Research Article

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Genetic Variability in the *MTHFR* Gene and Colorectal Cancer Risk Using the Colorectal Cancer Family Registry

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Abstract

Background: The *MTHFR C677T TT* genotype is associated with a 15% to 18% reduction in colorectal cancer risk, but it is not clear if other variants of the gene are associated with colorectal cancer risk.

Methods: We used a tagSNP approach to comprehensively evaluate associations between variation in the *MTHFR* gene and colorectal cancer risk using a large family-based case-control study of 1,750 population-based and 245 clinic-based families from the Colon Cancer Family Registry. We assessed 22 TagSNPs, selected based on pairwise r^2 >95%, using the Haploview Tagger and genotyped the TagSNPs on the Illumina GoldenGate or Sequenom platforms. The association between single nucleotide polymorphisms and colorectal cancer was assessed using log-additive, codominant, and recessive models.

Results: From studying the population-based families, the *C677T* (rs1801133) and *A1298C* (rs1801131) polymorphisms were associated with a decreased colorectal cancer risk overall [odds ratio (OR), 0.81; 95% confidence interval (95% CI), 0.63-1.04; and OR, 0.82; 95% CI, 0.64-1.07, respectively]. The *677 TT* genotype was associated with a decreased risk of microsatellite-stable/microsatellite-low tumors (OR, 0.69; 95% CI, 0.49-0.97) and an increased risk of microsatellite-high tumors (OR, 2.22; 95% CI, 0.91-5.43; $P_{interaction}$ = 0.01), as well as an increased risk of proximal cancers and a decreased risk of distal and rectal cancers ($P_{interaction}$ = 0.02). No other single nucleotide polymorphism was associated with risk overall or within subgroups.

Conclusion: The *677 TT* and *1298 CC* genotypes may each be associated with a decrease in colorectal cancer risk. We observed little evidence of additional genetic variability in the *MTHFR* gene relevant to colorectal cancer risk. *Cancer Epidemiol Biomarkers Prev; 19(1); 89–100. ©2010 AACR.*

Introduction

5,10-Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in folate-associated one-carbon metabolism. The MTHFR enzyme is a flavin adenine dinucleotide–dependent enzyme that irreversibly reduces 5,10-methyltetrahdrofolate to 5-methyltetrahydrofolate, acting at the junction of two critical uses for folate-associated one-carbon groups, nucleotide synthesis and synthesizing the universal methyl donor S-adenosylmethionine. The *MTHFR* gene is polymorphic and two common nonsynonymous single nucleotide polymorphisms (SNP), *C677T* (A222V; rs1801133) and *A1298C* (E429A; rs1801131), have been extensively studied for associations with colorectal cancer. Both genotypes have been associated with decreased enzyme function *in vitro*, with reductions of ~60% for the *677 TT* genotype (1, 2) and 30% for the *1298 CC* genotype (3, 4). Four recent meta-analyses of

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A.J. Levine and R.W. Haile conceived of the study, participated in its design, and drafted the manuscript. J.C. Figueredo, W. Lee, J.N. Poynter, and D. Conti structured the statistical analyses. J.C. Figueredo and W. Lee performed the statistical analyses. D.J. Duggan conducted the single nucleotide polymorphism selection and genotyping and assisted in drafting the manuscript. J.C. Figueredo, J.N. Poynter, P.T. Campbell, P. Newcomb, M.E. Martinez, J.L. Hopper, L.L. Merchand, J.A. Baron, P.J. Limburg, C.M. Ulrich, and R.W. Haile participated in the design of the study and made substantive comments in drafting the manuscript. All authors read and approved the final manuscript.

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these *MTHFR* genotypes and colorectal cancer risk overall, using data from 22 studies, reported a modest but statistically significant 15% to 18% decrease in risk for the *677 TT* genotype (5-8). A similar inverse association was also reported for the *1298 CC* genotype on colorectal cancer risk (5, 7). Many studies reported that the decreased colorectal cancer risk associated with the *677 TT* genotype was observed mainly in those with higher folate availability (9-14). However, all of these studies were conducted in populations not exposed to supplementation of the food supply with folic acid.

More recently, studies have assessed associations between an increased number of polymorphic loci in the *MTHFR* gene and other health outcomes using tagging SNP or haplotype-based approaches for SNP selection (15, 16). Liu et al. (15) reported statistically significant associations between two SNPs and lung cancer risk, whereas in another study, three *MTHFR* SNPs were significantly associated with lean body mass after correction for multiple testing (16). These data suggest that there may be additional functionally significant variants in the *MTHFR* gene, but whether such variants are important determinants of colorectal cancer risk is unknown.

In the present study, we used a tagSNP approach to comprehensively evaluate associations between variation in the *MTHFR* gene, including the two known functional polymorphisms, and colorectal cancer risk in a large family-based case-control study based on the Colon Cancer Family Registry (C-CFR). We also assessed whether associations between SNPs and colorectal cancer risk were modified by dietary or total folate intake, folate supplement, or multivitamin use and assessed potential heterogeneity of the *MTHFR* SNP to colorectal cancer associations by selected tumor characteristics.

Materials and Methods

Data for this study were obtained through the C-CFR, a National Cancer Institute (NCI)–funded registry of colorectal cancer cases, unaffected family members, and population-based controls, which uses comprehensive and standardized methods for data collection and genotyping. Detailed information about the C-CFR can be found in a recent report by Newcomb et al. (17) and at the CFR Web site.[12]

Case and Control Ascertainment

The C-CFR is an international collaborative study initiated in 1997 with the goal of creating a resource for the study of the genetic epidemiology of colorectal cancer. Subject recruitment at the different C-CFR sites is described in detail by Newcomb et al. (17) and is described only briefly here. Participants were recruited from six centers including centers in the University of Southern California Consortium (Arizona, Cleveland Clinic, Colorado, Dartmouth, Minnesota, North Carolina, and University of Southern California), Hawaii (Honolulu), Fred Hutchinson Cancer Research Center (Seattle, WA), Mayo Clinic (Rochester, MN), Cancer Care Ontario (Toronto, Canada), and University of Melbourne (Victoria, Australia) using population-based and clinic-based ascertainment strategies. All centers except Fred Hutchinson Cancer Research Center oversampled cases with multiple first-degree relatives reporting colorectal cancer or colorectal cancer cases diagnosed under age 50 y to target families with excess colorectal cancer risk. First-degree and some second-degree relatives with colorectal cancer were also recruited from families with multiple colorectal cancer cases. For all centers, unaffected siblings or, when necessary, second-degree relatives were recruited as controls. The clinic-based sample represents multiple-case families at high risk of Hereditary Non-Polyposis Colon Cancer or other familial colorectal cancer phenotypes.

We used a case/unaffected sibling control design with data from both population-based and clinic-based families in the main effect analyses. There were too few clinic-based case/control pairs for stratified analyses so all stratified analyses used the population-based families only. Cases were probands and siblings diagnosed with colorectal cancer and controls were siblings without colorectal cancer at the time of ascertainment. All cases were interviewed within 5 y of diagnosis (73% within 2 y). We excluded monozygous twins. In addition, we also genotyped a random set of unrelated population-based controls ($n = 265$) from one of the C-CFR sites (Fred Hutchinson Cancer Research Center). All subjects signed an informed consent before providing data to the C-CFR.

SNP Selection

TagSNPs were selected using Haploview Tagger (18) using the following criteria: minor allele frequency (MAF) of >5%, pairwise r^2 of >0.95, and distance from closest SNP of >60 bp on the Illumina platform. The linkage disequilibrium blocks were determined using data from HapMap data release no. 16c.1, June 2005, on National Center for Biotechnology Information B34 assembly, dbSNP b124. For each gene, we extended the 5′- and 3′-untranslated regions (UTR) to include the 5′-UTR and 3′-UTR most SNP within the linkage disequilibrium (LD) block (~10 kb upstream and 5 kb downstream). In regions of no or low LD, SNPs with an MAF of >5% at a density of ~1 per kb were selected from either HapMap or dbSNP. Finally, nonsynonymous SNPs and expert-curated SNPs regardless of MAF were included. In this analysis, we report results for 22 tagSNPs and 3 candidate nonsynonymous SNPs in one gene central to regulation of the folate pathway, *MTHFR*.

SNP Genotyping

SNPs were genotyped on the Illumina GoldenGate platform (19). We implemented a series of quality control checks based on the Illumina metrics, and SNPs were excluded from analysis based on the following criteria:

[12] http://epi.grants.cancer.gov/CFR/

GenTrain score of <0.4, 10%GC score of <0.25, AB T Dev of >0.1239, call rate of <0.95, more than two P-P-C errors, or less than three discordance with HapMap. Interplate and intraplate replicates were included, and SNPs were excluded from the analysis if there were more than two errors on the 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 three discordant genotypes were discovered in comparison with the genotypes from the International HapMap Project (20). We performed additional genotyping using Sequenom's iPLEX Gold for two SNPs (rs17376328 and rs2050265) that were not successfully genotyped on the Illumina platform. PCR and extension primers for these two SNPs were designed using the MassARRAY Assay Design 3.0 software (Sequenom, Inc.) and are available upon request. PCR amplification and single base extension reactions were done according to the manufacturer's instructions. Extension product sizes were determined by mass spectrometry using Sequenom's Compact matrix-assisted laser desorption/ionization–time-of-flight mass spectrometer. The resulting mass spectra were converted to genotype data using SpectroTYPER-RT software.

Two SNPs were excluded because they failed genotyping (rs17375901 and rs3753582—one nonsynonymous SNP (rs2274976, R593Q) was excluded due to missing genotypes for over 900 individuals and one SNP (rs7533315) was excluded because it was significantly out of Hardy-Weinberg equilibrium at a significance level of <0.001 (P = 4.74E-08). This analysis reports on the remaining 22 SNPs.

Microsatellite Instability Testing

All available tumors from the C-CFR's Jeremy Jass Memorial Pathology Bank were assayed for instability at the following 10 microsatellites: BAT25, BAT26, BAT40, BAT34C4, D5S346, D17S250, D18S55, D10S197, ACTC, and MYCL as described previously (17). Only subjects with clear results for at least four markers were included. Microsatellite Instability data were available for 1,200 (66.4%) of cases. Instability at >30% of the tested loci was defined as microsatellite instability high (MSI-H); instability at >10% of loci but <30% of loci was defined as microsatellite instability low (MSI-L); and those with instability at 0 loci were categorized as microsatellite stable (MSS). Due to the small numbers and the lack of evidence for a separate effect in these data, the MSI-L cases were combined with the MSS cases in the analysis.

Folate Supplement Use, Multivitamin Use, and Colon Subsite

A standard risk factor questionnaire was administered to all participants in both populations at the time of recruitment and was available for 1,782 cases and 2,815 controls (~98% of the study population for this analysis). The questionnaire collected information on demographic factors; personal and family cancer histories, including personal history of colorectal polyps, colorectal, and other cancers; and other risk factors including selected medication use, use of a folate supplement and multivitamin at least twice weekly for more than a month in the 2 y before recruitment; tobacco and alcohol use history; physical activity and selected dietary preferences (e.g., cooking preference for red meat); and reproductive history (in women). Alcohol use was queried for three time periods (20s and 30s, 40s, and since turning 50). In this analysis, we summed across all three periods to get an estimate of lifetime use. Weekly alcohol intake was calculated as the sum of drinks per week from beer, wine, and liquor.

Dietary Folate Intake

Estimated dietary folate intake during the same 2-year time period, available for 585 cases and 837 controls (approximately one-third of the study population), was estimated from a validated food frequency questionnaire developed at the University of Hawaii (21) and available only for subjects recruited in North America, excluding those from the Mayo clinic and Fred Hutchinson Cancer Research Center (which did not administer a food frequency questionnaire). All food frequency data were collected after 1998, when supplementation of the food supply with 140 μg folic acid per 100 g of cereal products became mandatory. Dietary folate intake was estimated using a food composition table that included that supplementation in one analysis and also, in a separate analysis, where we ignored this supplementation. For the postsupplementation analysis, dietary and total folate were estimated using dietary folate equivalents to allow for the different bioavailability of folic acid.

Tumor Subsite

Tumor subsite was obtained from the pathology report and was available for 1,734 (96.0%) of cases. Right colon was defined as occurring in the cecum through the splenic flexure; left colon included the descending colon through the sigmoid colon; and rectal tumors included the rectosigmoid junction and the rectum.

Statistical Analysis

MAF was estimated from the genotype data from unrelated population-based controls. Pairwise linkage disequilibrium between SNPs was estimated using the square of the correlation coefficient (R^2) between markers. In the analysis of main effects, the population- and clinic-based data were analyzed separately. We used multivariable conditional logistic regression with sibship as the matching factor to estimate main effects and stratum-specific odds ratios (OR). We controlled for age and sex in all analyses. Additional control for folic acid supplement use (yes/no), multivitamin use (yes/no), and alcohol intake (none, 1-7 drinks/wk and >7 drinks/wk) did not change the results. In addition, we present only the age- and sex-adjusted models here. Except for the *C677T* and *A1298C* polymorphisms, for which there

are data supporting a recessive model, we assumed a log-additive model to assess genotype/colorectal cancer associations. Because prior data suggest specific effects for *C677T* and *A1298C*, *P* values for these two SNPs were not corrected for multiple testing. For the log-additive model, *P* values for the remaining SNPs were adjusted for multiple testing, taking into account correlated tagSNPs, using a modified test of Conneely and Boehnke (22), which is valid only for one–degree of freedom (df) tests. As a secondary analysis, we assessed all the genotype effects in a codominant model, comparing heterozygotes and homozygotes for the minor allele to those homozygous for the major allele, using dummy variables to obtain the OR and 95% confidence interval (95% CI) for each genotype and a 2-df likelihood ratio to test to estimate *P* values for each comparison. Third, we assessed the possibility for recessive effects for all SNPs, comparing those homozygous for the minor allele to those with one or no minor alleles. The results using the recessive models were the same as those using the codominant models, and only the codominant model results are included here. For multiple df likelihood ratio tests, the Bonferroni method was used to reset the significance level to 0.00227.

All analyses within the exposure strata were specified in advance based on indications for potential effect modification in the literature. Stratum-specific ORs were estimated among population-based families to evaluate heterogeneity by MSI (MSS/MSI-L and MSI-H), tumor subsite (right, left, rectum/rectosigmoid junction), regular use of a folate or multivitamin supplement (yes or no), and dietary folate intake (dichotomized at the median of the control population). Because not all centers recruited subjects from populations with folic acid fortification of the food supply (i.e., Australia and New Zealand), we assessed potential heterogeneity by center. Finally, we considered whether inclusion of cases recruited >2 y after diagnosis resulted in biased estimates by comparing SNP OR estimates for cases diagnosed under and over 2 y after diagnosis. There was no heterogeneity for either variable. For all stratified analyses, we included interaction terms in the regression models to get the interaction *P* values and used a 2-df (MSI status) or 3-df (tumor subsite) likelihood ratio test to assess heterogeneity. All statistical analyses were conducted using the R programming language and SAS v9.1.

Results

Table 1 describes the *MTHFR* SNPs included in this study. Characteristics of the study population are presented in Table 2. There were 1,806 population-based cases with 2,879 sibling controls. For the clinic-based population, there were 269 cases with 475 sibling controls. Both study populations were mainly Caucasian:

Table 1. Primary SNP data for the 22 SNPs

SNP	Location in gene/ protein change	Position	Base change in assay	MAF* population-based families
rs11121832	Intron	11782707	A/G	0.25
rs12121543	Intron	11777258	A/G	0.24
rs12404124	Intron	11796456	C/T	0.41
rs13306556	Intron	11774697	A/G	0.10
rs1476413	Intron	11774887	A/G	0.27
rs17037390	Intron	11783430	A/G	0.16
rs17037396	Intron	11784634	C/T	0.11
rs17037425	Intron	11792970	C/T	0.14
rs17421462	Flanking 3′-UTR	11779434	A/G	0.08
rs17421511	Intron	11780375	A/C	0.16
rs1801131	E428A	11777063	A/G	0.31
rs1801133	A221V	11778956	C/T	0.32
rs1994798	Intron	11777342	A/G	0.41
rs3737964	Intron	11789631	C/T	0.25
rs3737965	Intron	11789038	C/T	0.06
rs4846048	Intron	11768839	C/T	0.29
rs4846049	Intron	11772952	C/T	0.32
rs4846054	Intron	11791817	G/T	0.40
rs6541003	3′-UTR	11778454	G/T	0.40
rs9651118	Intron	11784801	A/G	0.23
rs17376328	5′ UTR	11799249	G/A	0.05
rs2050265	5′UTR	11802286	T/C	0.16

*MAF was estimated from genotype data from unrelated population-based controls.

87.5% of cases and 87.3% of controls in the population-based sample and 97.3% of cases and 97.5% of controls in the clinic-based sample reported their race/ethnic group as white. In the population-based sample, 17% were from Ontario, Canada; 64% were from the four U.S. sites; and 19% were from Australia or New Zealand. For the clinic-based population, none of the participants were from Canada; 14% were from the USC consortium in the United States; 41% were from Australia or New Zealand; 45% were from the Mayo Clinic in the United States; and no cases or controls were from the Hawaii or Seattle sites. Sixty-five percent of population-based cases reported no family history of colorectal cancer; 30% reported at least one first-degree relative with a family colorectal cancer history; and family history data were missing for 4.6%. In the clinic-based cases, 37.5% reported no first-degree relative with colorectal cancer; 26% reported at least one first-degree relative with colorectal cancer; and family history was missing for 36.4%.

Approximately 47% of subjects with risk factor data in each population reported drinking a moderate amount of alcohol (1-7 drinks per week) and ~10% of each study population reported taking a folic acid supplement. In the population-based families, 54% of cases and 47% of controls reported taking a multivitamin regularly in the 2 years before enrollment in this study ($P < 0.01$). Population-based cases were significantly younger ($P < 0.01$), more likely to be male ($P < 0.01$), and had a significantly higher body mass index ($P < 0.01$). Among the subjects with food frequency questionnaire data (32.4% of cases and 29.1% of controls), cases were less likely to take calcium supplements ($P = 0.04$), had lower total folate intake ($P = 0.02$), and lower total B_{12} intake (0.019). In the clinic-based families, cases were significantly younger ($P < 0.01$), more likely to be smokers ($P < 0.01$), and more likely to be very active ($P < 0.01$). Cases in the clinic-based population were more likely to have tumors with MSI-H (20.4% and 9.9% in the clinic-based and population-based samples, respectively) and less likely to have MSS tumors (22.7% and 47.3%, respectively).

We evaluated 22 SNPs in *MTHFR* with MAFs ranging from 0.05 to 0.41 (Table 1). The results of the main effect analyses are presented in Table 3. We observed a borderline statistically significant decrease in colorectal cancer risk (OR, 0.81; 95% CI, 0.63-1.04; $P = 0.10$) for the *677 TT* genotype (rs1801133) relative to the *CC* and *CT* genotypes in the population-based series. There was a nonstatistically significant inverse association for the *677 TT* relative to the *CT* and *TT* genotypes in the clinic-based population (OR, 0.59; 95% CI, 0.31-1.12; $P = 0.11$). The *1298 CC* genotype (rs1801131) relative to the *AC* and *AA* genotypes was also associated with a borderline statistically significant decrease in colorectal cancer risk among the population-based families (OR, 0.82; 95% CI, 0.64-1.07; $P = 0.14$) but not among the clinic-based families (OR, 1.02; 95% CI, 0.53-1.98; $P = 0.95$). Under the assumption of a log-additive model, no other SNP was statistically significantly associated with colorectal cancer risk in either study population after the correction for multiple testing (Table 3). When we assessed associations assuming a codominant model, homozygosity for the minor allele was nominally associated with colorectal cancer risk for five SNPs in the population-based cases (rs12404124, rs1994798, rs4846048, rs4846049, and rs6541003), but no *P* value was significant at the Bonferroni-adjusted *P* value (Table 3). In the clinic-based cases, no SNPs were associated with risk in the codominant models. The results for the recessive models were essentially the same as those for the codominant models.

Among those with food frequency questionnaire data, we found no evidence of heterogeneity for *MTHFR* genotypes when stratified by dietary or total (food plus supplement) folate intake using either prefortification or postfortification estimates (data not shown). Folate and multivitamin supplement use (yes/no) in the 2 years before diagnosis or recruitment was available for the 98% of the study population with completed risk factor questionnaire data. The *677 TT* genotype was associated with a decrease in colorectal cancer risk only in nonusers of a folic acid supplement (OR, 0.78; 95% CI, 0.60-1.01; $P = 0.06$; and OR, 1.16; 95% CI, 0.65-2.08; $P = 0.40$ in folic acid supplement nonusers and users, respectively), although the group of folic acid supplement users was small and neither the interaction term ($P = 0.55$) nor the *P* for heterogeneity ($P = 0.08$) were statistically significant. The results were similar when we stratified on multivitamin use (OR, 0.68; 95% CI, 0.49-0.95; $P = 0.02$; and OR, 0.97; 95% CI, 0.72-1.32; $P = 0.87$ in multivitamin nonusers and users, respectively). Again, neither the *P* for the interaction ($P = 0.48$) nor heterogeneity ($P = 0.07$) were statistically significant. The same pattern was evident for the *1298 CC* genotype. There were no other interactions with folate or multivitamin supplement use for any SNP.

We also assessed heterogeneity of the *MTHFR* effect by tumor microsatellite instability among the population-based families (Table 4). The *677 TT* genotype was associated with decreased risk of MSS/MSI-L colorectal cancer (OR, 0.69; 95% CI, (0.50-0.97; $P = 0.03$) and increased risk of an MSI-H tumor (OR, 2.22; 95% CI, 0.91-5.43; $P = 0.08$; $P_{interaction} = 0.02$; $P_{heterogeneity} = 0.01$). The *1298 CC* genotype was associated with a nonstatistically significant decreased risk of MSS/MSI-L (OR, 0.77; 95% CI, 0.54-1.08; $P = 0.13$) and MSI-H (OR, 0.51; 95% CI, 0.24-1.08; $P = 0.08$) tumors. There was no heterogeneity by MSI status for the other SNPs.

The overall test for interaction between the three subsites for the *677 TT* genotype was statistically significant ($P_{interaction} = 0.01$; 3 df likelihood ratio *P* value for heterogeneity = 0.02). The *MTHFR 677 TT* genotype was associated with an increase in the risk of proximal tumors (OR, 1.40; 95% CI, 0.88-2.23; $P = 0.16$) and a decrease in the risk for distal (OR, 0.69; 95% CI, 0.43-1.10; $P = 0.12$) and rectal (OR, 0.62; 95% CI, 0.41-0.93; $P = 0.02$) tumors. There was no evidence for heterogeneity in risk by tumor subsite for the *MTHFR 1298 CC* genotype (OR, 0.70; 95%

Table 2. Selected characteristics of the study population

	Population-based families			Clinic-based families		
	Cases (*n* = 1,806)	Sibling controls (*n* = 2,879)	*P**	Cases (*n* = 269)	Sibling controls (*n* = 475)	*P*†
Person characteristic						
Mean age ± SD	53.5 ± 10.9	54.0 ± 11.8	<0.01	49.1 ± 11.4	51.4 ± 11.8	<0.01
Sex, no. (%)						
Male	927 (51.3)	1278 (44.4)	<0.01	133 (49.4)	204 (42.9)	0.19
Female	879 (48.7)	1601 (55.6)		136 (50.6)	271 (57.1)	
Race, no. (%)						
Non-Hispanic white	1580 (87.5)	2512 (87.3)	1.00	262 (97.4)	463 (97.5)	1.00
Black	32 (1.8)	42 (1.5)		1 (0.4)	2 (0.4)	
Asian	69 (3.8)	113 (3.9)		0 (0)	0 (0)	
Other†	104 (5.8)	189 (6.6)		5 (1.9)	9 (1.9)	
Unknown/missing	21 (1.2)	23 (0.8)		1 (0.4)	1 (0.2)	
Center, no. (%)						
Ontario, Canada	308 (17.1)	515 (17.9)	—	0 (0)	0 (0)	1.00
USC Consortium, U.S.	384 (21.3)	519 (18.0)		38 (14.1)	48 (10.1)	
Melbourne, Australia	344 (19.0)	611 (21.2)		110 (40.9)	213 (44.8)	
Hawaii, U.S.	63 (3.5)	103 (3.6)		0 (0)	0 (0)	
Mayo Foundation, U.S.	282 (15.6)	526 (18.3)		121 (45.0)	214 (45.1)	
Seattle, U.S.	425 (23.5)	605 (21.0)		0 (0)	0 (0)	
Family history of colorectal cancer, no. (%)						
No first -degree relative	1177 (65.2)			101 (37.5)		
At least first-degree relative	546 (30.2)	—	—	70 (26.0)	—	—
Unknown/Missing	83 (4.6)			98 (36.4)		
BMI (kg/m^2)‡						
15-18 (underweight)	22 (1.2)	25 (0.9)	<0.01	6 (2.2)	12 (2.5)	0.82
18-25 (normal)	629 (34.8)	1155 (40.1)		97 (36.1)	174 (36.6)	
25-30 (overweight)	670 (37.1)	1036 (36.0)		100 (37.2)	174 (36.6)	
30+ (obese)	422 (23.4)	594 (20.6)		53 (19.7)	92 (19.4)	
Unknown/missing	63 (3.5)	69 (2.4)		13 (4.8)	23 (4.8)	
Alcohol use (drinks/wk)						
None	467 (25.9)	829 (28.8)	0.08	76 (28.3)	132 (27.8)	0.43
1-7 (moderate)	857 (47.5)	1353 (47.0)		124 (46.1)	226 (47.2)	
8+ (heavy)	229 (12.7)	362 (12.6)		39 (14.5)	61 (12.8)	
Unknown/missing	253 (14.0)	335 (11.6)		30 (11.2)	56 (11.8)	
Smoking						
Never	781 (43.2)	1309 (45.5)	0.24	138 (51.3)	226 (47.6)	<0.01
Former	632 (35.0)	1001 (34.8)		58 (21.6)	153 (32.2)	
Current	343 (19.0)	509 (17.7)		66 (24.5)	87 (18.3)	
Unknown/missing	50 (2.8)	60 (2.1)		7 (2.6)	9 (1.9)	
Folate supplements§						
No	1586 (87.8)	2557 (88.8)	0.14	233 (86.6)	411 (91.3)	0.25
Yes	196 (10.9)	274 (9.5)		31 (11.5)	39 (8.7)	
Unknown/missing	24 (1.3)	48 (1.7)		5 (1.9)	11 (2.3)	
Multivitamins§						
No	820 (45.4)	1497 (52.0)	<0.01	138 (51.3)	267 (56.2)	0.27
Yes	971 (53.8)	1346 (46.8)		129 (48.0)	200 (42.1)	
Calcium supplements§						
No	1335 (73.9)	2063 (71.7)	0.03	208 (77.3)	345 (76.7)	0.33

(*Continued on the following page*)

Table 2. Selected characteristics of the study population (Cont'd)

	Population-based families			Clinic-based families		
	Cases (*n* = 1,806)	Sibling controls (*n* = 2,879)	*P**	Cases (*n* = 269)	Sibling controls (*n* = 475)	*P*†
Yes	459 (25.4)	785 (27.3)		56 (20.8)	105 (23.3)	
Unknown/missing	12 (0.7)	31 (1.1)		5 (1.9)	2 (0.4)	
Dietary folate (μg; mean ± SD)‖	327.4 ± 118.7	334.1 ± 126.8	0.32	349.6 ± 154.9	346.0 ± 145.0	0.95
Total folate DFE (mean ± SD)‖	477 ± 265.6	525.4 ± 439.7	<0.01	606.5 ± 463.1	549.6 ± 322.2	0.29
Dietary B_{12} (mean ± SD)‖	3.0 ± 1.2	2.9 ± 1.3	0.67	3.1 ± 1.4	3.0 ± 1.4	0.36
Total B_{12} (mean ± SD)‖	6.2 ± 6.4	7.4 ± 11.8	<0.01	10.0 ± 17.6	8.8 ± 10.0	0.41
Dietary B_6 (mean ± SD)‖	1.1 ± 0.4	1.1 ± 0.4	0.26	1.1 ± 0.5	1.1 ± 0.4	0.64
Total B_6 (mean ± SD)‖	1.9 ± 2.0	2.3 ± 3.8	<0.01	3.0 ± 6.0	2.6 ± 3.3	0.48
Tumor characteristics						
Site, no. (%)						
Right colon	598 (33.1)	—		85 (31.6)	—	
Left colon	525 (29.1)			44 (16.4)		
Rectum	593 (32.8)			77 (28.6)		
Unknown/missing	90 (5.0)			63 (23.4)		
MSI, no. (%)						
MSS	855 (47.3)	—		61 (22.7)	—	
MSI-L	151 (8.4)			14 (5.2)		
MSI-H	179 (9.9)			55 (20.4)		
Unknown/missing	621 (34.4)			139 (51.7)		

Abbreviations: DFE, dietary folate equivalent; BMI, body mass index.

*P values using a 1-df likelihood test from a conditional logistic regression model.

†Includes individuals who self-identified themselves as Hispanic, Native, Hawaiian/Pacific Islander, and mixed race.

‡Self-reported weight and height (2 y before the questionnaire completion date) used to calculate body mass index.

§Ever use of supplements regularly during the 2 y before recruitment (at least twice per week for more than a month).

‖Calorie-adjusted calculated from the food frequency questionnaire using postfortification food composition tables (*n* cases = 585; *n* controls = 837).

CI, 0.45-1.07 for proximal colon; OR, 1.22; 95% CI, 0.74-1.99 for distal colon; and OR, 0.69; 95% CI, 0.44-1.10 for rectal tumors; $P_{interaction} = 0.95$). No other SNP was differentially associated with tumor subsite.

We also conducted a stratified analysis to examine possible modification by age (<65/>65 years) or sex. There was no modification by age or sex for any SNP (data not shown).

Discussion

In the current study, we assessed a comprehensive set of SNPs that characterized the genetic variation of the *MTHFR* gene, including 10-kb 5′ of the transcription start site and 5-kb into the 3′-UTR. To our knowledge, this is the most comprehensive analysis of genetic variation in the *MTHFR* gene in relation to colorectal cancer risk completed to date. Our data from both the population- and clinic-based series are consistent with inverse associations for the *677 TT* (rs1801133; A222V) and the *1298 CC* genotypes (rs1801131; E429A). Stratification by folate and multivitamin supplement use suggested that *677 TT* and *1298 CC* genotypes may be associated with decreased colorectal cancer risk only among nonusers of folate or multivitamin supplements, but neither interaction was statistically significant. Our data also support a positive association between the *677 TT* genotype and the MSI-H phenotype and tumors of the proximal colon (wherein most MSI-H tumors are located); conversely, the results suggested an inverse association between this SNP and MSS/MSI-L tumors and tumors in the distal colon and rectum. No other SNPs were significantly associated with risk overall or in any subgroup.

Our data are consistent with four recent meta-analyses that suggested 15% to 18% reductions in colorectal cancer risk for the *677 TT* genotype (5-8). The *A1298C* polymorphism has been also well studied. Although the functional effects of the *C* allele are unclear (2), two meta-analyses (5, 7) suggest that homozygosity for the enzyme with a *C* at nucleotide *1298* is associated with an approximately 10% to 15% decrease in colorectal cancer risk, consistent with the estimate in the current study. Several studies published after the meta-analyses were completed have reported increased risk associated with the *677 TT* genotype

Table 3. Single SNP analysis: colorectal cancer risk by SNP, analysis model, and study population

SNP	Log-additive model[†]	Corrected *P*	Codominant model: heterozygotes[‡]	*P*	Codominant model: homozygotes	*P*	2-df LRT *P*
	Population-based sample*						
	OR (95% CI)		OR (95% CI)[†]		OR (95A% CI)		
rs11121832	0.95 (0.82-1.10)	0.93	0.96 (0.81-1.14)	0.63	0.89 (0.64-1.25)	0.51	0.79
rs12121543	0.94 (0.81-1.08)	0.94	0.89 (0.75-1.05)	0.17	1.00 (0.71-1.41)	1.00	0.34
rs12404124	0.90 (0.79-1.02)	0.54	0.96 (0.81-1.14)	0.62	0.77 (0.60-1.00)	0.05	0.13
rs13306556	1.01 (0.82-1.23)	0.99	0.98 (0.79-1.22)	0.86	1.22 (0.63-2.39)	0.56	0.83
rs1476413	0.95 (0.82-1.09)	0.95	0.93 (0.78-1.10)	0.38	0.93 (0.67-1.30)	0.68	0.68
rs17037390	0.86 (0.73-1.02)	0.56	0.86 (0.72-1.04)	0.13	0.75 (0.45-1.25)	0.26	0.26
rs17037396	1.00 (0.83-1.22)	0.98	0.94 (0.76-1.17)	0.59	1.34 (0.71-2.53)	0.37	0.50
rs17037425	0.90 (0.75-1.07)	0.80	0.91 (0.75-1.10)	0.34	0.74 (0.42-1.28)	0.27	0.46
rs17421462	1.01 (0.81-1.26)	1.00	0.94 (0.74-1.18)	0.59	1.97 (0.83-4.67)	0.12	0.24
rs17421511	0.93 (0.79-1.10)	0.95	0.93 (0.77-1.12)	0.46	0.88 (0.54-1.45)	0.63	0.74
rs1801131[§]	0.82 (0.64-1.07)	0.14[‖]	0.90 (0.76-1.07)	0.23	0.76 (0.56-1.02)	0.07	0.19
rs1801133[§]	0.81 (0.63-1.04)	0.10[‖]	1.10 (0.93-1.31)	0.26	0.88 (0.66-1.17)	0.36	0.14
rs1994798	0.89 (0.78-1.00)	0.44	0.95 (0.80-1.14)	0.59	0.76 (0.59-0.98)	0.03	0.08
rs3737964	0.95 (0.83-1.09)	0.93	0.96 (0.81-1.13)	0.61	0.89 (0.64-1.24)	0.50	0.78
rs3737965	0.90 (0.68-1.17)	0.95	0.90 (0.68-1.19)	0.45	0.77 (0.22-2.70)	0.69	0.73
rs4846048	0.87 (0.76-1.00)	0.42	0.93 (0.78-1.10)	0.37	0.71 (0.52-0.96)	0.03	0.09
rs4846049	0.87 (0.76-0.99)	0.37	0.89 (0.75-1.05)	0.16	0.74 (0.56-0.99)	0.04	0.13
rs4846054	0.91 (0.80-1.03)	0.64	0.97 (0.82-1.14)	0.75	0.79 (0.61-1.03)	0.08	0.17
rs6541003	0.89 (0.79-1.01)	0.52	0.96 (0.81-1.14)	0.62	0.77 (0.59-0.99)	0.05	0.12
rs9651118	1.15 (1.00-1.33)	0.45	1.26 (1.06-1.50)	0.01	1.09 (0.76-1.55)	0.65	0.03
rs17376328	1.04 (0.78-1.38)	1.00	1.06 (0.80-1.42)	0.67	0.43 (0.05-3.43)	0.43	0.67
rs2050265	0.87 (0.73-1.03)	0.58	0.88 (0.73-1.07)	0.21	0.68 (0.41-1.15)	0.15	0.26
	Clinic-based families[‖]						
rs11121832	1.09 (0.77-1.54)	1.00	1.28 (0.81-2.02)	0.30	0.91 (0.41-2.03)	0.83	0.83
rs12121543	1.12 (0.81-1.56)	0.99	1.05 (0.70-1.59)	0.81	1.41 (0.64-3.10)	0.39	0.71
rs12404124	1.00 (0.74-1.36)	1.00	0.94 (0.61-1.46)	0.79	1.02 (0.55-1.89)	0.95	0.93
rs13306556	1.12 (0.68-1.83)	0.93	1.12 (0.68-1.87)	0.65	1.14 (0.11-11.8)	0.91	0.91
rs1476413	1.03 (0.74-1.44)	1.00	1.02 (0.68-1.52)	0.93	1.09 (0.50-2.38)	0.84	0.98
rs17037390	0.90 (0.59-1.38)	1.00	0.90 (0.56-1.43)	0.65	0.83 (0.23-3.07)	0.78	0.89
rs17037396	1.07 (0.66-1.75)	1.00	1.11 (0.67-1.84)	0.69	0.73 (0.06-8.58)	0.81	0.89
rs17037425	0.99 (0.64-1.53)	1.00	1.01 (0.63-1.61)	0.97	0.79 (0.14-4.59)	0.79	0.95
rs17421462	1.32 (0.78-2.24)	0.87	1.16 (0.67-2.00)	0.59	9.91(1.25-78.4)	0.03	0.12
rs17421511	0.98 (0.64-1.50)	1.00	1.19 (0.77-1.83)	0.43	0.27 (0.05-1.48)	0.13	0.09
rs1801131*	1.02 (0.53-1.98)	0.95[‖]	0.98 (0.65-1.47)	0.92	1.01 (0.48-2.10)	0.99	0.98
rs1801133*	0.59 (0.31-1.12)	0.10[‖]	1.21 (0.82-1.80)	0.33	0.69 (0.33-1.44)	0.32	0.16
rs1994798	1.08 (0.78-1.49)	1.00	1.04 (0.67-1.61)	0.86	1.19 (0.62-2.27)	0.60	0.85
rs3737964	1.06 (0.76-1.47)	1.00	1.23 (0.78-1.93)	0.37	0.90 (0.43-1.87)	0.78	0.49
rs3737965	2.04 (1.07-3.88)	0.43	2.04 (1.07-3.88)	0.03	NA[¶]	NA	0.17
rs4846048	1.02 (0.72-1.44)	1.00	1.27 (0.81-1.98)	0.30	0.85 (0.42-1.74)	0.66	0.33
rs4846049	0.96 (0.69-1.34)	1.00	0.98 (0.65-1.48)	0.93	0.91 (0.44-1.88)	0.79	0.96
rs4846054	1.07 (0.78-1.45)	1.00	1.03 (0.67-1.60)	0.88	1.15 (0.62-2.15)	0.66	0.89
rs6541003	1.06 (0.76-1.46)	1.00	0.93 (0.60-1.43)	0.73	1.17 (0.61-2.25)	0.64	0.69

(*Continued on the following page*)

Table 3. Single SNP analysis: colorectal cancer risk by SNP, analysis model, and study population (Cont'd)

	Population-based sample*						
SNP	**Log-additive model†**	**Corrected *P***	**Codominant model: heterozygotes‡**	***P***	**Codominant model: homozygotes**	***P***	**2-df LRT *P***
	OR (95% CI)		**OR (95% CI)†**		**OR (95A% CI)**		
rs9651118	0.95 (0.68-1.33)	1.00	1.08 (0.70-1.68)	0.73	0.71 (0.35-1.48)	0.36	0.64
rs17376328	0.64 (0.32-1.32)	0.81	0.64 (0.32-1.32)	0.23	NA	NA	0.46
rs2050265	0.92 (0.60-1.40)	1.00	0.92 (0.57-1.46)	0.71	0.85 (0.23-3.12)	0.80	0.93

Abbreviations: LRT, likelihood ratio test; NA, not applicable.
*Based on a minimum of 1,702 cases and 27,26 controls from 1,647 discordant sibships.
†ORs estimated assuming a log-additive model and controlling for age and sex.
‡ORs estimated assuming a codominant model and controlling for age and sex.
§ORs estimate assuming a recessive genotype effect and controlling for age and sex.
‖*P* value not corrected for multiple testing.
¶OR could not be estimated because there were 0 subjects in the case or control group or the number was too small for a valid estimate.

(23-25), whereas others reported no association (26-28) or a decreased risk for those with the variant genotype (29). Chang et al. (9) reported a significant increase in risk for the *677 TT* genotype in those with low folate intake. For the *1298 CC* genotype, two studies reported an increase in risk (27, 28), one reported a decreased risk (29), and one reported no association with risk (9). Additionally, one recent study of a Hereditary Non-Polyposis Colon Cancer cohort reported an increase in the age of colorectal cancer onset among those with the *1298 CC* or joint *677T/1298C* genotypes, suggesting a protective effect of these variant alleles in Hereditary Non-Polyposis Colon Cancer kindreds (30). Variability in folate availability in these different source populations may explain the diverse findings, suggesting that future meta-analyses should account for such differences.

Data from observational studies suggest that the phenotype of the *MTHFR* valine protein (*677 TT* genotype) depends significantly on folate availability (31-34). A recent *in vitro* study in HCT116 colon carcinoma cells reported that the valine protein (*TT* genotype) was associated with increased genomic DNA methylation if folate is adequate but shows a significant decrease in genomic methylation when folate is deficient (35), supporting the impression that folate availability is a modifier of genotype effect. This is consistent with the biochemical changes in the valine-containing enzyme, which show that the enzyme is stabilized by the addition of 5-methyltetrahydrofolate to the culture medium (2). Therefore, we considered whether folate availability might modify associations between other SNPs and colorectal cancer risk as well. Although there was no heterogeneity by dietary or total folate consumption in the subgroup of participants with food frequency data, in the data from the whole study population, homozygosity for the *MTHFR 677 T* allele was significantly associated with decreased risk only in nonmultivitamin users, a result that was suggested for nonusers of folate supplements as well. These results conflict with most previous reports of nonfolate-supplemented populations, which suggest that the *677 TT* genotype is protective mainly for those with higher folate availability (9-14), although other studies have not observed this difference (29, 36-38). However, in the current study, neither the interactions nor the tests for heterogeneity were statistically significant. Therefore, this finding may be due to chance. On the other hand, one difference between our study population and those of the earlier studies is the likely higher folic acid levels to which most of our study population had been exposed during the 2 years preceding their recruitment, due to fortification of the food supply. It is likely that nonsupplement users in the current population have a greater folate intake than the subjects in the prefortification study populations (39) with levels more similar to those with higher folate intakes in previous studies. Subjects taking supplements in the current population showed little association with colorectal cancer risk, as one would expect if there were a stabilization of the enzyme in the presence of high-folate availability. Whether postsupplement folate levels are relevant to the lower risk we observed in *677 TT* homozygotes who were not taking folate or multivitamin supplements is unclear and must be assessed in future studies of populations with similar levels of fortification.

In stratified analyses, we found that the *677 TT* genotype was associated with a decreased risk of MSS tumors and tumors in the distal colon or rectum while being associated with an increased risk for MSI-H tumors and tumors in proximal colon. The literature on the relationship between the *677 TT* genotype and MSI status is mixed.

Table 4. Colorectal cancer risk by *MTHFR* genotype and MSI status

SNP	MSS + MSI-L*	*P*	MSI-H†	*P*	$P_{interaction}$‡
rs11121832	1.04 (0.86-1.27)§	0.66	1.05 (0.66-1.69)	0.23	0.97
rs12121543	0.94 (0.78-1.14)	0.54	0.97 (0.59-1.60)	0.83	0.91
rs12404124	0.96 (0.81-1.13)	0.61	0.90 (0.59-1.37)	0.61	0.77
rs13306556	1.03 (0.80-1.33)	0.83	0.93 (0.47-1.87)	0.85	0.79
rs1476413	0.97 (0.81-1.17)	0.75	1.05 (0.65-1.71)	0.83	0.74
rs17037390	0.88 (0.70-1.09)	0.25	0.76 (0.44-1.33)	0.34	0.64
rs17037396	0.97 (0.76-1.25)	0.84	1.11 (0.60-2.07)	0.74	0.69
rs17037425	0.95 (0.76-1.19)	0.65	0.82 (0.47-1.42)	0.48	0.63
rs17421462	1.25 (0.92-1.70)	0.16	1.00 (0.53-1.87)	0.99	0.56
rs17421511	0.97 (0.77-1.20)	0.75	1.08 (0.63-1.85)	0.78	0.71
rs1801131‖	0.77 (0.54-1.08)	0.13	0.51 (0.24-1.08)	0.08	0.36
rs1801133‖	0.69 (0.50-0.97)	0.03	2.22 (0.91-5.43)	0.08	0.01
rs1994798	0.94 (0.80-1.11)	0.50	0.85 (0.55-1.31)	0.47	0.65
rs3737964	1.01 (0.83-1.22)	0.92	1.08 (0.68-1.72)	0.75	0.80
rs3737965	0.98 (0.70-1.39)	0.92	0.81 (0.33-2.00)	0.66	0.69
rs4846048	0.94 (0.78-1.13)	0.52	0.92 (0.59-1.43)	0.71	0.93
rs4846049	0.91 (0.76-1.08)	0.27	0.85 (0.54-1.35)	0.49	0.79
rs4846054	0.99 (0.84-1.17)	0.88	0.91 (0.59-1.40)	0.68	0.74
rs6541003	0.98 (0.83-1.16)	0.86	0.87 (0.57-1.34)	0.54	0.61
rs9651118	1.07 (0.88-1.30)	0.48	0.68 (0.41-1.12)	0.13	0.12
rs17376328	0.86 (0.59-1.25)	0.43	1.41 (0.58-3.45)	0.45	0.32
rs2050265	0.87 (0.69-1.08)	0.21	0.90 (0.51-1.58)	0.71	0.91

*Based on a minimum of 958 cases and 1,532 controls from 931 discordant sibships.
†Based on a minimum of 175 cases and 255 controls from 166 discordant sibships.
‡*P* value for the multiplicative interaction term [MSI status * genotype (coded as 0, 1, or 2 for the presence of the minor allele)].
§Except as noted, all ORs were estimated assuming a log-additive model and controlling for age and sex.
‖ORs estimated from a recessive model and controlling for age and sex.

Most studies have reported an increased risk for MSI-H tumors in those homozygous for the *677 TT* genotype (9, 40-42), but some studies have reported no difference between MSI subgroups (43, 44) or a decrease in the risk of MSI-H tumors in those homozygous for the *677 T* allele (45, 46). Our data are also consistent with those of a recent study of Australian colorectal cancer patients, which reported a significant increase in the risk of proximal and decreased risk of distal colorectal cancers in those with the *677 TT* genotype (47), adding to the general consensus that those with the *677 TT* genotype may have an increased risk for tumors with the MSI-H phenotype and for tumors of the proximal colon. These two findings are likely to reflect the same association because data strongly suggest that the majority of MSI-H tumors develop in proximal colon and rarely in distal colon or rectum (47, 48). We did not observe any modification by MSI status for the *1298 CC* genotype, a result that is consistent with those of other reports (41, 45, 46). It is unclear why the association between the *1298* genotype and MSI status should be different from that of the *677* genotype.

This study has several strengths. In addition to the large sample size, we had a comprehensive approach to identifying genetic variation in the *MTHFR* gene, a family-based design which minimized the probability of population stratification, included detailed risk factor information on all our subjects, and had the ability to assess possible heterogeneity of genotype effects by folate nutrition, multivitamin use, MSI status, and tumor subsite. The ability to assess genotype associations in two separate samples is another strength.

Weaknesses of this study include the possibility that we have missed an important source of genetic variability because we used public databases to define SNPs; these databases are also incomplete. Additionally, although the case-unaffected sibling design is more powerful for assessing gene-environment interactions, it is less powerful for detecting main effects (49). Thus, our study may have been underpowered in the main effect analyses. In addition, we did not have the dietary data from all the participants and not all cases provided tumor tissue for MSI analysis, limiting the statistical power for analyses involving diet and MSI status; however, it is unlikely that the availability of dietary or MSI data are associated with genotype, so this should not have resulted in any bias in the observed ORs. As in any case-control study, dietary intake information was assessed after the diagnosis in cases, so the information may be affected by recall

bias. We were unable to genotype the rare R593Q (*G1793A*, rs2274976) polymorphism, so we could not assess whether this SNP was associated with risk overall or in any subgroup. The functional consequences of the R593Q polymorphism are not completely clear but some studies have suggested associations with various outcomes (50-54). Several studies have shown the *A* allele for this SNP to be in cis with the *1298 C* allele (53, 55, 56). Finally, many of the protocols used in the C-CFR were designed to oversample cases with a greater risk for a family history of colorectal cancer, which may decrease the generalizability of our findings. Additionally, in the population-based sample, ~90% of our study population was Caucasian and from the folate-supplemented populations in the United States or Canada, whereas this percentage was ~100% in the clinic-based subjects. Only 19% of the population-based sample was not of North American origin and none of the subjects in either population came from Latin America, South America, or Western Europe, all countries with significantly lower folate availability. To the extent that *MTHFR* genotype modifies risk differently in different countries, perhaps associated with differences in folate intake and the prevalence of smoking and alcohol use, our results may not generalize to all relevant populations.

Conclusion

In this study, using a tagSNP approach, we did not find strong evidence for additional functional genetic variation in the *MTHFR* gene for colorectal cancer risk. Our data suggest that the well-known *C677T* and *A1298C* variants are the most relevant *MTHFR* variants for colorectal cancer risk and that there may be heterogeneity in the risk associated with the *C677T TT* variant by MSI status, tumor subsite and, possibly, by folate or multivitamin supplement use in folic acid–supplemented populations.

Disclosure of Potential Conflicts of Interest

D. Conti is an advisor to Pfizer, Inc. P. Limburg is an advisor to Genomic Health, Inc.

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Genetic Variability in the *MTHFR* Gene and Colorectal Cancer Risk Using the Colorectal Cancer Family Registry

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