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DIETARY FOLATE, ALCOHOL, AND B VITAMINS IN RELATION TO LINE-1 HYPOMETHYLATION IN COLON CANCER

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Abstract

BACKGROUND AND AIMS—Although critical for methylation reactions, how dietary folate and B vitamins affect global DNA methylation level in colon cancers is currently unknown. Long interspersed nucleotide element-1 (LINE-1) is an emerging indicator of genome-wide DNA methylation level that has previously been linked to colon cancer survival.

METHODS—We examined the association between dietary intake of folate, alcohol, and B vitamins and LINE-1 hypomethylation in 609 incident colon cancers, utilizing the database of two independent prospective cohort studies.

RESULTS—Participants with ≥400μg folate intake per day were significantly less likely to develop LINE-1 hypomethylated colon cancers than those reporting <200μg of folate intake per day (Relative risk (RR)=0.57, 95% confidence interval (CI)=0.36–0.91) for <55% LINE-1 methylated colon tumors; RR=0.74, 95% CI=0.51–1.06 for 55–64% LINE-1 methylated colon tumors; and RR=1.08, 95% CI=0.66–1.75 for ≥65% LINE-1 methylated tumors; $P_{interaction}$=0.01). By contrast, high alcohol consumption conferred a higher risk of LINE-1 hypomethylated cancers (≥15g alcohol per day versus none, RR=1.67, 95% CI=1.04–2.67 for <55% LINE1 methylated tumors; and RR=1.55, 95% CI=1.10–2.18 for 55–64% LINE-1 methylated tumors) but had no association with ≥65% LINE-1 methylated tumors (RR=1.06, 95% CI=0.69–1.62). High intakes of vitamin B_6, B_{12}, or methionine were not significantly associated with colon cancers, regardless of LINE-1 methylation level.

CONCLUSION—The influence of dietary folate intake and alcohol consumption on colon cancer risk differs significantly according to tumoral LINE-1 methylation level.

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Keywords

methylgroup donors; folate; vitamin B6; colorectal cancer; DNA methylation

INTRODUCTION

DNA methylation is an important epigenetic mechanism that plays a major role in gene silencing, imprinting and repression of endogenous retroviruses [1,2,3]. Genome-wide DNA hypomethylation is believed to play an important role in genomic instability and carcinogenesis [4,5,6,7,8,9]. Several studies indicate a relation between global DNA hypomethylation and chromosomal instability (CIN) in tumor cells [5,8,9,10,11,12]. Moreover, global DNA hypomethylation as determined by repetitive nucleotide elements such as LINE-1 (long interspersed nucleotide element-1) methylation level is inversely correlated with microsatellite instability (MSI) and the CpG island methylator phenotype [CIMP; [13]] In a prior (and to our knowledge, the first) large-scale survival study of 643 colon cancer patients, LINE-1 hypomethylation was associated with poor prognosis [14].

Folic acid and related B vitamins (one-carbon nutrients) are essential for DNA methylation and nucleotide biosynthesis; it is therefore plausible that chronic folate deficiency may be associated with global DNA methylation level. Adequate dietary intake of these nutrients has previously been related to a lower colon cancer risk [15,16,17], whereas alcohol consumption increases colorectal cancer risk,[18] likely through its anti-folate effects [19]. Whether one-carbon nutrient intake differentially affects subtypes of colon cancer stratified by global DNA methylation level has not been studied. We therefore assessed whether the influence of folate and B vitamin intake on colon cancer risk differed according to LINE-1 methylation level in two prospective cohort studies in which folate intake has been inversely associated with the risk of colon cancer [18,20].

MATERIALS AND METHODS

Study Subjects and Covariate Assessment

Two independent prospective cohort studies, the Nurses' Health Study (121,701 women followed since 1976 [21]) and the Health Professionals Follow-up Study (51,529 men followed since 1986 [22]), formed the study population. Information on potential risk factors and newly diagnosed cases of cancer was updated biennially. Dietary intake of various nutrients including folate, vitamin B_6, B_{12}, and methionine as well as daily alcohol consumption were assessed by self-administered semiquantitative food frequency questionnaires (SFFQ; [23,24]) All nutrient contributions including those from supplements were added to the specific nutrient intake from foods to calculate a daily intake for each participant [23]. We assumed an ethanol content of 13.1 g for a 12-ounce (38-dl) can or bottle of beer, 11.0g for a 4-ounce (12-dl) glass of wine, and 14.0 g for a standard portion of spirits. After excluding participants who did not complete the baseline dietary questionnaire, or who reported a baseline history of cancer (except non-melanoma skin cancer), inflammatory bowel disease, hereditary nonpolyposis colorectal cancer, or a familial polyposis syndrome, 88,691 women and 47,365 men were eligible for analysis.

Ascertainment of Colon Cancer and Deaths

We included colon cancers reported in the NHS and the HPFS on biennial questionnaires between the return of the 1980 or 1986 questionnaires, respectively, and June 1, 2002. With permission from study participants, colon cancer was confirmed through physicians' review of the participants' medical records. If permission was denied, we attempted to confirm the

self-reported cancer with an additional letter or phone call. We also searched the National Death Index to identify deaths among nonrespondents. The computerized National Death Index is a highly sensitive method for identifying deaths in these cohorts [25]. For all deaths attributable to colon cancer, we requested permission from family members (subject to state regulation) to review the medical records. Colon cancer was considered the cause of death if the medical records or autopsy reports confirmed fatal colon cancer or if colon cancer was listed as the underlying cause of death without another more plausible cause. We collected paraffin-embedded tissue blocks from hospitals where colon cancer patients underwent resections of primary tumors [22]. In a previous analysis of these cohorts, folate intake was significantly associated with the risk of colon cancer but had no influence on the risk of rectal cancer [20]; as a result, we did not include incident rectal cancer among the study participants in this analysis. Like rectal cancer cases, cases of colon cancer for which we were unable to assess LINE-1 methylation level were censored from the analyses at their date of diagnosis and were not included as endpoints.

Quantification of tumoral LINE-1 methylation levels

Based on the availability of adequate tissue specimens, we analyzed 606 colon cancers for LINE-1 methylation level. Characteristics of those cancers for whom we did and did not analyze for molecular markers have previously been found to be very similar.[22] In order to accurately quantify relatively high methylation levels, we utilized Pyrosequencing technology using the PyroMark kit and the PSQ HS 96 System (Biotage, Uppsala, Sweden) as previously described [13]. The nucleotide dispensation order was: ACT CAG TGT GTC AGT CAG TTA GTC TG. Complete conversion of cytosine at a non-CpG site ensured successful bisulfite conversion. The percentage of C relative to the sum of the amounts of C and T at each CpG site was calculated. The average of the relative amounts of C in the 4 CpG sites was used as overall LINE-1 methylation level in a given sample. Pyrosequencing to measure LINE-1 methylation has been previously validated and shown to be a good indicator of cellular 5-methylcytocine level [13,26,27].

Statistical Analyses

We used a previously described method of competing risk analysis utilizing duplication method Cox regression to compare the specific association of baseline intake of folate and other nutrients with colon cancer risk according to three categories of LINE-1 methylation level (<55%; 55–64%; and ≥65%) [28,29]. We assessed the statistical significance of the difference between the risk estimates according to tumor type using a likelihood ratio test comparing the model that allowed for separate associations of folate and other nutrients according to LINE-1 methylation level with a model that assumed a common association. To represent interaction effects between dietary folate intake and LINE-1 methylation level, we created models with an indicator variable for LINE-1 methylation level in three categories as well as a product term of this indicator variable and dietary folate intake (continuously), and reported the Wald test statistic of this product term. Established or suspected risk factors for colon cancer were included in the multivariate models, as described at the bottom of Table 2. We used SAS version 9.1.3 (Cary, NC) for all analyses. Tissue collection and analyses were approved by the Harvard School of Public Health and Brigham and Women's Hospital Institutional Review Boards.

RESULTS

Among all 88,691 women and 47,363 men included in these analyses, those with a baseline folate intake of <200 μg/day were slightly more likely to eat meat and to smoke and less likely to exercise or use multivitamins (Table 1).

We documented 609 incident cases of colon cancer accessible for LINE-1 methylation data during 2,563,086 person-years. Of these, the LINE-1 methylation levels of 148 (24.3%) tumors were <55%, 265 (43.5%) were 55–64%, and 196 (32.2%) were ≥65%. LINE-1 methylation levels were approximately normally distributed (mean, 61.4%, median, 62.3%, standard deviation, 9.4). Median time interval between baseline folate intake and the diagnosis of incident colon cancer in our analyses was 17.2 years.

As in our previous studies [18,20,30,31], we identified an inverse association between folate and vitamin B_6 intake and colon cancer risk among all cases in this study. The multivariate relative risk of colon cancer was 0.76 (95% CI, 0.59–0.99) for total daily folate intake of ≥400 μg compared to <200 μg folate (Table 2). The influence of total folate intake differed according to LINE-1 methylation; comparing extreme categories of folate intake (≥ 400 μg/day versus <200 μg/day), the RR was 0.57 (95% CI, 0.36–0.91) for <55% LINE-1 methylated tumors, 0.74 (95% CI, 0.51–1.06) for 55–64% LINE-1 methylated tumors, and 1.08 (95% CI, 0.66–1.75) for ≥65% LINE-1 methylated tumors ($P_{interaction}$ = 0.01). In analyses restricted to folate from dietary sources, these RRs were generally similar albeit slightly weaker (data not shown). Similarly, using folate intake updated until up to 12 years before cancer diagnosis did not materially alter our results.

Next, we examined the influence of vitamin B_6 intake according to LINE-1 methylation (Table 3). The benefit of vitamin B_6 intake also appeared confined to <65% LINE-1 methylated tumors, though none of these associations was statistically significant. Of note, for both folate and B_6 intake, risk was principally elevated among participants in the lowest category, whereas the risk did not appear to decline substantially beyond the second category of exposure. The influence of intake of vitamin B_{12} and methionine (in quintiles) on colon cancer did not appear to differ by LINE-1 status (Table 3).

We further evaluated the influence of alcohol consumption on colon cancer risk according to LINE-1 methylation level. For daily alcohol consumption (Table 3), the overall increased risk of colon cancer with ≥15g alcohol compared to no alcohol consumption (RR 1.41; 95% CI, 1.11–1.79) appeared to be essentially restricted to <65% LINE-1 methylated tumors. Comparing extreme categories of alcohol consumption, the RR was 1.67 (95% CI, 1.04–2.67; P_{trend} = 0.02) for <55% LINE-1 methylated tumors, 1.55 (95% CI, 1.10–2.18; P_{trend} = 0.03) for 55–64%, and 1.06 (95% CI, 0.69–1.62; P_{trend} = 0.87) for ≥65% LINE-1 methylated tumors ($P_{interaction}$ = 0.13).

Because previous analyses have suggested that the association between alcohol consumption and colon carcinogenesis is increased in individuals with inadequate folate intake,[18] we examined dietary contrasts of total folate availability and daily alcohol consumption. The RR in participants with <299 μg folate intake/day and >5g alcohol consumption/day (i.e., dual depleted folate status) was 1.85 (95% 1.12–3.03) for <55% LINE-1 methylated tumors and 1.76 (95% CI, 1.17–2.64) for 55–64% LINE-1 methylated tumors, when compared to participants with ≥300 μg folate intake and <5g alcohol per day, whereas this RR was 1.04 (95% CI, 0.64–1.69) for ≥65% LINE-1 methylated tumors.

DISCUSSION

In this large prospective cohort study, we found that low folate and, to a lesser degree, vitamin B_6 intake and excess alcohol consumption were associated with increased risk of colon cancers with LINE-1 hypomethylation. The elevation in risk was principally limited to participants with the lowest levels of folate and vitamin B_6 intake, and no additional risk reduction was observed for intake beyond the second lowest category of consumption. Specifically, higher doses of either vitamin did not appear to confer any additional benefit. By contrast, the

increased risk with alcohol consumption appeared to follow a linear dose-response. Overall, our data support a possible etiologic link between deficiency in some one-carbon nutrients and genome-wide DNA hypomethylation during colorectal carcinogenesis.

To our knowledge, no prior study has assessed the influence of one-carbon nutrients on colon cancer risk according to LINE-1 methylation level, and only two previous studies have examined colon cancer survival according to LINE-1 methylation level. The larger study was based on data from our own cohorts, reporting LINE-1 hypomethylation to be an independent predictor of shorter survival in colon cancer patients [14]. Another much smaller study (with only 93 tumors) also identified a trend (albeit non-significant) towards poor survival in DNA-hypomethylated tumors [32].

We have previously shown evidence supporting that folate prevents p53 mutational events in colorectal carcinogenesis, but we did observe no influence of folate on p53-wild-type tumors [33]. The processes underlying aggressive tumor behavior in LINE-1 hypomethylated tumors are currently unknown. Possible mechanisms include activation of retroviruses at transposons, which may cause genomic instability, in particular chromosomal instability [34,35].

It is plausible that chronic folate deficiency may be associated with genome-wide DNA hypomethylation, given the importance of folate in DNA methylation and synthesis. Recent experimental data show a significant reduction in global DNA methylation level in colonic epithelial cells of mice with folate deficient diet [36]. A prior study explored the association between folate and other methyl donors and colon cancer subtypes [37]. While the overall inverse association between folate and colon cancer did not differ significantly according to microsatellite instability (MSI) status, there was the suggestion of a slightly stronger association between folate and MSI-high colon tumors (RR 0.79, 95% CI, 0.60–1.03 for microsatellite stable (MSS) /MSI-low colon cancers and RR 0.61, 95% CI, 0.37–1.02 for MSI-high colon cancers). Our current findings of a stronger association between low folate and LINE-1 hypomethylation are in line with a prior report that LINE-1 hypomethylation is inversely associated with MSI in these cohorts [13]. Further, as previously described, survival was poorer among colon cancer patients with deplete prediagnostic plasma folate in our cohorts [38]. If, as suggested by our data, LINE-1 hypomethylated colon tumors occur more frequently in folate deplete individuals, this provides compelling mechanistic support for the association between folate depletion and poor colon cancer survival.

In a recent report, Figueiredo et al. showed that folate supplementation did not alter LINE-1 methylation levels in normal colorectal mucosa [39]. Together with our data, this could suggest that folate levels may not be relevant in terms of LINE-1 methylation in normal mucosa, with relatively normal cellular kinetics, but once neoplasia develops some factor, possibly the increase in cellular proliferation, may reveal the relationship between folate and LINE-1 methylation.

Our study has several important strengths. First, because we collected detailed, updated information on a number of dietary and lifestyle covariates relevant to colon carcinogenesis over up to 22 years of follow-up and with high follow-up rates, we were able to examine long-term exposures to one-carbon nutrients and to take into consideration important confounding factors. Second, our study is prospective, eliminating concerns about differential recall bias, particularly with regard to our dietary assessments. Any remaining bias from exposure misclassification would thus be nondifferential by nature, biasing our results toward the null. Thirdly, our molecular characterization of colon cancer has proven very reliable, resulting in a number of interesting epidemiologic observations relating to colon cancer and tissue biomarkers [13,14,22,37].

Limitations of note relate to folate fortification, which became mandatory in 1998 [40]. We did obtain multiple assessments of one-carbon nutrient intakes prior to fortification. In addition, since the development of colon cancer likely requires some induction period before the onset of a clinically apparent tumor, it is unlikely that the post-fortification folate exposure would substantially influence colon cancer risk through 2002. Another potential limitation is that we were unable to obtain tumor tissue from all cases of confirmed colon cancer in the two cohorts. However, risk factors in cases unavailable for tissue analysis did not appreciably differ from those in cases with tumor tissue available.

In conclusion, we show that the reduced risk of colon cancer associated with replete folate status varies by LINE-1 methylation level, an indicator of global DNA methylation status. Thus, genome-wide DNA hypomethylation may be one mechanism by which folate affects colon cancer risk and survival, but additional studies are needed to further elucidate these preventive effects.

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Table 1

Age and age-standardized baseline characteristics of the Nurses' Health Study and Health Professionals Follow-up cohort*

	Energy-adjusted Folate Intake, µg/day							
	Women				Men			
Characteristic*	**<200**	**200–299**	**300–399**	**≥400**	**<200**	**200–299**	**300–399**	**≥400**
	N=20,907	**N=28,882**	**N=12,997**	**N=25,905**	**N=1,512**	**N=10,121**	**N=13,423**	**N=22,307**
Total intakeψ								
Folate (µg/day)	159	246	341	677	173	258	347	682
Vitamin B_6 (mg/day)	1.59	2.05	2.76	5.15	3.29	3.73	4.80	13.6
Vitamin B_{12} (mg/day)	5.55	6.45	7.78	15.1	7.89	8.79	9.78	16.4
Alcohol (g/day)	6.7	6.4	6.0	6.3	13.4	13.0	11.4	10.5
Methionine (mg/day)	1.74	1.86	1.95	1.93	2.03	2.13	2.20	2.21
Calcium (mg/day)	574	710	797	796	577	743	858	987
Beef, pork, or lamb as a main dish (servings/week)	3.1	2.6	2.3	2.3	2.4	2.1	1.8	1.5
Other characteristics*								
Median age (yr)	46.6	46.8	46.8	46.6	54.4	54.4	54.4	54.4
Former or current smoker (%)	60	56	54	55	60	55	51	50
Pack-yr †	23.3	20.4	18.7	19.2	31.7	27.2	24.1	23.1
Regular aspirin user	31	32	32	35	26	27	28	32
Body mass index (kg/m^2)‡	24.4	24.5	24.3	24.0	25.8	25.9	25.6	25.3
Physical activity, METS/wk (%) §	11.1	13.8	15.8	15.6	12.9	17.0	20.5	23.7
Post-menopausal (%) ¶	44	44	44	44	–	–	–	–
Never used hormones (%)	64	62	61	59	–	–	–	–
Past use of hormones (%)	18	19	19	19	–	–	–	–
Current use of hormones (%)	18	19	20	22	–	–	–	–
Current multivitamin use (%)	8	13	24	84	12	15	23	67
Prior lower endoscopy (%)	2	2	2	2	22	24	26	27
Colorectal cancer in a parent or sibling (%)	8	8	7	8	9	8	8	9

[*] Dietary intake and other characteristics at baseline questionnaire in 1980 (NHS) and 1986 (HPFS). Mean value, unless otherwise indicated. All values have been directly standardized according to the age distribution of the cohort.

[†] Pack-years were calculated for former and current smokers only.

[‡] The body-mass index is the weight in kilograms divided by the square of the height in meters.

[§] METS are metabolic equivalents. This was calculated based on the frequency of a range of physical activities (such as jogging) in 1986.

[¶] Hormones are defined as post-menopausal estrogen or estrogen/progesterone preparations. Percent of never, past, and current use was calculated among post-menopausal women only.

[ψ] Nutrient values (folate, vitamin B_6, B_{12}, methionine, and calcium) represent the mean of energy-adjusted intake.

Table 2

Relative risk of baseline folate intake and colon cancer according to LINE-1 methylation level among 88,691 women from the Nurses' Health Study (NHS) and 47,363 men from the Health Professionals Follow-up Study (HPFS).

	Energy-adjusted Folate Intake, μg/day				P_{trend}
	<200	200–299	300–399	≥400	
All cancer cases					
No. cases / Person-years	101 / 461357	166 / 756398	132 / 472313	210 / 873018	
Age-adjusted RR (95% CI)	1.0	0.84 (0.66–1.08)	0.91 (0.70–1.19)	0.79 (0.62–1.00)	0.07
Multivariate RR (95% CI)*	1.0	0.80 (0.62–1.03)	0.82 (0.62–1.09)	0.76 (0.59–0.99)	0.16
LINE-1 low (<55%) cancer cases†					
No. cases / Person-years	31 / 461416	34 / 756511	36 / 472406	47 / 873179	
Age-adjusted RR (95% CI)	1.0	0.57 (0.35–0.92)	0.83 (0.51–1.34)	0.58 (0.37–0.92)	0.05
Multivariate RR (95% CI)*	1.0	0.54 (0.33–0.88)	0.75 (0.46–1.23)	0.57 (0.36–0.91)	0.08
LINE-1 medium (55–64%) cancer cases†					
No. cases / Person-years	47 / 461399	77 / 756478	46 / 472394	95 / 873124	
Age-adjusted RR (95% CI)	1.0	0.84 (0.58–1.21)	0.68 (0.45–1.02)	0.76 (0.53–1.08)	0.21
Multivariate RR (95% CI)*	1.0	0.80 (0.55–1.15)	0.61 (0.40–0.93)	0.74 (0.51–1.06)	0.31
LINE-1 high (≥65%) cancer cases†					
No. cases / Person-years	23 / 461420	55 / 756494	50 / 472383	68 / 873145	
Age-adjusted RR (95% CI)	1.0	1.23 (0.75–2.00)	1.51 (0.92–2.48)	1.11 (0.69–1.79)	0.92
Multivariate RR (95% CI)*	1.0	1.17 (0.72–1.90)	1.36 (0.82–2.26)	1.08 (0.66–1.75)	0.94

*Multivariate models are adjusted for age (continuous), gender, energy intake (kcal), screening sigmoidoscopy or colonoscopy (yes/no), family history of colorectal cancer (yes/no), aspirin use (≥2 tablets/week or less), smoking (packyears), physical activity in METs (quintiles), body mass index in five categories (<21, 21–22.9, 23–24.9, 25–29.9, 30+), colon polyps (yes/no), beef intake (quintiles), calcium intake (quintiles), multi-vitamin use (yes/no), alcohol use (none, <5, 5–14.9, ≥15g/day), and intake of vitamin B_6, B_{12}, and methionine (quintiles).

†LINE-1, long interspersed nucleotide element-1.

Table 3

Risk of colon cancer according to baseline quintiles of one-carbon nutrient intake by tumoral LINE-1 methylation level among 88,691 women and 47,363 men (NHS and HPFS combined).

Energy-adjusted daily one-carbon nutrient intake			**RR (95% CI)***			**P_{trend}**
Methionine (g)	**Q1**	**Q2**	**Q3**	**Q4**	**Q5**	
All cancer cases						
Cases / Person-years	136 / 511094	140 / 512592	99 / 517670	125 / 512400	109 / 509330	
Age-adjusted	1.0	1.02 (0.81–1.30)	0.71 (0.55–0.92)	0.88 (0.69–1.12)	0.72 (0.56–0.93)	0.22
Multivariate*	1.0	1.03 (0.81–1.30)	0.72 (0.55–0.93)	0.91 (0.71–1.17)	0.77 (0.59–1.00)	0.10
LINE-1 low (<55%)†						
Cases / Person-years	33 / 511199	37 / 512685	29 / 517734	23 / 512491	26 / 509403	
Age-adjusted	1.0	1.12 (0.70–1.78)	0.86 (0.52–1.41)	0.67 (0.39–1.14)	0.71 (0.42–1.18)	0.51
Multivariate*	1.0	1.12 (0.70–1.79)	0.86 (0.52–1.43)	0.69 (0.40–1.18)	0.76 (0.45–1.27)	0.36
LINE-1 medium (55–64%)†						
Cases / Person-years	54 / 511174	71 / 512653	36 / 517727	60 / 512457	44 / 509383	
Age-adjusted	1.0	1.31 (0.92–1.86)	0.65 (0.42–0.99)	1.06 (0.74–1.53)	0.73 (0.49–1.09)	0.06
Multivariate*	1.0	1.31 (0.92–1.87)	0.66 (0.43–1.00)	1.10 (0.76–1.60)	0.78 (0.52–1.17)	0.03
LINE-1 high (≥65%)†						
Cases / Person-years	49 / 511171	32 / 514772	34 / 512686	42 / 517727	39 / 509393	
Age-adjusted	1.0	0.65 (0.42–1.02)	0.67 (0.44–1.04)	0.82 (0.54–1.24)	0.71 (0.47–1.09)	0.57
Multivariate	1.0	0.65 (0.42–1.02)	0.68 (0.44–1.06)	0.85 (0.56–1.29)	0.76 (0.50–1.17)	0.81
Vitamin B_6 (mg)	**Q1**	**Q2**	**Q3**	**Q4**	**Q5**	**P Trend**
All cancer cases						
Cases / Person-years	135 / 518267	134 / 514617	116 / 513711	98 / 510531	126 / 505964	
Age-adjusted	1.0	0.91 (0.71–1.15)	0.70 (0.55–0.90)	0.60 (0.47–0.78)	0.77 (0.60–0.98)	0.47
Multivariate*	1.0	0.92 (0.73–1.18)	0.75 (0.58–0.97)	0.66 (0.51–0.87)	0.88 (0.68–1.11)	0.40
LINE-1 low (<55%)†						
Cases / Person-years	36 / 518360	31 / 514695	27 / 513793	22 / 510606	32 / 506058	

Energy-adjusted daily one-carbon nutrient intake	RR (95% CI)*					P_{trend}
Methionine (g)	**Q1**	**Q2**	**Q3**	**Q4**	**Q5**	
Age-adjusted	1.0	0.79 (0.49–1.27)	0.61 (0.37–1.01)	0.51 (0.30–0.86)	0.73 (0.45–1.18)	0.88
Multivariate*	1.0	0.80 (0.50–1.30)	0.65 (0.40–1.08)	0.56 (0.33–0.95)	0.84 (0.52–1.36)	0.83
LINE-1 medium (55–64%)†						
Cases / Person-years	62 / 518333	59 / 514677	48 / 513771	49 / 510573	47 / 506040	
Age-adjusted	1.0	0.87 (0.61–1.24)	0.63 (0.43–0.92)	0.66 (0.45–0.96)	0.62 (0.43–0.91)	0.91
Multivariate*	1.0	0.89 (0.62–1.27)	0.68 (0.46–0.99)	0.72 (0.49–1.06)	0.71 (0.48–1.05)	0.86
LINE-1 high (≥65%)†						
Cases / Person-years	37 / 518353	44 / 514681	41 / 513775	27 / 510596	47 / 506037	
Age-adjusted	1.0	1.09 (0.70–1.68)	0.91 (0.51–1.42)	0.61 (0.37–1.00)	1.05 (0.68–1.61)	0.29
Multivariate	1.0	1.11 (0.72–1.72)	0.97 (0.62–1.52)	0.67 (0.41–1.10)	1.20 (0.77–1.86)	0.25
Vitamin B_{12} (g)	**Q1**	**Q2**	**Q3**	**Q4**	**Q5**	**P Trend**
All cancer cases						
Cases / Person-years	132 / 515939	120 / 515415	120 / 514236	123 / 509953	114 / 507543	
Age-adjusted	1.0	0.88 (0.69–1.13)	0.85 (0.66–1.09)	0.83 (0.65–1.06)	0.74 (0.58–0.96)	0.82
Multivariate*	1.0	0.87 (0.68–1.11)	0.84 (0.66–1.08)	0.86 (0.67–1.11)	0.77 (0.59–0.99)	0.86
LINE-1 low (<55%)†						
Cases / Person-years	34 / 516037	37 / 515487	26 / 514318	27 / 510040	24 / 507630	
Age-adjusted	1.0	1.05 (0.66–1.68)	0.71 (0.43–1.19)	0.71 (0.43–1.17)	0.61 (0.36–1.02)	0.88
Multivariate*	1.0	1.04 (0.65–1.65)	0.71 (0.43–1.18)	0.73 (0.44–1.22)	0.62 (0.37–1.06)	0.84
LINE-1 medium (55–64%)†						
Cases / Person-years	54 / 516011	49 / 515474	51 / 514301	57 / 510012	54 / 507596	
Age-adjusted	1.0	0.88 (0.60–1.30)	0.88 (0.60–1.29)	0.94 (0.65–1.37)	0.86 (0.59–1.25)	0.62
Multivariate*	1.0	0.87 (0.59–1.28)	0.88 (0.60–1.29)	0.97 (0.67–1.42)	0.89 (0.61–1.30)	0.61
LINE-1 high (≥65%)†						
Cases / Person-years	44 / 516016	34 / 515484	43 / 514300	39 / 510027	36 / 507614	
Age-adjusted	1.0	0.75 (0.48–1.17)	0.91 (0.60–1.39)	0.79 (0.52–1.22)	0.71 (0.45–1.10)	0.99
Multivariate	1.0	0.74 (0.47–1.16)	0.91 (0.60–1.39)	0.82 (0.53–1.27)	0.73 (0.47–1.13)	0.97

Energy-adjusted daily one-carbon nutrient intake	RR (95% CI)*				P_{trend}
Methionine (g)	**Q1**	**Q2**	**Q3**	**Q4**	**Q5**	
Alcohol (g)	**No alcohol**	**<5 g/day**	**5–14.9 g/day**	**≥15 g/day**	**P Trend**
All cancer cases					
Cases / Person-years	157 / 759573	165 / 800196	145 / 608441	142 / 395877	
Age-adjusted	1.0	1.06 (0.85–1.31)	1.12 (0.89–1.40)	1.51 (1.21–1.90)	0.0001
Multivariate*	1.0	1.08 (0.88–1.39)	1.10 (0.88–1.39)	1.41 (1.11–1.79)	0.01
LINE-1 low (<55%)†					
Cases / Person-years	35 / 758687	37 / 800305	39 / 608541	37 / 395979	
Age-adjusted	1.0	1.06 (0.67–1.69)	1.35 (0.86–2.13)	1.79 (1.12–2.84)	0.004
Multivariate*	1.0	1.07 (0.68–1.71)	1.34 (0.85–2.12)	1.67 (1.04–2.67)	0.02
LINE-1 medium (55–64%)†					
Cases / Person-years	69 / 758650	70 / 800273	57 / 608526	69 / 395945	
Age-adjusted	1.0	1.02 (0.73–1.42)	1.00 (0.70–1.41)	1.67 (1.19–2.33)	0.002
Multivariate*	1.0	1.03 (0.74–1.44)	0.99 (0.69–1.41)	1.55 (1.10–2.18)	0.03
LINE-1 high (≥65%)†					
Cases / Person-years	53 / 758658	58 / 800285	49 / 608528	36 / 395970	
Age-adjusted	1.0	1.10 (0.76–1.59)	1.11 (0.76–1.64)	1.13 (0.74–1.73)	0.48
Multivariate	1.0	1.12 (0.77–1.62)	1.10 (0.75–1.63)	1.06 (0.69–1.62)	0.87

*All models are adjusted for age (continuous), energy intake, gender, screening sigmoidoscopy or colonoscopy, family history of colorectal cancer, aspirin use, smoking, physical activity in METs, baseline body mass index, a history of colon polyps, beef intake, calcium, multi-vitamin use, and baseline folate, vitamin B_6, B_{12}, methionine, and alcohol if not primary exposure.

Abbreviations: LINE-1, long interspersed nucleotide element-1.