Research Article

Cancer Epidemiology, Biomarkers & Prevention

Prospective Study of Genomic Hypomethylation of Leukocyte DNA and Colorectal Cancer Risk

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Abstract

Background: Systematic genome-wide reductions of methylated cytosine (5-mC) levels have been observed in colorectal cancer tissue and are suspected to play a role in carcinogenesis, possibly as a consequence of inadequate folate intake. Reduced 5-mC levels in peripheral blood leukocytes have been associated with increased risk of colorectal cancer and adenoma in cross-sectional studies.

Methods: To minimize disease- and/or treatment-related effects, we studied leukocyte 5-mC levels in prospectively collected blood specimens of 370 cases and 493 controls who were cancer-free at blood collection from the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial. Leukocyte 5-mC level was determined by a high-pressure liquid chromatography (HPLC)/tandem mass spectrometry method and expressed as the relative amount of methyl to total cytosine residues, or %5-mC. We estimated the association between colorectal cancer risk and %5-mC categories by computing ORs and 95% confidence intervals (CI) through logistic regression modeling.

Results: We observed no dose-dependent association between colorectal cancer and%5-mC categories (lowest vs. highest tertile: OR, 1.14; 95% CI, 0.80–1.63; $P_{trend} = 0.51$). However, among subjects whose 5-mC levels were at the highest tertile, we observed an inverse association between natural folate intake and colorectal cancer (highest tertile of natural folate vs. lowest: OR, 0.35; 95% CI, 0.17–0.71; $P_{trend} = 0.003$; $P_{interaction} = 0.003$).

Conclusions: This prospective investigation show no clear association between leukocyte 5-mC level and subsequent colorectal cancer risk but a suggestive risk modification between 5-mC level and natural folate intake.

Impact: Adequate folate status may protect against colorectal carcinogenesis through mechanisms involving adequate DNA methylation in the genome. *Cancer Epidemiol Biomarkers Prev; 21(11); 2014–21. ©2012 AACR.*

Introduction

DNA methylation plays an important role in regulating a variety of cellular processes. The presence of DNA methylation is responsible for X-chromosome inactivation, gene silencing, imprinting, and repression of repetitive elements and endogenous retroviruses, whereas the absence of methylation in CpG islands near promoter regions is necessary for transcription of specific genes to proceed. Both systematic genome-wide (global) reductions of methylated cytosines and region-specific increases of methylated cytosines in CpG islands are common epigenetic events thought to play a role in carcinogenesis (1, 2). While hypermethylation occurs chiefly in gene promoter regions affecting gene control via impairing transcription, genomic hypomethylation occurs not only in transcription control regions such as promoters but also in repetitive DNA sequences, such as satellite and LINE repeats, retrotransposons, and endogenous retroviral elements, causing altered chromatin structure, chromosomal instability, aneuploidy, and higher mutation rates (3, 4).

Genomic DNA hypomethylation has been observed in somatic tissue of sporadic colorectal cancer (5), its precursor adenoma (6), and even adjacent normal-appearing mucosa of patients with colorectal neoplasia (7). It is thought to occur gradually in an age-dependent manner and to play an early role in the process of colorectal carcinogenesis (8). Genomic DNA methylation levels in colorectal tumors have been positively correlated with

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microsatellite instability and the CpG island methylator phenotype (9, 10), whereas hypomethylation has been associated with poor prognosis (11). It has been speculated that the effects of reduced DNA hypomethylation (i.e., sufficient methylation) may mediate the reported reduction in colorectal cancer risk associated with high dietary intake of folate, a B vitamin present in green leafy vegetables, fruits, dairy products, and potatoes that provides one-carbon groups for DNA synthesis, repair, and methylation (12, 13).

Two recent cross-sectional studies of colorectal cancer and adenoma measured genomic DNA methylation levels in peripheral blood leukocyte DNA as a surrogate for methylation in colorectal tissue; in both studies, hypomethylation was significantly associated with increased risk (14, 15). However, given that both studies used blood specimens collected after cancer diagnosis, the possibility that the associations reflect reverse causation due to disease-related effects cannot be ruled out.

In the present study, we evaluated genomic DNA hypomethylation in prospectively collected peripheral leukocytes in relation to subsequent colorectal cancer risk and its potential interrelationship with folate deficiency and other risk factors.

Materials and Methods

The PLCO trial

The Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial is a randomized trial of approximately 155,000 men and women ages 55 to 74, enrolled between 1993 and 2001 from 10 U.S. centers (Birmingham, AL; Denver, CO; Detroit, MI; Honolulu, HI; Marshfield, WI; Minneapolis, MN; Pittsburgh, PA; Salt Lake City, UT; St Louis, MO; and Washington, DC) to determine whether screening reduces the mortality from prostate, lung, colorectal, and ovarian cancers (16). Participants randomized to the screening arm underwent a 60-cm flexible sigmoidoscopy examination at study entry (T0) and at years 3 or 5 of the trial (T3/T5; the protocol for the second sigmoidoscopy changed from years 3 to 5 in 1999). Subjects with screen-detected abnormalities were referred to their personal physician for diagnostic follow-up (16). Subjects were also sent annual questionnaires asking about recent cancer diagnoses. Medical records were obtained for all subjects with an abnormal sigmoidoscopic examination, or all colorectal cancers reported on the questionnaires or through death certificates; therefore, all colorectal cancers were pathologically verified. Information on colorectal cancer location (proximal or distal) and stage (I, II, III, or IV) were based on pathology reports. In the present analysis, we considered cecum, appendix, ascending colon, hepatic flexure of colon, transverse colon, and splenic flexure of colon as proximal colon, descending colon, sigmoid colon, rectosigmoid junction as distal colon, and rectum. The Trial was approved by the Institutional Review Boards of the National Cancer Institute and the 10 screening centers.

Study population

Participants were eligible for this study if they (i) were randomized to the screening arm, (ii) consented to participate in PLCO ancillary epidemiologic studies of cancer and related diseases, (iii) completed a risk factor questionnaire, (iv) provided a blood sample with DNA source material available in the biorepository (17), and (v) reported a negative history of colorectal cancer. As a result, we identified 462 pathologically verified colorectal cancer cases and 61,445 healthy controls free of colorectal cancer, eligible for study, from whom 528 controls were selected, frequency-matched to the respective case series on age (55–59, 60–64, 65–69, 70–74 years), gender, race (white, black, other), year of randomization, and year since initial sigmoidoscopy screening. Subjects with colorectal cancer (61 cases) or any other cancer (6 cases and 9 controls) diagnosis before the leukocyte DNA blood draw or those with insufficient leukocyte DNA quantity or unsuccessful determination of genomic methylation status (25 cases and 26 controls) were further excluded, resulting in 370 cases and 493 controls for the present analysis. The length of follow-up for selected cases and controls from study entry until selection ranged between 0 and 10 years (median, 3; SD, 2.7).

Questionnaire data

Participants completed a baseline risk factor questionnaire, in which they reported their race, education, body size, use of tobacco, aspirin, ibuprofen, and female hormones, and personal and family history of medical conditions. Screening arm participants were also asked to complete a food frequency questionnaire at baseline, in which they reported their usual dietary intake over the 12 months before enrollment, including use of 137 food items, 12 types of single nutrient or multivitamin supplements, and alcohol. Details on how consumption amounts of nutrients from food and vitamin supplements were calculated are described elsewhere (18). We assessed both pre- and post-fortification folate intake in 3 variables in the present analysis: (i) natural folate (polyglutamates) found naturally in food, (ii) synthetic folic acid added to fortified food, and (iii) total folate equivalents (a combination of natural folate from food and folic acid in fortified food and vitamin supplements: natural folate + folic acid $\times$ 1.7 to reflect higher absorption of the latter). Natural food folate content was assigned using the pre-folic acid fortification (1998) database information from the 1994–1996 Continuing Survey of Food Intake by Individuals (19). Fortified food folate content was assigned on the basis of the post-folic acid grain fortification database information from the Nutrition Data System for Research (20). Considering the mandatory folic acid fortification of grains in the United States that began in 1998, we excluded cases diagnosed before 1998 ($n = 65$) and their matched controls ($n = 87$) in a sensitivity analysis and the results were very similar. Supplemental folic acid use and dose were derived from recent use (current or 2 years ago) of 4 multivitamins (one-a-day type, a therapeutic or high-dose

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type, Stresstabs, and B-complex), assigning a 200-μg folic acid dose for B-complex multivitamin and a 400-μg folic acid dose for the others.

Genomic DNA methylation data

DNA was extracted from stored blood samples using Qiagen standard protocols (QIAamp DNA Blood Midi or Maxi kit). Total genomic DNA methylation levels were determined by a high-pressure liquid chromatography (HPLC)/tandem mass spectrometry method (21) and expressed as the relative amount of methyl-cytosine to total cytosine residues or %5-mC. Internal laboratory quality controls consisted of 3 cell line and commercial samples with known 5-mC contents in every batch. In addition, blinded replicate samples interspersed with study samples (3 samples randomly placed in each of the 40 batches, $n = 120$) showed minimal between-batch variability [coefficient of variation (CV) = 4%].

Statistical analysis

To evaluate the correlation between control subjects' genomic leukocyte DNA methylation levels, and their demographic and lifestyle characteristics, Spearman correlation coefficients and P values were calculated. ORs and 95% confidence intervals (CI) were computed to evaluate the association between categories of %5-mC content, defined using the tertile cutoff points among control subjects, and colorectal cancer risk using unconditional logistic regression, adjusting for age (55–59, 60–64, 65–69, 70–74), gender, race, year of randomization, year since initial sigmoidoscopy screening, smoking, body mass index, use of nonsteroid anti-inflammatory drugs (NSAID), family history of colorectal cancer, and prior history of adenoma, hyperplastic polyps, or inflammatory bowel disease or polyposis syndrome (i.e., ulcerative colitis, Crohn disease, Gardner syndrome, and familial polyposis). Inclusion in the models of other potential confounders such as study center, education, and physical activity did not materially change the results. Trend tests were conducted for risks associated with tertiles of %5-mC content and folate intake using logistic regression models based on ordinal variables. Statistical significance of multiplicative interaction between %5-mC content and folate intake (both by tertile cutoffs) was tested using the Wald test for the interaction term in the logistic regression models.

Results

Cases had a lower level of education ($P = 0.02$) and were less likely to consume synthetic folate from diet ($P = 0.02$) than controls. Other characteristics were comparable between the study groups (Table 1). Among control subjects, modest correlations were found between genomic leukocyte DNA hypomethylation and having first-degree family history of colorectal cancer ($R = 0.097$, $P = 0.03$) and between genomic methylation levels and natural folate intake ($R = 0.095$, $P = 0.04$). No significant correlations with %5-mC level were observed for other nutrients thought to participate in the one-carbon metabolism pathway, such as synthetic folate from diet, total folate equivalents, vitamin B6, vitamin B12, methionine, and alcohol, nor with other potential risk factors, such as age, gender, race, college graduation, smoking status, regular use of aspirin or ibuprofen, hormone use among females, and body mass index.

Examining the %5-mC level as a continuous variable in the logistic regression model, DNA hypomethylation was not significantly associated with colorectal cancer risk (OR = 1.66 per 1.0 unit increase; 95% CI, 0.77–3.54). Categorizing the methylation levels by tertiles in control subjects, we observed no dose–response relationship with colorectal cancer risk ($P_{trend} = 0.51$; Table 2). Additional analyses of finer %5-mC categories, defined using quartiles and deciles as cutoff points, were similarly null (data not shown). Results from analyses stratifying by proximal colon versus distal colon or rectum cancers or by cancer stage were also null. Our findings did not noticeably change in sensitivity analyses including cases whose colorectal cancer diagnoses occurred before the blood draw ($n = 61$; OR, 1.03; 95% CI, 0.74–1.45 for the first vs. third tertile of 5-mC, $P_{trend} = 0.89$), excluding cases whose colorectal cancer diagnoses occurred within a year after the blood draw ($n = 193$; OR, 1.30; 95% CI, 0.79–2.14; $P_{trend} = 0.34$), excluding subjects with a prior history of adenoma or hyperplasic polyps (via pathologic confirmation during the trial or self-report on the baseline questionnaire; $n = 92$ cases and 83 controls; OR, 1.08; 95% CI, 0.69–1.67; $P_{trend} = 0.79$), and excluding cases diagnosed before 1998 (before the mandatory folic acid fortification of grains in the United States., $n = 110$) and matched controls ($n = 134$; OR, 1.15; 95% CI, 0.78–1.70; $P_{trend} = 0.53$).

Intake of natural folate was inversely but not significantly associated with colorectal cancer risk in our data (highest tertile vs. lowest: OR, 0.81; 95% CI, 0.57–1.15; $P_{trend} = 0.23$; Table 3). Upon stratifying by genomic leukocyte DNA methylation levels, we found that the decreasing risk associated with the increasing intake of natural folate was strongest and statistically significant within the stratum of subjects whose DNA methylation levels were in the highest (third) tertile (highest tertile of natural folate intake vs. lowest: OR, 0.35; 95% CI, 0.17–0.71; $P_{trend} = 0.003$; $P_{interaction} = 0.003$). Similarly, reduced DNA methylation level was significantly associated with colorectal cancer risk only among subjects whose natural folate intake levels were at the highest tertile (first tertile of 5-mC vs. third: OR, 2.65; 95% CI, 1.22–5.72; $P_{trend} = 0.02$, $P_{interaction} = 0.003$; Supplementary Table S1). No significant association was found for synthetic folic acid added to the diet, synthetic folic acid in supplements, or total folate equivalents from diet and supplements, irrespective of subjects' DNA methylation status ($P_{interaction}$ = 0.78, 0.12, and 0.38, respectively), nor for other nutrients in the one-carbon pathway such as vitamin B6, vitamin B12, methionine, and alcohol ($P_{interaction} = 0.07$–0.16; Supplementary Table S2). We note that the test of interaction between natural folate and DNA methylation was

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Table 1. Self-reported baseline and clinical characteristics of study subjects, the PLCO Cancer Screening Trial, 1993–2001

	Cases ($N = 370$) *n* (%) or mean (SD)	Controls ($N = 493$) *n* (%) or mean (SD)	*P*
Age group, y			
≤59	83 (22.4)	98 (19.9)	0.78
60–64	127 (34.3)	170 (34.5)	
65–69	93 (25.1)	126 (25.6)	
70–74	67 (18.1)	99 (20.1)	
Male	196 (53.0)	290 (58.8)	0.09
White	327 (88.4)	432 (87.6)	0.74
College graduated or higher	113 (30.6)	188 (38.4)	0.02
First-degree family history of colorectal cancer	50 (13.5)	59 (12.0)	0.50
Current cigarette smoker	41 (11.1)	52 (10.6)	0.86
Former cigarette smoker	162 (43.8)	225 (45.6)	
Regular aspirin or ibuprofen use (≥4/mo)	218 (58.9)	304 (61.7)	0.41
Current hormone use among females	88 (44.0)	112 (50.9)	0.21
Former hormone use among females	33 (16.5)	39 (17.7)	
Body mass index, kg/m^2	27.5 (4.6)	27.5 (5.0)	0.87
Alcohol, g/d	13.8 (29.4)	11.5 (20.4)	0.23
Natural food folate, μg/d	302.0 (121.2)	314.5 (125.8)	0.17
Fortified food folate, μg/d	163.7 (91.8)	181.9 (122.3)	0.02
Total folate equivalents (food and supplements), μg/d	1,056.8 (722.8)	1,088.8 (727.8)	0.54
Vitamin B6 (food and supplements), mg/d	2.37 (0.9)	2.47 (1.1)	0.16
Vitamin B12 (food and supplements), mg/d	5.7 (3.0)	5.9 (3.4)	0.40
Methionine (food), g/d	1.7 (0.7)	1.7 (0.7)	0.46
Distal carcinoma	238 (51.5)		
Stage I	82 (16.1)		
Stage II	184 (39.8)		
Stage III	111 (24.0)		
Stage IV	93 (20.1)		

Table 2. Risk of colorectal cancer associated with percent genomic leukocyte DNA methylation levels (or %5-mC), the PLCO Cancer Screening Trial, 1993–2001

	Cases ($N = 370$)	Controls ($N = 493$)		
%5-mC	*n*	*n*	%5-mC cutoff	OR (95% CI)
Tertile				
Third	104	160	≥4.11	1.00
Second	146	167	3.95–<4.11	1.44 (1.02–2.03)
First	120	166	<3.95	1.14 (0.80–1.63)
P_{trend}				0.51

NOTE: ORs and 95% CIs calculated by unconditional logistic regression adjusted for age, gender, race, time since initial sigmoidoscopy screening, year of randomization, smoking, body mass index, use of NSAIDs, a family history of colorectal cancer, and a prior history of adenoma, hyperplastic polyps, or inflammatory bowel disease or polyposis syndrome (i.e., ulcerative colitis, Crohn disease, Gardner syndrome, and familial polyposis).

significant at a Bonferroni corrected significance level accounted for all 7 of the evaluated one-carbon pathway factors ($\alpha = 0.007$). No methylation modifying effect was found for other suspected risk factors of colorectal cancer, such as smoking, obesity, and NSAID use ($P_{interaction} = 0.70$, 0.57, and 0.31, respectively).

Discussion

Findings from our study, to our knowledge, the first prospective investigation of DNA methylation and colorectal cancer, do not support an association between global genomic hypomethylation in leukocyte DNA and subsequent colorectal cancer risk. We did, however, observe a suggestive risk modification pattern of reduced colorectal cancer risk associated with increased natural folate intake most pronounced among subjects whose genomic leukocyte DNA methylation levels were in the highest tertile. No statistically significant evidence of interaction was found between DNA methylation level and synthetic folate, other nutrients related to methyl availability, or other suspected risk factors of colorectal cancer.

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Table 3. Risk of colorectal cancer associated with folate intake stratified by percent genomic leukocyte DNA methylation levels (or %5-mC), the PLCO Cancer Screening Trial, 1993–2001

	%5-mC								
	All		First tertile		Second tertile		Third tertile		
	n	OR (95% CI)	*n*	OR (95% CI)	*n*	OR (95% CI)	*n*	OR (95% CI)	*P* interaction
Natural food folate									
First tertile	165/201	1.00	50/86	1.00	59/60	1.00	56/55	1.00	
Second tertile	126/163	0.87 (0.62–1.23)	51/57	1.05 (0.58–1.91)	44/52	1.02 (0.56–1.85)	31/54	0.62 (0.33–1.15)	
Third tertile	104/155	0.81 (0.57–1.15)	44/49	1.33 (0.72–2.47)	43/55	0.87 (0.48–1.58)	17/51	0.35 (0.17–0.71)	
P_{trend}		0.23		0.38		0.64		0.003	0.003
Fortified food folate									
First tertile	143/202	1.00	51/78	1.00	48/65	1.00	44/59	1.00	
Second tertile	154/161	1.33 (0.95–1.85)	56/59	1.82 (1.03–3.23)	66/52	0.94 (0.50–1.76)	32/50	1.01 (0.55–1.84)	
Third tertile	98/156	0.98 (0.68–1.41)	38/55	1.02 (0.53–1.98)	32/50	0.99 (0.51–1.90)	28/51	1.02 (0.57–1.85)	
P_{trend}		0.93		0.90		0.77		0.94	0.78
Total folate equivalents (food + supplements)									
First tertile	164/200	1.00	59/78	1.00	59/68	1.00	46/54	1.00	
Second tertile	118/162	0.96 (0.69–1.35)	43/58	1.01 (0.55–1.84)	44/52	0.87 (0.49–1.56)	31/52	0.97 (0.51–1.85)	
Third tertile	113/157	0.92 (0.65–1.29)	43/56	1.02 (0.57–1.85)	43/47	1.18 (0.65–2.16)	27/54	0.67 (0.35–1.28)	0.38
P_{trend}		0.62		0.94		0.65		0.24	

NOTE: ORs and 95% CIs calculated by unconditional logistic regression adjusted for age, gender, race, time since initial sigmoidoscopy screening, year of randomization, smoking, body mass index, use of NSAIDs, a family history of colorectal cancer, and a prior history of adenoma, hyperplastic polyps, or inflammatory bowel disease or polyposis syndrome (i.e., ulcerative colitis, Crohn disease, Gardner syndrome, and familial polyposis).

Previously, Pufulete and colleagues (15) measured genomic DNA from colonic mucosal cells and leukocytes of 28 patients with colorectal cancer, 35 adenoma, and 76 controls recruited from patients referred for colonoscopy at King's College Hospital At London between August 2000 and May 2001 and found that increased levels of [3H] methyl incorporation into both colonic and leukocyte DNA (i.e., lower levels of DNA methylation) were associated with increased risk for adenoma ($P_{trend} = 0.02$ and 0.01, respectively) and a nonsignificantly increased risk for colorectal cancer ($P_{trend} = 0.09$ and 0.08, respectively). Similarly, Lim and colleagues (14), studying genomic DNA from leukocytes of 115 pairs of colorectal adenoma cases and matched controls among asymptomatic women who participated in a multicenter colonoscopy screening study conducted in the United States from 2000 to 2002, found an increased risk of adenoma associated with lower levels of %5-mC in leukocyte DNA using a reversed-phase HPLC and mass spectrometry method ($P_{trend} = 0.002$).

Unlike the previous studies (14, 15), we observed no clear dose-dependent risk associations between DNA hypomethylation and colorectal cancer risk. It is unlikely that error from poor assay performance is an explanation for our null finding, as we observed high assay reproducibility (CV = 4%) among quality control samples. Attenuation of a true association could in principle be caused by undetected colorectal neoplasia among cases and controls; however, analyses excluding subjects with a prior history of adenoma (via the screen detection in the trial or by self-report on baseline questionnaire) or adjusting for this factor in the logistic models resulted in no material change in our findings. A real association could be missed due to insufficient power; we have sufficient power (>90%) to detect the magnitude of associations reported in the previous studies (14, 15), although our power is insufficient to detect modest associations (e.g., OR below 1.6 for tertile exposure). Reverse causation is a possible explanation for the previous findings, that is, the lowered genomic methylation levels observed among diseased patients in the previous studies can potentially be a disease-induced result, although including cases with colorectal cancer before the blood collection ($n = 61$) or excluding cases whose colorectal cancer diagnoses occurred within a year of the blood draw ($n = 193$) resulted in no notable difference in our findings.

We found a positive correlation between consumption amounts of natural folate intake from food and 5-mC levels in leukocyte DNA and a protective effect of natural folate intake against colorectal cancer only among subjects with higher 5-mC content. A handful of studies have assessed the interrelationship between DNA methylation and folate intake for colorectal tumors, but results have been conflicting, partly due to the limitations of these studies being small in size (14, 22), cross-sectional in nature (14, 22), failing to assess natural folate (22) or differentiate natural from synthetic folate (23, 24), and measuring only a surrogate indicator of genomic DNA methylation level, for example, long interspersed

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nucleotide element-1 (LINE-1; refs. 23, 24). Lim and colleagues (14), assessing adenoma risk in a small cross-sectional study, found a nonsignificant risk reduction with food-derived folate intake but a significant risk modifying effect with DNA methylation for total folate intake (instead of food-derived folate), noting the possibility of chance findings due to the inconsistent findings among different indicators of folate intake. Measuring LINE-1 in somatic tissue of colorectal cancer, Schernhammer and colleagues (24) found that high folate intake protected against LINE-1 hypomethylated colon cancers, whereas measuring LINE-1 in normal colonic mucosa, Figueiredo and colleagues (23) found no association between LINE-1 methylation and risk of adenoma and no differential effect of folate on risk of adenoma according to extent of LINE-1 methylation; both studies did not clearly differentiate natural versus synthetic folate intake. Pufulete and colleagues (22), examining DNA methylation in leukocytes and colorectal mucosa of patients with adenoma before and after folic acid supplementation ($n \sim$ 15 pairs), found a positive correlation between folate supplementation and genomic DNA methylation levels in both leukocytes and colonic mucosa, although the effects of food-derived folate was not examined in the study. Similarly, other studies have associated localized (25) and dietary (26) folate levels with increased genomic DNA methylation in cancer tissues (25) and leukocytes of patients with cancer (26), consistent with animal studies (27).

Because folate and related B vitamins in one-carbon pathway are a source of the methyl group added to DNA-creating 5-methylcytosine, the potential that chronic folate status may influence global DNA methylation level is both plausible and the subject of many studies reviewed above. Folate is essential for DNA synthesis, repair, and methylation. Our findings lead us to speculate that adequate folate status may protect against colorectal carcinogenesis through mechanisms involving adequate DNA methylation in the genome, as methylation level affects DNA stability and gene expression.

Our findings of the methylation-mediating protection effect found only for folate derived from natural source of food (not synthetic folate) are in line with evidence provided by a meta-analysis of 5 cohort and 6 case–control studies reporting a colorectal cancer risk reduction only among subjects consuming higher intakes of food-derived folate (not total folate from foods and supplements; ref. 28) and 2 randomized trials finding no significant effect of folic acid supplementation against colorectal cancer after 3 years of treatment (29, 30). It was suggested that the smaller intake of food folate appeared to offer protection against colorectal cancer risk, whereas the use of (unphysiologically) large doses of folic acid in the intervention studies could result in unmetabolized folic acid in peripheral blood and cause potential adverse effects, for example, reduced cytotoxicity of natural killer cells (31). Despite the plausible mechanisms, we cannot dismiss the possibility that our findings are due to chance, given that we have examined the interrelationship of the methylation marker with a range of suspected risk factors.

Although our sample size ($n = 370$ colorectal cancer cases) is considerably larger than those of previous studies investigating genomic leukocyte DNA methylation levels and colorectal tumor risk [$n = 28$ colorectal cancer cases and 35 adenoma cases by Pufulete and colleagues (ref. 15) and $n = 115$ adenoma cases by Lim and colleagues (ref. 14), respectively] and we have sufficient power (>90%) to detect the magnitude of associations reported in the previous studies, we still do not have adequate power to detect modest associations (e.g., OR below 1.6 for tertile exposure). We have even less power to assess interactions and could have observed merely chance findings or have failed to detect weaker risk modification effects between DNA methylation status and one-carbon nutrients or other lifestyle factors. It would be important to evaluate tissue-specific changes in methylation. Although Pufulete and colleagues found similar positive correlations between folate supplementation and genomic DNA methylation levels in both leukocytes and colonic mucosa (22) and similar risk patterns of colorectal cancer with hypomethylation in both leukocyte and colonic DNA (15), data are currently lacking with respect to how methylation changes correlate between leukocyte and colorectal tumor tissue. We will have opportunities to address this question with the colorectal cancer tissue specimens recently collected in the PLCO Trial.

A major strength of our study includes its prospective design, examining DNA samples (i.e., 5-mC levels) collected before any cancer diagnosis and folate data collected before DNA samples and cancer diagnosis among cases and controls, to rule out reverse causation bias suspected in the previous cross-sectional studies (14, 15, 26, 32). In addition, cases and controls in this study came from 10 different screening centers representing a broad population distribution in the United States, and they were identified from the same source population, which was screened by a standardized procedure for colorectal cancer and adenoma (i.e., cases were not screened on the basis of symptoms and had an equal chance for disease detection as controls). The screen tests done in the trial also allowed us to identify the occurrence of adenoma that otherwise could have been missed in regular studies of colorectal cancer. Furthermore, we observed very small assay variability (CV = 0.04) for 5-mC content.

In this prospective investigation of DNA methylation and colorectal cancer, we found no clear dose-dependent risk patterns associated with the genomic leukocyte DNA methylation status. We did, however, observe that the genomic leukocyte DNA methylation levels were positively correlated with natural folate intake from food and significantly modified colorectal cancer risk associated with natural folate intake, suggesting a mediating role of DNA methylation for folate-related protective effects against colorectal cancer. Replication of this apparent

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interaction in future prospective studies with differentiation between natural versus synthetic folate intake in risk assessment is warranted. The findings may draw implications for future efforts in defining mechanisms of carcinogenesis and identifying biomarkers for colorectal cancer risk stratification.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: W.-Y. Huang, L.J. Su, S.I. Berndt
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