# 5,10-Methylenetetrahydrofolate Reductase 677 and 1298 Polymorphisms, Folate Intake, and Microsatellite Instability in Colon Cancer

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## Abstract

**The 5,10-methylenetetrahydrofolate reductase (*MTHFR*) gene plays a critical role in folate metabolism. Studies on the association between *MTHFR* polymorphisms and length changes in short tandem repeat DNA sequences [microsatellite instability (MSI)] are inconsistent. Using data from colon cancer cases ($n$ = 503) enrolled as part of an existing population-based case-control study, we investigated the association between *MTHFR 677* and *MTHFR 1298* polymorphisms and MSI. We also examined whether the association was modified by folate intake. Participants were case subjects enrolled as part of the North Carolina Colon Cancer Study. Consenting cases provided information about lifestyle and diet during in-home interviews as well as blood specimens and permission to obtain tumor blocks. DNA from normal and tumor tissue sections was used to determine microsatellite status (MSI). Tumors were classified as MSI if two or more microsatellite markers (BAT25, BAT26, D5S346, D2S123, and D17S250) had changes in the number of DNA sequence repeats compared with matched nontumor tissue. Tumors with one positive marker (MSI-low) or no positive markers (microsatellite stable) were grouped together as non-MSI tumors (microsatellite stable). *MTHFR 677* and *MTHFR 1298* genotypes were determined by real-time PCR using the 5′ exonuclease (Taqman) assay. Logistic regression was used to calculate odds ratio (OR) and 95% confidence intervals (95% CI). MSI was more common in proximal tumors (OR, 3.8; 95% CI, 1.7-8.4) and in current smokers (OR, 4.0; 95% CI, 1.6-9.7). Compared with *MTHFR 677* CC referent, *MTHFR 677* CT/TT genotype was inversely associated with MSI among White cases (OR, 0.36; 95% CI, 0.16-0.81) but not significant among African Americans. Although not statistically significant, a similar inverse association was observed between *MTHFR 677* CT/TT genotype and MSI among the entire case subjects (OR, 0.54; 95% CI, 0.26-1.10). Among those with adequate folate intake (>400 μg total folate), we found strong inverse associations between combined *MTHFR* genotypes and MSI (677 CC + 1298 AC/CC, OR, 0.09; 95% CI, 0.01-0.59; 677 CT/TT + 1298 AA, OR, 0.13; 95% CI, 0.02-0.85) compared with the combined wild-type genotypes (677 CC + 1298 AA). This protective effect was not evident among those with low folate (<400 μg total folate) intake. Our results suggest that *MTHFR* variant genotypes are associated with reduced risk of MSI tumors under conditions of adequate folate intake, although the data are imprecise due to small numbers. These results indicate that the relationship between *MTHFR* genotypes and MSI is influenced by folate status.** (Cancer Epidemiol Biomarkers Prev 2005;14(8):2023–9)

## Introduction

Colon cancer, a common cancer in the United States and other developed countries, is thought to have a strong environmental component. Among environmental exposures, diet has received a great deal of attention. There are at least three pathways to colon cancer: chromosomal instability, microsatellite instability (MSI), and CpG island methylator phenotype. Some sporadic colon cancers are associated with chromosomal instability, which involves allelic loss of tumor suppressor genes, such as *p53* or *APC*. Microsatellites are short tandem repeat DNA sequences. The change in length of these tandem repeats is termed MSI and is often associated with defects in DNA repair genes (1, 2). MSI is associated with 10% to 15% of sporadic colorectal carcinomas (3). Colon tumors harboring MSI more frequently present with proximal location, large tumor size, decreased likelihood of metastasis, less advanced stage at diagnosis, and better prognosis compared with microsatellite-stable (MSS) colon cancers (4, 5). Some colon cancers exhibit aberrant DNA methylation or CpG island methylator phenotype, which involves inactivation or silencing of genes by hypermethylation of promoter cytosine-guanosine (CpG) residues. Interestingly, MSI arises through aberrant methylation of the hMLH1 promoter in sporadic colon cancers (6, 7).

Folate is ingested through diet (e.g., dark green vegetables, eggs, fish, and wheat) or supplements. Evidence from epidemiologic studies suggest that adequate folate intake is associated with decreased risk of colorectal cancer (8-11), although not all studies support this association (12, 13). Folate is integral in the processes of both DNA synthesis and DNA methylation. The 5,10-methylenetetrahydrofolate reductase (MTHFR) enzyme plays an important role in folate metabolism and determines the balance between the different forms of folate for DNA synthesis and DNA methylation (14). MTHFR catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a substrate for the conversion of homocysteine to methionine. The latter is a precursor to *S*-adenosylmethionine, which is an important methyl donor for

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DNA methylation. In addition, 5,10-methylenetetrahydrofolate, a substrate of MTHFR, is also required for thymidylate and purine synthesis. However, low folate may lead to uracil misincorporation into DNA chromosomal breaks and increase the potential for premutagenic lesions (15, 16). Folate deficiency has also been linked to increased chromosomal aberrations in mammalian cells during mitosis (17). Altered folate metabolism lead to shifts in the balance between availability of 5-methyltetrahydrofolate for methylation reactions and 5,10-methylenetetrahydrofolate for DNA synthesis and repair (18, 19) may influence colorectal cancer risk.

Not surprisingly, polymorphisms in genes related to folate metabolism, specifically *MTHFR*, are thought to play a role in carcinogenesis of the large bowel. Two polymorphisms in the *MTHFR* gene that affect the efficiency of folate metabolism have been described (20-22). The *MTHFR 677* C>T transition in exon 4 and *MTHFR 1298* A>C transversion in exon 7 are associated with reduced enzyme activity resulting in slower folate metabolism. The *MTHFR 677* TT genotype results in 30% enzyme activity *in vitro* compared with the CC wild-type (23), whereas the *MTHFR 1298* CC genotype has been found to have 60% of the AA wild-type enzyme activity *in vitro* (22, 24). We have shown previously a significantly reduced risk of colon cancer among Whites with the *MTHFR 1298* CC variant genotype (20). Other studies also reported reduced risk of colorectal cancer among individuals homozygous for *MTHFR 677* TT genotype with high folate intake (10, 25, 26). Studies on colorectal adenomas, colorectal cancer precursors, suggest positive association between *MTHFR 677* TT genotype and adenoma risk, under inadequate folate status (27, 28). These observations indicate that the effect of MTHFR polymorphisms on colon adenoma and cancer risk is likely modifiable by folate status. *MTHFR 677* T or *MTHFR 1298* C polymorphisms combined with inadequate folate intake could lead to aberrant DNA methylation. MTHFR polymorphisms associated with reduced enzyme activity limit the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the form of folate required for DNA methylation. Interestingly, *MTHFR 677* TT genotype has been associated with reduced DNA methylation under low folate conditions (29, 30). On the other hand, MTHFR polymorphisms that are associated with reduced enzyme activity enhance the accumulation of 5,10-methylenetetrahydrofolate (31) for DNA synthesis by providing more nonmethylated folate for nucleotide synthesis and DNA repair (32). Low folate is associated with increased uracil misincorporation during DNA synthesis (33) and increased frequency of DNA strand breaks (15).

No studies have examined the relationship among *MTHFR* polymorphisms, MSI, and folate status in relation to colon cancer. The findings from the few studies that examined MSI and *MTHFR* polymorphisms are not consistent. A recent study reported a modest but not significant inverse association between MTHFR TT genotype and MSI (34), whereas another study observed a higher frequency of *MTHFR* TT genotype among colorectal cancer cases with MSI tumors (35). A third study found no association between *MTHFR* TT genotypes and MSI (36). In this study, we hypothesized that in colon cancer subjects *MTHFR 677* T and *MTHFR 1298* C polymorphisms would be associated with MSI and that the association between MTHFR and MSI would be modified by folate intake. We also hypothesized that there would be differences by race given the disparities in incidence and mortality and the distribution of *MTHFR* gene polymorphisms by race.

## Materials and Methods

**Study Design.** Participants in this study were colon cancer cases enrolled as part of a separate study, the North Carolina Colon Cancer Study, a population-based case-control study of colon cancer in 33 counties of central and eastern North Carolina. Details about the North Carolina Colon Cancer Study have been described previously (20). Colon cancer cases were identified through the rapid ascertainment system of the North Carolina Central Cancer Registry. All African Americans and a sample of Whites were recruited between October 1, 1996 and September 1, 2000. Colon cancer patients ages between 40 and 80 years residing in the 33-county area with confirmed invasive adenocarcinoma of the colon were considered eligible. A total of 676 interviews were completed among colon cancer cases in North Carolina Colon Cancer Study. The cooperation rate among cases [interviewed / (interviewed + refused)] was 84%. MSI data were collected on 503 cases. Of these, 486 had data on *MTHFR* genotype or folate intake and these were the people included in the analyses. Tissue specimens were prospectively collected under institutional review board approval as part of the North Carolina Colon Cancer Study. Formalin-fixed, paraffin-embedded colon blocks containing tumor or normal (tumor-free) tissue were identified from pathology reports and blocks were requested from participating hospitals. The study pathologist evaluated a reference slide from each block that was stained with H&E to confirm the initial diagnosis and also to identify areas of normal and tumor tissues for microdissection on additional serial tissue sections.

Trained nurse interviewers conducted interviews in the participants' homes. The interviews covered lifestyle and dietary habits, physical activity, smoking and alcohol use, family history of colon cancer, personal attributes, and nonsteroidal anti-inflammatory drug (NSAID) usage. Dietary information (frequency and serving size) was collected using a modified version of the semiquantitative Block food frequency questionnaire developed at the National Cancer Institute (NCI; ref. 37). The food questionnaire was modified by adding 29 food items consumed in North Carolina (38). Participants were asked to estimate their usual frequency of intake of various foods and portion sizes for the year before diagnosis. The 1-year period was selected to account for seasonal variations in dietary intake. We used the nutrients database program provided by NCI to compute estimates of usual intake for a variety of nutrients, including calcium and folate intake, for the year before diagnosis. In addition, use of NSAIDs during the previous 5 years was assessed. The University of North Carolina Medical School Institutional Review Board approved the study protocol.

**Blood Collection.** Blood was collected following the interview after receiving written consent from the participant. The blood specimens were kept at 4°C to 21°C for up to 48 hours during transit to the laboratory. DNA samples were extracted from the buffy coats using the Puregene DNA isolation kit following the manufacturer's recommendations (Gentra Systems, Inc., Minneapolis, MN).

**MSI Status.** DNA was extracted from normal and tumor tissue sections from formalin-fixed, paraffin-embedded tissue sections. A H&E-stained section was used as a guide for microdissection. Genomic DNA was extracted from the tissue by deparaffinization in xylene, purified with absolute alcohol, and pelleted at maximum speed in a microcentrifuge. The pellets were dried in a DNA SpeedVac (Savant, Inc., Farmingdale, NY) and resuspended in 15 μL Genereleaser (BioVentures, Inc., Murfeesboro, TN) according to the manufacturer's protocol. The resuspended DNA was then incubated overnight at 55°C, with 200 mg/mL proteinase K (Sigma, St. Louis, MO). This product was then used directly in the PCR reactions.

To determine MSI in the pathologic specimens, a reference panel of five NCI-recommended microsatellite markers (BAT25, BAT26, D5S346, D2S123, and D17S250) were used (39). The reaction to end-label one primer from each pair

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contained the primers, kinase buffer, T4 polynucleotide kinase, and $^{32}P/^{33}P$. The PCR reaction contained 0.125 pmol of each primer in the pair, 0.25 units Taq DNA polymerase, 40 mmol/L deoxynucleotide triphosphate mix, and 1.5 to 2.0 mmol/L $MgCl_2$. The PCR products were denatured in 95% formamide and used for electrophoresis on a 6% polyacrylamide/7.5 mol/L urea gel. Mutations, identified by changes in the electrophoretic mobility of the PCR products, were analyzed by autoradiography. Alleles in the tumor DNA were compared with nontumor DNA taken from the same specimen. The sample was defined as having high MSI if two or more of the five markers contained novel alleles in the tumor compared with matched nontumor tissue and as MSS if none of the markers had novel alleles. Specimens with one of five positive markers were defined as MSI-low and combined with the MSS group as non-MSI tumors (MSS). Among the 486 subjects with MSI, folate, and *MTHFR* genotype data, the frequencies of MSI and MSS were 10.1% and 89.9%, respectively.

**Genotyping.** Genotyping was done as reported previously (20) using the RFLP assay and the 5′ exonuclease (Taqman, Applied Biosystems, Foster City, CA) assays. Primers for the RFLP assay were *MTHFR 677* forward 5′-AGGACGGTGCGGTGAGAGTG-3′ and reverse 5′-TGAAGGAGAAGGTGTCTGCGG-3′ and *MTHFR 1298* forward 5′-CTTTGGGGAGCTGAAGGACTACTAC-3′ and reverse 5′-CACTTTGTGACCATTCCGGTTTG-3′. The 5′ exonuclease assay primer and probe sequences are as follows: *MTHFR 677* forward 5′-AGGCTGACCTGAAGCACTTGAA-3′ and reverse 5′-CTCAAAGAAAGCTGCGTGATGA-3′; probes end-labeled with the quencher and reporter dyes: VIC-TGTCTGCGGG AGCCCGATTTCA for the common allele and FAM-AGGTGTCTGCGGGAG*T*CGATTTCA for the variant allele; *MTHFR 1298* forward 5′-AAGGAGGAGCTGCTGAAGATGT-3′ and reverse 5′-TGTGACCATTCCGGTTTGG-3′; probes: VIC-AAGACACTT*T*CTTCACTG (A allele) and FAM-AGACACTT*G*CTTTCACT (C allele). At least four negative template controls as well as five positive controls for each allele were included with every assay. A random 10% repeat of samples was done using both methods, with 100% agreement.

**Statistical Analysis.** The response of interest was MSI. MSI was dichotomized into MSI versus non-MSS (MSI-low + MSS). The main exposures of interest in this study were folate intake and *MTHFR* genotype. Three different measures of folate intake were evaluated: total folate, dietary folate, and supplemental folate. The Block-NCI program was used to determine the amounts of dietary and supplemental folate intake. Total folate was the summation of the reported microgram amounts of supplemental folate and dietary folate intake. Dichotomous variables were created from the continuous data for both total folate and dietary folate. For total folate, 400 μg was chosen as the cut point because it is the current recommended daily allowance and it was used in previous studies (20). For dietary folate, we used the mean intake of 275 μg. The odds ratios (OR) did not differ when we used the median intake of dietary folate (257 μg) as the cut point (data not shown). Supplemental folate was collapsed into either yes, supplement taken, or no, supplement not taken.

There are three genotypes for each *MTHFR* locus: a homozygous wild-type or common allele, a homozygous variant allele, and a heterozygote, which has one copy of each allele. For both genotypes, heterozygotes and variant alleles were collapsed into one category to increase precision, thereby creating a dichotomous variable for each locus.

Covariates tested were total calcium intake (quartiles), multivitamin use [use regularly (>3 days a week), use occasionally (>1 day a month but <3 days a week), or do not use], family history (at least one first-degree relative with colorectal cancer: yes/no), alcohol use (nonuser, lower half of consumers, or upper half of consumers), smoking (never, former, or current), NSAID use during the past 5 years before diagnosis [never, occasionally (>1 day a month but <3 days a week), or frequently (≥3 days a week)], stage at time of diagnosis (local, regional, or distal), and tumor location (proximal or distal).

Proc Logistic (SAS 8.2, SAS Institute, Cary, NC) was used to calculate ORs and 95% confidence intervals (95% CI). Covariates were included if they resulted in at least a 10% difference between ORs for the main exposure of interest in the crude and adjusted models. *P*s were considered statistically significant at the <0.05 level. Interaction terms were created for the combinations for *MTHFR* and folate.

For all interaction models, race, sex, and age were included in the adjusted model. To test for multiplicative interaction, the interaction term was added to the model and evaluated using the likelihood ratio test. To test for additive interaction, the ORs and their variances from the model with the interaction term were used to find an interaction contrast ratio and its corresponding 95% CI and from that a *P* was generated test the null hypothesis that the interaction contrast ratio is equal to 0 (no interaction).

## Results

Table 1 provides the ORs with 95% CIs for the association between MSI and general characteristics among colon cancer cases. Age, White race, proximal location of tumor, high

**Table 1. General characteristics of colon cancer cases and associations with MSI**

| | MSS, $n$ = 437 (%) | MSI, $n$ = 49 (%) | OR (95% CI) |
|---|---|---|---|
| Age (median, 65 y) | | | |
| <65 | 212 (49) | 17 (35) | Reference |
| ≥65 | 225 (51) | 32 (65) | 1.77 (0.96-3.290) |
| Gender | | | |
| Female | 212 (49) | 29 (59) | Reference |
| Male | 225 (51) | 20 (41) | 0.65 (0.36-1.18) |
| Race | | | |
| Blacks | 204 (47) | 14 (29) | Reference |
| Whites | 233 (53) | 35 (71) | 2.19 (1.14-4.18) |
| Family history | | | |
| No | 350 (80) | 35 (71) | Reference |
| Yes | 87 (20) | 14 (29) | 1.61 (0.83-3.12) |
| Location of tumor | | | |
| Distal | 165 (45) | 8 (17) | Reference |
| Proximal | 205 (55) | 38 (83) | 3.82 (1.74-8.42) |
| Stage of cancer at time of diagnosis | | | |
| Local | 145 (39) | 17 (39) | Reference |
| Regional | 210 (59) | 26 (59) | 1.06 (0.55-2.02) |
| Distant | 41 (2) | 1 (2) | 0.21 (0.03-1.61) |
| Alcohol (category of use) | | | |
| None | 306 (71) | 27 (56) | Reference |
| Lower half | 55 (13) | 8 (17) | 1.65 (0.71-3.82) |
| Upper half | 73 (17) | 13 (27) | 2.02 (0.99-4.10) |
| Smoking status | | | |
| Never | 184 (42) | 9 (19) | Reference |
| Former | 184 (42) | 26 (54) | 2.89 (1.32-6.33) |
| Current | 67 (15) | 13 (27) | 3.97 (1.62-9.70) |
| NSAID use during past 5 y | | | |
| Never | 48 (11) | 10 (21) | Reference |
| Occasionally | 177 (40) | 23 (48) | 0.62 (0.28-1.40) |
| Frequently | 212 (49) | 15 (31) | 0.34 (0.14-0.80) |
| Total calcium intake | | | |
| Quartile 1 | 113 (26) | 7 (15) | Reference |
| Quartile 2 | 109 (25) | 12 (25) | 1.78 (0.68-4.68) |
| Quartile 3 | 105 (24) | 16 (33) | 2.46 (0.97-6.22) |
| Quartile 4 | 107 (25) | 13 (27) | 1.96 (0.75-5.10) |
| Vitamin/supplement use | | | |
| No/occasional | 264 (61) | 29 (62) | Reference |
| Regularly | 166 (39) | 18 (38) | 0.99 (0.53-1.83) |

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alcohol intake, and both former and current smoking were significantly associated with the likelihood of having MSI. Frequent use of NSAIDs versus no use over the past 5 years was associated with lower likelihood of MSI. Gender, family history, stage at the time of diagnosis, alcohol use, occasional NSAID use, calcium intake, and vitamin/supplement use were not significantly associated with MSI.

Table 2 provides the ORs for the association between MSI and total, dietary, and supplemental folate intake. There were no associations between MSI and the various forms of folate. We examined the relationship between MTHFR polymorphisms (*MTHFR 677* C>T and *MTHFR 1298* A>C) and MSI among the entire study subjects and by race. However, we had limited power due to small numbers. To increase precision, we combined the MTHFR heterozygotes and homozygous variant genotypes (677 CT + TT; 1298 AC + CC) for each polymorphism separately. Among the entire case subjects, there was a nonsignificant inverse association between *MTHFR* CT/TT genotype and MSI (677 CT/TT, OR, 0.54; 95% CI, 0.26-1.10 versus 677 CC wild-type referent). Among White subjects, those with MTHFR CT/TT genotype were significantly less likely to have MSI (OR, 0.36; 95% CI, 0.16-0.81) compared with the *MTHFR 677* CC wild-type referent. Among African Americans, there was no significant association between *MTHFR* CT/TT genotype and MSI. These data should be interpreted with caution because of unstable estimates due to small numbers. There was no association between *MTHFR 1298* AC/CC genotype and MSI among entire subjects and in stratified analysis by race. For *MTHFR 677* and *MTHFR 1298* haplotypes (677 CT/TT + 1298 AC/CC compared with wild-type 677 CC + 1298 AA) and MSI, the results were suggestive of inverse relation to MSI, but the results were not significant (OR, 0.51; 95% CI, 0.13-1.80).

Results for the relationship among *MTHFR* genotypes, total, dietary, or supplemental folate, and MSI are found in Table 3. Variables that resulted in at least a 10% difference between ORs for the main exposure of interest in the crude and adjusted models (age, race, tumor location, smoking status, total calcium intake, and vitamin/mineral supplement use) were included in the models. In all combinations, the *MTHFR* common wild-type genotypes 677 CC or 1298 AA and high total, dietary, or supplemental folate were used as the reference. Compared with the reference, those with *MTHFR 1298* AC/CC genotype and adequate total folate (>400 μg) were less likely to have MSI (OR, 0.18; 95% CI, 0.04-0.73). Similarly, those with *MTHFR 1298* AC/CC genotype and supplemental folate also showed significant inverse association to MSI (OR, 0.10; 95% CI, 0.01-0.81). Compared with the reference (677 CC + >275 μg dietary folate), subjects with *MTHFR 677* CT/TT genotype and dietary folate intake >275 μg were less likely to have MSI (OR, 0.11; 95% CI, 0.52), but there was no association between *MTHFR 1298* AC/CC genotype/high dietary folate and MSI (Table 3).

ORs for combined *MTHFR* genotypes and MSI among those with adequate folate are presented in Table 4A. Among those with high total folate (>400 μg), *MTHFR* variant genotypes were significantly associated with reduced risk of MSI, although some of the cell sizes are small. Among those with low folate intake, the results suggest a slight positive association between combined *MTHFR* variants and MSI, although not significant (Table 4B).

Interaction terms were created for the combinations for *MTHFR* and folate. For all interaction models, race, sex, and age were included in the adjusted model. There was evidence for multiplicative ($P = 0.01$) and additive ($P = 0.001$) interaction between *MTHFR 1298* genotype and total folate intake. The same was true for *MTHFR 1298* AC/CC and supplemental folate (additive $P = 0.001$, multiplicative $P = 0.02$) and *MTHFR 677* CT/TT and dietary folate (additive $P = 0.01$, multiplicative $P = 0.01$). None of the remaining interaction models, whether additive or multiplicative, resulted in a statistically significant result. We were unable to stratify the interactions by race due to low numbers in the African American category, which made the models unreliable.

**Table 2. Association between total, supplemental, and dietary folate and MSI**

| Variable | Folate intake | MSI, *n* (%) | MSS, *n* (%) | OR* (95% CI) |
|---|---|---|---|---|
| Total folate | ≥400 | 17 (38) | 128 (36) | Reference |
| | <400 | 28 (62) | 232 (64) | 1.06 (0.45-2.50) |
| Supplemental folate | Yes | 13 (29) | 104 (29) | Reference |
| | No | 32 (71) | 256 (71) | 0.98 (0.34-2.84) |
| Dietary folate | ≥275 | 19 (42) | 144 (40) | Reference |
| | <275 | 26 (58) | 216 (60) | 1.18 (0.6-2.50) |

*Adjusted for age, race, smoking status, total calcium intake, tumor location, and vitamin/mineral intake.

## Discussion

In this study, we investigated the association of two *MTHFR* polymorphisms, codons 677 C>T and 1298 A>C, folate intake, and MSI status among colon cancer cases. We hypothesized that *MTHFR* polymorphisms that are linked with reduced MTHFR enzyme activity would be associated with greater risk of colon tumors exhibiting MSI and that the association would be modified by folate status. We found that the relationship between *MTHFR* polymorphisms and MSI was influenced by folate status. Among subjects with adequate folate (>400 μg), the combined *MTHFR 677* and *MTHFR 1298* variant genotypes were associated with reduced risk of MSI tumors. This protective effect was not evident under low folate conditions. We examined the relationship between MTHFR genotypes and MSI among all case subjects and in stratified analysis by race. We observed inverse association between *MTHFR 677* CT/TT genotype and MSI for all case subjects, but the association was only significant among Whites. The relationship between *MTHFR 1298* AC/CC genotype and MSI was close to the null.

Several studies but not all have shown reduction in colorectal cancer risk among individuals homozygous for *MTHFR 677* TT under conditions of adequate folate intake (10, 25, 26, 40). These observations suggest that the protective effects of *MTHFR* variant genotypes on colon cancer risk may be modified by interactions with folate intake. In this study, combined *MTHFR* variant genotypes (677 CT/TT + 1298 AA; 677 CC + 1298 AC/CC; Table 4) were inversely related to the risk of MSI tumors in the presence of adequate folate intake. A possible explanation for this finding may relate to more efficient metabolism when folate intake is adequate. For instance, it is known that MTHFR variant genotypes with lower enzyme activity favor increased availability of the nonmethylated form of folate 5,10-methylenetetrahydrofolate for DNA synthesis and decreased levels of 5-methyltetrahydrofolate, the form of folate required for DNA methylation. However, when there is adequate supply of folate, although MTHFR activity is low, presumably, enough folate is being converted to 5-methyltetrahydrofolate for DNA methylation while at the same time shunting 5,10-methylenetetrahydrofolate toward DNA synthesis for the conversion of uracil to thymidine. However, *MTHFR 677* TT genotype in combination with low folate disrupts DNA methylation and may contribute to carcinogenesis (29). Indeed, emerging evidence implicates aberrant DNA methylation as an important component in the pathways leading to colorectal cancer. Aberrant DNA methylation, particularly in tumor suppressor and DNA mismatch

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**Table 3. Adjusted ORs and 95% CIs for MSI in relation to MTHFR genotypes and total, dietary, and supplemental folate**

| Genotype | Total folate (μg) | MSI | MSS | OR* (95% CI) | Dietary folate (μg) | MSI | MSS | OR* (95% CI) | Supplemental folate use | MSI | MSS | OR* (95% CI) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *MTHFR 1298* | | | | | | | | | | | | |
| AA | ≥400 | 11 | 63 | Reference | ≥275 | 5 | 89 | Reference | Yes | 10 | 58 | Reference |
| AC/CC | ≥400 | 3 | 73 | 0.18 (0.04-0.73) | ≥275 | 10 | 75 | 1.63 (0.49-5.44) | Yes | 1 | 53 | 0.10 (0.01-0.81) |
| AA | <400 | 12 | 147 | 0.51 (0.16-1.62) | <275 | 18 | 121 | 2.46 (0.79-7.67) | No | 13 | 152 | 0.62 (0.18-2.11) |
| AC/CC | <400 | 16 | 94 | 0.83 (0.26-2.64) | <275 | 9 | 92 | 1.17 (0.33-4.20) | No | 18 | 114 | 0.82 (0.24-2.76) |
| *MTHFR 677* | | | | | | | | | | | | |
| CC | ≥400 | 12 | 76 | Reference | ≥275 | 15 | 96 | Reference | Yes | 8 | 64 | Reference |
| CT/TT | ≥400 | 3 | 58 | 0.29 (0.07-1.17) | ≥275 | 2 | 66 | 0.11 (0.02-0.52) | Yes | 3 | 46 | 0.51 (0.12-2.23) |
| CC | <400 | 21 | 156 | 0.92 (0.33-2.59) | <275 | 18 | 136 | 0.67 (0.27-1.68) | No | 25 | 168 | 1.23 (0.34-4.41) |
| CT/TT | <400 | 8 | 85 | 0.53 (0.16-1.79) | <275 | 9 | 77 | 0.72 (0.26-2.00) | No | 8 | 97 | 0.54 (0.13-2.34) |

*Adjusted for age, race, tumor location, smoking status, total calcium intake, and vitamin/mineral supplement use.

repair genes, presumably acts through global hypomethylation or hypermethylation of specific CpG islands to promote gene inactivation. Hypermethylation of the hMLH1 promoter has been observed in most sporadic colorectal cancer with MSI (41) particularly when there is no evidence of mutations in DNA *MMR* genes (35, 42). However, we did not evaluate DNA methylation and mutations in *MMR* genes in this study.

Contrary to our predictions, we found an inverse relationship between *MTHFR* variant genotypes and MSI. *MTHFR 677* and *MTHFR 1298* variants are linked with reduced enzyme activity. The *MTHFR 677* TT genotype is associated with 30% reduced enzyme activity (21) compared with CC wild-type; therefore, our findings may be related to lower MTHFR enzyme activity. The increased availability of 5,10-methylenetetrahydrofolate associated with reduced MTHFR activity favors DNA synthesis and repair, thereby resulting in less uracil misincorporation and decreased DNA strand breaks (15) and presumably leading to more MSS tumors. The *MTHFR 1298* C variant affects enzyme activity to a lesser degree than *MTHFR 677* T variant (22). We did not observe a relationship between *MTHFR 1298* C variant and MSI. This finding could be related to higher *MTHFR 1298* enzyme activity. Although the two MTHFR variants are in linkage disequilibrium (43), we found no evidence for linkage disequilibrium between the two MTHFR polymorphisms in our study population. A recent study reported that the modest reduction in colorectal cancer risk associated with *MTHFR 1298* A>C may be independent of the *MTHFR 677* C>T (43).

Very few studies have examined the relationship between MTHFR polymorphisms and MSI. Toffoli et al. (34) observed a borderline inverse association between *MTHFR 677* TT genotype and MSI but found no significant association between *MTHFR 1298* CC genotype and MSI status. Our findings are similar but stronger than the results of the study by Toffoli et al. but conflict with two other studies. Shannon et al. (35), observed that MSI tumors were more common among older colorectal cancer cases with MTHFR TT genotype compared with those with MSS tumors, whereas Plaschke et al. (36) reported no significant associations between MTHFR genotypes and MSI. The reasons for these conflicting observations are not entirely clear. Our results differ from these two studies and may reflect differences in methodologies. For example, Shannon et al. (35) assessed MSI status using only one microsatellite marker (BAT26), whereas we used the panel of five microsatellite markers recommended by NCI. Differences in the classification of MSI resulting from testing a varying number of loci at different sites may contribute to conflicting reports in the literature (44). In our analysis, we used the common *MTHFR 677* wild-type genotypes (CC) as referent and the heterozygote and variant genotypes (CT + TT) were combined to increase precision. Shannon et al. (35) and Plaschke et al. (36) combined the common *MTHFR 677* wild-type and heterozygote genotypes (CT) as the referent group.

Not many studies have examined the relationship between dietary factors, such as folate, and colorectal tumors exhibiting MSI. Interestingly, we found no associations between MSI and folate intake (total, dietary, and supplemental). Our results agree with the observations of Slattery et al. (45), who evaluated the relationship between dietary intake of various nutrients and foods in relation to MSI and reported no associations between most dietary factors and MSI, except for alcohol consumption. The inverse associations that we observed between MTHFR haplotypes and MSI would suggest that, under conditions of adequate folate, the DNA synthesis and repair process is favored which likely leads to MSS tumors.

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**Table 4. Relationship between combined MTHFR genotypes and MSI by folate intake status**

| Combined MTHFR genotypes | | Total folate (μg) | MSI | MSS | OR* (95% CI) |
|---|---|---|---|---|---|
| (A) ORs and 95% CIs for the association between combined MTHFR genotypes and MSI among subjects with high folate intake | | | | | |
| *MTHFR 677* | *MTHFR 1298* | | | | |
| CC | AA | ≥400 | 8 | 23 | Reference |
| CC | AC/CC | ≥400 | 3 | 44 | 0.09 (0.01, 0.59) |
| CT/TT | AA | ≥400 | 3 | 28 | 0.13 (0.02, 0.85) |
| CT/TT | AC/CC | ≥400 | 0 | 15 | ND |
| (B) ORs and 95% CIs for the association between combined MTHFR genotypes and MSI among subjects with low folate intake | | | | | |
| *MTHFR 677* | *MTHFR 1298* | | | | |
| CC | AA | <400 | 6 | 64 | Reference |
| CC | AC/CC | <400 | 12 | 62 | 1.47 (0.43-4.99) |
| CT/TT | AA | <400 | 5 | 57 | 0.68 (0.16-2.78) |
| CT/TT | AC/CC | <400 | 3 | 17 | 1.20 (0.20-7.32) |

Abbreviation: ND, not determined.
*Adjusted for age, race, tumor location, smoking status, total calcium intake, and vitamin/mineral supplement use.

The strengths of this study include the effort to thoroughly collect pathologic specimens for MSI analysis, nutritional data, recruitment of cases from a region that contained both rural and urban areas, and the deliberate recruitment of both African Americans and Whites. To our knowledge, stratification by race for MSI studies has not been addressed before. The weaknesses of our study include the following issues. We estimated folate intake from the Block-NCI food frequency questionnaire. Estimation of nutrient intake from self-reported dietary information collected after diagnosis of disease in cases is subject to recall bias. In addition, we assumed in our study that folate supplement users took daily tablet containing 400 μg, which could have overestimated folate intake in some people. However, the results did not differ when we assumed lower values of folate for supplement users (data not shown). Many of the previous studies tested serum folate, which we were not able to do because blood samples were obtained after diagnosis in cases. One of the objectives of this study was to investigate differences among the Whites and African Americans. Unfortunately, the small numbers of African Americans with MSI were small, which led to imprecise estimates as evidenced by the large 95% CIs. These insufficient data made it difficult to formulate any clear-cut conclusions. Additional weaknesses include missing genotype data due to refusals for blood draw and the need to combine several categories of data due to small numbers. We examined only two polymorphic loci, and according to Little et al. (46), >30 different polymorphic loci are related to folate metabolism, and by looking at only a few of these loci, we may be missing information on the role of other important loci.

In conclusion, we found that *MTHFR* variant genotypes were inversely associated with MSI when folate intake was adequate. This would imply that, under conditions of reduced MTHFR enzyme activity and abundant folate, a balance in folate pools is maintained for DNA methylation and DNA synthesis. In light of a lack of survival advantage for patients with MSI colon tumors undergoing treatment with 5-fluorouracil (47, 48) whose metabolism involves folate intermediates, our findings could have potential implications for colon cancer treatment and prevention. Our observations need to be confirmed in larger studies.

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