin $B_{12}$, mean ± SD (mg/d) | | | |
| Alcohol (drinks/d), *n* (%) | 217 (31.5) | 123 (33.0) | 0.21* |
| None | 341 (49.4) | 165 (44.2) | |
| ≤1 | 132 (19.1) | 85 (22.8) | |
| ≥2 | | | |
| Adenoma characteristics (at baseline)‖ | | | |
| No. lifetime adenomas (mean ± SD) | 2.44 ± 2.30 | 2.22 ± 1.94 | 0.11§ |
| Large baseline adenomas (>1 cm), *n* (%) | 157 (21.4) | 96 (24.7) | 0.21* |
| Baseline adenomas with villous histology, *n* (%) | 107 (14.6) | 49 (12.6) | 0.37* |
| Baseline adenomas with proximal location, *n* (%) | 342 (46.7) | 164 (42.3) | 0.16* |

*$\chi^2$ test.
†Two-sample *t* test.
‡81 and 325 mg/d aspirin treatment groups combined.
§Nonparametric Mann-Whitney test.
‖Using standard definitions by Polyp Prevention Study Group (30, 31).

3-year colonoscopy, the difference was also minimal (mean, 63.83; 95% CI, 62.86-64.80 versus mean, 64.41; 95% CI, 64.14-64.69 for individuals with and without advanced lesions, respectively; *P* for the difference = 0.25). Individuals randomized to folic acid treatment had slightly higher LINE-1 methylation levels (mean, 64.53%; 95% CI, 64.16-64.90) than those in the placebo group (mean, 64.21%; 95% CI, 63.83-64.58), but this difference did not reach statistical significance ($P = 0.23$). Samples from the left side of the colon had significantly higher LINE-1 methylation levels than those on the right (left mean, 64.88%; 95% CI, 64.55-65.21 and right mean, 63.86%; 95% CI, 63.56-64.16; $P < 0.0001$), although this difference was modest in magnitude.

No significant associations were found between LINE-1 methylation and subject age, sex, or body mass index (Table 2). The association between LINE-1 methylation and smoking and alcohol appeared to be modified by site within the colon (right versus left): there were suggestions of an association on the right but none on the left. Hispanic and other racial groups had higher levels of LINE-1 methylation than White and Black individuals, particularly in samples from the right side.

Dietary folate intake at baseline was not associated with LINE-1 methylation overall or on the left side (Table 3). We observed an inverse association between LINE-1 methylation and folate from samples on the right, although there was no consistent trend over quartiles of intake. Furthermore, we did not observe any association between total folate intake and LINE-1 methylation. Multivitamin use and dietary intake of $B_2$ and $B_6$ were also not associated with LINE-1 methylation.

Circulating levels of folate (plasma and RBC), $B_2$ and $B_{12}$, and homocysteine showed no association with LINE-1 methylation overall or in specimens from the left or right side separately (Table 4). Individuals in the highest quartile of plasma $B_6$ had significantly higher levels of LINE-1 methylation than individuals in the lowest quartile, but this association was limited to samples taken from the left side ($P = 0.04$).

In addition, we found no evidence that LINE-1 methylation was significantly associated with genotypes in *MTHFR* (C677T or A1298C), *MTR* (A2756G), *MTRR* (A66G), and *CBS* (C1080T and C699T; Table 5).

Among all individuals, the adjusted relative risk (RR) of any adenoma occurrence associated with a 1 SD increase in LINE-1 methylation was 1.00 (95% CI, 0.91-1.10). For advanced lesions, the same increase in LINE-1 methylation was associated with a nonsignificantly reduced risk (adjusted RR, 0.79; 95% CI, 0.56-1.11). There was no evidence that the effect of folic acid differed between individuals in the lowest and highest tertiles of LINE-1 methylation [adjusted RR (95% CI) for folic acid treatment, 1.06 (0.83-1.36) and 0.94 (0.73-1.20), respectively; $P_{interaction} = 0.67$]. For advanced lesions, the risk associated with folic acid treatment was nonsignificantly greater among individuals in the lowest tertile of LINE-1 methylation than among those in

**Table 2. Association between LINE-1 methylation and selected demographic and lifestyle variables**

| | Counts* | Overall | | Left side | | Right side | | $P$ † |
|---|---|---|---|---|---|---|---|---|
| | $N_T/N_L/N_R$ | Mean (95% CI) | $P$ ‡ | Mean (95% CI) | $P$ ‡ | Mean (95% CI) | $P$ ‡ | |
| Age (y) | | | | | | | | |
| Q1 (30-52) | 107/107/106 | 64.75 (64.24-65.26) | 0.49 | 65.41 (64.81-66.00) | 0.63 | 64.09 (63.51-64.68) | 0.46 | 0.27 |
| Q2 (53-58) | 96/96/96 | 63.94 (63.40-64.48) | | 64.17 (63.55-64.78) | | 63.71 (63.05-64.38) | | |
| Q3 (59-64) | 93/92/92 | 64.39 (63.87-64.92) | | 64.93 (64.24-65.62) | | 63.87 (63.28-64.45) | | |
| Q4 (65-78) | 92/92/91 | 64.32 (63.80-64.85) | | 64.92 (64.21-65.62) | | 63.72 (63.17-64.27) | | |
| Sex | | | | | | | | |
| Male | 246/245/244 | 64.33 (64.02-64.64) | 0.72 | 64.86 (64.45-65.26) | 0.85 | 63.80 (63.44-64.17) | 0.66 | 0.81 |
| Female | 142/141/142 | 64.44 (63.95-64.93) | | 64.92 (64.36-65.48) | | 63.95 (63.43-64.46) | | |
| Body mass index ($kg/m^2$) | | | | | | | | |
| Normal | 113/113/112 | 64.37 (63.87-64.86) | 0.93 | 64.85 (64.28-65.43) | 0.70 | 63.89 (63.31-64.47) | 0.79 | 0.77 |
| Overweight | 195/195/193 | 64.37 (64.01-64.73) | | 64.84 (64.39-65.29) | | 63.90 (63.49-64.30) | | |
| Obese | 79/78/79 | 64.40 (63.78-65.02) | | 65.05 (64.24-65.86) | | 63.77 (63.10-64.44) | | |
| Smoking | | | | | | | | |
| Never | 164/164/163 | 64.39 (64.02-64.76) | 0.44 | 65.12 (64.66-65.57) | 0.62 | 63.65 (63.23-64.08) | 0.08 | 0.04 |
| Former | 163/163/163 | 64.24 (63.82-64.67) | | 64.65 (64.10-65.21) | | 63.84 (63.38-64.30) | | |
| Current | 60/59/58 | 64.73 (63.96-65.49) | | 64.88 (64.05-65.71) | | 64.57 (63.65-65.50) | | |
| Alcohol | | | | | | | | |
| Never | 123/123/122 | 64.54 (64.13-64.95) | 0.74 | 65.37 (64.78-65.95) | 0.14 | 63.70 (63.27-64.14) | 0.26 | 0.02 |
| 1 per day | 85/84/85 | 64.22 (63.85-64.59) | | 64.65 (64.23-65.08) | | 63.77 (63.30-64.24) | | |
| ≥2 per day | 165/165/163 | 64.40 (63.69-65.10) | | 64.61 (63.80-65.42) | | 64.19 (63.45-64.95) | | |
| Race | | | | | | | | |
| White | 325/324/324 | 64.27 (63.99-64.55) | 0.10 | 64.82 (64.47-65.16) | 0.43 | 63.72 (63.41-64.04) | 0.02 | 0.19 |
| Black | 22/22/21 | 64.03 (63.44-64.61) | | 64.84 (63.94-65.75) | | 63.21 (62.43-63.98) | | |
| Hispanic | 22/22/22 | 65.40 (63.85-66.96) | | 65.38 (63.47-67.28) | | 65.43 (63.81-67.05) | | |
| Other | 19/19/19 | 65.43 (63.61-67.25) | | 65.52 (63.48-67.55) | | 65.35 (63.56-67.13) | | |

*$N_T$ = total individuals; $N_L$ = total individuals with samples on the left side; $N_R$ = total individuals with samples on the right side.
†$P_{interaction}$ testing differences between right and left sides of the colorectum.
‡$P_{trend}$.

the highest tertile (adjusted RR, 1.41; 95% CI, 0.71-2.85 versus RR, 0.79; 95% CI, 0.33-1.88; $P_{interaction}$ = 0.30).

## Discussion

In this clinical trial of individuals randomly assigned to either 1 mg/d folic acid or placebo and prospectively followed for new colorectal adenomas, samples from the right side of the normal colon had significantly lower mean LINE-1 methylation levels than those on the left. Otherwise, LINE-1 methylation appeared very stable in the sense that it did not correlate significantly with many of the characteristics we studied including age, sex, body mass index, smoking status, alcohol use, dietary intake, and circulating levels of folate and other B vitamins,

**Table 3. Association between LINE-1 methylation and dietary intake of B-vitamin and multivitamin use**

| | Counts* | Overall | | Left side | | Right side | | $P$ † |
|---|---|---|---|---|---|---|---|---|
| | $N_T/N_L/N_R$ | Mean (95% CI) | $P$ ‡ | Mean (95% CI) | $P$ ‡ | Mean (95% CI) | $P$ ‡ | |
| Dietary folate (μg/d) | | | | | | | | |
| Q1 (78.08-205.86) | 94/94/93 | 64.66 (64.09-65.23) | 0.02 | 64.85 (64.18-65.51) | 0.32 | 64.47 (63.83-65.11) | <0.01 | 0.10 |
| Q2 (206.87-277.3) | 94/94/94 | 64.82 (64.69-65.32) | | 65.47 (64.79-66.16) | | 64.16 (63.59-64.73) | | |
| Q3 (277.31-373.65) | 94/94/93 | 64.05 (63.55-64.56) | | 64.57 (63.95-65.19) | | 63.53 (62.94-64.12) | | |
| Q4 (376.17-1,285.59) | 93/91/92 | 63.92 (63.36-64.48) | | 64.63 (63.96-65.31) | | 63.22 (62.61-63.82) | | |
| Dietary $B_2$ (mg/d) | | | | | | | | |
| Q1 (0.39-1.18) | 98/98/97 | 64.73 (64.18-65.28) | 0.57 | 65.22 (64.52-65.92) | 0.75 | 64.24 (63.67-64.82) | 0.16 | 0.12 |
| Q2 (1.19-1.57) | 92/92/92 | 64.17 (63.64-64.70) | | 64.36 (63.73-65.00) | | 63.97 (63.33-64.61) | | |
| Q3 (1.60-2.20) | 92/92/92 | 64.03 (63.56-64.48) | | 64.66 (64.03-65.30) | | 63.39 (62.89-63.89) | | |
| Q4 (2.21-6.56) | 93/91/91 | 64.53 (63.93-65.13) | | 65.29 (64.62-65.96) | | 63.77 (63.09-64.45) | | |
| Dietary $B_6$ (mg/d) | | | | | | | | |
| Q1 (0.33-1.08) | 99/99/99 | 64.72 (64.17-65.28) | 0.17 | 64.99 (64.31-65.66) | 0.77 | 64.46 (63.87-65.04) | 0.04 | 0.32 |
| Q2 (1.09-1.54) | 89/89/88 | 64.23 (63.68-64.78) | | 64.76 (64.13-65.39) | | 63.70 (63.05-64.35) | | |
| Q3 (1.55-2.00) | 95/95/94 | 64.38 (63.90-64.86) | | 65.02 (64.32-65.71) | | 63.76 (63.25-64.27) | | |
| Q4 (2.01-6.20) | 92/90/91 | 64.11 (63.56-64.65) | | 64.76 (64.12-65.39) | | 63.45 (62.80-64.11) | | |
| Multivitamin use | | | | | | | | |
| No | 256/255/253 | 64.22 (63.90-64.53) | 0.12 | 64.72 (64.31-65.13) | 0.18 | 63.71 (63.35-64.06) | 0.10 | 0.97 |
| Yes | 130/130/130 | 64.67 (64.20-65.13) | | 65.19 (64.65-65.72) | | 64.15 (63.61-64.70) | | |

NOTE: Quartiles of the residuals of the regression of the logarithm of the nutrient on the logarithm of kilocalories. Means are adjusted for energy intake using the logarithm of caloric intake. Quartiles ranges are based in a 2,000 calories/d diet.
*$N_T$ = total individuals; $N_L$ = total individuals with samples on the left side; $N_R$ = total individuals with samples on the right side.
†$P_{interaction}$ testing differences between right and left sides of the colorectum.
‡$P_{trend}$.

**Table 4. Association between LINE-1 methylation and circulating levels of B vitamins and homocysteine**

| | Counts* | Overall | | Left side | | Right side | | $P$ † |
|---|---|---|---|---|---|---|---|---|
| | $N_T/N_L/N_R$ | Mean (95% CI) | $P$ ‡ | Mean (95% CI) | $P$ ‡ | Mean (95% CI) | $P$ ‡ | |
| Plasma folate (nmol/L) | | | | | | | | |
| Q1 (3.14-10.02) | 93/93/91 | 64.80 (64.32-65.28) | 0.22 | 65.33 (64.69-65.97) | 0.43 | 64.26 (63.69-64.84) | 0.22 | 0.88 |
| Q2 (10.05-16.70) | 92/92/92 | 64.12 (63.53-64.70) | | 64.52 (63.80-65.24) | | 63.71 (63.09-64.33) | | |
| Q3 (16.73-27.26) | 93/92/92 | 64.20 (63.66-64.73) | | 64.70 (64.01-65.39) | | 63.71 (63.08-64.34) | | |
| Q4 (27.31-119.0) | 92/92/92 | 64.30 (63.77-64.83) | | 64.88 (64.26-65.50) | | 63.72 (63.13-64.31) | | |
| RBC folate (ng/mL) | | | | | | | | |
| Q1 (64.86-303.0) | 98/98/96 | 64.07 (63.69-64.46) | 0.99 | 64.41 (63.89-64.93) | 0.66 | 63.72 (63.23-64.22) | 0.66 | 0.68 |
| Q2 (304.0-383.0) | 96/96/95 | 64.58 (63.94-65.21) | | 65.13 (64.37-65.90) | | 64.02 (63.30-64.73) | | |
| Q3 (384.0-468.0) | 98/97/98 | 64.92 (64.32-65.53) | | 65.55 (64.83-66.26) | | 64.30 (63.64-64.95) | | |
| Q4 (469.0-952.0) | 95/94/95 | 63.95 (63.50-64.41) | | 64.47 (63.88-65.06) | | 63.45 (62.94-63.96) | | |
| Plasma $B_2$ (nmol/L) | | | | | | | | |
| Q1 (2.54-8.93) | 93/92/91 | 64.62 (63.99-65.24) | 0.45 | 64.81 (64.11-65.52) | 0.71 | 64.42 (63.68-65.17) | 0.08 | 0.17 |
| Q2 (9.10-14.70) | 93/93/92 | 64.31 (63.75-64.86) | | 64.88 (64.22-65.55) | | 63.72 (63.06-64.38) | | |
| Q3 (14.90-23.70) | 93/92/93 | 64.18 (63.73-64.64) | | 64.67 (64.07-65.27) | | 63.71 (63.19-64.22) | | |
| Q4 (23.80-468.5) | 92/92/92 | 64.34 (63.83-64.84) | | 65.08 (64.38-65.79) | | 63.59 (63.11-64.07) | | |
| Plasma $B_6$ (nmol/L) | | | | | | | | |
| Q1 (9.74-36.0) | 94/94/92 | 64.27 (63.69-64.84) | 0.29 | 64.38 (63.71-65.06) | 0.04 | 64.15 (63.44-64.84) | 0.76 | 0.11 |
| Q2 (36.1-51.2) | 93/92/92 | 64.08 (63.51-64.65) | | 64.69 (63.96-65.42) | | 63.47 (62.88-64.06) | | |
| Q3 (52.1-79.8) | 92/91/92 | 64.57 (64.06-65.08) | | 65.14 (64.51-65.77) | | 64.00 (63.40-64.60) | | |
| Q4 (80.7-857) | 92/92/92 | 64.53 (64.07-65.00) | | 65.25 (64.63-65.86) | | 63.83 (63.32-64.34) | | |
| Plasma $B_{12}$ (pmol/L) | | | | | | | | |
| Q1 (43.90-240.7) | 93/92/91 | 64.34 (63.69-64.99) | 0.58 | 64.68 (63.91-65.45) | 0.85 | 63.99 (63.29-64.69) | 0.45 | 0.75 |
| Q2 (242.7-307.1) | 93/93/93 | 64.62 (64.04-65.19) | | 65.25 (64.47-66.04) | | 63.98 (63.37-64.59) | | |
| Q3 (307.7-400.8) | 93/92/93 | 64.26 (63.83-64.69) | | 64.77 (64.22-65.31) | | 63.75 (63.20-64.30) | | |
| Q4 (400.9-2072) | 93/93/92 | 64.23 (63.78-64.67) | | 64.75 (64.22-65.27) | | 63.71 (63.16-64.26) | | |
| Homocysteine (μmol/L) | | | | | | | | |
| Q1 (4.73-7.92) | 93/93/92 | 64.23 (63.72-64.74) | 0.86 | 64.89 (64.29-65.48) | 0.82 | 63.58 (63.03-64.13) | 0.56 | 0.70 |
| Q2 (7.94-9.25) | 93/93/93 | 64.35 (63.85-64.85) | | 64.77 (64.07-65.46) | | 63.94 (63.93-64.49) | | |
| Q3 (9.29-11.10) | 93/91/92 | 64.62 (64.08-65.17) | | 65.14 (64.57-65.71) | | 64.11 (63.38-64.83) | | |
| Q4 (11.18-23.30) | 92/92/91 | 64.21 (63.62-64.79) | | 64.64 (63.85-65.44) | | 63.78 (63.22-64.33) | | |

*$N_T$ = total individuals; $N_L$ = total individuals with samples on the left side; $N_R$ = total individuals with samples on the right side.
†$P_{interaction}$ testing differences between right and left sides of the colorectum.
‡$P_{trend}$.

homocysteine, and selected genotypes. Higher levels of global hypomethylation were observed among Hispanics and other racial groups compared with White and Black individuals. Individuals who were randomized to folic acid did not have higher methylation in the normal colon after 3 years of follow-up than those in the placebo group. We also did not observe different levels of methylation in individuals with and without an adenoma at the examination at which the biopsies were taken. In addition, there was no indication that LINE-1 methylation significantly modified the association between folic acid treatment and risk of any adenomas or advanced lesions.

In agreement with our findings, a small study also suggested that global DNA methylation was lower on the right versus left side in the normal colon mucosa (29). Increases in global DNA methylation following folic acid supplementation were also found to be greater in the right colon than in the rectum in a clinical trial (18). However, other small studies failed to observe any association between LINE-1 methylation and site in the bowel (26, 37). The right side of the colorectum is hypothesized to be different from the left with respect to colon cancer etiology. The presence of BRAF mutations, the CpG island methylator phenotype and microsatellite instability, and absence of KRAS mutations are noted characteristics of cancers in the right compared with the left colon (38, 39) and previous studies have suggested that LINE-1 methylation is inversely correlated with CpG island methylator-high and microsatellite instability-high in colorectal cancers (26, 29, 40, 41).

Conflicting evidence exists on the relationship between folate and DNA methylation in the colorectum. Cravo et al. first proposed that folate deficiency may enhance colorectal carcinogenesis through an induction of genomic DNA hypomethylation (42). However, rodent studies examining folate deficiency have shown limited evidence of a significant change in genomic DNA methylation induced by folate deficiency in colon tissue (21). Human intervention studies of folate deficiency have suggested that lowered folate status may result in genomic DNA hypomethylation in peripheral blood lymphocytes (15). In addition, studies using the methyl acceptance assay have suggested the possibility that folate supplementation may increase global methylation in colorectal tissues in individuals with and without adenomas (18, 27, 28, 43), but this has not been seen in studies using other methylation measurements (20, 21). Our data provide no evidence that folic acid supplementation and dietary or circulating folate or other B-vitamin cofactors are associated with levels of global methylation in the normal colon among individuals with a history of adenomas. In addition, we investigated the association between LINE-1 methylation and serum homocysteine (a metabolite that inhibits *S*-adenosylmethionine-dependent methylation reactions) and observed no association. However, Friso et al. reported an inverse association between homocysteine and global DNA

methylation in human leucocytes (44). Together, these data support an alternative hypothesis proposed by Kim (45) that the effects of folate deficiency and supply of DNA methyl groups may be gene and site specific and depend on the cell type, organ, stage of transformation, degree, and duration of folate depletion. Alternatively, the effects of folate may be unrelated to DNA methylation.

Only a few polymorphic genes involved in the one-carbon metabolism have been studied with respect to measures of global DNA hypomethylation. *MTHFR*-677T/T and 1298C/C have been associated with lower global methylation in lymphocytic DNA (26, 44, 46-48). Furthermore, in a folate depletion-repletion study, women with *MTHFR*-677T/T genotype had a greater increase in leukocyte DNA methylation following repletion with folate than those who did not carry the variant (43). However, these results are in contrast with our findings in normal colonic mucosa as well as with a small study of polyp-free individuals (28). There is also conflicting evidence regarding the association between global DNA methylation and a polymorphism in *MTR*-A2756G (26, 28).

In agreement with our results, previous studies have reported no relationship with either age or gender and methylation in human leukocytes or colonic mucosa (24, 29, 37, 49-51). Alcohol consumption may decrease DNA methylation in hepatic tissue by antagonizing actions on folate metabolism and/or methionine synthetase or by decreasing the methyl group donor, *S*-adenosylmethionine, production. Indeed, alcohol ingestion has been shown to induce genomic DNA hypomethylation in the colonic mucosa of rats (22) and human leucocytes (52). However, at least one other study did not observe a significant change in global DNA methylation associated with alcohol use (18). Smoking has been linked with promoter methylation of specific genes and reduced levels of vitamin $B_{12}$, which is needed for the synthesis of *S*-adenosylmethionine. At least one study has noted significant differences in global DNA methylation by smoking in aerodigestive mucosal tissue (53). Previous studies have not reported on the potential variability in global hypomethylation across racial groups.

Some studies (24), but not all (41), have suggested that invasive cancers are more hypomethylated than adenomas and similarly for adenomas compared with normal tissue. Furthermore, hypomethylation appears to be detectable even in normal tissue far from the tumor site (37), suggesting that hypomethylation is an early event in colon carcinogenesis. We found no indication that normal tissue from individuals with adenomas had lower levels of LINE-1 methylation than those without adenomas.

A limitation of our findings is that we used a convenience sample. We selected the first 1,000 samples for analysis and therefore did not have all 4 samples for each of the 768 who consented for biopsy, nor did we measure LINE-1 methylation in adenomas. Also important to note is that our study was restricted to individuals with a recent history of adenomas, so we may have limited variability in methylation values. Furthermore, we used a pyrosequencing-based method to determine LINE-1 methylation. LINE-1 sequences are the most common repeat elements, constituting at least 18% of the human genome (54). LINE-1 elements are normally heavily methylated in somatic tissue, and their level of methylation is significantly correlated with genome-wide 5-methylcytosine content as measured by high-performance liquid chromatography (55). In a validation

**Table 5. Association between LINE-1 methylation and selected polymorphisms**

| | Counts* | Overall | | Left side | | Right side | | $P$ † |
|---|---|---|---|---|---|---|---|---|
| | $N_T/N_L/N_R$ | Mean (95% CI) | $P$ ‡ | Mean (95% CI) | $P$ ‡ | Mean (95% CI) | $P$ ‡ | |
| *MTHFR*-C677T | | | | | | | | |
| CC | 156/156/156 | 64.44 (64.02-64.86) | 0.53 | 65.05 (64.54-65.56) | 0.89 | 63.84 (63.34-64.34) | 0.32 | 0.26 |
| CT | 156/156/155 | 64.25 (63.85-64.65) | | 64.65 (64.14-65.16) | | 63.86 (63.42-64.29) | | |
| TT | 45/44/43 | 64.16 (63.38-64.94) | | 64.97 (63.98-65.95) | | 63.36 (62.56-64.16) | | |
| *MTHFR*-A1298C | | | | | | | | |
| AA | 174/173/172 | 64.04 (63.69-64.39) | 0.44 | 64.57 (64.16-64.98) | 0.30 | 63.52 (63.10-63.95) | 0.87 | 0.65 |
| AC | 142/142/141 | 64.64 (64.18-65.10) | | 65.12 (64.51-65.72) | | 64.16 (63.68-64.65) | | |
| CC | 39/39/39 | 64.42 (63.52-65.33) | | 65.21 (64.05-66.37) | | 63.62 (62.59-64.64) | | |
| *MTR*-A2756G | | | | | | | | |
| AA | 244/244/243 | 64.28 (63.95-64.61) | 0.39 | 64.78 (64.37-65.20) | 0.36 | 63.77 (63.41-64.14) | 0.88 | 0.72 |
| AG | 101/100/99 | 64.39 (63.87-64.91) | | 64.95 (64.33-65.58) | | 63.82 (63.22-64.43) | | |
| GG | 12/12/12 | 64.72 (63.78-65.67) | | 65.60 (63.91-67.29) | | 63.84 (63.07-64.61) | | |
| *MTRR*-A66G | | | | | | | | |
| AA | 88/88/86 | 64.59 (64.06-65.13) | 0.48 | 65.43 (64.75-66.11) | 0.13 | 63.76 (63.15-64.36) | 0.68 | 0.14 |
| AG | 168/168/168 | 64.19 (63.79-64.59) | | 64.64 (64.14-65.15) | | 63.74 (63.29-64.19) | | |
| GG | 99/98/98 | 64.33 (63.82-64.83) | | 64.72 (64.11-65.33) | | 63.93 (63.35-64.52) | | |
| *CBS*-C1080T | | | | | | | | |
| CC | 169/168/168 | 64.22 (63.81-64.62) | 0.86 | 64.84 (64.35-65.32) | 0.70 | 63.60 (63.12-64.07) | 0.44 | 0.55 |
| CT | 150/150/148 | 64.46 (64.04-64.88) | | 64.93 (64.39-65.48) | | 63.98 (63.53-64.42) | | |
| TT | 36/36/36 | 64.30 (63.53-65.06) | | 64.61 (63.59-65.64) | | 63.98 (63.15-64.81) | | |
| *CBS*-C699T | | | | | | | | |
| CC | 153/153/151 | 64.49 (64.07-64.91) | 0.74 | 64.88 (64.34-65.41) | 0.47 | 64.10 (63.65-64.55) | 0.82 | 0.37 |
| CT | 170/169/169 | 64.12 (63.73-64.50) | | 64.75 (64.27-65.22) | | 63.49 (63.05-63.93) | | |
| TT | 33/33/33 | 64.66 (63.80-65.51) | | 64.33 (64.21-66.45) | | 63.96 (62.84-65.09) | | |

*$N_T$ = total individuals; $N_L$ = total individuals with samples on the left side; $N_R$ = total individuals with samples on the right side.
†$P_{interaction}$ testing differences between right and left sides of the colorectum.
‡$P_{trend}$.

study, the LINE-1 assay was compared to a restriction digestion method, COBRA, in colon cancer cell lines treated with the methylation inhibitor 5-aza-2′-deoxycytidine; there was a 18% to 60% decrease in LINE-1 methylation after 3 days of treatment by either method (56). The relationship between the pyrosequencing-based LINE-1 assay and the methyl group acceptance assay used in some previous studies (13, 18, 27, 28, 43) is unknown.

In this study, assay replicates were highly correlated; however, different biopsies from the same subjects were not. The reasons for this discrepancy are not clear. This suggests that LINE-1 methylation varies randomly in a relatively narrow range in any one area of the bowel. There are no data using any methylation assay that have carefully looked at measurement issues.

Despite these limitations, our study has notable strengths. This study is based on a group of individuals randomized to folic acid supplementation and we had high response rates. The prospective design also assures that selection or recall biases are unlikely to influence our results.

In summary, we observed significant evidence to suggest that normal colonic mucosa from the right side of the colon has lower global hypomethylation compared with the left side in individuals with a previous history of adenomas. However, we also found that global DNA methylation is stable and shows no association with personal characteristics and folic acid supplementation. If indeed selected characteristics or folic acid supplementation are associated with colorectal neoplasia, they may not operate through genomic methylation. These implications merit further investigation.

## Disclosure of Potential Conflicts of Interest

Wyeth, which markets folate supplements, provided study agents for the clinical trial that this research is based on.

## Acknowledgments

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank all the individuals who participated in this clinical trial.

## References

1. Feinberg AP, Ohlsson R, Henikoff S. The epigenetic progenitor origin of human cancer. Nat Rev Genet 2006;7:21–33.
2. Feinberg AP, Vogelstein B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature 1983; 301:89–92.
3. Gama-Sosa MA, Midgett RM, Slagel VA, et al. Tissue-specific differences in DNA methylation in various mammals. Biochim Biophys Acta 1983;740:212–9.
4. Robertson KD. DNA methylation, methyltransferases, and cancer. Oncogene 2001;20:3139–55.
5. Gaudet F, Hodgson JG, Eden A, et al. Induction of tumors in mice by genomic hypomethylation. Science 2003;300:489–92.
6. Karpf AR, Matsui S. Genetic disruption of cytosine DNA methyltransferase enzymes induces chromosomal instability in human cancer cells. Cancer Res 2005;65:8635–9.
7. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R. DNA hypomethylation leads to elevated mutation rates. Nature 1998;395: 89–93.
8. Selhub J, Miller JW. The pathogenesis of homocysteinemia: interruption of the coordinate regulation by *S*-adenosylmethionine of the remethylation and transsulfuration of homocysteine. Am J Clin Nutr 1992;55:131–8.
9. Balaghi M, Horne DW, Wagner C. Hepatic one-carbon metabolism in early folate deficiency in rats. Biochem J 1993;291:145–9.
10. Kim YI, Christman JK, Fleet JC, et al. Moderate folate deficiency does not cause global hypomethylation of hepatic and colonic DNA or c-myc-specific hypomethylation of colonic DNA in rats. Am J Clin Nutr 1995;61:1083–90.
11. Kotsopoulos J, Sohn KJ, Kim YI. Postweaning dietary folate deficiency provided through childhood to puberty permanently increases genomic DNA methylation in adult rat liver. J Nutr 2008; 138:703–9.
12. Maloney CA, Hay SM, Rees WD. Folate deficiency during pregnancy impacts on methyl metabolism without affecting global DNA methylation in the rat fetus. Br J Nutr 2007;97:1090–8.
13. Kotsopoulos J, Sohn KJ, Martin R, et al. Dietary folate deficiency suppresses *N*-methyl-*N*-nitrosourea-induced mammary tumorigenesis in rats. Carcinogenesis 2003;24:937–44.
14. Stempak JM, Sohn KJ, Chiang EP, Shane B, Kim YI. Cell and stage of transformation-specific effects of folate deficiency on methionine cycle intermediates and DNA methylation in an *in vitro* model. Carcinogenesis 2005;26:981–90.
15. Jacob RA, Gretz DM, Taylor PC, et al. Moderate folate depletion increases plasma homocysteine and decreases lymphocyte DNA methylation in postmenopausal women. J Nutr 1998;128:1204–12.
16. Rampersaud GC, Kauwell GP, Hutson AD, Cerda JJ, Bailey LB. Genomic DNA methylation decreases in response to moderate folate depletion in elderly women. Am J Clin Nutr 2000;72:998–1003.
17. Ingrosso D, Cimmino A, Perna AF, et al. Folate treatment and unbalanced methylation and changes of allelic expression induced by hyperhomocysteinaemia in patients with uraemia. Lancet 2003;361: 1693–9.
18. Pufulete M, Al-Ghnaniem R, Khushal A, et al. Effect of folic acid supplementation on genomic DNA methylation in patients with colorectal adenoma. Gut 2005;54:648–53.
19. Axume J, Smith SS, Pogribny IP, Moriarty DJ, Caudill MA. The MTHFR 677TT genotype and folate intake interact to lower global leukocyte DNA methylation in young Mexican American women. Nutr Res 2007;27:1365–17.
20. Axume J, Smith SS, Pogribny IP, Moriarty DJ, Caudill MA. Global leukocyte DNA methylation is similar in African American and Caucasian women under conditions of controlled folate intake. Epigenetics 2007;2:66–8.
21. Kim YI. Folate and DNA methylation: a mechanistic link between folate deficiency and colorectal cancer? Cancer Epidemiol Biomarkers Prev 2004;13:511–9.
22. Choi SW, Stickel F, Baik HW, Kim YI, Seitz HK, Mason JB. Chronic alcohol consumption induces genomic but not p53-specific DNA hypomethylation in rat colon. J Nutr 1999;129:1945–50.
23. Keyes MK, Jang H, Mason JB, et al. Older age and dietary folate are determinants of genomic and p16-specific DNA methylation in mouse colon. J Nutr 2007;137:1713–7.
24. Chalitchagorn K, Shuangshoti S, Hourpai N, et al. Distinctive pattern of LINE-1 methylation level in normal tissues and the association with carcinogenesis. Oncogene 2004;23:8841–6.
25. El-Maarri O, Becker T, Junen J, et al. Gender specific differences in levels of DNA methylation at selected loci from human total blood: a tendency toward higher methylation levels in males. Hum Genet 2007;122:505–14.
26. Iacopetta B, Grieu F, Phillips M, et al. Methylation levels of LINE-1 repeats and CpG island loci are inversely related in normal colonic mucosa. Cancer Sci 2007;98:1454–60.
27. Pufulete M, Al-Ghnaniem R, Leather AJ, et al. Folate status, genomic DNA hypomethylation, and risk of colorectal adenoma and cancer: a case control study. Gastroenterology 2003;124:1240–8.
28. Pufulete M, Al-Ghnaniem R, Rennie JA, et al. Influence of folate status on genomic DNA methylation in colonic mucosa of subjects without colorectal adenoma or cancer. Br J Cancer 2005;92: 838–42.
29. Estecio MR, Gharibyan V, Shen L, et al. LINE-1 hypomethylation in cancer is highly variable and inversely correlated with microsatellite instability. PLoS ONE 2007;2:e399.
30. Baron JA, Cole BF, Sandler RS, et al. A randomized trial of aspirin to prevent colorectal adenomas. N Engl J Med 2003;348:891–9.
31. Cole BF, Baron JA, Sandler RS, et al. Folic acid for the prevention of colorectal adenomas: a randomized clinical trial. JAMA 2007;297: 2351–9.
32. Midttun O, Hustad S, Solheim E, Schneede J, Ueland PM. Multianalyte quantification of vitamin $B_6$ and $B_2$ species in the nanomolar

range in human plasma by liquid chromatography-tandem mass spectrometry. Clin Chem 2005;51:1206–16.
33. Molloy AM, Scott JM. Microbiological assay for serum, plasma, and red cell folate using cryopreserved, microtiter plate method. Methods Enzymol 1997;281:43–53.
34. Fiskerstrand T, Refsum H, Kvalheim G, Ueland PM. Homocysteine and other thiols in plasma and urine: automated determination and sample stability. Clin Chem 1993;39:263–71.
35. Figueiredo JC, Levine AJ, Grau MV, et al. Vitamins $B_2$, $B_6$, and $B_{12}$ and risk of new colorectal adenomas in a randomized trial of aspirin use and folic acid supplementation. Cancer Epidemiol Biomarkers Prev 2008;17:2136–45.
36. Toyota M, Ho C, Ahuja N, et al. Identification of differentially methylated sequences in colorectal cancer by methylated CpG island amplification. Cancer Res 1999;59:2307–12.
37. Suter CM, Martin DI, Ward RL. Hypomethylation of L1 retrotransposons in colorectal cancer and adjacent normal tissue. Int J Colorectal Dis 2004;19:95–101.
38. Shen L, Toyota M, Kondo Y, et al. Integrated genetic and epigenetic analysis identifies three different subclasses of colon cancer. Proc Natl Acad Sci U S A 2007;104:18654–9.
39. Nagasaka T, Koi M, Kloor M, et al. Mutations in both KRAS and BRAF may contribute to the methylator phenotype in colon cancer. Gastroenterology 2008;134:1950–60.
40. Ogino S, Kawasaki T, Kirkner GJ, Ohnishi M, Fuchs CS. 18q loss of heterozygosity in microsatellite stable colorectal cancer is correlated with CpG island methylator phenotype-negative (CIMP-0) and inversely with CIMP-low and CIMP-high. BMC Cancer 2007; 7:72.
41. Bariol C, Suter C, Cheong K, et al. The relationship between hypomethylation and CpG island methylation in colorectal neoplasia. Am J Pathol 2003;162:1361–71.
42. Cravo ML, Mason JB, Dayal Y, et al. Folate deficiency enhances the development of colonic neoplasia in dimethylhydrazine-treated rats. Cancer Res 1992;52:5002–6.
43. Shelnutt KP, Kauwell GP, Gregory JF III, et al. Methylenetetrahydrofolate reductase 677C->T polymorphism affects DNA methylation in response to controlled folate intake in young women. J Nutr Biochem 2004;15:554–60.
44. Friso S, Choi SW, Girelli D, et al. A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. Proc Natl Acad Sci U S A 2002;99:5606–11.
45. Kim YI. Nutritional epigenetics: impact of folate deficiency on DNA methylation and colon cancer susceptibility. J Nutr 2005;135:2703–9.
46. Friso S, Girelli D, Trabetti E, et al. The MTHFR 1298A>C polymorphism and genomic DNA methylation in human lymphocytes. Cancer Epidemiol Biomarkers Prev 2005;14:938–43.
47. Stern LL, Mason JB, Selhub J, Choi SW. Genomic DNA hypomethylation, a characteristic of most cancers, is present in peripheral leukocytes of individuals who are homozygous for the C677T polymorphism in the methylenetetrahydrofolate reductase gene. Cancer Epidemiol Biomarkers Prev 2000;9:849–53.
48. Castro R, Rivera I, Ravasco P, et al. 5,10-Methylenetetrahydrofolate reductase (MTHFR) 677C->T and 1298A->C mutations are associated with DNA hypomethylation. J Med Genet 2004;41:454–8.
49. Cho NY, Kim BH, Choi M, et al. Hypermethylation of CpG island loci and hypomethylation of LINE-1 and Alu repeats in prostate adenocarcinoma and their relationship to clinicopathological features. J Pathol 2007;211:269–77.
50. Moore LE, Pfeiffer RM, Poscablo C, et al. Genomic DNA hypomethylation as a biomarker for bladder cancer susceptibility in the Spanish Bladder Cancer Study: a case-control study. Lancet Oncol 2008;9:359–66.
51. Ogino S, Kawasaki T, Nosho K, et al. LINE-1 hypomethylation is inversely associated with microsatellite instability and CpG island methylator phenotype in colorectal cancer. Int J Cancer 2008;122:2767–73.
52. Cravo ML, Camilo ME. Hyperhomocysteinemia in chronic alcoholism: relations to folic acid and vitamins B(6) and B(12) status. Nutrition 2000;16:296–302.
53. Smith IM, Mydlarz WK, Mithani SK, Califano JA. DNA global hypomethylation in squamous cell head and neck cancer associated with smoking, alcohol consumption and stage. Int J Cancer 2007;121: 1724–8.
54. Kazazian HH, Jr. Mobile elements: drivers of genome evolution. Science 2004;303:1626–32.
55. Weisenberger DJ, Campan M, Long TI, et al. Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res 2005; 33:6823–36.
56. Yang AS, Estecio MR, Doshi K, Kondo Y, Tajara EH, Issa JP. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res 2004;32:e38.

# Global DNA Hypomethylation (LINE-1) in the Normal Colon and Lifestyle Characteristics and Dietary and Genetic Factors

Jane C. Figueiredo, Maria V. Grau, Kristin Wallace, et al.

*Cancer Epidemiol Biomarkers Prev* 2009;18:1041-1049.