Polymorphisms of thymidylate synthase in the 5'- and 3'-untranslated regions associated with risk of gastric cancer in South China: a case–control analysis

Zhengdong Zhang, Yaochu Xu, Jianwei Zhou, Xinru Wang, Liwei Wang, Xu Hu, Jiangtao Guo, Qingyi Wei and Hongbing Shen

Department of Molecular Cell Biology and Toxicology, Jiangsu Provincial Key Laboratories of Human Functional Genomics and of Applied Toxicology; School of Public Health, Nanjing Medical University, Nanjing, China; Yangzhong Cancer Research Institute, Yang-Zhong City, Jiangsu Province, China; Huai-An City, Jiangsu Province, China; Jianing Center for Disease Control and Prevention, Jinhua City, Jinhua Province, China and Department of Epidemiology, The University of Texas M. D. Anderson Cancer Center, Houston, TX, USA

*To whom correspondence and reprints should be addressed

Email: zdzhang@njmu.edu.cn, hbshen@njmu.edu.cn

Frutas y ciertos vegetales tienen un efecto protector sobre el cáncer gástrico (GC) y folato es uno de los nutrientes en frutas y vegetales. Se hipotetizó que los polimorfismos de thymidylate synthase (TYMS) geno asociados con folato en moléculas son asociados con GC. En una población en control de estudio de 337 GC casos y controles, frecuencia-matizada por edad, sexo y residencia en áreas en un surcoreano poblacional, se genotiparon los 28 bp tandem repeat en el TYMS 5'-untranslated enhanced region (TSER) y el 6 bp deletion/insertion at bp 1494 en el TYMS 3'-untranslated region (TS3'UTR). Se encontró que aunque el TS3'UTR polimorfismo no tuvo un efecto sobre GC, el TS3'UTR 6 bp/6 bp genotipo fue asociado con una significativamente incrementado riesgo de GC [razón de odds ajustada (OR) = 1.96, 95% intervalo de confianza (CI) = 1.18–3.25], especialmente el no-cardiac gastric cancer (2.16, 1.22–3.82), comparado con el 0 bp/0 bp genotipo. Sin embargo, cuando evaluamos estos dos polimorfismos juntos y usamos el genotipo combinado con un genotipo cero variante alelo (TSER 2R y TS3'UTR 6 bp variante alelos) como referencia, encontramos que el genotipo combinado con tres o cuatro variantes alelos fue asociado con un significativamente incrementado riesgo de GC (2.06, 1.12–3.79), especialmente en los casos de cáncer gástrico no-cardiac (2.33, 1.19–4.59), y este significativo asociación fue más pronunciado entre las mujeres (> 60 años) y los no-fumadores, y no bebe bebidas con alcohol. En conclusión, el TYMS polimorfismos, especialmente el TS3'UTR polimorfismo, se asocian con GC, especialmente en los casos de cáncer gástrico no-cardiac, y el TSER 2R y TS3'UTR 6 bp alelos pueden accidentalmente jugar un papel en la etiología de GC en el sur coreano poblacional. Estudios posteriores serán requeridos para verificar estos hallazgos.

Introduction

Although gastric cancer (GC) incidence and mortality rates have been fallen in recent decades, it remains the fourth most common cancer worldwide and there were estimated 876 000 newly diagnosed cases and 647 000 deaths in the year 2000 (1). In China, GC remains the leading cause of cancer-related mortality among men and women, and the crude mortality rate of GC was 25.2/100 000, which accounted for almost one-fourth of the total cancer deaths during 1990–1992 (2). Epidemiological studies have demonstrated that Helicobacter pylori (HP) infection, tobacco smoke, alcohol use and dietary factors (e.g. salty foods and N-nitroso compounds) remain the main risk factors for GC (3). However, only a fraction of individuals exposed to these factors develop GC, suggesting that individual susceptibility to GC may vary.

High consumption of fruits and vegetables is associated with decreased risk of GC (4). Folate is one of the constituents in fruits and vegetables and may provide protection against cancer, including GC (5). Thymidylate synthase (TYMS), as one of the several key enzymes, is known to be involved in folate metabolism (6). The TYMS gene is located on chromosome 18p11.32 and catalyzes the conversion of deoxyuridine monophosphate (dUMP) to deoxothymidine monophosphate (dTMP) using the 5,10-methylenetetrahydrofolate as a methyl donor (6). This reaction is the de novo source of cellular thymidine which is essential for the provision of nucleotides required for DNA synthesis and repair as well as a primary target for 5-fluorouracil (5-FU), the most common chemotherapy agent used to treat GC (7). Thymidylate deficiency may result in chromosomal breakage and fragile site induction (8,9), which may cause individual susceptibility to cancer.

The TYMS gene contains a series of 28 bp tandem repeats in the 5'-untranslated enhanced region (TSER). The double repeats (2R) or triple repeats (3R) are most common and known to be involved in modulation of TYMS mRNA expression (10). Several studies have investigated the association between the TSER polymorphism and risk of cancer, but the results were mixed (11–13). Recently, a novel polymorphism (rs16430), a 6 bp deletion/insertion at bp 1494 in the 3'-untranslated region of TYMS (TS3'UTR), was identified (14) and is thought to influence TYMS mRNA expression and stability (14,15). Studies on the association between the TS3'UTR polymorphism and risk of cancer also generated mixed results (12,16–20). In our previous studies, we found that the TS3'UTR polymorphism was associated with risks of squamous cell carcinoma of the head and neck (SCCHN) and lung cancer in Caucasians (21,22) and the TSER 3R and TS3'UTR 0 bp alleles appeared to jointly protect against SCCHN and progression of oral cavity cancer (21). In the present study, we further investigated the association between these two TYMS polymorphisms and GC risk in a southern Chinese population.

**Abbreviations:** CI, confidence interval; GC, gastric cancer; HP, Helicobacter pylori; OR, odds ratio; SCCHN, squamous cell carcinoma of the head and neck; TSER, thymidylate synthase in the 5'-untranslated enhanced region; TS3'UTR, thymidylate synthase in the 3'-untranslated region; TYMS, thymidylate synthase.
Materials and methods

Study population

The subject recruitment for this study has been described previously (23). In brief, 337 newly diagnosed GC cases and 326 cancer-free control subjects were recruited from Huai-An, Jin-Tan and Yang-Zhong cities in central Jiangsu Province, South China, between July 1997 and April 1999. These cities were known to have a high-mortality rate of GC. All cases were histologically confirmed gastric adenocarcinoma (132 cardiac and 205 non-cardiac gastric cancer) cases. Genetically unrelated cancer-free individuals were recruited as controls who were living in the same residential areas and frequency-matched to the cases on age (±5 years), sex, and residential areas. All case and control subjects provided informed consent to participate in the study and donated 5 ml of blood. Individuals who smoked one or more times a day for >1 year were defined as ever smokers, and individuals who consumed three or more alcohol drinks a week for >6 months were considered ever drinkers. Individuals who consumed tea every day were defined as ever tea drinkers. This research protocol was approved by the institutional review board of Nanjing Medical University.

Genotyping analysis

PCR and PCR-based restriction fragment length polymorphism (PCR-RFLP) assays were used to identify the TSER and TS3'UTR polymorphisms, respectively, as previously described (21). In brief, the primers of the TSER polymorphism were 5'-GGCTCCGAGCCGGCCACAGGCATGGCGCGG-3' (forward) and 5'-GGCTCCGAGCCGGCCACAGGCATGGCGCGG-3' (reverse) (10), which generated 243 bp (i.e. 3R) and 215 bp (i.e. 2R) fragments. The primers of the TS3'UTR polymorphism were 5'-CAATACTGAGGAGGCTGAGT-3' (forward) and 5'-CAGATACTGAGGCTGAGT-3' (reverse) (14), which generated 152 bp fragment for 6 bp deletion (i.e. 0 bp) or 158 bp for 6 bp insertion (i.e. 6 bp). The restriction enzyme DraI (New England BioLabs, Beverly, MA) was used to distinguish the TS3'UTR polymorphism, in which the presence of the 6 bp insertion creates a DraI restriction site, and the expected fragment sizes were 88 and 70 bp. Both the expected fragments of these two polymorphisms were separated on 3% NuSieve 3:1 agarose gel (Cambrex Bio Science Rockland, Rockland, ME). More than 10% of the samples were randomly selected for confirmation.

Statistical analysis

The \( \chi^2 \)-test was used to evaluate differences in the frequency distributions of selected demographic variables, smoking status, alcohol use, tea consumption, and each allele and genotype of the TSER and TS3'UTR polymorphisms between the cases and controls. The dummy coding scheme for each polymorphism was used for OR calculation but the nominal number was used as a continuous variable for the trend tests. Univariate and multivariate logistic regression analysis were used to obtain the crude and adjusted odds ratios (ORs) and 95% confidence intervals (CIs). The genotype data were further stratified by subgroups of age, sex, smoking status, alcohol use and tea consumption. In this study, we identified three of controls (0.9%) with the 4R allele (data not shown). Since they were rare, only 322 control subjects were included in the final analysis.

Results

Characteristics of the study population

The frequency distributions of selected characteristics of the cases and controls are presented in Table I. The frequency matching was effective, because there was no difference in sex and the mean age between the cases (58.7 ± 9.7 years; range, 24–82 years) and controls (58.1 ± 10.6 years; range, 32–87 years). In addition, there was no significant difference between the cases and controls by smoking status, alcohol use and residential areas (Table I). However, there were more never tea drinkers (65.0%) among the cases than among the controls (57.8%), but the difference was only borderline significant (\( P = 0.057 \)). Nevertheless, these variables were further adjusted in the multivariate logistic regression analysis. Because the cases and controls were selected from three different residential areas, the distributions of the genotypes were compared between the three areas among the cases and controls, although these three areas are geographically similar. However, we did not find any significant difference in the distributions among the three areas between the cases and controls. The genotype distributions of these two polymorphisms among the controls were in agreement with the Hardy–Weinberg equilibrium (Table II).

Genotype distributions of TYMS polymorphisms among the cases and controls

As shown in Table II, the genotype frequencies of the TS3'UTR polymorphism were 41.8, 42.5 and 15.7% for the 0 bp/0 bp, 0 bp/6 bp and 6 bp/6 bp genotypes, respectively, among the cases and 47.5, 43.2 and 9.3%, respectively, among the controls, and the difference was statistically significant (\( P = 0.037 \)). However, the difference in TSER genotype frequencies among the cases and controls was not significant. Similarly, a significant difference was observed in the frequency of the TS3'UTR 6 bp allele among the cases and controls. The genotype distributions of these two polymorphisms among the controls were in agreement with the Hardy–Weinberg equilibrium (Table II).

We then dichotomized the cases by anatomic subsites of stomach into non-cardiac gastric cancer and cardiac gastric cancer cases, and evaluated the genotype distributions of the TSER and TS3'UTR polymorphisms among these two case groups and controls. As shown in Table II, the genotype frequencies of TS3'UTR were 39.5, 44.4 and 16.1% for the 0 bp/0 bp, 6 bp/0 bp and 6 bp/6 bp genotypes, respectively, among
the non-cardiac gastric cancer cases and 45.4, 39.4 and 15.2%, respectively, among the cardiac gastric cancer cases, and the difference between the non-cardiac gastric cancer cases (but not the cardiac gastric cancer cases) and controls was statistically significant ($P = 0.036$). For the TSER polymorphism, however, the differences of genotype frequencies between both these two case groups and the controls were not significant. A significant difference was observed in the frequency of the TS3'UTR 6 bp allele between non-cardiac gastric cancer cases and controls.

We further analyzed the difference of the combined genotypes and risk of the cardiac gastric cancer cases in a dose–response manner (1.13, 0.72–1.77 for those with one variant allele, 1.77, 0.82–3.83 for those with three or four variant alleles; $P_{\text{trend}} = 0.048$) (Table II). However, no significant association between these two polymorphisms or the combined genotypes and risk of the cardiac gastric cancer cases was observed.

Stratification analysis of TS3'UTR polymorphism and risk of non-cardiac gastric cancer

In further stratification analysis as shown in Table III, the risk of non-cardiac gastric cancer associated with the TS3'UTR 6 bp/6 bp genotype was increased in a dose–response manner as the age increased (adjusted OR = 1.69, 95% CI, 0.51–5.61 for those <50 years old, 1.87, 0.62–5.66 for those 51–60 years old, and 2.42, 1.05–5.57 for those >60 years old). This increased risk was also more pronounced among women (6.59, 1.87–23.90), non-smokers (6.62, 2.50–17.53) and never tea drinkers (3.19, 1.54–6.60). However, we did not find any significant association between the TS3'UTR polymorphism and risk of non-cardiac gastric cancer.

---

### Table II. Frequency distributions of TSER and TS3'UTR alleles and genotypes and their associations with gastric cancer risk

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Controls (n = 322)</th>
<th>All gastric cancer cases (n = 337)</th>
<th>Non-cardiac gastric cancer cases (n = 205)</th>
<th>Cardiac gastric cancer cases (n = 132)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td><strong>TSER</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3R3R</td>
<td>203</td>
<td>63.0</td>
<td>217</td>
<td>64.4</td>
</tr>
<tr>
<td>2R3R</td>
<td>107</td>
<td>33.3</td>
<td>103</td>
<td>30.0</td>
</tr>
<tr>
<td>2R2R</td>
<td>12</td>
<td>3.7</td>
<td>19</td>
<td>5.6</td>
</tr>
<tr>
<td>2R</td>
<td>0.203</td>
<td>0.065</td>
<td>0.206</td>
<td>0.947</td>
</tr>
<tr>
<td><strong>TS3'UTR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 bp/0 bp</td>
<td>153</td>
<td>47.5</td>
<td>141</td>
<td>41.8</td>
</tr>
<tr>
<td>6 bp/0 bp</td>
<td>139</td>
<td>43.2</td>
<td>143</td>
<td>42.5</td>
</tr>
<tr>
<td>6 bp/6 bp</td>
<td>30</td>
<td>9.3</td>
<td>53</td>
<td>15.7</td>
</tr>
<tr>
<td>6 bp</td>
<td>0.309</td>
<td>0.369</td>
<td>0.369</td>
<td>0.025</td>
</tr>
<tr>
<td>Trend test</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>123</td>
<td>38.1</td>
<td>119</td>
<td>35.3</td>
</tr>
<tr>
<td>1</td>
<td>90</td>
<td>28.0</td>
<td>96</td>
<td>24.5</td>
</tr>
<tr>
<td>2</td>
<td>90</td>
<td>28.0</td>
<td>84</td>
<td>24.9</td>
</tr>
<tr>
<td>3 or 4</td>
<td>19</td>
<td>5.9</td>
<td>38</td>
<td>11.3</td>
</tr>
<tr>
<td>Trend test</td>
<td>0.158</td>
<td>0.048</td>
<td>0.048</td>
<td>0.928</td>
</tr>
</tbody>
</table>
and cardiac gastric cancer (data not shown). Furthermore, no statistical evidence for any interactions was found between these variables and the combined variant genotypes (data not shown).

**Statistical power**

It is obvious that this study did not have sufficient power to detect small ORs. For the TSER polymorphism, we had a 43% power (two-sided test, $\alpha = 0.05$) to detect an OR of 1.89 for 2R2R homozygotes (3.7% in the controls), if this variant genotype is a risk genotype, compared with the 3R3R + 2R3R genotype. Also, we only had a 5.7% power to detect an OR of 0.94 for both variant genotypes (2R3R + 2R2R; 37.0% in the controls) compared with the 3R3R genotype. Similarly, for the TS3'UTR polymorphism, we had a 75% power (two-sided test, $\alpha = 0.05$) to detect an OR of 1.87 for 6 bp/6 bp homozygotes (9.3% in the controls) but only a 31% power to detect an OR of 1.26 for the 6 bp/0 bp + 6 bp/6 bp genotype (6 bp/0 bp + 6 bp/6 bp; 52.5% in the controls).

**Discussion**

Studies have revealed that the number of the tandem repeats in TSER polymorphism can affect TYMS mRNA expression and protein translation efficiency (10,27,28). Recent studies have also demonstrated that TSER polymorphism may modulate the levels of plasma folate and homocysteine (16,29); thus, TSER is thought to be a functional polymorphism, and therefore may be a risk factor of cancer. To date, several studies investigated the association between the TSER polymorphism and risk of cancer, but the results were not consistent. One study found that the TSER 2R3R and 3R3R genotypes were protective against the adult acute lymphocytic leukemia compared with the 2R2R genotype in Caucasian subjects (11). Conversely but consistently, another study suggested that individuals with at least one TSER 2R allele had a significantly increased risk of malignant lymphoma compared with those without 2R allele in Japanese subjects (13). However, other studies did not find a significant association between the TSER polymorphism and risk of colorectal adenomas in Caucasians (12,24). In this study, we found no main effect of TSER polymorphism on increasing risk of GC, which is consistent with our previous studies on SCCHN and lung cancer in Caucasians (21,22) and other published data (12,24). However, the genotype frequencies of TSER among the control subjects in the present study differ from those in our previous studies. Studies reported that the frequency of tandem repeats in TSER polymorphism was varied among different ethnic groups or residential regions (10,27,30), and the frequency of TSER 3R allele in Chinese subjects was higher than that in Caucasians or Southwest Asians (30). Therefore, it is likely that these discrepancies in genotype frequencies may be because of ethnic or residential regional differences in allele frequencies.

The TS3'UTR is a novel polymorphism in the 3'-untranslated region of TYMS gene identified recently (14), and the function of TS3'UTR polymorphism is not known. Study revealed that the TS3'UTR polymorphism may affect the TYMS mRNA stability and translation (14), which in turn, affects TYMS protein expression levels. Therefore, TS3'UTR polymorphism is thought to be a potential functional polymorphism and might be associated with risk of cancer. Recently, several studies have investigated the association between the TS3'UTR polymorphism and the risk of cancer, but the results were mixed. Graziano et al. (17) reported that the TS3'UTR 6 bp/0 bp genotype was associated with a significantly increased GC risk in Caucasians (OR = 1.92, 95% CI, 1.13–3.28), compared with the 6 bp/6 bp genotype. However, this association was not observed in other studies (12,16,18,19).

In the present study, we found that the TS3'UTR 6 bp variant allele was associated with a significantly increased risk of GC in a dose–response manner. Specifically, the 6 bp/6 bp genotype was associated with 1.96-fold risk of GC compared with the 0 bp/0 bp genotype. Although the TSER
polymorphism had no main effect on risk of GC, the combined genotype with three or four variant alleles (TSER 2R and TS3'UTR 6 bp alleles) was associated with a significantly increased risk of GC. These findings suggest that the TSER 2R and TS3'UTR 6 bp alleles appeared to have a combined effect on increasing GC risk, which were consistent with our previous studies on SCCHN and lung cancer in Caucasians (21,22) and other published data in different ethnic groups including Caucasian, Japanese and northern Chinese populations (11,13,19), but not consistent with the study reported by Graziano et al. (17). The mechanisms of joint effect between the TSER and TS3'UTR polymorphisms on increasing risk of cancer warrant further investigations. Also, we found that the variant alleles of the TS3'UTR and the combined genotypes tended to increase the risk of non-cardiac gastric cancer in a dose–response manner. Specifically, the TS3'UTR 6 bp/6 bp genotype and the combined genotype with three or four variant alleles were associated with a significantly increased risk of non-cardiac gastric cancer. However, this increased risk was not observed in the cardiac gastric cancer cases. These findings suggest possible different genetic susceptibility to gastric cancer at different subsites of stomach (3).

In this study, subgroups of older, female and non-smoking subjects appeared to have higher risk of non-cardiac gastric cancer. This finding may reflect a high level of genetic susceptibility, possibly owing to reduced DNA repair capacity in these subgroups (31) or other unknown risk factors, which warrant further studies. Studies have revealed that increased green tea intake may reduce risk of cancer, including gastric cancer (32). Consistently, our data showed that tea consumption was a protective factor for GC in this study population, although only at a borderline significance.

The primary shortcoming of this study is the lack of data on detailed dietary intake of folate, plasma or erythrocyte folate levels and its precursors or metabolites such as homocysteine, because the effect of genetic variations in folate metabolic genes on cancer risk will depend on folate intake status (33,34). Therefore, our study could underestimate the risk in the presence of low dietary folate intake and could not evaluate possible gene–nutrient interaction. The strength of this study is that it is population-based and the genotype distributions in our study population are similar to reported distributions in other studies. For instance, the frequencies of the 0 bp/0 bp, 6 bp/0 bp and 6 bp/6 bp genotypes of the TS3'UTR among our 322 southern Chinese controls were 47.5, 43.2 and 9.3%, respectively, compared with 45.7, 44.5 and 9.8%, respectively, of 348 northern Chinese controls (19), although another smaller study with 223 Chinese controls had a different distribution, possibly owing to a selection bias because of a failure of the Hardy–Weinberg equilibrium test (20).

In conclusion, we found a significant association between the TS3'UTR polymorphism and GC risk, especially the risk of non-cardiac gastric cancer, in a case–control study of southern Chinese population, and the TSER 2R and TS3'UTR 6 bp variant alleles appeared to have a combined effect on the increasing risk of non-cardiac gastric cancer. Since we did not have the complete dataset on the HP infection status, we were not able to perform stratification analysis of HP infection on GC risk, which should be addressed in future study. Larger studies are needed to verify these findings, in which potential gene–gene and gene–environmental interactions on GC risk could be further examined.

Acknowledgements

This work was supported by National 973 Program of China: 2002CB512902; National Key Basic Research Program of China-special Grant: 200150; National Natural Science Foundation of China: 30271105; China Postdoctoral Science Foundation: 20040132 and National Natural Science Foundation of China: 30271148.

Conflict of Interest Statement: None declared.

References


Received February 4, 2005; revised May 9, 2005; accepted May 24, 2005