

## Variation in folate pathway genes and distal colorectal adenoma risk: a sigmoidoscopy-based case–control study

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### Abstract

**Background** Folate-associated one-carbon metabolism (FOCM) is an important pathway in colorectal neoplasia risk but data on genetic variation in this pathway are largely limited to studies of single SNPs in selected genes.

**Methods** We used a comprehensive tagSNP approach to study the association between genetic variation in 11 genes in the FOCM pathway and risk of incident distal colorectal adenomas in a sigmoidoscopy-based case–control study. We included 655 cases (one or more adenomas) and 695 controls (no adenomas) recruited from one of two Kaiser Permanente clinics between 1991 and 1995. We assessed a total of 159 tagSNPs selected using Haploview Tagger as well as selected non-synonymous SNPs. We used unconditional logistic regression to model the association between SNPs and risk of distal adenomas, assuming a log-additive model.

**Results** Five SNPs in the *SLC19A1* (*RFC1*) gene: rs1051266 (*G80A*), rs283895, rs2236484, rs12482346, and

rs2838958 were associated with adenoma risk after correction for multiple testing (all corrected  $p$  values  $\leq 0.043$ ). The non-synonymous *SLC19A1* SNP *G80A* interacted significantly with the *MTHFR C677T* genotype (interaction  $p$  value = 0.018).

**Conclusion** Our data suggest that genetic variation in *SLC19A1* may modify the risk of distal colorectal adenoma.

**Keywords** Folate · *MTHFR* · *SLC19A1* · TagSNP · Folate · Colorectal adenoma

### Introduction

Folate-associated one-carbon groups are essential for hundreds of intracellular transmethylation reactions including those involved in DNA methylation and DNA synthesis [1]. An inverse association between folate intake and carcinogenic changes in colorectal epithelium has been observed, both in vitro and in vivo, in both humans and animals [1–3]. However, clinical trials of folic acid supplementation [4] and animal experiments [5, 6] suggest that folate plays a dual role in the colorectum by both protecting against and promoting growth of neoplastic lesions depending on timing, dose, and source (diet vs. supplements).

Folate-associated one-carbon metabolism (FOCM) is a complex cycle of inter-related reactions that provide one-carbon groups needed for numerous intracellular processes. This pathway has been well characterized, and a mathematical model has been developed [7, 8]. Genetic variants in genes that play key roles in the FOCM pathway have been investigated as potential colorectal adenoma susceptibility genes, but studies have been limited to one or a few SNPs and a small number of genes, with mixed results [9, 10]. The *MTHFR C677T* and *A1298C* polymorphisms

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have been associated with CRC but not colorectal adenoma risk [11, 12]. A recent study of genetic variability in FOCM-related genes and adenoma risk focused on 24 non-synonymous SNPs in 13 genes, reported little evidence of a major role for the folate pathway genes included in their analysis [13].

In this study, we conducted a comprehensive analysis of the role of genetic variation in 11 genes that play key roles in the FOCM pathway and colorectal adenoma risk. Figure 1 defines the genes included in the current analysis and their roles in FOCM. We included enzymes involved in the uptake of folate, nucleotide synthesis, and *S*-adenosylmethionine (SAM) synthesis. We also included the gene for cystathionine- $\beta$ -synthase (CBS), an enzyme important for modulating intracellular homocysteine and that for gastric intrinsic factor (GIF), which is involved in the uptake of vitamin B<sub>12</sub>, a key co-factor in FOCM.

## Materials and methods

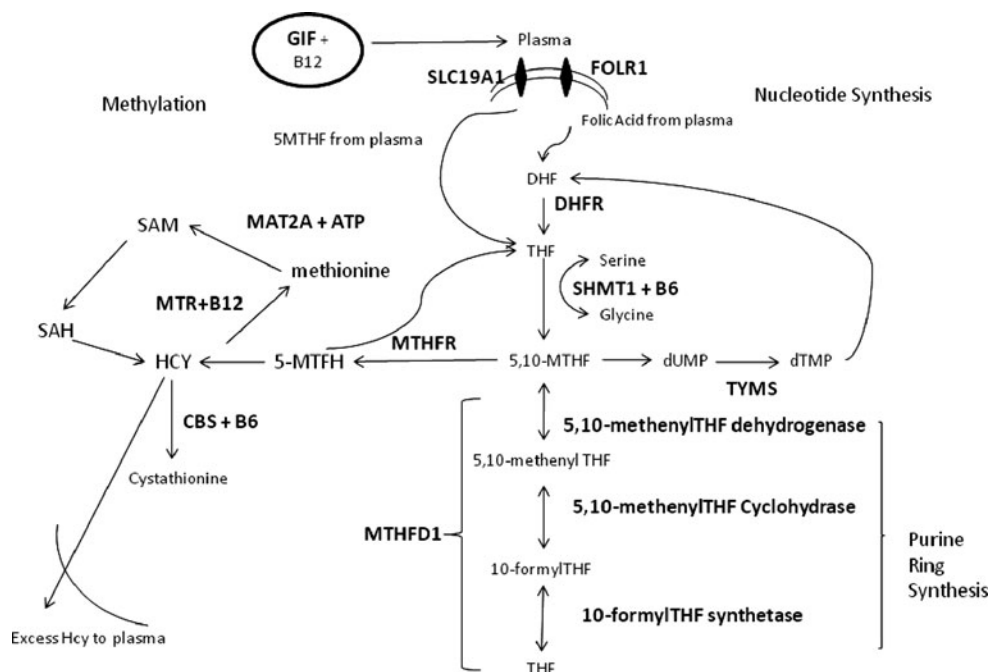
### Study subjects

The subjects in this study were participants in the USC/Kaiser Permanente study of risk factors for colorectal adenomas. Characteristics of this study population have

been previously described [14, 15]. Briefly, phase 1 subjects were recruited from one of two Kaiser Permanente clinics and received a sigmoidoscopy examination from 1991 to 1993, while phase 2 subjects were recruited from the same two clinics from 1993 to 1995. Identical criteria were used to recruit subjects from each phase. Eligible subjects were English-speaking, between the ages of 50–74 years, and living in the Los Angeles metropolitan area. Subjects were excluded if they had a history of invasive cancer, inflammatory bowel disease, familial polyposis, previous bowel surgery, or symptoms suggestive of gastrointestinal disease. Cases were those subjects who had at least one histologically confirmed adenoma during their sigmoidoscopy exam, and controls were those subjects with no evidence of an adenoma at sigmoidoscopy and who had no history of confirmed adenomas. Cases were individually matched to controls on age (within 5 years), gender, sigmoidoscopy date (within 3 months), and Kaiser Permanente clinic. All subjects signed an approved informed consent, donated a blood sample, and filled in two questionnaires.

### Risk factor data

The risk factor questionnaire queried demographic information, family cancer history, smoking history, history of



**Fig. 1** Genes involved in folate-associated one-carbon metabolism. Abbreviations: CBS cystathionine- $\beta$ -synthase, DHFR dihydrofolate reductase, FOLR1 folate receptor isoform 1, GIF gastric intrinsic factor, MAT2A methionine adenosyltransferase isoform 2 (non-liver), MTHFD1 methylenetetrahydrofolate dehydrogenase/methylnetra-

hydrofolatecyclohydrolase/formyltetrahydrofolate synthetase, MTHFR methylenetetrahydrofolate reductase, MTR methionine synthase, SHMT serine hydroxymethyltransferase, SLC19A1 solute carrier family 19 (folate transporter) member 1 (also called the reduced folate carrier 1 (RFC1)), TYMS thymidylate synthase

using selected over the counter and prescription drugs (e.g., aspirin and other non-steroidal anti-inflammatory agents, laxatives), specified dietary supplements (multivitamins, calcium), a physical activity history, a history of usual sun exposure, a brief description of their usual method for cooking red meats, and, for women, a brief reproductive history. A separate food frequency questionnaire (FFQ) was used to estimate nutrient intake. For phase 1 subjects, we used a modified form of the Block food frequency questionnaire [16]. For phase 2 subjects, we used a diet questionnaire developed and validated at the University of Hawaii [17]. The two questionnaires were similar with respect to the number and type of foods queried and the method for estimating nutrient intakes, in grams, milligrams, or micrograms per day, as appropriate for the nutrient. Nutrient intakes were based on expected nutrient levels for the stated portion size and estimated consumption frequency per week for specific foods and supplements using the nutritionist food and supplement databases developed by the designers of the FFQs. Dietary folate intake was categorized into quartiles using study phase-specific cutpoints and intakes from the controls. For alcohol, we used pre-defined cutpoints of 0, 0.1–10, 11–20, and >20 g/day.

#### Plasma folate

Blood samples were taken from fasting subjects into EDTA-coated tubes in the morning and immediately put on ice until processing to separate the plasma. In both study phases, samples were stored at  $-70^{\circ}\text{C}$  until processing. For phase 1 subjects, plasma folate was determined for the first 370 male subjects (52.3%) and the first 316 samples female subjects (86.8%) using the Quantaphase Radioassay as described [18]. For phase 2 subjects, plasma folate was estimated for 335 men (97.7%) and 199 women (97.5%) using the Quantaphase Radioassay II.

#### Plasma total homocysteine (tHcy)

Plasma tHcy was not estimated for phase 1 subjects. For phase 2 subjects plasma tHcy ( $\mu\text{mol/L}$ ) was estimated for 335 men (97.7%) and 199 women (97.5%). Blood samples were taken from fasting subjects into EDTA-coated tubes in the morning and immediately put on ice until processing to separate the plasma. In both study phases, samples were stored at  $-70^{\circ}\text{C}$  until processing. For determination of plasma tHcy, we used the reversed-phase HPLC method of Kuo et al. [19]. All chemicals were purchased from Sigma. Plasma thiols were measured using fluorescence detection after derivatization with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBDF). HPLC separation was carried out using gradient analysis with fluorescence detection (Varian Assoc., Sugar Land Texas) and the

Agilent Technology HPLC 1100 system (Agilent Technology, Wilmington DE). Thiols were separated using a Bakerbond C18  $4.6 \times 250$  mm column (Mallinckrodt Baker, Phillipsburg, NJ). The mobile phase consisted of 8% methanol to 40% at time 12 min. For quality control purposes, pooled plasma samples were analyzed with each batch of samples. The intra-assay coefficient of variance was 5.7% and inter-assay CV was 4.4% (8.6  $\mu\text{M}$ ).

#### SNP selection

We used a comprehensive tagSNP approach to assess gene-level risk in a pre-folic acid-fortification population of colorectal adenoma patients and controls. Supplemental Table 1 describes the SNPs included in this analysis by race/ethnic group. SNPs were selected using Haploview Tagger [20] and based on the CEPH data using the following criteria:  $\text{MAF} \geq 5\%$ , pairwise  $r^2 \geq 0.95$ , and distance from closest SNP greater than 60 base pairs on the Illumina platform. The linkage disequilibrium blocks were determined using data from HapMap data release #16c.1, June 2005, on NCBI B34 assembly, dbSNP b124. For each gene, we extended the 5'- and 3'-UTR regions to include the 5'- and 3'-most SNP within the LD block (approximately 10 kb upstream and 5 kb downstream). In regions of no- or low-LD, SNPs with an  $\text{MAF} \geq 5\%$  at a density of approximately 1 per kb were selected from either HapMap or dbSNP. Finally, non-synonymous and expert-curated SNPs regardless of MAF were included. The SNPs included in this study are listed in Supplementary Table 1.

#### SNP genotyping

Except for the *MTHFR C677T* polymorphism (described later), SNPs were genotyped on the Illumina GoldenGate platform [21]. We implemented a series of quality control checks based on Illumina metrics, and SNPs were excluded from analysis based on the following criteria: GenTrain score  $< 0.4$ , 10% GC score  $< 0.25$ , AB T Dev  $> 0.1239$ , call rate  $< 0.95$ , more than 2 Mendelian errors  $> 2$  or discordance with HapMap  $> 3$ . Inter- and intraplate replicates were included, and SNPs were excluded from the analysis if there were greater than 2 errors on replicate genotypes. In addition, genotype data from 30 CEPH trios (Coriell Cell Repository, Camden, NJ) were used to confirm reliability and reproducibility of the genotyping. SNPs were excluded from the analysis if more than 3 discordant genotypes were discovered in comparison with genotypes from the International HapMap Project [22]. After deleting SNPs with a minor allele frequency  $< 0.05$  in the total study sample and those with a  $p$  value for Hardy–Weinberg equilibrium (HWE)  $< 0.0003$  (the Bonferroni-corrected  $p$  value), we calculated effect estimates for 159 SNPs.

### *MTHFR C677T* genotype

We have previously reported on associations between folate and the *MTHFR C677T* genotype on adenoma risk in the phase 1 subjects [23]. For phase 1 subjects, the *MTHFR C677T* genotype (rs1801133) was determined by the PCR–RFLP method of Frosst et al. [24] using their published primer pairs. *MTHFR 677* genotyping was not available for 14 phase 1 subjects and 485 phase 2 subjects. For these subjects, the 677 genotype was determined by imputation using the MACH software [25]. Accuracy was 97.4% for non-Hispanic whites, 99.8% for Hispanics, 93.4% for African Americans, and 94.5% for Asians.

### Statistical analysis

Minor allele frequency was estimated from genotype data from cases and controls combined. Hardy–Weinberg equilibrium was assessed using a  $\chi^2$  test. Pairwise linkage disequilibrium between SNPs was estimated using Haploview [20]. We used the square of the correlation coefficient ( $r^2$ ) between markers to define linkage using the data from the study population. We have previously established that using unconditional logistic regression adjusting for the matching factors led to the same results as conditional logistic regression while allowing use of the entire study population [23]. Therefore, we used unconditional logistic regression controlling for the matching factors (age, sex, clinic and examination date), study phase and, in non-ethnic group-specific analyses, race/ethnic group to estimate main effects and stratum-specific odds ratios. Additional control for dietary folate (mcg/ml), total dietary fiber, multivitamin use (yes/no), and alcohol intake (0, >0–10, 11–20 and >20 g/day) did not change the results, and we present only the matching factor, race/ethnicity (where appropriate), and phase-adjusted models here.

Except for the *MTHFR C677T* (rs1801133) and *MTHFR A1298C* (rs1801131) polymorphisms, for which there are data supporting a recessive model, we assumed a log-additive model to assess genotype/adenoma associations. To minimize population stratification, we conducted all analyses separately by race/ethnic group. Since prior data suggest specific effects for *MTHFR C677T* and *A1298C*,  $p$  values for these two SNPs were not corrected for multiple testing in any analysis. Additionally, tests based on prior data from the literature (e.g., the interaction between *SLC19A1 G80A* genotype and *MTHFR C677T* genotype) were not corrected for multiple testing. For the log-additive model and within each gene,  $p$  values for all other SNPs were adjusted for multiple testing taking into account correlated tagSNPs using a modified test of Conneely and Boehnke ( $p_{\text{act}}$ ) [26]. The  $p_{\text{act}}$  also adjusts for the correlation

structure of each gene within each ethnic group, using the race/ethnic group-specific data to compute the underlying correlation structure specific to that group. For all stratified analyses, we included interaction terms in the regression models to get the interaction  $p$  values.  $p$  Values for trend were estimated by entering the quartile value (1–4) into the regression model. The likelihood ratio test was used to test for heterogeneity between strata. For these multiple degree of freedom likelihood ratio tests, the Bonferroni method was used to reset the significance level to 0.0003, based on a total of 159 SNPs.

We assessed the association between genotype and plasma tHcy using a multiple regression where plasma tHcy was modeled as a continuous variable, and the change in plasma tHcy per each variant allele (e.g., 0, 1, or 2) was assessed using a 1- $df$  likelihood ratio test, corrected for multiple testing using the method of Conneely and Boehnke [26]. Since plasma tHcy is not normally distributed, it was first transformed to its natural logarithm.

Comparisons between cases and controls for selected baseline characteristics and comparisons between subjects with and without genotyping data were made using multiple regression controlling for the matching factors, race/ethnicity, and study phase. For dietary factors, we also controlled for calories. All statistical analyses were conducted using the R programming language and SAS v9.1.

### Results

Genotyping results were available for a maximum of 1,354 of 1,621 subjects with questionnaire data (83.5%). Phase 2 subjects did not differ from phase 1 subjects in age, gender, ethnicity, and smoking patterns but had higher estimated dietary folate and alcohol intakes. The subjects with genotyping data did not differ significantly from those with no genotyping data on selected adenoma risk factors (data not shown). Since data on plasma folate was not available for all subjects, we compared those with data on plasma folate to those with no data on plasma folate. Those with plasma folate data differed significantly from those with no plasma folate data on a number of adenoma risk factors (e.g., dietary folate, total dietary fiber, and red meat intakes), and thus plasma folate was not used in any of the analyses.

The characteristics of the study population are presented in Table 1. Cases were more likely to be current smokers and to drink more alcohol, had less dietary folate and fiber intakes, more saturated fat intake, and ate more red meat. After adjustment for the matching factors, race/ethnic group, study phase and calories, smoking, dietary folate, and fiber intakes remained significantly different between cases and controls.

**Table 1** Selected characteristics of the study population

Variable	Controls <i>n</i> = 696	Cases <i>n</i> = 655	<i>p</i> Value <sup>a</sup>
Age mean (SD) <sup>b</sup>	61.59 (6.7)	61.72 (6.9)	0.66
Sex <i>n</i> (% male) <sup>b</sup>	458 (65.8)	418 (63.8)	0.42
Race/ethnic group <sup>b</sup>			
White	372 (53.5)	351 (53.6)	0.32
Black	108 (15.5)	117 (17.9)	
Hispanic	73 (10.4)	74 (11.3)	
Asian	114 (16.5)	81 (12.4)	
Other	29 (4.15)	32 (4.9)	
Calories mean (SD)	2,052 (960.7)	2,090 (939.2)	0.14
BMI mean (SD)	27.13 (4.6)	27.51 (4.5)	0.24
Alcohol (g/day) mean (SD) <sup>c,d</sup>	7.93 (27.2)	9.78 (29.5)	0.22
Smoking			
Never <i>n</i> (%)	283 (42.7)	233 (37.0)	<0.01
Former <i>n</i> (%)	314 (47.4)	284 (45.2)	
Current <i>n</i> (%)	66 (9.95)	112 (17.8)	
Missing/unknown	33	26	
Smoking (pack years) mean (SD)	16.85 (32.1)	18.83 (30.5)	0.01
Multivitamin use (% Yes) <sup>b</sup>	58.8	57.9	0.64
Dietary B <sub>12</sub> (mcg/day) mean (SD) <sup>b,c</sup>	6.14 (4.8)	6.09 (4.5)	0.59
Dietary folate (mcg/day) mean (SD) <sup>b,c</sup>	367.5 (212.5)	337.0 (181.9)	<0.01
Plasma total homocysteine (μmol/L) <sup>b</sup>	10.35 (3.75)	10.48 (4.73)	0.74
Total dietary fiber g/day mean (SD) <sup>b,c</sup>	22.84 (13.3)	21.46 (12.3)	<0.01
Vegetable fiber g/day mean (SD) <sup>b,c</sup>	8.38 (7.05)	7.51 (5.6)	<0.01
Saturated fat g/day mean (SD) <sup>b,c</sup>	23.45 (13.6)	24.56 (13.6)	0.12
Red meat g/day mean (SD) <sup>b,c</sup>	66.21 (67.4)	68.47 (56.1)	0.63
Number of adenomas <i>n</i> (%)			
1		698 (89.4)	
≥2		83 (10.6)	
Missing		1	
Adenoma size <i>n</i> (%)			
<1 cm		526 (68.1)	
≥1 cm		247 (31.9)	
Missing		9	
Adenoma site <i>n</i> (%)			
Rectum (<15 cm)		146 (18.8)	
Colon (≥15 cm)		631 (81.2)	
Missing		5	

<sup>a</sup> Comparisons between cases and controls for selected baseline characteristics were made using multiple regression controlling for the matching factors (age, sex, clinic, and examination date) and study phase

<sup>b</sup> 1 control was missing sex and age data, 2 controls were missing sex data, and 7 controls and 10 cases were missing data on race/ethnic group. For phase 2 subjects, 7 controls and 7 cases were missing plasma total homocysteine data

<sup>c</sup> All dietary intakes were defined as average use in the year before the sigmoidoscopy. Comparisons between cases and controls are adjusted for the matching factors, study phase, and calorie intake

<sup>d</sup> Sum of alcohol content of wine (10.9 g/serving), beer (12.8 g/serving), and spirits (15.0 g/serving) in the year before the sigmoidoscopy

### FOCM genes and adenoma risk

The per allele associations with adenoma risk (except for *MTHFR C677T* and *A1298C* as noted in the “Methods”

section) for all 159 SNPs analyzed, by race/ethnicity, are shown in Supplemental Table 2. When considering all subjects combined, we observed 5 SNPs in the *SLC19A1* gene that were statistically associated with adenoma risk



after correcting for multiple testing (Table 2). Three of these 5 SNPs, rs12482346 (OR = 1.24; 95% CI = 1.07–1.44;  $p_{\text{act}} = 0.032$ ), rs2838958 (OR = 0.79; 95% CI = 0.68–0.92;  $p_{\text{act}} = 0.02$ ) and rs1051266 (OR = 1.25; 95% CI = 1.07–1.45;  $p_{\text{act}} = 0.028$ ) were in moderate linkage disequilibrium (LD) in the total study population (rs12482346–rs2838958,  $r^2 = 0.63$ ; rs12482346–rs1051266,  $r^2 = 0.79$ ; rs2838958–rs1051266,  $r^2 = 0.69$ ). The remaining 2 tag-SNPs, rs2838951 (OR = 0.78; 95% CI = 0.67–0.91;  $p_{\text{act}} = 0.015$ ) and rs2236484 (OR = 1.25; 95% CI = 1.07–1.46;  $p_{\text{act}} = 0.027$ ), were not in linkage disequilibrium with these SNPs or each other. Heterogeneity across race/ethnicity in the total study population was statistically significant for one SNP (*MTHFD1* rs11627525;  $p = 0.0000886$ ). *MTHFD1* rs11627525 was not significantly associated with adenoma risk for any race/ethnic group in the main effects analysis.

When the analysis was restricted to non-Hispanic whites, a sixth SNP in the *SLC19A1* gene (rs7499) and two SNPs in the *MTHFD1* gene (rs11627387 and rs8016556) were associated significantly with risk (Table 2). *SLC19A1* rs7499 is in the same haplotype block as rs1051266. Among the SNPs significant in non-Hispanic whites, there was nominally significant heterogeneity across race/ethnic group for one SNP (*MTHFD1* 80116556;  $p = 0.03$ ).

Compared to those with at least one wild-type allele, neither the *MTHFR C677T* genotype (rs1801133) nor the *MTHFR A1298C CC* genotype (rs1801131) was associated with adenoma risk; OR = 0.93, 95% CI = 0.66–1.32 and OR = 0.97, 95% CI = 0.66–1.43 for the 677 *TT* and 1298 *CC* genotypes, respectively. Similarly, there were no associations between *MTR* D919G (rs1805087), *MTHFD1* R653Q (rs2236225), *SHMT1* L474F (rs1979277), or *MTHFR* A1793Q (rs2274976) and adenoma risk overall or in non-Hispanic whites (Supplementary Table 2).

### Dietary folate, alcohol, and adenoma risk

We assessed possible modifications of the genotype/adenoma associations by dietary folate intake and alcohol use for all 159 tagSNPs in the total study population and non-Hispanic whites. There was no interaction between any *SLC19A1* or *MTHFD1* SNP and dietary folate or alcohol in either the total study population or non-Hispanic whites (data not shown). The interactions between *MTHFR C677T* and *A1298C* and folate availability and alcohol use, assuming a recessive model, are shown in Table 3. Sample size was not sufficient to assess genotype effects for an index of folate and alcohol combined (there were 3 cells with fewer than 5 subjects, including cells with 0 subjects, in *MTHFR A1298C* and 1 such cell for *MTHFR C677T*). For the 677 *TT* genotype, there was no evidence of a linear

trend across quartiles of dietary folate intake ( $p$  value for trend = 0.793). When we stratified on alcohol intake, the OR for the 677 *TT* genotype and high alcohol consumption (>20 g/day) was non-significantly greater than 1.0 (OR = 1.46 (0.60–3.55) and about 1.0 for those drinking 0 g/day (OR = 0.96 (0.55–1.68), but, again, there was no linear trend ( $p = 0.68$ ). For *A1298C* (rs1801131), there was significant heterogeneity across strata of dietary folate ( $p = 0.004$ ) due to a significant decrease in risk for those in the second dietary folate quartile (OR = 0.22 (0.07–0.65) and an increase in risk for those in the highest dietary folate quartile (OR = 2.07 (0.92–4.64)). In the first and third quartiles, the ORs were 1.27 (0.57–2.85) and 1.15 (0.57–2.33), respectively ( $p$  value for linear trend = 0.12). There was no modification by alcohol consumption for *A1298C* (heterogeneity  $p$  value = 0.98; trend  $p$  value = 0.94).

There were no significant interactions between any of the remaining tagSNPs and dietary folate or alcohol (data not shown). No SNP significantly interacted with dietary B<sub>12</sub>. However, there was a nominally significant interaction between the vitamin B<sub>12</sub> transport protein *GIF* rs519221 and dietary vitamin B<sub>12</sub> (heterogeneity  $p$  value = 0.02; trend  $p$  value = 0.003). ORs were less than 1.0 in the lowest 3 B<sub>12</sub> quartiles and non-significantly greater than 1.0 in the highest B<sub>12</sub> quartile (ORs = 0.63 (0.43–0.92); 0.66 (0.46–0.95); 0.96 (0.67–1.39) and 1.33 (0.89–1.96) for the lowest intake to the highest intake quartiles, respectively).

### Interactions between FOCM genes and sex

There were no significant interactions between any SNPs and sex at the pre-defined level of significance except for *MTHFR A1298C*. *MTHFR A1298C* interacted with sex at a nominal level of significance (interaction  $p$  value 0.007). Assuming a recessive model, the association between homozygosity for the C allele, compared to those with at least one A allele, and adenoma risk was significantly decreased in women (OR = 0.50 (0.26–0.96) and non-significantly increased in men (OR = 1.52 (0.92–2.51).

### FOCM genes, plasma tHcy, and adenoma risk

The association between SNPs and plasma tHcy was assessed for phase 2 subjects (data not shown). One SNP in *MTHFR* was associated with plasma tHcy at a nominal level of significance (rs9651118;  $\beta = -1.09$ ,  $\pm 0.36$ ,  $p_{\text{act}} = 0.03$ ). There were no other associations with plasma tHcy overall. After stratifying on dietary folate quartiles, there was no longer an association between rs9651118 and

**Table 2** Single SNP analysis by race/ethnic group for SNPs significant in total population or non-Hispanic whites

Gene/SNP	Total <sup>a</sup>	Non-Hispanic White	Black	Hispanic	Asian	<i>p</i> Heterogeneity
<i>SLC19A1</i>						
rs2838951						
<i>n</i>	1,337	719	221	143	193	0.08
MAF <sup>b</sup>	0.37	0.41	0.28	0.35	0.38	
OR (95% CI) <sup>c</sup>	0.78 (0.67,0.92)	0.70 (0.57, 0.88)	1.28 (0.85, 1.92)	0.97 (0.58,1.62)	0.71 (0.46, 1.09)	
<i>p</i> <sub>act</sub> <sup>d</sup>	0.02	0.01	0.97	0.99	0.64	
rs2838958						
<i>n</i>	1,348	722	224	147	195	0.13
MAF	0.46	0.47	0.27	0.42	0.46	
OR (95% CI)	0.79 (0.68, 0.93)	0.73 (0.5, 0.90)	1.30 (0.85, 1.98)	0.85 (0.51, 1.42)	0.75 (0.49, 1.15)	
<i>p</i> <sub>act</sub>	0.02	0.02	0.85	0.99	0.91	
rs2236484						
<i>n</i>	1,325	712	223	142	188	0.14
MAF	0.46	0.42	0.47	0.47	0.47	
OR (95% CI)	1.26 (1.08, 1.47)	1.44 (1.16, 1.79)	0.90 (0.63, 1.28)	0.92 (0.57, 1.49)	1.39 (0.91, 2.13)	
<i>p</i> <sub>act</sub>	0.03	0.01	0.99	0.99	0.90	
rs1051266						
<i>n</i>	1,344	721	224	146	193	0.68
MAF	0.48	0.45	0.44	0.48	0.47	
OR (95% CI)	1.24 (1.06, 1.44)	1.34 (1.08, 1.65)	0.98 (0.68, 1.41)	1.14 (0.71, 1.82)	1.19 (0.79, 1.77)	
<i>p</i> <sub>act</sub>	0.04	0.03	0.99	0.99	0.98	
rs12482346						
<i>n</i>	1,340	721	222	143	194	0.23
MAF	0.47	0.45	0.49	0.50	0.49	
OR (95% CI)	1.24 (1.06, 1.44)	1.42 (1.15, 1.76)	0.88 (0.61, 1.26)	1.06 (0.66, 1.71)	1.19 (0.79, 1.80)	
<i>p</i> <sub>act</sub>	0.04	0.01	0.99	0.96	0.99	
rs7499						
<i>n</i>	1,334	714	223	144	192	0.35
MAF	0.43	0.41	0.41	0.46	0.46	
OR (95% CI)	1.22 (1.04, 1.42)	1.38 (1.11, 1.71)	0.92 (0.64, 1.32)	1.00 (0.62, 1.60)	1.21 (0.79, 1.86)	
<i>p</i> <sub>act</sub>	0.07	0.01	0.99	1.0	1.0	
<i>MTHFD1</i>						
rs11627387						
<i>n</i>	1,346	719	225	147	195	0.24
MAF	0.29	0.31	0.24	0.23	0.29	
OR (95% CI)	0.78 (0.66, 0.92)	0.68 (0.54, 0.85)	0.70 (0.45, 1.08)	1.01 (0.57, 1.81)	1.10 (0.71, 1.71)	
<i>p</i> <sub>act</sub>	0.07	0.01	0.96	0.99	0.96	
rs8016556						
<i>n</i>	1,348	722	225	145	195	0.03
MAF	0.33	0.35	0.26	0.25	0.39	
OR (95% CI)	0.87 (0.74, 1.02)	0.69 (0.55, 0.86)	1.32 (0.85, 2.04)	0.84 (0.47, 1.49)	1.20 (0.81, 1.77)	
<i>p</i> <sub>act</sub>	0.67	0.01	0.88	0.99	0.99	

<sup>a</sup> Includes 61 individuals who characterized their race/ethnicity as 'other'

<sup>b</sup> Minor allele frequency in cases and controls

<sup>c</sup> Odds ratio and 95% confidence interval estimated by logistic regression assuming a log-additive (per allele) model and controlling for the matching factors (age, sex, clinic, and examination date) and study phase. The estimates for the total study population are also adjusted for race/ethnicity

<sup>d</sup> Adjusted for multiple testing taking into account correlated tagSNPs using a modified test of Conneely and Boehnke [26]

**Table 3** Association between *MTHFR C677T* and *A1298C* genotypes and distal adenoma risk, for the total study population, by dietary folate and alcohol

Genotype	528–2,191 mcg/day <sup>a,b</sup>	360–527 mcg/day	262–359 mcg/day	42–261 mcg/day	<i>p</i> Trend	<i>p</i> Heterogeneity
Dietary folate quartiles						
<b>677TT/677CC + CT<sup>c</sup></b>						
CC + CT: cases/controls	124/145	127/149	122/152	187/144	0.70	0.12
TT: cases/controls	14/20	13/24	24/15	17/18		
OR (95% CI) <sup>d</sup>	0.91 (0.44, 1.9)	0.66 (0.32, 1.36)	2.01 (1.0, 4.05)	0.78 (0.39, 1.6)		
<b>1298CC/1298AA + AC</b>						
AA + AC: cases/controls	125/158	127/157	152/157	195/162	0.12	0.004
CC: cases/controls	14/20	13/24	24/15	17/18		
OR (95% CI)	2.07 (0.92, 4.6)	1.15 (0.57, 2.33)	0.22 (0.07, 0.65)	1.27 (0.57, 2.85)		
	0 g/day <sup>e</sup>	0.1–10 g/day	11–20 g/day	>20–600 g/day	<i>p</i> Trend	<i>p</i> Heterogeneity
Alcohol use						
<b>677TT/677CC + CT</b>						
CC + CT: cases/controls	265/277	153/196	60/50	80/67	0.52	0.68
TT: cases/controls	26/30	18/29	8/9	16/9		
OR (95% CI)	0.96 (0.55, 1.68)	0.83 (0.44, 1.56)	0.69 (0.24, 1.93)	1.46 (0.60, 3.55)		
<b>1298CC/1298AA + AC</b>						
AA + AC: cases/controls	278/297	163/210	63/53	94/74	0.94	0.98
CC: cases/controls	24/28	18/23	8/6	4/4		
OR (95% CI)	0.92 (0.52, 1.64)	1.11 (0.57, 2.13)	1.04 (0.33, 3.25)	0.88 (0.21, 3.67)		

<sup>a</sup> Quartile cutpoints were based on the distribution of the controls and were study phase specific

<sup>b</sup> Dietary folate values reflect reported average intake in the year before the sigmoidoscopy

<sup>c</sup> Numbers do not add up to total due to missing values for genotype for some whose race/ethnic group classification was 'other' (27 cases/29 controls for *C677T* and 2 cases/1 control for *A1298C*) or alcohol (1 case)

<sup>d</sup> All ORs and 95% confidence intervals were estimated by unconditional logistic regression assuming a recessive model and adjusting for the matching factors (age, sex, clinic, examination date), race/ethnic group and study phase

<sup>e</sup> Alcohol cutpoint values were pre-determined and not based on the population distribution of alcohol consumption

plasma tHcy in any quartile and no interaction between dietary folate and genotype (interaction  $p = 0.67$ ) on plasma tHcy levels. Neither *MTHFR C677T* (interaction  $p = 0.97$ , trend  $p = 0.68$ ) nor *MTHFR A1298C* (interaction  $p = 0.98$ , trend  $p = 0.87$ ) was associated with plasma tHcy in any dietary folate quartile. Mean plasma tHcy was not significantly higher for those with the *MTHFR 677 TT* or *1298 CC* genotypes and low dietary folate compared to those with the highest dietary folate (data not shown). There were no significant interactions between dietary folate and genotype for any other SNP (data not shown).

### Gene–gene interactions

Gene–gene interactions were assessed for genes for which there was prior data suggesting interactions with the *MTHFR C677T* genotype (Supplemental Table 3). This included SNPs in *CBS*, *MTR*, *TYMS*, and *SLC19A1*. In the total study

population, adenoma risk per A allele at *SLC19A1* rs1051266 was significantly increased for those with at least one *MTHFR 677 C* allele (OR = 1.33 (1.21–1.56)) but risk per A allele was decreased for those with the 677 TT genotype (OR = 0.74 (0.47–1.17)). The interaction  $p$  value was 0.018. Nominally significant interactions were also observed for 5 other *SLC19A1* SNPs (rs17004785, rs2297291, rs3788205, rs3939250, and rs3827266), none of which were in LD with rs1051266. There was no interaction between *MTR A2756G* (rs1805087) or any *CBS* SNP and the *MTHFR C677T* genotype. There were nominally significant interactions between the *MTHFR C677T* genotype and 9 SNPs in *MTR* (rs12759827, rs10737812, rs1252252, rs4659730, rs6676866, rs2385511, rs3768150, rs10802569, and rs12070633) and one SNP in *TYMS* (rs2298582). Except for rs12759827 and rs4659730, the remaining seven *MTR* SNPs were in LD ( $r^2 > 0.70$ ) with each other in non-Hispanic whites. Only one SNP, rs4659730, was in LD with rs1805087 and that only in non-Hispanic whites.



## Discussion

In this study, we conducted a comprehensive analysis of 159 tagSNPs in 11 genes involved in folate-associated one-carbon metabolism and the risk of distal colorectal adenomas. Our results suggest that genetic variability in *SLC19A1* (also known as *RFC1*) may be associated with distal colorectal adenoma risk. There was no significant modification by dietary folate intake or alcohol for any SNP except *MTHFR A1298C* and dietary folate. The data were consistent with a possible interaction between the *MTHFR 1298 CC* genotype and sex. There was a significant interaction between *SLC19A1* rs1051266 (*G80A*, *H27R*) and the *MTHFR C677T* genotype. Seven SNPs in the *MTR* gene that were within a single LD block among non-Hispanic whites, also interacted with the *MTHFR C677T* genotype.

To our knowledge, this is the most comprehensive study of genetic variation in the folate pathway and risk of colorectal adenoma conducted to date. Hazra et al. [13] assessed the association between 24 non-synonymous SNPs in 13 folate pathway genes and colorectal adenoma risk in the Nurses' Health Study. They reported a significant association only for the transcobalamin II (*TCN2*) P259R polymorphism, assuming a dominant model. As in our study, no associations were seen for non-synonymous SNPs in *MTHFR*, *MTHFD1*, *SHMT1*, or *MTR*.

*SLC19A1* codes for a ubiquitously expressed transmembrane protein necessary for the uptake of reduced folates such as 5-MTHF, the main circulating folate. The *SLC19A1* protein transports 5-MTHF from the lumen of the gut into the cells of the small intestine and from the blood stream into epithelial cells. 5-MTHF is the enzymatically active form of folate and is required for the synthesis of methionine from homocysteine, the regeneration of tetrahydrofolate, and nucleotide synthesis [27]. Thus, functional change in the activity of *SLC19A1* could modulate folate metabolism by increasing or decreasing intracellular folate availability. A plausible role for *SLC19A1* function and colorectal neoplasia risk is suggested by animal studies showing that mice with an inactive gene have an increased susceptibility to chemically induced CRC [28].

To our knowledge, this is the first study of the relationship between rs1051266 and colorectal adenomas. However, two previous studies, both also conducted in non-folic acid-supplemented populations, have reported on the association between this SNP and colorectal cancer [29, 30]. Contrary to our results (OR = 1.25 (95% CI 1.07–1.45) per A allele), there was no association with CRC risk in either study population but, unlike our data, both found a possible decrease in risk associated with the A allele in those with low folate intake. Further studies of this

SNP in other colorectal adenoma patients will be necessary before we can draw any conclusions.

Our data are consistent with previous studies of the association between the *MTHFR C677T* and *A1298C* polymorphisms and colorectal adenoma in suggesting that neither of these polymorphisms are associated with adenoma risk overall [11, 12]. Our data provide limited support for the hypothesis that the 677 *TT* genotype may increase colorectal adenoma risk when folate is low and decrease risk when folate is high as has been seen in some studies including our own analysis of the phase 1 subjects [23, 31–34]. However, our results are consistent with other studies not reporting such modification [35–38].

The data were consistent with a possible U-shaped relationship between the *MTHFR A1298C* genotype and dietary folate intake. A recent European study also observed an increased adenoma risk for the 1298 *CC* genotype and high folate intake [37]. However, our data are inconsistent with those of most other studies assessing this interaction for adenoma [36, 39] or colorectal cancer risk [40–43].

We also observed a significant interaction between the *MTHFR 1298* genotype and sex with a decreased risk for women and an increased risk for men with the *CC* genotype, relative to those with at least one A allele. These findings are consistent with those of two other adenoma studies [37, 39] but opposite to those of a recent CRC study [44] and inconsistent with studies that did not observe any modification by sex for CRC [45, 46] or rectal cancer [47]. Given these mixed results, future studies should assess such modification so that some consensus can be achieved.

Data from several studies have suggested a gene–gene interaction between the *SLC19A1 G80A* and *MTHFR C677T* genotypes [29, 48, 49]. In all studies, including our own, the data suggest that the A allele might be associated with lower risk in those with the 677 *TT* genotype. Although the *MTR A2756G* polymorphism did not interact significantly with *MTHFR C677T*, as suggested in some previous studies [50–54], we did observe nominally significant interactions for a set of seven linked *MTR* SNPs that may indicate some effect modification for this linkage block, at least in non-Hispanic whites. To our knowledge, this is the first study to suggest such an interaction, and it remains for future studies to further define this linkage block and replicate the interaction before any conclusions can be drawn.

Our study has several strengths including the large sample size and the comprehensive approach to defining genetic variation in a large number of folate pathway genes. In addition, we had data on adenoma risk factors and folate pathway intermediates including plasma tHcy. Our large sample size allowed us to conduct several stratified analyses with reasonable statistical power, including analyses by race/

ethnic group, folate intake, and alcohol use. Weaknesses of our study include the fact that we did not have data on all the genes relevant to the folate pathway. Also, because this was a sigmoidoscopy-based study, some controls may have had undetected adenomas in the proximal colon, and so our findings are limited to distal adenomas. Additionally, this study was conducted prior to fortification of the US food supply with folic acid and folate intakes were low compared to those of supplemented populations. Thus, our findings may be specific to unsupplemented populations. Finally, the use of a Bonferroni correction for multiple testing in all stratified analyses may have been too conservative, and so there may be some false-negative results. Alternatively, given the large number of total comparisons made, false-positive findings are also possible.

In conclusion, in this comprehensive tagSNP analysis of 11 folate pathway genes, we observed that variation in *SLC19A1* may play a role in colorectal adenoma risk in non-Hispanic whites. Our data suggest a possible interaction between the *MTHFR A1298C* genotype and folate intake as well as an interaction with sex. The *SLC19A1 G80A* polymorphism and seven SNPs defining a linkage disequilibrium block in the *MTR* gene in non-Hispanic whites may interact with *MTHFR C677T* genotype in determining distal colorectal adenoma risk.

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**Conflicts of interest** D. Conti is a consultant for Pfizer Inc.

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