RESEARCH COMMUNICATION

Polymorphisms in Thymidylate Synthase and Methylenetetrahydrofolate Reductase Genes and the Susceptibility to Esophageal and Stomach Cancer with Smoking

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Abstract

Thymidylate synthetase (TS) and methylenetetrahydrofolate reductase (MTHFR) are major enzymes in the metabolism of folates, involved in DNA 'breaks', instability and hypomethylation. To investigate the possible relations between the TS 3'-UTR and MTHFR C677T polymorphisms and environmental factors impacting on risk of esophageal and stomach cancers, we conducted a case-control study in a high incidence region of China for these cancers. We recruited 138 esophageal and 155 stomach cancer cases, and 223 controls. The TS 3'-UTR and MTHFR C677T genotypes were detected by RFLP assay, using PCR products. The frequency of the -6 bp homozygous TS 3'-UTR genotype was 37.7 % in controls, higher than in Caucasians, although the present distribution was not in Hardy-Weinberg equilibrium. Ever-smoking with the -6 bp/-6 bp TS genotype elevated the ORs (2.61, 1.24-5.49; 3.54, 1.60-7.82) for cases of esophageal and stomach cancers, respectively, when compared with never-smoking with the +6 bp/+6 bp and +6 bp/-6 bp genotypes. No combination between the TS and MTHFR genotypes gave increased ORs. The present results suggest that TS polymorphism may modify the risk of esophageal and stomach cancer with smoking, pointing to the necessity for further investigations with information on folate and methionine intake with a larger population.

Key words: Thymidylate synthase - methylenetetrahydrofolate reductase - polymorphism - stomach cancer - esophageal cancer - smoking

Asian Pacific J Cancer Prev, **5**, 133-138

Introduction

Thymidylate synthetase (TS) and methylene-tetrahydrofolate reductase (MTHFR) are major enzymes in the metabolism of folates (Horie et al., 1995; Marsh et al., 1999; Fodinger et al., 2000). TS catalyzes the intracellular conversion of deoxyuridylate to deoxythymidylate by simultaneous conversion of 5,10-methylenetetrahydrofolate to dihydrofolate, playing an important role in the provision of nucleotides required for both DNA synthesis and repair (Figure 1). MTHFR converts 5, 10-methylene-tetrrahydrofolate to 5-methyltetrrahydrofolate, a carbon donor for the remethylation of homocysteine to methionine.

Several polymorphisms exist for both genes, and MTHFR C677T and A1298C are related to variation in biological function (Frosst et al., 1995; van der Put et al., 1998; Lievers et al., 2001). Persons with the homozygote TT genotype show reduced enzyme activity, resulting in higher plasma homocysteine and lower circulation plasma folate, compared to CC homozygotes. The TS gene has a two or three 28-bp tandem repeat sequence in the promoter region, the number of this repeats being polymorphic (Horie et al., 1995). In addition, a 6-bp deletion polymorphism at bp 1494 in the 3'-untranslated region (UTR) has been reported (Ulrich et al., 2000; Kawakami et al., 1999; Kumagai et al., 2003). The TS 2-repeat sequence demonstrates smaller expression activity than that with the 3-repeats in a transient expression assay in cancer cells, and

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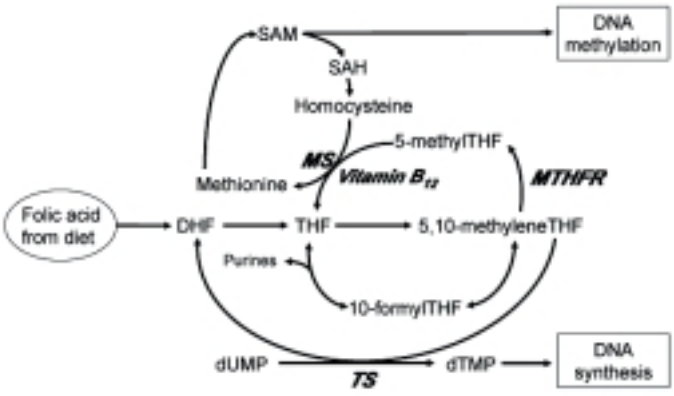

Figure 1. Overview of the Folate Cycle Pathway. DHF (dihydrotetrafolic acid); THF (tetrafolic acid); dUMP (deoxyuridine monophosphate); dTMP (deoxythymidine-5' -monophosphate); TS (thymidylate synthetase); MTHFR (methylenetetrahydrofolate reductase); MS (methionine synthetase); SAM (S-adenosylmethinine); SAH (S-adenosylhomocysteine).

differences in TS expression protein by the repeat length have also been observed in colorectal and stomach cancers (Frosst et al., 1995; Kawakami et al., 1999). The 6-bp deletion was reported to be associated with lower TS mRNA expression in tumor cells (Lenz,et al., Proc Am Assoc Cancer Res 43: abs. 660, 2002). Therefore, polymorphisms of the TS and MTHFR genes might impact on cancer susceptibility, in terms of DNA 'breaks', instability and hypomethylation.

Previous studies in the US and Japan reported a negative association between the MTHFR 677TT genotype and risk of colon cancer and malignant lymphomas (Chen et al., 1996; Ma et al., 1997; Matsuo et al., 2001). On the other hand, recent studies in China found a positive link between this genotype and esophageal and stomach cancer risk (Shen et al., 2001; Miao et al., 2002), confirmed by ourselves and especially in cases with smoking and drinking habits (Gao et al., 2002a; Wu et al., 2002). We are aware of no report on TS polymorphisms and cancer susceptibility, although several studies provided evidence that they might affect treatment efficacy and the toxicity of antifolate cancer therapeutics (Kawakami et al., 1999; Kumagai et al., 2003).

To investigate the possible relations between the TS 3'-UTR polymorphism and environmental factors for the risk of esophageal and stomach cancers, and combined effects with the MTHFR C677T polymorphism, we conducted the present case-control study in a high incidence region for these cancers, Huaian of China.

Subjects and Methods

Study Subjects

Huaian City, located in the northern part of Jiangsu Province, has relatively high incidence and mortality rates for both esophageal and stomach cancers, not only compared to other regions in the Province, but also in China as a whole (Gao et al., 2002b). We conducted a case-control study in Huaian to investigate how environmental and genetic factors might be involved in esophageal and stomach cancer development (Gao et al., 2002b; Takezaki et al., 2002; Gao et al., 2002c). In brief, we recruited cases from patients who visited Huaian City Municipal Hospital from December 1998 to March 2000 and who were histopathologically diagnosed as having primary esophageal or stomach cancer at the time of surgery. Doctors or nurses interviewed the subjects and collected blood samples after obtaining oral informed consent. Population-based controls were recruited from healthy residents in the villages or towns where cases resided. Doctors at public health centers randomly selected one control for each case, after matching for sex and age within 2 years of each case, using the records at the local governmental office. They also preformed interviews and blood collection in the same way. A few residents refused to participate in our study, but the response rate were over 90% for cases and controls. We used all controls combined in common for both stomach and esophageal cancers. The local government of Jiangsu Province and Huaian City gave permission for conducting the present study, although the cancer research institute of Jiangsu Province had no ethics committee.

Environmental Factors

The items of our questionnaire covered smoking and drinking habits, tea consumption, and intake of 12 foods. Smokers were divided into never- and ever-smokers (current and former). Former smokers were defined as persons who had quit smoking 1 year or more before the questionnaire survey. Alcohol drinkers were divided into non-regular drinkers (less than 2 times per week) and regular drinkers (2 times or more per week). Consumption of tea also was divided into two groups (>0 g/month and 0 g /month). Selected foods were divided into two groups according to intake frequency.

DNA Extraction and Genotyping of the TS and MTHFR

Whole blood was collected using EDTA-coated tubes and genomic DNA was isolated by standard methods, as described elsewhere (Gao et al., 2002b). The TS 3'-UTR genotypes were determined using a PCR-RFLP method (Ulrich et al., 2000). The probe sequences of the primers were 5'-CAAATCTGAGGGAGCTGAGT and 5'-CAGATAAGTGGCAGTACAGA. 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. MTHFR C677T genotypes were determined using PCR as described previously (Chen et al., 1996; Gao et al., 2002a) with probe sequences of 5' TGAAGGAGAAGGTGTCTGCGGGA-3' and 5'-AGGACGGTGCGGTGAGAGTG-3'. The PCR product was subjected to HinfI enzyme digestion, generating a single

band of 198 bp in length for the common allele (677C), and two bands of 23 and 175 bp for the rare allele (677T).

Statistical Analysis

Associations between the TS polymorphism and esophageal and stomach cancer risk were estimated by odds ratios (ORs), using the unconditional logistic regression model. We calculated adjusted ORs for age (continuous), sex, smoking and drinking habits, and consumption of tea, meat, raw vegetables and pickled vegetables to control for the effects of potential confounding factors. To investigate gene-environmental and gene-gene interactions, we calculated the ORs according to combinations of the TS and MTHFR genotypes, and smoking and alcohol drinking habits. The TS +6 bp allele carriers, i.e., 6 bp (+/+) and 6 bp (+/-), and the MTHFR 677T allele carriers, i.e., C/T and T/T, were combined into single groups according to the frequency of each allele. We used the chi-square test for categorical comparisons of the data. Statistical analyses were preformed with the statistical package Stata (ver 8.0; Stata Corporation, College Station, TX). The probability of Hardy-Weinberg equilibrium was assessed by the chi-square test with the statistical package STATA, too.

Results

The eligible subjects comprised 138 esophageal and 155 stomach cancer cases, and 223 controls (Table 1). One control 25 years old was excluded from the subjects, because of the unmatched age, so that the age distribution was very similar between each case group and controls. The proportion of male cases was higher for stomach than esophageal cancers (P<0.001). More smokers were included in cases than controls, and the difference was statistically significant for the esophageal and stomach cancer cases (P=0.034 and P=0.001). Ever-smoking demonstrated elevated ORs for esophageal and stomach cancers, with statistical significance. Regular drinking increased the OR for stomach cancer. The most common subsite for stomach cancer case was the cardia (57.4%) (data not shown in Table).

The proportional frequencies of the TS 3'-UTR genotypes were 8.1 % for the +6 bp homozygous (+6 bp/+6 bp), 54.3 % for the heterozygous (+6 bp/-6 bp) and 37.7 % for the -6 bp homozygous (-6 bp/-6 bp) forms in controls (Table 2). The +6 bp/-6 bp or -6 bp/-6 bp genotypes were not associated with increased ORs for esophageal and stomach cancer, when compared with the +6 bp/+6 bp, or the combined group of the +6 bp/+6 bp and +6 bp/-6 bp. This did not differ after adjustment for confounding factors. The allelic distribution of the TS 3'-UTR polymorphism for controls was not in Hardy-Weinberg equilibrium (P=0.005), but that of the MTHFR polymorphism for controls was in Hardy-Weinberg equilibrium (P=0.058) (data not shown in Table).

Ever-smokers with the -6 bp/-6 bp TS genotype demonstrated an increased OR for esophageal and stomach cancer with statistical significance, when compared with never-smokers with the +6 bp/+6 bp and +6 bp/-6 bp genotype (Table 3). Those with the +6 bp/+6 bp or +6 bp/-6 bp TS genotype also revealed an increased OR for stomach cancer. Regular drinking with the -6 bp/-6 bp TS genotype did not significantly elevate the ORs for either cancer.

We analyzed the combined effect of the TS and MTHFR genotypes. No increase was apparent in the OR for esophageal and stomach cancers with any combination of both genotypes (Table 4).

Table 1. Background Characteristics of Esophageal and Stomach Cancer Cases and Controls

	Controls		Esophageal cancer				Stomach cancer			
	N	%	N	%	OR[a]	(95% CI)	N	%	OR[a]	(95% CI)
Age in years										
36-50	40	17.9	15	10.9			18	11.6		
51-60	79	35.4	49	35.5			52	33.6		
61-70	79	35.4	60	43.5			66	42.6		
71-81	25	11.2	14	10.1			19	12.3		
Total	223		138				155			
Gender										
Male	147	65.9	74	53.6			120	77.4		
Female	76	34.1	64	46.4			35	22.6		
Smoking status[b]										
Never	98	44.1	45	32.6	1.00	Reference	33	21.3	1.00	Reference
Ever	120	54.1	91	65.9	2.03	(1.24-3.33)	121	78.1	2.66	(1.63-4.36)
Drinking staus[c]										
Non-regular	168	75.3	103	74.6	1.00	Reference	96	61.9	1.00	Reference
Regular	54	24.2	33	23.9	1.50	(0.85-2.67)	55	35.5	1.84	(1.13-3.02)

[a]Adjusted for age and sex.
[b]The numbers of missing values were 4, 2 and 1 for controls and cases of esophageal and stomach cancers, respectively.
[c]The numbers of missing values were 1, 2 and 4 for controls and cases of esophageal and stomach cancers, respectively.

Table 2. ORs for Esophageal and Stomach Cancer According to the TS 3'-UTR Polymorphism

TS 3'-UTR polymorphism	Controls		Cases					
	No	(%)	No	(%)	OR1[a]	(95% CI)	OR2[b]	(95% CI)
Esophageal cancer								
+6 bp/+6 bp	18	(8.1)	13	(9.4)	1.00	Reference	1.00	Reference
+6 bp/-6 bp	121	(54.3)	66	(47.8)	0.73	(0.33-1.59)	0.77	(0.33-1.83)
-6 bp/-6 bp	84	(37.7)	59	(42.8)	0.98	(0.66-1.45)	0.95	(0.61-1.49)
+6 bp/+6 bp & +6 bp/-6 bp	139	(62.3)	79	(57.3)	1.00	Reference	1.00	Reference
-6 bp/-6 bp	84	(37.7)	59	(42.8)	1.22	(0.79-1.90)	1.33	(0.82-2.15)
Stomach cancer								
+6 bp/+6 bp	18	(8.1)	10	(6.5)	1.00	Reference	1.00	Reference
+6 bp/-6 bp	121	(54.3)	80	(51.6)	1.12	(0.49-2.58)	1.40	(0.55-3.55)
-6 bp/-6 bp	84	(37.7)	65	(41.9)	1.04	(0.67-1.61)	1.29	(0.77-2.17)
+6 bp/+6 bp & +6 bp/-6 bp	139	(62.3)	90	(58.1)	1.00	Reference	1.00	Reference
-6 bp/-6 bp	84	(37.7)	65	(41.9)	1.11	(0.73-1.70)	1.12	(0.70-1.81)

[a]Adjusted for age and sex.
[b]Adjusted for age and sex, smoking, drinking and consumption of tea, meat, pickled vegetables and raw vegetables.

Discussion

The present study demonstrated that polymorphism of the TS 3'-UTR with smoking habit is associated with variation in risk of esophageal and stomach cancer. A positive association between the MTHFR 677TT polymorphism and stomach cancer risk has already been reported in China (Shen et al., 2001; Miao et al., 2002; Gao et al., 2002a; Wu et al., 2002), but the present observations provide the first evidence of an importance for the TS 3'-UTR polymorphism.

The 3'-UTR of a gene is not translated into protein, but it often involved in maintaining the secondary mRNA structure or mRNA stability, affecting protein expression levels, and a point-mutation polymorphism in the 3'-UTR of the N-acetyltransferase 1 gene is associated with altered enzyme activity in bladder and colon tissues (Bellet al., 1995). It is possible that the 6-bp deletion or insertion in 3'-UTR of the TS gene affect the secondary mRNA structure or mRNA stability and could thus ultimately affect TS protein levels. In fact, the allele of the 6-bp deletion is reported to be associated with lower TS mRNA expression in tumor cells (Lenz,et al., Proc Am Assoc Cancer Res 43: abs. 660, 2002).

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 at., 1995; Marsh et al., 1999; Ulrich et al., 2000). MTHFR is also involved in this cycle through remethylation of homocysteine to methionine (Fodinger et al., 2000). Functional variation of TS and MTHFR enzyme activities, thus could play a role in carcinogenesis by influencing DNA 'breaks', instability and hypomethylation. Tobacco smoking and alcohol drinking are associated with stomach and esophageal cancer risk through various mechanisms, including the production of oxyradicals and DNA adducts (Takezaki et al., 2000; La Vecchia et al., 1989; Ji et al., 1996; Inoue et al., 1994). The present findings suggest that the polymorphism of the TS 3'-UTR in the folate cycle modifies

Table 3. ORs for Esophageal and Stomach Cancer According to the Combination of Selected Environmental Factors and the TS 3'-UTR Polymorphism

Environmental Factors	TS 3'-UTR polymorphism	Controls No	Esophageal cancer No	OR[a]	(95% CI)	Stomach cancer No	OR[a]	(95% CI)
Smoking								
Never	+6 bp/+6 bp & +6 bp/-6 bp	59	28	1.00	Reference	19	1.00	Reference
Never	-6 bp/-6 bp	39	17	1.04	(0.46-2.37)	14	1.12	(0.46-2.74)
Ever	+6 bp/+6 bp & +6 bp/-6 bp	78	50	1.64	(0.85-3.14)	71	3.06	(1.55-6.16)
Ever	-6 bp/-6 bp	42	41	2.61	(1.24-5.49)	50	3.54	(1.60-7.82)
Drinking								
None	+6 bp/+6 bp & +6 bp/-6 bp	105	55	1.00	Reference	56	1.00	Reference
None	-6 bp/-6 bp	63	48	1.51	(0.88-2.60)	40	1.12	(0.64-1.94)
Regular	+6 bp/+6 bp & +6 bp/-6 bp	33	23	3.13	(1.00-4.52)	32	1.86	(0.97-3.56)
Regular	-6 bp/-6 bp	21	10	2.11	(0.77-5.79)	23	1.97	(0.88-4.22)

[a]Adjusted for age, sex and consumption of tea, meat, pickled vegetables and raw vegetables.

Table 4. ORs for Esophageal and Stomach Cancer According to the Combination of TS 3'-UTR and MTHFR Polymorphisms

TS 3'-UTR polymorphism	MTHFR	Controls	Esophageal cancer			Stomach cancer		
		No	No	OR[a]	(95% CI)	No	OR[a]	(95% CI)
+6bp/+6bp & +6bp/-6bp	C/C	43	25	1.00	Reference	21	1.00	Reference
+6bp/+6bp & +6bp/-6bp	C/T & T/T	23	21	1.63	(0.66-4.07)	17	1.89	(0.69-5.17)
-6bp/-6bp	C/C	96	54	1.19	(0.62-2.28)	69	1.52	(0.79-2.93)
-6bp/-6bp	C/T & T/T	60	38	1.52	(0.72-3.22)	48	1.46	(0.69-3.05)

[a]Adjusted for age and sex, smoking, drinking and consumption of tea, meat, pickled vegetables and raw vegetables.

the risk of esophageal and stomach cancer with smoking. In contrast, no interaction between these polymorphisms and drinking was apparent. Possible reasons include misclassification and limited variation in exposure level, because the drinking habit was divided into only two categories, and the proportion of habitual drinkers was relatively small, compared with ever-smokers.

One of limitations of the present investigation is the lack of information for dietary intake of folate and methionine. A previous study in the US found a negative association between the MTHFR 677TT genotype and colon cancer risk, especially in those with a high dietary supply of methionine (Chen et al., 1996; Ma et al., 1997). We used the dietary parameters of raw vegetable and meat consumption as major resources for these nutrients to adjust the ORs (Council Resources, Agency Science and Technology, Japan, 1992), although residual confounding may still exist, and no data are available to evaluate the intake of folate and methionine at the national level in China. Cigarette smoking may decrease folate in plasma and produce a localized deficiency of folic acid (Krumdieck, 1983). Alcohol can cleave folate, inhibit its absorption and utilization, and increase its excretion, with imbalanced DNA methylation (Shaw et al., 1989; Romero et al., 1981). Thus, a negative association between the MTHFR 677TT genotype and colon cancer risk was earlier found to be enhanced in alcoholic drinkers (Chen et al., 1996; Ma et al., 1997). A Chinese study conducted in Jiangsu Province, in the same region where the present work was conducted concordantly showed a positive association between the MTHFR 677TT genotype and stomach cancer risk, supporting our findings (Shen et al., 2001).

The distribution of the TS 3'-UTR polymorphism was not in Hardy-Weinberg equilibrium, although those of the present MTHFR polymorphism and the CYP2E1 polymorphism in our previous study, using similar population (Gao et al., 2002b) were in Hardy-Weinberg equilibrium. We recruited controls from the general population of Huaian, rural region of Jiangsu Province, including neither floating population nor different ethnicity. The potential of the methodological failure seems to be small, because concordant genotype frequencies of the TS 3'-UTR for cases and controls were observed between the previous Japanese and the present studies (Kumagai et al., 2003). We, however, need to examine this polymorphism for another population to assess the potential impact on cancer risk.

The methionine synthase (MS) that is also involved in DNA methylation of the folate cycle, and its gene polymorphism, MTR A2756G, was associated with colorectal cancer risk in Japanese (Matsuo et al., 2002). Further study is required to evaluate the combined effect of the polymorphisms of the MTHFR, MS and TS with sufficient number of subjects.

Interestingly, ethnic differences may exist regarding the distribution of the TS 3'-UTR polymorphism. Thus the genotype frequencies of the TS 3'-UTR among 95 Caucasian individuals and 167 Japanese individuals with rheumatoid arthritis were 48% vs. 5% for +6 bp/+6 bp, 44% vs. 44% for +6 bp/-6 bp, and 7% vs. 51% for –6 bp/-6 bp, respectively (Ulrich et al., 2000; Kumagai et al., 2003). The present results for cases and controls were concordant with the figures for Japanese, although the characteristics of the subjects were different. The frequency of the MTHFR 677TT does not significantly differ by ethnic group, although the proportions in Japanese and in the Chinese of the present study appear slightly higher than that for Caucasians (Hessner et al., 1999; Hamajima et al., 2002).

In summary, we here found that polymorphism of the TS 3'-UTR partially modifies the risk of esophageal and stomach cancer with the smoking habit, although the distribution of the polymorphism for controls was not in Hardy-Weinberg equilibrium. Ethnic variation is apparent in the distribution of the TS 3'-UTR polymorphism, deletion of 6 bp allele being relatively more common in the present population than in Caucasians. Further investigations with information on folate and methionine intake, and more subjects from different population are now needed to confirm the present results.

Acknowledgments

This study was supported in part by a Grant-in Aid for Scientific Research on Special Priority Areas of Cancer from the Japanese Ministry of Education, Culture, Sports, Science and Technology. The authors would like to thank Dr. Jiennong Zhou, President, Cancer Hospital and Institute of Jiangsu Province, for his cooperation. They are also grateful to the staff of the Huaian City Municipal Hospital and the Public Health Center of Huaian City for their assistance in data collection, and to Dr. Malcolm A Moore for his language assistance in preparing this manuscript.

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