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Impact of methylenetetrahydrofolate reductase polymorphisms and folate intake on the risk of gastric cancer and their association with *Helicobacter pylori* infection and tumor site

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ABSTRACT. Folic acid and methylenetetrahydrofolate reductase (MTHFR) may both affect the development of human cancer. We conducted a population-based case-control study in a Chinese population to investigate the potential role of folate intake and *MTHFR* gene polymorphisms in gastric cancer, and their interaction with infection by *Helicobacter pylori* and tumor location. A total of 767 patients with newly diagnosed gastric cancer and 775 controls were selected for this study. Genotyping of *MTHFR* C677T and A1298C was conducted by TaqMan assays using the ABI Prism 7911HT Sequence Detection System, and information

on folate intake was collected by questionnaire. Compared with the CC genotype of *MTHFR* C677T, the TT genotype was significantly associated with a decreased risk of gastric cancer when the analysis was adjusted for other potential risk factors. We found a marginal significantly decreased risk of gastric cancer for individuals carrying the T allele [adjusted odds ratio (OR) = 0.83; 95% confidence interval (CI) = 0.65-1.01]. We detected an inverse relationship between folate intake and risk of gastric cancer, and the adjusted ORs (95%CI) for moderate and high folate intake were 0.97 (0.74-1.25) and 0.64 (0.49-0.87), respectively. Moreover, *H. pylori* infection, folate intake, and location of the tumor showed a significant interaction with the *MTHFR* C677T polymorphism. Our study suggests a protective role of *MTHFR* 677TT and high folate intake against gastric cancer, and the effect of the *MTHFR* C677T genotype may differ by *H. pylori* infection, folate consumption, and tumor site.

Key words: Methylenetetrahydrofolate reductase; Polymorphisms; Folate intake; Gastric cancer

INTRODUCTION

Although gastric cancer incidence and mortality have been decreasing around the world, it is still the most common cause of cancer death in China for both genders (IARC, 2008). Although *Helicobacter pylori* strains have been proposed to be a major cause of gastric cancer, infections by *H. pylori* do not account completely for the incidence of gastric cancer. Epidemiological studies have indicated an association between folate intake and a decreased risk of certain cancers (Larsson et al., 2006; Yoo et al., 2012; de Cássia Carvalho et al., 2012; Promthet et al., 2012; Ding et al., 2012), including gastric cancer (Yoo et al., 2012), esophageal cancer (Larsson et al., 2006) and ovarian cancer (Ding et al., 2012). Folate is a key B vitamin required for 1-carbon metabolism and is critical for DNA methylation, synthesis, and repair (Jemal et al., 2007). Deficiency of folate has been suggested to increase the risk of colorectal cancer because folate deficiency results in irregular DNA methylation and imbalance in DNA precursors (Eichholzer et al., 2001; La Vecchia et al., 2002).

Methylenetetrahydrofolate reductase (MTHFR) is an enzyme that converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the prevalent form of circulating folate and the methyl donor for the conversion of homocysteine to methionine. Two common *MTHFR* gene polymorphisms, *MTHFR* C677T (rs1801133) and A1298C (rs1801131), have been studied extensively. The *MTHF* TT and CC variants result in low-activity MTHFR enzymes (Frosst et al., 1995; van der Put et al., 1998) resulting in low levels of circulating 5-methyltetrahydrofolate. The *MTHFR* 677TT genotype has been associated with a lower cancer risk compared with the homozygous CC genotype (Hubner and Houlston, 2007).

We have previously reported that folate intake and *MTHFR 677CT/TT* are associated with an increased risk of esophageal cancer, and that folate shows a significant interaction with the MTHFR C677T polymorphism (APJCP). The association of MTHFR with susceptibility to gastric cancer has been investigated by several recent studies, whose results, however, were inconclusive (Dong et al., 2010). Several studies have reported that the homozygous genotype of the

MTHFR C677T polymorphism is associated with an increased risk of gastric cancer (Graziano et al., 2006; Lacasana-Navarro et al., 2006), whereas other studies have reported that this polymorphism is associated with a decreased gastric cancer risk (Mu et al., 2004; Kim et al., 2005; Weng et al., 2006). However, these studies did not take into account possible interactions between *MTHFR* gene polymorphisms and folate intake, *H. pylori* infection, or tumor site. Therefore, we conducted a large-scale case-control study in a Chinese population to evaluate the potential role of folate intake and *MTHFR* gene polymorphisms in gastric cancer, and to investigate possible interactions of folate intake, *H. pylori* infection, and tumor sites with *MTHFR* polymorphisms.

MATERIAL AND METHODS

Study population

The study population comprised 767 patients with newly diagnosed gastric cancer and 775 population-based controls. All enrolled patients were confirmed for their pathological condition by the General Hospital of Chengdu Military Area between March 2008 and May 2011. Subjects with secondary or recurrent tumors were excluded. The tumor stages were classified according to the TNM classification, including clinical or pathological TNM stages. Gastric cancer was classified by anatomical site (cardia or non-cardia) and histological types (intestinal, diffuse, or mixed type).

The control group consisted of participants in the health examination center from April 2007 and April 2008, and they were matched with cancer patients by age and gender. All patients were asked to provide their peripheral blood, and they had read and signed an informed consent form.

Our study was approved by the ethics committee of the General Hospital of Chengdu Military Area in China.

Data collection

A self-administered structured questionnaire, consisting of 65 questions, was used in this study. Information collected included demographic data (age, gender, and family history of cancer) and clinical characteristics (histopathology, tumor location, and lymph modes status), tobacco use, smoking, and alcohol-consumption and dietary habits (including 45 foods/food groups). Cigarette smoking was measured in pack-years [number of cigarettes smoked per day/20 x smoking time (in years)] and divided into 2 categories: smokers who consumed less than 40 packs/year, and ≥40 packs/year or more. Alcohol consumption was calculated from the amount of alcohol consumed per day in grams. The subjects were classified into two categories: drinkers who consumed less than 22.8 g alcohol per day, and those consuming ≥22.8 alcohol per day.

For each food item, we collected the frequency and quantity of consumption, and calculated the daily intake by multiplying the frequency reported for the consumption of each food item by the specified portion size. The folate intake was computed by multiplying the food intake (in g) and the folate content (per g) of food in our questionnaire, and then the sum of the combined folate intake from various foods/food groups was calculated as the total folate intake. Trained interviewers conducted face-to-face interviews with all subjects to obtain the above information. Completed questionnaires were obtained from 767 cases and 775 controls. Cancer patients were asked to refer about habits a year before the disease diagnosed.

Diagnosis of *H. pylori* infection

Infection by *H. pylori* was diagnosed by enzyme-linked immunosorbent assay (ELISA) with IgG antibodies (HpIgG ELISA) using a commercially available kit (Genesis Diagnostics, Cambridgeshire, UK) according to manufacturer instructions on sera obtained from 5 mL of blood. The sensitivity and specificity of the kit was 91 and 100%, respectively.

Blood samples and DNA collection

All participants provided 5 mL of blood, which was stored at -20°C. Genomic DNA was extracted from whole-blood samples using the Qiagen Blood Kit (Qiagen, Chatsworth, CA, USA). Genotyping was conducted by TaqMan assays using the ABI Prism 7911HT Sequence Detection System (Applied Biosystems, Foster City, CA). Primer, probes, and reaction conditions were those described in our previous study (Jing et al., 2012). Briefly, a total PCR reaction volume of 10 uL contained 200 ng of genomic DNA and 20 pmol of each primer. The PCR conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 65 s, 60°C for 65 s, and 72°C for 90 s; a final extension was performed at 72°C for 5 min. After brief centrifugation, agarose gel electrophoresis was conducted. The PCR products included a 173-bp fragment of 677C/C wild-type homozygotes; 173-, 125-, and 48-bp fragments of 677C/T heterozygotes; and 125- and 48-bp fragments of 677T/T homozygotes. The genotyping was performed by laboratory personnel blinded to the case-control status. For quality-control purposes, we also genotyped internal positive control samples, used no-template controls, and replicated the genotyping of 10% of the samples.

Statistical analysis

The differences between the patients and controls were estimated by logistic regression analysis. Adjusted odds ratios (ORs) were calculated with a logistic regression model that controlled for gender and age and are indicated with their 95% confidence intervals (CIs). Subjects with the wild-type genotypes were considered the baseline group. The expected frequency of control genotypes was checked by the Hardy-Weinberg equilibrium test. Unconditional logistic regression was undertaken to estimate ORs and their 95%CIs after controlling for potentially confounding factors.

Folate intake was computed by multiplying food intake (μg/day) by folate content (μg/day) of the food; the sum of the folate intake from all foods was calculated to obtain the total intake. Folate intake was categorized as low, moderate, or high using tertiles as cut-off points. We assessed the role of folate intake in the association between MTHFR and gastric cancer by subgroup analysis of folate intake levels. All analyses were performed by using the the SPSS version 16.0 statistical software (SPSS, Chicago, IL, USA). The descriptive data for the major characteristics of study groups are expressed as means and percentages.

RESULTS

The general characteristics of the study population are shown in Table 1. The mean age of gastric cancer patients was 62.4 ± 12.3 years and that of controls 62.7 ± 11.8 years. Drink-

ers and smokers were at higher risk for gastric cancer than non-drinkers and non-smokers ($P < 0.05$). Patients with *H. pylori* infection were also at higher risk for gastric cancer ($P < 0.05$).

Table 1. General characteristics of subjects.

Characteristics	Cases N = 767	%	Controls N = 775	%	P value
Age (years)	62.4 ± 12.3		62.7 ± 11.8		
<60	270	35.2	283	36.5	
60-70	322	42	316	40.8	
>70	175	22.8	176	22.7	0.85
Gender					
Male	478	62.3	480	61.9	
Female	289	37.7	295	38.1	0.88
Smoking habit (package/year)					
<40	481	62.7	531	68.5	
≥40	286	37.3	244	31.5	0.016
Drinking habit (g/day)					
<22.8	503	65.6	564	72.8	
≥22.8	264	34.4	211	27.2	0.002
H. pylori infection					
Positive	549	71.6	392	50.6	
Negative	218	28.4	383	49.4	<0.001
TNM stage					
I	266	34.7			
II	208	27.1			
III	139	18.1			
IV	154	20.1			
Tumor site					
Cardiac	167	21.8			
Non-cardiac	600	78.2			
Histological type					
Intestinal	401	52.3			
Diffuse	297	38.7			
Mixed	69	9			

The distributions for the *MTHFR* C677T and A1298C genotypes and their adjusted ORs and 95%CIs in gastric cancer are shown in Table 2. The distributions of the *MTHFR* C677T and A1298C gene polymorphisms in the controls were in Hardy-Weinberg equilibrium. The frequencies of the *MTHFR* C677T CC, CT, and TT genotypes were 50.9, 37.7, and 11.4% among gastric cancer patients and 46.7, 39.7, and 13.6% among controls, respectively. The frequencies of the *MTHFR* A1298C AA, AC, and CC genotypes were 41.1, 46.9, and 12.0 among gastric cancer patients and 42.7, 46.2, and 11.1% among controls, respectively. For *MTHFR* C677T, the TT genotype was significantly associated with a decreased risk of gastric cancer when compared with the CC genotype and adjusted for potential risk factors. We found a marginal significantly decreased risk of gastric cancer for individuals with the T allele (adjusted OR, 0.83; 95%CI, 0.65-1.01). In contrast, none of the *MTHFR* A1298C polymorphisms was significantly associated with increased risk of gastric cancer.

The mean folate intakes in cancer patients and controls were 261.3 ± 24.7 μg/day and 295.5 ± 29.6 μg/day, respectively. We detected an inverse relationship between folate intake and risk of gastric cancer, and the adjusted OR and 95%CI for folate intakes of 230-310 μg/day and >310 μg/day were 0.97 (0.74-1.25) and 0.64 (0.49-0.87), respectively.

The effects of interactions of the *MTHFR* C677T polymorphisms with smoking, drinking, and *H. pylori* infection on the relative risk of developing gastric cancer is shown in

Table 3. When we used the *MTHFR* 677CC genotype as the reference, smoking and drinking did not modify the association between the *MTHFR* C677T polymorphism and the risk of gastric cancer. In contrast, *H. pylori* infection and folate intake each showed a significant interaction with the *MTHFR* C677T polymorphism: individuals who were not infected by *H. pylori* had a lower risk of developing gastric cancer when they had the *MTHFR* 677TT genotype (P for interaction <0.05). Moreover, higher folate intake was associated with a decreased risk of gastric cancer in individuals with the *MTHFR* 677CT and TT genotypes.

Table 2. Frequency distribution and association of methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C genotypes and folate intake levels with gastric cancer.

MTHFR	Cases N = 767	%	Controls N = 775	%	OR	Adjusted OR[1]
C677T						
CC	390	50.9	362	46.7	1.0 (Ref.)	1.0 (Ref.)
CT	289	37.7	307	39.7	0.87 (0.70-1.09)	0.84 (0.67-1.05)
TT	88	11.4	105	13.6	0.78 (0.56-1.08)	0.71 (0.51-0.97)
T allele	377	49.1	412	53.3	0.85 (0.69-1.04)	0.83 (0.65-1.01)
A1298C						
AA	316	41.1	331	42.7	1.0 (Ref.)	1.0 (Ref.)
AC	359	46.9	358	46.2	1.05 (0.84-1.31)	1.09 (0.88-1.52)
CC	92	12.0	86	11.1	1.12 (0.79-1.58)	1.40 (0.86-1.67)
C allele	451	58.9	444	57.3	1.06 (0.86-1.31)	1.13 (0.91-1.46)
Folate intake (µg/day)						
Means (SE)	261.3 ± 24.7		295.5 ± 29.6			
<230	335	43.7	300	38.7	1.0 (Ref.)	1.0 (Ref.)
230-310	296	38.6	280	36.1	0.96 (0.75-1.20)	0.97 (0.74-1.25)
>310	136	17.7	195	25.2	0.62 (0.47-0.82)	0.64 (0.49-0.87)

[1]Adjusted for age, gender, smoking, drinking, and *H. pylori* infection.

Table 3. Interaction between MTHFR C677T and MTHFR A1298C polymorphisms and smoking, drinking and *H. pylori* infection for gastric cancer risk.

Characteristics	CT *vs* CC OR (95%CI)[1]	TT *vs* CC OR (95%CI)[1]	P for interaction
Smoking habit (package/year)			
<40	0.81 (0.52-1.08)	0.59 (0.33-0.86)	
≥40	0.91 (0.62-1.21)	0.80 (0.52-1.02)	0.07
Drinking habit (g/day)			
<22.8	0.82 (0.61-1.06)	0.68 (0.47-0.95)	
≥22.8	0.88 (0.66-1.09)	0.73 (0.47-0.98)	0.38
H. pylori infection			
Positive	0.91 (0.68-1.13)	0.78 (0.53-1.04)	
Negative	0.75 (0.58-0.96)	0.63 (0.43-0.86)	0.012
Folate intake			
<230	0.96 (0.71-1.23)	0.85 (0.52-1.23)	
230-300	0.81 (0.57-0.96)	0.75 (0.53-1.04)	
>310	0.74 (0.45-0.87)	0.59 (0.41-0.86)	0.006

[1]Adjusted for age and gender.

The association between *MTHFR* C677T polymorphism and different locations of the gastric cancer tumors in the gastric system is shown in Table 4. The *MTHFR* C677T polymorphism was not associated with an increased risk of cardia cancer, whereas the *MTHFR* 677TT genotype was associated with a decreased risk of non-cardia cancer.

Table 4. MTHFR C677T and MTHFR A1298C genotype distributions and adjusted OR for different tumor sites.

	Controls	%	Cardiac cancer			Non-cardiac cancer		
			Cases N = 167	%	OR (95%CI)[1]	Cases N = 600	%	OR (95%CI)[1]
C677T								
CC	362	46.7	77	46.0	1.0 (Ref.)	313	52.2	1.0 (Ref.)
CT	307	39.7	66	39.4	1.01 (0.69-1.48)	223	37.2	0.84 (0.66-1.06)
TT	105	13.6	24	14.6	1.07 (0.62-1.82)	64	10.6	0.44 (0.27-0.68)

[1]Adjusted for age, gender, smoking, drinking, and *H. pylori* infection.

DISCUSSION

This study evaluated the association of the *MTHFR* C677T and *MTHFR* A1298C gene polymorphisms with increased susceptibility to gastric cancer. The *MTHFR* C677T polymorphism decreased the risk of gastric cancer, as did high folate consumption whose protective effect was modified by polymorphisms in the *MTHFR* C677T gene. Moreover, we observed that a *MTHFR* C677T polymorphism significantly interacted with *H. pylori* infection and folate intake in the development of gastric cancer.

Previous studies on the *MTHFR* C677T and *MTHFR* A1298C polymorphisms and their associations with gastric cancer risk have yielded inconsistent results. Studies conducted in China (Qin et al., 2008), Iran (Saberi et al., 2012), Korea (Cui et al., 2010), Mexico (Zuniga-Noriega et al., 2007; Galvan-Portillo et al., 2009), Norway (Vollset et al., 2007), and Denmark (Zacho et al., 2011) identified a significant association of the *MTHFR* C677T polymorphism with gastric cancer. Studies in China, Iran, and Denmark indicated a significantly increased risk of gastric cancer, with ORs ranging from 1.4-2.6 (Qin et al., 2008; Zacho et al., 2011; Saberi et al., 2012). In contrast, several other studies have indicated that *MTHFR* 677TT significantly protects against gastric cancer (Galvan-Portillo et al., 2009; Cui et al., 2010). Cui and colleagues, studying a Korean population, reported that the *MTHFR* 677T allele has a significant protective effect against gastric cancer, indicated by ORs of CT versus CC of 0.81 (95%CI, 0.69-0.94) (Cui et al., 2010). In a Mexican population, a significant reduction in gastric cancer risk was observed among individuals with high folate intake and who carried the *MTHFR* 677TT genotype (Galvan-Portillo et al., 2009). Two other studies detected a non-significant protective effect on gastric cancer risk (Zuniga-Noriega et al., 2007; Vollset et al., 2007). Here, we observed that *MTHFR* 677TT was associated with a reduced risk of gastric cancer, and the *MTHFR* 677T allele was associated with a marginally decreased risk. Moreover, the *MTHFR* 677TT genotype is associated with a protective effect against various cancers, including breast and colorectal cancers (Chou et al., 2006; Cui et al., 2010). The observed differences in the effects of the *MTHFR* C677T polymorphisms on gastric cancer risk in the different studies might be due to population background, sample sizes, or environmental factors and other factors. Further confirmation of the differential effects observed here is strongly needed.

Folate mediates the transfer of 1-carbon moieties in the synthesis of nucleotides required for DNA synthesis, replication, and repair, and also in DNA methylation reactions, and these processes may play a critical role in carcinogenesis (Wang et al., 2008; Mason, 2009). An abundant intake of folate-rich foodstuffs was more likely to convey protection against developing some cancers (Mason, 2009). Our study indicating a protective function of folate intake against increased gastric cancer risk has also been observed in previous epidemiological

studies (Chou et al., 2006; Vollset et al., 2007; Cui et al., 2010). Moreover, the results of these studies suggest that a higher intake of folate could greatly reduce the risk of cancer among individuals with the *MTHFR 677TT* genotype.

H. pylori infection is an important etiological factor in gastric cancer, and previous studies have indicated that *H. pylori* infection affects the induction of methylation in human gastric mucosa (Chan et al., 2003; Maekita et al., 2006; Tahara et al., 2009), and suggested that aberrant DNA methylation is one of the major events occurring early in tumorigenesis. The results of our study identified a significant interaction between *H. pylori* infection and *MTHFR* C677T polymorphisms on the effect on gastric cancer risk, which indicated that *H. pylori* infection of the gastric mucosa may lead to transcriptional inactivation of specific genes and may increase DNA damage, mutation, or chromosomal instability.

In summary, this case-control study has investigated the association between folate intake and the *MTHFR* C677T and *MTHFR* A1298C variant genotypes in the development of gastric cancer. Its results suggest a protective role of *MTHFR* 677TT and high folate intake against gastric cancer, and the effect of the *MTHFR* C677T genotype may differ by *H. pylori* infection, folate consumption, and different tumor sites. Gastric cancer appears to be the result of an interaction between genes and the environment, and further studies with larger sample sizes are needed to confirm any environmental and genetic associations in this disease.

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