Polymorphism in the Thymidylate Synthase Promoter Enhancer Region Modifies the Risk and Survival of Colorectal Cancer[1]

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Abstract

***Thymidylate synthase* (*TS*) converts dUMP to dTMP, the rate-limiting nucleotide in DNA synthesis. It is also the target for 5-flurorouracil, the most common chemotherapy agent for treatment of colorectal cancer (CRC). We designed a nested case-control study within the prospective Physicians' Health Study to investigate whether *TS* polymorphisms independently predict risk of CRC and simultaneously the overall survival after the disease in the same population. We also investigated influences of this polymorphism on plasma folate and homocysteine levels. The study consists of 270 incident CRC and 454 control subjects. Risk of CRC was estimated by use of conditional multiple logistic regression analysis. Survival was analyzed by Cox proportional hazards regression analysis. Compared with the *TS 3R/3R* genotype, the multivariate-adjusted risk ratio was 0.86 (0.59–1.25) for the *2R/3R* genotype and 0.59 (0.36–0.98) for the *2R/2R* genotype with *P* for trend of 0.03. The *TS 2R/2R* genotype was also associated with better survival, although the results were not significant. Compared with those with either the *3R/3R* or *2R/3R* genotypes, the age-adjusted hazard ratio for the *2R/2R* genotype was 0.57 (0.30–1.07). Individuals with the *2R/2R* genotype had significantly lower plasma folate levels than those with the *3R/3R* genotype, whereas their plasma homocysteine levels were unaffected by the *TS* promoter polymorphism. The deletion polymorphism at the *TS* 3′-untranslated region did not influence the CRC risk and survival, nor did it modify the plasma folate and total homocysteine levels. Given that individuals with high plasma folate had a better survival outcome with a hazard ratio of 0.68 (0.45–1.03) compared with those with low plasma folate, we conclude that the *TS* promoter polymorphism may modify both the risk and the survival of CRC; however, these effects do not appear to be mediated through its modulation of biological folate levels.**

Introduction

Low folate from diet or in circulation has been associated with increased risk of CRC[3] in several prospective studies (1, 2). The risk may be modified by functional polymorphisms in folate-metabolizing genes such as the *677C->T* polymorphism of the *MTHFR* gene (3, 4). *TS* competes with *MTHFR* for the 5-methyltetrahydrofolate as the substrate for intracellular conversion of dUMP to dTMP, a rate-limiting step in DNA synthesis (5). It is the primary target for fluoropyrimidine-based chemotherapy drugs, such as 5-FU, for treatment of CRC and other solid tumors (6). Overexpression of the TS protein has been associated with resistance to 5-FU-based treatment leading to poor survival outcomes (7).

A tandem repeat polymorphism has been identified in the 5′-UTR enhancer region of the *TS* promoter, which contains either triple (*TS*3R*) or double (*TS*2R*) repeats of a 28-bp sequence (8). The *TS*3R* allele is more common in Asians (~80%) than Caucasians (~60%; Ref. 9). Rare novel alleles containing 4 (*TS*4R*), 5 (*TS*5R*), or 9 (*TS*9R*) repeats have also been found (10, 11). Individuals homozygous for triple repeats (*TS 3R/3R*) have 3.6 times higher *TS* mRNA levels compared with those homozygous for the double repeat (*TS 2R/2R*) genotype (12). Effects of *TS* expression and polymorphism on CRC toxicity and survival have been reviewed by Ulrich *et al.* (13) Recent clinical studies have demonstrated that compared with patients with the *3R/3R* genotype, those with the *2R/2R* genotype had better response (decreased tumor burden) to 5-FU-based therapy (12, 14), as well as longer survival after the treatment (15, 16). For example, in an analysis of 50 metastatic CRC patients (12), response rates to 5-FU treatment ranged from 9% for the *3R/3R* genotype to 50% for the *2R/2R* genotype with respective median survival of 8.5 and 16.2 months. In a separate study on 211 Dukes' C-stage CRC patients, only individuals with *2R/2R* or *2R/3R* genotypes experienced survival benefits from the 5-FU treatment (odds ratio, 0.52; 95% CI, 0.33–0.82; Ref. 16). With respect to etiology of CRC, the only available data on this polymorphism comes from

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[3] The abbreviations used are: CRC, colorectal cancer; TS, thymidylate synthase; 5-FU, 5-flurorouracil; tHcy, total homocysteine; MTHFR, methylenetetrahydrofolate reductase; HR, hazard ratio; CI, confidence interval; UTR, untranslated region.

the recent study on colorectal adenomas, presumed precursors of CRC (17), in which the *TS* tandem repeat polymorphism was not an independent risk factor for colorectal adenomas but may modify the risk of colorectal adenomas associated with dietary folate intake. A recent study by Trinh *et al.* (18) demonstrated that the *TS* tandem repeat polymorphism was capable of modifying the plasma folate and tHcy levels. Given that these biomarkers have been associated with the risk of CRC, the *TS* promoter polymorphism may be a risk factor of the disease.

A novel 6-bp deletion in the 3′UTR of the *TS* gene (*TS 1494del6*) has also been identified (19). It was reported that individuals carrying one or two variant alleles (*−6bp*, 6-bp deleted) had 40% increased risk of colon cancer (odds ratio, 1.4; 95% CI, 0.99–1.98) compared with those with the homozygous wild-type genotype (*+6bp*, 6 bp present). Additionally, the tumor mRNA levels in patients with homozygous wild-type genotype (*+6bp/+6bp*) were reported to be 4.2-fold higher compared with those of patients with homozygous variant genotype (*−6bp/−6bp*). However, in a recent report from a large case-control study of colorectal adenomas, the *TS 1494del6* polymorphism was not associated with the disease (17).

In this study, we simultaneously examined the effect of both *TS* polymorphisms on the risk and survival of CRC in the prospective Physicians' Health Study. We also investigated whether these polymorphisms modified the risk and survival of CRC through its influences on plasma folate and tHcy levels.

Materials and Methods

The Study Population. The Physicians' Health Study was a double-blind trial of aspirin and β-carotene among 22,071 United States male physicians, 40–84 years of age in 1982. About 93% of participants were Caucasian-Americans. 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; or current use of vitamin A or β-carotene supplements. Before randomization, participants were asked to donate a blood sample, and 14,916 (68%) did so (20). Participants were monitored for incident cancer by biannual questionnaires and confirmed through medical records by the End Point Committee.

By the year 2000, 272 cases of CRC were identified and confirmed. Men who were free from diagnosed cancer at the time of cancer ascertainment (456) were selected as controls, and were matched on age (±1 year) and on smoking history at baseline (current, former, and never-smokers). We failed to genotype 2 cases and 2 controls on either polymorphism; thus, this analysis includes 270 cases and 454 controls.

Laboratory Analyses. Genotyping for the *TS* 5′-UTR polymorphism was carried out based on a method modified from that of Villafranca *et al.* (14). Primers with the sequences 5′-GCG GGA CGG CCG CGG GAA-3′ (sense) and 5′-TCC GAG CCG GCC AGG CAT GGC GCG G-3′ (antisense) were used in PCR reactions. PCR products were size-fractionated on 4% agarose gels. Genotyping for the *TS* 3′-UTR polymorphism was carried out based on a method modified from that of Ulrich *et al.* (19) Primers with the sequences 5′-CAA ATC TGA GGG AGC TGA GT-3′ (sense) and 5′CAG ATA AGT GGC AGT ACA GA3′ (antisense) were used in PCR reactions to generate a 152-bp fragment. The PCR products were digested with *Dra*I restriction enzyme and size fractionated on a 4% agarose gel. The expected fragment sizes are 70 bp and 88 bp for the wild-type *+6bp* allele and 152 bp for the variant *−6bp* allele. Laboratory personnel were blind to case-control status.

Plasma folate and tHcy levels were measured based on methods described previously (21, 22). All of the measurements of biomarkers were performed in the laboratory of the Jean Mayer United States Department of Agriculture Human Nutrition Research Center on Aging at Tufts University (Boston, MA). The intra-assay coefficient of variation was 6.8% for folate and 2.9% for tHcy.

Statistical Analyses. We calculated relative risk of CRC and 95% CI using conditional logistic regression. Potential confounding variables, such as multivitamin use (never, past, and current use) and aspirin assignment group in the trial were included in the regression models. We investigated the linkage disequilibrium between the two *TS* polymorphism by use of a linkage utility program (EH program developed at New York State Psychiatric Institute and Columbia University) based on Terwilliger and Ott (23).

To examine influences of *TS* genotype on plasma levels of folate and tHcy, ANOVA was used to calculate age-adjusted geometric means for these biomarkers within each stratum of the *TS* genotype, followed by comparison with a trend test. To improve the normality of the variables, plasma levels were $\log_e$-transformed. All of the probability values were two-tailed and based on $\log_e$-transformed values.

Kaplan-Meier survival curves were constructed and analyzed using the log-rank test. Survival time was defined as the number of years from when a patient was diagnosed with CRC until the death of the individual attributable to any cause. Cox proportional hazards regression was performed to compare age-adjusted estimates of the effect of the *TS* genotypes as well as plasma folate levels (below or above the median) on survival among CRC patients. HRs and 95% CIs were calculated from Cox multivariate regression models. Statistical analyses were performed using SAS Software, Release 8.1 (SAS Institute).

Results

Among the 270 CRC cases and 454 controls in the Physicians' Health Study, the *TS*3R* allele frequencies were 57% and 53%, respectively. The genotype distributions (Table 1) were in agreement with Hardy-Weinberg Equilibrium in both cases ($P = 0.98$) and controls ($P = 0.87$). Compared with those with the *TS 3R/3R* genotype, individuals with *TS*2R* allele had nonsignificantly lower risk of CRC with multivariate-adjusted relative risk of 0.86 (95% CI, 0.59–1.25) for the *2R/3R* genotype and 0.59 (95% CI, 0.36–0.98) for the *2R/2R* genotype. The *P* for trend was 0.03 across the *TS* promoter genotype. This association was unchanged after additional adjusting for β-carotene intake, and the *MTHFR 677C->T* and *1298A->T* genotypes (data not shown).

The frequencies for the variant *−6bp* allele of the *TS 1494del6* polymorphism were 33% and 35% in the cases and controls, respectively. This polymorphism was not associated with CRC risk (Table 1). Additionally, this polymorphism is genetically linked with the *TS* promoter polymorphism in the study population. For example, only 3 (21 expected) individuals, all of which are controls, were homozygous variant at both loci. A formal test based on Terwilliger and Ott (23) revealed that the two polymorphisms were at linkage disequilibrium ($D' = -0.6$; $P < 0.001$). None of the compound genotypes significantly influenced the risk of CRC (data not shown).

The relationship of the *TS* promoter polymorphism and plasma folate, and tHcy levels is reported in Table 2. Compared with the geometric mean folate level of 6.24 ng/ml among

Table 1 Association of *TS* polymorphisms and risk of colorectal cancer in the Physicians' Health Study

Genotype	No. cases (%)	No. controls (%)	RR[a] (95% confidence interval)	*P* trend
TS promoter polymorphism				
3R/3R	87 (32)	132 (29)	(referent)	
2R/3R	135 (50)	218 (48)	0.86 (0.59–1.25)	
2R/2R	48 (18)	104 (23)	0.59 (0.36–0.98)	0.03
TS 3′-UTR polymorphism				
+6bp/+6bp	124 (46)	201 (44)	(referent)	
+6bp/−6bp	113 (42)	191 (42)	0.82 (0.59–1.16)	
−6bp/−6bp	33 (12)	62 (14)	0.81 (0.47–1.38)	0.28

[a] Relative risk from conditional regression model with matching variables of age, aspirin assignment groups, and use of cigarettes and multivitamins.

Table 2 Relation of *TS* and *MTHFR* polymorphisms and geometric mean levels of plasma folate and tHcy in the Physicians' Health Study

Genotype	Folate (ng/ml)			tHcy (nmol/ml)		
	n	Mean	*P*	*n*	Mean	*P*
TS promoter						
3R/3R	169	6.24	(referent)	146	11.5	(referent)
3R/2R	262	5.79	0.23	222	11.7	0.64
2R/2R	114	5.29	0.03	99	11.5	0.96
TS 5′-UTR						
+6bp/+6bp	238	5.62	(referent)	202	11.6	(referent)
+6bp/−6bp	234	6.00	0.26	202	11.6	0.98
−6bp/−6bp	72	6.05	0.39	61	11.4	0.70
MTHFR						
CC	221	6.19	(referent)	214	11.4	(referent)
CT	199	5.79	0.24	192	11.4	0.87
TT	61	5.10	0.03	58	12.8	0.03
TS-MTHFR						
3R/3R-CC	74	7.06	(referent)	65	11.8	(referent)
2R/3R-CC	112	6.25	0.20	101	11.2	0.33
2R/2R-CC	46	6.20	0.28	44	10.5	0.09
3R/3R-CT	64	6.43	0.38	60	10.7	0.11
2R/3R-CT	112	6.46	0.36	94	11.6	0.75
2R/2R-CT	44	5.42	0.03	39	12.0	0.81
3R/3R-TT	22	6.14	0.37	19	11.3	0.63
2R/3R-TT	28	4.63	<0.001	25	13.3	0.18
2R/2R-TT	15	6.26	0.50	13	12.6	0.56

[a] Geometric means adjusted for age.
[b] Test for linear trend on the continuous log-transformed plasma folate and total homocysteine levels.

individuals with the *3R/3R* genotype, those with the *2R/3R* (5.79 ng/ml; $P = 0.23$) and *2R/2R* (5.29 ng/ml; $P = 0.03$) genotypes had lower levels of plasma folate with a *P* for trend of 0.03. Meanwhile, no effect of this polymorphism on plasma tHcy levels was apparent. Additionally, no effects of the *TS 1494del6* polymorphism on plasma folate and tHcy levels were seen (Table 2). These associations remained unchanged after adjusting for smoking, use of multivitamin supplements, aspirin and β-carotene, and other folate gene polymorphisms (*i.e. MTHFR 677C->T* and *1298A->C*). Because the *TS* competes with the *MTHFR* for 5-methyltetrahydrofolate as substrate for thymidylate synthesis, and we reported previously that the *MTHFR 677C->T* polymorphism was capable of modifying the plasma folate and tHcy levels in this population (4), we examined the association of compound *MTHFR-TS* promoter genotypes and plasma folate, and tHcy levels (Table 2). Individuals with homozygous wild-type genotypes at both loci (*i.e. 3R/3R-CC*) had highest levels of plasma folate. Compared with these individuals, those who were variant homozygotes at one locus and heterozygotes at another (*i.e. 2R/2R-CT* or *2R/3R-TT*) had significantly lower plasma folate levels; however, individuals who are variant homozygotes at both loci had lower plasma levels, but the reduction was not significant. Compound heterozygosity (*i.e. 2R/3R-CT*) was also associated with a nonsignificant reduction in plasma folate levels. Meanwhile, no clear association with plasma tHcy was apparent.

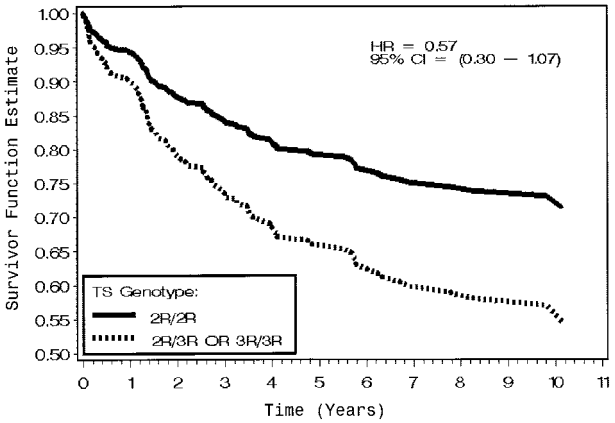

Fig. 1. Overall survival of the CRC cases ($n = 270$) in the Physicians' Health Study affected by the *TS* genotypes: *2R/2R versus 2R/3R* or *3R/3R*.

By May 21, 1999, 91 of the 270 CRC patients had died. The survival time since diagnosis among patients who died ranged from 0.20 to 121.5 months. The frequency of the *2R/2R* genotype among these patients was 12%, almost half that among 179 CRC patients who were alive at the end of follow-up (21%). Compared with those with the *3R/3R* genotype, age-adjusted HR of dying was 1.04 (95% CI, 0.66–1.65) for the *2R/3R* genotype and 0.63 (95% CI, 0.32–1.28) for the *2R/2R* genotype. We combined the *2R/3R* and *3R/3R* genotypes as a single risk group to increase the statistical power. Compared with this combined reference group, the age-adjusted HR for *2R/2R* was 0.57 (95% CI, 0.30–1.07). Adjusted Kaplan-Meier survival curves are presented in Fig. 1. Meanwhile, the *TS 1494del6* polymorphism was not associated with overall survival after the diagnosis of CRC (data not shown).

To investigate whether influences, if any, of the *TS* promoter polymorphism on CRC survival are mediated through modulation of biological levels of folate, we examined whether plasma folate levels were predictive of overall survival after CRC (Fig. 2). Compared with those with plasma folate below the median level of 5.64 ng/ml, individuals with plasma folate above the median level had better overall survival at borderline significance (HR, 0.68; 95% CI, 0.45–1.03).

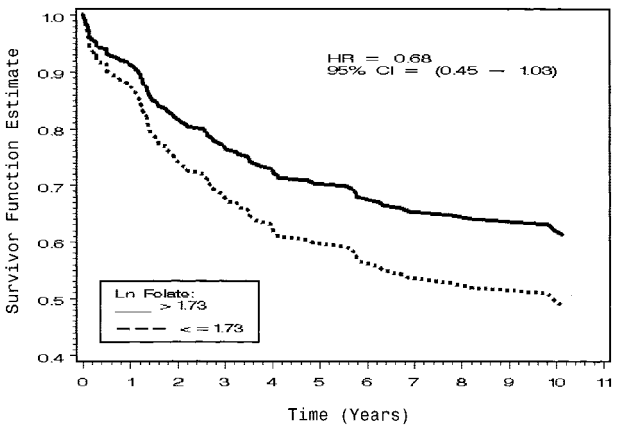

Fig. 2. Overall survival of the CRC cases ($n = 270$) in the Physicians' Health Study stratified at the median plasma folate levels.

Discussion

TS catalyzes the transformation of dUMP to dTMP, the only *de novo* source of thymidylate for DNA biosynthesis (24). The human *TS* promoter has been characterized, and mechanisms of gene regulation have been identified (25). A tandem repeat polymorphism, containing either double (*TS*2R*) or triple (*TS*3R*) repeats of a 28-bp sequence, has been identified near the initiation start site of the *TS* promoter (8), which acts as a *cis*-acting enhancer element of the *TS* gene (26). The prevalence of the *TS* polymorphism varies worldwide with the *TS*3R* allele frequency ranging from 49% in Kenyans to 82% in Chinese (9). In our population of United States physicians (>93% Caucasian), the *TS*3R* allele frequency was 55%, similar to a previous report on Caucasian populations by Marsh *et al.* (9). A new polymorphism at the 3′UTR region of the gene, *TS 1494del6*, has been identified recently (19); the *+6bp/+6bp* genotype was shown to be associated with high TS expression in CRC tissues. However, reports on direct associations of the *TS* polymorphisms and risk of CRC are sparse and controversial (17, 27).

Our study is the first to investigate prospectively the association of the *TS* polymorphism and risk of CRC. Our results suggest that the *TS 2R/2R* genotype may be associated with reduced risk of CRC. Meanwhile, individuals with the *2R/2R* genotype had significantly lower plasma folate levels compared with those with the *3R/3R* genotype. These findings suggest that the reduced risk is not mediated by the influence of *TS*2R* on plasma folate levels, as lower levels increased the risk of CRC (2). In a recent case-control study of 510 colorectal polyps and 604 polyp-free controls, the *3R/3R* genotype significantly increase the risk of polyps among individuals with low and medium folate consumption (17).

The *TS* gene is self-regulated at the translational level, with the protein binding to its mRNA and inhibiting translation (28). The tight regulation of *TS* activity suggests the importance of regulating the balance between dUMP and dTMP, hence, between dTTP and the dUTP that escapes dUTPase activity. Inhibitor of *TS* results in depletion of intracellular dTTP pools and elevation in dUMP (29), and, in turn, DNA damage (30). Alternatively, high *TS* activity could lead to a higher dTTP concentration, which may lead, through a complex feedback mechanism, to resetting the dNTP pool and possibly to increased DNA replication errors (31) leading to increased cancer risk. TS can also bind to other downstream mRNAs and inhibit their translation, including *c-myc*, *TP53*, and *p21*, all of which play critical roles in regulating cell cycle progression and DNA biosynthesis (28, 32). As a result, enhanced expression by *TS*3R* may stimulate cell proliferation (33, 34) and prevent apoptosis (35).

TS is an important target of primary chemotherapy drugs for treatment of CRC, such as 5-FU. Overexpression of *TS* may explain the recent findings that the *TS*3R* allele is predictive of poor response to 5-FU treatment in terms of poor tumor shrinkage and shorter survival (14–16). Our study obtained similar findings in that the *TS 2R/2R* conferred better survival. Because no information on specific cancer treatments was available for this population, treatment (especially 5-FU)-related outcomes could not be examined. Data from the National Cancer Data Base (based on hospital registries across the United States) indicate that use of chemotherapy ranged from 10% in 1985 to 26% in 1993, although the use varied by tumor stage, age, geographic locations, sizes of the hospital, and so forth (36). The fact that we observed a direct association of the *TS* polymorphism and CRC survival suggests that modified response to chemotherapy may only explain part of the association. The *TS* polymorphism may also predispose individuals to having tumors with poor prognosis markers independent of response to treatment (*e.g.* being poorly differentiated and microsatellite stable). Meanwhile, the influence of the *TS* promoter polymorphism on CRC survival is unlikely to be mediated through its effect on biological folate levels, because better survival status was associated with the *TS*2R* allele, which is related to lower plasma folate levels.

The findings of elevated plasma folate levels associated with the *TS 3R/3R* genotype in the current study contradict the latest findings from a Chinese population in Singapore (18). Although the genotype distribution was drastically different between the two populations (*e.g.* the *2R/2R* frequency of 3% in the Chinese population *versus* 23% in our Caucasians population), the opposite effect of the *TS* promoter polymorphism cannot be readily explained. It is possible that the two populations may exhibit a different extent of linkage disequilibrium with another, as yet unidentified, functional polymorphism. In addition, folate intake was not assessed in the Physicians' Health Study, because the dietary information collected from the participants is not comprehensive. Consequently, stratified analyses by dietary folate intake as was done in Trinh *et al.* (18) cannot be performed. These findings need to be confirmed in additional studies.

The finding of modulation of plasma folate by the *TS* promoter polymorphism is of interest, because low plasma folate is a risk factor for several other human diseases including cardiovascular disease (20) and neural tube defects (37). One might speculate that although *TS 2R/2R* may protect against CRC, it could be a risk factor for other diseases. The novel finding of better CRC survival associated with high plasma folate levels also merits attention. It suggests that survival of CRC may be improved by dietary modulation such as folate-rich diet or vitamin supplementation. It is also possible that instead of being directly related to good CRC prognosis, a high plasma folate level may be a surrogate for a healthy lifestyle that results in favorable disease outcome. The prospective design of this study is of importance because the levels of these biomarkers were obtained before diagnosis of CRC; thus, any influence of treatment or change of diet because of the disease is unlikely.

How the *TS* genotype that confers high *TS* activity (*3R/3R*) is associated with high plasma folate levels but poor survival cannot be readily explained. One possibility is that overex-

pressed TS may interact with *S*-adenosylmethionine and remove its feedback inhibition on *MTHFR* leading to enhanced synthesis of 5-methyltetrahydrofolate, the circulating form of folate. This hypothetical mechanism merits additional investigation.

In summary, the novel tandem repeat polymorphism in the *TS* promoter may be a modest predictor of CRC risk and survival. The reduced risk and better survival of CRC associated with *TS*2R* allele may be mediated through mechanisms other than its genetic modification of plasma folate levels.

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