Methylenetetrahydrofolate Reductase Polymorphisms Increase Risk of Esophageal Squamous Cell Carcinoma in a Chinese Population

Chunying Song, Deyin Xing, Wen Tan, Qingyi Wei, and Dongxin Lin

Department of Etiology and Carcinogenesis, Cancer Institute, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China [C. S., D. X., W. T., D. L.]; and Department of Epidemiology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030 [Q. W.]

Abstract

Methylenetetrahydrofolate reductase (MTHFR) plays a central role in folate metabolism that affects DNA methylation and synthesis. Because germ-line mutations at nucleotides 677 (C→T) and 1298 (A→C) in the MTHFR gene cause diminished enzyme activity, and aberrant DNA methylation is oncogenic, we examined the relationship between these two MTHFR polymorphisms and susceptibility to esophageal squamous cell carcinoma (ESCC) in 240 ESCC cases and 360 age- and sex-matched controls in northern China. We found that the allele frequency of MTHFR 677T was significantly higher among cases than among controls (63% versus 41%, \(P < 0.001\)). Subjects with the 677TT genotype had a more than 6-fold increased risk of developing ESCC [adjusted odds ratio (OR), 6.18; 95% confidence interval (CI), 3.32–11.51] compared with those who had the 677CC genotype. Furthermore, the elevated ESCC risk associated with the 677 polymorphism was in an allele-dose relationship (trend test, \(P = 0.0001\)) with ORs of 1.00, 3.14 (95% CI, 1.94–5.08), and 6.18 (95% CI, 3.32–11.51) for the CC, CT, and TT genotypes, respectively, after adjustment for age, sex, smoking status, and the MTHFR 1298 polymorphism. The allele frequency for the MTHFR 1298C was 14% among cases and 17% among controls. The 1298CC genotype was extremely rare in both controls (1.4%) and cases (2.9%) and was also associated with an elevated risk of ESCC [adjusted OR, 4.43; 95% CI, 1.23–16.02] compared with the 1298AA genotype, whereas the 1298AC genotype had no effect on the risk of ESCC. Thus, our findings support the hypothesis that genetic polymorphisms in the MTHFR gene may contribute to susceptibility to carcinogenesis of the esophagus in the at-risk Chinese population.

Introduction

Folate deficiency resulting from low consumption of vegetables and fruits is associated with increased risk of several cancers, including esophageal cancer (1–4). An important biological function of folate is to provide methyl groups required for intracellular methylation reactions and de novo deoxynucleoside triphosphate synthesis; therefore, folate deficiency is thought to be carcinogenic through disruption of DNA methylation and synthesis and impaired DNA repair (5). However, folate requires metabolic transformations catalyzed by several enzymes including MTHFR.3

MTHFR is responsible for circulating form of folate, 5-methyltetrahydrofolate, which converts methionine to 5-adenosylmethionine, the universal methyl donor for various intracellular methylation reactions, particularly DNA methylation (6). Two germ-line mutations have been identified in the MTHFR locus at nucleotides 677 (C677T) and 1298 (A1298C), and the variant genotypes are associated with an increased thermolability and significantly diminished specific activity of the enzyme (7, 8). It has been shown recently that individuals with the 677TT genotype had significantly elevated homocysteine levels, indicating a decline in remethylation of homocysteine to methionine, in the plasma compared with those with the wild-type genotype (CC; Refs. 9–11) and diminished genomic DNA methylation, particularly when folate intake is inadequate (12).

ESCC is one of the most prevalent cancers in China and the world, and there are about 250,000 ESCC cases diagnosed each year in China, accounting for half of the world’s cases. Epidemiological and ecological studies of ESCC in high-risk areas in northern China have identified some environmental risk factors (13–15), including nutritional deficiency. Because low consumption of vegetables and fruits, a major source of folate, has been consistently associated with an increased risk of ESCC in the at-risk populations (16–18), and folate deficiency is implicated in human carcinogenesis, we hypothesized that MTHFR polymorphisms are associated with an increased risk of developing ESCC. Therefore, we conducted a hospital-based case-control study to examine the association between genetic polymorphisms in MTHFR and risk of ESCC.

Materials and Methods

Subjects. This analysis included 240 ESCC patients and 360 healthy controls. All subjects were unrelated ethnic Chinese. Patients were consecutive cases newly diagnosed with histologically confirmed primary ESCC at the Cancer Hospital, Chinese Academy of Medical Sciences (Beijing, China) between July 1998 and July 2000. All cases were from Beijing and the surrounding regions. Because this was a study of genotype, and the marker was a constitutional one, both incident and prevalent cases were included. Patients with primary tumors outside the esophagus or of unknown origin were excluded. Healthy control subjects were accrued from a nutritional survey conducted in the same regions. The selection of these control subjects was described previously in a case-control study of CYP1A1 genotype and lung cancer in the same regions (19). Briefly, these controls were randomly selected from a nutritional survey data base for health examination. The selection criteria included no individual history of cancer and frequency matching on gender and age distribution of the case group. At recruitment, each participant was personally interviewed to obtain detailed information on demographic characteristics, lifetime history of tobacco use, and family history of cancer. Of the total 404 controls used in the previous study (19), 44 subjects were excluded because their DNA samples were no longer available. This study was approved by the Institutional Review Board of the Chinese Academy of Medical Sciences Cancer Institute.

MTHFR Genotyping. Genomic DNA was isolated, using standard methods (20), from peripheral blood of the controls or from surgically resected “normal” tissues adjacent to the tumors of patients with esophageal cancer. The MTHFR genotypes at the C677T and A1298C sites were analyzed by PCR-based RFLP methods as described previously (21). PCR was performed in a GeneAmp 2400 thermocycler (Perkin-Elmer, Norwalk, CT), and the profile consisted of an initial melting step of 2 min at 94°C; followed by 35 cycles of
though there were more smokers in the case group (59.2%) than in the controls (47.8%). In both controls and cases, the distribution of age for the 30 s at 94°C, 30 s at 61°C, and 30 s at 72°C; and a final elongation step of 7 min at 72°C.

The restriction enzyme Hinfl (New England BioLabs, Beverly, MA) was used to distinguish the C677T polymorphism, and the gain of a Hinfl restriction site occurs in the polymorphic allele. The wild-type allele (C677C) and a 15% masked, random sample of cases (n = 36) was also used in accordance with the Hardy-Weinberg equilibrium. The allele frequency for the MTHFR 1298C was 14% among cases and 17% among controls. However, subjects homozygous for the MTHFR 1298 variant allele (CC) were extremely rare in both controls (1.4%) and cases (2.9%). The OR adjusted for sex, age, and smoking status was not elevated for the MTHFR 1298CC genotype compared with the 1298AA genotype (OR = 0.88; 95% CI, 0.59–1.32) (adjusted for age, sex, smoking status and the MTHFR 677 polymorphism).

The potential interaction between the MTHFR 677 and 1298 polymorphisms on the risk of ESCC was further examined. Table 3 shows the ORs of ESCC associated with the MTHFR 677 and 1298 genotypes. Although there was no evidence of interaction between these two polymorphisms, a joint effect between the MTHFR 677 and 1298 polymorphisms on risk of ESCC was observed. Among individuals who carried both MTHFR 677CT and 1298AA genotypes, the OR of ESCC was 2.30 (95% CI, 1.21–4.36); however, the OR increased to 19.2 (95% CI, 1.99–infinity; P = 0.048) among individuals who carried both the MTHFR 677CT and 1298AA genotypes, which were significantly associated with risk of ESCC.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Cases (n = 240)</th>
<th>Controls (n = 360)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>177</td>
<td>258</td>
<td>0.576</td>
</tr>
<tr>
<td>Female</td>
<td>63</td>
<td>102</td>
<td></td>
</tr>
</tbody>
</table>

* For χ² test.

**Results**

A summary of selected characteristics including smoking status of the cases and controls is shown in Table 1. No statistical differences were observed between cases and controls in the distribution of age and sex, suggesting that the frequency matching was adequate. Although there were more smokers in the case group (59.2%) than in the control group (51.9%; P = 0.082), there was no significant difference between cases and controls in years of smoking (15.5 ± 15.5 and 17.9 ± 18.8 years, respectively; P = 0.326). No family history of ESCC was reported among controls or cases.

The allele frequencies for MTHFR 677C and 677T were 59% and 41%, respectively, among the 360 controls and 37% and 63%, respectively, among the 240 ESCC cases (Table 2). These differences were statistically significant (P < 0.001). However, the observed frequencies of three MTHFR 677T genotypes among controls (CC, 35.0%; CT, 47.8%; and TT, 17.2%) were not different from those expected from the Hardy-Weinberg equilibrium (34.7%, 48.4%, and 16.9%, respectively; P = 0.988). A total of 38.8% of the cases were homozygous for the variant allele (TT), which was significantly higher (P < 0.001) than that of the controls (17.2%). Subjects with the MTHFR 677TT genotype had a more than 6-fold increased risk of developing ESCC (adjusted OR, 6.18; 95% CI, 3.32–11.51) compared with subjects with the MTHFR 677CC genotype. Furthermore, the elevated risk of ESCC associated with the MTHFR 677T polymorphism was in an allele-dose relationship (trend test, P = 0.0001) with ORs of 1.00, 3.14 (95% CI, 1.94–5.08), and 6.18 (95% CI, 3.32–11.51) for the CC, CT, and TT genotype, respectively. In the stratification analysis, age, sex, and smoking status had no effect on the risk of ESCC related to this polymorphism (data not shown). The fact that a single MTHFR variant allele (i.e., CT genotype) was associated with a significantly increased ESCC risk (3-fold) suggests that this polymorphism had a dominant effect on the risk of ESCC.

The distribution of MTHFR 1298AA, 1298AC, and 1298CC genotypes among controls was 67.2%, 31.4%, and 1.4%, respectively (Table 2) and was also in accordance with the Hardy-Weinberg equilibrium. The allele frequency for the MTHFR 1298C was 14% among cases and 17% among controls. However, subjects homozygous for the MTHFR 1298 variant allele (CC) were extremely rare in both controls (1.4%) and cases (2.9%). The OR adjusted for sex, age, and smoking status was not elevated for the MTHFR 1298CC genotype compared with the 1298AA genotype (OR = 0.88; 95% CI, 0.59–1.32) (adjusted for age, sex, smoking status and the MTHFR 677 polymorphism).

**Statistical Analysis.** The association between the MTHFR polymorphisms and risk of ESCC was estimated by ORs and their 95% CIs, which were calculated by unconditional logistic regression. Because of the use frequency matching, the ORs were also adjusted for age, gender, and smoking status. Tests for independence and interaction between the MTHFR 677 and 1298 polymorphisms were performed by using the likelihood ratio test (22). All analyses were performed using Statistical Analysis System software (version 6.12; SAS Institute, Cary, NC).

<table>
<thead>
<tr>
<th>MTHFR genotype</th>
<th>Cases (n = 240)</th>
<th>Controls (n = 360)</th>
<th>OR* (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR 677</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>29 (12.0)</td>
<td>126 (35.0)</td>
<td>1.00</td>
</tr>
<tr>
<td>CT</td>
<td>118 (49.2)</td>
<td>172 (47.8)</td>
<td>3.14 (1.94–5.08)</td>
</tr>
<tr>
<td>TT</td>
<td>93 (38.8)</td>
<td>62 (17.2)</td>
<td>6.18 (3.32–11.51)</td>
</tr>
<tr>
<td>MTHFR 1298</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>179 (74.6)</td>
<td>242 (67.2)</td>
<td>1.00</td>
</tr>
<tr>
<td>AC</td>
<td>54 (22.5)</td>
<td>113 (31.4)</td>
<td>0.88 (0.59–1.32)</td>
</tr>
<tr>
<td>CC</td>
<td>7 (2.9)</td>
<td>5 (1.4)</td>
<td>4.43 (1.23–16.02)</td>
</tr>
</tbody>
</table>

* ORs and 95% CIs were calculated in a logistic regression model with MTHFR 677CC or 1298AA as the reference group and adjustment for age, sex, smoking status, and relevant polymorphism.

**Table 3 Risk of ESCC associated with the MTHFR C677T genotypes by A1298C genotypes in a Chinese population**

<table>
<thead>
<tr>
<th>MTHFR genotype</th>
<th>Cases (%)</th>
<th>Controls (%)</th>
<th>OR* (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>AA</td>
<td>15 (6.2)</td>
<td>59 (16.4)</td>
</tr>
<tr>
<td>CC</td>
<td>AC</td>
<td>9 (3.8)</td>
<td>62 (17.2)</td>
</tr>
<tr>
<td>CC</td>
<td>AC</td>
<td>5 (2.1)</td>
<td>5 (1.4)</td>
</tr>
<tr>
<td>CT</td>
<td>AA</td>
<td>75 (31.3)</td>
<td>123 (34.2)</td>
</tr>
<tr>
<td>CT</td>
<td>AC</td>
<td>41 (17.1)</td>
<td>49 (13.6)</td>
</tr>
<tr>
<td>CT</td>
<td>CC</td>
<td>2 (0.8)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>TT</td>
<td>AA</td>
<td>89 (37.1)</td>
<td>60 (16.7)</td>
</tr>
<tr>
<td>TT</td>
<td>AC</td>
<td>4 (1.6)</td>
<td>2 (0.6)</td>
</tr>
<tr>
<td>TT</td>
<td>CC</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>

* ORs and 95% CIs were calculated in a logistic regression model with both MTHFR 677CC and 1298AA as the reference group and adjustment for age, sex, and smoking status.

* Test of significance and the 95% CI were based on the exact conditional distribution.  
  
  
P = 0.048.
and 1298CC genotypes. The upper limit of the CI for the OR was infinite because no control subject carried both variant 677CT and 1298CC genotypes. We also found the existence of the 677TT/1298AC or 677CT/1298CC variants to be rare (0.6% in controls and 2.4% in cases; Table 3) and a complete absence of 677TT/1298CC genotypes in our study populations, which are consistent with findings reported by other investigators (8, 21), indicating a possible lethal effect of the 677TT/1298CC genotype.

**Discussion**

In the present study, we investigated whether genetic polymorphisms in *MTHFR*, a gene that plays a central role in folate metabolism, could have an impact on the risk of developing ESCC. We observed a significant difference in the distribution of *MTHFR* C677T and *MTHFR* A1298C genotype frequencies among healthy controls and ESCC patients. Subjects homozygous for the *MTHFR* 677TT or 1298C allele had a >6-fold or a >4-fold increased risk of developing ESCC. Furthermore, the elevated risk of ESCC associated with the *MTHFR* C677T polymorphism was in an allele-dose relationship, suggesting a dominant effect of this polymorphism on susceptibility to ESCC. We also found a joint effect between *MTHFR* 677CT and 1298CC variant genotypes on risk of ESCC, although this finding is limited due to the small number of subjects included in the analysis. These data clearly demonstrate that the *MTHFR* polymorphisms are a genetic determinant in the development of ESCC in the at-risk Chinese population. The impact of these genetic alternations on ESCC could be substantial because the *MTHFR* polymorphic alleles, especially the 677TT allele, are prevalent in this study population. Because ESCC is a malignant disease that has been linked to low consumption of dietary folate in high-risk populations in China, our findings are biologically plausible and therefore etiologically significant.

Although the study subjects included in this hospital-based case-control study are not representative of the general Chinese population, the results of this study, which had a relatively large number of subjects, solid and reproducible genotyping techniques, and significantly increased odds ratios with small P values, are unlikely to be due to selection bias. The fact that allele and genotype frequencies among our controls are consistent with those derived from the Hardy-Weinberg equilibrium and those reported previously in the Chinese population by other investigators (23) further supports the randomness of our control selection. Furthermore, the observed effect was not affected by other potential predictors of ESCC risk such as age, sex, and smoking. Therefore, it is unlikely that subject selection or unknown confounding factors could have biased our results in this study.

More importantly, our findings are biologically plausible because both variant *MTHFR* 677T and 1298G genotypes result in diminished activity of *MTHFR* (7, 8), which plays a central role in regulating folate metabolism toward methyl donor formation and de novo nucleotide synthesis. This metabolic pathway is believed to be critical in maintaining normal DNA methylation and DNA synthesis and repair (5). Studies have suggested that cancer risk associated with the *MTHFR* polymorphisms may exhibit a gene-nutrient interaction that depends on the level of folate intake (24, 25). Based on this hypothesis, when folate intake is sufficient, individuals with the variant *MTHFR* genotypes may have a reduced risk because under these conditions, adequate provision of methyl donors could still be ensured, which would enhance DNA synthesis affected by inhibition of the 5-methyl-tetrahydrofolate pathway due to diminished MTHFR enzyme activity and result in a decreased risk of DNA damage. However, in the presence of low folate intake, both impaired DNA methylation and DNA synthesis/repair may become the primary mechanism of carcinogenesis in those who have the variant *MTHFR* genotypes. In agreement with this hypothesis, several case-control studies have shown that in those with the variant *MTHFR* 677T allele, decreased risk of colorectal neoplasia was observed among subjects with adequate folate levels, and elevated risk was observed among subjects with low folate intake (25). Furthermore, there is evidence that the polymorphic *MTHFR* locus was associated with an increased risk of endometrial cancer (20), cervical intraepithelial neoplasia (26), and breast and/or ovarian cancer (27). Therefore, our findings in the present study are generally consistent with these observations and provide evidence for the first time that folate metabolism may also play an important role in carcinogenesis of the esophagus.

Although we do not have the data for folate intake in the present study to further examine the gene-nutrient interaction, previous epidemiological studies (16–18) conducted in this Chinese population have indicated an inverse association between consumption of vegetables and fruits, a major source of folate, and risk of ESCC. One of the molecular mechanisms through which the *MTHFR* polymorphisms increase the risk of developing ESCC may be DNA hypomethylation. In a recent study (12), it was shown that genomic DNA methylation was significantly lower in the subjects with the *MTHFR* 677TT genotype compared with those with the 677CC genotype, and the methylation status in subjects with the *MTHFR* 677TT genotype was directly correlated with RBC folate levels. Another molecular mechanism is that the *MTHFR* polymorphisms may result in increased DNA damage and impaired DNA repair, leading to esophageal carcinogenesis. For example, folate deficiency has also been shown to diminish the DNA repair capacity of human lymphocytes in vitro and the mismatch repair system in ulcerative colitis patients (28, 29). Taken together, these data provide very plausible molecular mechanisms through which suboptimal cellular folate levels and the *MTHFR* polymorphisms could increase the risk for development of ESCC.

In conclusion, this study demonstrates a significant association between the *MTHFR* polymorphisms and risk of ESCC in a high-risk population in China, providing a genetic basis for the hypothesis that low folate intake and/or impaired folate metabolism may play a role in carcinogenesis in the esophagus. Because this is the first report on the association between genetic polymorphisms in *MTHFR* and susceptibility to ESCC, additional studies on the role of gene (the *MTHFR* polymorphisms)-environment (folate and alcohol intake) interaction are needed to confirm the role of these genetic polymorphisms in the etiology of ESCC. Because folate supplementation may overcome the effects of genetically determined reduction of *MTHFR* activity, our results also suggest a potential role of folate supplement in chemoprevention of ESCC in an at-risk population of individuals carrying the variant *MTHFR* alleles.

**References**

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