Published in final edited form as:
Cancer Epidemiol Biomarkers Prev. 2009 April ; 18(4): 1041–1049. doi:10.1158/1055-9965.EPI-08-0926.

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

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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 approximately three years after the qualifying exam, we obtained two biopsies of the normal-appearing mucosa from the right colon and two 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 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.

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

Keywords

Global LINE-1 methylation; Folate; Folic Acid; Clinical Trial; Colon; Lifestyle; Dietary; Polymorphisms; Adenomas

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 (MTHF), is involved in the remethylation of homocysteine (Hcy) to methionine, the precursor of S-adenosylmethionine (SAM), 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 tissue 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, 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 co-factors 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 to left side of the colon (29).

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.

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 three-by-two factorial design, with 1 mg of 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 prior to recruitment, one or more histologically-confirmed adenomas removed within 16 months prior to 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 prior to recruitment. Follow-up colonoscopy was obtained from 1,081 individuals approximately 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 IRB approval at hospital (n=3), unknown (n=1). Our analysis includes data from 1000 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, one subject had only one biopsy from the left colon, 274 subjects had two biopsies (273 from both sides of the colon and one from the right colon only), one subject had three biopsies (one from the right and two 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 and one from the left colon).

The samples were collected in 2cc 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 tissue 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 non-neoplastic. Advanced lesions were defined as invasive carcinoma or adenomas with at least 25% villous component, high grade dysplasia, or estimated size of 1 centimeter or greater.

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 has been described previously[1]. 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.

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

Measurement of Baseline Circulating Levels—Blood samples from non-fasting 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 1100 g for 10 minutes. 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 B_6 (pyridoxal 5'phosphate, PLP, 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 sulphate resistant strain of Lactobacillus leichmannii (33) and red blood cell (RBC) folate was determined by the ACS:180® folate assay, a competitive immunoassay using direct chemi-luminescent technology (Bayer Corporation, Tarrytown NY). Plasma homocysteine was analyzed by HPLC 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-beta-synthase (*CBS*)-1080 C>T, A360A (rs1801181) and *CBS*-699 C>T, Y233Y (rs234706), methylenetetrahydrofolate reductase (*MTHFR*)-677 C>T, V222A (rs1801133) and -1298 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, Foster City, CA). Each assay contained quality control DNAs 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 (long interspersed nucleotide elements) 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 performed as previously reported (36). Methylation analysis of LINE-1 promoter (GenBank accession number X58075) was investigated using a pyrosequencing-based methylation analysis. We carried out 50 μl PCR in 60 mM Tris HCl pH 8.5, 15 mM ammonium sulfate, 2 mM MgCl2, 10% DMSO, 1 mM dNTP mix, 1 unit of 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 of bisulfite-treated genomic DNA. PCR cycling conditions were 95°C for 30s, 50°C for 30s and 72°C for 30s 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 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, Inc., Westborough, MA). In brief, 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. Then, 0.3 μM pyrosequencing primer (5'-GGGTGGGAGTGAT-3') was annealed to the purified single-stranded PCR product and pyrosequencing was performed using the PSQ HS 96 Pyrosequencing System (Pyrosequencing, Inc.).

Statistical Analysis

We compared individuals included in this study with those that we were unable to assay regarding selected characteristics using a chi-squared 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. Since 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, 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 1 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 prior to 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, 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 co-dominant model.

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

Over-dispersed 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 (i.e., greater than 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 with 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 to 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 3-year colonoscopy, the difference was also minimal (mean=63.83, 95% CI=62.86-64.80 vs. 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; 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 BMI (Table 2). The association between LINE-1 methylation and smoking and alcohol appeared to be modified by site within the colon (right vs. 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, 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 RR of any adenoma occurrence associated with a one standard deviation increase in LINE1-methylation was 1.00 (95% CI=0.91-1.10). For advanced lesions, the same increase in LINE-1 methylation was associated with a non-significantly 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's for folic acid treatment =1.06 (95% CI=0.83-1.36) and 0.94 (95% CI=0.73-1.20) respectively, p-interaction=0.67). For advanced lesions, the risk associated with folic acid treatment was non-significantly greater among individuals in the lowest tertile of LINE-1 methylation than among those in the highest tertile (adjusted RR=1.41, 95% CI=0.71-2.85 vs. 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/day of 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, homocysteine, and selected genotypes. Higher levels of global hypomethylation were observed among Hispanics and other racial groups compared to 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 (CIMP) phenotype and microsatellite instability (MSI) and absence of KRAS mutations are noted characteristics of cancers in the right compared to the left colon (38,39) and previous studies have suggested that LINE-1 methylation is inversely correlated with CIMP-high and MSI-high in colorectal cancers (26,29,40,41).

Conflicting evidence exists on the relationship between folate and DNA methylation in the colorectum. Cravo and Mason 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, dietary or circulating folate or other B-vitamin co-factors 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-adenosyl-L-methionine (SAM)-dependent methylation reactions) and observed no association. However, Friso 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 upon 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 methionine synthase (*MTR*-A2756G) (26,28).

In agreement with our results, previous studies have reported no relationship with either age and 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 (SAM), 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 SAM. 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 to 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 1000 samples for analysis, and therefore did not have all four 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 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 18-60% 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 to 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.

Acknowledgments

This work was supported in part by grants (R01-CA-105346, R01-CA-059005, U54-CA-100971) from the National Institutes of Health. J.C.F. is supported in part by a post-PhD Research Fellowship from the National Cancer Institute of Canada (#017602). We thank all the individuals who participated in this clinical trial.

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Abbreviations

CI
: confidence interval

PLP
: pyridoxal 5'phosphate

RBC
: red blood cell

RR
: rate ratio

Table 1

Comparison of individuals in the Aspirin/Folate Polyp Prevention Trial who participated and did not participate in this Study

Characteristic	Individuals excluded in this study	Individuals included in this study	p-value**
No. of participants	733	388	
Age at baseline, mean ± SD (y)	57.3 ± 9.9	57.8 ± 9.1	*0.83*#
Male Sex, No. (%)	466 (63.6)	246 (63.4)	*0.96***
Body-mass index > 30 kg/m^2, No. (%)	176 (24.0)	77 (19.9)	*0.12***
Current cigarette smoker, No. (%)	106 (14.5)	58 (15.0)	*0.97***
Colorectal cancer in first-degree relative, No. (%)	224 (36.9)	117 (37.9)	*0.78***
Self identified as White, No. (%)	633 (86.4)	325 (83.8)	*0.24***
Aspirin Treatment Group¥, No. (%)	489 (66.7)	260 (67.0)	*0.92***
Folate Treatment Group, No. (%)	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 Hcy, mean ± SD (μmol/L)	10.1 ± 4.2	9.9 ± 2.95	*0.80*¶
Multivitamin use, No. (%)	265 (36.3)	130 (33.5)	*0.36***
Dietary intake			
Folate intake, mean ± SD (mcg/day)	327.9 ± 6.1	305.0 ± 7.7	*0.01*#
Vitamin B_2, mean ± SD (mg/day)	1.79 ± 0.03	1.72 ± 0.43	*0.11*#
Vitamin B_6, mean ± SD (mg/day)	1.67 ± 0.03	1.64 ± 0.04	*0.26*#
Vitamin B_{12}, mean ± SD (mg/day)			
Alcohol (drinks per day), No (%)	217 (31.5)	123 (33.0)	*0.21***
None	341 (49.4)	165 (44.2)	
1 or less	132 (19.1)	85 (22.8)	
2 or more			
Adenoma Characteristics (at baseline) §			
Number lifetime adenomas (mean ± SD)	2.44 ± 2.30	2.22 ± 1.94	*0.11*¶
Large baseline adenomas (>1 cm), No. (%)	157 (21.4)	96 (24.7)	*0.21***
Baseline adenomas with villous histology, No. (%)	*107 (14.6)*	*49 (12.6)*	*0.37***
Baseline adenomas with proximal location, No. (%)	342 (46.7)	164 (42.3)	*0.16***

¥ 81 and 325 mg/day aspirin treatment groups combined

§ using standard definitions by Polyp Prevention Study Group (30,31)

** chi-square test

two sample t-test

¶non-parametric Mann-Whitney test

Table 2

Association between LINE-1 methylation and selected demographic and lifestyle variables

	Counts[‡]	Overall		Left side		Right side		p-value[¥]
	$N_T/N_L/N_R$	Mean (95% CI)	p-value[§]	Mean (95% CI)	p-value[§]	Mean (95% CI)	p-value[§]	
Age (yr)								
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)		
BMI (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-value for trend;

¥ p-value for interaction testing differences between right and left side of the colorectum.

Table 3

Association between LINE-1 methylation and dietary intake of B-vitamins and multivitamin use

	Counts‡	Overall		Left side		Right side		p-value¥
	$N_T/N_L/N_R$	Mean (95% CI)	p-value§	Mean (95% CI)	p-value§	Mean (95% CI)	p-value§	
Dietary folate (mcg/day)								
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 (376.17-1285.59)	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/day)								
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/day)								
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)		

‡ 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-value for trend;

¥ p-value for interaction testing differences between right and left side of the colorectum.

ǂ 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 2000 calories/day diet.

Table 4

Association between LINE-1 methylation and circulating levels of B vitamins and homocysteine

	Counts‡	Overall		Left side		Right side		p-value¥
	$N_T/N_L/N_R$	Mean (95% CI)	p-value§	Mean (95% CI)	p-value§	Mean (95% CI)	p-value§	
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*

	Counts‡	Overall		Left side		Right side		p-value¥
	$N_T/N_L/N_R$	Mean (95% CI)	p-value§	Mean (95% CI)	p-value§	Mean (95% CI)	p-value§	
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-value for trend;

¥p-value for interaction testing differences between right and left side of the colorectum.

Table 5

Association between LINE-1 methylation and selected polymorphisms

	Counts[‡]	Overall		Left side		Right side		p-value[¥]
	$N_T/N_L/N_R$	Mean (95% CI)	p-value[§]	Mean (95% CI)	p-value[§]	Mean (95% CI)	p-value[§]	
***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-value for trend;

¥ p-value for interaction testing differences between right and left side of the colorectum.