Aberrant DNA methylation of the *P16*, *MGMT*, and *hMLH1* genes in combination with the methylenetetrahydrofolate reductase C677T genetic polymorphism and folate intake in gastric cancer

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ABSTRACT. Epidemiological studies have indicated that folate metabolism is correlated with increased risk of gastric cancer. Since methylenetetrahydrofolate reductase (MTHFR) is an important enzyme involved in folate metabolism, in this study, we examined whether polymorphisms and haplotypes of *MTHFR* are correlated with the risk of gastric cancer. The polymorphisms *MTHFR* C677T and *MTHFR* A1298C were genotyped by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis in 285 patients and 570 healthy controls. Association analyses based on binary logistic regression were conducted to determine the odds ratio (OR) and its 95% confidence interval (95%CI) for each genotype. The *MTHFR* 677TT genotype was significantly related with a reduced risk of gastric cancer (OR = 0.60, 95%CI = 0.39-0.92) compared to the CC genotype. Similarly, the *MTHFR* 1298CC genotype was significantly

associated with a decreased risk of cancer (OR = 0.52, 95%CI = 0.32-0.81). Haplotype analysis showed that the TC haplotype was associated with a reduced risk of gastric cancer compared to the most common haplotype, CA (OR = 0.28, 95%CI = 0.12-0.60). Our results suggest that the *MTHFR* C677T and *MTHFR* A1298C polymorphisms are related to gastric cancer susceptibility in the Chinese population.

Key words: Methylenetetrahydrofolate reductase; Gastric cancer; Susceptibility; Haplotype analysis

INTRODUCTION

Gastric cancer is one of the most common types of tumors worldwide, and it ranks as the second leading cause of cancer-related deaths (Lao-Sirieix et al., 2010). Its incidence shows wide geographical variation, and almost two-thirds of all gastric cancer cases occur in developing countries, with 42% of them occurring in China alone. Based on the incidence and mortality rates of gastric cancer, the absolute number of cases is predicted to increase by the year 2050 (Forman and Burley, 2006). Gastric cancer is caused by multiple factors, including environmental and genetic factors, which may interact to affect tumor promotion and progression (Hamajima et al., 2006; Bai et al., 2008; Pan et al., 2012). Epidemiological studies have indicated that folate metabolism is correlated with an increased risk of gastric cancer, and that low intake of folate and polymorphisms in folate-metabolizing genes affect folate metabolism, and thus affect the susceptibility to gastric cancer (Choi and Mason, 2000; Miao et al., 2002; Götze et al., 2007).

Methylenetetrahydrofolate reductase (MTHFR), an important enzyme in folate metabolism, has a major impact on the regulation of the folic acid pathway due to the conversion of 5,10 methylenetetrahydrofolate (methylene-THF) to 5-methyl-THF. The most common polymorphisms in the *MTHFR* gene are C677T and A1298C, and consequent variations of the protein could change the enzyme activity. In particular, decreases in methyl-THF are associated with hyperhomocysteinemia, especially in a folate deficiency state. The *MTHFR* C677T (rs1801133) and *MTHFR* A1298C (rs1801131) variants have been associated with decreased MTHFR activity, increased homocysteine levels, and an altered mechanism of folate (Frosst et al., 1995; Bagley and Selhub, 1998; Brattström et al., 1998). The frequencies of *MTHFR* C677T (rs1801133) and *MTHFR* A1298C (rs1801131) vary across different ethnic groups, and variants of the two proteins commonly occur in Caucasian, Asian, and Hispanic populations, with a variation rate of 25 to 45% (Botto and Yang, 2000; Inoue et al., 2007). Previous experimental studies indicated that the low activity of *MTHFR* C677T (rs1801133) and *MTHFR* A1298C (rs1801131) are correlated with DNA hypomethylation, which may cause genomic instability and inactivate the tumor suppression genes (Brockton, 2006). Previous epidemiological studies have shown that variants of *MTHFR* might play an important role in the susceptibility to cancers and some disorders, such as colorectal cancer, cervical cancer, gastric cancer, vascular diseases, and neural tube defects (Neves et al., 2010; Teng et al., 2013; van Huis-Tanja et al., 2013; Yu et al., 2013). The association between gastric cancer risk and variants of *MTHFR* has been widely investigated; however, the results are inconsistent (Cui et al., 2010; Dong et al., 2010; Neves et al., 2010). Small sample sizes; inclusion of various ethnic groups; and variations in diet, environmental factors, and study methods

may contribute to the discrepancies across these studies. We conducted a case-control study to determine whether polymorphisms and haplotypes of *MTHFR* are correlated with the risk of gastric cancer.

The *P16*, *MGMT*, and *HMLH1* genes are important tumor suppression and DNA repair genes, and previous studies have shown that inactivation of these genes could increase the carcinogenesis of various cancers, including esophageal cancer. However, there is limited evidence about the role of the aberrant hypermethylation of these cancer-related genes (*P16*, *MGMT*, and *HMLH1*) in the Chinese population. Here, we report the results of a case-control study that aimed to explore the association of *MTHFR* genetic polymorphisms to cancer and the relationship of *P16*, *MGMT*, and *HMLH1* to *MTHFR*.

MATERIAL AND METHODS

Subjects

A total of 325 patients with gastric cancer confirmed by pathological diagnosis were recruited in The First Affiliated Hospital of Shantou University between January 2008 and December 2011. The age of patients ranged from 35 to 77 years. The exclusion criteria were patients with cardiac adenocarcinoma, secondary or recurrent tumors, a history of other malignant neoplasms, and previous eradication therapy for *Helicobacter pylori* infection. Ultimately, 285 patients were included in the final statistical analysis, with a participation rate of 87.7%.

The control group comprised 570 subjects who were randomly selected from individuals who underwent health examinations during the same period with no history of cancer or other digestive system disease. All controls were frequency-matched by age (within 5 years) and gender with cases. The present study was approved by the Hospital Ethics Committee of the First Affiliated Hospital of Shantou University Medical College in China. All patients signed informed consent forms.

Data extraction and quantification

The questionnaire data and blood samples were obtained upon initial recruitment of cases and controls. Information about gender, age, and family history of cancer, tobacco usage, and smoking were collected from medical records by doctors or nurses.

Evidence of *H. pylori* infection was determined using the enzyme-linked immunosorbent assay (ELISA) for immunoglobulin G (IgG) antibodies (HpIgG ELISA) with a commercially available kit (Genesis Diagnostics; Cambridgeshire, UK) on sera obtained from 5 mL blood according to manufacturer instructions.

Determination of folate concentration

Folate intake was estimated through a comprehensive quantitative food-frequency questionnaire (FFQ) designed by the US National Institutes of Health and modified according to common Chinese food items and cooking habits. The food questionnaire contained 95 single food items with quantity and frequencies in five categories. The daily intake of folate was calculated by multiplying the folate content of the food by the total food intake (in grams)

reported by the subjects. We then summarized the total folate intake by summing the separate folate intakes from various foods. Subjects were divided into two groups depending on folate intake: low intake (≤260 μg/day) and high intake (>260 μg/day).

Genotyping

All participants were asked to provide 5 mL venous blood, and genomic DNA was extracted from the blood samples using the Qiagen Blood Kit (Qiagen; Chatsworth, CA, USA). The forward and reverse primers of *MTHFR* C677T and *MTHFR* A1298C were designed by using Premier 5.0 software (Table 1). Genotyping of *MTHFR* C677T and *MTHFR* A1298C were accomplished by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis. The PCR conditions were as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, annealing at 60°C for 65 s, extension at 72°C for 90 s, and a final extension at 72°C for 5 min. A random sample of 5% of cases and controls was genotyped again by different researchers. The reproducibility was 100%.

Table 1. Primer of MTHFR C677T and MTHFR A1298C.

MTHFR		Primers	Fragment length (bp)	Annealing temperature (°C)
C 677T	Forward	5'-CGTGGCTCCTGCGTTTCC-3'	387	56
	Reverse	5'-GAGCCGGCCACAGGCAT-3'		
A1298C	Forward	5'-CAAATCTGAGGGAGCTGAGT-3'	168	55
	Reverse	5'-CAGATAAGTGGCAGTACAGA-3'		

Statistical analysis

All analyses were performed with the SPSS version 13.0 statistical software (SPSS; Chicago, IL, USA). Demographic characteristics were compared between cases and controls by means of a chi-squared test. The distributions of *MTHFR* C677T (rs1801133) and *MTHFR* A1298C (rs1801131) genotypes between cases and controls were compared by the chi-squared test, and the association between the *MTHFR* polymorphisms and gastric cancer risk was estimated by unconditional logistic regression analysis. The odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by using a conditional logistic regression model that adjusted for potential risk factors. Subjects with the wild-type genotype were considered as the reference group. Deviations from Hardy-Weinberg equilibrium (HWE) were checked for in the control group with the chi-squared test. All comparisons were two-sided, and $P < 0.05$ was considered to be statistically significant.

RESULTS

The characteristics of the study population are presented in Table 2. A total of 285 cases and 570 controls were included in our analysis. We did not find significant differences between cases and controls in the distributions of age and gender, which suggested adequate frequency matching. We found that patients were more likely to have a smoking habit compared to controls, but there was no significant difference in drinking status between cases and controls.

Table 2. Characteristics of the study population.

	Cases	%	Controls	%	χ^2	P
	N = 285		N = 570			
Age (years)						
Mean age	57.3 ± 5.6		58.7 ± 6.1			
<55	118	41.5	249	43.6		
≥55	167	58.5	321	56.4	0.25	0.61
Gender						
Male	186	65.3	372	65.3		
Female	99	34.7	198	34.7	-	-
Tobacco smoking						
Never	161	56.5	386	67.8		
Ever	124	43.5	184	32.2	7.64	<0.05
Alcohol drinking						
Never	166	58.2	356	62.4		
Ever	119	41.8	214	37.6	1.06	0.31
Family history of cancer						
No	27	9.6	20	3.5		
Yes	258	90.4	550	96.5	13.01	<0.001
Folate intake (µg/d)						
≤260	182	63.8	286	50.2		
>260	103	36.2	284	49.8	10.9	<0.05
H. pylori infection						
Positive	115	40.3	299	52.5		
Negative	170	59.7	271	47.5	11.15	<0.001

Table 3 shows the genotype distributions for *MTHFR* and their corresponding ORs and 95%CIs for gastric cancer risk. The distributions of the *MTHFR* C677T and *MTHFR* A1298C polymorphisms in the controls conformed to HWE (P > 0.05). The frequencies of the *MTHFR* 677CC, CT, and TT genotypes were 51.4, 33.9, and 14.7% in patients with gastric cancer, and 43.5, 35.7, and 20.8% in healthy controls, respectively. The frequencies of the *MTHFR* 1298AA, AC, and CC genotypes were 47.6, 40.3, and 12.1% in patients with gastric cancer, and 39.5, 41.2, and 19.3% in healthy controls, respectively. We found a significant difference in the frequencies of the *MTHFR* C677T and *MTHFR* A1298C polymorphisms between groups. The frequencies of the *MTHFR* 677T allele and 1298C allele were significantly lower in gastric cancer patients than in controls (P < 0.05). Multiple logistic regression analysis showed that compared with the CC genotype, the *MTHFR* 677TT genotype was significantly related with a reduced risk of gastric cancer (OR = 0.60, 95%CI = 0.39-0.92, P < 0.05) after adjusting for gender, age, tobacco smoking, family history of cancer, folate intake, and *H. pylori* infection. Similarly, compared with the 1298AA genotype, we found that the *MTHFR* 1298CC genotype was significantly associated with a decreased risk of cancer (OR = 0.52, 95%CI = 0.32-0.81, P < 0.05), and that the C allele also showed a reduced risk (OR = 0.73, 95%CI = 0.54-0.97, P < 0.05).

Linkage disequilibrium (LD) and haplotype analysis

We conducted haplotype analysis using the SHEsis software. The *MTHFR* C677T and *MTHFR* A1298C polymorphisms were in high LD (D' = 1.0) (Figure 1). Four main haplotypes of *MTHFR* were considered in the analysis of all subjects. The results of the individual-level haplotype analysis and gastric cancer risk are shown in Table 4. The CA haplotype was found to be the most common in both the case and control groups. We did not find significant differences in the distributions of haplotypes CA or TC between the two groups (P < 0.05).

Haplotype TC was associated with a reduced risk of gastric cancer compared to the most common haplotype, CA (OR = 0.58, 95%CI = 0.39-0.89, P < 0.05).

Table 3. MTHFR C677T and MTHFR A1298C genotype distributions and odds ratio for gastric cancer.

MTHFR	Cases	%	Controls	%	χ^2	P	Adjusted OR (95%CI)
	N = 285		N = 570				
C677T							
CC	146	51.4	248	43.5			1.0
CT	97	33.9	203	35.7			0.81 (0.58-1.13)
TT	42	14.7	119	20.8	6.4	<0.05	0.60 (0.39-0.92)
T-allele	139	48.6	304	53.3			0.77 (0.58-1.05)
A1298C							
AA	136	47.6	225	39.5			1.0
AC	115	40.3	235	41.2			0.81 (0.59-1.12)
CC	34	12.1	110	19.3	9.22	<0.05	0.52 (0.32-0.81)
C-allele	149	52.4	327	57.3			0.73 (0.54-0.97)

Figure 1. Linkage disequilibrium of the two SNPs by the SHEsis software.

Table 4. Association between haplotypes and gastric cancer for two SNPs in MTHFR.

Haplotype	Cases	%	Controls	%	χ^2	P	Adjusted OR (95%CI)
CA	134	47.1	219	40.5			1
CC	98	23.2	202	25.4	1.98	0.16	0.79 (0.57-1.11)
TA	53	33.7	149	27.7	2.68	0.11	0.58 (0.39-0.89)
TC	0	0	0	0	NA	NA	NA

DISCUSSION

Gastric cancer is a complex disease that is caused by genetic and environmental factors and their interactions (Hamajima et al., 2006; Bai et al., 2008; Pan et al., 2012; Bastos et al., 2013). Increasing evidence indicates that the *MTHFR* C677T and *MTHFR* A1298C polymorphisms influence the risk of cancer (Sun et al., 2008; Tahara et al., 2009; Neves et

al., 2010; Saberi et al., 2012). Folate mediates the transfer of one-carbon moieties during the process of DNA synthesis, replication, and repair, as well as DNA methylation reactions (Wang et al., 2008). MTHFR is a key enzyme in one-carbon metabolism, and the MTHFR variants could influence the levels of folate and homocysteine, and thus influence individual susceptibility to cancer.

In the present case-control study, we investigated the association between the *MTHFR* C677T and *MTHFR* A1298C polymorphisms and the risk of gastric cancer. Our results indicated that the frequencies of *MTHFR* C677T and *MTHFR* A1298C were significantly different between cases and controls. We found that the *MTHFR* 677TT and *MTHFR* 1298CC genotypes were associated with a reduced risk of gastric cancer, and that high folate consumption was correlated with a decreased risk of cancer. Moreover, haplotype analysis of the *MTHFR* C677T and A1298C polymorphisms showed that the TC haplotype was correlated with a reduced risk of gastric cancer when compared to the CA haplotype.

The association between the single-nucleotide polymorphisms (SNPs) in *MTHFR* and susceptibility to gastric cancer has been widely investigated, but the results are inconsistent (Sun et al., 2008; Dong et al., 2010). Several studies conducted in Chinese, Caucasian, and Mexican populations have shown that the *MTHFR* C677T polymorphism does not influence the risk of gastric cancer (Weng et al., 2006; Götze et al., 2007; Zhang et al., 2007). However, several studies conducted in Korean, Chinese, and Caucasian populations indicated the *MTHFR* 677TT genotype has a protective effect on the risk of gastric cancer. A meta-analysis of 22 studies including 4070 cases and 6462 controls suggested the *MTHFR* 677TT genotype influences susceptibility to gastric cancer, but no association was found between the *MTHFR* A1298C polymorphism and gastric cancer risk (Dong et al., 2010). By contrast, several studies observed positive associations between the *MTHFR* 677TT and 1298CC genotypes and gastric cancer risk (Boccia et al., 2007; Vollset et al., 2007; Yoo et al., 2012). In our study, we found that *MTHFR* 677TT and 1298CC had a 0.6-fold and 0.52-fold risk of gastric cancer, respectively. The discrepancies of these results may be caused by different population backgrounds, sample sizes, control selection criteria, and interactions with other environmental factors. Therefore, further confirmation of this association is greatly needed.

To the best of our knowledge, our study was the first to investigate the association between polymorphisms in *MTHFR* and gastric cancer risk based on haplotype analysis. We found that the *MTHFR* C677T and *MTHFR* A1298C were in high LD (D' = 0.956), suggesting that the two SNPs are located in the same haplotype region. The results of SHEsis analysis showed that CA was the most common haplotype. The TC haplotype was associated with a reduced risk of gastric cancer in the Chinese population. These results indicated that the two SNPs, *MTHFR* C677T (rs1801133) and *MTHFR* A1298C (rs1801131), may interact, and that both might influence the susceptibility to gastric cancer.

Three limitations of our study should be considered. First, the cases and controls were only selected from one hospital, and the sample may therefore lack representation of the general population. Second, gastric cancer is a complex disease that is caused by both genetic and environmental factors. Other genetic and environmental factors may interact with the *MTHFR* polymorphisms. Third, the sample size was relatively small in our study, and further studies using large sample sizes are warranted to verify these results.

In conclusion, the present case-control study in a Chinese population found a protective effect of the *MTHFR* C677T and A1298C variant genotypes for gastric cancer, and we also found that the TC haplotype was significantly associated with reduced risk of gastric cancer.

Our results suggest the *MTHFR* C677T and A1298C variants could influence the susceptibility to gastric cancer, and provide important information for biomarker research, therapeutic, and preventative strategies by molecular methods for gastric cancer.

Conflicts of interest

The authors declare no conflict of interest.

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