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Original Paper

A Functional Variant at the miR-214 Binding Site in the Methylenetetrahydrofolatereductase Gene Alters Susceptibility to Gastric Cancer in a Chinese Han Population

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Key Words
Gastric cancer • miR-214 • MTHFR • Polymorphism

Abstract
Background and Aims: Single nucleotide polymorphisms in miRNA binding sites, which are located in mRNA 3' untranslated regions (3'-UTRs), were recently found to influence microRNA-target interactions. Specifically, such polymorphisms can modulatebinding affinity or create or destroy miRNA-binding sites;such variants have also been found to be associated with cancer risk. In this study, we explored the effect of a functional variant at the miR-214 binding site in the methylenetetrahydrofolate reductase gene (rs114673809) on gastric cancer (GC) risk in a hospital-based case-control study in a Chinese Han population. ***Methods and Results***: We genotyped the rs114673809 polymorphism in 345 gastric cancer patients and 376 cancer-free controls using the polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) technique. The functions of rs114673809 were investigated using a luciferase activity assay and validated by immunoblotting. We found that participants carrying the rs114673809 AA genotype or A allele had a significantly increased risk of gastric cancer (OR = 1.667, 95% CI = 1.044–2.660, P = 0.034; OR = 1.261, 95% CI = 1.017–1.563, P = 0.037, respectively) compared to those carrying the GG genotype and G allele. In addition, rs114673809 modified the binding of hsa-miR-214 to MTHFR as well as MTHFR protein levels in gastric cancer patients. ***Conclusion***: Our data suggested that rs114673809, which is located at the miR-214 binding site in the 3'-UTR of MTHFR, may play an important role in the development of gastric cancer in a Chinese Han population.

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Introduction

Gastric cancer (GC) remains the second leading cause of cancer-related mortality worldwide, although both its incidence and mortality have been declining in the past decade [1-3]. Epidemiological studies have suggested that gastric carcinogenesis is a complex, multistep and multifactorial process involving genetic and epigenetic alterations of protein-coding proto-oncogenes and tumor-suppressor genes [4]. Environmental factors, including Helicobacter pylori (H. pylori) infection, alcohol consumption, tobacco use and a diet high in salted and nitrated foods, are important factors for the etiology of gastric cancer [5]. Studies have found not everyone with these *lifestyle factors* or who are exposed to *similar* environmental risk factors eventually develop gastric cancer, suggesting that host or genetic factors may also play a role in the etiology of the disease [6]. *Genetic polymorphisms have been* implicated in the development of various diseases, including gastric cancer [7]. Although the precise molecular mechanism remains incompletely understood, genetic polymorphisms are thought to play important roles in gastric carcinogenesis [8].

MiRNAs, a type of endogenous small non-coding RNAs, are approximately 22 nucleotides in length and function as negative regulators of post-transcriptional gene expression [9]. Mature miRNAs usually regulate *target* genes by binding to the 3′ untranslated region (3′UTR) of their target mRNA, leading to mRNA degradation or the suppression of translation [10]. Some studies have demonstrated that several microRNAs are associated with development of cancers [11, 12]. Genetic variants in miRNA target sites in the 3′ UTRs may affect miRNA target recognition and miRNA regulation [13]. Several studies have determined that single nucleotide polymorphisms (SNPs) in the miRNA target sites are associated with the risk of cancers, including leukemias [14, 15] and lung [16], colorectal [17], bladder [18], oral [19], thyroid [20], breast [21] and gastric cancers[22]. However, the role of miRSNPs in the susceptibility of cancer is largely unknown.

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme that plays an important role in the metabolism of intracellular folate and catalyzes the irreversible reduction of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate [23]. A C to T *substitution* at position *677* in MTHFR (C677T) results in reduced plasma folate levels and increased genetic susceptibility to gastric cancer [23, 24]. A bioinformatics analysis found that MTHFR is a target gene of miR-214. A recent study explored the relationship between miRNA expression and the progression of gastric cancer. This previous report showed that sixteen microRNAs were up-regulated in gastric cancer, including miR-214 [25]. Yang et al. also found that miR-214 was highly overexpressed in gastric cancer tissues and cell lines [26]. However, the specific role of miR-214 and its molecular mechanism in the context of gastric cancer cells remains unknown.

Based on these observations, we hypothesized that a functional variant at the miR-214 binding site in the methylenetetrahydrofolate reductase gene may be associated with the risk of gastric cancer. The present report is a case-control study to test this hypothesis.

Materials and Methods

Study Subjects

This study consisted of 345 patients with histologically confirmed gastric adenocarcinoma and 376 cancer-free controls. *All of the* study participants were unrelated ethnic Han Chinese and residents in Jiangsu Province, China. All of the patients were recruited between March 2011 and January 2013 from the Department of Oncology, Danyang People's Hospital and Taixing People's Hospital. Moreover, all demographic and clinical information, including age, sex, smoking and alcohol use, were obtained using a short questionnaire and clinical medical records. The age- (±5 years) and sex-matched controls to these cases were selected from individuals receiving routine medical examinations in these hospitals. Each participant signed a written informed consent, and 5 ml venous blood was obtained from each participant for genomic DNA extraction. The research protocol was approved by the institutional review board of Danyang People's Hospital and Taixing People's Hospital.

DNA Extraction and Genotyping

Genomic DNA was extracted from whole blood specimens using the Blood Mini Kit(Qiagen, Valencia, CA) according to the manufacturer's instructions. The DNA purity and concentrations were determined by spectrophotometric measurement of absorbance at 260 and 280 nm using a UV spectrophotometer. The isolated DNA was dissolved in TE buffer and stored at -20 °C before analysis. The rs114673809 polymorphism was genotyped using the polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) technique. Briefly, the following PCR assay was performed using a Bio-Rad S-1000 thermocycler (Bio-Rad Laboratories, Hercules, CA). The primers 5′-AATCAGCTCCTTGGGACACG-3′ and 5′-CACCCTGGAAAGGGGAGTTG-3′ were used to amplify the target region containing the rs114673809 polymorphism. The amplification reaction was carried out in the following conditions: an initial melting step of 3 min at 94 °C, followed by 35 cycles of 30 sec at 94 °C, 30 sec at 58 °C and 30 sec at 72 °C, with a final elongation of 10 min at 72 °C. The reaction yielded a 304-bp amplicon. The presence of the G allele creates an TauI (New England Biolabs, Ipswich, MA) restriction site; digested amplicons from GG homozygotes appear as a 172 bp and a 132 bp band, homozygotes for the A allele appear as a 304 bp band, and heterozygotes have all three of these bands. In addition, genotyping results were validated by direct DNA sequencing in a randomly selected 10% of samples for quality control. The genotype concordance was 100%.

Vector construction and luciferase reporter assays

The *dual luciferase assay* was *performed* using the Dual-Luciferase Reporter Assay System (psiCHECK-2 vector, Promega). A 850-bp fragment of the MTHFR 3' UTR was amplified from genomic DNA using the following primer sequences: forward, 5′-GGACTAGTGGTTGTTGCCAACTAAGCCC-3′; reverse, 5′- TTCAAGCTTTCCAGGGAGTGATGACAGAG-3′. The PCR product was inserted into the psiCHECK-2 vector downstream of the luciferase gene sequence. All of the constructs were verified by DNA sequencing. According to the MTHFR genotypes, constructed vectors were termed psiCHECK-AA and psiCHECK-GG, respectively. HEK-293 cells were plated in 96-well clusters. Lipofectamine 2000 was used to transfect the cells with 80 ng of psiCHECK-AA or psiCHECK-GG reporter constructs,(Invitrogen, USA)hsa-miR-214 or controls (all at 30 nM, Ambion, USA), and 80 ng of a psiCHECK-2 luciferase vector. After 48 hoftransfection, the cells were washed twice and lysed with passive lysis buffer. Firefly luciferase activity was determined using the dual-luciferase reporter assay system and a luminometer (Promega), as we previously described [27]. The relative luciferase *activity* was calculated by normalizing the firefly luciferase activity against the internal control luciferase activity.

Immunoblot analyses

The samples were obtained from peripheral blood mononuclear cells (PBMCs). The cells were disrupted in RIPA buffer, and the protein concentrations were determined using the BCA assay according to the manufacturer's instruction (Beyotime, Haimen, Jiangsu) using bovine serum albumin as a standard. An equal amount of cell lysates (40 μgprotein) were denatured in 2× SDS-PAGE sample buffer and electrophoresed for 3 h at 20 mA on 10% polyacrylamide gels. The separated proteins were then transferred into polyvinylidenedifluoride (PVDF) membranes (Roche Diagnosis, Indianapolis, IN, USA). The membrane was then blocked in a TBST solution containing 5% nonfat dry milk for 4 h at room temperature. After incubation with an anti-human MTHFR (1:1000; Abcam, Cambridge, MA, USA) primary antibody under blocking conditions, the membrane was washed with TBST three times for 10 min each, followed by incubation with goat anti-mouse IgG secondary antibody (1/100000, Zhongshan Goden Bridge Biotechnology Co., Ltd, Beijing) for 1 h at room temperature. The proteins were detected using the ECL Kit (*Pierce*, *Rockford*, *IL*, *USA*). The bands were quantified by normalization to GAPDH using Image J Software (National Institutes of Health Bethesda, MD, *USA*).

Homocysteine determination

Plasma homocysteine levels were determined using an enzyme immunoassay method described by Frantzen et al. [28] using commercially available kit from Hopeyearmed Ltd, Tianjin, China.

Statistical analysis

The data were *analyzed* using the Statistical Package for Social Sciences version 12.0 (SPSS, Chicago, IL). The *Hardy-Weinberg equilibrium* was *tested* using the Chi-squared test. Differences between genotype

distribution and allele frequency were tested by Fisher's exact test. Odds ratios (OR) and 95% confidence intervals (CI) were examined by logistic regression analysis. The Student's t-test was used to compare the MTHFR protein levels between genotypes. All of the statistical hypothesis tests were two-sided, with *P* value <0.05 representing statistical significance.

Results

Characteristics of the Study Population

The demographic characteristics and other selected characteristics of the participants are presented in Table 1. There were no significant differences in the distribution of age, sex, or family history of gastric cancer between the controls and gastric cancer cases. However, cases were more likely to have *H. pylori* infection (*P*=0.039), more likely to smoke (*P* = 0.037), and more likely to drink alcohol (*P* = 0.041) than controls. The patient's information indicated that smoking, drinking and *H.pylori* infection may be associated with the development of gastric cancer.

Association between the rs114673809 polymorphism and GC risk

Table 2 shows the genotype distribution of rs114673809 among GC cases and controls as well as the association of the polymorphisms with GC risk. The SNP genotype frequencies in the study population fit the Hardy-Weinberg equilibrium (χ^2=0.031, *P* = 0.860 in cases; χ^2 = 0.264, *P* = 0.607 in controls). Pearson's χ^2 test and logistic regression analysis were used to assess the association between the rs114673809 polymorphism and gastric cancer risk. The participants carrying the rs114673809 AA genotype had a significantly increased risk of developing gastric cancer (OR = 1.667, 95% CI = 1.044–2.660, *P*=0.034) compared to the GG genotype. Similar associations were observed in participants carrying the rs114673809 A allele (A allele vs. G allele: OR = 1.261, 95% CI = 1.017–1.563, *P* = 0.037).

Stratification analysis of GC risk by the polymorphism rs114673809

We further stratified by selected variables, including age, sex, smoking status and drinking status, to evaluate the association between rs114673809 genotypes and gastric cancer risk. As shown in Table 3, the stratification analysis indicated that the detrimental genotypes rs114673809 AG/AA were more pronounced in males (OR = 1.564, 95% CI = 1.068–2.289, P=0.027) and in younger (OR = 1.661, 95% CI = 1.063-2.595, P=0.033), never-

Table 1. Distributions of selected variables in GC cases and controls

	Controls No. (%)	Cases No. (%)	P
Number	376	345	
Age, yr (mean ± SD)	61.3±12.7	62.1±13.1	0.406
＜59 (median)	179(47.6)	160(46.4)	
≥59 (median)	197(52.4)	185(53.6)	0.741
Sex			
Males	237(63.0)	221(64.1)	
Females	139(37.0)	124(35.9)	0.775
Family history of GC			
Positive	30(8.0)	27(7.8)	
Negative	346(92.0)	318(92.2)	0.940
H. pylori infection			
Positive	241(64.1)	246(71.3)	
Negative	135(35.9)	99(28.7)	0.039
Smoking Status			
Never	196(52.1)	153(44.3)	
Ever	180(47.9)	192(55.7)	0.037
Drinking Status			
Never	191(50.8)	149(43.2)	
Ever	185(49.2)	196(56.2)	0.041

Table 2. Distribution of the rs114673809 polymorphism genotypes in GC cases and controls

		Control n(%)	Cases n (%)	OR (95%CI)	P
rs 114673809	GG	162(43.1)	128(37.1)		
	GA	173(46.0)	163(47.2)	1.192 (0.870-1.635)	0.296
	AA	41(10.9)	54(15.6)	1.667 (1.044-2.660)	0.034
	Gallele	497(66.1)	419(60.7)		
	A allele	255(33.9)	271(39.3)	1.261 (1.017-1.563)	0.037

Table 3. Stratification analysis GC risk by the polymorphism rs114673809

	cases/controls		OR	P
	GG	AG+AA	(95% CI)	
Age, yr				
＜59	50/77	110/102	1.661 (1.063-2.595)	0.033
≥59	78/85	107/112	1.041 (0.694-1.562)	0.918
Sex				
Males	77/102	144/135	1.564 (1.068-2.289)	0.027
Females	51/60	73/79	1.087 (0.666-1.775)	0.803
Smoking Status				
Never	38/70	115/126	1.681 (1.052-2.687)	0.036
Ever	90/92	102/88	1.185 (0.789-1.780)	0.468
Drinking Status				
Never	45/82	104/109	1.739 (1.106-2.732)	0.018
Ever	83/80	113/105	1.037 (0.691-1.557)	0.918
H. pylori infection				
Positive	100/104	146/137	1.108 (0.773-1.589)	0.583
Negative	28/58	71/77	1.910 (1.097-3.325)	0.028
Family history of GC				
Positive	10/13	17/17	1.300(0.449-3.766)	0.788
Negative	118/149	200/197	1.282(0.939-1.750)	0.132

smoking (OR = 1.681, 95% CI = 1.052-2.687, P=0.036), never-drinking OR = 1.739, 95% CI = 1.106-2.732, P=0.018) and *H. pylori*-negative individuals(OR = 1.910, 95% CI = 1.097-3.325, P=0.028).

rs114673809 modified the binding of hsa-miR-214 to MTHFR

Using an online tool miRNASNP 2.0 (http://www.bioguo.org/miRNASNP/), we found the rs114673809 *created* a miRNA-mRNA *binding site* (Fig. 1A).We cloned and transfected the luciferase reporter vector of the 3´UTR of MTHFR gene with either the -A or –G rs114673809 allele into HEK 239 cells. The observed activity was lower in the reporter vector carrying-A allele than the reporter vector carrying-G allele ($p< 0.05$) (Fig. 1B).To determine the effect of rs114673809 on MTHFR expression, we performed expression analysis in peripheral blood mononuclear cells from GC patients with GG, AG or AA rs114673809 genotypes. Using western blot analyses, we observed a significant decrease in MTHFR protein levels in AA carriers relative to the GG carriers ($p < 0.05$) (Fig. 1Cand 1D).We next detected plasma homocysteine levels in GC cases and controls. We found significantly higher plasma homocysteine levels in GC cases relative to controls. Furthermore, we observed a significant increase plasma homocysteine levels in GC cases carrying AA genotype relative to GC cases carrying the GG genotype (Fig. 1F).

Discussion

In the present hospital-based case-control study containing a total of 345 GC patients and 376 healthy controls, we found that the SNP rs114673809A>G is associated with the risk

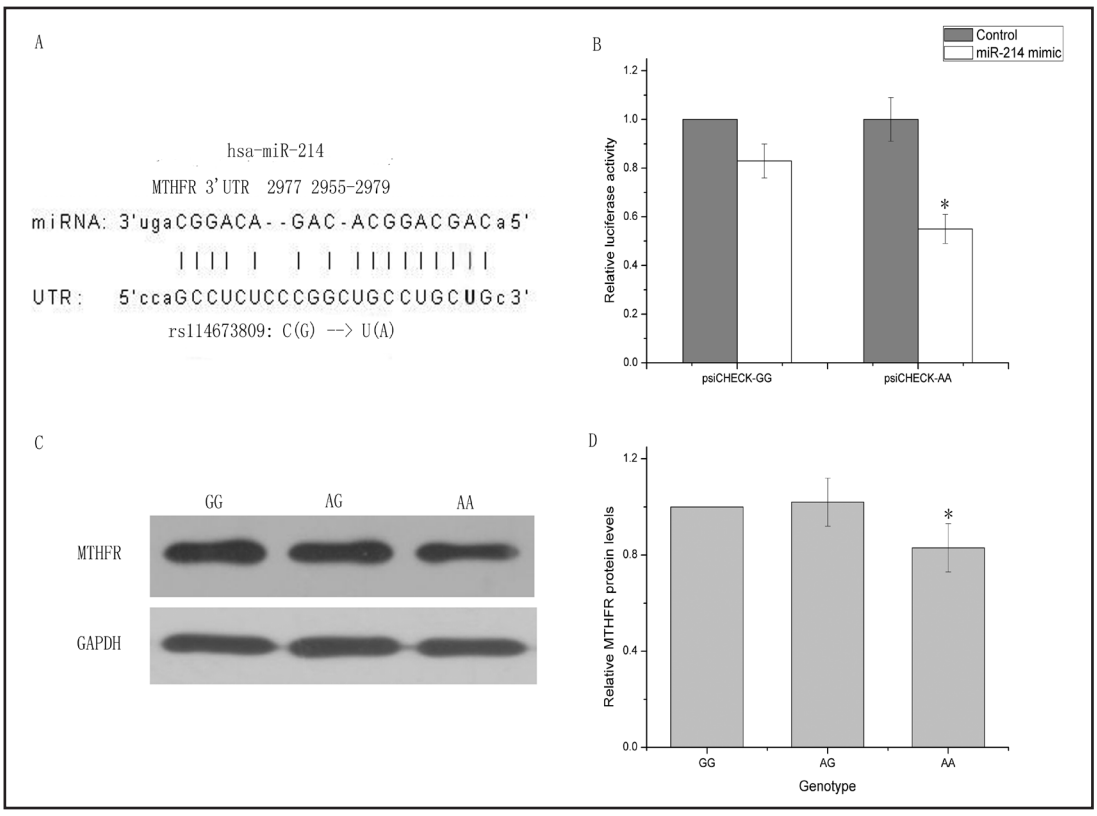

Fig. 1. Modification of MTHFR expression based on the rs114673809 genotype. A: rs114673809 created a miRNA-mRNA binding site. *B:* Luciferase assay for miR-214 targeting a MTHFR 3´UTR vector with either a -G or -A allele. HEK293 cells were cotransfected with miR-214 (30 nM) or negative controls. C, D: MTHFR protein levels were detected in GC patients with GG, AG or AA rs114673809 genotypes. E: Plasma homocysteine levels were detected in GC cases and controls. F: Plasma homocysteine levels were detected in patients with GG, GA or AA rs114673809 genotypes. **P*<0.05.

of gastric cancer. Our data showed that subjects carrying the rs114673809AA genotype had a significantly increased risk for gastric cancer compared with the GG genotype (*P*<0.05). Additionally, it appeared that the increased risk caused by this polymorphism was more pronounced in males and in younger, never-smoking, never-drinking and *H. pylori*-negative individuals. To our knowledge, this is the first study to explore the association between the rs114673809 variant and the risk of GC.

Increasing evidence indicates that miRNAsare dysregulated in various types of human cancers, and altered miRNA expression might contribute to human carcinogenesis via dysregulation of target gene expression [29-31]. Hence, the identification of these miRNAs and *their* mRNA *targets*that are associated with tumorigenesis would provide valuable insight into the *di*agnostic and treatment of patients with human malignancies. It was previously reported that miR-214 was up-regulated in human ovarian cancer and induces cell survival and cisplatin resistance by targeting the PTEN/Akt pathway [29]. In addition, miR-214 expression was up-regulated in pancreatic cancer tissues compared with matched normal pancreatic tissues, and this up-regulation was observed induce the resistance of the pancreatic cancer cells to gemcitabine by targeting ING4 mRNA [32]. However, Zhang et al. found that miR-214 is down-regulated in human cervical cancer tissue and that it negatively regulates HeLa cell proliferation by targeting MEK3 and JNK1 mRNAs [33]. Yang et al found that miR-214 acts as a tumor suppressor and regulates proliferation, migration and invasion by targeting PTEN in gastric cancer [26]. In the present study, we found that a functional variant at the miR-214 binding site in the methylenetetrahydrofolate reductase

gene modified the binding of hsa-miR-214 to MTHFR. This genetic variant may influence the function of MTHFR and was associated with the development of malignancies.

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme that plays an essential role in the metabolism of folate. Folate deficiency may cause DNA instability, defective DNA repair, and aberrant DNA methylation; such effects can be involved in carcinogenesis, including GC [34, 35]. The *MTHFR 677 C to T* substitution in MTHFR (C677T) results in an *alanine to valine* substitution. This polymorphism is associated with reduced activation of MTHFR that leads to reduced plasma folate levels [36]. Low enzyme activity of *MTHFR* TT *genotype* is *related* to *DNA hypomethylation,* which may induce *genomein stability* and thereby influence the expression of oncogenes or tumor suppressors. An increasing amount of evidence has indicated the association of folate and aberrant DNA methylation with the risk of human cancers. Previous studies of the MTHFR polymorphism and their associations with gastric cancer were quite inconsistent. Of the published studies, the MTHFR 677TT genotype was found to be a strong risk factor for gastric cancer in Chinese and East Asian populations [37, 38]. This same polymorphism was also reported to have no association with GC [39-41]and was even suggested to confer a decreased risk in a Mexican population [42]. Galvan-Portillo et al. reported that the MTHFR 677 TT genotype among individuals with high consumption of folate was associated with decreased gastric cancer risk (OR = 0.23; 95% CI 0.06-0.84) compared to wild-type homozygous and heterozygous genotypes combined. In the present study, we found that participants carrying the rs114673809 AA genotype had a significantly increased risk of developing gastric cancer (OR = 1.667, 95% CI = 1.044–2.660, P = 0.034) compared to GG genotype. In addition, rs114673809 modified the binding of hsa-miR-214 to MTHFR and altered MTHFR protein levels in gastric cancer patients. A significant association between the MTHFR 677T allele and increased Hcy levels was found in different ethnic groups [43]. Zacho J et al. reported that MTHFR c.677C>T homozygosity and lifelong hyperhomocysteinemia, and hence hypomethylation, was associated with an increased risk of esophagus and gastric cancer [44]. We therefore hypothesize that rs4846049 modulates the risk of GC through homocysteine levels. We observed a significant increase plasma homocysteine levels in GC cases carrying the AA genotype relative to cases carrying the GG genotype. The results indicated that the miR-214/MTHFR axis may play an important role in the development of GC.

In conclusion, our study provides the first evidence that a functional variant at the miR-214 binding site in the methylenetetrahydrofolate reductase gene plays an important role in mediating an individual's susceptibility to GC. Our studyfurther supports the hypothesis that mutations in microRNAs or microRNA binding sites may affect microRNA-mediated regulation and are associated with the risk of cancer, including gastric cancer. Additional larger, preferably population-based, case-control studies are required to validate the present findings and to understand the mechanisms of the rs114673809 variant.

Disclosure Statement

The authors declare no conflict of interest.

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