Methylenetetrahydrofolate reductase tagging polymorphisms are associated with risk of esophagogastric junction adenocarcinoma: a case-control study involving 2,740 Chinese Han subjects

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ABSTRACT

In this study, we aimed to determine the potential association of MTHFR tagging single nucleotide polymorphisms (SNPs) with risk of developing esophagogastric junction adenocarcinoma (EGJA). MTHFR rs1801133 G>A, rs3753584 T>C, rs4845882 G>A, rs4846048 A>G and rs9651118 T>C polymorphisms were genotyped in 1,677 healthy individuals and 1,063 patients with EGJA. We found that MTHFR rs1801133 G>A polymorphism was significantly associated with the risk of developing EGJA (AA vs. GG: adjusted P = 0.001; GA/AA vs. GG: adjusted P = 0.007 and AA vs. GA/GG: adjusted P = 0.001). However, for MTHFR rs4845882 G>A polymorphism, the decreased risk of EGJA was found in two genetic models (AA vs. GG: adjusted P = 0.002 and AA vs. GA/GG: adjusted P = 0.005). In addition, for MTHFR rs3753584 T>C and rs9651118 T>C polymorphisms, a tendency to decreased risk of EGJA was noted. In a subgroup analysis, a significantly decreased risk of EGJA in <64 years subgroup was identified. We found that MTHFR G<sub>rs1801133</sub>T<sub>rs3753584</sub>G<sub>rs4845882</sub>A<sub>rs4846048</sub>C<sub>rs9651118</sub> and G<sub>rs1801133</sub>T<sub>rs3753584</sub>A<sub>rs4845882</sub>G<sub>rs4846048</sub>T<sub>rs9651118</sub> haplotypes significantly decreased the risk of EGJA (P = 0.002, P < 0.001 and P = 0.038, respectively). In conclusion, our study demonstrates that MTHFR rs1801133 G>A may be associated with the increased risk of EGJA. Meanwhile, MTHFR rs3753584 T>C, rs4845882 G>A and rs9651118 T>C polymorphisms and haplotypes may decrease the risk of EGJA in Eastern Chinese Han population. Further studies with large sample size and detailed gene-environmental data are needed to validate our conclusion.
INTRODUCTION

The increasing incidence of esophagogastric junction adenocarcinoma (EGJA) was observed worldwide [1–3] and was considered to have different etiology and risk factor compared with distal gastric carcinoma (GC) [4]. EGJA remains poor prognosis [5] and is a common public health problem. The vital risk factors contributing to the development of EGJA are obesity, gastro-esophageal reflux disease, smoking, foods preserved by salting and low intake of fruits and vegetables et al [6, 7]. However, these observed risk factors could not interpret the overall susceptibility to EGJA. Recently, more and more epidemiologic studies suggested that individual’s genetic factor might influence the pathogenesis of EGJA.

Accumulating evidences indicate that folate insufficiency may increase the susceptibility of multiple malignancies [8, 9]. In humans, the majority of methyl groups may be presented by folic acid for endocellular methylation reactions and DNA de novo deoxynucleoside synthesis. During DNA synthesis, lack of folate can cause uracil misincorporation and then affect the stability of DNA [10]. In folate metabolism and DNA synthesis, methylenetetrahydrofolate reductase (MTHFR) is an important enzyme which catalyzes the reviversification of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. And 5-methyltetrahydrofolate is a main circulating and existing form of folate and is the methyl donor for DNA methylation and remethylation procedure of homocysteine to methionine. Based on the important role of participation in both DNA synthesis and methylation, any variant of MTHFR gene may involve in the carcinogenesis.

Human MTHFR is composed of 656 amino acids. MTHFR gene is located on the short arm of Chromosome 1. The human MTHFR gene is very polymorphic (http://www.ncbi.nlm.nih.gov/SNP) and a number of loci have been established, such as rs1537514, rs3753584, rs9651118, rs1537516, rs4845882, rs1801131, rs1801133, rs2066462, rs4846048 and rs3737967 polymorphisms, etc. Interestingly, many previous case-control studies demonstrated that MTHFR polymorphisms were correlated with the risk of multiple human malignancies [e.g., esophageal squamous cell carcinoma (ESCC) [11], gastric cardia adenocarcinoma (GCA) [12], cervicalcancer [13], breast cancer [14, 15] and childhood acute lymphoblastic leukemia [16 et al)]. Thus, the single nucleotide polymorphisms (SNPs) in MTHFR genes on EGJA risk attracted our interest. Exploring the potential association of MTHFR SNPs with EGJA susceptibility may be conducive to the prevention and personalized diagnosis. In this study, we selected MTHFR tagging SNPs (rs1801133 G>A, rs3753584 T>C, rs4845882 G>A, rs4846048 A>G and rs9651118 T>C) and performed a case-control study to evaluate the effect of MTHFR genotypes for EGJA risk.

RESULTS

Baseline characteristics

A total of 1,063 sporadic patients with EGJA and 1,677 normal controls were recruited. Of the EGJA patients, 759 were male and 304 were female, with a mean age (± standard deviation) of 64.19 ± 8.63 years. The normal controls comprised of 1,194 males and 483 females with a mean age of 63.91 ±10.22 years. The demographics (age and sex) was well matched (P = 0.165 and P = 0.909, respectively; Table 1). Of the smoking and alcohol consumption, a significant difference was observed between EGJA patients and controls (P < 0.001, Table 1). The frequency distribution of MTHFR genotypes was determined after genotyping the 2,740 study subjects. For MTHFR rs1801133 G>A, rs3753584 T>C, rs4845882 G>A, rs4846048 A>G and rs9651118 T>C polymorphisms, success rates of genotyping were 99.01%, 99.09%, 99.05%, 99.09% and 98.98%, respectively (Table 2). In controls, the distribution of MTHFR genotype frequencies accorded with Hardy–Weinberg equilibrium (HWE), except for MTHFR rs4846048 A>G polymorphism (Table 2).

Association of MTHFR rs1801133 G>A, rs3753584 T>C, rs4845882 G>A, rs4846048 A>G and rs9651118 T>C polymorphisms with EGJA

The genotypes of MTHFR rs1801133 G>A, rs3753584 T>C, rs4845882 G>A, rs4846048 A>G and rs9651118 T>C polymorphisms are presented in Table 3. For MTHFR rs1801133 G>A polymorphism, the risk of developing EGJA was significant in three genetic models [AA vs. GG: crude odds ratio (OR) = 1.50, 95% confidence interval (CI): 1.19–1.90, P = 0.001; GA/AA vs. GG: crude OR = 1.27, 95% CI: 1.08–1.49, P = 0.004 and AA vs. GA/AA: crude OR = 1.45, 95% CI: 1.17–1.80, P = 0.001; Table 3]. Adjustment for age, sex, smoking and drinking, the similar results were also found (AA vs. GG: adjusted OR = 1.47, 95% CI: 1.16–1.86, P = 0.001; GA/AA vs. GG: adjusted OR = 1.25, 95% CI: 1.06–1.47, P = 0.007 and AA vs. GA/AA: adjusted OR = 1.43, 95% CI: 1.15–1.77, P = 0.001; Table 3). For MTHFR rs3753584 T>C polymorphism, the decreased risk of EGJA was found in two genetic models (AA vs. GG: crude OR = 0.47, 95% CI: 0.29–0.75, P = 0.002 and AA vs. GA/ GG: crude OR = 0.50, 95% CI: 0.31–0.80, P = 0.004; Table 3). Adjustment for age, sex, smoking and drinking, the results were not materially changed (AA vs. GG: adjusted OR = 0.47, 95% CI: 0.29–0.76, P = 0.002 and AA vs. GA/ GG: adjusted OR = 0.50, 95% CI: 0.31–0.81, P = 0.005; Table 3). In addition, these associations were still significant after a Bonferroni correction for multiple comparisons.
Association of MTHFR rs1801133 G>A, rs3753584 T>C, rs4845882 G>A, rs4846048 A>G and rs9651118 T>C polymorphisms with EGJA in Different Stratification Groups

In the stratified analyses by sex, age, drinking and smoking, the genotype frequencies of MTHFR rs1801133 G>A polymorphism are listed in Table 4. After adjustment by logistic regression analysis, the association of MTHFR rs1801133 G>A variants with EGJA risk was evident in some subgroups [male group: AA vs. GG: adjusted OR = 1.66, 95% CI 1.26–2.20, P < 0.001, GA/AA vs. GG: adjusted OR = 1.27, 95% CI 1.05–1.53, P = 0.015 and AA vs. GA/GG: adjusted OR = 1.61, 95% CI 1.24–2.08, P < 0.001; <64 years subgroup: AA vs. GG: adjusted OR = 1.51, 95% CI 1.06–2.14, P = 0.022, GA/AA vs. GG: adjusted OR = 1.38, 95% CI 1.09–1.74, P = 0.007 and AA vs. GA/GG: adjusted OR = 1.39, 95% CI 1.00–1.92, P = 0.049; ≥64 years subgroup: AA vs. GG: adjusted OR = 1.42, 95% CI 1.03–1.95, P = 0.032 and AA vs. GA/GG: adjusted OR = 1.47, 95% CI 1.10–1.96, P = 0.010; never smoking group: AA vs. GG: adjusted OR = 1.43, 95% CI 1.08–1.87, P = 0.012, GA/AA vs. GG: adjusted OR = 1.32, 95% CI 1.10–1.59, P = 0.004 and AA vs. GA/GG:

Table 1: Distribution of selected demographic variables and risk factors in EGJA cases and controls

| Variable                  | Overall Cases (n=1,063) | Overall Controls (n=1,677) | P * |
|---------------------------|-------------------------|---------------------------|-----|
| Age (years)               | 64.19 ±8.63             | 63.91 ±10.22              | 0.451 |
| Age (years)               |                         |                          | 0.165 |
| < 64                      | 494 (46.47)             | 825 (49.19)               |     |
| ≥64                       | 569 (53.53)             | 852 (50.81)               |     |
| Sex                       |                         |                          | 0.909 |
| Male                      | 759 (71.40)             | 1194 (71.20)              |     |
| Female                    | 304 (28.60)             | 483 (28.80)               |     |
| Smoking status            |                         |                          | <0.001 |
| Never                     | 773 (72.72)             | 1323 (78.89)              |     |
| Ever                      | 290 (27.28)             | 354 (21.11)               |     |
| Alcohol use               |                         |                          | <0.001 |
| Never                     | 908 (85.42)             | 1507 (89.86)              |     |
| Ever                      | 155 (14.58)             | 170 (10.14)               |     |

* Two-sided χ² test and Student t test.

Table 2: Primary information for MTHFR polymorphisms (rs1801133 G>A, rs3753584 T>C, rs4845882 G>A, rs4846048 A>G and rs9651118 T>C)

| Genotyped SNPs | rs1801133 G>A | rs3753584 T>C | rs4845882 G>A | rs4846048 A>G | rs96511118 T>C |
|----------------|--------------|--------------|--------------|--------------|----------------|
| Chromosome     | 1            | 1            | 1            | 1            | 1              |
| Function       | Missense     | NearGene-5   | Intron       | Intron       | Intron         |
| Chr Pos (Genome Build 36.3) | 11778965 | 11787173 | 11765754 | 11768839 | 11784801 |
| MAF* for Chinese in database | 0.439 | 0.093 | 0.198 | 0.105 | 0.382 |
| MAF in our controls (n = 1,677) | 0.359 | 0.108 | 0.209 | 0.096 | 0.378 |
| P value for HWE* test in our controls | 0.679 | 0.691 | 0.972 | 0.014 | 0.270 |
| Genotyping method | SNPscan | SNPscan | SNPscan | SNPscan | SNPscan |
| % Genotyping value | 99.01% | 99.09% | 99.05% | 99.09% | 98.98% |

*MAF: minor allele frequency.
*HWE: Hardy–Weinberg equilibrium.
Table 3: Logistic regression analyses of associations between *MTHFR* rs1801133 G>A, rs3753584 T>C, rs4845882 G>A, rs4846048 A>G and rs9651118 T>C polymorphisms and the risk of EGJA

| Genotype                  | Cases (n=1,063) | Controls (n=1,677) | Crude OR (95%CI) | P     | Adjusted OR a (95%CI) | P     |
|---------------------------|-----------------|--------------------|------------------|-------|-----------------------|-------|
|                           | n   | %    | n   | %    |                     |       |
| **MTHFR rs1801133 G>A**   |     |      |     |      |                     |       |
| GG                        | 367 | 35.29| 683 | 40.82| 1.00                 | 1.00  |
| GA                        | 492 | 47.31| 778 | 46.50| 1.11 (0.94-1.32)     | 0.208 |
| AA                        | 181 | 17.40| 212 | 12.67| 1.50 (1.19-1.90)     | 0.001 |
| GA + AA                   | 673 | 64.71| 990 | 59.18| 1.27 (1.08-1.49)     | 0.004 |
| GG + GA                   | 859 | 82.60| 1,461| 87.33| 1.00                 | 1.00  |
| AA                        | 181 | 17.40| 212 | 12.67| 1.45 (1.17-1.80)     | 0.001 |
| A allele                  | 854 | 41.06| 1,202| 35.92|                     |       |
| **MTHFR rs3753584 T>C**   |     |      |     |      |                     |       |
| TT                        | 855 | 82.13| 1,330| 79.45| 1.00                 | 1.00  |
| CT                        | 177 | 17.00| 326 | 19.47| 0.83 (0.67-1.01)     | 0.062 |
| CC                        | 9   | 0.86 | 18  | 1.08 | 0.76 (0.34-1.70)     | 0.504 |
| CT + CC                   | 186 | 17.87| 344 | 20.55| 0.84 (0.69-1.03)     | 0.087 |
| TT + CT                   | 1032| 99.14| 1,656| 98.92| 1.00                 | 1.00  |
| CC                        | 9   | 0.86 | 18  | 1.08 | 0.80 (0.36-1.79)     | 0.591 |
| C allele                  | 195 | 9.37 | 362 | 10.81|                     |       |
| **MTHFR rs4845882 G>A**   |     |      |     |      |                     |       |
| GG                        | 687 | 66.06| 1,049| 62.66| 1.00                 | 1.00  |
| GA                        | 330 | 31.73| 552 | 32.97| 0.89 (0.75-1.05)     | 0.153 |
| AA                        | 23  | 2.21 | 73  | 4.36 | **0.47 (0.29-0.75)** | 0.002 |
| GA + AA                   | 353 | 33.94| 625 | 37.34| 0.86 (0.73-1.01)     | 0.074 |
| GG + GA                   | 1,017| 97.79| 1,601| 95.64| 1.00                 | 1.00  |
| AA                        | 23  | 2.21 | 73  | 4.36 | **0.50 (0.31-0.80)** | 0.004 |
| A allele                  | 376 | 18.08| 698 | 20.85|                     |       |
| **MTHFR rs4846048 A>G**   |     |      |     |      |                     |       |
| AA                        | 860 | 82.61| 1,378| 82.32| 1.00                 | 1.00  |
| AG                        | 171 | 16.43| 272 | 16.25| 0.98 (0.80-1.21)     | 0.883 |
| GG                        | 10  | 0.96 | 24  | 1.43 | 0.65 (0.31-1.37)     | 0.260 |
| AG + GG                   | 181 | 17.39| 296 | 17.68| 0.98 (0.80-1.20)     | 0.845 |
| AA + AG                   | 1,031| 99.04| 1,650| 98.57| 1.00                 | 1.00  |
| GG                        | 10  | 0.96 | 24  | 1.43 | 0.67 (0.32-1.40)     | 0.284 |
| G allele                  | 191 | 9.17 | 320 | 9.56 |                     |       |
| **MTHFR rs9651118 T>C**   |     |      |     |      |                     |       |
| TT                        | 423 | 40.75| 638 | 38.11| 1.00                 | 1.00  |
| TC                        | 486 | 46.82| 808 | 48.27| 0.86 (0.73-1.02)     | 0.075 |

(Continued)
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Table 5 summarizes the results of association between MTHFR rs3753584 T>C polymorphism and EGJA risk by sex, age, smoking status and alcohol consumption.

| Genotype | Cases (n=1,063) | Controls (n=1,677) | Crude OR (95%CI) | P  | Adjusted OR \(^{a}\) (95%CI) | P  |
|----------|----------------|-------------------|-----------------|----|-----------------------------|----|
| CC       | 129 12.43      | 228 13.62         | 0.81 (0.63-1.04); \(P<0.001\) | 0.094| 0.83 (0.65-1.07) | 0.150|
| TC+CC    | 615 59.25      | 1,036 61.89       | 0.90 (0.76-1.05); \(P=0.171\) | 0.171| 0.91 (0.78-1.07) | 0.260|
| TT+TC    | 909 87.57      | 1,446 86.38       | 1.00             | \(P=1.0)\) | 1.00             | \(P=1.0)\) |
| CC       | 129 12.43      | 228 13.62         | 0.90 (0.71-1.13); \(P=0.372\) | 0.372| 0.92 (0.73-1.16) | 0.489|
| C allele | 744 35.84      | 1,264 37.75       | 1.10             | \(P=0.094\) | 0.83 (0.65-1.07) | 0.150|

\(^{a}\) Adjusted for age, sex, smoking and drinking status; Bold values are statistically significant \((P<0.05)\).

Table 4: Stratified analyses between MTHFR rs1801133 G>A polymorphism and EGJA risk by sex, age, smoking status and alcohol consumption

| Variable | MTHFR rs1801133 G>A (case/control) | Adjusted OR\(^{b}\) (95% CI); \(P\) |
|----------|-----------------------------------|--------------------------------------|
| Sex      |                                   | GG  GA  AA                          |
| Male     |                                   | GG  GA  AA                          |
|          |                                   | 1.10 (0.90-1.34); \(P=0.360\)       |
|          |                                   | 1.66 (1.26-2.20); \(P<0.001\)       |
|          |                                   | 1.27 (1.05-1.53); \(P=0.015\)       |
|          |                                   | 1.61 (1.24-2.08); \(P<0.001\)       |
| Female   |                                   | GG  GA  AA                          |
|          |                                   | 1.10 (0.80-1.50); \(P=0.575\)       |
|          |                                   | 1.03 (0.66-1.60); \(P=0.909\)       |
|          |                                   | 1.17 (0.86-1.59); \(P=0.311\)       |
|          |                                   | 1.01 (0.67-1.53); \(P=0.954\)       |
| Age      |                                   | GG  GA  AA                          |
| <64      |                                   | 1.24 (0.97-1.58); \(P=0.089\)       |
|          |                                   | 1.51 (1.06-2.14); \(P=0.022\)       |
|          |                                   | 1.38 (1.09-1.74); \(P=0.007\)       |
|          |                                   | 1.39 (1.00-1.92); \(P=0.049\)       |
| ≥64      |                                   | 0.97 (0.77-1.23); \(P=0.822\)       |
|          |                                   | 1.42 (1.03-1.95); \(P=0.032\)       |
|          |                                   | 1.12 (0.90-1.40); \(P=0.312\)       |
|          |                                   | 1.47 (1.10-1.96); \(P=0.010\)       |
| Smoking status |                     | GG  GA  AA                          |
| Never    |                                   | 1.19 (0.98-1.44); \(P=0.085\)       |
|          |                                   | 1.43 (1.08-1.87); \(P=0.012\)       |
|          |                                   | 1.32 (1.10-1.59); \(P=0.004\)       |
|          |                                   | 1.33 (1.04-1.72); \(P=0.026\)       |
| Ever     |                                   | 0.86 (0.61-1.22); \(P=0.396\)       |
|          |                                   | 1.62 (1.01-2.61); \(P=0.046\)       |
|          |                                   | 1.04 (0.75-1.45); \(P=0.805\)       |
|          |                                   | 1.79 (1.15-2.76); \(P=0.009\)       |
| Alcohol consumption |                   | GG  GA  AA                          |
| Never    |                                   | 1.19 (0.99-1.42); \(P=0.065\)       |
|          |                                   | 1.54 (1.20-1.98); \(P=0.001\)       |
|          |                                   | 1.35 (1.13-1.60); \(P=0.001\)       |
|          |                                   | 1.45 (1.15-1.82); \(P=0.002\)       |
| Ever     |                                   | 0.60 (0.36-1.00); \(P=0.051\)       |
|          |                                   | 1.10 (0.56-2.19); \(P=0.780\)       |
|          |                                   | 0.70 (0.43-1.13); \(P=0.138\)       |
|          |                                   | 1.47 (0.80-2.73); \(P=0.217\)       |

\(^{a}\) The genotyping was successful in 1063 (97.84%) EGJA cases, and 1677 (99.76%) controls for MTHFR rs1801133 G>A;

\(^{b}\) Adjusted for age, sex, smoking status and alcohol consumption (besides stratified factors accordingly) in a logistic regression model;

adjusted OR = 1.33, 95% CI 1.04–1.72, \(P=0.026\); ever smoking group: AA vs. GG: adjusted OR = 1.62, 95% CI 1.01–2.61, \(P=0.046\) and AA vs. GA/GG: adjusted OR = 1.79, 95% CI 1.15–2.76, \(P=0.009\) and never drinking group: AA vs. GG: adjusted OR = 1.54, 95% CI 1.20–1.98, \(P=0.001\), GA/AA vs. GG: adjusted OR = 1.35, 95% CI 1.13–1.60, \(P=0.001\) and AA vs. GA/GG: adjusted OR = 1.45, 95% CI 1.15–1.82, \(P=0.002\); Table 4).

Table 5 summarizes the results of association between MTHFR rs3753584 T>C polymorphism and...
EGJA risk in the stratified analysis. We found that MTHFR rs3753584 T>C polymorphism was associated with the decreased risk of EGJA in <64 years subgroup [TC vs. TT: adjusted OR = 0.70, 95% CI 0.52–0.93, \( P = 0.016 \) and TC/CC vs. TT: adjusted OR = 0.73, 95% CI 0.55–0.97, \( P = 0.032 \) (Table 5)].

The results of association between MTHFR rs4845882 G>A polymorphism and EGJA risk in the stratified analyses are summarized in Table 6. We found that MTHFR rs4845882 G>A polymorphism decreased the risk of EGJA in several subgroups [male group: AA vs. GG: adjusted OR = 0.47, 95% CI 0.27–0.83, \( P = 0.016 \) and AA vs. GA/GG: adjusted OR = 0.50, 95% CI 0.23–1.52, \( P = 0.052 \); smoking status: Never: adjusted OR = 0.49, 95% CI 0.16–1.51, \( P = 0.216 \) and Ever: adjusted OR = 1.29, 95% CI 0.33–5.04, \( P = 0.711 \); alcohol consumption: Never: adjusted OR = 0.63, 95% CI 0.24–1.61, \( P = 0.331 \) and Ever: adjusted OR = 1.49, 95% CI 0.24–9.44, \( P = 0.669 \)] (Table 6).

Table 7 lists MTHFR rs4846048 A>G genotype frequencies in the stratified analysis. We found no significant difference in genotype distribution of MTHFR rs4846048 A>G polymorphism among EGJA cases and non-cancer controls.

The results of relationship between MTHFR rs9651118 T>C polymorphism and EGJA risk in the stratified analyses are summarized in Table 8. We found that MTHFR rs9651118 T>C polymorphism was associated with the decreased risk of EGJA in <64 years subgroup [TC vs. TT: adjusted OR = 0.78, 95% CI 0.61–0.99, \( P = 0.040 \) (Table 8)].

### Table 5: Stratified analyses between MTHFR rs3753584 T>C polymorphism and EGJA risk by sex, age, smoking status and alcohol consumption

| Variable          | MTHFR rs3753584 T>C (case/control) | Adjusted OR (95% CI); \( P \) |
|-------------------|-------------------------------------|-------------------------------|
|                   | TT/CC                              | TT/CC                         |
| **Sex**           |                                     |                               |
| Male              | 613/950 126/226 7/15               | 0.85 (0.67–1.08) 0.69 (0.28–1.70) 0.85 (0.67–1.08); \( P = 0.177 \) 0.415 \( P = 0.184 \) 0.72 (0.29–1.78); \( P = 0.471 \) |
| Female            | 242/380 51/100 2/3                 | 0.80 (0.55–1.17) 0.61 (0.08–4.43) 0.83 (0.57–1.20); \( P = 0.252 \) 0.622 \( P = 0.319 \) 0.64 (0.09–4.72); \( P = 0.663 \) |
| **Age**           |                                     |                               |
| <64               | 398/640 79/177 5/7                 | 0.70 (0.52–0.93); 0.99 (0.31–3.19); 0.73 (0.55–0.97); \( P = 0.016 \) 0.987 \( P = 0.032 \) 1.08 (0.34–3.47); \( P = 0.899 \) |
| ≥64               | 457/691 98/149 4/11                | 0.98 (0.74–1.30) 0.53 (0.17–1.68) 0.97 (0.73–1.28); \( P = 0.881 \) 0.282 \( P = 0.813 \) 0.54 (0.17–1.71); \( P = 0.296 \) |
| **Smoking status**|                                     |                               |
| Never             | 619/1,058 131/249 4/14             | 0.88 (0.70–1.11) 0.47 (0.16–1.45) 0.88 (0.70–1.11); \( P = 0.283 \) 0.189 \( P = 0.288 \) 0.49 (0.16–1.51); \( P = 0.216 \) |
| Ever              | 236/272 46/77 5/4                  | 0.74 (0.49–1.11) 1.22 (0.31–4.74) 0.77 (0.52–1.15); \( P = 0.144 \) 0.776 \( P = 0.202 \) 1.29 (0.33–5.04); \( P = 0.711 \) |
| **Alcohol consumption** |                                     |                               |
| Never             | 729/1,190 152/299 6/16              | 0.81 (0.65–1.00) 0.59 (0.23–1.52) 0.82 (0.66–1.01); \( P = 0.052 \) 0.273 \( P = 0.064 \) 0.63 (0.24–1.61); \( P = 0.331 \) |
| Ever              | 126/140 25/27 3/2                   | 1.11 (0.60–2.06) 1.52 (0.24–9.63) 1.14 (0.63–2.07); \( P = 0.748 \) 0.657 \( P = 0.668 \) 1.49 (0.24–9.44); \( P = 0.669 \) |

\( a \) The genotyping was successful in 1063 (97.93%) EGJA cases, and 1677 (99.82%) controls for MTHFR rs3753584 T>C; \( b \) Adjusted for age, sex, smoking status and alcohol consumption (besides stratified factors accordingly) in a logistic regression model;
We used a SHESIS software (http://analysis.bio-x.cn/myAnalysis.php)\cite{17} to construct haplotypes of \textit{MTHFR} gene (Table 9). Finally, five \textit{MTHFR} haplotypes were identified. When \textit{MTHFR} $A_{rs1801133}$ $G_{rs3753584}$ $A_{rs4845882}$ $T_{rs9651118}$ haplotype was used as reference, we found that \textit{MTHFR} $G_{rs1801133}$ $T_{rs3753584}$ $C_{rs4845882}$ $G_{rs9651118}$, $G_{rs1801133}$ $G_{rs3753584}$ $A_{rs4845882}$ $T_{rs9651118}$ and $G_{rs1801133}$ $G_{rs3753584}$ $C_{rs4845882}$ $G_{rs9651118}$ haplotypes significantly decreased the risk of EGJA ($P = 0.002$, $P < 0.001$ and $P = 0.038$, respectively, Table 9).

### DISCUSSION

Incidence of EGJA has increased over the past two decades\cite{18, 19}. Many studies demonstrated that the morbidity of EGJA was increased in Asian countries, such as China, Korea and Japan\cite{19–21}. However, the etiology of EGJA remains unknown. In this study, we explored the association between \textit{MTHFR} $rs1801133$ G>A, $rs3753584$ T>C, $rs4845882$ G>A and $rs9651118$ T>C polymorphisms and EGJA risk in Eastern Chinese Han population. We found that \textit{MTHFR} $rs1801133$ G>A might be associated with the increased risk of EGJA. Meanwhile, \textit{MTHFR} $rs3753584$ T>C, $rs4845882$ G>A and $rs9651118$ T>C polymorphisms decreased the risk of EGJA.

\textit{MTHFR} gene lies in 1p36.3 and contains 11 exons with a length of about 1980 bp. In exon 4, a G to A variant at nucleotide 677 locus ($rs1801133$ G>A) directly leads to valine substitution for alanine, which is relevant to a reduction of \textit{MTHFR} activity\cite{22}. The individuals who carry heterozygous genotype (GA genotype) of \textit{MTHFR} $rs1801133$ G>A polymorphism have 70% of normal enzyme activity, however, those who carry homozygous

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**Table 6: Stratified analyses between \textit{MTHFR} rs4845882 G>A polymorphism and EGJA risk by sex, age, smoking status and alcohol consumption**

| Variable | \textit{MTHFR} rs4845882 G>A (case/control)a | Adjusted ORb (95% CI); $P$ |
|----------|---------------------------------------------|---------------------------|
|          |  | GG | GA | AA | GG | GA | AA | GA/AA | AA vs. (GA/GG) |
| Sex      | Male | 492/746 | 237/391 | 17/54 | 1.00 | 0.89 (0.73-1.09); $P$: 0.268 | 0.47 (0.27-0.83); $P$: 0.009 | 0.86 (0.71-1.05); $P$: 0.113 | 0.50 (0.29-0.87); $P$: 0.014 |
|          | Female | 195/303 | 93/161 | 6/19 | 1.00 | 0.86 (0.63-1.18); $P$: 0.354 | 0.43 (0.16-1.12); $P$: 0.084 | 0.86 (0.63-1.17); $P$: 0.330 | 0.46 (0.18-1.21); $P$: 0.117 |
| Age      | <64 | 320/507 | 152/279 | 10/37 | 1.00 | 0.83 (0.65-1.06); $P$: 0.132 | 0.41 (0.20-0.84); $P$: 0.015 | 0.81 (0.64-1.02); $P$: 0.077 | 0.45 (0.22-0.91); $P$: 0.027 |
|          | ≥64 | 367/542 | 178/273 | 13/36 | 1.00 | 0.94 (0.74-1.18); $P$: 0.566 | 0.53 (0.28-1.01); $P$: 0.052 | 0.91 (0.73-1.14); $P$: 0.424 | 0.55 (0.29-1.04); $P$: 0.068 |
| Smoking status | Never | 496/832 | 243/427 | 14/62 | 1.00 | 0.92 (0.76-1.12); $P$: 0.416 | 0.37 (0.21-0.67); $P$: 0.001 | 0.89 (0.73-1.07); $P$: 0.207 | 0.39 (0.22-0.70); $P$: 0.002 |
|          | Ever | 191/217 | 87/125 | 9/11 | 1.00 | 0.81 (0.58-1.14); $P$: 0.220 | 0.96 (0.38-2.40); $P$: 0.927 | 0.83 (0.60-1.16); $P$: 0.269 | 1.04 (0.42-2.59); $P$: 0.939 |
| Alcohol consumption | Never | 591/938 | 276/501 | 19/66 | 1.00 | 0.85 (0.71-1.01); $P$: 0.065 | 0.44 (0.26-0.74); $P$: 0.002 | 0.83 (0.69-0.98); $P$: 0.032 | 0.48 (0.29-0.80); $P$: 0.005 |
|          | Ever | 96/111 | 54/51 | 4/7 | 1.00 | 1.30 (0.80-2.12); $P$: 0.293 | 0.85 (0.23-3.19); $P$: 0.813 | 1.25 (0.78-2.02); $P$: 0.355 | 0.78 (0.21-2.87); $P$: 0.708 |

\textit{a} The genotyping was successful in 1063 (97.84%) EGJA cases, and 1677 (99.82%) controls for \textit{MTHFR} rs4845882 G>A; 
\textit{b} Adjusted for age, sex, smoking status and alcohol consumption (besides stratified factors accordingly) in a logistic regression model;
genotype (AA genotype) have only 30% of normal enzyme activity [23]. A case-control study reported that rs1801133 AA genotype was associated an increased risk of GCA [24]. Another case-control study also found that \( MTHFR \) rs1801133 AA and GA genotypes were associated the increased risk of GCA [25]. These results were in accordance with our conclusions. In the future, more replicated study should be conducted to verify these primary findings.

\( MTHFR \) rs3753584 T>C is situated in the intron region of \( MTHFR \) gene. There were only a few studies focusing on the association between \( MTHFR \) rs3753584 T>C and cancer risk. A previous study found that there was an increased lung cancer risk in carriers of \( MTHFR \) rs3753584 CC genotype compared with carriers of rs3753584 TT genotype [26]. However, no association was found between ESCC risk and \( MTHFR \) rs3753584 T>C polymorphism [11]. In addition, Wang et al. also reported that \( MTHFR \) rs3753584 T>C was not associated with GCA risk [12]. The present study concluded that rs3753584 TC and CC genotypes were related to a decreased EGJA risk in <64 years subgroup. These apparent discrepancy findings may be due to the insufficient sample size. In the future, more studies with large sample size and detailed environmental factors are indispensable to explore the relationship between \( MTHFR \) rs3753584 T>C and the risk of different cancers.

\( MTHFR \) rs4845882 G>A polymorphism lies in a intron region and is almost complete linkage disequilibrium (LD) with \( MTHFR \) rs1801131 A>C locus. Shen et al. found there was no significant relationship between \( MTHFR \) rs4845882 G>A polymorphism and gastric cancer risk [27]. Additionally, the association between \( MTHFR \) rs4845882 G>A and GCA risk was not concluded in a recent study [12]. However, our study saw a decreased EGJA risk in the individuals carrying

| Variable | \( MTHFR \) rs4846048 A>G (case/control) | \( MTHFR \) rs4846048 A>G (case/control) | Adjusted OR (95% CI); \( P \) | \( MTHFR \) rs4846048 A>G (case/control) | \( MTHFR \) rs4846048 A>G (case/control) | \( MTHFR \) rs4846048 A>G (case/control) | \( MTHFR \) rs4846048 A>G (case/control) | \( MTHFR \) rs4846048 A>G (case/control) |
|----------|---------------------------------|---------------------------------|-------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Sex      |                                 |                                 |                         |                                 |                                 |                                 |                                 |                                 |
| Male     | 615/984 124/189 7/18             | 1.04 (0.81-1.33); \( P \): 0.772 | 0.58 (0.24-1.40); \( P \): 0.227 | 1.02 (0.80-1.29); \( P \): 0.904 | 0.58 (0.24-1.41); \( P \): 0.233 |
| Female   | 245/394 47/83 3/6               | 0.88 (0.59-1.30); \( P \): 0.522 | 0.73 (0.18-2.96); \( P \): 0.659 | 0.90 (0.61-1.32); \( P \): 0.597 | 0.77 (0.19-3.11); \( P \): 0.711 |
| Age      |                                 |                                 |                         |                                 |                                 |                                 |                                 |                                 |
| <64      | 398/677 78/134 6/12             | 0.99 (0.73-1.34); \( P \): 0.927 | 0.74 (0.27-2.02); \( P \): 0.562 | 0.99 (0.74-1.34); \( P \): 0.955 | 0.76 (0.28-2.07); \( P \): 0.590 |
| ≥64      | 462/701 93/138 4/12             | 1.00 (0.75-1.33); \( P \): 0.981 | 0.48 (0.15-1.51); \( P \): 0.210 | 0.97 (0.74-1.29); \( P \): 0.855 | 0.49 (0.16-1.53); \( P \): 0.221 |
| Smoking status |             |                                 |                         |                                 |                                 |                                 |                                 |                                 |
| Never    | 624/1,081 125/220 5/20        | 0.96 (0.75-1.22); \( P \): 0.725 | 0.43 (0.16-1.16); \( P \): 0.095 | 0.94 (0.74-1.19); \( P \): 0.611 | 0.45 (0.17-1.19); \( P \): 0.107 |
| Ever     | 236/297 46/52 5/4            | 1.13 (0.73-1.75); \( P \): 0.582 | 1.49 (0.39-5.72); \( P \): 0.563 | 1.17 (0.77-1.78); \( P \): 0.473 | 1.47 (0.38-5.66); \( P \): 0.572 |
| Alcohol consumption |             |                                 |                         |                                 |                                 |                                 |                                 |                                 |
| Never    | 736/1,236 145/248 6/21      | 0.96 (0.77-1.20); \( P \): 0.709 | 0.47 (0.19-1.16); \( P \): 0.101 | 0.95 (0.76-1.18); \( P \): 0.613 | 0.48 (0.19-1.19); \( P \): 0.115 |
| Ever     | 124/142 26/24 4/3          | 1.31 (0.70-2.46); \( P \): 0.406 | 1.77 (0.36-8.68); \( P \): 0.483 | 1.35 (0.74-2.46); \( P \): 0.323 | 1.70 (0.35-8.32); \( P \): 0.512 |

* The genotyping was successful in 1063 (97.93%) EGJA cases, and 1677 (99.82%) controls for \( MTHFR \) rs4846048 A>G;

* Adjusted for age, sex, smoking status and alcohol consumption (besides stratified factors accordingly) in a logistic regression model;
MTHFR rs9651118 AA genotype in male and <64 years subgroups. MTHFR rs9651118 T>C is situated in intron 2 and possesses low LD with rs1801133 G>A (r² < 0.30). Functional annotation by HapReg demonstrated that MTHFR rs9651118 T>C coincides with MTHFR enhancers or promoters, which may correspond to the regions of open chromatin [28]. Several studies implicated that MTHFR rs9651118 C allele was associated with a reduced risk of lung cancer and prostate cancer [28, 29]. In addition, MTHFR rs9651118 C allele was associated with a decreased risk of breast cancer [30]. Our results suggested that MTHFR rs9651118 TC genotype may reduce EGJA susceptibility in <64 years subgroup, which were very similar to the findings of previous studies. In the future, these potential should be confirmed by functional studies.

In this case-control study, we constructed five MTHFR haplotypes to assess the potential inherited patterns of haplotype. We found that MTHFR G<sub>rs1801133</sub>T<sub>s3753584</sub>G<sub>rs4845882</sub>A<sub>rs4846048</sub>C<sub>rs9651118</sub>G<sub>rs1801133</sub>C<sub>rs3753584</sub>A<sub>rs4845882</sub>A<sub>rs4846048</sub>T<sub>rs9651118</sub> haplotypes significantly decreased the risk of EGJA. To the best of our knowledge, we first explore the relationship of haplotypes in MTHFR rs1801133 G>A, rs3753584 T>C, rs4845882 G>A, rs4846048 A>G and rs9651118 T>C polymorphisms with EGJA susceptibility. We also found that MTHFR rs1801133 G and rs4845882 A alleles might be protective factors for haplotype to EGJA.

However, several limitations in our study should be presented. First, for the controls were recruited from the local hospitals, the selection bias of the study population should not be ignored. Second, the data of plasma folate level were not available, which may affect the association between MTHFR SNPs and EGJA susceptibility. Thirdly, for lack of cancer stage, disease progression and overall survival data, we did not consider the influence of MTHFR.

### Table 8: Stratified analyses between MTHFR rs9651118 T>C polymorphism and EGJA risk by sex, age, smoking status and alcohol consumption

| Variable | MTHFR rs9651118 T>C | Adjusted OR<sup>b</sup> (95% CI); P |
|----------|---------------------|-------------------------------------|
|          | TT  | TC  | CC  | TT  | TC  | CC  | TT / CC | CC vs. (TC/TT) |
| Sex      |     |     |     |     |     |     |         |               |
| Male     | 309/447 | 339/574 | 95/170 | 0.82 (0.68-1.00); 0.80 (0.60-1.07); 0.86 (0.71-1.04); 0.91 (0.69-1.19); | 0.054 | 0.134 | 0.109 | 0.492 |
| Female   | 114/191 | 147/234 | 34/58 | 1.03 (0.76-1.40); 0.99 (0.61-1.61); 1.11 (0.82-1.50); 1.01 (0.64-1.60); | 0.857 | 0.970 | 0.501 | 0.956 |
| Age      |     |     |     |     |     |     |         |               |
| <64      | 190/288 | 231/424 | 59/111 | 0.78 (0.61-0.99); 0.80 (0.55-1.15); 0.83 (0.66-1.05); 0.95 (0.68-1.34); | 0.040 | 0.225 | 0.128 | 0.767 |
| ≥64      | 233/350 | 255/384 | 70/117 | 0.96 (0.76-1.21); 0.89 (0.63-1.25); 0.99 (0.80-1.23); 0.93 (0.68-1.28); | 0.728 | 0.502 | 0.914 | 0.651 |
| Smoking status |     |     |     |     |     |     |         |               |
| Never   | 299/499 | 357/636 | 95/186 | 0.88 (0.73-1.06); 0.80 (0.60-1.06); 0.92 (0.77-1.11); 0.89 (0.68-1.16); | 0.182 | 0.126 | 0.380 | 0.374 |
| Ever    | 124/140 | 129/173 | 34/43 | 0.83 (0.59-1.16); 0.95 (0.56-1.59); 0.87 (0.63-1.19); 1.05 (0.64-1.72); | 0.270 | 0.835 | 0.378 | 0.843 |
| Alcohol consumption |     |     |     |     |     |     |         |               |
| Never   | 357/564 | 415/728 | 113/213 | 0.86 (0.72-1.03); 0.80 (0.62-1.04); 0.90 (0.76-1.06); 0.89 (0.70-1.14); | 0.092 | 0.096 | 0.214 | 0.369 |
| Ever    | 66/74 | 71/80 | 16/15 | 0.93 (0.57-1.49); 1.12 (0.50-2.51); 0.97 (0.61-1.54); 1.17 (0.54-2.53); | 0.750 | 0.783 | 0.907 | 0.686 |

<sup>a</sup> The genotyping was successful in 1063 (97.65%) EGJA cases, and 1677 (99.82%) controls for MTHFR rs9651118 T>C;
<sup>b</sup> Adjusted for age, sex, smoking status and alcohol consumption (besides stratified factors accordingly) in a logistic regression model;
SNPs on progress and prognosis of EGJA. Last but not least, other environmental and genetic factors were not considered. Further studies are necessary to explore the effect of interactions between environment and gene factors on EGJA risk.

In conclusion, our study demonstrates that MTHFR rs1801133 G>A may be associated with the increased risk of EGJA. Meanwhile, MTHFR rs3753584 T>C, rs4845882 G>A and rs9651118 T>C polymorphisms decrease the risk of EGJA in Eastern Chinese Han population. The further case-control studies are needed to confirm our findings.

MATERIALS AND METHODS

Subjects

Study conducted at the Affiliated Union Hospital of Fujian Medical University, Fujian Medical University Cancer Hospital and the Affiliated People’s Hospital of Jiangsu University was approved by the Ethics Committee of Fujian Medical University (Fuzhou, China) and Jiangsu University (Zhenjiang, China). Subjects were enrolled from three hospitals in Eastern China. Our study involved 2,740 study participants, comprising 1,063 histopathologically confirmed sporadic EGJA patients and 1,677 healthy normal controls. Among them, 280 EGJA patients and 840 controls were enrolled from Fujian Medical University Union Hospital and Cancer Hospital of Fujian Medical University from January 2014 to May 2016. In addition, 783 EGJA patients and 837 controls were enrolled from the Affiliated People’s Hospital of Jiangsu University between January 2008 and November 2016. All EGJA patients were Siewert type II. The control group involved normal individuals who visited these hospitals for health check. The healthy normal controls were unrelated to the EGJA patients and were cancer-free individuals. Data of demographic details and risk factors was obtained using a structured questionnaire. The definition of ‘ever smokers’ were subjects who smoked at least one cigarette per day over 1 year [11], and ‘ever drinkers’ were subjects who drank no less than three times a week for more than 6 months [11]. The corresponding data are listed in Table 1. The Ethical Committee of Fujian Medical University and Jiangsu University approved the study protocols (No. SQ2015-006-01 and No. 20150083, respectively).

Selection of SNPs

The MTHFR tagging SNPs (upstream and downstream of MTHFR gene extending 5 Kb, respectively) were selected from the database of CHB population using the HapMap Project (http://hapmap.ncbi.nlm.nih.gov/index.html.en) and Haploview 4.2 software. The major criterion were: (a) MAF ≥ 0.05 and call rate ≥ 95 %, (b) a HWE P ≥ 0.05, (c) a pairwise linkage disequilibrium (LD) r^2 threshold of 0.8 between polymorphisms (r^2 > 0.8) [11, 31, 32]. Finally, five MTHFR tagging SNPs (rs1801133 G>A, rs3753584 T>C, rs4845882 G>A, rs4846048 A>G and rs9651118 T>C) were eligible and included in this case-control study to evaluate the effect of MTHFR polymorphisms with EGJA risk. The primary information of MTHFR tagging SNPs is presented in Table 2.

DNA extraction and genotyping

Each participant donated 2ml blood sample which was stored in an EDTA-anticoagulated tube. We use the Promega Genomic DNA Purification Kit (Promega, Madison, USA) to extract the genomic DNA. SNPscan™ genotyping assay (Genesky Biotechnologies Inc., Shanghai, China) [33, 34] was harnessed to determine
the genotyping of \textit{MTHFR} rs1801133 G>A, rs3753584 T>C, rs4845882 G>A, rs4846048 A>G and rs9651118 T>C polymorphisms. Briefly, 150ng DNA sample was denatured at 98°C for 5min. The ligation reaction was done in an ABI 2720 thermal cycler. We used a 48-plex fluorescence PCR reaction for each ligation product amplification. In an ABI 3730XL sequencer, PCR products were analyzed by capillary electrophoresis. The obtained raw data were conducted by GeneMapper 4.1 software (Applied Biosystems, USA). One hundred and ten DNA samples (4%) were randomly selected to reanalyze the genotypes by different laboratory technicians and the reproducibility was 100%.

**Statistical analysis**

Age of EGJA patients and controls was expressed as mean ± standard deviation. And a Student’s t-test was harnessed to assess the difference for age. The Chi-square test ($\chi^2$) was used to compare age, sex, smoking, drinking and the genotypes distribution of \textit{MTHFR} SNPs in patients and controls. We used multivariate logistic regression analysis to assess the risk of \textit{MTHFR} rs1801133 G>A, rs3753584 T>C, rs4845882 G>A, rs4846048 A>G and rs9651118 T>C polymorphisms and considered the confounders such as sex, age, smoking and drinking status. The crude/adjusted ORs and 95% CIs were calculated using the SAS software (Version 9.4; SAS Institute Inc., Cary, NC, USA). A $P < 0.05$ (two sided) was considered as statistical significance. In this study, multiple comparisons were conducted by Bonferroni correction [35]. We used a SHESIS software (http://analysis.bio-x.cn/myAnalysis.php) [17] to construct \textit{MTHFR} haplotypes.

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**CONFLICTS OF INTEREST**

The authors have no potential conflicts of interest.

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