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Thymidylate Synthase Promoter Polymorphism, Interaction with Folate Intake, and Risk of Colorectal Adenomas

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Abstract

Thymidylate synthase (TS) is a key enzyme in folate metabolism and the primary target of 5-fluorouracil. A repeat polymorphism in the *TS* promoter enhancer region (2rpt *versus* 3rpt of 28 bp) is associated with decreased expression, and a 6-bp deletion in the 3′untranslated region may affect RNA stability. We investigated the role of *TS* polymorphisms in a case control study of adenomatous polyps (510 cases and 604 polyp-free controls). Multivariate-adjusted odds ratios (ORs; 95% confidence interval) for *TSER* 2rpt/3rpt and 2rpt/2rpt compared with 3rpt/3rpt were 0.8 (0.6–1.2) and 0.9 (0.6–1.3), respectively. We observed a significant gene-nutrient interaction between the *TSER* polymorphism and folate intake: among 3rpt/3rpt individuals (greater expression), folate intake > 440 μg/day (highest tertile) *versus* ≤440 μg/day was associated with a 2-fold decreased risk [ORs 1.0 (reference group) *versus* 0.5 (0.3–0.9)]. However, among 2rpt/2rpt individuals, high folate intake was associated with a 1.5-fold increased risk [ORs 0.6 (0.4–0.9) *versus* 0.9 (0.5–1.5; *P* for interaction = 0.03)]. Vitamin B_{12} showed a similar trend (*P* = 0.08). No clear pattern was seen with the *TS* 1494del6 polymorphism. These findings raise questions regarding the molecular pathways linking folate metabolism and colorectal carcinogenesis, including whether high folate is beneficial in the presence of all metabolic genotypes.

Introduction

TS[2] is a key enzyme in folate metabolism and catalyzes the conversion of dUMP to dTMP. This conversion is essential for the provision of thymidine, a nucleotide needed for DNA synthesis and repair. TS is also a target for major chemotherapeutic drugs, including 5-fluorouracil. The substrate for TS, 5,10-methylene-THF, is a central metabolite in folate metabolism and is diverted into different pathways, including thymidine synthesis, purine synthesis, and toward the provision of methyl groups for DNA methylation. Colon cells are subject to rapid turnover and, thus, are the site of high rates of DNA synthesis. Furthermore, DNA repair is essential in the colonic environment, and DNA repair mechanisms can be affected by nucleotide availability (1–3). Folate deficiency has been shown to increase several measures of DNA damage (4, 5), and a low folate status is consistently associated with an increased risk of colorectal cancer (6, 7). The human *TS* promoter region includes several mechanisms for gene regulation, including a *cis*-acting enhancer element (8). This *TSER* is polymorphic, containing two or three 28-bp tandem repeats among Caucasian and Asian populations (9, 10). The less common double repeat is associated with 2.6-fold lower TS expression in HeLaS3 cells than the triple repeat (9), and *in vivo* TS mRNA levels in tumor tissue were 3.6-fold lower among 2rpt/2rpt individuals compared with 3rpt/3rpt individuals (11). We also reported recently on a second *TS* polymorphism, a 6-bp deletion at bp 1494 in the 3′UTR that may be associated with decreased mRNA levels in colorectal tumors (12, 13). We hypothesized that a change in *TS* expression may affect colon carcinogenesis and investigated the risk of colorectal adenomas associated with the two *TS* polymorphisms in a large case control study of colonoscopically screened men and women.

Materials and Methods

Study Subjects. Subject recruitment for the Minnesota Cancer Prevention Research Unit case control study has been described previously (14). Briefly, cases and controls were recruited through a multiclinic private gastroenterology practice in metropolitan Minneapolis, Digestive Healthcare. Patients (age 30–74 years) who were scheduled for colonoscopy between April 1991 and April 1994 were screened for eligibility and recruited prior to colonoscopy. Recruitment at all 10 sites was initiated at the time of scheduling with the intention of recruiting subjects with both patient and recruiter blind to the final diagnosis. The study protocol was approved by the internal review boards of the University of Minnesota, and each DH endoscopy site and written informed consent was given by each study participant.

Eligibility criteria for both cases and controls were: (*a*) resident of Twin cities metropolitan area; (*b*) age 30–74 years; (*c*) English speaking; (*d*) no known genetic syndrome associated with colonic neoplasia; and (*e*) no history of cancer (except nonmelanoma skin cancer), adenomatous polyps, or inflammatory bowel disease. Indications for colonoscopy have been published previously (15), and 68% of all colonoscoped patients participated.

At the colonoscopy visit, questionnaires were collected, blood was drawn, and the colonoscopy findings were recorded. Only participants with a complete colonoscopy reaching the cecum were eligible. All polyps were removed and evaluated histologically. Patients with polyps showing invasive carcinoma were not included. Adenomatous polyp cases are those found to have ≥1 adenomatous polyp; controls were polyp free at colonoscopy.

Data Collection. Data collection included information on dietary intake, physical activity, smoking habits, anthropometric measurements, medical information, demographic information, and reproductive history. When data were incomplete, study staff followed up by phone. The dietary questionnaire was an adaptation of the Willett food-frequency questionnaire, which has been studied previously for validity and repeatability. Furthermore, folate values derived from this questionnaire correlated with RBC folate levels (an indicator of long-term folate status $r = 0.56$).

Genotyping. Genomic DNA from lymphocytes was extracted using the PureGene kit (Gentra Systems, Inc., Minneapolis, MN). For the analysis of the *TSER* 28-bp repeat polymorphism, a fragment containing the repeats was amplified using primers TS forward primer: 5′-GTGGCTCCTGCGTTTC-CCCC-3′ and TS reverse primer: 5′-GGCTCCGAGCCGGCCACAGGCATG-GCGCGG-3′ (9). The PCR reactions contained 1 × GeneAmp buffer (Applied Biosystems, Foster City, CA), 1.5 mM $MgCl_2$, 200 μM deoxyribonucleotide triphosphates, 100 nM each primer, 10% DMSO, 1 unit of Amplitaq DNA polymerase (Applied Biosystems), and 100 ng of genomic DNA. Cycling conditions were one cycle of 94°C for 2 min, 35 cycles of 94°C for 30 s, 63°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. The

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[2] The abbreviations used are: TS, thymidylate synthase; THF, tetrahydrofolate; MTHFR, 5,10-methylenetetrahydrofolate reductase; TSER, thymidylate synthase enhancer region; OR, odds ratio; UTR, untranslated region.

Table 1 *Selected characteristics of the study population in the Minnesota Cancer Prevention Research Unit Polyp Study*

Characteristic	Adenomatous polyps (n = 523)	Controls (n = 625)
Age[a] (years)	58.0 ± 9.7 (31–74)	52.9 ± 11.0 (30–74)
Sex		
Men	62%	38%
Women	38%	62%
Race/ethnicity		
Caucasian	98%	97%
Other[b]	2%	3%
Thymidylate synthase genotypes		
TSER polymorphism		
3rpt/3rpt	30%	26%
3rpt/2rpt	49%	52%
2rpt/2rpt (decreased expression)	21%	21%
2rpt/4rpt		0.3%[c]
3′UTR polymorphism		
6bp/6bp	45%	50%
6bp/0bp	44%	40%
0bp/0bp	11%	10%

[a] Mean ± SD (range).
[b] Not included in further analyses.
[c] Not Caucasian, not included in further analyses.

amplified fragments were separated on a 3% NuSieve agarose gel (Biowhittacker, Rockland, ME). The fragment containing three and two repeats were 243 and 215 bp, respectively.

The 3′UTR polymorphism was analyzed by RFLP as described (12). Briefly, a fragment containing the 6-bp deletion was amplified using primers 5′-CAAATCTGAGGGAGCTGAGT-3′ and 5′-CAGATAAGTGGCAGTACAGA-3′ in a reaction containing 1 × GeneAmp buffer, 2.5 mM MgCl2, 150 mM deoxyribonucleotide triphosphates, 300 nM each primer, 1 unit of AmpliTaq DNA polymerase, and 100 ng of genomic DNA. The cycling conditions were 1 cycle of 94°C for 5 min, 30 cycles of 94°C for 30 s, 58°C for 45 s, and 72°C for 45 s, and a final extension at 72°C for 5 min. The amplified fragments were digested with *DraI*, and the products were separated on a 3% NuSieve agarose gel. The expected fragment sizes were 70 and 88 bp for the wild-type allele and 152 bp for the variant allele.

Statistical Data Analysis. Unconditional logistic regression models were used to obtain maximum likelihood estimates, ORs, and 95% confidence intervals. Multivariate adjustment included age, sex, body mass index (kg/m^2), percentage of calories from fat, hormone replacement therapy (ever/never), and pack-years of smoking. These variables altered some risk estimates by >10%. In general, confounding effects were small and often only apparent in stratified analyses; nonetheless, a consistent multivariate adjustment was maintained throughout all analyses presented. Other factors evaluated but not found to be confounders were waist:hip ratio, regular use of aspirin or nonsteroidal anti-inflammatory drugs (at least once/week *versus* never), hours of physical activity, and the dietary intake variables kcal, dietary fiber, vitamin B_6, vitamin B_{12}, and alcohol. The MTHFR C677T polymorphism was also not found to confound the associations.

Effect modification of the relation between nutrient intakes and risk of adenomas by genotype was evaluated by testing for different slopes with increasing nutrient intake across genotypes. Because the allele frequency of the TSER polymorphism is known to vary by race (10), we restricted the study population to Caucasians (who comprised 97% of the original population) for a sample size of 510 cases and 604 controls. All tests of statistical significance were two sided. SAS, version 8.2 (SAS Institute Inc., Cary, NC), was used for the analyses.

Results

The study population and their dietary intakes have been described previously (15), and selected characteristics are presented in Table 1. The allele frequency of the *TSER* polymorphism among the controls was 0.48 with 52% heterozygotes (2rpt/3rpt) and 21% homozygous variant (2rpt/2rpt). The allele frequency of the *TS* 1494 6-bp deletion was 0.30 with 40% heterozygotes (6 bp/0 bp) and 10% homozygous variant (0 bp/0 bp). The two polymorphisms were in linkage disequilibrium: among those with a TSER 3rpt/3rpt genotype, 29% were found to have the TS 6 bp/6 bp genotype and 26% the 0 bp/0 bp genotype; among individuals with a TSER 2rpt/2rpt genotype, these proportions were 73 and 3%, respectively ($P < 0.001$). However, all genotype combinations were observed. Both variants were in Hardy-Weinberg equilibrium.

TSER Polymorphism. Multivariate adjusted ORs (95% confidence interval) for 2rpt/3rpt and 2rpt/2rpt compared with 3rpt/3rpt were 0.8 (0.6–1.2) and 0.9 (0.6–1.3), respectively. Risk estimates were similar among men and women and among individuals either greater than age 60 or younger. As shown in Table 2 and Fig. 1, we observed a statistically significant gene-nutrient interaction with dietary folate intake: among individuals with the 3rpt/3rpt genotype, folate intake > 440 μg/day (highest tertile) *versus* ≤440 μg/day was associated with a 2-fold decreased risk [ORs 1 (ref; low intake) *versus* 0.5 (0.3–0.9; high intake)]. However, among individuals with the 2rpt/2rpt variant, a 1.5-fold increased risk associated with high folate intake was observed [ORs 0.6 (0.4–0.9) *versus* 0.9 (0.5–1.5); P for interaction = 0.03]. A similar trend was observed for vitamin B_{12} intake: among individuals with the 3rpt/3rpt and 2rpt/3rpt genotypes, risk was reduced 2-fold with intakes in the highest tertile (≥9.78 μg/day, compared with <9.78 μg/day), whereas a slight increase in risk (1.3-fold) with intakes ≥ 9.78 μg/day was observed among individuals with the 2rpt/2rpt genotype (P for interaction = 0.08). Folate intake did not confound this association, nor did vitamin B_{12} confound the folate/*TS* association. The associations with the *TS* polymorphism did not differ by intakes of vitamin B_6, methionine, or alcohol (data not shown).

Because we have shown previously that the *MTHFR* C677T polymorphism affects risk of adenomas in this population (15), we stratified the analysis further by *MTHFR* genotype (Table 3). Although the cell sizes are small, and the results should be considered exploratory, some trends are suggested: (*a*) the decrease in risk associated with high folate intakes was again confined to individuals with greater *TS* expression (3rpt/3rpt genotype), irrespective of *MTHFR* genotype; and (*b*) the previously reported increase in adenoma risk associated with low folate intake among individuals with the *MTHFR* TT genotype (15, 16) was only seen among those with the 3rpt/3rpt genotype (>3-fold). Lower *TS* expression (2rpt/3rpt or 2rpt/2rpt genotypes)

Table 2 *Association between the TSER polymorphism and adenomatous polyps—stratified by dietary intakes of folate and vitamin B_{12}*[a]

Nutrient intakes from both diet and supplements	(n cases/n controls)	*TSER* genotype			P for interaction[b]
		3rpt/3rpt	3rpt/2rpt	2rpt/2rpt	
Folate					
Low/medium	347/381	1.0 (ref)	0.70 (0.48–1.02)	0.58 (0.36–0.94)	
High	158/207	0.53 (0.31–0.91)	0.63 (0.40–0.99)	0.88 (0.51–1.53)	P = 0.03
Vitamin B_{12}					
Low/medium	354/382	1.0 (ref)	0.75 (0.52–1.10)	0.62 (0.39–1.00)	
High	151/206	0.52 (0.31–0.90)	0.51 (0.33–0.81)	0.82 (0.45–1.49)	P = 0.08

[a] Multivariate adjustment for age, sex, body mass index, hormone replacement therapy (y/n), percentage of kcal from fat, and pack-years of smoking.
[b] Testing for different slopes associated with nutrient intake across genotypes. Cutpoints for dietary intakes: folate (440 μg) and vitamin B_{12} (9.78 μg).

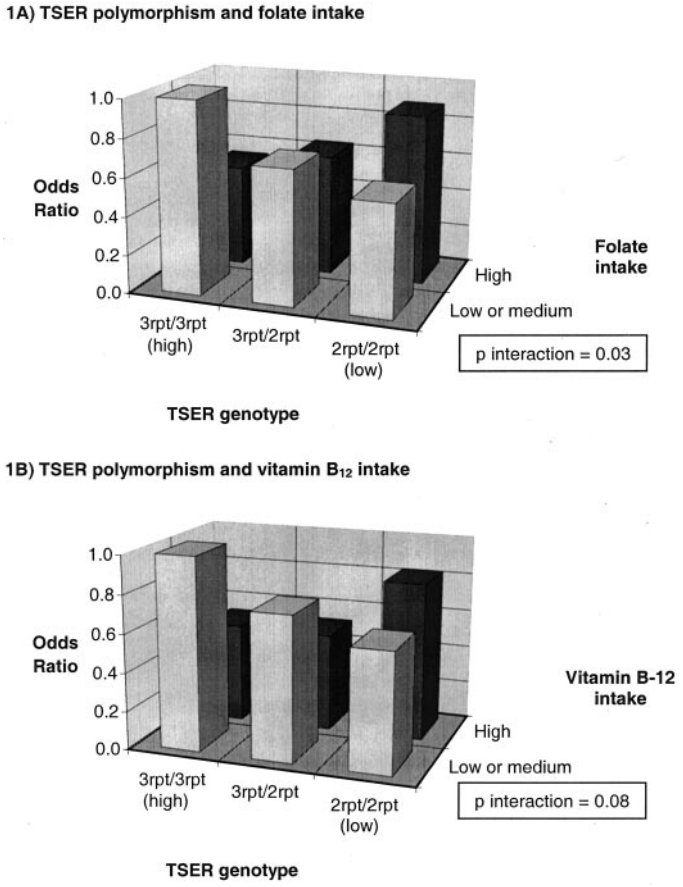

Fig. 1. Risk of colorectal polyps associated with the TSER polymorphism, stratified by folate (*A*) or vitamin B-12 (*B*) intake.

appeared to be inversely associated with risk under conditions of low-folate intakes, most prominently so among individuals with the *MTHFR* TT genotype. When folate intakes were not considered, the combination of a TSER 2rpt/2rpt genotype and MTHFR TT genotype was the lowest risk group compared with all other genotype combinations (data not shown).

TS 1494del6 Polymorphism. The *TS* 6-bp deletion polymorphism at bp 1494 in the 3′UTR was not associated significantly with risk of colorectal adenomas [compared with 6bp/6bp wild-type OR = 1 (reference): 6bp/0bp OR = 1.22 (0.93–1.61); 0 bp/0 bp OR = 1.13 (0.73–1.74)]. When stratified by dietary intakes of folate or vitamin B_{12}, or by MTHFR genotype, no consistent patterns were apparent (data not shown). Although one might expect results similar to those for the *TSER* polymorphism, because of linkage between the two polymorphisms, these findings illustrate that "imperfect" disequilibrium may result in different associations with disease phenotypes. Adjustment for the *TSER* polymorphism did not modify the associations. We investigated the risk of adenomas associated with all possible *TSER/TS* 1494del6 genotype combinations, and none of the risks was statistically different from 1 (with a *TSER* 3rpt/3rpt *TS* 6bp/6bp genotype as referent; data not shown).

Discussion

To our knowledge, this is the first study investigating the relevance of TS polymorphisms in colorectal carcinogenesis. As with other polymorphisms in folate metabolism (15, 17), upon stratification by a relevant exposure (folate), an informative pattern emerged. We observed a statistically significant gene environment interaction, with a decreased adenoma risk associated with high folate intakes among individuals with the *TSER* wild-type (3rpt/3rpt) genotype and the opposite association (increased risk with high folate intake) among individuals with lower *TS* expression (2rpt/2rpt). Similar trends were observed for vitamin B-12 intake, which is an essential cofactor for another key enzyme in folate metabolism, methionine synthase.

Under low or medium folate intakes and in the presence of a *MTHFR* TT genotype, which can be considered to further impair folate status, the *TSER* variant genotypes were associated with a decreased risk of colorectal adenoma. Thus, we hypothesize that the lower *TS* expression (2rpt/2rpt or 3rpt/2rpt genotypes) may have a "folate-sparing" effect that reduces risk of adenoma.

The finding of a decreased risk associated with lower *TS* expression in the presence of low-folate intake (Fig. 1) was unexpected. Previously, several groups have hypothesized that the inverse association between the C677T MTHFR polymorphism and colorectal carcinogenesis may be attributable to a greater diversion of the relevant substrate (5,10-methylene-THF toward nucleotide synthesis; TS and several enzymes involved in purine synthesis; Refs. 15, 17, and 18). TS converts dUMP to dTMP, and it has been shown that folate deficiency can lead to uracil misincorporation associated with an increased risk of double-strand breaks (4, 5). Our findings, however, suggest that the purine synthesis pathway may be a relevant mechanism linking folate metabolism to colorectal carcinogenesis.

Two potential explanations could reconcile our findings with the existing knowledge about this pathway: (*a*) it is likely that, within the folate pathway, a balance exists between the provision of 5,10-methylene-THF for thymidine synthesis, purine synthesis, and the provision of methyl groups for methylation reactions. On the basis of biochemical models in pig liver, the cellular concentration of 5,10-methylene-THF regulates the flux of this metabolite into the pathways leading to nucleotide synthesis and methionine regeneration (19). It is unclear whether variations in enzyme function could affect these regulatory mechanisms; and (*b*) it may be that an adequate provision of 5,10-methylene-THF for purine synthesis is key. Depurination is the most common form of spontaneous DNA damage, with an estimated 10,000 depurinations/cell/day (20, 21). Although an abundance of apurinic endonucleases efficiently repairs this damage, quantitative measurements show that abasic sites are present in cellular DNA, with a steady-state level of 5–10,000 lesions/cell/day (21). Thus, depurination may be the major challenge the body faces on a continuous basis, and the provision of an adequate supply of purines may be an important prerequisite for repair. Accordingly, in the presence of low folate, it may be that purine synthesis is the most protected pathway.

Perturbations of nucleotide pools attributable to folate deficiency have been associated with inhibited DNA repair and increased mutation rates (1, 2). However, the role of purine depletion in mutagenesis

Table 3 *Association between TS genotypes and adenomatous polyps—stratified by MTHFR C677T genotypes and folate intake*[a]

		TSER polymorphism	
(*n* cases/*n* controls)		3rpt/3rpt	3rpt/2rpt or 2rpt/2rpt
MTHFR C677T polymorphism			
CC			
Low/medium folate	168/182	1.81 (1.07–3.06)	1.0 (ref)
High folate	76/95	0.91 (0.49–1.71)	1.17 (0.71–1.93)
CT			
Low/medium folate	145/160	1.15 (0.65–2.02)	1.03 (0.69–1.53)
High folate	62/81	0.74 (0.32–1.68)	1.14 (0.70–1.87)
TT			
Low/medium folate	31/38	2.22 (0.85–5.84)	0.88 (0.43–1.80)
High folate	19/30	0.60 (0.14–2.50)	0.71 (0.33–1.50)

[a] Multivariate adjustment for age, sex, body mass index, hormone replacement therapy (y/n), percentage of kcal from fat, and pack-years of smoking. Cutpoints for dietary intakes: folate (440 μg), vitamin B_{12} (9.78 μg), and vitamin B_6 (3.17 mg).

is unclear. There is no evidence that folate deficiency results in a greater prevalence of apurinic sites, but, to our knowledge, this has not yet been investigated. An effect of folate deficiency on purine pools has been documented in one study (22). The most promising evidence in support of a link between folate deficiency, purine synthesis, and DNA damage stems from investigations in an *in vitro* model of folate-deficient erythropiesis, indicating that decreased *de novo* synthesis of purines plays an equally important role as decreased *de novo* synthesis of thymidylate in the pathogenesis of megaloblastic anemia (23).

Our study had several limitations. The study population was comprised of individuals undergoing screening by colonoscopy and was thus not necessarily representative of the general population. The major advantage of this clinic-based approach, nonetheless, is the unambiguous distinction between patients with adenomas and polyp-free controls. The indications for colonoscopy were not related to the *TS* genotype or to intakes of the nutrients investigated. Thus, a bias attributable to differences in indication for colonoscopy is unlikely. A strength of the study is the relatively large study size, permitting us to investigate gene-nutrient interactions. However, for analyses investigating genotype combinations (*MTHFR* and *TS*) in conjunction with nutritional status, our statistical power was limited.

In summary, previous research on *MTHFR* polymorphisms has illustrated the importance of genetic variability in the folate pathway in colorectal carcinogenesis. We here extend these findings to a common polymorphism in TS, which modifies risk depending on folate status (and possibly vitamin B-12 status). Our findings raise questions regarding the molecular pathways linking folate metabolism to colorectal carcinogenesis, especially including whether folate is beneficial in the presence of all metabolic genotypes. It may also have implications for the pharmacogenetics of antifolate drugs, such as 5-fluorouracil.

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