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

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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-flurouracil, the most common chemotherapeutic 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 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 3R/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
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 stroke, or transient ischemic attack; cancer except nonmelanoma skin cancer. 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 Villafraanca et al. (14). Primers with the sequences 5′-GCC GGA CGG CCG CGG GAA-3′ (sense) and 3′-TCC GAG CCG GCC AGG CAT GCC 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 GAA-3′ (antisense) were used in PCR reactions to generate a 152-bp fragment. The PCR products were digested with DraI 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,-transformed. All of the probability values were two-tailed and based on log,-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
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.

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 of 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).
The tight regulation of with the protein binding to its mRNA and inhibiting translation results suggest that the TS 2R/2R with reduced risk of CRC. Meanwhile, individuals with the risk of CRC (2). In a recent case-control study of 510 colorectal and elevation in dUMP (29), and, in turn, DNA damage (30).

Inhibitor of TS*2R suggests that the reduced risk is not mediated by the influence of 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 1494del66, 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 Caucasian population), the opposite effect of the TS promoter polymorphism cannot be readily explained. It is possible that the two populations may exhibit 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 5-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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References

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