RESEARCH ARTICLE

Diet Folate, DNA Methylation and Polymorphisms in Methylenetetrahydrofolate Reductase in Association with the Susceptibility to Gastric Cancer

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Abstract

Methylenetetrahydrofolate reductase (MTHFR) has been reported to be associated with DNA methylation, an epigenetic feature frequently found in gastric cancer. We conducted a case-control study to explore the association of MTHFR C677T polymorphisms with gastric cancer risk and its relation with the DNA methylation of COX-2, MGMT, and hMLH1 genes. Genotyping of P16, MGMT and HMLH1 was determined by methylation-specific PCR after sodium bisulfate modification of DNA, and genotyping of MTHFR C677T was conducted by TaqMan assays using the ABI Prism 7911HT Sequence Detection System. Folate intake was calculated with the aid of a questionnaire. Compared with the MTHFR 677CC genotype, the TT genotype was significantly associated with 2.08 fold risk of gastric cancer when adjusting for potential risk factors. Individuals who had an intake of folate above 310 μg/day showed protective effects against gastric cancer risk. The effect of MTHFR C677T polymorphisms on the risk of gastric cancer was modified by folate intake and methylation status of MGMT (P for interaction <0.05).

Keywords: Methylenetetrahydrofolate reductase - DNA methylation - folate intake - gastric cancer risk

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Introduction

Gastric cancer is one of the most common malignant diseases worldwide, which is a disease of multiple etiologic factors involving infectious, dietary, environmental and genetic factors (IRAC, 2008). Helicobacter pylori (H. pylori) infection has been proved to be associated with gastric cancer and duodenal ulcer (Taylor and Blaser, 1991; Munoz, 1994). Folate is a water-soluble vitamin naturally found in green leafy vegetables, cereals and fruits, which is a key B vitamin in one-carbon mechanism and plays an important role in DNA methylation, and DNA synthesis and repair (Jernal et al., 2007). Deficiency of folate has been suggested to increase risk of various cancers through aberrations in DNA methylation and imbalance of DNA precursors (Eichholzer et al., 2001; La et al., 2002; Chen et al., 2002).

Methylenetetrahydrofolate reductase (MTHFR) acts centrally in folate metabolism, catalyzing the 5-methylenetetrahydrofolate synthesis reaction, the predominant circulatory form of folate and carbon donor for the remethylation of homocysteine to methionine, which leads to the production of S-adenosylmethionine for DNA methylation (Shen et al., 2001). Two common MTHFR polymorphisms, MTHFR C677T and A1298C, could influence the status of DNA methylation and would explain individual susceptibility to gastric cancer (Wang et al., 2008).

DNA methylation is an important epigenetic feature of DNA that plays critical roles in gene regulation and cellular differentiation mechanisms. Methylation of promoter in the CpG island culminates in gene silencing. However, gastric cancer has higher number of silenced genes by methylation of their CpG islands (Perri et al., 2007). There was little evidence on the association of MTHFR C677T polymorphism and aberrant DNA methylation with gastric cancer. We therefore conducted a case-control study to explore the association of MTHFR C677T polymorphisms with gastric cancer risk and its relation with the DNA methylation of COX-2, MGMT and hMLH1 genes.

Materials and Methods

The study population consists of 264 patients with newly diagnosed gastric cancer, and 535 population-based controls. All enrolled patients were pathologically confirmed in Affiliated Hospital of Inner Mongolia Medical University during January. 2008 and April. 2012. The patients ranged from 28 to 76 years old. The exclusion

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criteria were patients with cardiac adenocarcinoma, secondary or recurrent tumors, a history of other malignant neoplasms, and previous eradication therapy for H.pylori. Tumor stages were classified according to the TNM classification. Gastric cancer was classified by histological types (intestinal, diffuse or mixed type).

The controls were selected from participants who take the health examination during January 2008 and December 2011, and cases and control were frequency-matched by age (within 5-years) and sex. The present study was approved by the Hospital Ethics Committee in the Affiliated Hospital of Inner Mongolia Medical University in China. All patients were signed the informed consent.

Data extraction and quantification

The questionnaire data and blood samples were obtained at the initial recruitment of cases and controls. A face to face investigation was conducted to all patients and controls enrolled in our study. Information were collected about demographic (sex, age and family history of gastric cancer) and clinical characteristics (histopathology and lymph modes status), tobacco usage, smoking, alcohol-drinking habits and dietary habits (including 45 foods/food groups). Cigarette smoking was measured as ever smokers and non-smokers. Alcohol consumption was measured as ever drinkers and non-drinkers. All patients were completed questionnaires. Cancer patients were asked to refer about their dietary habits half a year before the cancer diagnosed.

The folate intake was calculated by multiplying the food intake (in grams) and the folate content (per gram) of each food from the questionnaires, and the total folate intake was summed by calculating the folate intake from various foods/food groups. The H. pylori infection was determined using the enzyme linked immunoabsorbent assay (ELISA) for IgG antibodies (HpIgG ELISA) with commercially available kit (Genesis Diagnostics, Cambridgeshire, UK) on sera obtained from 5 mL blood according to the manufacturer's instructions.

All participants were asked to provide 5ml peripheral bloods, and the bloods were stored at -20°C. Genomic DNA was extracted from blood samples using a salt exact with Qiagen Blood Kit (Qiagen, Chatsworth, CA). For the methylation at the promoter region of COX-2, MGMT and hMLH1, the pairs of amplification primer and an extension primer were designed using Assay Design 3.1 software (Sequenom, San Diego, CA, USA). The genotyping of them was determined by methylation-specific PCR after sodium bisulfate modification of DNA (Herman et al., 1996; Wang et al., 2008). The genomic DNA was incubated with NaOH at 37°C for 10 minutes, and the freshly prepared hydroquinone and NaHSO3 were used for them. Samples were incubated under mineral oil at 50°C for 16 hours. Modified DNA was purified and eluted with 50ul preheated TE solution. Modification was completed by the treatment of 5.5ul of 3 mol/L NaOH (final concentration 0.3 M) for 20 minutes. DNA was precipitated by ethanol and resolved in TE. After these procedures, the unmethylated cytosine would be converted to uracil and determined as thymine by Taq polymerase during the PCR process. Genotyping of MTHFR C677T polyomrphisms was conducted by TaqMan assays using the ABI Prism 7911HT Sequence Detection System (Applied Biosystems, Foster City, CA). The forward primer and the backward primer of MTHFR C677T were based on a previous study (Jing et al., 2012). Briefly, a total volume of 10 ul of PCR product was obtained through 200ng of genomic DNA and 20 pmol of each primer. 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, and extension at 72°C for 90 s, and final extension at 72°C for 5 minutes. After transient centrifugation, agarose electrophoresis was conducted. For quality control, genotyping was performed without knowledge of the case/control status of the subjects, and a random sample of 5% of cases and controls was genotyped again by different researchers. The reproducibility was 100%.

Statistical analysis

All analysis was performed by using SPSS version 13.0 statistical software (SPSS, Chicago, IL, USA). The continuous variable of folate intake was categorized as low, moderate, or high using tertiles as cut-off points. We assessed the role folate intake on the association between MTHFR and gastric cancer was assessed by subgroup analysis of folate intake levels. Demographic characteristics were compared between cases and controls by means of a Chi-square test or Student's t test. The differences of P16, MGMT, hMLH1 and MTHFR C677T between the patients and controls were estimated by unconditional logistic regression analysis. Adjusted odds ratios (OR) and 95% confidence intervals (CIs) were calculated by logistic regression model that adjusted for potential risk factors. Subjects with the wild-type genotype were considered as the reference group. The Hardy-Weinberg equilibrium (HWE) was checked for controls with the chi-square test. All comparisons were two-sided, and $P<0.05$ was regarded as statistically significant.

Results

A total of 264 gastric cancer cases (138 intestinal types, 101 diffuse types and 25 mixed types) and 535 healthy controls were collected (Table 1). As shown in Table 1, the mean age of gastric cancer patients and controls were 60.4±13.5 and 62.6±12.8 years old, respectively. The distributions of sex, age and smoking habit were not seemed a large extent between cases and controls. Individuals with consumption of alcohol above 22.8 g/day were more likely to have higher risk of gastric cancer ($P<0.05$). Individuals infected with H.pylori showed an increased risk of gastric cancer than non-infection ones ($P<0.05$).

The distributions of the MTHFR C677T polymorphisms in the controls were in line with Hardy-Weinberg equilibrium. The frequencies of MTHFR 677 CC, CT and TT genotypes were 43.6%, 39.6% and 16.8% among gastric cancer patients, 51.8%, 38.7% and 9.5% among controls, respectively. Compared with CC genotype of MTHFR C677T, the TT genotype was significantly associated with 2.08 fold risk of gastric cancer when

Table 1. Characteristics of Gastric Cancer and Controls

	Cases N=264	%	Controls N=535	%	*P* value
Age (years)					
<60	97	36.8	159	36.5	
≥60	167	63.2	276	63.5	0.96
Sex					
Male	164	62.3	269	61.9	
Female	100	37.7	166	38.1	0.94
Smoking					
Ever	166	62.7	298	68.5	
Never	98	37.3	137	31.5	0.127
Drinking					
Ever	173	65.6	317	72.8	
Never	91	34.4	118	27.2	0.04
H.pylori infection					
Positive	189	71.6	220	50.6	
Negative	75	28.4	215	49.4	<0.001
TNM stage					
I-II	163	61.8			
III	48	18.1			
IV	53	20.1			
Lauren's histotype					
Intestinal	138	52.3			
Diffuse	101	38.2			
Mixed	25	9.5			
COX-2					
Methylated	104	39.5			
Unmethylated	160	60.5			
MGMT					
Methylated	151	57.2			
Unmethylated	113	42.8			
hMLH1					
Methylated	172	65.2			
Unmethylated	92	34.8			

adjusting for potential risk factors. The average folate intake in cases and controls were 254.4±34.4 μg/day and 287.4±41.6 μg/day, respectively. We found an inverse association between folate intake and cancer risk, individuals who had an intake of folate above 310 μg/day showed 0.54 fold risk of gastric cancer risk.

The interaction of MTHFR C677T polymorphisms with folate intake, H.pylori infection and promoter methylation status was shown in table 3. When compared with MTHFR 677CC genotype, individuals with low intake of folate intake and carrying MTHFR 677TT had higher risk of gastric cancer risk (OR=2.65, 95%CI=0.52-3.78, P for interaction<0.05). Similarly, individuals with methylated MGMT and carrying MTHFR 677TT showed a higher risk of gastric cancer (OR=2.71, 95%CI=1.62-4.17, P for interaction<0.05). MTHFR C677T polymorphisms presented significant interaction with folate intake and methylation status of MGMT. However, MTHFR C677T did not show significant interaction with H.pylori infection and methylation status of COX-2 and hMLH1.

Discussion

The relationship between DNA methylation of COX-2, MGMT, and hMLH1 genes and MTHFR C677T polymorphisms with the cancer risk has been pointed in many studies (Lee et al., 2011; Chen et al., 2012; Nakai et al., 2012). However, their association with gastric cancer risk in Chinese population is still unknown. In our study, we found the MTHFR 677TT genotype showed an increased risk of gastric cancer, and high folate consumption was observed a reduced risk of gastric cancer. Moreover, we found MTHFR C677T polymorphisms were significantly interaction with folate intake and methylation status of MGMT on the development of gastric cancer.

The association of MTHFR C677T polymorphisms with gastric cancer risk has been explored in many studies (Zuniga-Noriega et al., 2007; Qin et al., 2008; Cui et al., 2010; Zacho et al., 2011; Saberi et al., 2012), and their results are inconsistent. Some results demonstrated the genetic polymorphisms significantly increased the risk of gastric cancer in studies conducted in China (Qin et al., 2008), Iran (Saberi et al., 2012) and Dnemark (Zacho et al., 2011). However, several studies have indicated MTHFR 677TT genotype plays an protective role in the development of gastric cancer in Korea (Cui et al., 2010) and Mexico (Galvan-Portillo et al., 2009). Moreover, there are two studies which have shown a non-significant association of MTHFR C677T with gastric cancer risk (Vollset et al., 2007; Zuniga-Noriega et al., 2007). In our study, we found MTHFR 677TT was related to an increased risk of gastric cancer. The inconsistency of these results might be due to the difference in study design, ethnicities of subjects, sample size and by chance. Further large sample size studies are needed to confirm the results.

Table 2. Association of MTHFR C677T Genotype and Folate Intake Levels with Gastric Cancer

	Cases N=264	%	Controls N=535	%	Adjusted OR(95% CI)[1]
MTHFRC677T					
CC	115	43.6	277	51.8	-
CT	105	39.6	207	38.7	1.22(0.88-1.70)
TT	44	16.8	51	9.5	2.08(1.28-3.66)
Folate intake(μg/day)					
<230	129	48.7	211	39.4	-
230-310	89	33.7	194	36.3	0.75(0.52-1.06)
>310	46	17.6	130	24.3	0.54(0.34-0.83)

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

Table 3. Association of MTHFR C677T Polymorphisms with Promoter Methylation Status, Folate Intake and *H.pylori* Infection

	CT vs CC OR(95% CI)[1]	TT vs CC OR(95% CI)[1]	P for interaction
Folate intake			
<230	1.05(0.77-1.84)	2.65(1.52-3.78)	
230-300	0.81(0.57-1.43)	2.24(1.33-3.42)	
>310	0.75(0.45-1.07)	1.53(0.87-2.35)	0.005
H.pylori infection			
Positive	1.51(0.98-1.83)	2.35(1.32-3.15)	
Negative	1.06(0.70-1.51)	1.64(0.94-2.87)	0.07
COX-2			
Methylated	1.45(0.92-1.96)	2.21(1.34-3.87)	
Unmethylated	1.17(0.73-1.57)	1.79(1.12-3.43)	0.22
MGMT			
Methylated	1.76(1.05-2.06)	2.71(1.62-4.17)	
Unmethylated	1.02(0.65-1.53)	1.50(0.93-2.54)	0.003
hMLH1			
Methylated	1.31(0.87-1.87)	2.14(1.32-3.75)	
Unmethylated	1.16(0.84-1.68)	1.83(1.05-2.40)	0.08

[1]Adjusted for age, sex, smoking and drinking

DNA methylation is considered as the an important form of epigenetic modification, and results in the addition of a methyl (CH3) group at the carbon 5 position of the cytosine ring (Das et al., 2004). In human, the DNA methylation primarily affects cytosine of the symmetrical dinucleotide CpG (Issa et al., 2004) and its subsequent pattern is transmitted through mitosis and maintained after DNA replication (Gius et al., 2005). Aberrant CpG island methylation plays an important role in the carcinogenic process. A previous study have indicated that the aberrant methylation occurred in cancers includes global hypomethylation in genomic DNA and hypermethylation in specific gene promoters (Momparler et al., 2000). In our study, we found individuals with methylation of MGMT might increase the risk of gastric cancer, which indicates DNA methylation may play a role in the development of gastric cancer.

Folate mediates the transfer of one-carbon moieties in the synthesis of nucleotides necessary for DNA synthesis, replication and repair, and it also has a role in DNA methylation reactions, and the two functions may increase the carcinogenesis of gastric cancer (Wang et al., 2008; Mason et al., 2009). Our study has indicated high intake of folate intake prevent the development of gastric cancer, and this effect of folate supplement on carcinogenesis was pointed by previous studies (Vollset et al., 2007; Cui et al., 2010; Zhao et al., 2011).

In conclusion, the results of our study have indicated the MTHFR C677T polymorphisms have an significant association with gastric cancer risk, and the effect of MTHFR C677T polymorpihsms may interact with folate consumption and methylation of MGMT. Further large sample studies are still needed to verify the environmental and genetic association of gastric cancer risk.

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References

Chen HTK, Graubard BI, Heineman EF, et al (2002). Nutrient intakes and adenocarcinoma of the esophagus and distal stomach. *Nutr Cancer*, **42**, 33-40.

Chen J, Huang ZJ, Duan YQ, et al (2012). Aberrant DNA Methylation of P16, MGMT, and hMLH1 Genes in combination with MTHFR C677T Genetic Polymorphism and Folate Intake in Esophageal Squamous Cell Carcinoma. *Asian Pac J Cancer Prev*, **13**, 5303-6.

Cui LH, Shin MH, Kweon SS, Kim HN, et al (2010). Methylenetetrahydrofolate reductase C677T polymorphism in patients with gastric and colorectal cancer in a Korean population. *BMC Cancer*, **10**, 236.

Das PM, Singal R (2004). DNA methylation and cancer. *J Clin Oncol*, **22**, 4632-42.

DE Cássia Carvalho Barbosa R, DA Costa DM, Cordeiro DE, et al (2012). Interaction of MTHFR C677T and A1298C, and MTR A2756G Gene Polymorphisms in Breast Cancer Risk in a Population in Northeast Brazil. *Anticancer Res*, **32**, 4805-11.

Eichholzer M, Luthy J, Moser U, et al (2001). Folate and the risk of colorectal, breast and cervix cancer: the epidemiological evidence. *Swiss Med Wkly*, **131**, 539-49.

Galvan-Portillo MV, Cantoral A, Onate-Ocana LF, et al (2009): Gastric cancer in relation to the intake of nutrients involved in onecarbon metabolism among MTHFR 677 TT carriers. *Eur J Nutr*, **48**, 269-76.

Gius D, Bradbury CM, Sun L, et al (2005). The epigenome as a molecular marker and target. *Cancer*, **104**, 1789-93.

International Agency for Research on Cancer (2008). Globocan 2008: Stomach Cancer incidence, Mortality and Prevalence Worldwide in 2008. *IARC*.

Issa JP (2004). CpG island methylator phenotype in cancer. *Nat Rev Cancer*, **4**, 988-93.

Jernal A, Siegel R, Ward E, Murray T, et al (2007). Cancer statistics, 2007. *CA Cancer J Clin*, **57**, 43-66.

Jing C, Huang Z, Duan Y, et al (2012). Folate intake, methylenetetrahydrofolate reductase polymorphisms in association with the prognosis of esophageal squamous cell carcinoma. *Asian Pac J Cancer Prev*, **13**, 647-51.

La Vecchia C, Negri E, Pelucchi C, Franceschi S (2002). Dietary folate and colorectal cancer. *Int J Cancer*, **102**, 545-7.

Lee KH, Lee JS, Nam JH, et al (2011). Promoter methylation status of hMLH1, hMSH2, and MGMT genes in colorectal cancer associated with adenoma-carcinoma sequence. *Langenbecks Arch Surg*, **396**, 1017-26.

Mason JB (2009). Folate, cancer risk, and the Greek god, Proteus: A tale of two chameleons. *Nutr Rev*, **67**, 206-12.

Momparler RL, Bovenzi V (2000). DNA methylation and cancer. *J Cell Physiol*, **183**, 145-54.

Nakai K, Mitomi H, Alkam Y, et al (2012). Predictive value of MGMT, hMLH1, hMSH2 and BRCA1 protein expression for pathological complete response to neoadjuvant chemotherapy in basal-like breast cancer patients. *Cancer Chemother Pharmacol*, **69**, 923-30.

Qin JM, Wang XM, Chen B, et al (2008). Study on the ingestion of folate and polymorphism of MTHFR C677T with esophageal cancer in Xinjiang Kazakh. *Zhonghua Liu Xing Bing Xue Za Zhi*, **29**, 30-3.

Saberi S, Zendehdel K, Jahangiri S, et al (2012). Impact of Methylenetetrahydrofolate Reductase C677T Polymorphism on the Risk of Gastric Cancer and Its Interaction with Helicobacter pylori Infection. *Iran Biomed J*, **16**, 179-84.

Munoz N (1994). Is Helicobacter pylori a cause of gastric cancer? An appraisal of the seroepidemiological evidence. *Cancer Epidemiol Biomarkers Prev*, **3**, 445-51.

Perri F, Cotugno R, Piepoli A, et al (2007). Aberrant DNA methylation in non-neoplastic gastric mucosa of H. pylori infected patients and effect of eradication. *Am J Gastroenterol*, **102**, 1361-71.

Shen H, Xu Y, Zheng Y, et al (2001). Polymorphisms of 5, 10-methylenetetrahydrofolate reductase and risk of gastric cancer in a Chinese population: a case-control study. *Int J Cancer*, 5, 332-6.

Taylor DN, Blaser MJ (1991). The epidemiology of Helicobacter pylori infection. *Epidemiol Rev*, **13**, 42-59.

Vollset SE, Igland J, Jenab M, et al (2007). The association of gastric cancer risk with plasma folate, cobalamin, and methylenetetrahydrofolate reductase polymorphisms in the European Prospective Investigation into Cancer and Nutrition. *Cancer Epidemiol Biomarkers Prev*, **16**, 2416-24.

Wang J, Sasco AJ, Fu C, et al (2008). Aberrant DNA methylation of P16, MGMT, and hMLH1 genes in combination with MTHFR C677T genetic polymorphism in esophageal squamous cell carcinoma. *Cancer Epidemiol Biomark Prev*, **17**, 118-25.

Zacho J, Yazdanyar S, Bojesen SE, et al (2011). Hyperhomocysteinemia, methylenetetrahydrofolate reductase c.677C>T polymorphism and risk of cancer: cross-sectional and prospective studies and meta-analyses of 75,000 cases and 93,000 controls. *Int J Cancer*, **128**, 644-52.

Zhao P, Lin F, Li Z, et al (2011). Folate intake, methylenetetrahydrofolate reductase polymorphisms, and risk of esophageal cancer. *Asian Pac J Cancer Prev*, **12**, 2019-23.

Zúñiga-Noriega JR, Velazco-Campos Mdel R, Aguirre-Rodríguez A, et al (2007). C677T polymorphism of the MTHFR gene and the risk of developing distal gastric cancer in a Mexican population. *Rev Gastroenterol Mex*, **72**, 355-8.