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Association between a microRNA-214 binding site polymorphism in the methylenetetrahydrofolate reductase gene and esophageal squamous cell carcinoma

G.R. Shen[1]*, W.Z. Li[2]*, Y.C. Liu[3], X.P. Li[1] and H.Y. Yuan[2]

[1]Medical Laboratory Department,
The First People's Hospital of Wujiang District, Nantong University, Suzhou, China
[2]Clinical Laboratory Department, Taixing People's Hospital, Taixing, Jiangsu, China
[3]Department of Oncology, Taixing People's Hospital, Taixing, Jiangsu, China

*These authors contributed equally to this study.
Corresponding author: H.Y. Yuan
E-mail: 18151959756@163.com

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ABSTRACT. MicroRNAs (miRNAs) are key regulators of gene expression and play an important role in the development and progression of various diseases including esophageal squamous cell carcinoma (ESCC). In this study, we determined whether a polymorphism at the miR-214 binding site in the 3'-untranslated region (3'-UTR) of the methylenetetrahydrofolate reductase gene (*MTHFR*) is associated with susceptibility to ESCC. A total of 448 ESCC cases and 460 gender- and age-matched subjects were recruited for the study. The genotypes of the rs114673809 single nucleotide polymorphism (SNP) were determined by polymerase chain reaction sequencing. Associations between genotypes of *MTHFR* rs114673809 and ESCC risk were

determined using logistic regression analyses. In the recessive model, when the *MTHFR* rs114673809 GG homozygote genotype was used as the reference group, the GA genotype was not associated with the risk of ESCC (GA *vs* GG: OR = 1.261, 95%CI = 0.960-1.657, P = 0.110), but the AA genotype was associated with increased risk of ECSS (AA *vs* GG: OR = 1.752, 95%CI = 1.076-2.853, P = 0.027). Additionally, the rs114673809 A allele carriers also showed a 1.286-fold increased ESCC risk compared with those carrying the rs114673809 G allele genotype. Furthermore, we observed a significant increase in plasma homocysteine levels in ESCC cases carrying the AA genotype relative to ESCC cases carrying the GG genotype. Our data demonstrate that a polymorphism at the miR-214 binding site in the 3'-UTR of *MTHFR* is an ESCC susceptibility SNP in the Chinese population.

Key words: Esophageal squamous cell carcinoma; Polymorphism; Methylenetetrahydrofolate reductase

INTRODUCTION

Esophageal carcinoma (EC) is a serious threat to human health worldwide; it is the eighth most common human cancer and accounts for the sixth highest rate of cancer mortality. Histologically, EC consists of two major types: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (Bandla et al., 2012). Each year, approximately 50% of newly diagnosed esophageal carcinoma cases occur in China (Holmes and Vaughan, 2007). The carcinogenesis of EC is a complex process that is related to multiple etiologic factors, including dietary factors, infection, alcohol consumption, cigarette smoking, and obesity (Vaughan et al., 1995; Mayne et al., 2001; Wang et al., 2013). However, only a small portion of individuals who have been exposed to the same risk factors develop EC. This suggests that genetic factors play an important role in the development of EC (Fan et al., 2013; Zhang et al., 2013; Ye et al., 2014).

Methylenetetrahydrofolate reductase (MTHFR) is a key regulatory enzyme in folate and homocysteine metabolism, and catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a methyl-group donor in the conversion of homocysteine to methionine. The gene that encodes MTHFR has the chromosomal locus 1p36.3 and comprises 11 exons. Two common variant genotypes of *MTHFR*, C677T and A1298C, are associated with a significant reduction in enzyme activity (Martin et al., 2006; Saberi et al., 2012). The *MTHFR* genotypes 677TT and 1298CC are associated with higher homocysteine levels and lower folate levels, which may lead to DNA hypomethylation and an increased risk of cancer.

MicroRNAs (miRNAs), a class of small (20-22 nucleotide) non-coding RNA molecules, negatively regulate gene expression at the post-transcriptional level by partially binding to complementary sequences in the 3'-untranslated region (3'-UTR) of the targeted mRNA. Recent studies have shown that miRNAs are involved in many biological processes, including cell proliferation, differentiation, cell cycle progression, and apoptosis. Many studies have found that dysregulation of miRNAs is associated with a variety of human diseases including cancer. Owing to their potential effects, single nucleotide polymorphisms

(SNPs) in pre-miRNA or mature miRNA sequences, or miRNA-binding sites, may modulate miRNA-target interactions and the expression of target genes. SNPs in pre-miRNA or mature miRNA sequences, or miRNA-binding sites have been implicated in cancer susceptibility. For example, an miR-184 binding site polymorphism in *TNFAIP2* (rs8126) is associated with the risk of gastric cancer (Xu et al., 2013). A genetic variation in an miR-191 binding site in *MDM4* contributes to susceptibility to ESCC (Zhou et al., 2013).

In our previous study, we found an miR-214 binding site SNP (rs114673809) in the 3'-UTR region of *MTHFR* through bioinformatic analysis. However, the role of this polymorphism in MTHFR activity and cancer susceptibility remains unclear. In this study, we explored the association between rs114673809 and susceptibility to ESCC in a Chinese population.

MATERIAL AND METHODS

Study subjects

To estimate the association between *MTHFR* gene polymorphisms and the risk of ESCC, 448 southern Han Chinese patients with ESCC and 460 gender- and age-matched (± 5 years) control subjects were recruited from Wujiang People's Hospital (Wujiang, Jiangsu Province, China) and Taixing People's Hospital (Taixing, Jiangsu Province, China) between January 2013 and October 2015. The control subjects were individuals who went to either hospital for a routine health check and were free from any type of cancer. A written questionnaire was used to obtain demographic and risk factor information, including smoking, alcohol consumption, and family history of malignancy. Smokers were classified as individuals who had smoked at least once during the past 30 days. Individuals were defined as alcohol consumers if they drank alcohol at least once every week. Written informed consent was obtained from all the subjects enrolled in the study. This study was approved by the ethics review boards of Wujiang People's Hospital and Taixing People's Hospital.

DNA extraction and genotyping

Peripheral blood (5 mL) was collected from each subject. DNA was obtained from peripheral lymphocytes using a QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer instructions. The primers for the polymerase chain reaction (PCR) analysis of *MTHFR* polymorphism rs114673809 were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and were synthesized commercially by Sangon Biotech (Shanghai, China). The primer sequences were as follows: forward: 5'-AATCAGCTCCTTGGGACACG-3'; reverse: 5'-CACCCTGGAAAGGGGAGTTG-3'. PCR amplification was performed in a 25-μL reaction volume containing 1 μL genomic DNA, 2 μL 2.5 mM dNTPs, 2.5 μL 10X rTaq buffer, 1 μL each primer (10 mM), and 0.125 μL rTaq. PCRs were performed under the following conditions: initial denaturation at 94°C for 3 min, followed by 30 cycles of 15 s at 94°C, annealing at 62°C for 10 s, polymerization at 72°C for 15 s, with a final polymerization step at 72°C for 7 min. The PCR products were purified and sequenced by Sangon Biotech.

Homocysteine determination

Plasma homocysteine levels were determined using the enzyme immunoassay method

described by Frantzen et al. (1998) using a commercially available kit from Jianglai Tech Ltd., Shanghai, China.

Statistical analysis

The distribution differences of the categorical variables between cases and controls were assessed by the chi-square test. A goodness-of-fit chi-square test was used to test the Hardy-Weinberg equilibrium (HWE) of the cancer-free control genotype distributions. Group comparisons were analyzed using the Student *t*-test (two-sided). The association analysis between genetic variants and ESCC risk, and the evaluation of odds ratios (ORs) and 95% confidence intervals (95%CIs) were conducted using unconditional logistic regression, and were adjusted for age and gender. All statistical analyses were performed with the SPSS software (v.12.0) (SPSS Inc., Chicago, IL, USA).

RESULTS

Characteristics of ESCC patients and controls

The selected characteristics of the 448 ESCC cases and 460 cancer-free controls are summarized in Table 1. No statistically significant differences were found between the ESCC cases and the healthy controls in terms of age and gender distributions ($P > 0.05$), indicating that the frequency matching was adequate. However, there were more smokers, alcohol consumers, and individuals with a family history of malignancy among the ESCC patients than among the cancer-free control subjects.

Table 1. Distribution of selected characteristics among esophageal squamous cell carcinoma (ESCC) patients and controls.

Variable	ESCC patients [N (%)] N = 448	Controls [N (%)] N = 460	P
Age (years)			0.640
≤59	193 (43.1)	206 (44.8)	
>59	255 (56.9)	254 (55.2)	
Gender			0.608
Female	125 (27.9)	136 (29.6)	
Male	323 (72.1)	324 (70.4)	
Smoking status			0.038
No	293 (65.4)	331 (72.0)	
Yes	155 (34.6)	129 (28.0)	
Drinking status			0.011
No	282 (62.9)	327 (71.1)	
Yes	166 (37.1)	133 (28.9)	
Family history of malignancy			0.000
No	381 (85.0)	428 (93.0)	
Yes	67 (15.0)	32 (7.0)	

Association between the rs114673809 polymorphism and risk of ESCC

The genotype distributions of the rs114673809 polymorphism in the cases and controls are shown in Table 2. All observed genotype frequencies in both controls and cases conformed to HWE (control: $\chi^2 = 0.814$, $P = 0.367$; ESCC cases: $\chi^2 = 0.296$, $P = 0.586$). The genotype frequencies of rs114673809 were 44.0% (GG), 45.5% (GA), and 10.5% (AA) in

the ESCC patients, and 51.1% (GG), 42.0% (GA), and 7.0% (AA) in the control subjects. When the rs114673809 GG homozygote genotype was used as the reference group, the AA genotype was associated with a significantly increased risk of ESCC (AA *vs* GG: OR = 1.752, 95%CI =1.076-2.853, P = 0.027). However, the GA genotype was not associated with the risk for ESCC (GA *vs* GG: OR = 1.261, 95%CI = 0.960-1.657, P = 0.110). When the rs114673809 G allele was used as the reference group, the T allele was associated with a significantly increased risk of ESCC (A *vs* G: OR = 1.286, 95%CI = 1.052-1.570, P =0.014).

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

Genotypes	Cases [N (%)] N = 448	Controls [N (%)] N = 460	OR (95%CI)	P
GG	197 (44.0)	235 (51.1)		
GA	204 (45.5)	193 (42.0)	1.261 (0.960-1.657)	0.110
AA	47 (10.5)	32 (7.0)	1.752 (1.076-2.853)	0.027
G allele	598 (66.7)	663 (72.1)		
A allele	298 (33.2)	257 (27.9)	1.286 (1.052-1.570)	0.014

Stratification analyses of *MTHFR* rs114673809 and risk of ESCC

We then further evaluated the effect of *MTHFR* rs114673809 on the risk of ESCC stratified by age, gender, smoking habits, alcohol consumption, and family history of malignancy. As shown in Table 3, the association between *MTHFR* rs114673809 and the risk of ESCC appeared stronger in the subgroups of younger subjects (OR = 1.500, 95%CI = 1.009-2.229, P = 0.045), males (OR = 1.463, 95%CI = 1.073-1.996, P = 0.018), non-smokers (OR = 1.442, 95%CI = 1.050-1.979, P = 0.025), non-drinkers (OR = 1.430, 95%CI = 1.072-1.907, P = 0.028), and individuals with a family history of malignancy (OR = 1.401, 95%CI = 1.062-1.849, P = 0.020).

Table 3. Stratification analysis of esophageal squamous cell carcinoma (ESCC) risk associated with the rs114673809 polymorphism.

	Cases/controls		OR (95%CI)	P
	GG	GA+AA		
Age				
<59	79/105	114/101	1.500 (1.009-2.229)	0.045
≥59	118/130	137/124	1.217 (0.859-1.724)	0.228
Gender				
Males	135/166	188/158	1.463 (1.073-1.996)	0.018
Females	62/69	63/67	1.046 (0.644-1.701)	0.902
Smoking status				
No	123/169	170/162	1.442 (1.050-1.979)	0.025
Yes	74/66	81/63	1.147 (0.719-1.830)	0.634
Drinking status				
No	118/167	164/160	1.430 (1.072-1.907)	0.028
Yes	79/68	87/65	1.152 (0.730-1.819)	0.562
Family history of malignancy				
No	163/218	220/210	1.401 (1.062-1.849)	0.020
Yes	34/17	33/15	1.100 (0.473-2.557)	0.834

Detection of plasma homocysteine levels

We next detected plasma homocysteine levels in ESCC cases and controls. We found significantly higher plasma homocysteine levels in the ESCC cases relative to the controls

(Figure 1A). Furthermore, we observed a significant increase in plasma homocysteine levels in the ESCC cases carrying the AA genotype relative to the ESCC cases carrying the GG genotype (Figure 1B).

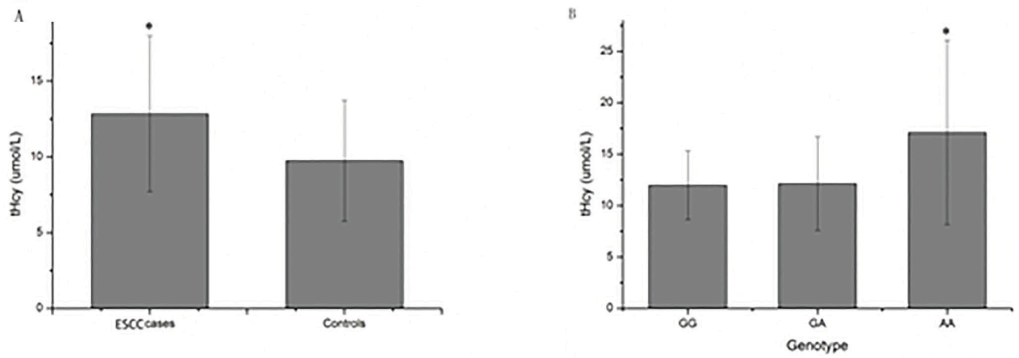

Figure 1. Detection of plasma homocysteine levels. **A.** Plasma homocysteine levels were detected in esophageal squamous cell carcinoma (ESCC) cases and controls. **B.** Plasma homocysteine levels were detected in patients with GG, GA, or AA rs114673809 genotypes. *P < 0.05.

DISCUSSION

In the current study, we investigated the association between the *MTHFR* rs114673809 functional SNP and ESCC risk using a case-control approach. We observed that individuals with *MTHFR* rs114673809 AA genotypes showed significantly increased ESCC risk compared with the GG genotype carriers. These results highlight the involvement of functional genetic variants in miRNA-binding sites in ESCC etiology.

Folic acid (FA) is a major component of leafy green vegetables and citrus fruit, and its deficiency has been implicated in the development of a variety of cancers, including ESCC. FA is thought to play an important role in DNA synthesis and gene activation by influencing DNA methylation and promoting *de novo* deoxynucleoside synthesis (Choi and Mason, 2000). Several mechanisms could underlie FA deficiency-associated cancers, including aberrant DNA methylation, DNA strand breaks, and impaired DNA repair. MTHFR is the key enzyme involved in FA metabolism and works by modulating the levels of the circulating form of MTHFR, 5-methyl-tetrahydrofolate (5MTHF). The precursor of 5-MTHF(5,10MTHF) is crucial for DNA repair because it provides a supply of the precursors for DNA synthesis (Keld et al., 2014). There are two well-studied polymorphisms of the *MTHFR* gene: C677T and A1298C (Weisberg et al., 1998). In this study, we found that individuals with *MTHFR* rs114673809 AA genotypes showed significantly increased ESCC risk compared with the GG genotype carriers. Several studies have demonstrated that individuals with the *MTHFR* 677TT genotype have higher plasma homocysteine levels compared with the wild-type *MTHFR* 677CC, and are therefore associated with diminished genomic DNA methylation, especially when FA intake is insufficient (Li et al., 2005; Wang et al., 2005). In the present study, we observed a significant increase in plasma homocysteine levels in ESCC cases carrying the AA genotype relative to the ESCC cases carrying the GG genotype. Our findings indicate that the *MTHFR* rs114673809 functional SNP might influence homocysteine levels.

In summary, our results suggest that the functional *MTHFR* rs114673809 SNP is

associated with a significantly increased ESCC risk in Chinese populations. Further effort is needed to determine whether the *MTHFR* rs114673809 genetic polymorphism can be used as a potential diagnostic marker for ESCC.

Conflicts of interest

The authors declare no conflict of interest.

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