RESEARCH ARTICLE

Polymorphisms in the Thymidylate Synthase Gene and Risk of Colorectal Cancer

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Abstract

To evaluate the relationship between polymorphisms (28 bp repeated sequences in 5'-UTR and 6-bp ins/del in 3'-UTR) in then thymidylate synthetase gene (TS) and risk of colorectal, colon and rectal cancers, we conducted a case-control study with 315 cases of colorectal cancer and 439 population-based controls in Jiangsu province, China. TS genotypes were identified using PCR–RFLP (restriction fragment length polymorphism) methods. Odds ratios (ORs) were estimated with an unconditional logistic regression model. We found that the distributions of 5'-UTR genotypes in TS were significantly different between controls and male colon cases ($\chi 2$ =8.25, P = 0.016). Compared with 3R/3R genotype, individuals with the 2R allele were at an increased risk of colon cancer (age-, BMI-, smoking- and alcohol drinking-adjusted OR=1.98, 95%CI: 1.11-3.53) among men. In ccontrast, the 6-bp ins/del polymorphism at the TS 3'- UTR did not influence risk of the colorectal, colon and rectal cancers. When combined genotypes for both TS 5'-UTR and 3'-UTR polymorphisms were evaluated, individuals with the 5'-UTR 2R allele had a OR of 3.61 (95%CI: 1.38-9.49) for colon cancer among men with the 3'-UTR –6bp/-6bp genotype. These results show that the polymorphism of the 28 bp repeated sequences in TS 5'-UTR could influence susceptibility to colon cancer and that there was a coordinated effect between TS 3'-UTR and 5'-UTR polymorphisms in increasing risk of colon cancer among Chinese men.

Keywords: Colorectal cancer - thymidylate synthetase - genetic polymorphism

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Introduction

Thymidylate synthetase (TS) is a key enzyme in folate metabolism catalysing the conversion of deoxyuridine monophosphate to deoxythymidine monophosphate, providing the sole de novo source of thymidine required for DNA synthesis and repair (Choi et al., 2000). TS gene is polymorphic. Horie et al. (1995) reported a functional polymorphism of the tandemly repeated sequences in the 5'-terminal regulatory region of TS promoter (5'-UTR), which contains either triple (TS 3R) or double (TS 2R) repeats of a 28-bp sequence. The TS 3R allele is more common in Asians (80%) than Caucasians (60%). 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 (Pullarkat et al., 2001). Ulrich et al. (2000) identified a 6-bp ins/del variation at bp 1494 in the 3'-untranslated region (3'-UTR) of the TS mRNA by screening public databases of the expressed sequence tag. Presence of the deleted allele (–6bp) has been reported to result in enhanced TS mRNA degradation in vitro, and clinical studies have observed reduced TS mRNA expression in colorectal tumours of –6bp/-6bp homozygote patients (Mandola et al., 2004). Polymorphisms in the TS gene may result in altered activity level of enzyme, and affect the DNA methylation and synthesis, and in turn affect cancer susceptibility (Ulrich et al., 2002; Chen et al., 2003; Matsuo et al., 2005; Ulrich et al., 2005; Curtin et al., 2007; Hubner et al., 2007; van den Donk et al., 2007; Carmona et al., 2008). Our previous studies have shown the relationships between genetic polymorphisms of TS 3'-UTR and the susceptibility of stomach and esophageal cancer (Gao et al., 2004). To investigate possible relations between genetic polymorphisms of TS and the susceptibility of colorectal cancer, we conducted a case-control study in Jiangsu province, China.

Materials and Methods

Study Subjects

We recruited colorectal cancer cases using data of Cancer Registry in Huian and Jintan Cities of Jiangsu Province of China, also recruited cases from patients who visited Jiangsu Provincial Caner Hospital from these cities between the period of Aug. 2000 and Sept. 2002. Cases

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were histopathologically diagnosed as having primary colorectal cancer. Physicians at hospital or families of patients asked eligible cases to participate in our study, and doctors or nurses interviewed the subjects and collected their blood samples after obtaining informed consent. Population-based controls were selected from healthy residents in eight villages or towns of Huian and Jintan Cities. Doctors of the public health center randomly selected one or two control for each case, after matching for ethnicity, sex and age within 2 years of each case using the records of residents at the local governmental office, and then asked eligible residents for their participation, and performed interviews and collected blood samples by the same manner. We selected controls not only women, but also the numbers of men was higher in controls than in cases. But among selected male controls, a part of peasants have gone to cities for business, so we could not interview them in their home. Ten patients and 33 residents refused to participate in our study, but the final response rate were 97% for cases and 93% for controls. The ethical committee of Jiangsu Provincial Institute of Cancer Research approved the present study.

DNA Extraction and Genotyping of the TS

Whole blood was collected into EDTA-coated tubes and centrifuged for 15 min, and the buffy coat layer was isolated. Genomic DNA was extracted from 200μl of buffy coat using a Qiagen QIAamp DNA Blood Mini Kit (QIAGEN Inc., Valencia, CA). Genotyping for the TS 5`-UTR polymorphism was carried out based on a method modified from that of Villafranca et al. (2001). Primers with the sequences 5'-GCG GGA CGG CCG CGG GAA (sense) and 5'-TCCGAG CCG GCC AGG CAT GGC GCG G (antisense) were used in PCR reactions. PCR products were size-fractionated on 3% agarose gels. The expected fragment sizes are 220 bp (2R allele) and 248 bp (3R allele). Genotyping for the TS 3'-UTR polymorphism was carried out based on a method modified from that of Ulrich et al. (2000). The probe sequences of the primers for TS 3'-UTR genotypes were 5'-CAAATCTGAGGGAGCTGAGT (sense) and 5'-CAGATAAGTGGCAGTACAGA (antisense). The PCR product was subjected to DraI enzyme digestion, and samples were then analyzed by electrophoresis in 3% agarose gels. The expected fragment sizes are 70 bp and 88 bp for the presence of the 6 bp (+6 bp) allele and 152 bp for the absence of the 6 bp (-6 bp) allele.

Statistical Analysis

Associations between the TS polymorphism and colorectal cancer risk were estimated by odds ratios (ORs), using the unconditional logistic regression model. We calculated adjusted ORs for age, sex, smoking and drinking habits, and BMI to control for the effects of potential confounding factors. In the multivariate analysis, the TS 5'-UTR 2R allele carriers (2R/2R and 2R/3R) and the TS 3'-UTR +6bp allele carriers (+6bp/+6bp and +6bp/-6bp) were combined into a same group, respectively, because the number of the homozygous genotype (2R/2R, +6bp/+6bp) carriers was small. The procedure LOGISTIC from the statistical package SAS (SAS Institute Inc., USA) was used for the calculations. The probability of Hardy-Weinberg equilibrium was assessed by x^2 test.

Results

Numbers of subjects were 315 cases with colorectal cancer (105 colon and 210 rectum) and 439 controls. The background characteristics of cases and controls have been described previously (Gao et al., 2010). The proportional distribution of females in controls was significantly higher than that in colorectal cases. The mean age and BMI did not significantly differ between controls and colorectal cancer cases. The proportional distributions of smokers and drinkers were significantly higher in colorectal cancer cases than in controls.

Data for associations between TS 5'-UTR polymorphism and colorectal, colon and rectal cancer risk are presented Table 1. In total of men and women, the distributions of 5'-UTR 3R/3R, 3R/2R and 2R/2R genotypes were not significantly different between controls and cases of colorectal cancer (χ^2 = 0.89, P =

Table 1. TS 5'-UTR Genotypes and Risk of Colorectal Cancer

Genotype	Controls	Colorectum		Colon		Rectum	
	n (%)	n (%)	OR (95%CI)	n (%)	OR (95%CI)	n (%)	OR (95%CI)
Total[a]							
3R/3R	290 (66.1)	204 (64.8)	1.00	62(59.1)	1.00	142(67.6)	1.00
2R/3R	129 (29.4)	100 (31.8)	1.12(0.81-1.54)	41(39.0)	1.51(0.96-2.38)	59(28.1)	0.96(0.66-1.39)
2R/2R	20 (4.6)	11 (3.5)	0.85(0.40-1.84)	2(1.9)	0.49(0.11-2.16)	9(4.3)	0.97(0.42-2.22)
2R/3R & 2R/2R	149(33.9)	111(35.2)	1.08(0.79-1.47)	43(40.9)	1.39(0.89-2.16)	68(32.4)	0.96(0.67-1.37)
Males[b]							
3R/3R	151(67.7)	123(64.7)	1.00	35(53.9)	1.00	88(70.4)	1.00
2R/3R	60(26.9)	63(33.2)	1.36(0.88-2.11)	29(44.6)	2.24(1.24-4.05)	34(27.2)	1.04(0.63-1.74)
2R/2R	12(5.4)	4(2.1)	0.54(0.16-1.74)	1(1.5)	0.41(0.05-3.40)	3(2.4)	0.55(0.15-2.05)
2R/3R & 2R/2R	72(32.9)	67(35.3)	1.24(0.81-1.89)	30(46.1)	1.98(1.11-3.53)	37(29.6)	0.98(0.60-1.59)
Females[b]							
3R/3R	139(64.4)	81(64.8)	1.00	27(67.5)	1.00	54(63.5)	1.00
2R/3R	69(31.9)	37(29.6)	0.89(0.54-1.45)	12(30.0)	0.92(0.43-1.97)	25(29.4)	0.88(0.50-1.55)
2R/2R	8(3.7)	7(5.6)	1.49(0.51-4.37)	1(2.5)	0.92(0.11-7.96)	6(7.1)	1.62(0.51-5.10)
2R/3R & 2R/2R	77(35.6)	44(35.2)	0.95(0.59-1.51)	13(32.5)	0.93(0.44-1.94)	31(36.5)	0.96(0.56-1.64)

[a]ORs are adjusted for sex, age, smoking, alcohol drinking and BMI; [b]ORs are adjusted for age, smoking, alcohol drinking and BMI

Table 2. TS 3'-UTR Genotypes and Risk of Colorectal Cancer

Genotype	Controls	Colorectum		Colon		Rectum	
	n (%)	n (%)	OR (95%CI)	n (%)	OR (95%CI)	n (%)	OR (95%CI)
Total[a]							
-6bp/-6bp	199(45.3)	155(49.2)	1.00	49(46.7)	1.00	106(50.5)	1.00
+6bp/-6bp	197(44.9)	134(42.5)	0.86(0.63-1.17)	48(45.7)	0.94(0.61-1.48)	86(41.0)	0.81(0.57-1.16)
+6bp/+6bp	43(9.8)	26(8.3)	0.78(0.45-1.34)	8(7.6)	0.78(0.34-1.78)	18(8.6)	0.77(0.42-1.43)
Males[b]							
-6bp/-6bp	103(46.2)	98(51.6)	1.00	30(46.2)	1.00	68(54.4)	1.00
+6bp/-6bp	99(44.4)	77(40.5)	0.80(0.53-1.22)	30(46.2)	0.99(0.55-1.79)	47(37.6)	0.73(0.45-1.19)
+6bp/+6bp	21(9.4)	15(7.9)	0.74(0.35-1.54)	5(7.7)	0.72(0.24-2.12)	10(8.0)	0.72(0.31-1.68)
Females[b]							
-6bp/-6bp	96(44.4)	57(45.6)	1.00	19(47.5)	1.00	38(44.7)	1.00
+6bp/-6bp	98(45.4)	57(45.6)	1.04(0.65-1.67)	18(45.0)	0.92(0.44-1.91)	39(45.9)	1.08(0.63-1.86)
+6bp/+6bp	22(10.2)	11(8.8)	0.83(0.36-1.93)	3(7.5)	0.73(0.18-2.96)	8(9.4)	0.83(0.32-2.14)

[a]ORs are adjusted for sex, age, smoking, alcohol drinking and BMI; [b]ORs are adjusted for age, smoking, alcohol drinking and BMI

Table 3. ORs for Colorectal Cancer According to the Combination of TS 5'-UTR and 3'-UTR Polymorphisms

TS 5'-UTR	TS 3'-UTR	Controls	Colorectum		Colon		Rectum	
Genotype	Genotype	n	n	OR (95%CI)	n	OR (95%CI)	n	OR (95%CI)
Total[a]								
3R/3R	-6bp/-6bp	163	118	1.00	34	1.00	84	1.00
2R/3R & 2R/2R	-6bp/-6bp	36	37	1.47(0.86-2.49)	15	2.02(0.98-4.15)	22	1.30(0.70-2.39)
3R/3R	-6bp/+6bp & +6bp/+6bp	127	86	0.95(0.66-1.37)	28	1.06(0.61-1.85)	58	0.91(0.60-1.38)
2R/3R & 2R/2R	-6bp/+6bp & +6bp/+6bp	113	74	0.93(0.64-1.37)	28	1.21(0.69-2.11)	46	0.83(0.53-1.38)
Males[b]								
3R/3R	-6bp/-6bp	87	75	1.00	19	1.00	56	1.00
2R/3R & 2R/2R	-6bp/-6bp	16	23	1.90(0.91-3.96)	11	3.61(1.38-9.49)	12	1.44(0.61-3.39)
3R/3R	-6bp/+6bp & +6bp/+6bp	64	48	0.90(0.55-1.49)	16	1.13(0.53-2.42)	32	0.84(0.48-1.47)
2R/3R & 2R/2R	-6bp/+6bp & +6bp/+6bp	56	44	0.97(0.58-1.62)	19	1.61(0.78-3.33)	25	0.75(0.42-1.37)
Females[b]								
3R/3R	-6bp/-6bp	76	43	1.00	15	1.00	28	1.00
2R/3R & 2R/2R	-6bp/-6bp	20	14	1.00(0.43-2.32)	4	0.78(0.19-3.23)	10	1.07(0.43-2.71)
3R/3R	-6bp/+6bp & +6bp/+6bp	63	38	1.07(0.61-1.87)	12	0.95(0.41-2.21)	26	1.10(0.57-2.09)
2R/3R & 2R/2R	-6bp/+6bp & +6bp/+6bp	57	30	0.94(0.52-1.70)	9	0.90(0.35-2.27)	21	0.97(0.50-1.90)

[a]ORs are adjusted for sex, age, smoking, alcohol drinking and BMI; [b]ORs are adjusted for age, smoking, alcohol drinking and BMI

0.642), colon cancer (χ^2 = 4.64, P = 0.098), and rectal cancer (χ^2 = 0.16, P = 0.925). The allele frequencies of the 3R and 2R were 81% and 19% in controls and colorectal cancer cases, 79% and 21% in cases with colon cancer, 82% and 18% in cases with rectal cancer, respectively. The genotype distributions were in Hardy-Weinberg Equilibrium in both controls and cases (all P value > 0.05). It shows that subjects from population are representative. Compared with those with 3R/3R genotype, individuals with 2R/3R genotype had nonsignificantly increased risk of colon cancer with age, sex, BMI, smoking and alcohol drinking-adjusted OR of 1.51 (95% CI, 0.96–2.38). When the data were further stratified analyzed by gender, we observed a significant difference in TS 5'-UTR genotype distributions between male controls and cases with colon cancer (χ^2 = 8.25, P = 0.016). Compared with those with 3R/3R genotype, individuals with 2R/3R genotype had a significantly increased risk of colon cancer (OR=2.24, 95% CI: 1.24-4.05), individuals with 2R allele showed an OR of 1.98 (95% CI: 1.11-3.53) for colon cancer among men. No such risk of increase for colorectal, colon and rectal cancers were observed among women (Table 1).

Data for associations between TS 3'-UTR polymorphism and colorectal, colon and rectal cancer risk are presented Table 2. The distributions of 3'-UTR –6bp/-6bp, –6bp/+6bp and +6bp/+6bp genotypes were not significantly different between controls and cases of colorectal cancer (χ^2 = 1.29, P = 0.525), colon cancer (χ^2 = 0.47, P = 0.789), and rectal cancer (χ^2 = 1.53, P = 0.466). The allele frequencies of the –6bp and +6bp were 68% and 32% in controls, 70% and 30% in cases with colorectal or colon cancer, 71% and 29% in cases with rectal cancer, respectively. The genotype distributions were in Hardy-Weinberg Equilibrium in both controls and cases (all P value > 0.05). This polymorphism was not associated with risk of colorectal, colon and rectal cancers (Table 2).

The data were further analyzed to examine the combined effects of TS 5'-UTR and 3'-UTR genotypes for risk of colorectal, colon and rectal cancers (Table 3). Among men, compared with the 5'-UTR 3R/3R and 3'-UTR –6bp/-6bp genotype, individuals with 5'-UTR 2R allele and 3'-UTR –6bp/-6bp genotype showed a significantly increased OR (3.61, 95%CI: 1.38-9.49) for colon cancer.

Discussion

TS is not only essential in regulation of a balanced supply of the nucleotides required for DNA replication and DNA repair, but also plays an important role in the

folate cycle (Horie et al., 1005; Marsh et al., 1999; Ulrich et al., 2000). Low folate from diet or in circulation has been associated with increased risk of colorectal cancer in several prospective studies (Giovannucci et al., 1995; Kato et al., 1999) In a cohort of 505 Chinese from Singapore, Trinh et al. (2002) found that TS 3R/3R genotype was associated with reduced plasma folate and, among individuals with low dietary folate intake, with elevated plasma homocysteine levels. These results suggest that TS and methylenetetrahydrofolate reductase compete for limiting supplies of folate required for the remethylation of homocysteine.

A number of studies have evaluated the relationship between TS polymorphisms and colorectal cancer, but the results are inconsistent. Chen et al. (2003) among US male physicians observed no association between the risk of colorectal cancer and the TS 3'-UTR 6 bp/del polymorphism, but, individuals with the 5'-UTR 2R/2R genotype had a decreased risk of colorectal cancer compared to those with the 3R/3R genotype. Additionally, none of the compound genotypes significantly influenced the risk of colorectal cancer, nor was any modulating effect of folate status observed. Cornelia et al. (2005) among African American, Caucasian, or Hispanic subjects from US found that TS 5'-UTR 2R/2R genotype was associated with a reduced colon cancer risk among men but not women. When combined genotypes for both TS 5'-UTR and 3'-UTR polymorphisms were evaluated, they found that ORs for variant genotypes were generally below 1.0, with statistically significantly reduced risks for colon cancer among women. Matsuo et al. (2005) in Japanese found that TS tandem repeats polymorphism as a risk factor for colorectal cancer did not show statistical significance by genotype alone, whereas interaction with drinking was significant (P = 0.028). Carmona et al. (2008) in Portugal subjects found that the TS 5'-UTR polymorphism was not associated with risk of colorectal cancer, but individuals homozygous or heterozygous for TS 3'-UTR 6bp variant carried a significantly and unequivocally lower risk of developing colorectal cancer. Furthermore, by combining the protective effects of the 6 bp/del allele and the 28 bp 2R allele, Carmona B et al found a combined genotype of the TS gene (2R/2R, 6 bpdel/del+6 bpdel), which was associated with a 58% decreased risk of developing colorectal cancer. In this study, we detected that a polymorphism in the TS 5'-UTR might be associated with risk of colon cancer in Chinese men, that is, the TS 5'-UTR 2R allele is significantly increases the risk of colon cancer among men, but not among women. When combined genotypes for both TS 5'-UTR and 3'-UTR polymorphisms were evaluated, we found that individuals with the 5'-UTR 2R allele had a OR of 3.61 (95%CI: 1.38-9.49) for colon cancer among men with the 3'-UTR –6bp/-6bp genotype. The reasons for the discrepancies observed among these studies are not readily apparent. However, ethnic variation are apparent in the distribution of the TS 3'-UTR and 5'-UTR polymorphisms, the 3'-UTR -6 bp allele and the 5'-UTR 3R allele being relatively more common in the present population than in Caucasians. It is also worth noting that all previous studies on the influence of these genetic polymorphisms on the risk of developing colorectal cancer were performed in different countries, where the diet is certainly very different from the average dietary intake in China.

Finally, some limitations in the present study require further discussion. The sample size in the present study was not sufficient for stratified subgroup analyses, with consequent reduction in the magnitude of statistical power and increase in the potential for random error. Another possible problem is selection bias for controls, these being recruited by local health staff, albeit from the general population with a high response rate. The proportional distribution of female in controls was higher than that in colorectal cases, which may have caused a lower prevalence of smokers and alcohol drinkers in the present controls, though we adjusted for sex and age in all statistical analyses.

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