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Nutrients in folate-mediated, one-carbon metabolism and risk of rectal tumors in men and women

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Abstract

In an investigation of rectal tumors characterized by CpG Island Methylator Phenotype (CIMP), *KRAS2* mutation, and *TP53* mutation, we examined associations with dietary and supplemental folate, riboflavin, vitamins B₆ and B₁₂, and methionine, nutrients involved in folate-mediated one-carbon metabolism. We also examined folate intake and common *MTHFR* polymorphisms in relation to CIMP. Data from a population-based study of 951 cases (750 with tumor markers) and 1,205 controls were evaluated using multiple logistic regression models and generalized estimating equations. Reduced risk of methylated tumors was suggested in women with upper tertiles of folate intake (≥ 0.42 mg/day) vs. lower tertile: OR=0.6, 95% CI 0.3–1.2. In men, a significant 3-fold increased risk of CIMP+ tumor was observed for the upper tertile of folate (≥ 0.75 mg/day) vs. the lower tertile (< 0.44 mg/day): OR=3.2, 95% CI 1.5–6.7. These men consumed a greater proportion of folic-acid fortified foods relative to natural, primarily plant-based sources (52% vs. 48%) than women with CIMP+ tumor (22% vs. 78%). *MTHFR* 1298A>C influenced folate in male CIMP+ risk (P -interaction<0.01). Our findings suggest folate supplementation effects may differ between genders, perhaps due to variation in *MTHFR* and/or endogenous/exogenous hormones, and may be important in the initiation and progression of methylated rectal tumors in men.

Keywords

Folate-mediated one-carbon metabolism (FOCM); diet; CpG island methylator phenotype (CIMP); *TP53*; *KRAS2*; rectal cancer

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INTRODUCTION

Colorectal tumorigenesis appears to occur via distinct molecular pathways (1–3). A specific molecular pathway which has recently gained interest is characterized by a large number of hypermethylated CpG islands with subsequent epigenetic transcriptional silencing (4, 5). A large fraction (~30–40%) of proximal site colon tumors and, to a lesser extent, distal colon and rectal tumors (~3–12%) are characterized by a CpG island methylator phenotype (CIMP + or CIMP high), in which numerous CpG islands are methylated and tumor suppressor genes such as the cell-cycle regulator *CDKN2A* (p16) are inactivated (2, 6–9). In colorectal carcinogenesis, both global DNA hypomethylation and CpG island promoter hypermethylation occur concurrently (10). S-Adenosylmethionine (SAM), the universal donor of methyl groups in humans, and S-Adenosylhomocysteine (SAH), the product of and an inhibitor of DNA methyltransferases, provide connections between folate-mediated one-carbon metabolism (FOCM) and DNA methylation (11, 12). Diets low in folate, vitamins B₆ and B₁₂, methionine, and high in alcohol intake may disturb DNA methylation, resulting in global DNA hypomethylation concurrently with a greater risk of cancers with CpG island methylation (13, 14). Global DNA hypomethylation has been linked with low folate intakes in both animal models and human studies; however, the role of folate status and that of other nutrients in folate-mediated one-carbon metabolism (B vitamins, methionine) in the etiology of promoter-specific DNA methylation is less clear (11). Given the heterogeneity of acquired mutations in CRC, several studies have examined associations of folate and other FOCM-related nutrients in adenomas and tumors that exhibit CIMP (15–18) and other molecular characteristics, including mutation of the *KRAS2* oncogene (19, 20) and *TP53* overexpression, a surrogate marker for mutational status (21).

In their recent review, Jacobs, et al. conclude that for many nutrients, conflicting reports exist of associations with colorectal neoplasia and that discrepant observations may be related to differing effects by sex. Thus further research on sex-specific dietary effects in CRC are warranted (22). We previously reported distinct differences between men and women in diet and lifestyle associations with rectal cancer (23, 24). In the current investigation, we examine folate, riboflavin, vitamins B₆ and B₁₂, and methionine as potential nutrients affecting methylation, in a gender-specific investigation of rectal tumors characterized by CIMP, *KRAS2*, and *TP53* mutation status. Furthermore, it has been suggested that genetic polymorphisms in *MTHFR*, a key enzyme regulating folate metabolism potentially modified by FOCM diet, may affect promoter methylation in the development of CRC (25, 26); thus we examine the interaction of folate and *MTHFR* 677C>T and 1298A>C coding polymorphisms in CIMP+ tumors. To our knowledge, our study is the first to evaluate these associations in a large case-control study of rectal tumors in men and women, in combination and by gender to examine sex-specific differences.

MATERIALS AND METHODS

Study population

Participants in the study were from the Kaiser Permanente Medical Care Program of Northern California (KPMCP), and the state of Utah. All eligible incident cases within these defined populations were identified and recruited for the study. Cases with a first primary tumor in the recto-sigmoid junction or rectum were identified between May 1997 and May 2001 using a rapid-reporting system. Case eligibility was determined by the Surveillance Epidemiology and End Results (SEER) Cancer Registries in Utah and Northern California. Cases with a previous colorectal tumor, familial adenomatous polyposis, ulcerative colitis, or Crohn's disease documented on the pathology report were not eligible for the study. In addition to these criteria, participants were between 30 and 79 years of age at time of diagnosis, English speaking, and mentally competent to complete the interview. Using the

same eligibility criteria, controls were frequency matched to cases by sex and by five-year age cohort with a ratio of cases to controls of 1:1. At KPMCP, controls were randomly selected from membership lists. At Utah, controls younger than 65 were randomly selected from driver's license lists and controls 65 years and older were randomly selected from social security lists. Additionally, controls could not have had a previous colon or rectal cancer. The race/ethnicity of the study population, reported at the time of interview, was primarily white, non-Hispanic (Table 1). Response rates were 65% for both cases and controls; cooperation rates, the number of people who participated of those we were able to contact, were 73% for cases and 69% for controls (27). Institution review board approval was obtained from the University of Utah and KPMCP. A total of 951 incident rectal cancer cases (750 with tumor marker data) and 1,205 matched controls were included in the study.

Data collection

Data were collected for cases and controls by trained and certified interviewers for a calendar-year referent period that occurred one to two years prior to year of diagnosis or selection; rigorous quality control methods were employed (28). A detailed interviewer-administered dietary history questionnaire (DHQ) was used to assess diet and supplement intake. The DHQ was adapted and computerized from the CARDIA dietary history (29–31). Participants were asked to recall foods eaten, the frequency which they were eaten, serving size, and if fats were added in the preparation. Nutrient intake was calculated using the University of Minnesota Nutrition Coordinating Center's Nutrition Data System for Research (NDS-R), Database version 4.02_30, © Regents of the University of Minnesota, and include folic acid fortified foods and vitamins.

Dietary folate intake reflects actual dietary intake of folate, including synthetic folic acid from fortified foods. Supplemental intake reflects folic acid from both multivitamins and single vitamin supplements. Data were ascertained both before and after folate fortification (140 mcg folic acid per 100 g of enriched cereal-grain flour products, for example) became mandatory as of January 1, 1998 (32, 33). However, in 1996, several food manufacturers began to comply with this requirement (34). As shown in Table 1, only a small proportion of data (5% of controls and 8% of cases) were collected for the period prior to 1996 when the food supply nationwide was unfortified. Therefore, dietary folate (including synthetic folates in fortified foods consumed by the majority of participants) and total folate from all sources (dietary folate and supplements) are presented. Anthropometric measures, personal and family medical history, and a screening history were collected. Height was measured at the time of interview and weight was reported for the two years prior to interview. Long-term vigorous physical activity data were collected using a detailed physical activity questionnaire (35). Participants were asked to report number of years they smoked, age stopped smoking (if former smoker), and usual number of cigarettes smoked per day while smoking regularly. Participants who reported non-steroidal anti-inflammatory medications (NSAIDs), ibuprofen or aspirin used regularly for one month or more, within two years of the referent year were considered recent users. Estrogen-positive women were those taking synthetic estrogen and women who had not gone through menopause (naturally or surgically). Estrogen negative women were post-menopause and were not taking hormone replacement therapy.

Tumor analysis

Tumor DNA was obtained from paraffin-embedded tissue as described (36). Tumors were characterized by their genetic profile that included: sequence data for exons 5 through 8, the mutation hotspots of the *TP53* gene; sequence data for *KRAS2* codons 12 and 13; methylation specific PCR of sodium bisulfite modified DNA for five CpG Island markers, *CDKN2A*, *MLH1* and methylated in tumors (MINT) 1, 2 and 31 (6). Tumors with two or

more methylated CpG islands were scored as CIMP+. At this time, there is no consensus CpG island panel or method of detection to determine CIMP. However, we have used our panel to demonstrate significant relationships between CIMP and numerous clinicopathologic variables, including the BRAF V600E mutation and cigarette smoking which were independent of microsatellite instability (6, 37, 38); this work has supported the legitimacy of the CIMP concept in CRC (4, 5).

MTHFR Genotyping

Genotyping of the 677C>T (rs1801133, aka A222V 665C>T) and 1298A>C (rs1801131, aka E429A 1286A>C) coding polymorphisms was performed on DNA from blood collected at the time of the study interview, as previously described in detail. Both polymorphisms satisfied Hardy-Weinberg equilibrium and had minor allele frequencies of 0.32 for 677 T and 0.33 for 1298 C in study controls. For each marker, control samples representing all possible genotypes and internal blinded replicates representing >1% of the sample were included in each tray. The concordance rate was >99%. Of study participants, 791 cases and 1008 controls were successfully genotyped.

Statistical analysis

All statistical analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC). Tumors were defined by specific alterations detected; any *TP53* mutation, any *KRAS2* mutation or *KRAS2* transversion mutations, or CIMP+ defined as methylation of 2 or more of 5 markers. Population-based controls were used to assess associations for the population overall while examining multiple outcomes defined by tumor status. A multiple logistic regression model was used to compare all interviewed cases, regardless of whether or not tumor tissue was obtained, to controls. In order to compare specific types of mutations to controls while adjusting for the other tumor mutations simultaneously, a generalized estimating equation (GEE) with a multinomial outcome was used as case subjects could contribute from one to three outcome observations depending upon how many tumor mutations (CIMP+, *KRAS2*, *TP53*) an individual had (39). The GEE accounts for correlation introduced by including subjects multiple times and was implemented using the GENMOD procedure as described by Kuss and McLerran (40). All models were adjusted for sex (where appropriate), age at diagnosis or selection, study center, race/ethnicity, recent estrogen within 2 years of the referent year (women), total energy (kcal), and fiber which has been previously shown to have a protective effect in rectal cancer and may modify the effect of folate (15, 41). Additionally, a fully-adjusted model included other factors that have been shown to be related to distal CRC including screening (sigmoidoscopy), smoking status (within 5 years of the referent year), recent NSAID use (within 2 years of the referent year), long-term vigorous physical activity, and referent year (pre-/peri- or post-fortification of folate). Potential confounding variables that did not impact odds ratio (OR) estimates substantively (<5%) when added to the model, and thus were not included in the final analyses presented, were family history of CRC, calcium, long-term alcohol, and body mass index in kg/m².

We assessed associations, ORs and 95% confidence intervals (95% CIs) in models with folate, riboflavin, vitamins B₆ and B₁₂, and methionine from diet and from all sources (combined dietary and supplemental intake for folate, riboflavin, and vitamins B₆ and B₁₂). Tertiles of nutrients were calculated for men and women separately in study controls. *P* for trend was assessed over using ordered categories of variables and comparing the likelihood ratio of a model with the variable to the likelihood ratio of a model without the variable using a chi-squared test with one degree of freedom. *P* for interaction was determined by comparing a full model including an ordinal multiplicative interaction term to a reduced model without an interaction term, using a likelihood ratio test.

RESULTS

Characteristics of the study population are shown in Table 1. Overall, 59% of rectal cases were male. The mean age at diagnosis was 61 for both men and women. The majority of study participants (83% of controls and 81% of cases) reported that they were white, non-Hispanic. Although fewer cases reported regular use of vitamin and mineral supplements than controls, use among female cases was higher than in male cases (52% and 37%, respectively; $p < 0.01$). Similarly, mean level of supplemental folate was higher in women than in men with rectal cancer (0.23 vs. 0.16 mg/day, respectively; $P < 0.01$). However, among only those cases who reported using vitamin and supplements, mean intake between women and men did not differ (0.44 vs. 0.43 mg/day, $P = 0.90$). The proportion of women who were estrogen positive (see Methods) within two years of the referent year was higher in controls than in cases. As previously reported, male controls were significantly more likely to undergo sigmoidoscopy screening than female controls (42). Rectal cases reported sigmoidoscopy during the 10 years prior to diagnosis about half as frequently as controls; however, rates between men and women did not differ significantly.

In Table 2, associations between dietary factors involved in FOCM and rectal cancer in a case-control comparison are presented. In rectal tumors overall, high folate from all sources (including vitamin supplements) was not associated with rectal cancer (upper compared to lower tertile of intake). Likewise, intakes of other B vitamins and methionine from diet and supplements were not associated overall or by gender, similar to our previous report (43). Risk estimates for FOCM-related nutrients from dietary sources only, excluding supplements, were similar and thus not shown.

In regard to risk of CIMP+, *KRAS2*-mutated, and *TP53*-mutated rectal cancers, we hypothesized that dietary factors involved in FOCM may be most relevant in the etiology of highly methylated tumors. Generally, total intake of folate, riboflavin, vitamins B6 and B12, and methionine were not associated with CIMP+ rectal cancer (Table 2). In women, a decreased risk associated with higher levels of dietary and supplemental folate intake and CIMP+ tumors did not reach statistical significance (Table 2, upper two tertiles). It has been shown that CIMP is more common in proximal colon cancers than in distal CRC (2, 8, 9, 44). The relatively small number of methylated rectal tumors in women may have limited our power to detect a significant protective association. Conversely, in men, we observed a significant 3-fold increased risk of CIMP+ tumors in the upper tertile of folate intake from all sources (≥ 0.75 mg/day) compared to the lower tertile (< 0.44 mg/day, P -trend < 0.01). Similarly, the upper tertile of intake (compared to the lower tertile) of riboflavin, B6, and B12, when supplements were included, resulted in a ~2-fold increased risk of having a methylated rectal tumor in men (P -trends = 0.10, 0.07, and 0.03 respectively). We found no significant association between FOCM dietary factors and *KRAS2* or *TP53* rectal tumor mutations overall (Supplemental Table S1, online), or by gender (data not shown). As previously hypothesized in colorectal adenomas (19), we observed a trend toward decreased risk of *KRAS2* G>C/T transversion mutations (one-third of all point mutations in codons 12 and 13) associated with higher levels of FOCM nutrients, which was significant for methionine (OR 0.4, 95% CI 0.2–0.9, upper tertile vs. lower tertile; P -trend = 0.04); see Supplemental Table S1.

Results of the interaction of total folate intake from all sources (diet and supplemental folate) and gender associated with CIMP+ rectal cancer, both for a case-control and case-case comparison, are shown in Table 3. Due to small cell sizes in interaction models, tertiles of folate intake were not assessed. Rather, dichotomous categories of total folate from diet and supplements were examined. To recognize greater bioavailability of synthetic folic acid in vitamin supplements, a conversion factor of 1.7 was applied to supplement intake to

estimate dietary folate equivalents (DFE). The following cutpoints were used in the analyses: 0.4 mg/day, to mirror the recommended daily allowance; and 1.0 mg/day, which approximates an upper tertile cutpoint for total folate when folic acid in supplements is DFE-adjusted. For intake categorized as <1.0 or ≥ 1.0 mg/day, *P*-interaction with gender was significant in either a case-control or case-case comparison of CIMP+ rectal cancer (0.03 and 0.005, respectively).

We examined whether *MTHFR* genotype modified the association of folate in CIMP+ rectal tumors in men and women (Table 4). The 677C>T genotype did not interact with folate consumption. Our findings suggest, however, that 1298A>C may interact with folate (0.4 mg/day) in men with CIMP+ tumors (*P*-interaction <0.01; case-control comparison). The increased risk of CIMP+ tumor observed in men in the upper tertiles of folate consumption was confined to those carrying the *MTHFR* 1298 AA genotype, whereas genotypes containing the variant C allele did not confer an increase in risk.

To examine potential differences between men and women in sources of dietary folate, a detailed examination of food sources comprising the majority of folate intake by gender and tumor status was undertaken. In Figure 1, the major sources of dietary folate from foods (adjusted for total energy), are shown for primarily unfortified natural sources (plant based; e.g. salad greens including spinach, vegetables, fresh fruit and fruit juice, beans and nuts) and sources fortified with synthetic folic acid (refined and whole grains and grain products). For male CIMP+ cases in the upper tertile of total intake (1a), 52% of major sources of dietary folate were grain-based and 41% were plant-based foods. In contrast, men with CIMP- tumors in the upper tertile of total intake consumed 44% grain-based and 51% plant-based foods (data not shown). For male CIMP+ cases in the lower two tertiles of total intake, diet composition also shifts away from grain based to a majority of foods that are plant based (43% and 53%, respectively; data not shown). For female CIMP+ cases (and for women in general), a majority (76%) of major sources of dietary folate in the upper tertile of total intake (1b) was comprised of plant-based foods, in particular salad greens including spinach. These findings suggest that in addition to folic acid from vitamins and supplements, grain-based sources of synthetic folate in fortified foods may increase risk of CIMP+ rectal tumors in men whereas for in women, high folate intake from natural sources may have a protective effect in CIMP+ tumors as hypothesized.

It has been suggested that diets reflecting high or low methyl donor status may influence FOCM in colorectal cancer (11, 43). We examined a combination of nutrient intakes contributing to methyl donor status, i.e. alcohol, folate, and methionine with rectal tumor subtypes. A 'low risk' diet high in folate (~0.4 mg/day) and methionine and no or moderate longterm alcohol consumption (high methyl donor status) compared to a 'high-risk' diet low in folate or methionine and high longterm use of alcohol (low methyl donor status) conferred a similar modest but statistically non-significant decreased likelihood of having a CIMP+ rectal tumor (data not shown). We also examined tertiles of a composite micronutrient score similar to (45), comprised of standardized total folate, riboflavin, vitamins B₆ and B₁₂, and methionine intake; no association with CIMP+ rectal tumors was observed (data not shown).

DISCUSSION

Similar to our previous study of colon cancers (15), we found that few of the dietary factors hypothesized to affect DNA methylation were associated more strongly with CIMP+ than non-CIMP rectal tumors. However, statistically significant sex-specific differences in risk of CIMP+ rectal cancer and folate intake were observed. There is currently no consensus as to a safe upper limit of folate intake in the post-fortification era, and in the current study we

were able to evaluate fortified folate intake including folic acid from multivitamins and supplements, which are commonly used in the U.S. Thus our finding of greatly increased risk of CIMP+ rectal tumors in men associated with high intake of natural and synthetic folate from diet and supplements (and other B vitamins to a lesser extent, as the trend is less pronounced and not apparent when supplements are not included), is intriguing.

Evidence that high intake of folate (usually thought of as chemoprotective) in the post-fortification era may promote tumor progression is accumulating. Data from animal studies have shown that administration of folate after neoplastic foci are established can promote CRC tumors (46). In a large chemoprevention trial in human participants, an increased risk of 67% in advanced colorectal adenomas was observed with 1 mg/day folic acid supplementation in individuals with an adenoma history (47). It is possible that the timing of folate is important (48). The possibility that high folate intake might be detrimental suggests that further study is needed to evaluate the impact of folate on polyp progression.

We previously reported the *MTHFR* 677C>T CT and TT genotypes were associated with decreased rectal cancer risk in women, and that the 1298A>C homozygous variant CC genotype decreased risk in both men and women (43). No interaction with intake of folate or other nutrient intakes or risk for methylation diet with *MTHFR* polymorphisms in rectal cancer overall was observed (43). Likewise, there was no significant interaction of the 677 C>T and 1298 A>C polymorphisms in CIMP+ rectal cancer. Thus it is not surprising that in the current study, the *MTHFR* 1298 polymorphism appears to influence risk of male CIMP+ rectal cancer, while *MTHFR* 677 C>T does not play a significant role. Our earlier finding that 1298A>C, interacting with diet, may be involved in the development of highly methylated colon cancers was also seen in male rectal cases (25).

Although CIMP+ rectal cancer is equally as uncommon in men and women (~11% of tumors with marker data available), the effect of diet relevant to FOCM differs in women and men, perhaps due to endogenous or exogenous hormones. In contrast to men, *MTHFR* 1298 AA genotype did not confer increased risk of CIMP+ rectal tumor in a high folate environment in women, although power to detect an association was limited. We found no significant interaction between recent estrogen status and folate intake in women (data not shown). The proportion of CIMP+ male cases who reported undergoing sigmoidoscopy screening prior to diagnosis relative to male cases with non-CIMP+ tumors is smaller than in females (men, 7% vs. 21%, respectively; women, 12% vs. 15%, respectively). In minimally-adjusted models in which screening was not included as a covariate, the observed gender difference in regard to men and women and high folate intake remained significant and was of similar effect size.

FOCM-related diet generally did not appear to influence *KRAS2* mutational status in rectal cancer, similar to findings of a prospective study of these nutrients in relation to colon cancer (49). When transition or transversion mutations in *KRAS2* were examined, based on a previous report that folate may be more strongly inversely related to risk of G>T transversions in adenomas (19), a trend toward reduced risk of *KRAS2* transversions in rectal cancer and higher intake was significant for methionine, but not folate (Supplemental Table 1). A mechanism by which deficiency of FOCM nutrients might be associated with *KRAS2* mutations in colorectal cancer is unclear; however, as folate and B vitamins are important for DNA methylation and synthesis, it is plausible that inadequate intakes may be associated with tumor mutations.

This study has several strengths, including a large sample of cases with rectal tumor data and detailed nutrient intakes from a validated, interviewer-administered diet history; however, these findings must be interpreted cautiously. As a number of comparisons were

made in our hypothesis-based investigation, it is possible that our observation of high folate and increased risk of CIMP+ rectal tumor in men was a spurious association. Another limitation was our ability to accurately assess greater bioavailability of synthetic folate, particularly from dietary sources, as DFEs were not available for specific foods in our nutrient database. However, as most study interviews were conducted during and after fortification of the food supply, an association of high folate based on tertile intake of DFE and rectal cancer would likely show similar increased risk in men.

In summary, a hypothesized reduction in risk of highly methylated rectal tumors at higher levels of total folate intake was not observed. An unexpected significant 3-fold increased risk of a highly methylated tumor in men was observed in the upper tertile of folate from natural and synthetic sources including supplements. These men appeared to consume a greater proportion of folic-acid fortified foods relative to natural sources of folate from vegetables, fruits, beans, and legumes. As a CIMP+ phenotype is relatively uncommon in rectal cancer, confirmation is needed perhaps in a larger consortium including cases assessed for tumor markers and well-characterized for diet. Our findings suggest that the effect of folate supplementation may have different effects between women and men. Additionally, we offer some support that a diet rich in FOCM nutrients may reduce risk of *KRAS*2 transversion mutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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1(a)

1(b)

Figure 1. Comparison of major sources of folate: (a) Male CIMP+ cases in upper tertile of total intake (≥ 0.75 mg/d); (b) Female CIMP+ cases in upper tertile of folate intake (≥ 0.74 mg/d).

Table 1

Description of the study population.

Characteristic	Controls		Cases	
	<i>n</i>	%	<i>n</i>	%
Total	1205	100.0	951	100.0
Sex				
Male	673	55.9	559	58.8
Female	532	44.1	392	41.2
Age (y)				
30–39	33	2.7	25	2.6
40–49	145	12.0	122	12.8
50–59	304	25.2	248	26.1
60–69	392	32.5	318	33.4
70–79	331	27.5	238	25.0
Center				
KPMCP	746	61.9	614	64.6
Utah	459	38.1	337	35.4
Race/ethnicity				
White, non-Hispanic	999	82.9	766	80.5
Hispanic	91	7.6	81	8.5
Black	50	4.1	38	4.0
Asian Mixed	47	3.9	53	5.6
race/ethnicity	16	1.3	12	1.3
Referent year (-folic acid fortification) ^I				
1995 (pre-)	57	4.7	76	8.0
1996–1997 (peri-)	511	42.4	492	51.7
1998–2000 (post-)	637	52.9	383	40.3
Vitamins and supplements				
Not used	612	50.8	543	57.1
Regular use	593	49.2	408	42.9
Supplemental folate (mg/day), mean (SE)				
Men	0.23	(0.02)	0.16	(0.01)
Women	0.27	(0.02)	0.23	(0.02)
Dietary folate (mg/day), mean (SE)				
Men	0.46	(0.01)	0.45	(0.01)
Women	0.39	(0.01)	0.39	(0.01)
Recent estrogen status				
Negative	218	41.0	198	50.5
Positive	314	59.0	193	49.2
Screening				
Not screened	745	63.4	784	83.0
Yes, sigmoidoscopy	430	36.6	161	17.0

Characteristic	Controls		Cases	
	<i>n</i>	%	<i>n</i>	%
Body mass index				
<25	395	33.0	308	32.5
25–29	517	43.2	370	39.0
30 or greater	285	23.8	270	28.4
CIMP status ² (n=670)				
CIMP 0/–	-	-	596	89.0
CIMP+	-	-	74	11.0
<i>KRAS2</i> status (n=743)				
No mutation	-	-	528	71.1
Mutation	-	-	215	28.9
<i>TP53</i> status (n=704)				
No mutation	-	-	364	51.7
Mutation	-	-	340	48.3

¹ Reporting of diet history in relation to government-mandated folic acid fortification.

² CIMP+ was defined as 2 or more of 5 CpG islands methylated (see Methods).

Table 2
Associations between tertile intake of folate-mediated one-carbon metabolism diet and CpG-island methylation in rectal cancer

	All cases ^I			CIMP+			CIMP+, men only			CIMP+, women only		
	Control n	Case n	OR (95% CI)	Case n	OR (95% CI)	Case n	OR (95% CI)	Case n	OR (95% CI)	Case n	OR (95% CI)	
Tertile intake men/women ²												
Folate (mg/day)												
<0.44/<0.42	398	373	1	Referent	29	1	Referent	13	1	Referent	16	1
≥0.44/≥0.42	408	295	0.9	(0.7, 1.1)	17	0.8	(0.4, 1.5)	9	1.3	(0.5, 3.3)	8	0.6
≥0.75/≥0.74	399	283	0.9	(0.7, 1.2)	27	1.5	(0.8, 2.5)	18	3.2	(1.5, 6.7)	9	0.6
P trend			0.58			0.20			<0.01			0.44
Riboflavin (mg/day)												
<2.5/<2.4	392	331	1	Referent	28	1	Referent	14	1	Referent	14	1
≥2.5/≥2.4	404	310	0.9	(0.8, 1.2)	19	0.8	(0.4, 1.5)	10	1.1	(0.5, 2.7)	9	0.6
≥4.1/≥3.9	398	302	0.9	(0.7, 1.2)	26	1.2	(0.7, 2.3)	16	2.2	(1.1, 4.7)	10	0.7
P trend			0.48			0.52			0.10			0.51
Vitamin B6 (mg/day)												
<2.5/<2.3	391	343	1	Referent	29	1	Referent	13	1	Referent	16	1
≥2.5/≥2.3	406	304	0.9	(0.7, 1.2)	19	0.8	(0.4, 1.4)	11	1.3	(0.6, 3.1)	8	0.4
≥4.1/≥4.0	397	296	1.0	(0.7, 1.2)	25	1.2	(0.7, 2.1)	16	2.2	(1.0, 4.7)	9	0.5
P trend			0.76			0.61			0.07			0.27
Vitamin B12 (mcg/day)												
<6.4/<5.7	391	335	1	Referent	23	1	Referent	11	1	Referent	12	1
≥6.4/≥5.7	405	315	1.0	(0.8, 1.2)	23	1.2	(0.7, 2.2)	13	1.8	(0.8, 4.0)	10	0.9
≥11.7/≥10.9	398	293	0.9	(0.7, 1.1)	27	1.6	(0.9, 2.9)	16	2.7	(1.3, 5.9)	11	1.0
P trend			0.33			0.14			0.03			0.98
Methionine (gm/day)												
<1.7/<1.4	391	299	1	Referent	26	1	Referent	15	1	Referent	11	1
≥1.7/≥1.4	408	302	0.9	(0.7, 1.2)	29	1.2	(0.7, 2.1)	17	1.4	(0.7, 3.0)	12	1.0
≥2.5/≥2.0	395	342	1.1	(0.8, 1.5)	18	0.9	(0.4, 2.1)	8	0.8	(0.3, 2.5)	10	0.8
P trend			0.72			0.69			0.86			0.63

NOTE: adjusted for sex, age, race, center, energy, fiber, screening, referent year, smoking, recent NSAID use, physical activity, and recent estrogen (women).

^IIncludes rectal cases without tumor marker data.

² Cutpoints based on total intake from diet and supplements in control subjects.

Table 3
Interaction between folate and sex associated with CpG-island methylation in rectal cancer

	Rectal cases ¹ vs. controls			CIMP+ cases vs. controls			CIMP+ vs. CIMP- cases		
	Men	Women		Men	Women		Men	Women	
Folate intake, diet and supplements²	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	
<0.4 mg/day	1	Referent	0.7 (0.5, 1.0)	1	Referent	1.6 (0.8, 3.4)	1	Referent	2.0 (0.9, 4.0)
≥0.4 mg/day	0.8 (0.6, 1.0)	0.7 (0.5, 0.1)	1.5 (0.7, 3.0)	1.0 (0.5, 2.0)	1.7 (0.8, 3.4)	1.2 (0.6, 1.0)			
<i>P</i> interaction		0.25		0.13		0.04			
<1.0 mg/day	1	Referent	0.8 (0.6, 1.0)	1	Referent	1.3 (0.7, 2.4)	1	Referent	1.5 (0.9, 2.8)
≥1.0 mg/day	0.9 (0.7, 1.2)	0.8 (0.6, 1.1)	2.5 (1.4, 4.5)	1.0 (0.4, 2.2)	2.8 (1.5, 5.1)	1.2 (0.5, 2.6)			
<i>P</i> interaction		0.61		0.03		0.005			

NOTE: Adjusted for age, race, center, energy, fiber, screening, referent year, smoking, recent NSAID use, physical activity, and recent estrogen (women).

¹Includes rectal cases without tumor marker data.

²Folate intake from vitamin supplements is DFE-adjusted.

Table 4
Interaction between folate and *MTHFR* 1298A>C genotype associated with CpG-island methylation in rectal cancer

	CIMP+ cases ¹ vs. controls			CIMP+ cases vs. controls, men			CIMP+ cases vs. controls, women		
	1298 AA	OR	(95% CI)	1298 AC/CC	OR	(95% CI)	1298 AA	OR	(95% CI)
Folate intake, diet and supplements²									
<0.4 mg/day	1	Referent		0.9	(0.4, 2.0)	1	Referent	0.4	(0.1, 1.4)
≥0.4 mg/day	1.5	(0.7, 3.1)		0.4	(0.2, 1.0)	3.9	(1.2, 12.3)	0.2	(0.1, 0.7)
<i>P</i> interaction				0.04		0.008		0.80	
<1.0 mg/day	1	Referent		0.6	(0.3, 1.2)	1	Referent	0.5	(0.2, 1.3)
≥1.0 mg/day	2.4	(1.3, 4.6)		0.6	(0.2, 1.6)	5.4	(2.3, 12.5)	0.2	(0.1, 1.2)
<i>P</i> interaction				0.15		0.19		0.27	

NOTE: Adjusted for sex, age, race, center, energy, fiber, screening, referent year, smoking, recent NSAID use, physical activity, and recent estrogen (women).

¹Includes rectal cases without tumor marker data.

²Folate intake from vitamin supplements is DFE-adjusted.