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B Vitamins, Methionine and Alcohol Intake and Risk of Colon Cancer in Relation to *BRAF* Mutation and CpG Island Methylator Phenotype (CIMP)

Eva S. Schernhammer[1,2,3,4]*, Edward Giovannucci[4,5], Yoshifumi Baba[6], Charles S. Fuchs[1,6], Shuji Ogino[6,7]

1 Channing Laboratory, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts, United States of America, 2 Ludwig Boltzmann-Institute for Applied Cancer Research, KFJ-Spital, Vienna, Austria, 3 Applied Cancer Research - Institute for Translational Research Vienna (ACR–ITR VIEnna), Vienna, Austria, 4 Department of Epidemiology, Harvard School of Public Health, Boston, Massachusetts, United States of America, 5 Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts, United States of America, 6 Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts, United States of America, 7 Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts, United States of America

Abstract

Background: One-carbon metabolism appears to play an important role in DNA methylation reaction. Evidence suggests that a low intake of B vitamins or high alcohol consumption increases colorectal cancer risk. How one-carbon nutrients affect the CpG island methylator phenotype (CIMP) or *BRAF* mutation status in colon cancer remains uncertain.

Methods: Utilizing incident colon cancers in a large prospective cohort of women (the Nurses' Health Study), we determined *BRAF* status (N = 386) and CIMP status (N = 375) by 8 CIMP-specific markers [*CACNA1G*, *CDKN2A* (p16), *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3*, and *SOCS1*], and 8 other CpG islands (*CHFR*, *HIC1*, *IGFBP3*, *MGMT*, MINT-1, MINT-31, p14, and *WRN*). We examined the relationship between intake of one-carbon nutrients and alcohol and colon cancer risk, by *BRAF* mutation or CIMP status.

Results: Higher folate intake was associated with a trend towards low risk of CIMP-low/0 tumors [total folate intake ≥400 µg/day vs. <200 µg/day; the multivariate relative risk = 0.73; 95% CI = 0.53–1.02], whereas total folate intake had no influence on CIMP-high tumor risks ($P_{heterogeneity}$ = 0.73). Neither vitamin B_6, methionine or alcohol intake appeared to differentially influence risks for CIMP-high and CIMP-low/0 tumors. Using the 16-marker CIMP panel did not substantially alter our results. B vitamins, methionine or alcohol intake did not affect colon cancer risk differentially by *BRAF* status.

Conclusions: This molecular pathological epidemiology study suggests that low level intake of folate may be associated with an increased risk of CIMP-low/0 colon tumors, but not that of CIMP-high tumors. However, the difference between CIMP-high and CIMP-low/0 cancer risks was not statistically significant, and additional studies are necessary to confirm these observations.

Introduction

DNA methylation is an important epigenetic mechanism in gene silencing and imprinting [1,2,3]. Aberrant epigenetic silencing of tumor suppressors by CpG island hypermethylation is commonly observed in human malignancies. The CpG island methylator phenotype (CIMP) is characterized by propensity for widespread CpG island hypermethylation [4,5]. High degree of CIMP (CIMP-high) is a distinct phenotype in colorectal cancer, associated with older age, female gender, proximal tumor location, microsatellite instability, *BRAF* mutation, and high-level tumor LINE-1 methylation [6,7,8,9]. However, etiologic factors for CIMP-high remain largely speculative. Despite a strong association between CIMP and *BRAF* mutation in colorectal cancer

[5,6,7,8], the hypothesis that *BRAF* mutation causes aberrant CpG island methylation [10] has been highly controversial [11]. Recently, DNMT3B has emerged as one of causes of CpG island methylation in tumor [12,13,14]; nonetheless, the association between DNMT3B and CIMP in colorectal cancer is at most modest [13] and tumor CpG island methylation appears to be influenced by additional factors.

Folic acid and related vitamins B_2, B_6 and B_{12}, are essential for DNA methylation and the production of purine and pyrimidine nucleotides required for DNA synthesis. Considerable epidemiological evidence suggests that a low-folate diet is associated with an increased risk of colorectal cancer [15,16,17]. Likewise, alcohol consumption increases colorectal cancer risk [18], likely through its anti-folate effects [19]. We have recently shown that adequate

folate intake and low consumption of alcohol are protective against LINE-1 hypomethylated colon cancer, but not against LINE-1 hypermethylated colon cancer [20]. While a recent study demonstrated an association between folate and CpG island methylation in normal colorectal tissue [21], how folate or other B vitamin intake influences CpG island methylation or *BRAF* mutation in tumor remains less well understood, and the literature data are somewhat limited [22,23,24,25].

We therefore assessed whether the influence of folate on colon cancer risk differed according to CIMP and *BRAF* status. For this purpose, we used tumor specimens from a prospective cohort study that has previously shown that folate intake was inversely associated with the risk of colon cancer [26]. The availability of detailed and updated information on folate intake and tumor specimens within this cohort permitted a more comprehensive examination of the effect of folate intake on risk of developing CIMP-high and non-CIMP-high colon cancers as well as by *BRAF* mutational status. Specificity in the association between folate and colon cancer to particular tumor phenotypes would further enhance the case for causality and would provide important insights into the carcinogenic mechanisms of folate deficiency.

Materials and Methods

Study Subjects

The Nurses' Health Study (NHS) was established in 1976 when 121,701 U.S. female registered nurses, 30 to 55 years of age, completed a mailed questionnaire [27]. Follow-up within the cohort currently exceeds 92%. We mailed biennial questionnaires to update information and identify newly diagnosed cases of cancer. In 1980, the NHS questionnaire was expanded to include a validated assessment of diet, and updated dietary assessments have been conducted every four years [28]. The institutional review boards at Brigham and Women's Hospital and the Harvard School of Public Health approved this study and the consent procedure.

Assessment of Nutrients

Dietary intake of various nutrients including folate, vitamin B$_6$, B$_{12}$, and methionine were assessed by self-administered semi-quantitative food frequency questionnaires (SFFQ), which were completed in 1980, 1984, 1986, 1990, 1994, and 1998. Nutrient intakes were calculated by multiplying the reported frequency of consumption of each specified food item by the nutrient content of the specified portion size and then summing these products for all food items. Information on multivitamin use and the use of other supplements was also collected, including details on which brand name and type. An extensive database of supplement formulation was then used to calculate specific nutrient contributions from these supplemental sources. These nutrient contributions were subsequently added to the specific nutrient intake from foods to calculate a daily intake for each woman. This method of dietary assessment has been extensively validated and its reliability evaluated [28]. The correlation coefficient was 0.55 for the correlation between total folate intake calculated from the 1980 questionnaire and erythrocyte folate concentrations measured in 1987 in this cohort [29]. Moreover, vitamin B$_6$ intake as assessed by 1980, 1984, and 1986 SFFQs has been shown to correlate with one-week diet records, with correlations ranging from 0.54 to 0.58 [28,30].

Assessment of Other Covariates

Alcohol consumption was the sum of the values for three types of beverages: beer, wine, and spirits. We assumed an ethanol content of 13.1 g for a 12-ounce (38-dl) can or bottle of beer, 11.0 g for a 4-ounce (12-dl) glass of wine, and 14.0 g for a standard portion of spirits. In validation studies, the correlation coefficient for the correlation between alcohol consumption derived from the 1980 SFFQ and the average of four one-week diet records was 0.90 [31]. Other risk factors for colon cancer such as physical exercise and body mass index have generally been assessed biennially on the main questionnaires.

Ascertainment of Colon Cancer Cases and Tumor Tissue Procurement

We included colon cancers reported on the biennial questionnaires between the return of the 1980 questionnaire and June 1, 2004. With permission from study participants, colon cancer was confirmed through physicians' review of the nurses' 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 the nonrespondents to each two-year questionnaire. The computerized National Death Index is a highly sensitive method for identifying deaths in this cohort [32]. For all deaths attributable to colon cancer, we requested permission from family members (subject to state regulation) to review the medical records. We successfully obtained specimens for 58% of cases (n = 527) over 22 years of follow-up in NHS. There was no significant difference in demographic or exposure variables between cases with available tissue and those without available tissue [33].

Genomic DNA extraction and sequencing of *BRAF*

Genomic DNA from paraffin-embedded tissue was extracted, and whole genome amplification was performed by PCR using random 15-mer primers [34]. PCR and sequencing targeted for *BRAF* codon 600 were performed as previously described [35].

Real-time PCR (MethyLight) for quantitative DNA methylation

To determine CIMP status, we quantified DNA methylation in eight CIMP-specific promoters. Bisulfite treatment on genomic DNA and subsequent real-time PCR (MethyLight) [36] were validated and performed as previously described [37]. The eight CIMP-specific promotors that we quantified (*CACNA1G*, *CDKN2A* (p16), *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3*, and *SOCS1*) [5,38] were selected from screening of 195 CpG islands [5,39]. CIMP-high was defined as ≥6/8 methylated markers using the eight-marker CIMP panel, and CIMP-low/0 was defined as 0/8 to 5/8 methylated markers, according to the previously established criteria [38]. In secondary analyses, we added eight more markers (*CHFR*, *MGMT*, *P14*, *WRN*, *HTC1*, *MINT1*, *MINT31*, *IGFBP3*) [7] for a 16-marker panel. Using this 16-marker panel, we created two categories of CIMP (CIMP-high defined as ≥11/16 methylated markers; and CIMP-low/0 as 0/16 to 10/16 methylated markers). Concordance of CIMP-high diagnosis between the 8-marker and 16-marker panels was high (0.97; k = 0.89) [7].

Statistical Analysis

We excluded women who did not complete the baseline 1980 dietary questionnaire or recorded implausible dietary data (n = 29,279), reported a baseline history of cancer (except non-melanoma skin cancer; n = 3,627), inflammatory bowel disease, hereditary nonpolyposis colon cancer, or a familial polyposis syndrome (n = 103), or had died prior to baseline (n = 1). After these exclusions, 88,691 women were eligible for analysis and

Table 1. Baseline characteristics of the Nurses' Health Study cohort[*].

Characteristic[*]	Energy-adjusted Folate Intake, μg/day			
	<200	200–299	300–399	≥400
	N = 20,907	N = 28,882	N = 12,997	N = 25,905
Dietary Intake[Ψ]				
Folate (μg/day)	159	246	341	677
Vitamin B$_6$ (mg/day)	1.59	2.05	2.76	2.15
Vitamin B$_{12}$ (mg/day)	5.55	6.45	7.78	15.06
Alcohol (g/day)	6.35	6.37	6.00	6.33
Methionine (mg/day)	1.74	1.86	1.95	1.93
Calcium (mg/day)	607	723	798	812
Beef, pork, or lamb as a main dish (servings per week)	0.44	0.37	0.32	0.33
Other Characteristics[*]				
Median age (yr)	46.6	46.8	46.8	46.6
Former or current smoker (%)	60	56	54	55
Pack-years[†]	23.3	20.4	18.7	19.2
Regular aspirin user (%)	31	32	32	35
Body mass index (kg/m^2)[‡]	24.4	24.5	24.3	24.0
Physical activity, METS/wk (%)[§]	11.1	13.8	15.8	15.6
Post-menopausal (%)	44	44	44	44
Never used hormones (%)	64	62	61	59
Past use of hormones (%)	18	19	20	22
Current use of hormones (%)	18	19	19	19
Current multivitamin use (%)	8	12	23	84
Prior lower endoscopy (%)	2	2	2	2
Colorectal cancer in a parent or sibling (%)	2	2	2	2
History of prior polyps (%)	8	8	8	8

[*]Dietary intake and other characteristics at baseline questionnaire in 1980 (mean value, unless otherwise indicated).
[Ψ]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 physical activities (such as jogging) in 1986.
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accrued follow-up time beginning on the month of return of their baseline questionnaire and ending on the month of diagnosis of colon cancer, death from other causes, or June 1, 2002, whichever came first. In a previous analysis of this cohort, folate intake was significantly associated with the risk of colon cancer but had no influence on the risk of rectal cancer [26]; as a result, we did not consider incident rectal cancer among the study participants in this analysis. Like rectal cancer cases, cases of colon cancer for which we were unable to quantified DNA methylation were censored from the analyses at their date of diagnosis and were not included as endpoints.

We calculated incidence rates of colon cancer for participants in a specific category of folate intake by dividing the number of incident cases by the number of person-years. We computed relative risks (RR) by dividing the incidence rate in one category by the incidence rate in the reference category and used Cox proportional hazards modeling to control for multiple variables simultaneously and to compute 95% confidence intervals (CI). With the exception of folate, vitamin B$_6$, methionine, and alcohol, for which we used baseline information in our primary analyses, we used the most updated information for all covariates prior to each two-year interval.

To compare the specific effect of intake of folate and other nutrients on colon cancer risk according to CIMP and *BRAF* status, we employed a previously described method of competing risk analysis utilizing duplication method Cox regression [40,41]. This methodology permits estimation of separate regression coefficients for nutrient intake stratified by the type of outcome [e.g. CIMP-high cancer vs. CIMP-low/0 cancer; *BRAF* mutation (+) vs. (−)]. We assessed the statistical significance of the interaction between the risk estimates according to tumor type using a likelihood ratio test that compared the model that allowed for separate associations of folate and other nutrients according to CIMP/*BRAF* status with a model that assumed a common association. We conservatively interpreted statistical significance of findings because of multiple hypothesis testing. To take into account multiple testing (five one-carbon nutrients were examined) we adjusted our significance level to p = 0.01 (= 0.05/5). We used SAS version 9.1.3 (Cary, NC) for all analyses. All P values are two-sided.

Results

Among 88,691 women included in these analyses, those with a baseline folate intake of less than 200 μg/day were slightly more

Table 2. Risk of colon cancer according to baseline quintiles of one-carbon nutrient intake by CIMP expression in tumors among 88,691 women in the Nurses' Health Study (1980–2002).

Energy-adjusted daily one-carbon nutrient intake	RR (95% CI)*					P Trend
Folate (μg)	**Q1**	**Q2**	**Q3**	**Q4**		
	<200	200–299	300–399	≥400		
Cases/Person-years	100/439203	120/606372	55/273313	100/543046		
All cancers‡	1.0	0.78 (0.60–1.02)	0.75 (0.54–1.04)	0.71 (0.53–0.93)		0.06
All cancers*	1.0	0.81 (0.62–1.06)	0.80 (0.57–1.12)	0.78 (0.59–1.05)		0.23
Cases/Person-years	80/439218	92/606395	41/273323	75/543075		
CIMP-low/0‡	1.0	0.75 (0.55–1.01)	0.70 (0.48–1.01)	0.66 (0.48–0.91)		0.04
CIMP-low/0*	1.0	0.78 (0.58–1.05)	0.75 (0.51–1.10)	0.73 (0.53–1.02)		0.15
Cases/Person-years	20/439270	28/606450	14/273348	25/534107		
CIMP-high‡	1.0	0.91 (0.51–1.61)	0.95 (0.48–1.88)	0.88 (0.49–1.59)		0.82
CIMP-high*	1.0	0.95 (0.53–1.68)	1.02 (0.51–2.02)	0.98 (0.54–1.77)		0.94
Methionine (g)	**Q1**	**Q2**	**Q3**	**Q4**	**Q5**	
	≤1.50	1.51–1.70	1.71–1.90	1.91–2.20	≥2.21	
Cases/Person-years	78/371363	87/371303	63/375394	62/372422	85/371052	
All cancers‡	1.0	1.1 (0.81–1.49)	0.77 (0.55–1.07)	0.75 (0.54–1.04)	0.96 (0.71–1.31)	0.81
All cancers*	1.0	1.09 (0.80–1.49)	0.76 (0.54–1.06)	0.74 (0.53–1.04)	0.96 (0.70–1.32)	0.87
Cases/Person-years	56/371384	76/371713	51/375402	39/372445	66/371068	
CIMP-low/0‡	1.0	0.90 (0.64–1.27)	1.03 (0.70–1.51)	0.74 (0.49–1.11)	1.00 (0.69–1.43)	0.44
CIMP-low/0*	1.0	0.75 (0.53–1.07)	0.85 (0.58–1.25)	0.65 (0.43–0.98)	1.04 (0.73–1.49)	0.48
Cases/Person-years	22/371411	11/371771	12/375349	23/372449	19/371104	
CIMP-high‡	1.0	0.39 (0.19–0.81)	0.56 (0.28–1.13)	1.06 (0.59–1.91)	0.69 (0.37–1.27)	0.37
CIMP-high*	1.0	0.49 (0.24–1.01)	0.51 (0.25–1.04)	0.97 (0.54–1.75)	0.77 (0.41–1.42)	0.35
Vitamin B6 (mg)	**Q1**	**Q2**	**Q3**	**Q4**	**Q5**	
	≤1.30	1.31–1.60	1.61–2.00	2.01–3.50	≥3.51	
Cases/Person-years	89/375530	75/372933	70/3744309	64/371267	21/367894	
All cancers‡	1.0	0.78 (0.58–1.06)	0.66 (0.48–0.98)	0.63 (0.46–0.87)	0.74 (0.54–1.00)	0.48
All cancers*	1.0	0.80 (0.58–1.11)	0.70 (0.48–1.01)	0.70 (0.45–1.10)	0.86 (0.56–1.33)	0.22
Cases/Person-years	72/375549	56/372946	55/374322	49/371278	56/367915	
CIMP-low/0‡	1.0	1.35 (0.98–1.96)	0.65 (0.45–0.92)	0.59 (0.41–0.86)	0.66 (0.46–0.94)	0.12
CIMP-low/0*	1.0	1.36 (0.94–1.96)	0.68 (0.45–1.01)	0.66 (0.41–1.07)	0.77 (0.48–1.23)	0.43
Cases/Person-years	17/375591	19/372973	15/374359	15/371307	21/367944	
CIMP-high‡	1.0	1.04 (0.54–1.99)	0.74 (0.37–1.49)	0.77 (0.38–1.54)	1.05 (0.55–1.99)	0.57
CIMP-high*	1.0	1.06 (0.55–2.06)	0.79 (0.38–1.62)	0.86 (0.40–1.85)	1.24 (0.61–2.52)	0.31
Vitamin B12 (g)	**Q1**	**Q2**	**Q3**	**Q4**	**Q5**	
	≤4.0	4.1–5.0	5.1–7.0	7.1–11.0	≥11.1	
Cases/Person-years	98/481642	60/293090	75/317891	64/391812	78/377499	
All cancers‡	1.0	0.95 (0.69–1.31)	1.08 (0.80–1.46)	0.74 (0.54–1.01)	0.89 (0.66–1.20)	0.77
All cancers*	1.0	0.94 (0.68–1.30)	1.08 (0.80–1.47)	0.76 (0.55–1.05)	0.94 (0.69–1.28)	0.94
Cases/Person-years	75/481663	48/293100	55/317904	47/391828	63/377516	
CIMP-low/0‡	1.0	1.01 (0.70–1.45)	1.04 (0.73–1.47)	0.71 (0.49–1.02)	0.94 (0.67–1.31)	0.79
CIMP-low/0*	1.0	1.01 (0.71–1.46)	1.04 (0.73–1.47)	0.73 (0.50–1.05)	0.99 (0.70–1.39)	0.93
Cases/Person-years	23/481706	12/293130	20/317936	17/391850	15/377553	
CIMP-high‡	1.0	0.81 (0.40–1.62)	1.23 (0.68–2.24)	0.83 (0.44–1.56)	0.73 (0.38–1.40)	0.91
CIMP-high*	1.0	0.80 (0.40–1.62)	1.23 (0.68–2.25)	0.86 (0.46–1.62)	0.77 (0.40–1.49)	1.00
Alcohol (g)	**Q1**	**Q2**	**Q3**	**Q4**		
	No alcohol	<5 g/day	5–14.9 g/day	≥15 g/day		
Cases/Person-years	111/595227	139/629283	77/416138	48/221285		
All cancers‡	1.0	1.25 (0.98–1.61)	0.99 (0.74–1.33)	1.12 (0.80–1.57)		0.88

Table 2. Cont.

Energy-adjusted daily one-carbon nutrient intake	RR (95% CI)[*]				P Trend
All cancers[*]	1.0	1.27 (0.99–1.63)	1.01 (0.75–1.36)	1.11 (0.78–1.58)	0.98
Cases/Person-years	80/595254	113/629305	59/416154	36/221297	
CIMP-low/0[‡]	1.0	0.71 (0.53–0.94)	1.06 (0.76–1.48)	1.16 (0.79–1.72)	0.91
CIMP-low/0[*]	1.0	0.70 (0.52–0.93)	1.08 (0.77–1.52)	1.16 (0.77–1.73)	0.79
Cases/Person-years	31/595293	26/629378	18/416189	12/221315	
CIMP-high[‡]	1.0	0.84 (0.50–1.41)	0.83 (0.46–1.48)	1.00 (0.51–1.95)	0.60
CIMP-high[*]	1.0	0.85 (0.50–1.43)	0.84 (0.47–1.51)	1.00 (0.51–1.95)	0.67

[‡]Age adjusted only.
[*]All models are adjusted for age (continuous), energy intake, gender, screening sigmoidoscopy, 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 B6, B12, methionine, and alcohol if not primary exposure. P for heterogeneity of the association for folate intake and CIMP-low/0 colon cancer versus folate intake and CIMP-high colon cancers = 0.73 ($\chi^2 = 1.31$, 3 d.f.).
P for heterogeneity of the association for vitamin B₆ intake and CIMP-high colon cancer and vitamin B₆ intake and CIMP-low/0 colon cancers = 0.63 ($\chi^2 = 2.6$, 4 d.f.) and for vitamin B₁₂ intake = 0.94 ($\chi^2 = 0.79$, 4 d.f.). P for heterogeneity of the association for methionine intake and CIMP-high colon cancer and methionine intake and CIMP-low/0 colon cancers = 0.007 ($\chi^2 = 14.097$, 4 d.f.), and for alcohol intake = 0.32 ($\chi^2 = 3.52$, 3 d.f.).
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likely to smoke and to be sedentary, compared to women with 400 µg daily folate intake or more (Table 1). They were also less likely to use aspirin or postmenopausal hormones regularly. In addition, only 8% of women with less than 200 µg/day folate intake used multivitamins, whereas among those with 400 µg folate or more daily, 84% reported current multivitamin use. Further details have been reported elsewhere [42].

We documented 375 incident cases of colon cancer accessible for quantifying DNA methylation during 1,861,934 person-years. Using DNA methylation assays on these 375 colon cancers, 87 (23%) tumors were found to be CIMP-high. Similarly, BRAF mutational status was available for 386 accessible incident colon cancer cases documented during 1,861,927 person years, of which 86 (22%) were BRAF mutated.

As in our previous studies [18,26,43,44], we observed an inverse association between folate and vitamin B₆ intake and colon cancer risk among all cases in our cohort (Tables 1 and 2).

We further evaluated the influence of one-carbon nutrients on colon cancer risk according to CIMP status in colon cancer. Overall, none of the associations examined reached statistical significance at the conservative significance level (p = 0.01). For total folate intake (Table 2), the benefit of consumption beyond 200 µg per day appeared to be restricted to CIMP-low/0 cancers. Compared to women with less than 200 µg folate per day, the multivariate RR for the development of CIMP-low/0 colon cancer among those reporting 400 µg or more of folate intake per day was 0.73 (95% CI, 0.53 to 1.02; P for trend = 0.15) though statistical significance was not reached. In contrast, total folate intake had no influence on the risk of CIMP-high tumors (RR, 0.98; 95% CI, 0.54–1.77).

We further examined the influence of vitamin B₆ and B₁₂ intake according to CIMP status (Table 2). The benefit of vitamin B₆ intake also appeared confined to CIMP-low/0 cancer (multivariate RR 0.77; 95% CI, 0.48 to 1.23 comparing extreme quintiles), whereas B₆ intake had no influence on the risk of CIMP-high tumors. However, there was no differential effect of vitamin B₁₂ on colon cancer risk by tumoral CIMP status and none of the observed associations reached statistical significance.

We also examined the influence of methionine intake and alcohol intake according to CIMP status (Table 2). There appeared to be a modestly greater reduction in the risk of CIMP-high cancers with increasing methionine intake. We did not observe a significant influence of alcohol intake on colon cancer risk, and the effect did not appear to differ according to CIMP status.

When using our 16-marker panel, overall, results remained unchanged (data not shown).

Similarly, for BRAF mutation status, none of the examined nutrients displayed a significant effect overall, or by mutational status (Table 3).

Discussion

Our current study is a prototypical study in "*Molecular Pathological Epidemiology*", the concept of which has been recently described and consolidated by Ogino et al. [45]. It represents a relatively new, specialized field of epidemiology based on the molecular classification of cancer. In molecular pathologic epidemiology, a known or suspected etiologic factor is examined in relation to a specific somatic molecular change, in order to gain insights into the carcinogenic mechanism [45]. Assume, a given etiologic factor (lifestyle, dietary, environmental or genetic) is hypothesized to cause a certain somatic molecular change. If we can demonstrate a specific relationship between the etiologic factor and the molecular change, it can provide evidence for causality. In addition, for an individual who has a susceptibility to a specific somatic molecular change leading to cancer, we may be able to develop a personalized preventive measure, which targets specific molecules or pathways [45]. Therefore, molecular pathologic epidemiology research can contribute to deciphering the carcinogenic process as well as optimizing preventive strategies [45].

In this prospective cohort study, we found suggestions for both low folate and vitamin B₆ intakes to be associated with an increased risk of CIMP-low/0 colon cancers but not with that of CIMP-high tumors. The elevation in risk was principally limited to participants with the lowest levels of folate and vitamin B₆ intake. In addition, we observed that the effect of a higher methionine intake on colon cancer risk modestly differed by tumoral CIMP status, with the beneficial effect of methionine being principally limited to CIMP-high cancer. There was no difference in the effects of folate or B vitamins on colon cancers stratified by BRAF status. Moreover, none of the observed associations reached statistical significance.

Table 3. Risk of colon cancer according to baseline quintiles of one-carbon nutrient intake by BRAF mutation status in tumors among 88,691 women in the Nurses' Health Study (1980–2002).

Energy-adjusted daily one-carbon nutrient intake	RR (95% CI)*					P Trend
Folate (µg)	**Q1**	**Q2**	**Q3**	**Q4**	**Q5**	
	<200	200–299	300–399	≥400		
Cases/Person-years	102/439203	124/606368	55/273313	105/543043		
All cancers[‡]	1.0	0.79 (0.61–1.03)	0.73 (0.53–1.02)	0.73 (0.55–0.95)		0.10
All cancers*	1.0	0.83 (0.63–1.08)	0.79 (0.57–1.11)	0.81 (0.61–1.08)		0.37
Cases/Person-years	79/439222	99/606387	43/273321	79/543064		
BRAF mutation (−)[‡]	1.0	0.70 (0.40–1.24)	0.71 (0.35–1.42)	0.80 (0.45–1.39)		0.86
BRAF mutation (−)*	1.0	0.74 (0.42–1.30)	0.76 (0.38–1.54)	0.89 (0.51–1.57)		0.87
Cases/Person-years	23/439265	25/606455	12/273350	26/543113		
BRAF mutation (+)[‡]	1.0	0.81 (0.60–1.09)	0.74 (0.51–1.07)	0.71 (0.52–0.96)		0.08
BRAF mutation (+)*	1.0	0.85 (0.63–1.15)	0.80 (0.55–1.17)	0.80 (0.57–1.09)		0.26
Methionine (g)	**Q1**	**Q2**	**Q3**	**Q4**	**Q5**	
	≤1.50	1.51–1.70	1.71–1.90	1.91–2.20	≥2.21	
Cases/Person-years	82/371361	90/371701	65/375393	63/372420	86/371052	
All cancers[‡]	1.0	1.08 (0.80–1.46)	0.76 (0.55–1.05)	0.72 (0.52–1.00)	0.92 (0.68–1.25)	0.62
All cancers*	1.0	1.07 (0.79–1.45)	0.74 (0.53–1.02)	0.70 (0.50–0.98)	0.91 (0.66–1.24)	0.61
Cases/Person-years	24/371412	13/371772	12/375440	19/372454	18/371107	
BRAF mutation (−)[‡]	1.0	1.87 (0.95–3.68)	0.48 (0.24–0.95)	0.74 (0.41–1.36)	0.66 (0.36–1.22)	0.88
BRAF mutation (−)*	1.0	1.90 (0.96–3.73)	0.46 (0.23–0.93)	0.73 (0.40–1.33)	0.65 (0.35–1.20)	0.87
Cases/Person-years	58/371382	77/371710	53/375400	44/372438	68/371064	
BRAF mutation (+)[‡]	1.0	1.31 (0.93–1.84)	0.87 (0.60–1.26)	0.71 (0.48–1.06)	1.03 (0.73–1.47)	0.64
BRAF mutation (+)*	1.0	1.29 (0.92–1.82)	0.85 (0.58–1.24)	0.70 (0.47–1.03)	1.01 (0.71–1.45)	0.62
Vitamin B6 (mg)	**Q1**	**Q2**	**Q3**	**Q4**	**Q5**	
	≤1.30	1.31–1.60	1.61–2.00	2.01–3.50	≥3.51	
Cases/Person-years	93/375527	77/372932	70/374309	66/371265	80/367894	
All cancers[‡]	1.0	0.77 (0.57–1.04)	0.63 (0.46–0.87)	0.62 (0.45–0.85)	0.73 (0.54–0.99)	0.85
All cancers*	1.0	0.77 (0.56–1.07)	0.66 (0.46–0.94)	0.66 (0.42–1.03)	0.82 (0.53–1.26)	0.52
Cases/Person-years	19/375591	15/372978	16/374359	13/371309	23/367948	
BRAF mutation (−)[‡]	1.0	1.37 (0.70–2.69)	0.71 (0.36–1.37)	0.60 (0.29–1.21)	1.03 (0.56–1.89)	0.28
BRAF mutation (−)*	1.0	1.36 (0.68–2.69)	0.73 (0.37–1.47)	0.64 (0.30–1.38)	1.15 (0.58–2.28)	0.19
Cases/Person-years	74/375546	62/372940	54/374323	53/371275	57/367911	
BRAF mutation (+)[‡]	1.0	0.78 (0.56–1.09)	0.62 (0.43–0.87)	0.62 (0.44–0.89)	0.66 (0.46–0.93)	0.62
BRAF mutation (+)*	1.0	0.78 (0.55–1.12)	0.64 (0.43–0.95)	0.67 (0.42–1.07)	0.73 (0.46–1.16)	0.89
Vitamin B12 (g)	**Q1**	**Q2**	**Q3**	**Q4**	**Q5**	
	≤4.0	4.1–5.0	5.1–7.0	7.1–11.0	≥11.1	
Cases/Person-years	104/481636	61/293088	75/317892	68/391811	78/377500	
All cancers[‡]	1.0	0.91 (0.66–1.24)	1.02 (0.76–1.37)	0.74 (0.54–1.00)	0.84 (0.62–1.12)	0.57
All cancers*	1.0	0.90 (0.66–1.24)	1.02 (0.75–1.37)	0.76 (0.56–1.03)	0.88 (0.65–1.19)	0.72
Cases/Person-years	24/481709	11/293131	18/317938	17/391847	16/377559	
BRAF mutation (−)[‡]	1.0	1.41 (0.69–2.89)	1.06 (0.58–1.95)	0.80 (0.43–1.48)	0.74 (0.40–1.40)	0.95
BRAF mutation (−)*	1.0	1.42 (0.70–2.91)	1.06 (0.57–1.95)	0.82 (0.44–1.53)	0.78 (0.42–1.48)	0.86
Cases/Person-years	80/481653	50/293098	57/317903	51/391829	62/377510	
BRAF mutation (+)[‡]	1.0	0.97 (0.68–1.38)	1.01 (0.72–1.42)	0.72 (0.51–1.02)	0.86 (0.62–1.21)	0.46
BRAF mutation (+)*	1.0	0.96 (0.67–1.37)	1.00 (0.71–1.41)	0.74 (0.52–1.05)	0.92 (0.65–1.28)	0.60
Alcohol (g)	**Q1**	**Q2**	**Q3**	**Q4**		
	No alcohol	<5 g/day	5–14.9 g/day	≥15 g/day		
Cases/Person-years	112/595229	145/629277	81/416136	48/221285		
All cancers[‡]	1.0	1.29 (1.01–1.66)	1.04 (0.78–1.38)	1.11 (0.79–1.55)		0.98

Table 3. Cont.

Energy-adjusted daily one-carbon nutrient intake	RR (95% CI)*				P Trend
All cancers*	1.0	1.32 (1.03–1.70)	1.07 (0.80–1.43)	1.11 (0.79–1.58)	0.87
Cases/Person-years	23/595301	33/629373	18/416192	12/221319	
BRAF mutation (−)‡	1.0	0.38 (0.24–0.59)	1.21 (0.61–2.08)	1.35 (0.67–2.71)	0.75
BRAF mutation (−)*	1.0	0.68 (0.40–1.17)	1.16 (0.62–2.15)	1.36 (0.67–2.74)	0.80
Cases/Person-years	89/595247	112/629303	63/416150	36/221294	
BRAF mutation (+)‡	1.0	1.84 (1.42–2.38)	1.48 (1.09–2.00)	1.52 (1.05–2.21)	0.84
BRAF mutation (+)*	1.0	1.29 (0.97–1.70)	1.05 (0.75–1.46)	1.05 (0.71–1.56)	0.75

‡Age adjusted only.
*All models are adjusted for age (continuous), energy intake, gender, screening sigmoidoscopy, 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 B₆, B₁₂, methionine, and alcohol if not primary exposure.
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Mechanistically, it appears plausible that chronic folate deficiency may be involved in CpG island methylation [46]. In a Japanese population, reduced activity of the enzyme methylenetrahydrofolate reductase (MTHFR) due to the MTHFR codon 429 variant (rs1801131), thereby impairing folate metabolism, has been associated with CIMP-high tumors in the proximal colon [47]. In a Caucasian population, genetic variation in the MTHFR rs1801131 variant appears to increase risk of CIMP-high colon tumors [48,49] although this has not been confirmed by the Netherlands Cohort Study data [22,50]. The relation between the MTHFR SNP and CIMP in colon cancer is strong in combination with low folate and methionine intake as well as high alcohol consumption [49,51]. A more recent study suggests that low expression of gamma-glutamyl hydrolase (GGH) is strongly associated with CIMP-high tumors, providing further support for a role of one-carbon metabolism in this phenotype [52].

A prior case-control study of 1,154 colon tumors assessed the influence of one-carbon nutrient intake on CIMP as well as BRAF status in these tumors and found them not to be associated with either CIMP-high or BRAF mutation [51]. However, compared to our current study, nonquantitative methylation-specific PCR on a different CpG island panel, and a less stringent definition of CIMP-high (2 or more of 5 markers methylated) were used in that study. Interestingly, the authors also observed that obesity was associated with a two-fold risk of having a non-CIMP-high tumor [51]. In our study, after carefully adjusting for obesity, both folate and vitamin B₆ (the intake of which is highly correlated in our population) were associated with risk of CIMP-low/0 tumors.

In another case-case study within the Netherlands Cohort Study, colorectal cancer patients in the low folate/high alcohol intake group are more likely to have promoter hypermethylation than patients in the high folate/low alcohol group [53]. Notably, the Netherlands Cohort Study has shown a possible association between vitamin B₆ and MLH1 methylation [22] and an inverse association between MTR rs1805087 polymorphism and CIMP in men [22].

In one of the most recent study to evaluate associations between one-carbon metabolism and colon cancer by CIMP/BRAF status, to date, utilizing a nested case-control approach within the Northern Sweden Health and Disease study (190 cases and 1:2 matched controls) [24], van Guelpen et al. show a lower risk of CIMP-low/CIMP-high colorectal tumors with very low folate levels. Collectively, there is accumulating evidence for the hypothesis that a balance between various metabolic intermediates

of methyl-group influences locus-specific CpG island methylation reaction. Utilizing data from an adenoma prevention trial of folic acid and aspirin, finally, higher RBC folate levels, but not higher dietary folate consumption, was associated with higher methylation levels [21].

Besides influence of one-carbon nutrients, evidence suggests that local DNA sequence context may influence assembly of a methylation reaction machinery and locus-specific DNA methylation. Studies have shown that cis-acting elements cause allele-specific methylation in the mammalian genome [54,55,56]. Thus, germline variations in cis-acting elements may influence epigenetic status including DNA methylation. The MLH1 rs1800734 promoter SNP has been associated with MLH1 promoter methylation [57,58] and MSI [59] in colorectal cancer. Another study has shown that MGMT rs16906252 promoter SNP is strongly associated with MGMT promoter methylation and loss of expression in colorectal cancer [60], and with MGMT methylation in peripheral blood cells and normal colonic mucosa in individuals without colorectal cancer [61,62]. It remains to be investigated whether these or other cis-acting germline variants interact with other one-carbon-related factors to modify susceptibility to aberrant DNA 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 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 on differential recall bias, particularly with regard to our dietary assessments. Any remaining bias from exposure misclassification were thus likely nondifferential by nature, biasing our results only toward the null. Further, we have successfully linked these nutrients—as assessed via a semiquantitative food frequency questionnaire (SFFQ)—to other relevant endpoints in prior analyses, indicating that measurement error is not large enough to hide any real associations.

Limitations of note relate to folate fortification, which became mandatory in 1998 [63]. We have multiple assessments of one-carbon nutrient intakes prior to fortification. Further, our results remained unchanged when restricted to cases that arose prior to folate fortification (1998), which has also been demonstrated in prior analyses [20,42,64]. Other limitations and caveats specific to molecular pathologic epidemiology in general have been discussed elsewhere [45].

We cannot exclude the possibility of residual confounding as a potential explanation for our findings; nonetheless, in our multivariate analyses which included many known or suspected risk factors for colon cancer, the multivariate risk estimates did not materially differ from the age-adjusted results. Further, we were unable to obtain tumor tissue from all cases of confirmed colon cancer detected in the Nurses' Health Study cohort, but the risk factors in these cases did not appreciably differ from those in cases with tumor tissue available. Finally, even prior to mandated fortification in 1998 [63], our participants still had relatively high folate and vitamin B$_6$ levels. It is therefore possible that we might have found even stronger associations in the present study if our folate levels would have included an even lower range.

In summary, we demonstrate that the reduced risk of colon cancer associated with replete folate status is somewhat limited to CIMP-low/0 cancers. Additional studies are necessary to elucidate the exact mechanism of an abnormality in one-carbon metabolism leading to aberrant CpG island methylation.

Acknowledgments

Author Contributions

Conceived and designed the experiments: ES EG CF SO. Performed the experiments: SO YB. Analyzed the data: ES SO. Wrote the paper: ES EG YB CF SO.

References

1. Jones PA, Baylin SB (2007) The epigenomics of cancer. Cell 128: 683–692.
2. Feinberg AP, Ohlsson R, Henikoff S (2006) The epigenetic progenitor origin of human cancer. Nat Rev Genet 7: 21–33.
3. Gosden RG, Feinberg AP (2007) Genetics and epigenetics–nature's pen-and-pencil set. N Engl J Med 356: 731–733.
4. Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, et al. (1999) CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci U S A 96: 8681–8686.
5. Weisenberger DJ, Siegmund KD, Campan M, Young J, Long TI, et al. (2006) CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet 38: 787–793.
6. Samowitz WS, Albertsen H, Herrick J, Levin TR, Sweeney C, et al. (2005) Evaluation of a large, population-based sample supports a CpG island methylator phenotype in colon cancer. Gastroenterology 129: 837–845.
7. Nosho K, Irahara N, Shima K, Kure S, Kirkner GJ, et al. (2008) Comprehensive biostatistical analysis of CpG island methylator phenotype in colorectal cancer using a large population-based sample. PLoS One 3: e3698.
8. Barault L, Charon-Barra C, Jooste V, de la Vega MF, Martin L, et al. (2008) Hypermethylator phenotype in sporadic colon cancer: study on a population-based series of 582 cases. Cancer Res 68: 8541–8546.
9. Ogino S, Goel A (2008) Molecular classification and correlates in colorectal cancer. J Mol Diagn 10: 13–27.
10. Minoo P, Moyer MP, Jass JR (2007) Role of BRAF-V600E in the serrated pathway of colorectal tumourigenesis. J Pathol 212: 124–133.
11. Hinoue T, Weisenberger DJ, Pan F, Campan M, Kim M, et al. (2009) Analysis of the association between CIMP and BRAF in colorectal cancer by DNA methylation profiling. PLoS One 4: e8357.
12. Linhart HG, Lin H, Yamada Y, Moran E, Steine EJ, et al. (2007) Dnmt3b promotes tumorigenesis in vivo by gene-specific de novo methylation and transcriptional silencing. Genes Dev 21: 3110–3122.
13. Nosho K, Shima K, Irahara N, Kure S, Baba Y, et al. (2009) DNMT3B expression might contribute to CpG island methylator phenotype in colorectal cancer. Clin Cancer Res 15: 3663–3671.
14. Karpinski P, Myszka A, Ramsey D, Misiak B, Gil J, et al. (2010) Polymorphisms in methyl-group metabolism genes and risk of sporadic colorectal cancer with relation to the CpG island methylator phenotype. Cancer Epidemiol 34: 338–344.
15. Kim YI (2004) Folate, colorectal carcinogenesis, and DNA methylation: lessons from animal studies. Environ Mol Mutagen 44: 10–25.
16. Giovannucci E (2002) Epidemiologic studies of folate and colorectal neoplasia: a review. J Nutr 132: 2350S–2355S.
17. Harnack L, Jacobs DR, Jr., Nicodemus K, Lazovich D, Anderson K, et al. (2002) Relationship of folate, vitamin B-6, vitamin B-12, and methionine intake to incidence of colorectal cancers. Nutr Cancer 43: 152–158.
18. Giovannucci E, Rimm EB, Ascherio A, Stampfer MJ, Colditz GA, et al. (1995) Alcohol, low-methionine–low-folate diets, and risk of colon cancer in men. J Natl Cancer Inst 87: 265–273.
19. Ma J, Stampfer MJ, Giovannucci E, Artigas C, Hunter DJ, et al. (1997) Methylenetetrahydrofolate reductase polymorphism, dietary interactions, and risk of colorectal cancer. Cancer Res 57: 1098–1102.
20. Schernhammer ES, Giovannucci E, Kawasaki T, Rosner B, Fuchs CS, et al. (2010) Dietary folate, alcohol and B vitamins in relation to LINE-1 hypomethylation in colon cancer. Gut 59: 794–799.
21. Wallace K, Grau MV, Levine AJ, Shen L, Hamdan R, et al. (2010) Association between folate levels and CpG Island hypermethylation in normal colorectal mucosa. Cancer Prev Res (Phila) 3: 1552–1564.
22. de Vogel S, Bongaerts BW, Wouters KA, Kester AD, Schouten LJ, et al. (2008) Associations of dietary methyl donor intake with MLH1 promoter hypermethylation and related molecular phenotypes in sporadic colorectal cancer. Carcinogenesis 29: 1765–1773.
23. Slattery ML, Curtin K, Wolff RK, Herrick JS, Caan BJ, et al. (2010) Diet, physical activity, and body size associations with rectal tumor mutations and epigenetic changes. Cancer Causes Control 21: 1237–1245.
24. Van Guelpen B, Dahlin AM, Hultdin J, Eklof V, Johansson I, et al. (2010) One-carbon metabolism and CpG island methylator phenotype status in incident colorectal cancer: a nested case-referent study. Cancer Causes Control 21: 557–566.
25. Curtin K, Samowitz WS, Ulrich CM, Wolff RK, Herrick JS, et al. (2011) Nutrients in Folate-Mediated, One-Carbon Metabolism and the Risk of Rectal Tumors in Men and Women. Nutr Cancer 63: 357–366.
26. Giovannucci E, Stampfer MJ, Colditz GA, Hunter DJ, Fuchs C, et al. (1998) Multivitamin use, folate, and colon cancer in women in the Nurses' Health Study. Ann Intern Med 129: 517–524.
27. Chen J, Giovannucci E, Hankinson SE, Ma J, Willett WC, et al. (1998) A prospective study of methylenetetrahydrofolate reductase and methionine synthase gene polymorphisms, and risk of colorectal adenoma. Carcinogenesis 19: 2129–2132.
28. Willett WC, Sampson L, Stampfer MJ, Rosner B, Bain C, et al. (1985) Reproducibility and validity of a semiquantitative food frequency questionnaire. Am J Epidemiol 122: 51–65.
29. Giovannucci E, Stampfer MJ, Colditz GA, Rimm EB, Trichopoulos D, et al. (1993) Folate, methionine, and alcohol intake and risk of colorectal adenoma. J Natl Cancer Inst 85: 875–884.
30. Willett WC, Sampson L, Browne ML, Stampfer MJ, Rosner B, et al. (1988) The use of a self-administered questionnaire to assess diet four years in the past. Am J Epidemiol 127: 188–199.
31. Willett WC, Stampfer MJ, Colditz GA, Rosner BA, Hennekens CH, et al. (1987) Moderate alcohol consumption and the risk of breast cancer. N Engl J Med 316: 1174–1180.
32. Stampfer MJ, Willett WC, Speizer FE, Dysert DC, Lipnick R, et al. (1984) Test of the National Death Index. Am J Epidemiol 119: 837–839.
33. Chan AT, Ogino S, Fuchs CS (2007) Aspirin and the risk of colorectal cancer in relation to the expression of COX-2. N Engl J Med 356: 2131–2142.
34. Ogino S, Kawasaki T, Brahmandam M, Yan L, Cantor M, et al. (2005) Sensitive sequencing method for KRAS mutation detection by Pyrosequencing. J Mol Diagn 7: 413–421.
35. Ogino S, Kawasaki T, Kirkner GJ, Loda M, Fuchs CS (2006) CpG island methylator phenotype-low (CIMP-low) in colorectal cancer: possible associations with male sex and KRAS mutations. J Mol Diagn 8: 582–588.
36. Eads CA, Danenberg KD, Kawakami K, Saltz LB, Blake C, et al. (2000) MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res 28: E32.
37. Ogino S, Kawasaki T, Brahmandam M, Cantor M, Kirkner GJ, et al. (2006) Precision and performance characteristics of bisulfite conversion and real-time PCR (MethyLight) for quantitative DNA methylation analysis. J Mol Diagn 8: 209–217.
38. Ogino S, Kawasaki T, Kirkner GJ, Kraft P, Loda M, et al. (2007) Evaluation of markers for CpG island methylator phenotype (CIMP) in colorectal cancer by a large population-based sample. J Mol Diagn 9: 305–314.
39. Ogino S, Cantor M, Kawasaki T, Brahmandam M, Kirkner GJ, et al. (2006) CpG island methylator phenotype (CIMP) of colorectal cancer is best characterised by quantitative DNA methylation analysis and prospective cohort studies. Gut 55: 1000–1006.
40. Lunn M, McNeil D (1995) Applying Cox regression to competing risks. Biometrics 51: 524–532.
41. Glynn RJ, Rosner B (2005) Comparison of risk factors for the competing risks of coronary heart disease, stroke, and venous thromboembolism. Am J Epidemiol 162: 975–982.
42. Schernhammer ES, Ogino S, Fuchs CS (2008) Folate and vitamin B6 intake and risk of colon cancer in relation to p53 expression. Gastroenterology 135: 770–780.

43. Wei EK, Giovannucci E, Selhub J, Fuchs CS, Hankinson SE, et al. (2005) Plasma vitamin B6 and the risk of colorectal cancer and adenoma in women. J Natl Cancer Inst 97: 684–692.
44. Fuchs CS, Willett WC, Colditz GA, Hunter DJ, Stampfer MJ, et al. (2002) The influence of folate and multivitamin use on the familial risk of colon cancer in women. Cancer Epidemiol Biomarkers Prev 11: 227–234.
45. Ogino S, Chan AT, Fuchs CS, Giovannucci E (2011) Molecular pathological epidemiology of colorectal neoplasia: an emerging transdisciplinary and interdisciplinary field. Gut 60: 397–411.
46. Suzuki H, Toyota M, Sato H, Sonoda T, Sakauchi F, et al. (2006) Roles and causes of abnormal DNA methylation in gastrointestinal cancers. Asian Pac J Cancer Prev 7: 177–185.
47. Oyama K, Kawakami K, Maeda K, Ishiguro K, Watanabe G (2004) The association between methylenetetrahydrofolate reductase polymorphism and promoter methylation in proximal colon cancer. Anticancer Res 24: 649–654.
48. Hazra A, Fuchs CS, Kawasaki T, Kirkner GJ, Hunter DJ, et al. (2010) Germline polymorphisms in the one-carbon metabolism pathway and DNA methylation in colorectal cancer. Cancer Causes Control 21: 331–345.
49. Curtin K, Slattery ML, Ulrich CM, Bigler J, Levin TR, et al. (2007) Genetic polymorphisms in one-carbon metabolism: associations with CpG island methylator phenotype (CIMP) in colon cancer and the modifying effects of diet. Carcinogenesis 28: 1672–1679.
50. de Vogel S, Wouters KA, Gottschalk RW, van Schooten FJ, de Goeij AF, et al. (2011) Dietary methyl donors, methyl metabolizing enzymes, and epigenetic regulators: diet-gene interactions and promoter CpG island hypermethylation in colorectal cancer. Cancer Causes Control 22: 1–12.
51. Slattery ML, Curtin K, Sweeney C, Levin TR, Potter J, et al. (2007) Diet and lifestyle factor associations with CpG island methylator phenotype and BRAF mutations in colon cancer. Int J Cancer 120: 656–663.
52. Kawakami K, Ooyama A, Ruszkiewicz A, Jin M, Watanabe G, et al. (2008) Low expression of gamma-glutamyl hydrolase mRNA in primary colorectal cancer with the CpG island methylator phenotype. Br J Cancer 98: 1555–1561.
53. van Engeland M, Weijenberg MP, Roemen GM, Brink M, de Bruine AP, et al. (2003) Effects of dietary folate and alcohol intake on promoter methylation in sporadic colorectal cancer: the Netherlands cohort study on diet and cancer. Cancer Res 63: 3133–3137.
54. Kerkel K, Spadola A, Yuan E, Kosek J, Jiang L, et al. (2008) Genomic surveys by methylation-sensitive SNP analysis identify sequence-dependent allele-specific DNA methylation. Nat Genet 40: 904–908.
55. Schilling E, El Chartouni C, Rehli M (2009) Allele-specific DNA methylation in mouse strains is mainly determined by cis-acting sequences. Genome Res 19: 2028–2035.
56. Zhang Y, Rohde C, Reinhardt R, Voelcker-Rehage C, Jeltsch A (2009) Non-imprinted allele-specific DNA methylation on human autosomes. Genome Biol 10: R138.
57. Chen H, Taylor NP, Sotamaa KM, Mutch DG, Powell MA, et al. (2007) Evidence for heritable predisposition to epigenetic silencing of MLH1. Int J Cancer 120: 1684–1688.
58. Samowitz WS, Curtin K, Wolff RK, Albertsen H, Sweeney C, et al. (2008) The MLH1 −93 G>A promoter polymorphism and genetic and epigenetic alterations in colon cancer. Genes Chromosomes Cancer 47: 835–844.
59. Raptis S, Mrkonjic M, Green RC, Pethe VV, Monga N, et al. (2007) MLH1 −93G>A promoter polymorphism and the risk of microsatellite-unstable colorectal cancer. J Natl Cancer Inst 99: 463–474.
60. Ogino S, Hazra A, Tranah GJ, Kirkner GJ, Kawasaki T, et al. (2007) MGMT germline polymorphism is associated with somatic MGMT promoter methylation and gene silencing in colorectal cancer. Carcinogenesis 28: 1985–1990.
61. Candiloro IL, Dobrovic A (2009) Detection of MGMT promoter methylation in normal individuals is strongly associated with the T allele of the rs16906252 MGMT promoter single nucleotide polymorphism. Cancer Prev Res (Phila Pa) 2: 862–867.
62. Hawkins NJ, Lee JH, Wong JJ, Kwok CT, Ward RL, et al. (2009) MGMT methylation is associated primarily with the germline C>T SNP (rs16906252) in colorectal cancer and normal colonic mucosa. Mod Pathol 22: 1588–1599.
63. Food and Drug Administration (1996) Food standards: amendment of standards of identity ro enriched grain products to require addition of folic acid. Final rule. Fed Regist. 21 CFR Parts 136, 137, and 139 21 CFR Parts 136, 137, and 139. pp 8797–8807.
64. Schernhammer ES, Giovannucci E, Fuchs CS, Ogino S (2008) A prospective study of dietary folate and vitamin B and colon cancer according to microsatellite instability and KRAS mutational status. Cancer Epidemiol Biomarkers Prev 17: 2895–2898.