Susceptibility to Gastric Cardia Adenocarcinoma and Genetic Polymorphisms in Methylenetetrahydrofolate Reductase in an At-Risk Chinese Population[1]

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Abstract

Methylenetetrahydrofolate reductase (MTHFR) plays a central role in converting folate to methyl donor for DNA methylation, an epigenetic modification known to be dysregulated in carcinogenesis. Our previous study revealed that *MTHFR* polymorphisms contribute to a great risk of esophageal cancer in a Chinese population. This case-control study was to examine the association between *MTHFR* polymorphisms and gastric cardia adenocarcinoma (GCA), which is also prevalent in high-risk areas of esophageal cancer and thus may share common etiological factors with esophageal cancer in this population. The study subjects were 217 patients with GCA and 468 population controls matched on sex and age. The *MTHFR* C677T and A1298C genotypes were detected by a PCR-based RFLP assay. It was found that subjects with the *MTHFR* 677TT variant genotype had a 2-fold increased risk of GCA (odds ratio, 2.04; 95% confidence interval, 1.28–3.26). Moreover, a significantly elevated risk was also seen among the *MTHFR* 677CT heterozygotes (odds ratio, 1.56; 95% confidence interval, 1.03–2.36). The *MTHFR* A1298C polymorphism had no effect on risk of GCA. These findings are generally consistent with our initial observation for esophageal cancer and suggest that the *MTHFR* genotype may be a determinant of GCA among this at-risk Chinese population.

Introduction

Epidemiological studies have shown an association between low consumption of vegetables and fruits and increased risk of cancers including gastroesophageal cancer (1–3). Folate is one of the important constituents of vegetables and fruits that may provide protection against cancer. An important function of folate is to provide methyl groups required for intracellular methylation reactions and *de novo* deoxynucleoside synthesis. Therefore, folate deficiency is thought to be carcinogenic through disruption of DNA methylation, synthesis, and repair (4, 5). However, to serve as a mediator of methylation, folate requires metabolism catalyzed by several enzymes. Thus, it is likely that not only folate deficiency but also functional polymorphisms in genes associated with impaired folate metabolism may contribute to cancer risk.

MTHFR[3] plays a central role in biotransformation of folate to form *S*-adenomethionine, the universal methyl donor in cells (6). Two single nucleotide polymorphisms in the *MTHFR* gene, 677C→T and 1298A→C, have been identified, and the variant genotypes are associated with a significant reduction of enzyme activity (7, 8). Individuals with the *MTHFR* variant genotypes have elevated plasma homocysteine levels compared with the wild-type genotype, indicating a decline in remethylation of homocysteine to methionine (9, 10). It has been shown that genomic DNA methylation is diminished in subjects with the *MTHFR* 677TT genotype, particularly when folate intake is inadequate (11, 12). DNA hypomethylation has been linked to the activation of oncogenes and chromosome instability (13, 14), which are common events in carcinogenesis.

GCA is a common cancer in China as well as in the rest of the world. In China, this cancer is more prevalent in areas of high-risk of esophageal cancer. For example, in Linxian, a well-known high-risk area for esophageal cancer, one-third of the gastroesophageal cancers occurred in the gastric cardia (15). GCA differs from gastric cancer at other sites in epidemiological characteristics, etiology, pathogenesis, and clinical behavior (16) but may share common risk factors with esophageal cancer for carcinogenesis. Epidemiological studies have identified some environmental risk factors for esophageal cancer and GCA, which include nutritional deficiency, especially low consumption of vegetables and fruits, a major source of folate (1–3, 17–19). Because folate deficiency is linked to carcinogenesis, we hypothesized that the *MTHFR* polymorphisms, which disrupt folate metabolism, may play a role in developing gastroesophageal cancer. Recently, we have shown a strong association between *MTHFR* C677T and A1298C polymorphisms and increased risk of esophageal cancer in a high-risk population in China (20). This report described a case-control study that aimed to test the hypothesis in GCA in the same population.

Materials and Methods

Study Subjects. This case-control study consisted of 217 patients with GCA and 468 population controls. All subjects were

Received 3/22/02; revised 6/19/02; accepted 8/5/02.
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[1] Supported by Grant 39825112 from the National Natural Science Foundation and Grant G1998051204 from State Key Basic Research Program.
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[3] The abbreviations used are: MTHFR, methylenetetrahydrofolate reductase; GCA, gastric cardia adenocarcinoma; OR, odds ratio; CI, confidence interval.

Table 1 Select characteristics and smoking status in patients with GCA and controls

Variable	Cases (n = 217)	Controls (n = 468)	P^a
Age, *n* (%)			0.68
≤55 years	79 (36.4)	178 (38.0)	
>55 years	138 (63.6)	290 (62.0)	
Sex, *n* (%)			0.25
Male	178 (82.0)	366 (78.2)	
Female	39 (18.0)	102 (21.8)	
Smoking status, *n* (%)			0.81
Never	92 (42.4)	203 (43.4)	
Ever	125 (57.6)	265 (56.6)	

[a] Two-sided χ^2 test.

ethnic Han Chinese and residents of Beijing and the surrounding regions. Patients were recruited between July 1999 and July 2001 at the Cancer Hospital, Chinese Academy of Medical Sciences (Beijing, China). All patients with histologically confirmed GCA were enrolled, yielding a 100% response rate. Patients with primary tumors outside the gastric cardia or of unknown origin were excluded. Control subjects were cancer-free individuals living in the same region as the cases. They were selected from a community cancer screening program for early detection of cancer conducted during the same period as the cases were collected. These control subjects were randomly selected from a pool of 2800 individuals based on a physical examination, and the response rate was 96%. The selection criteria included no individual history of cancer and frequency matching to cases by sex and age (±5 years). At recruitment, each control subject was solicited to donate a 2-ml blood sample. Personal data from each participant regarding demographic characteristics such as sex and age and related risk factors including tobacco smoking and family history of gastroesophageal cancer were collected by questionnaire. This study was approved by the institutional review board of the Chinese Academy of Medical Sciences Cancer Institute.

MTHFR Genotyping. Genomic DNA was extracted from blood samples of the controls and from surgically resected "normal" tissues adjacent to the tumor of GCA patients. *MTHFR* genotypes at C677T and A1298C sites were analyzed by PCR-based RFLP methods as described previously (20). Briefly, the PCR primers for the C677T site were 677F (5′-TGA-AGG-AGA-AGG-TGT-CTG-CGG-GA-3′) and 677R (5′-AGG-ACG-GTG-CGG-TGA-GAG-TG-3′), which produce a 198-bp fragment. The primers for the A1298C site were 1298F (5′-CTT-TGG-GGA-GCT-GAA-GGA-CTA-CTA-C-3′) and 1298R (5′-CAC-TTT-GTG-ACC-ATT-CCG-GTT-TG-3′), which generate a 163-bp fragment. These fragments were amplified separately but under the same conditions, as follows: a 25-μl reaction mixture consisted of ~100 ng of template DNA, 0.5 μM each primer, 0.2 mM each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, and 1.2 units of Taq DNA polymerase with 1× Reaction Buffer (Promega, Madison, WI). The PCR profile consisted of an initial melting step of 2 min at 94°C; followed by 35 cycles of 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 enzymes *Hin*fI and *Mbo*II (New England Biolabs, Beverly, MA) were used to distinguish the C677T and A1298C polymorphisms, respectively. The 677CC wild-type homozygotes were identified by the presence of only a 198-bp fragment; 677CT heterozygotes were identified by the presence of 198-, 175-, and 23-bp fragments; and 677TT homozygotes were identified by the presence of 175- and 23-bp fragments. The 1298AA wild-type homozygotes produce five fragments of 56, 31, 30, 28, and 18 bp; 1298AC heterozygotes produce six fragments of 84, 56, 31, 30, 28, and 18 bp; and 1298CC homozygous variants produce four fragments of 84, 31, 30, and 18 bp. To ensure quality control, genotyping was performed with blinding to case/control status, and a 15% random sample of cases and controls was genotyped twice by different persons, and the reproducibility was 100%.

Table 2 Genotype frequencies of *MTHFR* 677 and 1298 polymorphisms among cases with GCA and controls and their contributions to the risk of GCA

MTHFR	Cases (n = 217)	Controls (n = 468)	OR (95% CI)[a]
	n (%)	*n* (%)	
C677T genotype			
CC	47 (21.7)	151 (32.3)	Reference
CT	107 (49.3)	217 (46.4)	1.56 (1.03–2.36)
TT	63 (29.0)	100 (21.3)	2.04 (1.28–3.26)
T allele frequency	0.54	0.44	
A1298C genotype			
AA	150 (69.1)	324 (69.2)	Reference
AC	64 (29.5)	139 (29.7)	1.19 (0.81–1.74)
CC	3 (1.4)	5 (1.1)	2.41 (0.51–11.37)
C allele frequency	0.16	0.16	

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

Statistical Analysis. Pearson's χ^2 test was used to examine differences in demographic variables, smoking, and distributions of genotypes between cases and controls. The association between the *MTHFR* polymorphisms and risk of GCA was estimated using ORs and their 95% CIs, which were calculated by unconditional logistic regression and adjusted for age, sex, and smoking status. Tests for interaction between the *MTHFR* 677 and 1298 polymorphisms were performed by using the likelihood ratio test. All analyses were performed with Statistical Analysis System software (version 6.12; SAS Institute, Cary, NC).

Results

The relevant characteristics of the study subjects are shown in Table 1. The distributions of age and gender among cases and controls were not statistically different. The median age was 56.5 years (range, 41–72 years) for the case group and 54.1 years (range, 45–76 years) for the control group. Eighty-two percent of cases and 78.2% of controls were male. The distribution of smokers was not significantly different between cases (57.6%) and controls (56.6%). No familial GCA was reported both in cases and in controls.

Genotyping results (Table 2) show that the allele frequency for *MTHFR* 677T was 0.54 among GCA patients compared with 0.44 among controls. The observed frequencies of three *MTHFR* C677T genotypes among controls (*CC*, 32.3%; *CT*, 46.4%; and *TT*, 21.3%) were not different from those expected from the Hardy-Weinberg equilibrium (P = 0.65). However, they were significantly different from those among cases (*CC*, 21.7%; *CT*, 49.3%; and *TT*, 29.0%; χ^2 = 9.7; P = 0.008). Subjects who carried the *MTHFR* 677TT genotype were at a 2-fold increased risk for developing GCA (adjusted OR, 2.04; 95% CI, 1.28–3.26) compared with subjects who carried the *MTHFR* 677CC genotype. Furthermore, a significantly elevated risk of GCA was also observed among subjects carrying

the *MTHFR* 677CT genotype (adjusted OR, 1.56; 95% CI, 1.03–2.36).

The allele frequencies for *MTHFR* 1298C were 0.16 in both GCA patients and controls. The distribution of A1298C genotypes among controls (*AA*, 69.2%; *AC*, 29.7%; and *CC*, 1.1%) was also in accordance with the Hardy-Weinberg equilibrium ($P = 0.17$) and was not significantly different from that among cases (*AA*, 69.1%; *AC*, 29.5%; and *CC*, 1.4%). Although the adjusted ORs of GCA for the *MTHFR* 1298AC and 1298CC genotypes were 1.19 (95% CI, 0.81–1.74) and 2.41 (95% CI, 0.51–11.37) compared with the 1298AA genotype, none of them reached statistical significance (Table 2). In the stratification analysis, sex, age, and smoking status had no effect on the risk of GCA related to the two polymorphisms in the *MTHFR* gene, whereas adjustment for the *MTHFR* 677 polymorphism had great effect on the OR for the 1298CC genotype (OR increased from 1.30 to 2.41).

Results of the analysis of the combined effect of the *MTHFR* 677 genotypes and 1298 genotypes on risk of GCA are shown in Table 3. No subject in our study was homozygous for the mutant alleles at both sites (677TT/1298CC). Although the existence of the 677CT/1298CC or 677TT/1298AC variants was rare, cases appeared more likely to carry both of these variant genotypes than their corresponding controls. Among subjects carrying both *MTHFR* 677CT and 1298AA genotypes, the OR was 2.47 (95% CI, 1.33–4.60); however, the OR was 13.46 (95% CI, 1.19–infinity) among individuals who carried both 677CT and 1298CCgenotypes. Similarly, the OR for individuals carrying the 677TT/1298AC genotype was 11.03 (95% CI, 1.37–88.89) compared with 2.80 (95% CI, 1.50–5.24) for those carrying the 677TT/1298AA genotype. However, a homogeneity test showed that none of these differences was statistically significant, probably due to the very small number of both variant alleles in analysis.

Table 3 Risk of GCA associated with the *MTHFR* C677T genotypes by A1298C genotypes

MTHFR 677	*MTHFR* 1298	Cases (%)	Controls (%)	OR (95% CI)[a]
CC	*AA*	17 (7.8)	78 (16.7)	Reference
CC	*AC*	28 (12.9)	68 (14.5)	1.99 (0.98–4.06)
CC	*CC*	2 (0.9)	5 (1.1)	1.55 (0.25–9.78)
CT	*AA*	73 (33.5)	148 (31.6)	2.47 (1.33–4.60)
CT	*AC*	33 (15.2)	69 (14.8)	2.23 (1.12–4.46)
CT	*CC*	1 (0.5)	0 (0.0)	13.46[b,c] (1.19–infinity)
TT	*AA*	60 (27.8)	98 (20.9)	2.80 (1.50–5.24)
TT	*AC*	3 (1.4)	2 (0.4)	11.03[b,d] (1.37–88.89)
TT	*CC*	0 (0.0)	0 (0.0)	

[a] ORs and 95% CIs were calculated in a logistic regression model with both *MTHFR* 677CC and 1298AA as the reference group and adjusted for sex, age, and smoking status.
[b] Test of significance and the 95% CI were based on the exact conditional distribution.
[c] $P = 0.19$.
[d] $P = 0.008$.

Discussion

We have shown in our previous study that the *MTHFR* 677 and 1298 polymorphisms were strong genetic risk factors for esophageal cancer in a high-risk Chinese population (20). Starting with the hypothesis that GCA may share common etiological factors with esophageal cancer in this population, we examined whether these *MTHFR* polymorphisms could also have impact on risk of GCA. On the basis of this study, a 2-fold increased risk of GCA was observed among subjects with the *MTHFR* 677TT genotype. In addition, elevated GCA risk was also found among the *MTHFR* 677CT heterozygotes, indicating a dominant effect of this polymorphism on the disease. These results are generally in agreement with our previous findings for esophageal cancer (20) and demonstrate that the *MTHFR* genotype is a determinant not only for esophageal cancer but also for GCA.

Recently, Shen *et al.* (21) reported a similar case-control study conducted in Jiangsu Province in southeastern China. Their findings are very similar to ours, showing that an increased risk of GCA (OR, 2.47; 95% CI, 1.14–5.32) was associated with the *MTHFR* 677TT genotype. However, they did not observe the 677CT genotype as a risk genotype for GCA. The reason for this inconsistency could be simply due to small sample size (only 82 GCA cases and 166 controls) in their study. Because the heterozygous alleles (677CT) also have significantly reduced MTHFR activity (7, 10, 22), it was not surprising to observe a higher risk of GCA for subjects with this genotype as compared with those with the wild-type genotype. In the present study, we did not find any association of the A1298C polymorphism with risk of GCA and joint effect between A1298C and C677T polymorphisms, which is consistent with that reported by Shen *et al.* (21) for GCA but is not in agreement with our previous observation for esophageal cancer (20). A possible explanation for this difference between GCA and esophageal cancer could be that the esophageal tissue may be more susceptible than gastric cardia to carcinogenesis due to *MTHFR* polymorphisms because reduction of MTHFR functional activity caused by the 1298A→C mutation is significantly less than that caused by the 677C→T mutation (10). After this paper had been submitted for publication, Stolzenberg-Solomon *et al.* (23) reported a similar result in a cohort study in Linxian, China, a high-risk area for esophageal squamous cell carcinoma and GCA. They showed that individuals with the *MTHFR* 677TT genotype had significantly higher risk for these two cancers (relative risk, 1.45; 95% CI, 1.02–2.05). In addition, they showed that an increased risk of esophageal cancer was also related to a polymorphism in *MTRR*, a gene encoding for methionine synthase reductase, for which folate and vitamin B_{12} are cofactors. These findings further support our observations in the present study and previous one (20).

Although selection bias and/or systematic error might occur in a case-control study due to inappropriate selection of subjects and the presence of other confounding factors, the results in this study, which had a relatively large number of subjects, solid and reproducible genotyping techniques, and significantly increased ORs with small *P*s, are unlikely to be due to selection bias. The fact that allele and genotype frequencies among our controls in this independent study are consistent with those in our previous study (20) and those reported in the Chinese population by other investigators (21, 24) further supports the randomness of our control selection. In addition, the observed risk effect of the variant *MTHFR* 677 genotypes was not influenced by other potential predictors of GCA risk such as age, sex, and smoking. Thus, it is unlikely that subject selection or unknown confounding factors could have biased our results in this study.

Because folate deficiency is associated with cancer, and folate requires metabolism for its functions, an impact on cancer risk by impaired folate metabolism resulting from polymorphisms of *MTHFR* is biologically plausible. MTHFR plays a central role in folate metabolism, and low MTHFR activity may prevent the shunting of methyl groups from *de novo* dTMP synthesis, a rate-limiting step for DNA synthesis, to methyla-

tion pathways (6). Although these two pathways are both important in protecting against carcinogenesis, different mechanisms might exist to influence susceptibility to carcinogenesis via balances between the methyl group provision and dTMP synthesis in individuals with the variant *MTHFR* genotypes. It has been suggested that cancer risk associated with *MTHFR* polymorphisms may be modulated by folate intake (25, 26). When folate intake is sufficient, individuals carrying the variant *MTHFR* genotypes may have a decreased risk because under these conditions, while adequate provision of methyl donors could still be ensured, enhanced genomic integrality would be achieved via conserving folate within a cyclic pathway inside cells by shunting methyl groups toward nucleotide synthesis due to diminished MTHFR activity. However, in the population where folate intake is low, both DNA methylation and DNA synthesis/repair might be impaired in the carriers of variant *MTHFR* genotypes, which, in turn, results in increased risk of carcinogenesis. This hypothesis for gene-nutrient interaction may explain the conflicting reports showing reduced risk of leukemia (27, 28) and colorectal cancer (25, 26) or elevated risk of endometrial cancer (29), cervical intraepithelial neoplasia (30), breast and/or ovarian cancer (31), esophageal cancer (20), and GCA (Ref. 21 and this study). However, alternative mechanisms may also exist to explain these conflicting results. For example, this may reflect the variation of different cancer sites and histology in response to the aberrant methylation resulting from *MTHFR* polymorphisms. It is possible that in the case of cancer types for which *MTHFR* polymorphisms increase the risk, activation of proto-oncogenes by aberrant methylation may be predominant mechanism, whereas in the case of cancers for which *MTHFR* polymorphisms reduce the risk, deficiency in methyl donors may protect against hypermethylation-induced silencing of tumor suppressor genes and thus lower the risk. It is interesting to note that folic acid deficiency-induced uracil incorporation into primary human lymphocyte DNA was not altered *in vitro* by the *MTHFR* C677T polymorphism (32). Additional studies to test the effects of *MTHFR* polymorphisms and folate deficiency on different types of cells would be helpful to clarify the mechanisms.

Folate deficiency has been shown to be common in various regions of China including Beijing (33–35), and a number of epidemiological studies conducted in the Chinese population have consistently indicated an inverse association between consumption of vegetables and fruits, a major source of folate, and risk of gastroesophageal cancer (2, 3, 19). Based on these data and findings observed in our present study, DNA hypomethylation should be considered as a possible molecular mechanism by which *MTHFR* polymorphisms increase the risk of GCA development. Consistent with this postulation, a recent study has shown that genomic DNA methylation was significantly lower in subjects with the *MTHFR* 677TT genotype compared with those with the 677CC genotype and that the methylation status in subjects with the *MTHFR* 677TT genotype was directly correlated with RBC folate levels (11, 12). DNA hypomethylation can result in chromosome instability and aberrant gene expression, which are commonly observed in many human cancers and in the early stages of carcinogenesis (13, 14, 36, 37). For instance, it has been shown that global genomic DNA hypomethylation caused by folate depletion is often accompanied by overexpression of oncogenes such as c-*myc* and c-Ha-*ras* (38, 39) and mutations in tumor suppressor genes such as *Apc* and *p53* (40) in carcinogenesis. Taken together, these data provide very plausible molecular mechanisms through which suboptimal cellular folate levels and *MTHFR* polymorphisms could increase the risk for development of GCA.

In conclusion, our study demonstrates a significant association between the *MTHFR* C677T variant genotypes and risk of GCA in an at-risk Chinese population. These results are consistent with the findings in our previous study for esophageal cancer and strongly indicate that folate deficiency and/or impaired folate metabolism may play a role in gastroesophageal carcinogenesis. A larger population-based case-control study designed to determine interactions between folate/homocysteine metabolism genes and between these genes and folate intake on elevated risk of gastroesophageal cancers is under way in our laboratory.

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