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Methylenetetrahydrofolate Reductase Polymorphism, Dietary Interactions, and Risk of Colorectal Cancer[1]

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ABSTRACT

Folate derivatives are important in experimental colorectal carcinogenesis; low folate intake, particularly with substantial alcohol intake, is associated with increased risk. The enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) catalyzes the conversion of 5,10-methylenetetrahydrofolate, required for purine and thymidine syntheses, to 5-methyltetrahydrofolate, the primary circulatory form of folate necessary for methionine synthesis. A common mutation (677C→T) in MTHFR reduces enzyme activity, leading to lower levels of 5-methyltetrahydrofolate. To evaluate the role of folate metabolism in human carcinogenesis, we examined the associations of MTHFR mutation, plasma folate levels, and their interaction with risk of colon cancer. We also examined the interaction between genotype and alcohol intake.

We used a nested case-control design within the Physicians' Health Study. Participants were ages 40–84 at baseline when alcohol intake was ascertained and blood samples were drawn. During 12 years of follow-up, we identified 202 colorectal cancer cases and matched them to 326 cancer-free controls by age and smoking status. We genotyped for the MTHFR polymorphism and measured plasma folate levels.

Men with the homozygous mutation (15% in controls) had half the risk of colorectal cancer [odds ratio (OR), 0.49; 95% confidence interval (CI), 0.27–0.87] compared with the homozygous normal or heterozygous genotypes. Overall, we observed a marginal significant increased risk of colorectal cancer (OR, 1.78; 95% CI, 0.93–3.42) among those whose plasma folate levels indicated deficiency (<3 ng/ml) compared with men with adequate folate levels. Among men with adequate folate levels, we observed a 3-fold decrease in risk (OR, 0.32; 95% CI, 0.15–0.68) among men with the homozygous mutation compared with those with the homozygous normal or heterozygous genotypes. However, the protection due to the mutation was absent in men with folate deficiency. In men with the homozygous normal genotype who drank little or no alcohol as reference, those with the homozygous mutation who drank little or no alcohol had an 8-fold decrease in risk (OR, 0.12; 95% CI, 0.03–0.57), and for moderate drinkers, a 2-fold decrease in risk (OR, 0.42; 95% CI, 0.15–1.20); no decrease in risk was seen in those drinking 1 or more drinks/day.

Our findings provide support for an important role of folate metabolism in colon carcinogenesis. In particular, these results suggest that the 677C→T mutation in MTHFR reduces colon cancer risk, perhaps by increasing 5,10-methylenetetrahydrofolate levels for DNA synthesis, but that low folate intake or high alcohol consumption may negate some of the protective effect.

INTRODUCTION

Animal and cell models suggest a role for folate in reducing colon carcinogenesis (1, 2). In epidemiological studies, diets low in folate, particularly in combination with substantial alcohol intake, are associated with increased risks of colon cancer and its precursor, the adenomatous polyp (3–9). MTHFR[3] is a critical enzyme in folate metabolism (Fig. 1; Ref. 10). Its product, 5-methylTHF, is the predominant form of folate in plasma, whereas the enzyme substrate, 5,10-methyleneTHF, is found mainly intracellularly. 5-methylTHF provides the methyl group for *de novo* methionine synthesis and DNA methylation (11). Imbalanced DNA methylation, characterized by global genomic hypomethylation (12, 13) and methylation of usually unmethylated CpG sites (14, 15), is observed consistently in colonic neoplasia (16). A decreased 5-methylTHF pool may affect DNA methylation and thereby contribute to carcinogenesis. On the other hand, the substrate for MTHFR, 5,10-methyleneTHF, is required for conversion of deoxyuridylate to thymidylate, and, thus, depletion of this form of folate may interfere with the thymidylate biosynthesis and result in development of deoxynucleotide pool imbalances (17). This leads to accumulation of deoxyuridylate in DNA (17) and removal of this abnormal base might labilize DNA to strand breaks (18–25). Chromosome breaks appear to be important in nearly all human cancers and are especially common in colorectal cancer (26–28). Alcohol decreases folate absorption, alters its metabolism, increases its excretion, and therefore may interfere with both DNA methylation and thymidylate synthesis (29–33).

A common mutation (677C→T alanine-to-valine) has been identified in the MTHFR gene (34, 35). This mutation causes reduced enzyme activity (35), leading to reduced plasma folate levels and increased plasma homocysteine levels (35–38). It therefore has been proposed as a risk factor for cardiovascular disease and for neural tube defects when folate intake is low (35–38). This mutation is known to cause lower levels of circulating folate (5-methylTHF) and is postulated to lead to an accumulation of 5,10-methyleneTHF. Exploration of the association of this mutation with colorectal cancer therefore permits a further assessment of the role of folate in carcinogenesis and the relative importance of the alternate folate metabolic pathways. We examined the association of MTHFR genotype with risk of colorectal cancer, as well as the interaction of the genotype with plasma folate and alcohol intake in a nested case-control study among the Physicians' Health Study participants.

PATIENTS AND METHODS

This is a prospective case-control study nested in the Physicians' Health Study, a randomized, double-blind, placebo-controlled 2 × 2 factorial trial of low-dose aspirin (Bufferin, Bristol-Myers Products, 325 mg every other day) and β-carotene (Lurotin, BASF, 50 mg on alternate days) among 22,071 healthy United States male physicians ages 40–84 years (39). Men were excluded if they had a history of myocardial infarction; stroke or transient ischemic attack; cancer (except nonmelanoma skin cancer); current renal or liver disease; peptic ulcer or gout; and current use of a vitamin A or β-carotene

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[3] The abbreviations used are: MTHFR, 5,10-methylenetetrahydrofolate reductase; 5-methylTHF, 5-methyltetrahydrofolate; 5,10-methyleneTHF, 5,10-methylenetetrahydrofolate; OR, odds ratio; CI, confidence interval.

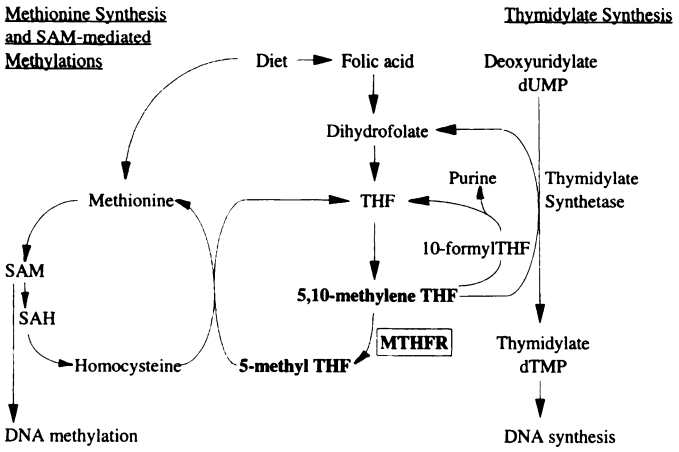

Fig. 1. Competing pathways in folate metabolism. *THF*, tetrahydrofolate; *SAM*, *S*-adenosylmethionine.

supplement. Alcohol intake (beer, wine, or liquor) was ascertained from the baseline questionnaire by asking the participants to check one of the following seven categories: 2 or more/day, daily, 5–6/week, 2–4/week, 1/week, 1–3/month, and rarely/never.

Blood samples (60% nonfasting) were collected at baseline, in 1982, as described previously (38, 40). We received specimens from 14,916 (68%) of the randomized physicians. The men were followed through biannual mailed questionnaires. Between 1982 and 1995, we confirmed 202 colorectal cancer diagnoses by medical records, and matched these men with 326 cancer-free controls by age and smoking status. Controls were alive and free of colorectal cancer when their matched cases were diagnosed. To increase the statistical power, we attempted to match two controls for each case but could find a second control for only 124 cases.

DNA from these cases and controls was extracted from baseline blood, and MTHFR genotype was analyzed in Dr. Rozen's laboratory; the investigators and laboratory personnel were blinded to case-control status. The presence of the mutation was determined, as described previously (35), by PCR amplification of genomic DNA using an exonic (5′-TGAAGGAGAA GGTGTCT-GCG GGA-3′) and an intronic (5′-AGGACGGTGCGGTGAGAGTG-3′) primer. Thirty-five cycles of PCR were performed for 1 min at 94°C, 1 min at 68°C, and 2 min at 72°C, with a 7-min elongation at 72°C at the end of the cycles. The PCR products were digested with *Hin*fI, since the mutation creates a *Hin*fI restriction site.

Plasma levels of folate were measured microbiologically using a 96-well plate and manganese supplementation, as described by Tamura *et al.* (41), in the laboratory of J. S. Sixteen blind paired quality control samples were interspersed at random among the specimens. The quality control samples were aliquots of a large, well-mixed plasma pool from healthy volunteers and were treated in the exact same manner as the samples collected from the participants. The mean within-pair coefficient of variation in these paired quality control specimens was 16% for folate.

We assessed whether the MTHFR mutation and plasma folate were related to risk of colorectal cancer. All of the statistical analyses were done using Statistic Analysis Software (42). Because the distribution of plasma folate was skewed, we used a natural log transformation. We calculated the age-adjusted geometric mean of plasma folate concentration within strata of MTHFR genotype and case-control status using analysis of covariance. We calculated the age- and smoking-adjusted OR to estimate the relative risk of developing colorectal cancer using unconditional logistic regression analysis. We addressed potential confounding by alcohol consumption, multivitamin use, body mass index, and exercise level on the baseline questionnaire, and aspirin assignment group, by including these as covariates in multivariate models. We assessed the age-adjusted OR for the joint effect of MTHFR genotype and folate status (folate deficiency was defined as plasma folate levels <3 ng/ml; Ref. 43) or alcohol consumption (0–0.14 drinks/day, 0.15–0.8/day, and 0.9–2+/day) using an indicator variable for each category in logistic regression models. Because matched and unmatched analysis yielded virtually the same results, we show results from the unconditional logistic regression models. All *P* values are two sided.

RESULTS

Table 1 shows the general characteristics of the study participants. The frequency of the homozygous mutant Val/Val genotype among controls was 15%. The age- and smoking-adjusted OR of colorectal cancer was 0.50 (95% CI, 0.27–0.92) for men with genotype Val/Val compared with those with homozygous normal Ala/Ala genotype (Table 2). The apparent protective effect was similar (OR, 0.45; 95% CI, 0.24–0.86) after further adjusting for alcohol consumption, multivitamin use, exercise, body mass index, and aspirin assignment. The mutation appears to be recessive; we and others have found that individuals with the heterozygous genotype Ala/Val have plasma homocysteine levels similar to those with the Ala/Ala genotype (26, 35, 37, 38). The age- and smoking-adjusted OR was 0.49 (95% CI, 0.27–0.87) and multivariate-adjusted OR was 0.46 (95% CI, 0.25–0.84) when men with Ala/Ala or Ala/Val genotype were combined for use as the reference group. To increase the statistical power, we added 286 additional controls from our previous study of the MTHFR polymorphism and myocardial infarction from the same population (38). The age- and smoking-adjusted OR did not change materially (OR, 0.56; 95% CI, 0.33–0.96) comparing Val/Val men to those with Ala/Ala or Ala/Val genotype. The associations did not vary materially by colon (OR, 0.48; 95% CI, 0.25–0.91) or rectal cancer (OR, 0.55; 95% CI, 0.13–2.27), although the latter was not statistically significant, presumably due to small numbers. The association was slightly and nonsignificantly stronger for older men (60–84 years; $n = 100$ cases; OR, 0.41; 95% CI, 0.19–0.90) than younger men (40–59 years; $n = 102$ cases; OR, 0.58; 95% CI, 0.24–1.40).

To examine the impact of plasma folate, we compared men with adequate levels to those with deficient levels (<3 ng/ml). Overall, we observed a marginally significant increased risk of colorectal cancer (OR, 1.78; 95% CI, 0.93–3.42) among those whose plasma folate indicated a deficiency compared with those with adequate folate levels. However, because the mutation is associated with a reduced risk of colon cancer and with low plasma folate levels, it is possible that a protective effect of folate on risk of colon cancer might be partly obscured by these two effects of the mutation. To separate the effects of the mutation and of folate intake on risk of colon cancer, we first assessed plasma folate levels by case-control status within strata of MTHFR genotypes. As expected, men with the Val/Val genotype had significantly lower levels of folate (case and control combined geometric mean, 3.43 ng/ml; $P < 0.01$) compared with those with the homozygous normal (6.44 ng/ml) or heterozygous genotypes (6.10 ng/ml). However, we observed that cases with the Val/Val genotype

Table 1 *Means (±SD) and proportions of risk factors for colorectal cancer in the Physicians' Health Study*

Risk factor	Cases (n = 202)	Controls (n = 326)	P value
Age (years)	60 ± 9	57 ± 8	Matching factor
Quetelet index [weight (kg)/height (m^2)]	25.3 ± 3.2	24.9 ± 3.0	0.14
Geometric mean folate (ng/mL)[a]	5.45	5.84	0.19
Alcohol intake (drinks/day)	0.60 ± 0.46	0.57 ± 0.48	0.77
Exercise (times/day)	0.36 ± 0.30	0.35 ± 0.28	0.80
Folate deficiency (%)[a] (<3 ng/ml)	13	9	0.36
Cigarette smoking (%)			
Past	55	53	
Current	8	8	Matching factor
Multivitamin use (%)			
Past	16	15	
Current	22	22	0.94
Aspirin assignment (%)	50	53	
Colon cancer (%)	79		
Rectal cancer (%)	21		

[a] Only 170 cases and 294 controls had plasma folate levels.

Table 2 *Frequency of the MTHFR genotype and risk of colorectal cancer among United States physicians*

	Cases		Controls		Age and smoking-adjusted OR	Multivariate-adjusted OR[a]
MTHFR Genotype	*n*	(%)	*n*	(%)	(95% CI)	(95% CI)
Homozygous normal (Ala/Ala)	92	45.5	145	44.5	1.00 (reference)	1.00 (reference)
Heterozygous (Ala/Val)	92	45.5	132	40.5	1.05 (0.72–1.54)	0.98 (0.67–1.45)
Homozygous mutant (Val/Val)	18	9	49	15	0.50 (0.27–0.92)	0.45 (0.24–0.86)
With genotype Ala/Ala and Ala/Val as reference group					0.49 (0.27–0.87)	0.46 (0.25–0.84)
Total	202		326			

[a] Adjusted for age, smoking status, alcohol consumption, multivitamin use, exercise, body mass index, and aspirin use.

Table 3 *Age-adjusted geometric mean plasma folate levels (ng/ml) according to MTHFR genotype and case-control status among United States physicians*

MTHFR genotype	Cases	Controls
Homozygous normal (Ala/Ala)	6.63	6.26
Heterozygous (Ala/Val)	5.82	6.39
Homozygous mutant (Val/Val)	2.40[a,b]	4.90[a]

[a] $P \leq 0.05$ for genotype (Val/Val) *versus* (Ala/Ala), or for genotype (Val/Val) *versus* (Ala/Val).
[b] $P \leq 0.01$ for cases *versus* controls.

had significantly lower folate levels (2.40 ng/ml) than controls with this genotype (4.90 ng/ml; $P < 0.01$; Table 3).

Low folate levels were associated with higher risk of colorectal cancer for all MTHFR genotypes, but the effect of folate deficiency was especially strong in the Val/Val group (Fig. 2). Moreover, the protective effect of the mutation was apparent only among those with adequate folate. A similar, although less distinct pattern was observed using multivitamin supplements in place of plasma folate levels (data not shown). The results of the multivitamin analysis were weaker, presumably because they did not adequately identify those with a folate deficiency.

We then examined whether the protective effect of the mutation varied by alcohol consumption. In men with the Ala/Ala genotype who drank little or no alcohol as reference, those with the Val/Val genotype at the same drinking level had an 8-fold decrease in risk (OR, 0.12; 95% CI, 0.03–0.57). Although only 2 cases had the Val/Val genotype in the low alcohol category, this small number reflects the major reduction in risk because in the absence of any effect, one would expect about 16 cases in that group. Moderate drinkers with the Val/Val genotype had a 2-fold decrease in risk (OR, 0.42; 95% CI, 0.15–1.20). A decrease in risk was not seen in men in the highest drinking category (1 or more drinks/day; Table 4). Viewed conversely, the possible association between alcohol intake and colon cancer was the most evident and strongest among men with the Val/Val genotype.

DISCUSSION

In this study of United States physicians, we observed that the 677C→T mutation in MTHFR was associated with half the risk of colorectal cancer. Because the homozygous mutant genotype is common, with 15% of controls in this study and 13% of controls in our previous study of myocardial infarction (38), the impact of this variant on colorectal cancer is substantial. This selective advantage may explain, at least in part, the high frequency (approximately 35% of alleles; Refs. 35 and 38) of this mutation in the general population. We observed a similar (but not statistically significant) effect of the mutation in the Health Professionals Follow-up Study (44). However, the finding requires further confirmation in other populations, especially among those with low folate intake.

Overall, we observed a marginally significant inverse association between plasma folate levels and risk of colorectal cancer. This association was obscured by the effect of the MTHFR mutation on both colon cancer risk and plasma folate levels, because the mutation reduces plasma folate (predominately 5-methylTHF) levels (36, 38). Consistent with this finding is the observation by van der Put *et al.* (36) that red blood cell folate levels (possibly reflecting the tissue concentration) were significantly higher in those with the homozygous mutation. Also consistent is the finding by Glynn *et al.* (9) of an inverse association between risk of colon cancer and dietary folate intake but little association with serum folate. We observed a strong inverse association with colorectal cancer (3-fold reduced risk) in men with the homozygous mutant genotype and adequate folate levels, but the protection due to the mutation was absent in men with folate deficiency. In fact, folate deficiency may even increase risk of colorectal cancer among those with the homozygous mutant genotype, but our nonsignificant results need to be confirmed by a larger study. Because plasma folate level is regulated both by MTHFR enzyme and dietary intake, individuals with the mutation and adequate folate intake appear to have the benefit.

The strongest apparent protective effect of the homozygous mutant genotype, with an 8-fold decrease in risk, was found among men who drink little or no alcohol. No protection was observed among drinkers who had 1 or more drinks/day. We observed a similar significant interaction in the Health Professional Follow-up Study (44). High alcohol consumption may overcome the apparent protective effect of the mutation because ethanol can cleave folate (29), inhibit its absorption and utilization (30, 31), and increase its excretion (32). Furthermore, alcohol, as a methyl group antagonist, may cause imbalanced DNA methylation (33).

Numerous clinical observations and epidemiological studies support an inverse association between low folate intake, assessed by

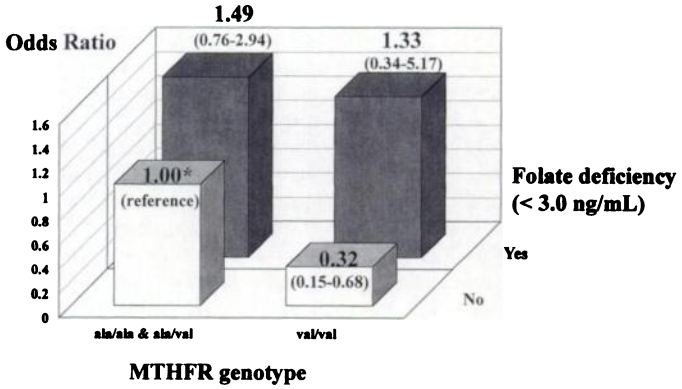

Fig. 2. Age-adjusted OR of colorectal cancer by MTHFR genotype and plasma folate status among United States physicians. Number of cases/controls: 139/229 for men with Ala/Ala or Ala/Val genotype and adequate folate; 18/21 for men with the same genotype and folate deficiency; 9/39 for men with the Val/Val genotype with adequate folate; and 4/5 for men with the Val/Val genotype and folate deficiency. *, OR (95% CI).

Table 4 *Age-adjusted OR[a] of colorectal cancer according to MTHFR genotype and alcohol intake status among United States physicians*

	Alcohol intake[b]		
MTHFR genotype	Low (0–0.14 drinks/day)	Medium (0.15–0.8 drinks/day)	High (0.9–2+ drinks/day)
Homozygous normal (Ala/Ala)			
No.[c]	32/49	36/48	24/46
OR	1.00	1.24	0.72
(95% CI)	(Reference)	(0.66–2.34)	(0.37–1.42)
Heterozygous (Ala/Val)			
No.[c]	23/49	40/43	29/40
OR	0.69	1.40	1.00
(95% CI)	(0.35–1.37)	(0.74–2.63)	(0.51–1.94)
Homozygous mutant (Val/Val)			
No.[c]	2/21	6/18	10/10
OR	0.12	0.42	1.31
(95% CI)	(0.03–0.57)	(0.15–1.20)	(0.48–3.58)

[a] $P < 0.01$ test for interaction.
[b] Two controls had missing alcohol consumption information.
[c] Number of cases/controls.

dietary intake or by measurement of blood or red blood cell levels, and an increased risk of colorectal adenoma or cancer (1, 3–6). A cause-and-effect relationship was also seen in a rodent model of colorectal neoplasia (2). The mechanism(s) remains unclear. Because the different forms of folate participate in distinct pathways of single-carbon metabolism, several mechanisms may be responsible (Fig. 1; Ref. 1). Our findings suggest that availability of 5,10-methyleneTHF, which is necessary for DNA synthesis, may be critical. 5,10-MethyleneTHF occupies a central role because its one-carbon moiety has several metabolic possibilities: (*a*) it can be transferred directly to deoxyuridylate in thymidylate synthesis; (*b*) it can be reduced to 5-methylTHF by MTHFR for *de novo* methionine synthesis; and (*c*) it can be oxidized to 10-formyltetrahydrofolate for *de novo* purine synthesis (10). The conversion of 5,10-methyleneTHF to 5-methylTHF is essentially irreversible under physiological conditions (10). A reduced 5-methylTHF pool and an increased level of intracellular 5,10-methyleneTHF might be expected because of the mutant MTHFR (35, 36, 38).

Sufficient methionine intake may also reduce the risk of colorectal cancer (7, 8) by providing the methyl groups for DNA methylation and by increasing intracellular 5,10-methyleneTHF. The latter effect occurs because increased *S*-adenosylmethionine formed from methionine may inhibit MTHFR activity (11) as a feedback control of methyl group formation. However, the increased intracellular level of 5,10-methyleneTHF by either MTHFR mutation or increased methionine intake depends on overall folate status. When folate intake is low or folate antagonists such as alcohol are present, the protection might be diminished due to folate deficiency.

Severe depletion of folate can lead to decreased levels of *S*-adenosylmethionine, which is necessary for DNA methylation (16). However, moderate folate deficiency does not cause global hypomethylation of hepatic and colonic DNA or c-*myc*-specific hypomethylation of colonic DNA in rats (45). In our study, higher folate concentrations beyond the deficiency range did not appear to confer increasing benefits. A relationship between a folate-deficient diet and incidence of chemically induced tumors in rats may simply be due to an effect on DNA synthesis rather than DNA methylation (46). In the majority of the well-nourished physicians in this study, low folate and methionine intake is likely to be uncommon. With sufficient intake of these nutrients, the effect of the MTHFR mutation on DNA methylation is likely to be small, whereas a reduction in flux through the MTHFR pathway among those with the mutation may be important by reducing erroneous incorporation of deoxyuridylate during DNA synthesis (17, 18, 19). However, in the presence of insufficient intake of methionine and folate, especially with high alcohol consumption, reduced levels of 5,10-methyleneTHF may cause accumulation of deoxyuridylate into DNA, leading to chromosome breaks (17–22, 24–28). On the other hand, DNA methylation may be affected as well by reduced levels of 5-methylTHF and *S*-adenosylmethionine due to insufficient supply from diet (7–9) and reduced *de novo* methionine synthesis due to the MTHFR mutation.

Our findings provide support for an important role of folic acid in colon carcinogenesis. In particular, these results suggest that the common 677C→T mutation in MTHFR reduces colon cancer risk, perhaps by increasing 5,10-methyleneTHF levels for DNA synthesis, but that low folate intake or high alcohol consumption appear to reduce the potential benefit.

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