Polymorphisms in the Reduced Folate Carrier, Thymidylate Synthase, or Methionine Synthase and Risk of Colon Cancer

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Abstract

Folate metabolism supports the synthesis of nucleotides as well as the transfer of methyl groups. Polymorphisms in folate-metabolizing enzymes have been shown to affect risk of colorectal neoplasia and other malignancies. Using data from a population-based incident case-control study (1,600 cases and 1,962 controls), we investigated associations between genetic variants in the reduced folate carrier (*RFC*), thymidylate synthase (*TS*), methionine synthase (*MTR*), and 5,10-methylenetetrahydrofolate reductase (*MTHFR*) and colon cancer risk. The *TS* enhancer region (*TSER*) variant was associated with a reduced risk among men [2rpt/2rpt versus 3rpt/3rpt wild-type; odds ratio (OR), 0.7; 95% confidence interval, 0.6-0.98] but not women. When combined genotypes for both *TS* polymorphisms (*TSER* and 3′-untranslated region 1494delTTAAAG) were evaluated, ORs for variant genotypes were generally below 1.0, with statistically significantly reduced risks among women. Neither *MTR* D919G nor *RFC* 80G>A polymorphisms were associated with altered colon cancer risk. Because folate metabolism is characterized by interrelated reactions, we evaluated gene-gene interactions. Genotypes resulting in reduced MTHFR activity in conjunction with low TS expression were associated with a reduced risk of colon cancer. When dietary intakes were taken into account, individuals with at least one variant *TSER* allele (3rpt/2rpt or 2rpt/2rpt) were at reduced risk in the presence of a low folate intake. This study supports findings from adenoma studies indicating that purine synthesis may be a relevant biological mechanism linking folate metabolism to colon cancer risk. A pathway-based approach to data analysis is needed to help discern the independent and combined effects of dietary intakes and genetic variability in folate metabolism. (Cancer Epidemiol Biomarkers Prev 2005; 14(11):2509–16)

Introduction

Folate is an essential micronutrient in humans, the primary function of which is as a carrier of single-carbon units. Folate-dependent reactions include the biosynthesis of thymidylate, purines, methionine, and glycine thus linking it to nucleotide synthesis as well as the provision of methyl groups (1). High dietary folate intakes, or biomarkers thereof, have been associated with a reduced risk of colon cancer or its precursors in most, although not all, studies (2-5). Several studies showing associations with genetic polymorphisms in folate-metabolizing enzymes lend support to a causal relationship between folate and colorectal carcinogenesis (6-10). Biological mechanisms linking folate to colorectal carcinogenesis include an altered provision of *S*-adenosylmethionine for methylation reactions, including DNA methylation, and changes in the availability of nucleotides, such as thymidylate, for DNA synthesis and repair (11, 12).

We have previously reported on associations between polymorphisms in 5,10-methylenetetrahydrofolate reductase (MTHFR) and risk of colon cancer (13). Here, we extend this work to common genetic variants in thymidylate synthase (TS), the reduced folate carrier (RFC), and methionine synthase (MTR) in relation to colon cancer risk. TS is a key enzyme in folate metabolism that catalyzes the conversion of dUMP to dTMP for the provision of thymidine, a rate-limiting nucleotide essential for DNA synthesis and repair (see Fig. 1). TS is also a primary target for major chemotherapeutic agents, including 5-fluorouracil. We investigated the role of a polymorphism in the 5′-untranslated region (5′-UTR) enhancer region (three or two repeats of a 28-bp sequence), resulting in reduced TS expression among those with fewer repeats (14) and a 6-bp insertion or deletion (1,494 bp in the 3′-UTR) that affects mRNA stability (15, 16).

RFC is responsible for the active transport of 5-methyl-tetrahydrofolate from plasma to cytosol. A polymorphism in the *RFC* gene (80G>A, $Arg^{27}His$) seems associated with a higher affinity for folate (17). Among 169 healthy individuals who were stratified by *MTHFR* 677C>T genotype, the variant A allele was consistently and linearly associated with higher plasma folate concentrations (17). Furthermore, concentrations of methotrexate 24 to 48 hours after administration were higher among children with acute leukemia homozygous for the variant A allele, providing additional support for differential carrier activity among those with variant genotypes (18).

MTR catalyzes the methylation of homocysteine to methionine with simultaneous conversion of 5-methyl-tetrahydrofolate to tetrahydrofolate (Fig. 1). A variant in the *MTR* gene (2756A>G, $Asp^{919}Gly$; ref. 19) may affect plasma homocysteine concentrations. Some studies (20, 21) but not others (22-24) have found that homocysteine concentrations tend to decrease linearly across genotypes, with the AA genotype associated with the highest homocysteine concentrations. Studies on colorectal neoplasia have been inconsistent: the GG genotype has been associated with a somewhat reduced risk of colorectal cancer (22, 25) yet a possible increased risk of colorectal adenoma (26).

In this large population-based case-control study of colon cancer, we sought to evaluate the role of these polymorphisms in defining colon cancer risk, either alone or in interaction with specific nutrient intakes and other genotypes. Furthermore, as

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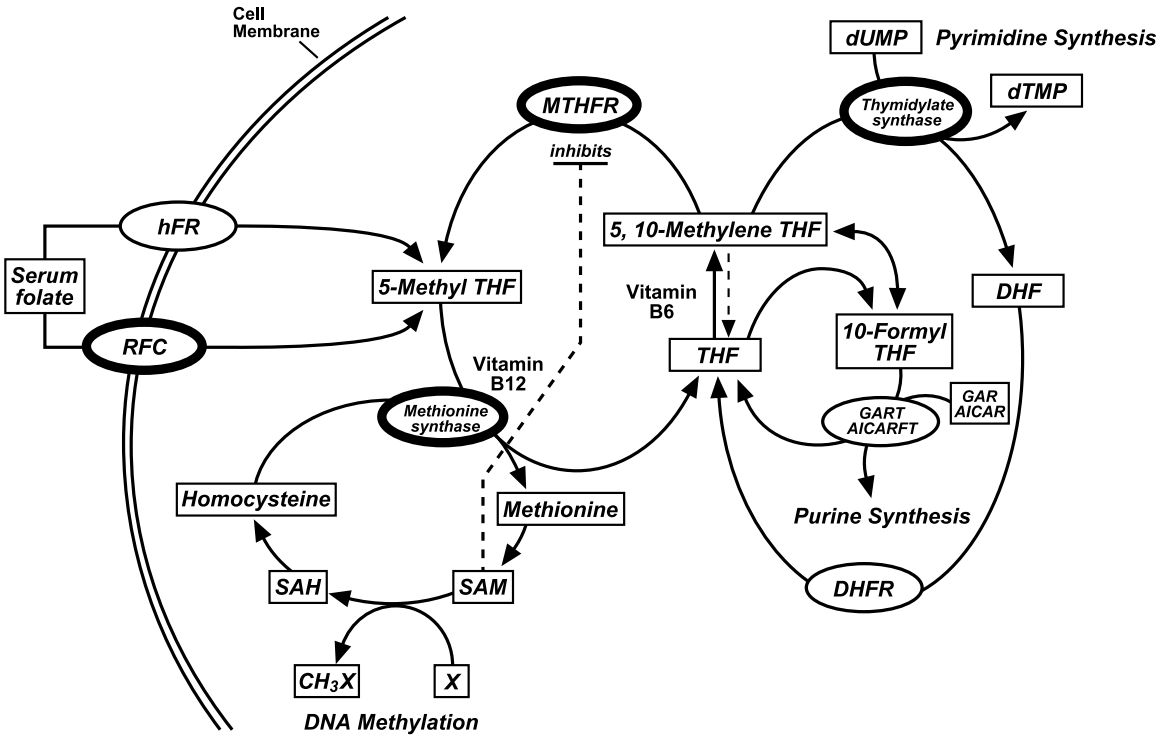

Figure 1. Simplified version of folate-mediated one-carbon metabolism, highlighting proteins with polymorphisms investigated in this study (figure modified from ref. 36). Key enzymes are denoted as ovals, substrates as rectangles. THF, tetrahydrofolate; DHF, dihydrofolate; DHFR, dihydrofolate reductase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate; X, a variety of substrates for methylation; RFC, reduced folate carrier; hFR, human folate receeptor; MTHFR, 5,10-methylenetetrahydrofolate reductase; GART, phosphoribosylglycinamide formyltransferase; AICARFT, 5-aminomidazole-4-carboxamide ribonucleotide; 5-FU, 5-fluorouracil.

some of the associations of folate metabolism may differ by estrogen exposure (13), possibly because of mechanisms attributable to hypermethylation of the estrogen receptor (27), we evaluated interactions with postmenopausal hormone (PMH) use.

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 (*International Classification of Diseases for Oncology*, 2nd edition codes 18.0, 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) or with known familial adenomatous polyposis, ulcerative colitis, or Crohn's disease were not eligible. Of all cases identified, 65% of those contacted consented to participate in the study. Controls who had never had a previous colorectal tumor were randomly selected in proportion to the cases within the geographically defined areas from Kaiser Permanente Medical Care Program membership lists in California; driver's license lists, random digit dialing, or Centers for Medicare and Medicaid Services lists, formerly known as the Health Care Finance Administration, for Utah; and driver's license or state identification lists in Minnesota. Controls were frequency matched to cases by sex and 5-year age group. These methods have been described in detail (28). Of all controls selected, 64% participated.

Data Collection. Trained interviewers collected diet and lifestyle data in person using laptop computers. Study quality control methods have been described (29, 30). The reference 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 Coronary Artery Risk Development in Young Adults diet history questionnaire (31). 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.

***TS, MTR*, and *RFC* genotyping.** Of 4,403 cases and controls with valid study data, 3,680 (84%) had blood collected primarily during the in-person interview, or during a clinical visit (83% of cases and 85% of controls). Genomic DNA was extracted using methods described in (refs. 14, 32). All samples were genotyped for two polymorphisms in the *TS* gene (*TSER*, 3′-UTR 1494delTTAAAG), *MTR* D919G, and *RFC* 80G>A. A total of 3,562 (97% of cases and 97% of controls with blood collected) had genotype information for both *TSER* and *3′-UTR 1494delTTAAAG*. 5′-Nuclease assays that had been previously used to genotype other polymorphisms in the folate pathway (*MTHFR* 677C>T, *MTHFR* 1298A>C, and *MTR* D919G) have been described (13, 26).

Both TS polymorphisms were analyzed using fluorescent size discrimination. For the analysis of the *TSER* 28-bp repeat polymorphism, a fragment containing the repeats was

amplified using the following primers: forward primer, 5′-6FAM-GTGGCTCCTGCGTTTCCCCC-3′; reverse primer, 5′-GGCTCCGAGCCGGCCACAGGCATGGCGCGG-3′(14). The PCR reactions contained 1× GeneAmp buffer (Applied Biosystems, Foster City, CA), 1.5 mmol/L $MgCl_2$, 200 μmol/L deoxyribonucleotide triphosphates, 100 nmol/L each primer, 10% DMSO, 1 unit AmpliTaq DNA polymerase (Applied Biosystems), and 100 ng of genomic DNA. Cycling conditions were one cycle of 94°C for 2 minutes; 35 cycles of 94°C for 30 seconds, 63°C for 30 seconds, and 72°C for 30 seconds; and a final extension at 72°C for 5 minutes. The amplified fragments were analyzed on an ABI 3100 genetic analyzer. A fragment containing the 3′-UTR deletion was amplified using the following primers: forward primer, 5′-6FAM-CAAATCTGAGGGAGCTGAGT-3′; reverse primer, 5′-CAGA-TAAGTGGCAGTACAGA-3′. The PCR reactions contained 1× GeneAmp buffer, 2 mmol/L $MgCl_2$, 150 μmol/L deoxynucleotide triphosphates, 300 nmol/L each primer, 1 unit AmpliTaq DNA polymerase, and 50 ng genomic DNA. Cycling conditions were one cycle of 94°C for 5 minutes; 30 cycles of 94°C for 30 seconds, 60°C for 45 seconds, and 72°C for 60 seconds; and a final extension at 72°C for 10 minutes. The amplified fragments were analyzed on an ABI 3100 genetic analyzer. For both *TS* polymorphisms, the correlation between fragment size and repeat number was confirmed by sequencing.

The 80G>A polymorphism in *RFC* was detected by allelic discrimination using the 5′ nuclease assay on a 7900HT sequence detection system (ABI). The 5′-nuclease genotyping assay was validated by genotyping 100 individuals by both 5′-nuclease assay and RFLP. There were no discrepancies between the two assays. Genotyping of the 80G>A polymorphism was done in 20-μL reactions containing 1× Taqman PCR core reagents (ABI), 3 mmol/L $MgCl_2$, 200 nmol/L each PCR primer (forward primer, 5′-AGCCCAGCGGTGGA-GAAG-3′ and reverse primer, 5′-AGCCGTAGAAGCAAAGG-TAGCA-3′), 150 nmol/L MGB probe 5′-VIC-TCCTGGC-GGCGCC-3′ (Applied Biosystems; G allele), 100 nmol/L MGB probe 5′-6-FAM-TGGCGGCACCTCG-3′ (A allele), 0.5 unit AmpliTaq Gold, 0.2 unit AmpErase UNG, and 5 ng genomic DNA. The amplification cycles were 50°C for 5 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Positive controls for all the genotypes as well as four negative controls were included in each plate. For quality control of all the polymorphisms, genotyping for 94 randomly selected samples was repeated. There were no discrepancies.

Statistical Methods. Logistic regression models were used to estimate associations in various ways. We stratified the data by sex and estimated the risk of colon cancer given a certain *TS, MTR*, or *RFC* genotype and examined risk estimates further stratified by other population characteristics (e.g., tumor site and age). The combined effects of *TSER* and *3′-UTR 1494delTTAAAG* were calculated using individuals who were homozygous for the common allele at both loci as the reference group. We assessed the joint interaction between genotype and level of nutrient intake by using those with low nutrient intake and homozygous for the most common (wild type) allele for *TSER, 3′-UTR, MTR*, or *RFC* as a common reference point. We also assessed gene-gene interactions in the folate pathway using the homozygous genotype for the most common allele as the reference. Similarly, the interaction between genotype and recent estrogen status in postmenopausal women was assessed using as the reference group no PMH use and wild-type *TS, MTR*, or *RFC* genotype.

Maximum likelihood estimates of population *TS* haplotype frequencies from unphased genotype data were obtained from an expectation maximization algorithm, assuming Hardy-Weinberg equilibrium, according to Excoffier and Slatkin (33) using SAS/Genetics software, 2002 (SAS/Genetics, Cary, NC).

Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated from unconditional logistic regression models. In these models, age at diagnosis or selection, body mass index reported for the reference period (kg/m^2), long-term vigorous leisure time physical activity, total energy intake, dietary fiber, dietary calcium, and number of cigarettes smoked per day on a regular basis were included as covariates to adjust for potential confounding. Haplotype-specific relative risks were assessed according to methods described in Stram et al. (34) using logistic regression software (SAS, release 8.2).

Separate analyses were done 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, and the risk of colon cancer were based on departure from additive risks using the relative risk due to interaction formulation of Hosmer and Lemeshow extended to more than two allelic combinations and/or environmental exposures (35). Interaction using a multiplicative scale was also examined. Interactions between

Table 1. Characteristics of the study population (n = 3,562)

	Cases (n = 1,600)	Controls (n = 1,962)	P*
Tumor site, *n* (%)			
Proximal	791 (49)		
Distal	771 (48)		
Unknown	38 (3)		
Age at diagnosis or selection (range, 30-79), y†	64.9 ± 9.8	65.0 ± 10.2	0.86
Sex, *n* (%)			
Men	897 (56)	1,036 (53)	
Women	703 (44)	926 (47)	0.05
Race/ethnicity, *n* (%)			
Non-Hispanic White	1,462 (91)	1,825 (93)	
Other	138 (9)	137 (7)	0.05
Recent PMH use, postmenopausal women‡, *n* (%)			
No	467 (77)	549 (69)	
Yes	143 (23)	242 (31)	<0.01
Kilocalories†			
Men	2,773 ± 1,217	2,638 ± 1,162	0.01
Women	2,046 ± 874	1,974 ± 832	0.09
TSER genotype, *n* (%)			
3/3 repeats	488 (30)	542 (28)	
3/2 repeats	764 (48)	983 (50)	
2/2 repeats	348 (22)	437 (22)	0.16
Frequency, 2 repeat allele§	0.46	0.48	0.15
TS 3′UTR 1494delTTAAAG genotype, *n* (%)			
ins/ins	720 (45)	881 (45)	
ins/del	690 (43)	866 (44)	
del/del	190 (12)	215 (11)	0.65
Frequency, del allele§	0.32	0.32	0.77
MTR D919G genotype, *n* (%)			
DD	1,015 (63)	1,264 (64)	
DG	529 (33)	608 (31)	
GG	56 (4)	90 (5)	0.15
Frequency, G allele§	0.20	0.20	0.97
RFC 80G>A genotype, *n* (%)			
GG	513 (32)	585 (30)	
GA	788 (49)	976 (50)	
AA	299 (19)	401 (20)	0.24
Frequency, A allele	0.43	0.45	0.10
TS haplotype, estimated (expected)‖,¶			
3 repeat/ins	0.30 (0.36)	0.28 (0.35)	
3 repeat/del	0.24 (0.17)	0.24 (0.17)	
2 repeat/ins	0.38 (0.31)	0.40 (0.33)	
2 repeat/del	0.08 (0.15)	0.08 (0.15)	0.56

*Based on χ^2 or t test.
†Mean ± SD.
‡Postmenopausal hormone use within 2 years of diagnosis/selection.
§Allele frequencies are reported for non-Hispanic Whites.
‖Maximum likelihood estimate (expected assuming linkage equilibrium).
¶$P < 0.01$ (χ^2 test of linkage disequilibrium for cases or controls, separately).

genotypes and PMH use in postmenopausal women were assessed using a Wald χ^2 test of the difference between slopes from the (assumed linear) change in ORs, keeping the wild-type *TS* genotype constant across the varying genotypes for the respective other *TS* polymorphism.

Results

Selected characteristics of the study population and *MTHFR* genotype frequencies by case or control status are presented in Table 1. The study participants were predominantly self-identified as non-Hispanic Caucasian (92%), with the remainder Hispanic (4%) and African American (4%). All genotypes were in Hardy-Weinberg equilibrium (assessed separately for cases and controls), and allele frequencies were consistent with previous reports (36). The *TS* polymorphisms were in linkage disequilibrium ($D' = 0.46$ among non-Hispanic Caucasians).

Table 2 describes the main associations seen with the polymorphisms, stratified by gender. Among men, *TSER* variant genotypes were associated with a significantly decreased risk (3rpt/2rpt: OR, 0.8; 95% CI, 0.6-0.98; 2rpt/2rpt: OR, 0.7; 95% CI, 0.6-0.98). No such risk reduction was observed among women. When combined genotypes (or diplotypes) for both *TS* polymorphisms were considered, almost all of the ORs for variant genotypes among both men and women were below 1.0, with statistically significantly reduced risks for women. Risk estimates for *TS* haplotypes, including variant alleles, compared with wild-type haplotype were not significantly different from 1.0 (data not shown).

Neither the *MTR* D919G nor *RFC* 80G>A polymorphism was associated with altered colon cancer risk among men or women (Table 2).

Folate metabolism involves circulation of folate metabolites through multiple cycles, as well as feedback mechanisms between these cycles (Fig. 1). Therefore, we evaluated gene-gene interactions between the polymorphisms investigated here, as well as those we have reported on previously (8, 13). ORs different from 1.0 were seen largely for stratifications of *TS, RFC*, or *MTR* by *MTHFR* 677C>T or 1298A>C genotypes, and these are presented in Table 3. Among men, reduced risks associated with variant *TS* genotypes (e.g., the presence of *TSER* 2rpt/2rpt or *TS* 3′-UTR deletion) were most pronounced for those with *MTHFR* TT genotypes. The *MTHFR* 1298CC genotype was associated with a decreased risk among women; however, this risk reduction seemed independent of TS genotypes. There was no evidence for interactions between *MTR* D919G or *RFC* 80G>A and *MTHFR* genotypes.

We also investigated whether risk estimates associated with polymorphisms in these folate-metabolizing enzymes differed by dietary intakes of folate, methionine, alcohol, or vitamins B6, B2, or B12. Consistent with our previous report on colorectal adenoma (37) among men, the *TSER* variant conferred a reduced risk in the presence of low folate intake (lowest tertile < 318 μg/d; *TS* 2rpt/2rpt or 2rpt/3rpt: OR, 0.7; 95% CI, 0.5-0.9 compared with wild-type 3rpt/3rpt). A similar risk reduction with the *TSER* variant genotypes was observed among men with low methionine intakes (<2.0 g/d): *TS* 2rpt/2rpt or 2rpt/3rpt (OR, 0.6; 95% CI, 0.4-0.9) compared with wild-type 3rpt/3rpt. However, none of these patterns was observed among women for the *TS* genotypes nor for *TS* haplotypes in either sex. There were no clear patterns or associations following stratification by vitamin B6 or B12 intake. No meaningful differences in risk were observed for *MTR* genotypes when stratified by nutrient intakes.

Among women in the lowest tertile of folate intake (≤273 μg/d), the *RFC* variant genotypes were associated with a decreased risk (wild-type GG: OR, 1.0; GA or AA: OR, 0.7; 95% CI, 0.5-1.0). Among women, we observed a significant gene-nutrient interaction in that only those with the GG genotype benefited from a diet higher in folate, whereas no difference in risk with variable folate intake was seen among those with the combined GA or AA genotypes ($P_{interaction} = 0.04$, multiplicative scale; $P = 0.01$, additive scale). This pattern was not seen among men.

Because of the observed differences in risk patterns among men and women and our past findings regarding an interaction between postmenopausal hormone use (PMH use) and

Table 2. Association between *TS, MTR*, and *RFC* genotypes and colon cancer

	Men			Women		
Genotype	Cases (*n*)	Controls (*n*)	OR (95% CI)	Cases (*n*)	Controls (*n*)	OR (95%CI)
TSER						
3rpt/3rpt	295	283	1.0 (reference)	190	254	1.0 (reference)
3rpt/2rpt	421	519	0.8 (0.6 to <1.0)	337	462	0.9 (0.7-1.2)
2rpt/2rpt	173	231	0.7 (0.6 to <1.0)	171	205	1.1 (0.8-1.5)
TS 3′-UTR 1494delTTAAAG						
ins/ins	387	468	1.0 (reference)	328	409	1.0 (reference)
ins/del	392	465	1.0 (0.8-1.2)	293	398	0.9 (0.7-1.1)
del/del	110	100	1.2 (0.9-1.7)	77	114	0.8 (0.6-1.2)
Combined *TSER* and *TS* 3′-UTR						
3rpt/3rpt						
ins/ins	77	82	1.0 (reference)	71	61	1.0 (reference)
ins/del or del/del	218	201	1.2 (0.8-1.7)	119	193	0.5 (0.3-0.8)
3rpt/2rpt or 2rpt/2rpt						
ins/ins	310	386	0.9 (0.6-1.2)	257	348	0.6 (0.4-0.9)
ins/del or del/del	284	364	0.8 (0.6-1.2)	251	319	0.7 (0.5 to <1.0)
MTR D919G						
DD	555	668	1.0 (reference)	458	600	1.0 (reference)
DG	308	319	1.1 (0.9-1.4)	220	288	1.0 (0.8-1.3)
GG	30	54	0.7 (0.4-1.1)	25	37	0.9 (0.5-1.5)
RFC 80G>A*						
GG	301	317	1.0 (reference)	210	267	1.0 (reference)
GA	425	511	0.8 (0.7-1.0)	361	468	1.0 (0.8-1.2)
AA	167	211	0.8 (0.6-1.1)	132	190	0.9 (0.6-1.1)

NOTE: Adjusted for age, body mass index, lifetime vigorous leisure activity, energy intake, dietary fiber, dietary calcium, usual number of cigarettes smoked, and other *TS* polymorphism where appropriate (*TSER, TS* 3′-UTR).

*Men and women combined: GG, OR = 1.0 (reference); GA, OR = 0.9 (95% CI, 0.8-1.0); AA, OR = 0.8 (95% CI, 0.7-1.0).

Table 3. Association between polymorphisms in the folate pathway and colon cancer

Genotype		Men			Women		
		Cases (*n*)	Controls (*n*)	OR (95% CI)	Cases (*n*)	Controls (*n*)	OR (95% CI)
TSER	MTHFR 677C>T						
3rpt/3rpt	CC or CT	266	242	1.0 (reference)	172	226	1.0 (reference)
	TT	28	41	0.6 (0.4-1.0)	18	28	0.9 (0.5-1.7)
3/2rpt or 2/2rpt	CC or CT	544	668	0.7 (0.6-0.9)	456	592	1.0 (0.8-1.3)
	TT	50	81	0.6 (0.4-0.9)	52	75	0.9 (0.6-1.4)
	MTHFR 1298A>C						
3rpt/3rpt	AA or AC	261	254	1.0 (reference)	175	219	1.0 (reference)
	CC	34	29	1.2 (0.7-2.0)	15	35	0.5 (0.3 to <1.0)
3/2 or 2/2rpt	AA or AC	535	676	0.8 (0.6 to <1.0)	463	589	1.0 (0.8-1.2)
	CC	59	74	0.8 (0.5-1.1)	45	78	0.7 (0.5-1.1)
TS 3′-UTR	*MTHFR* 677C>T						
ins/ins	CC or CT	347	418	1.0 (reference)	297	359	1.0 (reference)
	TT	40	49	1.0 (0.6-1.6)	31	50	0.8 (0.5-1.2)
Any deletion	CC or CT	463	492	1.1 (0.9-1.3)	331	459	0.9 (0.7-1.1)
	TT	38	73	0.6 (0.4-0.9)	39	53	0.9 (0.6-1.4)
	MTHFR 1298A>C						
ins/ins	AA or AC	346	420	1.0 (reference)	298	354	1.0 (reference)
	CC	41	48	1.0 (0.6-1.5)	30	55	0.6 (0.4-1.0)
Any deletion	AA or AC	450	510	1.0 (0.8-1.2)	340	454	0.9 (0.7-1.1)
	CC	52	55	1.1 (0.7-1.7)	30	58	0.6 (0.4 to <1.0)
MTR D919G	*MTHFR* 677C>T						
DD	CC or CT	507	583	1.0 (reference)	402	533	1.0 (reference)
	TT	47	83	0.7 (0.5 to <1.0)	56	67	1.1 (0.8-1.6)
DG or GG	CC or CT	307	333	1.0 (0.9-1.3)	230	289	1.1 (0.9-1.3)
	TT	31	40	0.9 (0.6-1.5)	15	36	0.6 (0.3-1.1)
	MTHFR 1298A>C						
DD	AA or AC	495	597	1.0 (reference)	417	523	1.0 (reference)
	CC	60	70	1.0 (0.7-1.5)	41	77	0.7 (0.4 to <1.0)
DG or GG	AA or AC	304	340	1.1 (0.9-1.3)	226	289	1.0 (0.8-1.2)
	CC	34	33	1.2 (0.7-2.0)	19	36	0.7 (0.4-1.2)
RFC 80G>A	*MTHFR* 677C>T						
GG	CC or CT	274	279	1.0 (reference)	194	236	1.0 (reference)
	TT	27	38	0.7 (0.4-1.1)	16	31	0.7 (0.3-1.2)
GA or AA	CC or CT	540	636	0.8 (0.7-1.0)	438	586	0.9 (0.7-1.1)
	TT	51	85	0.6 (0.4-0.9)	55	72	0.9 (0.6-1.4)
	MTHFR 1298A>C						
GG	AA or AC	267	283	1.0 (reference)	194	236	1.0 (reference)
	CC	34	34	1.1 (0.7-1.9)	16	31	0.6 (0.3-1.1)
GA or AA	AA or AC	532	653	0.8 (0.7 to >1.0)	449	576	0.9 (0.7-1.2)
	CC	60	69	0.9 (0.6-1.3)	44	82	0.6 (0.4 to <1.0)

NOTE: Adjusted for age, BMI, lifetime vigorous leisure activity, energy intake, dietary fiber, dietary calcium, usual number of cigarettes smoked, and other *TS* polymorphisms where appropriate (*TSER, TS*3′UTR). $P_{interaction}$ values of gene-gene associations were not statistically significant on an additive or multiplicative scale (data not shown).

MTHFR genotype, we investigated whether risk estimates of *TS, MTR*, or *RFC* genotypes differed by PMH use. Among PMH users, the variant *TS* genotypes were associated with substantially reduced risk of colon cancer, whereas much weaker associations were observed among non-PMH users (Table 4). No such interactions were observed for *MTR* or *RFC* (data not shown).

Discussion

Within this large population-based study of colon cancer, we investigated polymorphisms in three folate-metabolizing enzymes (TS, MTR, and MTHFR), as well as the relevant carrier protein (RFC), thus addressing genetic variability in multiple key proteins in this biological pathway. There is strong evidence for the functional effect of *MTHFR* 677C>T and *TSER* variant genotypes (14, 38-41), with some, yet less well defined, evidence for the *in vivo* functional relevance of *MTR, RFC*, and the *TS* 3′-UTR variant (16-18, 20-23, 42). Our evaluations of colon cancer risk confirm this assessment, in that there were no significant alterations in risk for *MTR, RFC*, and *TS* polymorphisms, with the exception of a risk reduction associated with the *TSER* variants among men. The ORs for *TSER* are comparable with those from a previous report by Chen et al. (43) among male physicians (OR, 0.9; 95% CI, 0.6-1.3 for the 2rpt/3rpt genotype and OR, 0.6; 95% CI, 0.4-0.98 for the 2rpt/2rpt genotype). A statistically significant trend towards reduced risk with the variant *TSER* alleles was observed both here and in that population (43). These results indicate that the variant *TSER* 2rpt/2rpt genotype reduces risk of colon or colorectal cancer among men to a degree comparable with that of the *MTHFR* 677TT genotype (44). As risk reductions for *TSER* variants were only seen among women who are on postmenopausal hormones but not among women overall, estrogen status may play a role.

Further complexity is added as a result of the presence of a second functionally relevant polymorphism in *TS* (15, 16). We evaluated the combined effects of these genotypes as well as haplotypes to discern possible risk patterns. The combined *TS* wild type/wild type genotype constitutes only 8.3% of our population. When compared with this wild type/wild type reference group of putatively highest *TS* expression and *TS* mRNA stability, the variant *TSER* genotypes were associated with statistically significantly reduced risk among women (OR, 0.6; 95% CI, 0.4-0.9) yet not among men. Our sample sizes for these sex-specific associations were limited, and results should be followed up in large study populations that have the ability to investigate combined genotypes as well as sex-specific ORs.

Table 4. Association among combined *TS* genotype, postmenopausal hormone (PMH) use, and colon cancer in postmenopausal women

	TS 3′-UTR 1494delTTAAAG genotype					
	ins/ins			ins/del and del/del		
TSER genotype	Cases (*n*)	Controls (*n*)	OR (995% CI)	Cases (*n*)	Controls (*n*)	OR (95% CI)
No, PMH						
3rpt/3rpt	41	42	1.0 (reference)	80	113	0.7 (0.4-1.2)
3/2 or 2/2rpt	178	208	0.9 (0.5-1.4)	164	182	0.9 (0.5-1.4)
Yes, PMH						
3rpt/3rpt	21	9	2.2 (0.9-5.4)	21	56	0.4 (0.2-0.7)
3/2 or 2/2rpt	48	89	0.5 (0.3 to <1.0)	53	87	0.6 (0.3 to > 1.0)

NOTE: Adjusted for age, body mass index, lifetime vigorous activity, energy intake, dietary fiber, dietary calcium, and usual number of cigarettes smoked. $P < 0.01$ (Wald χ^2 test of slopes) for *TSER* 3rpt/3rpt genotype across 3′-UTR genotypes. $P = 0.03$ (Wald χ^2 test of slopes) for 3′-UTR insertion/insertion genotype across *TSER* genotypes.

The presence of two common functional variants within *TS* suggests that it is essential to take both of these into account simultaneously.

The *MTR* polymorphism is less common (allele frequency = 0.20) and has been investigated in three epidemiologic studies, including a large Norwegian cohort (7, 25, 45). Similar to our findings, Le Marchand et al. (45) and Chen et al. (7) did not report any associations between this variant and colon cancer risk, whereas Ulvik et al. (25) observed a significantly reduced risk among those with a GG genotype compared with wild-type AA (OR, 0.65; 95% CI, 0.47-0.90). We observed reduced risks among men (OR, 0.7), but the 95% CI included 1.0. As only about 5% of the population have the homozygous variant genotype, very large studies are needed to quantify the strength of this association.

For gene-gene interactions, only combinations with the *MTHFR* polymorphisms showed interesting patterns. This is not surprising, because MTHFR is a key regulatory enzyme in folate-mediated one-carbon metabolism, the activity of which determines the distribution of folate metabolites toward nucleotide synthesis or methylation reactions. There is strong evidence that the *MTHFR* 677C>T variant alters the balance of metabolites within the pathway (39, 46). In combined analyses of *TS* and *MTHFR* polymorphisms, we observed that men carrying at least one variant *TS* allele (either *TSER* 2rpt or *TS* 1494del) in addition to the *MTHFR* 677TT genotype were at relatively lowest risk compared with all other groups (both OR, 0.6; 95% CI, 0.4-0.9). This confirms our previous observation in colorectal adenoma, where individuals with low TS expression and low MTHFR activity genotypes also experienced the lowest adenoma risk (OR, 0.56; ref. 10). If this statistical interaction reflects biological mechanisms, then we may hypothesize that the observed pattern suggests that a greater diversion of folate metabolites (specifically 5,10-methylene-tetrahydrofolate) toward purine synthesis is protective for the development of colorectal neoplasia. Recent findings by Quinlivan et al. suggest that folate depletion adversely affects purine synthesis in humans and a greater relative rate of adenine synthesis among individuals with the *MTHFR* TT genotype (46). Depurination is the most common type of DNA damage with ~10,000 depurinations/cell/d (47, 48). Although efficiently repaired, apurinic sites are present in DNA. We recognize that one other study did not observe this risk pattern, but their sample size was limited to 270 cases, with consequent restricted power for studying gene-gene interactions (43).

Our investigations of gene-diet interactions confirmed, to some extent, associations we have previously observed with respect to *TSER*, and folate intake that reduced *TS* expression (*TSER* 2rpt/2rpt) is associated with a reduced risk in the presence of a low folate intake (10). However, this pattern was seen only among men and also has not been observed in the Health Professionals study (43). Again, if that pattern reflects a biological mechanism, it would point toward purine synthesis as a key link between one-carbon metabolism and colorectal neoplasia. We were unable to confirm previously observed gene-diet interactions for *MTR* in colorectal adenoma (26) and did not see a clear pattern for *RFC*-diet interactions. However, the RFC is the transporter for naturally occurring folates (in the form of 5-methyl-tetrahydrofolate) but plays a smaller role in the transport of folic acid (49). Thus, in populations, such as the one described here, where folate intake from supplements in the form of folic acid comprises a substantial proportion of the overall folate intake, genetic variability in the RFC may not be as relevant for the overall supply of folate metabolites. Unfortunately, no quantitative information on supplement use was available for this population.

Lastly, we observed differences in risk patterns dependent on the past use of postmenopausal hormones. Interactions between folate metabolism and PMH use are not implausible, as there are links between homocysteine concentrations and PMH use (50-53), and methylation of the estrogen receptor is an early event in colorectal carcinogenesis, which may less frequently occur in the presence of PMH (27, 54). We have previously reported on a significant difference in risk patterns of PMH-associated risks by *MTHFR* genotypes (13). However, these interactions need to be confirmed by others, because sample sizes were in parts insufficient to yield stable estimates.

Although this study is quite comprehensive with respect to investigations of genetic variability in one-carbon metabolism and risk of colon cancer, there are three important limitations. First, our investigations did not include other genetic polymorphisms in folate-metabolizing enzymes that may be of possible relevance, such as methionine-synthase reductase (MTRR) or serine-hydroxymethyltransferase (SHMT). Thus far, *MTRR* does not seem related to colon cancer risk (45), and the functional relevance of the *cSHMT* polymorphisms is unclear. The study presented here focused on candidate polymorphisms in key enzymes with substantial evidence for functional effect; we hope to expand our investigations to other relevant candidate polymorphisms as they are reported.

Second, there is now strong evidence that a subset of colorectal cancer cases arises as part of a CpG island methylator phenotype (55, 56). Information on CpG island methylator phenotype status should be taken into account in future studies investigating links between genetic variability in folate metabolism and risk of colorectal cancer.

A final limitation is our current inability to integrate knowledge of biochemical relationships within the pathway into the statistical analysis. Although an approach that uses stratification for gene-nutrient or gene-gene interactions is valuable, in that it allows for an empirical investigation of the associations, it is also limited in that statistical power for higher-order interactions is lacking, even within this large

study population. Because folate metabolism consists of several interconnected cycles (see Fig. 1), such interactions are to be expected. Our approach toward solving this problem is to use, in the future, results from a mathematical model of one-carbon biochemistry for investigations of multiple genetic variants on selected biomarkers. Although this model is still under development, preliminary results show that it replicates the biochemical relationships in the folate cycle and methionine cycle with reasonable accuracy (57, 58). Furthermore, our group and others are developing methods to address this key problem for molecular epidemiologic studies (59). Thus, we hope that in the future, we will be able to achieve closer integration of the biochemistry and statistical analysis. Because there is strong evidence that disturbances in this biochemical pathway can modify risk of several types of malignancies (60-63), birth outcomes (64-66), and possibly cardiovascular disease (67) and autism (68), a more thorough understanding of the interplay of multiple genetic polymorphisms under specific dietary conditions and their combined effect on biomarkers and disease end points will be highly relevant.

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