

MTHFR C677T and A1298C Polymorphisms: Diet, Estrogen, and Risk of Colon Cancer

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Abstract

5,10-methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in folate metabolism, diverting metabolites toward methylation reactions or nucleotide synthesis. Using data from an incident case-control study (1608 cases and 1972 controls) we investigated two polymorphisms in the MTHFR gene, C677T and A1298C, and their associations with risk of colon cancer. All of the combined genotypes were evaluated separately, and the 1298AA/677CC (wild-type/wild-type) group was considered the reference group. Among both men and women, the 677TT/1298AA (variant/wild-type) genotype was associated with a small reduction in risk [men: odds ratio (OR), 0.7, 95% confidence interval (CI), 0.5–1.0; women: OR, 0.8, 95% CI, 0.5–1.2]. However, the 677CC/1298CC (wild-type/variant) genotype was associated with a statistically significant lower risk among women (OR, 0.6; 95% CI, 0.4–0.9) but not men. When the polymorphisms were considered individually, for A1298C a significant risk reduction associated with the homozygous variant CC genotype was seen among women only (OR, 0.6; 95% CI, 0.5–0.9), and nonstatistically significant reduced risks were observed for the variant 677 TT genotypes among both men and women. Stratification by nutrient intakes showed inverse associations with higher intakes of folate, vitamin B₂, B₆, B₁₂, and methionine among women with the MTHFR 677CC/1298AA genotypes, but not those with 677TT/1298AA. We observed opposite risk trends for both MTHFR variants, depending on whether women used hormone-replacement therapy or not (*P* for interaction = <.01).

In summary, this study supports recent findings that the MTHFR A1298C polymorphism may be a predictor of colon cancer risk and have functional relevance. The possible interaction with hormone-replacement therapy warrants additional investigation.

Received 5/19/03; revised 10/9/03; accepted 10/22/03.

Grant support: NIH Grants R01 CA48998 and R01 CA59045.

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Introduction

Folate acts as a one-carbon donor and is an essential nutrient for the synthesis of nucleotides (*e.g.*, thymidine and purines) and the provision of methyl groups (1). Dietary folate intakes have been found associated with a reduced risk of colorectal neoplasia in most, although not all, studies (2–9). Recent studies on genetic polymorphisms in folate-metabolizing enzymes lend support to a causal relationship between folate and colorectal carcinogenesis (9–13). Potential mechanisms for the observed associations include an altered provision of *S*-adenosylmethionine for methylation reactions, including DNA methylation, and changes in the availability of nucleotides for DNA synthesis and repair (14, 15).

MTHFR (5,10-methylenetetrahydrofolate reductase) plays a central role in folate metabolism. This enzyme catalyzes the irreversible reaction of 5,10-methylene-tetrahydrofolate to 5-methyl tetrahydrofolate, which serves as a substrate for the remethylation of homocysteine to methionine, with the subsequent synthesis of *S*-adenosylmethionine. However, the substrate of MTHFR, 5,10-methylene-tetrahydrofolate, is also required for thymidine synthesis via thymidylate synthase, and indirectly for purine synthesis. Two common polymorphisms have been described in the *MTHFR* gene, both single nucleotide substitutions resulting in amino acid changes (C677T → Ala222Val and A1298C → Glu429Ala; Refs. 16, 17). Whereas C677T unequivocally affects enzyme function and has been associated with increased plasma homocysteine concentrations and an altered balance of folate metabolites (16, 18), the *in vivo* functional relevance of the A1298C variant is less well defined. A1298C affects *in vitro* enzyme function to a lesser degree, and individuals carrying the variant have frequently normal homocysteine and plasma folate concentrations (19, 20). However, some studies have noted a substantially decreased risk of acute lymphocytic or hyperdiploid pediatric leukemia associated with the variant *MTHFR* 1298 allele (21, 22), and Keku *et al.* (23) reported recently a significantly decreased risk of colon cancer among whites with the 1298CC genotype. *Glu429Ala* is located in the regulatory domain of the human enzyme (17, 24). It is unclear whether the substitution affects folate metabolism under specific physiological conditions, *e.g.*, under low nutrient intakes.

We have reported previously on the association between the *MTHFR* C677T polymorphism and risk of colon cancer (9). Here we extend these findings to the A1298C polymorphism, combined genotypes, dietary associations, and colon cancer risk.

Materials and Methods

Participants were African-American, Caucasian, or Hispanic subjects from the Kaiser Permanente Medical Care Program of Northern California, an eight county area in Utah, and the metropolitan Twin Cities area of Minnesota. Eligibility criteria for cases included diagnosis with first-primary incident colon

cancer (ICD-O 2nd edition codes 18.0 and 18.2–18.9) between October 1, 1991 and September 30, 1994; between 30 and 79 years of age at time of diagnosis; and mentally competent to complete the interview. Proximal tumors were defined as cecum through transverse colon; tumors in the splenic flexure, and descending and sigmoid colon were categorized as distal. Cases with adenocarcinoma or carcinoma of the rectosigmoid junction or rectum (defined as the first 15 cm from the anal opening), with known familial adenomatous polyposis, ulcerative colitis, or Crohn's disease were not eligible. Of all of the cases identified, 65% of those contacted consented to participate in the study. Controls who had never had a previous colorectal tumor were selected from Kaiser Permanente Medical Care Program membership lists in California, driver's license lists, random-digit-dialing, or Centers for Medicare & Medicaid Services lists (formerly known as the Health Care Finance Administration), for Utah, and driver's license or state identification lists in Minnesota. These methods have been described in detail (25). Of all of the controls selected, 64% participated.

Data Collection. Trained interviewers collected diet and lifestyle data using laptop computers. Study quality control methods have been described (26, 27). The referent period for the study was the calendar year ~2 years before date of diagnosis (cases) or date of selection (controls).

Dietary intake data were ascertained using an adaptation of the validated CARDIA diet history questionnaire (27, 28). Participants were asked to determine which foods were eaten and the frequency with which foods were eaten. Nutrients were calculated using the Minnesota Nutrition Coordinating Center's nutrient database version 19. Study participants were asked if they took multivitamins and other vitamin or mineral supplements on a regular basis. Multivitamin and supplement use was categorized as described previously (29). One third of participants reported taking a multivitamin regularly. Less than 1% reported taking individual supplements of folate, vitamin B6, and vitamin B12. About 1% reported taking a B complex supplement.

MTHFR Genotyping. Of 4403 cases and controls with valid study data, 3680 (84%) had blood collected. Genomic DNA was extracted using methods described previously (30). Of individuals with DNA, originally 3283 had *MTHFR* 677 genotype data. For this analysis, results for an additional 297 samples not available previously for *MTHFR* 677 genotyping were assayed from DNA stored at the Fred Hutchinson Cancer Research Center, and subsequently all of the samples were genotyped for *MTHFR* 1298 resulting in a total of 3580 cases and controls with genotype information for both *MTHFR* 677 and *MTHFR* 1298.

The *C677T* and *A1298C* polymorphisms were detected by allelic discrimination using the 5' nuclease assay on a 7900HT sequence detection system (Applied Biosystems, Foster City, CA). The 5' nuclease genotyping assays were validated by genotyping 100 individuals by both 5' nuclease assay and RFLP (16, 24). There were no discrepancies. Genotyping of the *C677T* polymorphism was performed in 20 μ l reactions containing 1 \times Taqman PCR core reagents (Applied Biosystems), 5 mM MgCl₂, 200 nM each PCR primer (forward primer 5'CCGAAGCAGGGAGCTTTG 3' and reverse primer 5'CGGTGCATGCCTTCAAA 3'), 100 nM MGB-probes (Applied Biosystems; C-allele 5'VIC-AAATCGgCTCCCGCAG3', T-allele 5'FAM-TGAAATCGaCTCCCGCA3'), 0.5 units AmpliTaq Gold, 0.2 units AmpErase UNG, and 5 ng genomic DNA. Genotyping of the *A1298C* polymorphism was performed in 20

μ l reactions containing 1 \times Taqman PCR core reagents (Applied Biosystems), 3 mM MgCl₂, 200 nM each PCR primer (forward primer 5'AGAGCAAGTCCCCAAGGA 3' and reverse primer 5'CTTTGTGACCATTCCGGTTTG3'), 100 nM MGB probes (A-allele 5'VIC-AGTGAAGaAAGTGTCTTT3' and C-allele 5' 6-FAM-AGTGAAGcAAGTGTCTT3') 0.5 units AmpliTaq Gold, 0.2 units AmpErase UNG, and 5 ng genomic DNA. The amplification cycles were 50°C for 5 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s, and 60°C for 1 min. Positive controls for all of the genotypes as well as four negative controls were included in each plate. For quality control purposes, genotyping for 94 randomly selected samples was repeated. There were no discrepancies. Genotype frequencies among cases and controls for both *MTHFR* 677 and *MTHFR* 1298 were compatible with Hardy-Weinberg equilibrium (χ^2 test).

Statistical Methods. Logistic regression models were used to estimate associations in various ways. We stratified the data by combined *MTHFR* 677 and 1298 genotype and estimated the risk of colon cancer given a certain genotype and population characteristics (e.g., proximal or distal tumor site). The combined effects of *MTHFR* 677 and 1298 were calculated using individuals who were homozygous wild-type for enzyme activity at both loci as the referent group. We assessed the joint interaction between genotype and specific level of nutrient intake by using those at the highest risk as a common referent point (low nutrient intake and wild-type for both *MTHFR* 677 and 1298). Similarly, the interaction between genotype and recent estrogen status in postmenopausal women was assessed using those at the highest risk as the referent group [no hormone replacement therapy (HRT) use] and wild-type for both *MTHFR* 677 and 1298.

Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated from unconditional logistic regression models. In these models, age at diagnosis or selection, body mass index reported for the referent period (kg/m²), long-term vigorous leisure-time physical activity, total energy intake, dietary fiber, dietary calcium, and usual number of cigarettes smoked per day on a regular basis were included as covariates to adjust for potential confounding. In models of risk of colon cancer, given genotype and nutrient intake, other B vitamins (dietary folate, B₂, B₆, and B₁₂), methionine, and current alcohol consumption were also included as covariates where appropriate. Categories of exposure for nutrients were based on the distribution of the control population for men and women separately. Tertile cut-points were used to designate low (lowest tertile), intermediate (middle tertile), or high intake (upper tertile). Long-term alcohol exposure was based on gram amount from reported wine, beer, and liquor consumption averaged for 10 and 20 years ago. The cut-point for high exposure was ≥ 20 g/day for men and ≥ 10 g/day for women, corresponding approximately with the upper tertile.

Separate analyses were performed for men and women to determine whether differences existed by sex, as most of the literature has focused on either men or women. Assessment of interactions among genotypes, diet, HRT, and the risk of colon cancer were based on departure from additive risks using the relative excess risk due to interaction formulation of Hosmer and Lemeshow (31) extended to more than two allelic combinations and/or environmental exposures. In addition to relative risk due to interaction, interactions between genotypes and hormone replacement therapy were assessed using a Wald χ^2 test of the difference between slopes from the (assumed linear) change in ORs holding either *MTHFR* 677 or 1298 constant

(wild-type) and varying the other *MTHFR* genotype from wild-type to heterozygous to homozygous variant.

Results

Selected characteristics of the study population and *MTHFR* genotype frequencies by case and control status are presented in Table 1. The study participants were predominantly self-identified as Caucasian (92%), with the remainder Hispanic (4%) and African-American (4%). For African-Americans in the study population ($n = 130$ with *MTHFR* data) variant genotypes 677 *TT* or 1298 *CC* were rarely observed (frequencies of 2% and 5%, respectively).

The distribution of cases and controls for *MTHFR* genotypes shows that C677T and A1298C are in complete linkage disequilibrium (χ^2 test, $P < 0.0001$), with no evidence of the existence of an allele that carries both variants [inferred *MTHFR* alleles: 677C/1298A (wild-type), 677T/1298A, and 677C/1298C]. When polymorphisms were evaluated sepa-

rately, with respect to the C677T polymorphism the homozygous variant genotype was associated with a slightly reduced risk in men (*CT* versus *CC* wild-type: OR, 1.1, 95% CI, 0.9–1.3; *TT* versus *CC*: OR, 0.8, 95% CI, 0.5–1.1) and women (*CT* versus *CC*: OR, 0.9, 95% CI, 0.7–1.1; *TT* versus *CC*: OR, 0.8, 95% CI, 0.5–1.1). For A1298C no risk reduction was observed in men (*AC* versus *AA* wild-type: OR, 1.0, 95% CI, 0.8–1.2; *CC* versus *AA*: OR, 1.0, 95% CI, 0.7–1.4), but a reduced risk was seen for women (*AC* versus *AA* wild-type: OR, 1.0, 95% CI, 0.8–1.2; *CC* versus *AA*: OR, 0.6, 95% CI, 0.5–0.9). These risk estimates included the respective other polymorphism in the multivariate adjustment. Subsequently, we evaluated associations of *MTHFR* 677/1298 genotypes compared with a reference group of individuals carrying two wild-type alleles (*MTHFR* 677 *CC*/1298 *AA*). Overall colon cancer risk estimates, stratified by sex, are shown in Table 2. Men who were homozygous variant 677 *TT* and wild-type 1298 *AA* showed the lowest risk in relation to the reference group (OR, 0.7; 95% CI, 0.5–1.0). When stratified by age, this association was observed only among men ≥ 65 years of age (OR, 0.6; 95% CI, 0.3–0.9). In men, the A1298C variant allele was not associated with colon cancer risk. Conversely in women, the 1298 *CC* variant genotype (always occurring with wild-type 677 *CC* genotype) was associated with a decreased risk (OR, 0.6; 95% CI, 0.4–0.9), and this association did not differ by age.

No appreciable difference in risk was seen for tumor site and either the *MTHFR* 677 or 1298 genotypes (data not shown). Although allele frequencies for African-Americans were significantly different from those of Caucasians or Hispanics, stratification by race was not useful in determining associations for African-American participants due to small sample size and imprecise estimates (data not shown). The distribution of *MTHFR* 677 and *MTHFR* 1298 genotypes among African-Americans or Hispanics did not differ between cases and controls.

Associations between several dietary factors relevant to folate metabolism (folate, vitamin B₁₂, and alcohol) and combined *MTHFR* genotype in men and women are shown in Table 3. Due to the imprecise measure of intakes from supplements, this stratified analysis was restricted to individuals who indicated no regular intakes of vitamin supplements (1088 cases and 1315 controls), and, thus, reflects total dietary intakes.

In this study, we did not observe statistically significant alterations in colon-cancer risk associated with folate intake or *MTHFR* genotype in men. Trends toward a reduction in risk with high folate intake were observed only among those with *MTHFR* 677CC/1298AA (wild-type/wild-type) genotypes or those who carried a heterozygous/wild-type combination. Among women, any possible risk reduction associated with high-versus-low folate intakes was seen only among individuals with the *MTHFR* 677 *CC* genotype [note for example, among *MTHFR* 677CC/1298AA: low folate intake OR, 1.0 (ref); high folate intake OR, 0.5 and 95% CI, 0.2–1.3]. The lowest risk of colon cancer was seen among women with 677 *CC* and 1298 *CC* (wild-type/variant) genotypes who consumed a diet high in folate (OR, 0.2; 95% CI, 0.1–0.7; P interaction = 0.09).

With respect to stratification by vitamin B₁₂ and *MTHFR* genotypes, no significant differences in colon cancer risk were seen for men. Among women, patterns were similar to those observed for folate intake, with a risk reduction suggested among those with the *MTHFR* 1298 *CC* variant genotype, in combination with a 677 *CC* wild-type genotype (low vitamin B₁₂ intake: OR, 0.8 and 95% CI, 0.3–2.1; high vitamin B₁₂ intake OR, 0.2 and 95% CI, 0.1–0.7).

Table 1 Characteristics of the study population ($n = 3580$)

| | Cases ($n = 1608$) | Controls ($n = 1972$) | P^a |
|---|-------------------------|----------------------------|-------|
| Tumor site | | | |
| Proximal | 794 (49%) | | |
| Distal | 775 (48%) | | |
| Unknown site | 39 (3%) | | |
| Age at diagnosis or selection (range, 30–79) | | | |
| Years ^b | 64.4 +/- 9.8 | 64.5 +/- 10.1 | 0.77 |
| Sex | | | |
| Men | 900 (56%) | 1042 (53%) | |
| Women | 708 (44%) | 930 (47%) | 0.06 |
| Recent hormone-replacement therapy use, postmenopausal women ^c | | | |
| No | 470 (77%) | 551 (69%) | |
| Yes | 144 (23%) | 243 (31%) | <0.01 |
| Kilocalories ^b | | | |
| Men | 2773 +/- 1217 | 2637 +/- 1160 | 0.01 |
| Women | 2048 +/- 876 | 1972 +/- 831 | 0.07 |
| Folate (mcg/day) ^d | | | |
| Men | 387 (215) | 393 (214) | 0.80 |
| Women | 320 (177) | 321 (174) | 0.68 |
| Vitamin B ₁₂ (mcg/day) ^d | | | |
| Men | 6.5 (4.9) | 6.3 (4.7) | 0.18 |
| Women | 4.5 (3.4) | 4.5 (3.4) | 0.61 |
| Long-term alcohol intake (gm/day) ^d | | | |
| Men | 14.7 (27.0) | 12.0 (24.8) | 0.08 |
| Women | 3.7 (9.2) | 3.7 (9.0) | 0.43 |
| <i>MTHFR</i> 677 and 1298 genotypes ^e | | | |
| 677 <i>CC</i> (wt) and 1298 <i>AA</i> (wt) | 223 (14%) | 247 (13%) | |
| 677 <i>CT</i> (het) and 1298 <i>AA</i> (wt) | 384 (24%) | 455 (23%) | |
| 677 <i>TT</i> (var) and 1298 <i>AA</i> (wt) | 150 (9%) | 227 (12%) | |
| 677 <i>CC</i> (wt) and 1298 <i>AC</i> (het) | 358 (22%) | 424 (21%) | |
| 677 <i>CT</i> (het) and 1298 <i>AC</i> (het) | 340 (21%) | 403 (20%) | |
| 677 <i>TT</i> (var) and 1298 <i>AC</i> (het) | — | — | |
| 677 <i>CC</i> (wt) and 1298 <i>CC</i> (var) | 153 (10%) | 216 (11%) | |
| 677 <i>CT</i> (het) and 1298 <i>CC</i> (var) | — | — | |
| 677 <i>TT</i> (var) and 1298 <i>CC</i> (var) | — | — | 0.17 |

^a Based on χ^2 or t test.

^b Mean +/- SD.

^c Hormone replacement therapy within 2 years of diagnosis/selection.

^d Median (interquartile range) for dietary intake. Long-term alcohol intake based on consumption of wine, beer, and liquor for drinkers reported 10 and 20 years ago, averaged.

^e Some genotypes of *MTHFR* 677 and 1298 were not observed, presumably due to complete linkage disequilibrium.

Table 2 Association between MTHFR genotype and colon cancer risk stratified by sex and age^{a,b}

| MTHFR 677 | AA | | | | MTHFR 1298 AC | | | | CC | | | |
|--|-----------|--------------|-----------------|-----------|---------------|--------------|-----|-----------|-----------|--------------|-----|-----------|
| | Cases (n) | Controls (n) | OR ^c | 95% CI | Cases (n) | Controls (n) | OR | 95% CI | Cases (n) | Controls (n) | OR | 95% CI |
| Men | | | | | | | | | | | | |
| CC | 131 | 145 | 1.0 | — | 178 | 217 | 0.9 | (0.6–1.2) | 93 | 103 | 1.0 | (0.7–1.4) |
| CT | 211 | 236 | 1.0 | (0.7–1.3) | 201 | 215 | 1.0 | (0.7–1.4) | | | | |
| TT | 78 | 123 | 0.7 | (0.5–1.0) | | | | | | | | |
| Age of diagnosis/selection <65 years | | | | | | | | | | | | |
| CC | 52 | 66 | 1.0 | — | 70 | 89 | 1.0 | (0.6–1.6) | 38 | 34 | 1.3 | (0.7–2.4) |
| CT | 82 | 107 | 0.9 | (0.5–1.4) | 102 | 92 | 1.3 | (0.8–2.1) | | | | |
| TT | 32 | 37 | 1.1 | (0.6–2.0) | | | | | | | | |
| Age of diagnosis/selection 65–79 years | | | | | | | | | | | | |
| CC | 79 | 79 | 1.0 | — | 108 | 128 | 0.8 | (0.5–1.2) | 55 | 69 | 0.8 | (0.5–1.3) |
| CT | 129 | 129 | 1.1 | (0.7–1.6) | 99 | 123 | 0.9 | (0.6–1.3) | | | | |
| TT | 46 | 86 | 0.6 | (0.3–0.9) | | | | | | | | |
| <i>P</i> interaction, age category and MTHFR genotype = 0.34 | | | | | | | | | | | | |
| Women | | | | | | | | | | | | |
| CC | 89 | 102 | 1.0 | — | 178 | 206 | 1.0 | (0.7–1.4) | 60 | 113 | 0.6 | (0.4–0.9) |
| CT | 171 | 217 | 0.9 | (0.6–1.3) | 134 | 184 | 0.9 | (0.6–1.2) | | | | |
| TT | 71 | 103 | 0.8 | (0.5–1.2) | | | | | | | | |
| Age of diagnosis/selection <65 years | | | | | | | | | | | | |
| CC | 38 | 47 | 1.0 | — | 78 | 72 | 1.4 | (0.8–2.4) | 21 | 39 | 0.7 | (0.3–1.4) |
| CT | 72 | 88 | 1.1 | (0.6–1.8) | 52 | 81 | 0.9 | (0.5–1.7) | | | | |
| TT | 27 | 38 | 1.0 | (0.5–1.9) | | | | | | | | |
| Age of diagnosis/selection 65–79 years | | | | | | | | | | | | |
| CC | 51 | 55 | 1.0 | — | 100 | 134 | 0.8 | (0.5–1.3) | 39 | 74 | 0.6 | (0.3–1.0) |
| CT | 99 | 129 | 0.8 | (0.5–1.3) | 82 | 103 | 0.8 | (0.5–1.4) | | | | |
| TT | 44 | 65 | 0.7 | (0.4–1.2) | | | | | | | | |
| <i>P</i> interaction, age category and MTHFR genotype = 0.86 | | | | | | | | | | | | |

^a Adjusted for age, body mass index, lifetime vigorous activity, energy intake, dietary fiber, dietary calcium, and usual number of cigarettes smoked. Participants with missing data for any of these variables were excluded (11 men and 10 women).

^b MTHFR 677/1298 genotypes CT/CC, TT/AC, and TT/CC were not observed in the study population.

^c OR, odds ratio; CI, confidence interval.

A significant interaction was seen in men for long-term alcohol intake and MTHFR genotypes ($P = 0.03$). Men with the 677 TT/1298 AA genotype and alcohol intake of <20 g/day experienced a reduced risk (OR, 0.3; 95% CI, 0.1–0.7) compared with 677 CC/1298AA (wild-type/wild-type) and no alcohol intake. In women, alcohol intake of at least 10 g/day was associated with reduced risk among those with the 677 CC/1298 CC genotype (OR, 0.2; 95% CI, 0–0.8); the P for interaction was not significant.

Associations among vitamins B₂, B₆, and methionine, and MTHFR genotype were similar to folate results in both men and women (data not shown); among women, apparent risk reductions with higher intakes were limited to those with 677 CC genotypes, predominantly those with the combined 677CC/1298CC (wild-type/variant) genotype (data not shown).

Analyses using nutrients based on quartile categories or collapsed quintile categories (extreme quintiles and the middle three groups combined) were not different from those using categories based on tertile cutoff points. When nutrient densities (per 1000 kcal) were used to stratify intake category, results were also not materially different.

Due to the observed differences in MTHFR genotype risk among men and women, and because recent studies suggest a relationship between HRT and homocysteine concentrations (32–36), we investigated associations between MTHFR genotype and HRT use within the past 2 years in postmenopausal women (Table 4). For MTHFR C677T, any risk reduction found with use of HRT was limited to individuals with both wild-type MTHFR 677 CC/1298 AA genotypes (no HRT use: OR, 1.0, ref versus HRT use: OR, 0.3; 95% CI, 0.1–0.6), but not seen among those homozygous for the variant 677 T allele (no HRT

use: OR, 0.6, 95% CI, 0.4–1.1 versus HRT use: OR, 0.6, 95% CI, 0.3–1.2). The same relationship was observed for the MTHFR A1298C polymorphism: HRT was associated with a decreased risk among those with the wild-type 677CC/1298AA (no HRT use: OR, 1.0, ref versus HRT use: OR, 0.3, 95% CI, 0.1–0.6) but not among those with the 677CC/1298CC (wild-type/homozygous variant) genotype (no HRT use: OR, 0.4, 95% CI, 0.2–0.7 versus HRT use: OR, 0.5, 95% CI, 0.2–1.1). Concurrently, we observed opposite trends associated with both variant MTHFR alleles compared with joint wild-type among postmenopausal women who used HRT within the past 2 years versus those who did not. The interaction between combined MTHFR genotypes and HRT use was statistically significant calculated as relative excess risk of interaction ($P < 0.01$). A test for interaction based on comparison of slopes was also statistically significant for the A1298C/HRT use relationship (two-sided $P = 0.04$ for Wald χ^2 test of slopes). Although these analyses are based on relatively small numbers of participants, their consistency across both MTHFR variants may be suggestive of a true interaction between hormone replacement therapy and MTHFR genotype.

Discussion

In this large multicenter case-control study of colon cancer, we were able to investigate both common MTHFR polymorphisms (C677T and A1298C) by stratifying on the combined genotypes, and to undertake subgroup analyses by nutrients and hormone-replacement therapy. Overall, we showed similar risk estimates for the C677T variant (see also Ref. 9) as other groups, indicating a slight inverse association with colon cancer

Table 3 Associations between MTHFR genotypes and colon cancer risk, stratified by dietary nutrient intakes (nonsupplement users)^{a,b}

| MTHFR 677 | MTHFR 1298 AA | | | | AC | | | | CC | | | |
|---|---------------|--------------|-----------------|-----------|-----------|--------------|-----|-----------|-----------|--------------|-----|-----------|
| | Cases (n) | Controls (n) | OR ^c | 95% CI | Cases (n) | Controls (n) | OR | 95% CI | Cases (n) | Controls (n) | OR | 95% CI |
| Men | | | | | | | | | | | | |
| Folate ^d P interaction = 0.90 ^e | | | | | | | | | | | | |
| CC | | | | | | | | | | | | |
| Low | 32 | 33 | 1.0 | — | 38 | 50 | 0.8 | (0.4–1.6) | 20 | 23 | 1.0 | (0.4–2.1) |
| Int | 35 | 30 | 1.3 | (0.6–2.5) | 53 | 55 | 1.0 | (0.5–1.9) | 27 | 21 | 1.3 | (0.6–2.9) |
| High | 28 | 39 | 0.7 | (0.3–1.5) | 37 | 53 | 0.6 | (0.3–1.3) | 24 | 26 | 1.0 | (0.4–2.3) |
| CT | | | | | | | | | | | | |
| Low | 54 | 54 | 1.1 | (0.6–2.1) | 45 | 48 | 1.1 | (0.6–2.0) | | | | |
| Int | 56 | 64 | 0.9 | (0.5–1.7) | 51 | 56 | 1.0 | (0.5–1.9) | | | | |
| High | 37 | 53 | 0.7 | (0.3–1.4) | 52 | 51 | 1.1 | (0.5–2.2) | | | | |
| TT | | | | | | | | | | | | |
| Low | 18 | 23 | 0.8 | (0.4–1.9) | | | | | | | | |
| Int | 18 | 36 | 0.6 | (0.3–1.2) | | | | | | | | |
| High | 20 | 26 | 0.8 | (0.4–1.8) | | | | | | | | |
| Vitamin B ₁₂ ^d —P interaction = 0.90 ^e | | | | | | | | | | | | |
| CC | | | | | | | | | | | | |
| Low | 25 | 33 | 1.0 | — | 32 | 61 | 0.7 | (0.4–1.4) | 19 | 19 | 1.5 | (0.6–3.4) |
| Int | 33 | 37 | 1.3 | (0.6–2.6) | 54 | 44 | 1.7 | (0.9–3.3) | 29 | 31 | 1.4 | (0.7–2.9) |
| High | 37 | 32 | 1.6 | (0.7–3.4) | 42 | 53 | 1.0 | (0.5–2.2) | 23 | 20 | 1.4 | (0.6–3.4) |
| CT | | | | | | | | | | | | |
| Low | 35 | 51 | 1.0 | (0.5–2.1) | 41 | 44 | 1.4 | (0.7–2.8) | | | | |
| Int | 55 | 54 | 1.4 | (0.7–2.7) | 59 | 62 | 1.4 | (0.7–2.7) | | | | |
| High | 57 | 66 | 1.1 | (0.6–2.2) | 48 | 49 | 1.3 | (0.8–2.7) | | | | |
| TT | | | | | | | | | | | | |
| Low | 16 | 27 | 0.9 | (0.5–1.9) | | | | | | | | |
| Int | 19 | 34 | 0.8 | (0.4–1.8) | | | | | | | | |
| High | 21 | 24 | 1.1 | (0.5–2.6) | | | | | | | | |
| Long-term alcohol intake ^f P interaction = 0.03 ^e | | | | | | | | | | | | |
| CC | | | | | | | | | | | | |
| None | 31 | 25 | 1.0 | — | 28 | 40 | 0.5 | (0.3–1.1) | 14 | 21 | 0.5 | (0.2–1.3) |
| Mod | 31 | 44 | 0.6 | (0.3–1.1) | 59 | 67 | 0.6 | (0.3–1.2) | 32 | 30 | 0.8 | (0.4–1.7) |
| High | 33 | 33 | 0.6 | (0.3–1.3) | 41 | 51 | 0.5 | (0.3–1.0) | 25 | 19 | 0.8 | (0.4–1.9) |
| CT | | | | | | | | | | | | |
| None | 38 | 41 | 0.8 | (0.4–1.5) | 38 | 48 | 0.7 | (0.3–1.3) | | | | |
| Mod | 63 | 70 | 0.7 | (0.4–1.3) | 64 | 74 | 0.7 | (0.3–1.2) | | | | |
| High | 46 | 60 | 0.5 | (0.2–0.9) | 46 | 33 | 0.9 | (0.4–1.9) | | | | |
| TT | | | | | | | | | | | | |
| None | 21 | 26 | 0.6 | (0.3–1.4) | | | | | | | | |
| Mod | 17 | 43 | 0.3 | (0.1–0.7) | | | | | | | | |
| High | 18 | 16 | 0.7 | (0.3–1.8) | | | | | | | | |
| Women | | | | | | | | | | | | |
| Folate ^d —P interaction = 0.09 ^e | | | | | | | | | | | | |
| CC | | | | | | | | | | | | |
| Low | 21 | 14 | 1.0 | — | 35 | 46 | 0.5 | (0.2–1.1) | 16 | 18 | 0.6 | (0.2–1.7) |
| Int | 19 | 29 | 0.5 | (0.2–0.8) | 36 | 42 | 0.6 | (0.3–1.4) | 12 | 26 | 0.3 | (0.2–0.8) |
| High | 16 | 20 | 0.5 | (0.2–1.3) | 37 | 37 | 0.7 | (0.3–1.7) | 8 | 20 | 0.2 | (0.1–0.7) |
| CT | | | | | | | | | | | | |
| Low | 44 | 58 | 0.5 | (0.3–1.1) | 28 | 37 | 0.5 | (0.2–1.2) | | | | |
| Int | 28 | 43 | 0.4 | (0.2–0.9) | 28 | 42 | 0.5 | (0.2–1.1) | | | | |
| High | 34 | 35 | 0.7 | (0.3–1.1) | 26 | 37 | 0.5 | (0.2–1.4) | | | | |
| TT | | | | | | | | | | | | |
| Low | 16 | 27 | 0.4 | (0.2–0.9) | | | | | | | | |
| Int | 13 | 17 | 0.5 | (0.3–1.3) | | | | | | | | |
| High | 15 | 23 | 0.4 | (0.2–1.0) | | | | | | | | |
| Vitamin B ₁₂ ^d —P interaction = 0.90 ^e | | | | | | | | | | | | |
| CC | | | | | | | | | | | | |
| Low | 22 | 19 | 1.0 | — | 37 | 45 | 0.7 | (0.3–1.6) | 14 | 17 | 0.8 | (0.3–2.1) |
| Int | 16 | 25 | 0.6 | (0.2–1.4) | 27 | 50 | 0.4 | (0.2–1.0) | 15 | 25 | 0.4 | (0.2–1.1) |
| High | 18 | 19 | 0.7 | (0.3–1.3) | 44 | 30 | 1.2 | (0.5–2.8) | 7 | 22 | 0.2 | (0.1–0.7) |
| CT | | | | | | | | | | | | |
| Low | 32 | 44 | 0.6 | (0.3–1.4) | 28 | 41 | 0.6 | (0.3–1.3) | | | | |
| Int | 40 | 41 | 0.8 | (0.4–1.8) | 30 | 37 | 0.7 | (0.3–1.6) | | | | |
| High | 34 | 51 | 0.5 | (0.2–1.2) | 24 | 38 | 0.6 | (0.2–1.4) | | | | |
| TT | | | | | | | | | | | | |
| Low | 19 | 26 | 0.7 | (0.3–1.5) | | | | | | | | |
| Int | 11 | 25 | 0.4 | (0.1–1.0) | | | | | | | | |
| High | 14 | 16 | 0.7 | (0.3–2.1) | | | | | | | | |

Table 3 Continued

| MTHFR 677 | MTHFR 1298 AA | | | | AC | | | | CC | | | |
|--|---------------|--------------|-----------------|-----------|-----------|--------------|-----|-----------|-----------|--------------|-----|-----------|
| | Cases (n) | Controls (n) | OR ^c | 95% CI | Cases (n) | Controls (n) | OR | 95% CI | Cases (n) | Controls (n) | OR | 95% CI |
| Long-term alcohol intake ^f – P interaction = 0.62 | | | | | | | | | | | | |
| CC | | | | | | | | | | | | |
| None | 33 | 34 | 1.0 | — | 51 | 67 | 0.8 | (0.4–1.5) | 25 | 32 | 0.8 | (0.4–1.6) |
| Mod | 15 | 21 | 0.7 | (0.3–1.6) | 39 | 42 | 0.9 | (0.5–1.7) | 9 | 20 | 0.4 | (0.2–1.0) |
| High | 8 | 8 | 1.1 | (0.3–3.2) | 18 | 16 | 1.1 | (0.5–2.6) | 2 | 12 | 0.2 | (0.0–0.8) |
| CT | | | | | | | | | | | | |
| None | 49 | 58 | 0.8 | (0.4–1.5) | 64 | 62 | 0.7 | (0.4–1.4) | | | | |
| Mod | 36 | 60 | 0.6 | (0.3–1.2) | 50 | 42 | 0.9 | (0.4–1.7) | | | | |
| High | 21 | 18 | 1.2 | (0.5–2.7) | 20 | 12 | 0.6 | (0.2–1.8) | | | | |
| TT | | | | | | | | | | | | |
| None | 26 | 37 | 0.7 | (0.4–1.5) | | | | | | | | |
| Mod | 15 | 21 | 0.8 | (0.3–1.8) | | | | | | | | |
| High | 3 | 9 | 0.3 | (0.1–1.3) | | | | | | | | |

^a Adjusted for age, body mass index, lifetime vigorous activity, energy intake, dietary fiber, calcium, usual number of cigarettes and folate, vitamin B₂, vitamin B₆, vitamin B₁₂, methionine, and alcohol, where appropriate.

^b MTHFR 677/1298 genotypes CT/CC, TT/AC, and TT/CC were not observed in the study population.

^c OR, odds ratio; CI, confidence interval.

^d Tertile cut-off points for men were: folate, 318/459 mcg/day; vitamin B₁₂, 4.8/7.8 mcg/day. Tertile cut-off points for women were: folate, 273/388 mcg/day; vitamin B₁₂, 3.5, 5.7 mcg/day.

^e Relative excess risk due to interaction (31).

^f High alcohol intake from consumption of beer, wine, and liquor averaged for 10 and 20 years ago: men, ≥ 20 gm/day; women, ≥ 10 gm/day.

risk (10, 37, 38). Surprisingly, the 1298 CC variant genotype was associated with a significantly decreased risk among women (overall CC versus AA OR, 0.6; 95% CI, 0.4–0.9), and reduced risk estimates more strongly than the C677T polymorphism (TT versus CC OR, 0.8; 95% CI, 0.5–1.1). The biochemical relevance of the A1298C polymorphism is not well defined; although the variant allele has been shown to reduce MTHFR activity modestly *in vivo* and *in vitro* in lymphocyte extracts, a thorough biochemical evaluation of the human recombinant MTHFR protein by Yamada *et al.* (19) did not show a difference in its biochemical phenotype. *In vivo*, its effects on enzyme function appear less pronounced than those of the C677T polymorphism, as evidenced by little alteration of homocysteine or serum folate concentrations (17, 24). However, in studies of adult and childhood acute leukemia (21, 22) the A1298C variant has been found to be associated with reduced risk, at least of some subtypes. Keku *et al.* (23) reported recently a statistically significantly reduced risk of colon cancer among whites with the 677 CC/1298 CC genotype (OR, 0.5; 95% CI, 0.2–0.8); these findings are consistent with the risk reduction seen in our study among women (OR, 0.6) but not men (OR, 1.0). Within the Physician's Health Study, risk for this subgroup of individuals was reported as OR, 0.78 (95% CI, 0.36–1.72; Ref. 39); these confidence intervals were wide because the risk estimate was based on 15 cases and 28 controls. Nonetheless, the findings are certainly consistent with the larger case-control study by Keku *et al.* (23) and the one presented here. Interestingly, within the Physician's Health Study, individuals with the genotype combination of wild-type 677 CC and variant 1298 CC had marginally elevated homocysteine concentrations (12.3 versus 10.8 nmol/ml for w/w), which lends support to an *in vivo* phenotype for the A1298C polymorphism (39).

The amino acid affected by this single nucleotide substitution, Glu429, is located near the binding site for the allosteric MTHFR inhibitor S-adenosyl-methionine, and, thus, may possibly affect feedback inhibition. Although current experimental data do not show differences of S-adenosyl-methionine binding, studies of the impact of A1298C under different nutritional

status have not yet been undertaken. It has been suggested that the decreased levels of activity stem from differences in protein stability rather than from the properties of the purified enzyme *per se* (19).

For both MTHFR polymorphisms, risk estimates associated with the variant alleles were lower among older men and women (Table 2). These findings suggest that other factors associated with aging interact with MTHFR. However, the relationships observed are inconsistent with our study on colorectal adenoma (12).

Because risk of colorectal neoplasia associated with MTHFR activity has been shown previously to differ by nutritional status (10–12, 40, 41), we stratified the analyses of genotype combinations by dietary intakes of folate and nutrients involved in folate metabolism (vitamin B₁₂, vitamin B₆, vitamin B₂, alcohol, and methionine). Among individuals who reported never having taken multivitamins or other supplements regularly (67%), associations were similar to reports by others (10–12), indicating that a reduced risk associated with higher folate intakes was observed only among those with MTHFR 677 CC (wild-type) or CT genotype, but not TT with reduced enzyme activity. The study population was recruited before any fortification of food sources, and the nutritional assessment included a detailed in-person dietary interview with food models; results include only individuals with a complete diet history. Our study supports a reduced risk associated with higher vitamin B₁₂ intakes among women with wild-type 677CC genotype, particularly when combined with a 1298CC variant genotype. However, no such associations were seen among men, and the risk estimates were fairly unstable due to the few individuals in each cell.

Among women, high alcohol intake (≥ 10 g/day) was associated with a decreased risk for carriers of the homozygous variant MTHFR genotypes (677 CC/1298 CC: OR, 0.2, 95% CI, 0.0–0.8; 677TT/1298AA: OR, 0.3, 95% CI, 0.1–1.3, both compared with 677CC/1298AA reference group). For men, a significant risk reduction with moderate alcohol consumption (< 20 g/day) was seen among individuals with the variant 677TT/1298AA genotype, but not for the variant 677CC/

Table 4 Association between MTHFR genotypes and colon cancer risk, stratified by hormone replacement therapy (postmenopausal women)^a

| Recent hormone replacement therapy | MTHFR 677 | MTHFR 1298 | | | | | | | | | | | |
|------------------------------------|-----------|------------|-----------|------------|-------------------------|-----------|-----------|------------|-------------------------|-----------|-----------|------------|-------------------------|
| | | AA | | | | AC | | | | CC | | | |
| | | Cases (n) | Ctrls (n) | Odds ratio | 95% confidence interval | Cases (n) | Ctrls (n) | Odds ratio | 95% confidence interval | Cases (n) | Ctrls (n) | Odds ratio | 95% confidence interval |
| No | CC | 70 | 61 | 1.0 | — | 107 | 120 | 0.8 | (0.5–1.2) | 35 | 76 | 0.4 | (0.2–0.7) |
| | CT | 115 | 127 | 0.8 | (0.5–1.2) | 92 | 100 | 0.8 | (0.5–1.3) | | | | |
| | TT | 46 | 64 | 0.6 | (0.4–1.1) | | | | | | | | |
| Yes | CC | 10 | 31 | 0.3 | (0.1–0.6) | 41 | 61 | 0.6 | (0.3–1.0) | 12 | 23 | 0.5 | (0.2–1.1) |
| | CT | 33 | 58 | 0.5 | (0.3–0.9) | 31 | 43 | 0.6 | (0.4–1.1) | | | | |
| | TT | 17 | 26 | 0.6 | (0.3–1.2) | | | | | | | | |

^a Adjusted for age, body mass index, lifetime vigorous activity, energy intake, diet fiber, calcium, usual number of cigarettes smoked. Relative excess risk due to interaction $P < 0.01$. Wald χ^2 test of slopes, A1298C/HRT use relationship $P = 0.04$. Wald χ^2 test of slopes, C677T/HRT use relationship $P = 0.09$.

1298CC genotype. These results are consistent with our findings for colorectal adenomas, showing a reduced risk among moderate drinkers with the *MTHFR* 677TT genotypes (12). However, other groups, particularly those involving prospective data collection, have reported opposite trends (e.g., increased risks with higher alcohol consumption and 677 TT genotypes; Refs. 10, 37, 41). The underlying biological mechanisms with respect to an interaction between folate and moderate alcohol consumption are unclear; most studies on the effects of alcohol consumption on folate absorption or metabolism have focused on very high, chronic alcohol intakes (42–44).

Our study showed some evidence for an interaction between estrogen status and *MTHFR* genotypes. Inverse associations between HRT and colon cancer incidence were only evident among women carrying both *MTHFR* wild-type alleles. Similarly, reduced risks associated with both *MTHFR* variants were limited to women who did not report current or recent use of hormone replacement therapy. Several recent studies show reductions of homocysteine concentrations under HRT (32–34, 36), or variations in homocysteine concentrations among women of different ages and lifestyles that are consistent with effects attributable to estrogen status (35). One study has investigated modifying effects of the *MTHFR* C677T polymorphism on the HRT-homocysteine relationship and observed no discernable differences, yet did not appear to have sufficient statistical power to test for such an interaction (34).

MTHFR genotypes can affect methylation capacity, as illustrated by studies on overall methylation patterns in lymphocytes (45, 46). It is also known that methylation of the estrogen receptor is an early event in colorectal carcinogenesis, which may be less likely to occur in the presence of HRT or endogenous estrogens (47–49). However, there is currently no clear biological rationale linking overall methylation capacity (based on the availability of the methyl-donor *S*-adenosyl-methionine) to hypermethylation of a specific gene. Our study provides support for a possible link between *MTHFR* or folate and estrogen status in colorectal carcinogenesis, which should be pursued in further studies.

In summary, the *MTHFR* A1298C polymorphism may be as relevant in predicting colon cancer risk as the C677T variant, at least among women. This finding raises questions about the possible impact of the A1298C polymorphism on the function of *MTHFR* or its regulation; the interaction with use of HRT warrants additional investigation.

Acknowledgments

We thank Sandra Edwards and Leslie Palmer at the University of Utah for the data collection efforts of this study, and Juanita Leija for assistance with genotyping.

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