Letter to the Editor

Serum Folate, *MTHFR* C677T Polymorphism and Esophageal Squamous Cell Carcinoma Risk*

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This study examined associations between *MTHFR* C677T polymorphism and serum folate concentrations with the risk of esophageal precancerous lesions (EPL) and esophageal squamous cell carcinoma (ESCC). The highest quartile of serum folate concentration significantly decreased the risk of ESCC compared with the lowest quartile (OR=0.11; 95% CI, 0.04-0.33; *P*<0.05). *MTHFR* 677 C>T polymorphism was associated with the risk of ESCC by using chi-square tests (*P*<0.05). For the CT genotype, the risk of ESCC significantly increased in study participants with low serum folate concentrations (≤26.92 μg/L) compared with participants with high serum folate concentrations (>26.92 μg/L) by using multinomial logistic regression models. The *MTHFR* genotype may further modify associations between serum folate concentrations and the risk of ESCC, but it was not significantly associated with the risk of EPL.

Esophageal cancer is one of the most common upper gastrointestinal malignancies, and causes significant morbidity and mortality worldwide. There are 250 000 new cases diagnosed yearly in China, which contributes to more than 50% of all cases globally[1]. It has been reported that esophageal carcinogenesis is a multi-stage process, with most patients detected in the later period, and the 5-year survival rate is less than 10%. Early detection and treatment is critical for the prevention of esophageal cancer.

Folate plays a key role in the one-carbon metabolism pathway, which is involved in DNA methylation, synthesis, and repair[2]. Folate can be transformed to tetrahydrofolate in the body, which is the carrier of single-carbon fragments of metabolism. Tetrahydrofolate is then methylated into 5,10-methylenetetrahydrofolate, which participates in the synthesis of pyrimidine and purine. Conversely, the enzyme 5,10-methylenetetrahydrofolate reductase (*MTHFR*) irreversibly catalyzes conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the methyl donor in DNA methylation. Therefore, folate deficiency may induce chromosomal damage, formation of fragile sites, and micronuclei, often associated with tumorigenesis[3]. Uracil misincorporation into DNA due to low serum folate may lead to not only chromosomal aberrations but also DNA repair disruption[4]. Previous studies on relationships between folate and cancer have mainly focused on colorectal cancer and breast cancer and less on esophageal cancer; although some studies have reported that dietary folate was inversely related to the risk of esophageal cancer[5]. Studies on the role of serum folate in the development of esophageal cancer are limited.

Methylenetetrahydrofolate reductase (*MTHFR*) is a critical enzyme in folate metabolism and is essential for DNA synthesis, and decreased folate may adversely affect DNA synthesis. Reduction in *MTHFR* activity may result in accumulation of 5,10-methylenetetrahydrofolate, which may accelerate the methylation of uridylate to thymidylate, leading to misincorporation of uracil in DNA and contributing to overall DNA damage in cells.

The C677T polymorphism is the most common functional variant, leading to a reduction in enzyme activity. Several studies have found associations between *MTHFR* genotypes and esophageal cancer, supporting the hypothesis that the *MTHFR* polymorphism may play a role in carcinogenesis of the esophagus[6]. Effects of *MTHFR* polymorphisms

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on other cancers are not consistent[7]. Few studies have investigated the effects of serum folate concentrations on the risk of esophageal cancer in conjunction with genetic polymorphisms.

Few relationships between folate and esophageal precancerous lesions have been reported. Esophageal epithelial dysplasia was defined as esophageal precancerous lesions, and is a reversible tissue lesion. Prevention or treatment of esophageal epithelial dysplasia is critical to prevent the occurrence of esophageal cancer. In this study, we assessed associations between serum folate concentrations and the risk of esophageal precancerous lesions (EPL) and esophageal squamous cell carcinoma (ESCC), and determined whether the associations can be modified by the *MTH*FR genotype.

All participants were unrelated ethnic Han Chinese and were residents in a high esophageal cancer incidence region of northern Jiangsu in Huai'an District, Huai'an, China. We selected new cases of ESCC from a database of the Malignant Tumor Registry of Huai'an Center for Disease Control and Prevention. Eligible cases were newly diagnosed ESCC by gastroscopy or X-ray at local hospitals and confirmed by clinical histopathology between 2010 and 2012. EPL and healthy individuals were also identified during the same period and randomly screened by gastroscopy and histopathology in early diagnosis and early treatment projects for esophageal cancer in Jiangsu province. Eligibility criteria for both cases and controls included not being pregnant or taking folate supplements at the time of recruitment, and no prior history of cancer and/or esophageal surgery and chemotherapy or radiation therapy. Participants with ESCC, EPL, and healthy controls were included in the study. Doctors were provided with training for the study, and gastroscopies were conducted and questionnaires from face-to-face surveys were collected. Spot checks were conducted on a regular basis. In addition to epidemiological and food frequency questionnaires, two 5 mL blood samples were collected from each participant following fasting for more than 8 h, and separated into white blood cells and serum and stored at -80 °C until analyzed. Informed consent was obtained from each participant. The study was approved by the Institutional Review Board of Southeast University Zhongda Hospital.

Serum folate concentrations were measured and quantified using an ELISA kit (Kexing, Shanghai, China), according to the manufacturer's instructions. Genomic DNA was extracted from white blood cells using a Wizard® Genomic DNA Purification kit (A1120; Promega, WI, USA), according to the manufacturer's instructions. Genotyping for the *MTH*FR C677T polymorphism was performed using the PCR-restriction fragment length polymorphism methods. PCR amplification primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). A 233 bp product was amplified using forward primer 5'-CGA AGC AGG GAG CTT TGA GGC TG-3', and reverse primer 5'-AGG ACG GTG CGG TGA GAG TG-3', as described by Wang YM et al.[8]. Briefly, PCR amplification was carried out using a PCR premix kit (SK2072; Sangon, Shanghai, China) in a total volume of 20 μL that contained DNA 0.5 μL, 2× PCR buffer 12.5 μL, 0.5 μL primers (10 μmol/L) and 6.5 μL ddH_2O. Amplification was run in an automated thermocycler (AG6321; Eppendorf, Hamburg, Germany). The mixture was initially denatured at 94 °C for 5 min, followed by 35 cycles for 30 s at 94 °C, 30 s at 67 °C, 60 s at 72 °C, and a 10 min final extension at 72 °C[8]. PCR products were run on 2% agarose gels (111860, Biowest, Madrid, Spain) and stained with 1 μg/mL ethidium bromide. The amplified 233 bp PCR products were digested with *Hinf*I (R0155V, New England Biolabs, America) restriction enzyme, according to the manufacturer's instructions. The digested PCR products were separated on 3% agarose gels and visualized with ethidium bromide staining. A single undigested band at 233 bp represented a homozygous wild-type allele, and two bands at 176 and 57 bp represented a homozygous mutant allele.

A database was established with EpiData software and analyzed using SPSS 17.0 software. χ^2 tests were used to compare differences in gender, smoking, drinking and genetic polymorphisms among control, ESCC and EPL participants. Mean values and standard deviations were calculated for age, with mean differences tested by ANOVA. The median values (*M*) and the 25th and 75th percentiles (25th-75th) were calculated for serum folate concentrations, and ANOVA was used to compare mean values of serum concentrations of folate after log transformation. Serum folate concentrations were categorized into quartiles based on normal control participants. Multinomial logistic regression models were used to estimate odds ratios (OR) and corresponding 95% confidence intervals (CI) and to assess whether serum folate concentrations were associated with the risk of EPL and ESCC.

The relationship between the polymorphism of

*MTH*FR C677T and EPL and ESCC was assessed using chi-square tests. Risk estimations were calculated using the wild-type as a reference. Tests for Hardy-Weinberg equilibrium among the controls were conducted using observed genotype frequencies and a χ^2 test with 1 degree of freedom.

Gene-nutrient interactions were evaluated using multinomial logistic regression models by combining the *MTH*FR C677T polymorphism with serum folate concentrations. We evaluated whether the associations between folate concentrations and the risk of EPL and ESCC could be modified by genotypes of *MTH*FR.

The characteristic profiles of 167 normal controls, 109 patients with EPL and 126 new cases of ESCC are presented in Table 1. The average age was 59.17±6.48 years for the control group, 59.20±6.79 years for the EPL group, and 60.75±5.54 for the ESCC group. There was no significant difference in ages with a balance test (F=2.67; P>0.05). Males accounted for 53.9% of the control group, 48.6% of the EPL group, and 57.9% of the ESCC group. There was no significant difference in gender with a chi-square test (P>0.05). The proportion of smokers (Ever smoker: 40.12%, 35.78%, 33.02; Passive smoking: 35.33%, 34.86%, 48.11%) and drinkers (Ever drinker: 18.56%, 19.27%, 30.19%) in control, EPL and ESCC groups, respectively, showed no significant differences with a chi-square test (P>0.05).

In this human-based, case-control study, we investigated the risk factors for ESCC. No significant differences in age, gender, tobacco, and alcohol consumption among EPL, ESCC and controls were observed. *M* (25th-75th) for serum folate concentrations [26.92 (17.04-34.19) μg/L, 25.74 (17.63-31.14) μg/L and 19.56 (14.51-29.08) μg/L] in the control, EPL, and ESCC groups, respectively, showed significant differences with ANOVA tests (P<0.05). The ESCC group showed significantly lower serum folate concentrations compared with the EPL and control groups, results that are similar to a previous case-control study[9].

Serum folate concentrations were further classified into quartiles to evaluate associations between serum folate levels and the risk of EPL and ESCC. The data indicated that the highest quartile of serum folate concentration significantly decreased the risk of ESCC compared with the lowest quartile (OR=0.11; 95% CI, 0.04-0.33; P<0.05) (Table 1).

The frequencies of the *MTH*FR genotypes and associations between *MTH*FR genotypes and the risk of EPL and ESCC are presented in Table 2. There was a significant difference in genotype distribution among the control, EPL, and ESCC groups (χ^2=14.80, P<0.05). The *MTH*FR C677T genotype showed no association with EPL. However, *MTH*FR 677 C>T polymorphism was significantly associated with the risk of ESCC (P<0.05), and the CT+TT genotype significantly increased the risk of ESCC compared with the CC genotype (OR=2.18; 95% CI, 1.35-3.51).

The main effect of each factor was analyzed by multinomial logistic regression. No significant differences between age, gender, smoking, drinking, and the risk of EPL and ESCC were observed (P>0.05). Therefore, interactions between serum folate concentrations and *MTH*FR polymorphism on the risk of EPL and ESCC were analyzed after adjustment for age, gender, smoking habit, and drinking (Table 3). Interactions between serum folate concentrations and common *MTH*FR polymorphisms for the risk of EPL were not statistically significant, but the *MTH*FR polymorphism modified the association between serum folate concentrations and the risk of ESCC. Compared with participants with serum folate concentrations higher than the median value (>26.92 μg/L) and the *MTH*FR 677CT genotype, those with a low serum folate concentration (≤26.92 μg/L) and the *MTH*FR 677CT

Table 1. Odds Ratios and 95% Confidence Intervals for EPL and ESCC by Folate Quartiles

Folate Quartiles (μg/L)	Control *n* (%)	EPL			ESCC		
		n (%)	OR (95%CI)[a]	P^c	*n* (%)	OR (95%CI)[a]	P^c
Q1 (<17.04)	42 (25.1)	26 (23.9)	1.0[b]		44 (34.9)	1.0[b]	
Q2 (17.04-26.92)	42 (25.1)	34 (31.2)	1.29 (0.66-2.54)	0.45	48 (38.1)	1.08 (0.57-2.07)	0.81
Q3 (26.92-34.19)	42 (25.1)	29 (26.6)	1.12 (0.56-2.23)	0.75	28 (22.2)	0.55 (0.27-1.12)	0.1
Q4 (>34.19)	41 (24.6)	20 (18.4)	0.76 (0.36-1.61)	0.48	6 (4.8)	0.11 (0.04-0.33)	0.0

Note. [a]OR and 95% CI calculated by multinomial logistic regression, adjusted for age, gender (male vs. female), tobacco, and alcohol consumption status (ever *vs*. never). [b]Lowest quartile is the reference category. [c]*P* values are from logistic regression models.

Table 2. Odds Ratios and 95% Confidence Intervals for the 677 C>T *MTH*FR Polymorphism with Risk of EPL and ESCC

*MTH*FR[a] C677T Genotypes	Control *n* (%)	EPL		ESCC	
		n (%)	OR (95% CI)[b]	*n* (%)	OR (95% CI)[b]
CC	90 (53.9)	52 (47.7)	1.0[d]	44 (34.9)	1.0[d]
CT	60 (35.9)	37 (33.9)	1.07 (0.63-1.82)	65 (51.6)	2.22 (1.34-2.67)
TT	17 (10.2)	20 (18.3)	2.04 (0.98-4.23)	17 (13.5)	2.05 (0.95-4.39)
P[c]			0.1		0.01
CC or CT	150 (89.8)	89 (81.7)	1.0[d]	109 (86.5)	1.0[d]
TT	17 (10.2)	20 (18.3)	1.98 (0.99-2.98)	17 (13.5)	1.38 (0.67-2.82)
CC	90 (53.9)	52 (47.7)	1.0[d]	44 (34.9)	1.0[d]
CT or TT	77 (46.1)	57 (52.3)	1.28 (0.79-2.08)	82 (65.1)	2.18 (1.35-3.51)

Note. [a]*MTH*FR: methylenetetrahydrofolate reductase. [b]OR and 95% CI calculated by chi-square tests. [c]*P* values (linear-by-linear association) are from chi-square tests. [d]Reference category.

Table 3. Joint Associations between Folate and the *MTH*FR C677T Genotype with Risk of EPL and ESCC

Folate Quartiles (μg/L)[a]	EPL, [OR (95% CI)[b], *P* Interaction[d]]			ESCC, [OR (95% CI)[b], *P* Interaction[d]]		
	CC	CT	TT	CC	CT	TT
Q1 (<17.04)	1.0[c]	1.77 (0.58-5.41) 0.31	2.17 (0.53-8.93) 0.28	1.0[c]	3.74 (1.36-10.27) 0.01	2.17 (0.53-8.93) 0.28
Q2 (17.04-26.92)	1.67 (0.66-4.25) 0.28	1.59 (0.56-4.48) 0.38	2.60 (0.66-10.23) 0.17	1.58 0.62-4.03) 0.34	2.89 (1.11-7.52) 0.03	3.03 (0.80-11.54) 0.10
Q3 (26.92-34.19)	1.38 (0.53-3.59) 0.51	1.15 (0.40-3.31) 0.80	4.33 (0.92-20.33) 0.06	0.79 (0.27-2.27) 0.66	1.40 (0.51-3.89) 0.52	2.17 (0.38-12.35) 0.38
Q4 (>34.19)	0.98 (0.34-2.77) 0.96	1.02 (0.35-3.01) 0.97	1.63 (0.31-8.43) 0.56	0.11 (0.01-0.90) 0.04	0.38 (0.09-1.56) 0.18	0.54 (0.06-5.38) 0.60

Note. [a]Serum folate levels based on the quartiles of the control group. [b]OR and 95% CI calculated by multinomial logistic regression, adjusted for age, gender (male *vs.* female), tobacco, and alcohol consumption status (ever *vs.* never). [c]Reference category. [d]Based on likelihood ratio tests and multinomial logistic regression models with the interaction term between serum folate and *MTH*FR C677T genotype.

genotype had a significantly increased risk of ESCC [(OR=3.74; 95% CI, 1.36-10.27) and (OR=2.89; 95% CI, 1.11-7.52)]. Those participants with the highest folate concentrations (>34.19 μg/L) and the *MTH*FR 677CC genotype appeared to have a protective factor for ESCC risk (OR=0.11; 95% CI, 0.01-0.90).

The small sample size of this study may reduce the credibility of the results by introducing imprecision into measurements, and may have limited more precise estimations between associations. A large scale study with greater statistical power is needed to better detect associations, particularly for the subgroup analyses of the interaction tests. However, a statistical significance between serum folate concentrations and the *MTH*FR genotypes among the different study groups was observed, but a study with a larger sample size is needed to confirm possible interactions between the risk of esophageal cancer and the nutritional factors that the *MTH*FR genotype may modify.

In summary, our findings indicated that serum folate concentrations were inversely associated with the risk of EPL and ESCC. The *MTH*FR 677 C>T polymorphism was significantly associated with the risk of ESCC. There was a significant difference in *MTH*FR genotype distribution among the control, EPL and ESCC groups. *MTH*FR genotypes may modify associations between serum folate concentrations and the risk of ESCC. Associations between folate

concentrations and the risk of EPL were not significant.

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