Association of the thymidylate synthase polymorphisms with esophageal squamous cell carcinoma and gastric cardiac adenocarcinoma

Jianhui Zhang1,3, Yajing Cui1, Gang Kuang1, Yan Li1, Na Wang1, Rui Wang2, Wei Guo1, Denggui Wen1, Lizhen Wei1, Fengling Yu1 and Shijie Wang1,2

1Hebei Cancer Institute and 2The Fourth Affiliated Hospital, Hebei Medical University, Shijiazhuang 050011, Hebei Province, China
3To whom correspondence should be addressed
Email: jianhuizhi@hotmail.com

Polymorphisms in the untranslated regions (UTRs) of the thymidylate synthase (TS) gene, which may modulate TS transcription and expression, have been associated with susceptibility and prognosis of several tumors. However, their effects on the development and clinical staging of esophageal squamous cell carcinoma (ESCC) and gastric cardiac adenocarcinoma (GCA) have not been assessed so far. In this study, the 28-bp tandem repeat and the G/C single nucleotide polymorphism in the TS 5'UTR, the 6-bp deletion (6 bp−) polymorphism in the TS 3'UTR, were genotyped in 465 cancer patients (232 ESCC, 233 GCA) and 348 control subjects in North China. The genotype and allele type distribution of the TS variants in ESCC, GCA patients and controls did not show significant difference. However, the frequency of the 6 bp−/2R haplotype in ESCC and GCA patients was marginally or significantly lower than that in controls (P = 0.05 and 0.006, respectively). Thus, the 6 bp−/2R significantly reduced the risk to ESCC and GCA, compared with the 6 bp−/3G haplotype (odds ratio (OR) = 0.61 and 0.48, 95% confidence interval (CI) = 0.37–1.00 and 0.28–0.81, respectively). In addition, the 6 bp+/3G haplotype in ESCC patients was also significantly less common than in controls (P = 0.002). Compared with the 6 bp−/3G haplotype, the 6 bp+/3G significantly reduced the risk to ESCC (OR = 0.30, 95% CI = 0.14–0.67). Moreover, the TS 2R/3G genotype frequency in ESCC patients with and without lymphatic metastasis was significantly different (27.1 versus 4.9%, P < 0.001). Therefore, the 2R/3G genotype had an ~11-fold increase in the risk of lymphatic metastasis of ESCC, compared with the 3G/3G genotype (95% CI = 2.67–49.74). The results suggested that the TS polymorphisms might be associated with the susceptibility to ESCC and GCA, and the 2R/3G genotype might be a candidate marker to predict the potential of lymphatic metastasis in ESCC.

Introduction

Thymidylate synthase (TS), whose gene is located on chromosome 18p11.32, is a critical enzyme in maintaining a balanced supply of deoxynucleotides required for DNA synthesis and repair (1). This enzyme catalyzes the conversion of deoxyuridine monophosphate to deoxythymidine monophosphate, reducing the level of uracil that might otherwise be incorporated into DNA. Impairment of the TS enzyme has been associated with chromosome damage and fragile site induction (2,3), TS is also a target for major chemotherapeutic drugs, including 5-fluorouracil (1,4). Levels of TS expression have been shown to vary significantly in human tumors and the sensitivity of tumor towards 5-fluorouracil-based chemotherapy has been associated with the intra-tumoral level of TS (5,6).

Several polymorphisms in the TS untranslated regions (UTRs), which may influence TS mRNA transcription, message stability or protein expression, have been described recently. One of them is a unique tandem repeat sequence in the TS 5'UTR immediately upstream of the ATG codon initiation site that contains two, three or infrequently more 28-bp repeats (7). The presence of the triple (3R) versus double (2R) 28-bp repeat sequence has been shown to enhance mRNA transcription and protein expression in in vitro and in vivo studies (8,9). A novel G→C single nucleotide polymorphism (SNP) in the second repeat of the 3R alleles has been identified recently. The 3R sequence with guanine (3G) shows three to four times greater efficiency of translation than the 3R with cytidine (3C) and the 2R sequence (10,11). Another TS polymorphism, a 6-bp deletion in the 3'UTR, was discovered by searching the expressed sequence Tag database (12). It has been reported recently that the allele with a 6-bp deletion (6 bp−) is associated with decreased mRNA stability in vitro and lower intra-tumoral TS expression in vivo (13). The association of the TS polymorphisms with the occurrence and prognosis of several tumor types has been investigated. The TS 5'UTR tandem repeat polymorphism has been associated with susceptibility to colorectal adenoma (14,15), malignant lymphoma (16) and lymphocytic leukemia (17), and may modify disease prognosis of colorectal cancer (14), gastric cancer (18) and leukemia (19). The relationship between the TS 5'UTR polymorphism and sensitivity and toxicity to 5-fluorouracil-based chemotherapy has also been reported in colorectal cancer (20,21). Moreover, it has been suggested that the double polymorphisms in the TS 5'UTR, the tandem repeat variation and the G to C SNP, might provide a potential for a more effective prediction of clinical outcome of 5-fluorouracil-based chemotherapy (10). Conversely, no association between the 6 bp− polymorphism in the TS 3'UTR, alone or in combination with the 5'UTR variants, and susceptibility to colorectal adenomas was observed (15).

Esophageal squamous-cell carcinoma (ESCC) and gastric cardiac adenocarcinoma (GCA) are very common in certain regions of China. Although several exogenous factors are linked to the development of these two tumor types (22–27), genetic background has been suggested to play important roles in carcinogenesis (28–32). Since TS activity is involved in

Abbreviations: 6 bp−, 6 bp deletion; 6 bp+, 6 bp sequence; CI, confidence interval; ESCC, esophageal squamous-cell carcinoma; GCA, gastric cardiac adenocarcinoma; LOH, loss of heterozygosity; OR, odds ratio; SNP, single nucleotide polymorphism; TS, thymidylate synthase; UGIC, upper gastrointestinal cancers; UTR, untranslated regions.
tumor development and chemotheraphy efficacy, we hypothesize that the polymorphisms resulting in reduced TS expression may affect risk of the occurrence and progression of ESCC and GCA through impaired supply of deoxynucleotides required for DNA synthesis and repair in epithelium cells of upper gastrointestinal tract. This report describes our case-control study of ESCC and GCA in a Chinese population aimed to examine this hypothesis.

Materials and methods

Subjects
This study included 465 cancer patients (232 with ESCC and 233 with GCA) and 348 healthy controls. The cases were outpatients for endoscopic examination or inpatients for tumor resection in the Fourth Affiliated Hospital, Hebei Medical University between 2001 and 2003. Histological tumor typying was carried out on the basis of biopsies or resected specimens in the Department of Pathology of the same hospital. All esophageal carcinomas were squamous-cell carcinomas and all gastric cardiac carcinomas were adenocarcinomas with their epicenters at the gastroesophageal junction, i.e. from 1 cm above until 2 cm below the junction between the end of the tubular esophagus and the beginning of the saccular stomach (33). The healthy subjects were recruited between 2001 and 2003 from individuals who visited the same hospital for physical examination and volunteered to join the epidemiology studies. All of the healthy controls have no history of diagnosis or cancer and genetic diseases. The cancer patients and controls were all unrelated Han nationality and from Shijiazhuang city or its surrounding regions. Two professional interviewers, directly after sampling, obtained information on sex, age, smoking habit and family history of cancers from patients and controls. For smoking habit, the former and present smoking status, the numbers of cigarette per day and the time of starting and quitting were inquired. Individuals who formerly or currently smoked 5 cigarettes/day for at least 2 years were defined as smokers. The participants were also asked for family history of cancers, including if there are/were cancer patients in their family, the relationship of the cancer patients to the participants and what type of cancer they have/had. Individuals with at least one first-degree relative or at least two second-degree relatives having esophageal/cardiac/gastric cancer were defined as having family history of upper gastrointestinal cancers (UGIC). Information on TNM staging from 141 ESCC and 94 GCA patients who underwent surgical treatment was available from hospital recordings and pathological diagnosis. The study was approved by the Ethics Committee of Hebei Cancer Institute and informed consent was obtained from all recruited subjects.

DNA extraction
Five milliliters of venous blood from each subject was drawn in Vacutainer tubes containing EDTA and stored at 4°C. Genomic DNA was extracted within 1 week after sampling by using proteinase K (Merck, Darmstadt, Germany) digestion followed by a salting out procedure according to the method published by Miller et al. (34). To analyze the genomic balance status in ESCC tissues, the resected tumor samples from a subset of the ESCC patients were subjected to microdissection under light microscopy. Five to ten micrometer tissue sections were prepared and one section was stained with H&E to simplify the recognition of the interest regions. After deparaffinization, the sections were lightly counterstained with hemalum. The tumor cells and corresponding normal epithelium cells were scraped out of the sections with a sterilized ophthalmic scalpel and collected separately into Eppendorf tubes.

Materials and methods

DNA extraction
Five milliliters of venous blood from each subject was drawn in Vacutainer tubes containing EDTA and stored at 4°C. Genomic DNA was extracted within 1 week after sampling by using proteinase K (Merck, Darmstadt, Germany) digestion followed by a salting out procedure according to the method published by Miller et al. (34). To analyze the genomic balance status in ESCC tissues, the resected tumor samples from a subset of the ESCC patients were subjected to microdissection under light microscopy. Five to ten micrometer tissue sections were prepared and one section was stained with H&E to simplify the recognition of the interest regions. After deparaffinization, the sections were lightly counterstained with hemalum. The tumor cells and corresponding normal epithelium cells were scraped out of the sections with a sterilized ophthalmic scalpel and collected separately into Eppendorf tubes. The cells were suspended in TE-buffer (10 mM Tris-Cl, pH 7.5, 0.1 mM EDTA) and incubated overnight at 55°C with proteinase K at a concentration of 2.5 mg/ml. The samples were subsequently heated at 94°C for 8 min to inactivate proteinase K. The supernatants, which contain DNAs of the tumor cells, were concentrated by the speed vac evaporator (Savant, Farmingdale, NY) followed by purification with DNA Clean-Up Kit (Wizard SV Gel and PCR Purification Kit, Promega). The fragments were amplified again for DNA sequencing with genotyping primers. DNA sequences of the PCR products were determined using the PCR method (Applied Biosystems Model 377 Sequencer (PE Applied Biosystems, Warrington, UK).

Statistical analysis
Statistical analysis was performed using SPSS10.0 software package (SPSS Company, Chicago, IL). The statistical analysis was performed to compare the observed and expected genotype frequencies using χ2 test. Comparison of the TS genotype, allele frequency and haplotype distribution in the study groups was performed by means of two-sided contingency tables using χ2 test. The TS haplotype frequencies and linkage disequilibrium coefficient were estimated by using EH linkage software (1.2 version, Rockefeller University, NY). The odds ratio (OR) and 95% confidence interval (CI) were calculated using an unconditional logistic regression model adjusted by age and sex accordingly. A probability level of 5% was considered significant for all statistical analyses.

Results

Subject characteristics
As shown in Table I, the mean age of ESCC and GCA patients was comparable with that of healthy controls. The gender distribution in ESCC and GCA patients was also similar to that in healthy controls. Among control subjects, information on smoking status from 62 individuals was unavailable and family history of UGIC from 204 individuals was unclear or failed to be recorded. The proportion of smokers in ESCC and GCA patients (56.9 and 56.7%, respectively) was not significantly different from that in healthy controls (P = 0.06 and 0.07, respectively). However, the frequency of positive family history of UGIC in ESCC (31.0%) and GCA (34.3%) patients was significantly higher than that in healthy controls (2.4%) (P < 0.0001). Thus, family history of UGIC significantly increased the risk of developing ESCC and GCA in this population, with age and sex adjusted OR of 9.87 (95% CI = 3.99-24.43) and 11.69 (95% CI = 5.72-24.25), respectively. Among 141 ESCC and 94 GCA patients with available two repeats were expected as 243 and 215 bp, respectively. To analyze the G→C SNP in the 3R alleles, 8 μl of the PCR products, which showed genotypes with the 3R allele were further subject to digestion with HaeIII restriction enzyme (TakaRa Biotechnology, Dalian, China) in a 10-μl volume, followed by electrophoresis on a 4% agarose gel stained with ethidium bromide. HaeIII digestion produced 66-, 47-, 45-, 44-, 28- and 13-bp bands for the 3G allele and 94-, 47-, 45-, 44- and 13-bp bands for the 3C allele. The TS 3′UTR polymorphism was analyzed by PCR-RFLP as described by Ulrich et al. (12). Briefly, the fragment containing the 6-bp sequence was amplified using primers 5′-CAATCTGAGGAGCTGAGT-3′ and 5′-CAAGAAGTGGCACTACAGA-3′ in a 25-μl mixture containing 10× buffer, 100 ng of DNA, 2.5 mmol of MgCl2, 1 U of Taq-DNA-polymerase, 200 μmol of dNTPs and 300 μmol of sense and antisense primer. The cycling conditions were 1 cycle of 94°C for 5 min, 30 cycles of 94°C for 30 s, 58°C for 45 s and 72°C for 45 s, and a final extension at 72°C for 5 min. The amplified fragments were digested overnight with DraI (TakaRa Biotechnology, Dalian, China) and the products were separated on a 5% agarose gel. The expected fragments were 70 and 88 bp for the allele with 6 bp sequence (6 bp-) and 152 bp for the variant with 6 bp deletion (6 bp+).

To analyze the genomic balance status in ESCC tissues, the TS 5′UTR polymorphisms were genotyped in both of the tumor cells and corresponding normal epithelium cells. For an internal control, the β-actin gene was amplified from tissue DNA in a separate PCR reaction by using primers 5′-ATCA-TTTTGAACCTCAAT-3′ and 5′-CATCTCTGTCGCAAATC-3′, which results in a fragment of 317 bp.

For a negative control, distilled water instead of DNA in the reaction system was used for each panel of PCR. For 10% of samples, PCR was repeated once for quality control.

DNA sequence analysis
DNA sequencing analysis was used to confirm the results of TS genotyping at the 5′UTR tandem repeat locus in a subset of 12 representative samples. For the heterozygotes, each PCR fragment was recovered from agarose gel followed by purification with DNA Clean-Up Kit (Wizard SV Gel and PCR Clean-up System, Promega). The fragments were amplified again for DNA sequencing with genotyping primers. DNA sequences of the PCR products were determined using the PCR sense primer with an Applied Biosystems Model 377 Sequencer (PE Applied Biosystems, Warrington, UK).

Statistical analysis
Statistical analysis was performed using SPSS10.0 software package (SPSS Company, Chicago, IL). Hardy–Weinberg analysis was performed to compare the observed and expected genotype frequencies using χ2 test. Comparison of the TS genotype, allele frequency and haplotype distribution in the study groups was performed by means of two-sided contingency tables using χ2 test. The TS haplotype frequencies and linkage disequilibrium coefficient were estimated by using EH linkage software (1.2 version, Rockefeller University, NY). The odds ratio (OR) and 95% confidence interval (CI) were calculated using an unconditional logistic regression model adjusted by age and sex accordingly. A probability level of 5% was considered significant for all statistical analyses.

Results

Subject characteristics
As shown in Table I, the mean age of ESCC and GCA patients was comparable with that of healthy controls. The gender distribution in ESCC and GCA patients was also similar to that in healthy controls. Among control subjects, information on smoking status from 62 individuals was unavailable and family history of UGIC from 204 individuals was unclear or failed to be recorded. The proportion of smokers in ESCC and GCA patients (56.9 and 56.7%, respectively) was not significantly different from that in healthy controls (48.6%) (P = 0.06 and 0.07, respectively). However, the frequency of positive family history of UGIC in ESCC (31.0%) and GCA (34.3%) patients was significantly higher than that in healthy controls (4%) (P < 0.0001). Thus, family history of UGIC significantly increased the risk of developing ESCC and GCA in this population, with age and sex adjusted OR of 9.87 (95% CI = 3.99-24.43) and 11.69 (95% CI = 5.72-24.25), respectively. Among 141 ESCC and 94 GCA patients with available
Table I. Selected characteristics in ESCC, GCA patients and healthy controls

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control n (%)</th>
<th>ESCC n (%)</th>
<th>P-value*</th>
<th>GCA n (%)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>244 (70.1)</td>
<td>168 (72.4)</td>
<td>0.55</td>
<td>178 (76.4)</td>
<td>0.10</td>
</tr>
<tr>
<td>Female</td>
<td>104 (29.9)</td>
<td>64 (27.6)</td>
<td></td>
<td>55 (23.6)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50</td>
<td>129 (37.1)</td>
<td>69 (29.7)</td>
<td>0.07</td>
<td>72 (30.9)</td>
<td>0.13</td>
</tr>
<tr>
<td>&gt;50</td>
<td>219 (62.9)</td>
<td>163 (70.3)</td>
<td></td>
<td>161 (69.1)</td>
<td></td>
</tr>
<tr>
<td>Mean age in years (SD)</td>
<td>51.3 (10.7)</td>
<td>54.8 (10.2)</td>
<td>0.06b</td>
<td>55.0 (10.5)</td>
<td>0.06b</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ex- or current smoker</td>
<td>138 (48.6)</td>
<td>132 (56.9)</td>
<td>0.06</td>
<td>132 (56.7)</td>
<td>0.07</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>146 (51.4)</td>
<td>100 (43.1)</td>
<td></td>
<td>101 (43.3)</td>
<td></td>
</tr>
<tr>
<td>Family history of UGIC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>6 (4.2)</td>
<td>72 (31.0)</td>
<td>&lt;0.0001</td>
<td>80 (34.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Negative</td>
<td>138 (95.8)</td>
<td>160 (69.0)</td>
<td></td>
<td>153 (65.7)</td>
<td></td>
</tr>
<tr>
<td>5′UTR genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3G/3G</td>
<td>61 (17.5)</td>
<td>37 (16.0)</td>
<td>0.90</td>
<td>43 (18.5)</td>
<td>0.84</td>
</tr>
<tr>
<td>3G/3C</td>
<td>60 (17.3)</td>
<td>37 (16.0)</td>
<td></td>
<td>44 (18.9)</td>
<td></td>
</tr>
<tr>
<td>3C/3C</td>
<td>102 (29.3)</td>
<td>68 (29.3)</td>
<td></td>
<td>61 (26.2)</td>
<td></td>
</tr>
<tr>
<td>2R/3G</td>
<td>45 (12.9)</td>
<td>32 (13.8)</td>
<td></td>
<td>29 (12.4)</td>
<td></td>
</tr>
<tr>
<td>2R/3C</td>
<td>62 (17.8)</td>
<td>41 (17.6)</td>
<td></td>
<td>47 (20.2)</td>
<td></td>
</tr>
<tr>
<td>2R/2R</td>
<td>13 (3.7)</td>
<td>10 (4.3)</td>
<td></td>
<td>8 (3.4)</td>
<td></td>
</tr>
<tr>
<td>Othersa</td>
<td>5 (1.5)</td>
<td>7 (3.0)</td>
<td></td>
<td>1 (0.4)</td>
<td></td>
</tr>
<tr>
<td>5′UTR allele type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3G</td>
<td>228 (32.8)</td>
<td>145 (31.2)</td>
<td>0.58</td>
<td>159 (34.1)</td>
<td>0.62</td>
</tr>
<tr>
<td>3C</td>
<td>327 (47.0)</td>
<td>217 (46.8)</td>
<td></td>
<td>214 (45.9)</td>
<td></td>
</tr>
<tr>
<td>2R</td>
<td>136 (20.5)</td>
<td>75 (15.8)</td>
<td></td>
<td>75 (15.2)</td>
<td></td>
</tr>
<tr>
<td>4R + 5R</td>
<td>5 (0.7)</td>
<td>7 (1.5)</td>
<td></td>
<td>1 (0.2)</td>
<td></td>
</tr>
<tr>
<td>3′UTR genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 bp−/6 bp−</td>
<td>159 (45.7)</td>
<td>102 (44.0)</td>
<td>0.92</td>
<td>104 (44.6)</td>
<td>0.96</td>
</tr>
<tr>
<td>6 bp−/6 bp+</td>
<td>155 (44.5)</td>
<td>107 (46.1)</td>
<td></td>
<td>105 (45.1)</td>
<td></td>
</tr>
<tr>
<td>6 bp+/6 bp+</td>
<td>34 (9.8)</td>
<td>23 (9.9)</td>
<td></td>
<td>24 (10.5)</td>
<td></td>
</tr>
<tr>
<td>3′UTR allele type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 bp−</td>
<td>473 (68.0)</td>
<td>311 (67.0)</td>
<td>0.74</td>
<td>313 (67.2)</td>
<td>0.78</td>
</tr>
<tr>
<td>6 bp+</td>
<td>223 (32.0)</td>
<td>153 (33.0)</td>
<td></td>
<td>153 (32.8)</td>
<td></td>
</tr>
</tbody>
</table>

aP-value for χ² test.
bP-value for t-test.
cInformation on smoking status and family history was available from a subset of healthy controls.
dTS genotypes including repeat numbers of >3 (4R and 5R) were combined because of their infrequency.

tNM information, invasion of adjacent structure was reported in 53 and 37 cases and lymphatic metastasis was in 59 and 46 cases, respectively. No distant metastasis was reported in both the ESCC and GCA patients.

**Distribution of the TS polymorphisms in cases and controls**

All of the cancer patients and healthy controls were successfully genotyped for the three TS polymorphisms. The results from the re-genotyped samples completely matched the original ones. For the TS 5′UTR tandem repeat polymorphism, four different PCR fragments of ~210, 240, 270 and 300 bp in length were observed (Figure 1). Sequencing analyses showed that these four fragments contained the same sequence except for two, three, four and five copies of the 28-bp repeat sequence, respectively (data not shown). For genotypes with the 3R allele, the 28-bp tandem repeat polymorphism and the G→C SNP (Figure 2) were analyzed in combination to characterize the polymorphisms in the TS 5′UTR. As shown in Table I, the frequencies of the TS 5′UTR alleles in healthy subjects were 32.8 (3G), 47.0 (3C), 19.5 (2R) and 0.7% (4R or 5R), respectively. The genotype distribution of the TS 5′UTR polymorphisms in healthy controls was 17.5 (3G/3G), 17.3 (3G/3C), 29.3 (3C/3C), 12.9 (2R/3G), 17.8 (2R/3C) and 3.7% (2R/2R), whereas the 2R/4R (0.3%), 3C/4R (0.6%), 2R/5R (0.6%) and 3R/5R (0.0%) genotypes were very rare. For the 6 bp variant in the TS 3′UTR (Figure 3), the frequency of the 6 bp−/6 bp−, 6 bp−/6 bp+ and 6 bp+/6 bp+ genotype was 45.7, 44.5 and 9.8%, whereas the prevalence of the 6 bp− and 6 bp+ allele was 68.0 and 32.0%, respectively. The distribution of the three TS variants was not influenced by gender and age and did not significantly deviate from those expected from Hardy–Weinberg equilibrium (data not shown). Haplotype analyses showed that the 6 bp−/3C was the most frequent haplotype in the population (30.7%), followed by 6 bp−/3G

**Fig. 1.** TS 5′UTR genotyping by PCR amplification followed by separation on 3% agarose gel as described in the text. Lane 1, 100 bp ladder; lane 2, 3R/3R; lane 3, 2R/2R; lane 4, 2R/3R; lane 5, 2R/5R; lane 6, 3R/4R; lane 7, 3R/5R; lane 8, 2R/4R.

**Fig. 2.** TS 3′UTR genotypes were determined by PCR amplification (above) and HaeIII restriction enzyme digestion (below). The PCR products were additionally digested by HaeIII only if the genotypes contain the 3R allele to determine the SNP type, as described in the text. Lane 1, 100 bp ladder; lane 2, 3G/3G; lane 3, 3G/3C; lane 4, 3C/3C; lane 5, 2R/3G; lane 6, 2R/3C.

**Fig. 3.** TS 3′UTR genotyping by PCR-RFLP analysis followed by separation on 3% agarose gel as described in the text. Lane 1, 100 bp ladder; lanes 2 and 3, 6 bp−/6 bp−; lanes 4 and 5, 6 bp−/6 bp+; lanes 6 and 7, 6 bp+/6 bp+. An extra band ~158 bp always existed with the heterozygous genotype, probably due to incomplete digestion of the 6 bp− allele caused by second structure formed by the heterozygous DNA strands.
To analyze the combined effects of the TS 5’UTR and 3’UTR polymorphisms on the susceptibility to ESCC and GCA, we calculated the relative risk of each haplotype by using the 6 bp haplotype as the reference group because (i) the 6 bp–3G may produce a high level of TS due to the existence of the 3G allele and (ii) the 6 bp– allele tends to be in linkage disequilibrium with the 3G in the population. As a result, the 6 bp–2R haplotype significantly reduced the risk of developing GCA (OR = 0.48, 95% CI = 0.28–0.81) and ESCC (OR = 0.61, 95% CI = 0.37–1.00). Additionally, individuals with the 6 bp–3G haplotype had a significantly reduced risk for the development of ESCC (OR = 0.30, 95% CI = 0.14–0.67) and a similar trend was seen for GCA (OR = 0.58, 95% CI = 0.31–1.08), although the frequency difference between GCA patients and healthy controls did not reach significant level (\(\chi^2 = 3.01, P = 0.08\)).
in colorectal cancer and might be associated with the prognosis of this tumor type (35,36). We speculated that the genomic imbalance at the TS 5’UTR locus might also be a frequent event and therefore influence the significance of blood DNA genotyping in the prediction of lymphatic metastasis. Thus, tumor cells and corresponding normal epithelium cells from 66 tumor tissues were separated by microdissection and 60 pairs of DNA were successfully prepared for genotype stability analysis. The other six pairs of samples failed in DNA preparation from either tumor or normal cells, because the β-actin gene could not be successfully amplified. Among 32 pairs of samples showing TS homozygous genotypes (28 cases with 3R/3R and four with 2R/2R) in blood DNA, the genotypes obtained from tumor and normal epithelium cell DNAs were exactly the same as those found with blood DNA. However, among 28 pairs of informative samples, LOH was found in two ESCC tissues (7.1%) and both lost their 3C allele in tumor cells (Figure 4). One ESCC patient with LOH was reported to have lymphatic metastasis and another one was lymph node negative.

Discussion

Micronutrient deficiency, alone or in combination with exposure to chemical carcinogens, may induce DNA damage in epithelial cells of the esophagus (37). Therefore, DNA repair mechanisms, which can be affected by nucleotide availability, are essential for protecting epithelial cells from malignant conversion, suggesting that TS may be involved in carcinogenesis of upper gastrointestinal tumors such as ESCC and GCA. Therefore, it is interesting to know whether TS polymorphism is a valuable marker to predict risk for the occurrence and progression of ESCC and GCA, since it can be tested directly from blood DNA at any stage of the diseases.

The present case-control study shows that neither the combined polymorphisms in the TS 5’UTR nor the 6 bp− polymorphism in the TS 3’UTR is independently associated with susceptibility to ESCC and GCA, although the risk of several tumor types has been associated with the TS 5’UTR tandem repeat polymorphism (14–17). However, association of the TS haplotypes with the disposition to ESCC and GCA is observed. Since the combined effects of the TS alleles on TS transcription and translation have not been investigated so far, we used the 6 bp−/3G as the reference haplotype because the 3G allele was reported to have three to four times higher translation efficiency than the 3C and 2R alleles (10,11) and was in linkage disequilibrium with the 6 bp− allele in the study population. Our results indicate that the 6 bp+/3G haplotype had an ~3-fold decrease in risk of developing ESCC and had a similar trend to reduce the risk of GCA. Since the 6 bp+ allele has been reported to increase mRNA stability and enhance TS expression (13), we speculate that the 6 bp+/3G haplotype may produce higher TS activity than the 6 bp−/3G haplotype, providing a sufficient supply of deoxynucleotides for DNA synthesis and repair in the epithelium cells and subsequently reduce the risk of ESCC and perhaps GCA. However, the combined effects of the TS alleles on TS transcription and translation might not be simply additive, because in contrast to our expectation, the 6 bp−/2R haplotype, which theoretically may produce low levels of TS due to the co-existence of the 6 bp− and 2R alleles, also showed a significantly reduced risk for the development of ESCC and GCA. Although we cannot rule out the possibility that the results might be a skew due to a relatively small sample in the analysis, the significant difference in the haplotype frequency between GCA patients and controls (P = 0.006) and the similar trend observed in both ESCC and GCA strongly suggest that these TS polymorphisms might have joint effects on mRNA transcription, message stability and protein expression. Unfortunately, this genotype and phenotype association was not exploited in the present study because of the unavailability of the tissue samples.

Epidemiological studies have shown that ESCC and GCA may share similar etiological factors (22–27). However, the genetic susceptibility factors for ESCC and GCA have rarely been compared. The present study demonstrates that the TS 5’UTR and 3’UTR polymorphisms may play a role in the development of both ESCC and GCA. These results encourage
us to conduct further comparative studies to understand the carcinogenesis of these two cancers.

Local invasion and lymphatic metastasis are main factors influencing prognosis and survival of upper gastrointestinal tumors. To make individualized treatment plans, it is worthwhile to find out molecular markers that may predict the potential of tumor invasion and/or metastasis. One important observation in our study is that the 5′UTR 2R/3G genotype has >11-fold higher risk for developing metastatic disease of ESCC, compared with the 3G/3G genotype. This result indicates that the low level of TS expression may facilitate spreading of tumor cells into lymphatic vessels and therefore may predict a worse prognosis of ESCC. To evaluate the significance of TS genotypes in predicting clinical stages of ESCC, several issues need to be clarified. First, although the information on TNM staging was only available in a subset of cancer patients, the similar distribution of the TS polymorphisms between cancer patients with and without information on TNM suggests that selection bias did not occur. Secondly, the great difference in the 2R/3G frequency between ESCC patients with and without lymphatic metastasis (27.7 versus 4.9%) demonstrates a strong association of the TS 5′UTR polymorphism with the clinical staging of ESCC. Thirdly, two studies in Japanese reported that LOH at the TS 5′UTR tandem repeat locus occurred frequently (62 and 77%, respectively) in colorectal cancer tissues (35,36), which may predict sensitivity to 5-fluorouracil-based chemotherapy (36). The present study did find that LOH at the TS 5′UTR locus occurs in ESCC tissues (7.1%), but the frequency is far less from that reported in colorectal cancer. Although the significance of TS 5′UTR LOH in the progression of ESCC is unclear yet, its infrequency indicates that it is impossible to be a confounding factor for the association between the TS genotype identified using blood DNA and the risk of metastatic disease. Taken together, these results suggest that ESCC patients with the 2R/3G genotype may need to be treated more actively in lymph node resection and need trimodality treatment after operation. Another interesting finding in the present study is that the TS genotypes may not modify the clinical progression of GCA, although the similar role of the TS polymorphisms in risk of developing ESCC and GCA was observed. However, since this is a retrospective study and the sample size may not have power to detect small effect, further investigations with larger sample size are necessary to clarify this important issue.

In summary, this preliminary study demonstrates that the polymorphisms in the TS 5′UTR and 3′UTR may be associated with risk of developing ESCC and GCA. In addition, the TS 5′UTR 2R/3G genotype appears to be a useful molecular marker for predicting lymphatic metastasis of ESCC.

Acknowledgements

We gratefully acknowledge Mrs Liwei Zhang, Mrs Xiaoxing Guo, Mr Ming He and Mr Mingli Wu in the Fourth Affiliated Hospital of Hebei Medical University, China, for their assistance in recruiting study subjects. Supported by Grants from the National Natural Science Foundation (No. 30371591) and the Educational Department of Hebei Province (No. 2001150), China.

References


Received March 9, 2004; revised July 19, 2004; accepted July 21, 2004

Downloaded from https://academic.oup.com/carcin/article-abstract/25/12/2479/2475876 by guest on 07 March 2019