Int J Clin Exp Med 2015;8(10):18936-18944
www.ijcem.com /ISSN:1940-5901/IJCEM0009903

Original Article

Genetic variations in *MTHFR* and gastric cardia adenocarcinoma susceptibility in the Chinese Han population

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Received May 5, 2015; Accepted October 7, 2015; Epub October 15, 2015; Published October 30, 2015

Abstract: *Methylenetetrahydrofolate reductase* (*MTHFR*) gene polymorphisms are associated with many types of cancers. The purpose of our study was to evaluate the effect of *MTHFR* single nucleotide polymorphisms (SNPs) on gastric cardia adenocarcinoma (GCA). We conducted a hospital-based case-control study. Three hundred and thirty cases with GCA and 608 controls were recruited. The ligation detection reaction (LDR) method was used to determine genotypes. The genotype *MTHFR* rs1801133 TT was significantly more frequent in cases than in controls (adjusted odds ratio (OR) = 1.46, 95% confidence interval (CI) = 1.04-2.05, $P = 0.029$) in a recessive model, after adjusting for age, sex and smoking and alcohol use. The haplotype *MTHFR* $G_{rs4845882}A_{rs4846048}T_{rs1801133}C_{rs9651118}A_{rs3753584}$ was more frequent in cases than in controls (crude OR = 5.32, 95% CI = 2.34-12.10, $P < 0.001$). No association between other genotypes and haplotypes was observed. Our results suggest that the genotype *MTHFR* rs1801133 TT and the *MTHFR* $G_{rs4845882}A_{rs4846048}T_{rs1801133}C_{rs9651118}A_{rs3753584}$ haplotype may be associated with susceptibility to GCA. Further studies are needed to confirm these findings.

Keywords: Polymorphism, MTHFR, gastric cardia adenocarcinoma, susceptibility

Introduction

Gastric cardia adenocarcinoma (GCA) is a lethal malignancy common in Chinese population. Epidemiological studies have shown a steady decline in non-cardia gastric cancer but a continuous increase in morbidity and mortality of GCA. This underlines the importance of preventative strategies for GCA undertaken in the past twenty years [1]. Many studies have demonstrated the importance of various environmental factors [2]. Genetic factors, including single nucleotide polymorphisms (SNPs), may also be important. It has been suggested that SNPs might partly explain differences in individual susceptibility to GCA [3].

DNA methylation is one of the principal mechanisms leading to loss of gene function [4]. DNA methylation involves cytosine on carbon 5. It is one of the epigenetic mechanisms currently being researched most intensively in mammals. It regulates the transcriptional plasticity of the mammalian genome. It plays a vital role in diverse cellular processes, including gene expression and regulation, and in the control of cell differentiation. During normal cell aging and differentiation, variations in DNA methylation may contribute to tumorigenesis [5]. DNA methylation is facilitated and regulated by the level of the methyl donor, S-adenosylmethionine (SAM), and in return DNA methylation affects the level of SAM [6]. SAM is synthesized by SAM synthetase from ATP and methionine. As a precursor of SAM, methionine is regulated by several pathways, including the methionine salvage pathway, the folate pathway and the diet and transmethylation pathway. The folate pathway is an important factor in one-carbon (C1) metabolism [7].

Folate is an important nutrient. It has roles in a number of fundamental physiological processes, including cell division, DNA synthesis and

methylation [8]. It is catalyzed by dihydrofolate reductase to form tetrahydrofolate (H_4F). Once formed, H_4F enters C1 metabolism and form methylene-H_4F [9]. Catalyzed by methylenetetrahydrofolate reductase (MTHFR), methylene-H4F takes part in methionine synthesis.

MTHFR is a key enzyme that catalyzes the transformation of 5, 10-methylenetetrahydrofolate into 5-methyltetrahydrofolate. This is a crucial hydrolysis step in the re-methylation of homocysteine into methionine and folate [10]. The gene coding MTHFR is located on 1p36.3 and contains 11 exons. In humans, the MTHFR enzyme is made up of dimers, each of which has a C-terminal regulatory domain and an N-terminal catalytic domain [11]. Functional polymorphisms of *MTHFR* may result in reduced enzyme activity and decreased plasma levels of 5-methyl tetrahydrofolic acid. This leads to decreased transformation of homocysteine to methionine, and may play a role in carcinogenesis [12]. The gene coding for MTHFR contains more than 20 SNPs, some of which are non-synonymous. These are the most frequently studied.

Given the biological and pathologic importance of *MTHFR*, functional genetic variations in *MTHFR* may contribute to the development of GCA. To explore the association between *MTHFR* tagging SNPs rs9651118 T>C, rs4846048 A>G, rs1801133 C>T, rs4845882 G>A and rs3753584 A>G and susceptibility to GCA, we performed a hospital-based case-control study in the Chinese Han population.

Materials and methods

Subjects

The study was approved by the Institutional Review Board of Jiangsu University (Zhenjiang, China). All subjects in the study, including the controls, were of Chinese Han origin. Participants were recruited consecutively from the Affiliated People's Hospital of Jiangsu University and the Affiliated Hospital of Jiangsu University (Zhenjiang City, China) between October 2008 and June 2013. In all of the cases, the diagnosis of GCA cases has been established by post-operative pathological studies. Potential participants were excluded if they had a past history of cancer, an autoimmune disorder, or had received chemotherapy or radiotherapy. Controls were recruited randomly. Most of them were hospitalized due to trauma. Those with a past history of any form of malignancy were ineligible to be controls. The controls were matched to the study participants for ethnicity, sex and age (±5 years).

A previously piloted questionnaire was administered to the participants and to the controls by one of two specially trained interviewers. This was used to obtain demographic data and data on known risk factors for GCA, including cigarette smoking and alcohol drinking, as has been previously described [13].

DNA extraction

Each subject donated 2 ml peripheral venous blood, which was stored in tubes containing ethylene diamine tetraacetic acid (EDTA) disodium salt at 4°C. Genomic DNA was extracted using a commercially available DNA Blood Mini Kit (Qiagen, Berlin, Germany), within a week of blood sampling.

Polymorphism genotyping

For our study we selected *MTHFR* tagging SNPs according to the HapMap Project and Haploview 4.2 software described previously (**Figure 1**) [13]. The *MTHFR* SNPs mentioned above genotyping was performed utilizing using the ligase detection reaction (LDR) method. Technical support was provided by Shanghai Biowing Applied Biotechnology Company [13-15]. For quality control purpose, we randomly selected 110 samples for repeat test. This confirmed an accuracy rate of 100%. We used the SHEsis Program (Bio-X Inc., Shanghai, China, available at http://202.120.7.14/analasis/myAnalasis.php) to construct haplotypes of the five SNPs [16].

Statistical analysis

The ages of the cases and controls were compared using the t-test. Deviations from the Hardy-Weinberg equilibrium (HWE) in controls were tested using an internet-based HWE calculator (available at http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) [15]. Differences in the genotype, haplotype and demographic characteristics between cases and controls were estimated using the χ^2 test. Unconditional logistic regression analysis was used to evaluate associa-

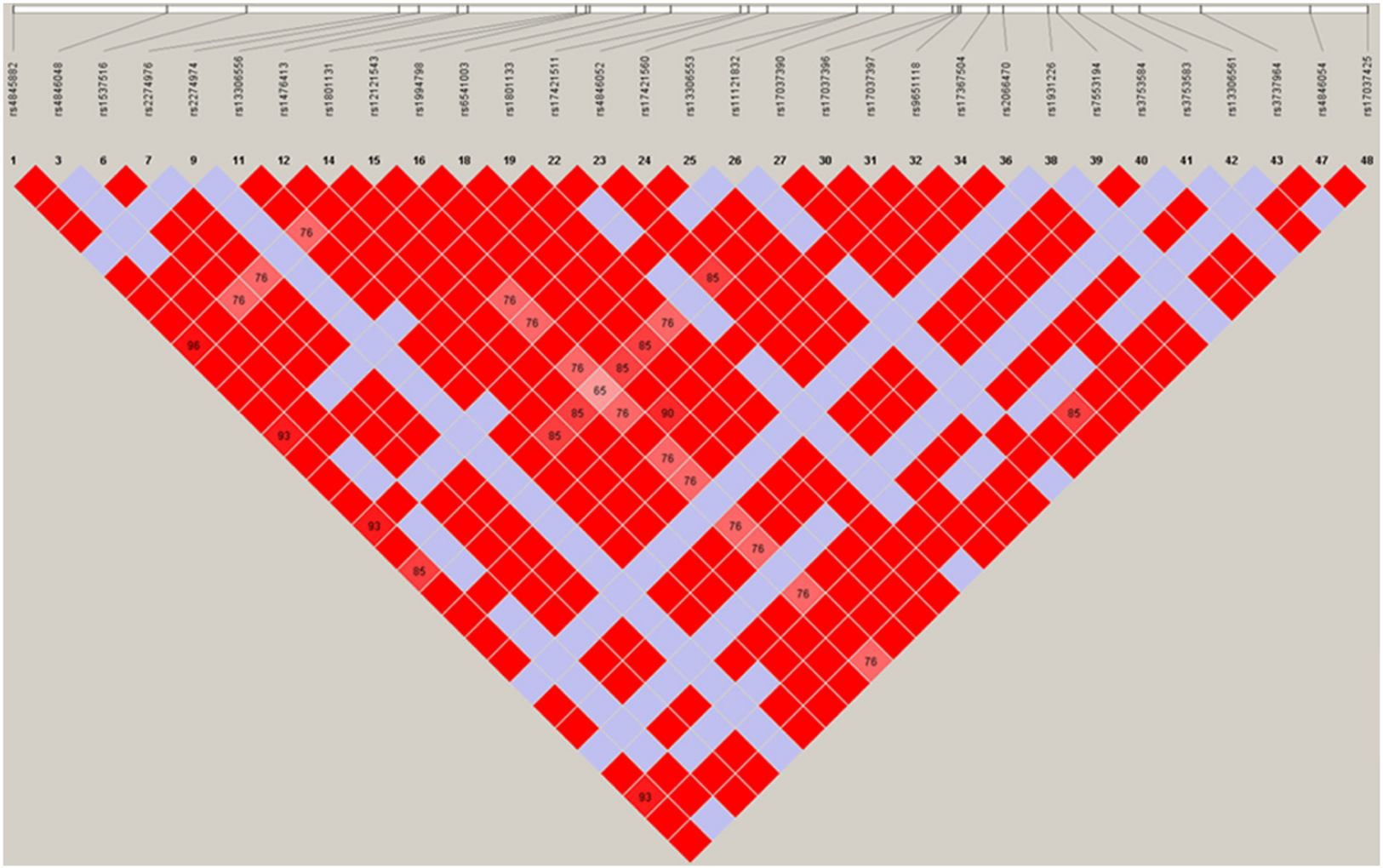

Figure 1. Linkage disequilibrium (LD) plot of MTHFR: the plot was drawn by Haploview 4.2 with D'Color Scheme. The cells are gradient color representing strength of LD between five polymorphisms. Light-colored cells indicate low LD and dark-colored indicate high LD.

Table 1. Distribution of selected demographic variables and risk factors in GCA cases and controls

Variable	Cases (n = 330)		Controls (n = 608)		P^a
	n	%	n	%	
Age (years) mean ± SD	65.06 (±8.37)		64.19 (±6.66)		0.103
Age (years)					0.746
< 60	89	26.97	170	27.96	
≥ 60	241	73.03	438	72.04	
Sex					0.965
Male	223	67.58	410	67.43	
Female	107	32.42	198	32.57	
Tobacco use					**0.006**
Never	209	63.33	438	72.04	
Ever	121	36.67	170	27.96	
Alcohol use					0.072
Never	233	70.61	462	75.99	
Ever	97	29.39	146	24.01	

[a]Two-sided χ^2 test and student t test; Bold values are statistically significant ($P < 0.05$).

tions between the *MTHFR* genotypes and susceptibility to GCA by computing odds ratios (Ors, crude or adjusted appropriate) and 95% confidence intervals (CIs). Statistical analysis was performed using the SAS 9.1.3 software (SAS Institute, Cary, NC). Differences were considered statistically significant when $P < 0.05$; with two-sided probabilities.

Results

Subject characteristics

A total of 330 cases and 608 controls were included in the study. Their demographic characteristics and risk factors for GCA are shown in **Table 1**. The cases and controls were matched for age and sex. There was no statistical difference between them in alcohol use, but GCA cases were significantly more likely to use tobacco.

Associations between MTHFR polymorphisms and GCA risk

Our principle findings concerning MTHFR rs9651118 T>C, rs4846048 A>G, rs1801133 C>T, rs4845882 G>A and rs3753584 A>G polymorphisms are shown in Table S1. With the exception rs1801133 C>T (P = 0.033), the genotype distribution of these SNPs in the controls conformed to the HWE (P > 0.05).

With regard to rs1801133 C>T, in the recessive model, the TT homozygote genotype was associated with a borderline statistically increased risk of GCA ($P = 0.054$). In the same model, and after adjusted for age, sex and tobacco and alcohol use, the TT genotype increased the risk of GCA (adjusted OR = 1.46, $P = 0.029$; **Table 2**).

The genotype frequencies of *MTHFR* rs9651118 T>C, rs4846048 A>G, rs4845882 G>A and rs3753584 A>G polymorphisms were not statistically different between the cases and the controls ($P = 0.912$, $P = 0.473$, $P = 0.421$ and $P = 0.324$, respectively).

Further analysis of the association between the haplotypes of these SNPs and the susceptibility to GCA was further performed. Compared with the $G_{rs4845882}A_{rs4846048}T_{rs1801133}T_{rs9651118}A_{rs3753584}$ haplotype, the $G_{rs4845882}A_{rs4846048}T_{rs1801133}C_{rs9651118}A_{rs3753584}$ haplotype was associated with an increased susceptibility to GCA (crude OR = 5.32, 95% CI = 2.34-12.10, $P < 0.0001$; **Table 3**). No significant associations were observed between other haplotypes and GCA risk.

Discussion

In this study, we performed a hospital-based case-control study to investigate whether functional SNPs in *MTHFR* affect the susceptibility of the Chinese Han population to GCA. We found evidence that *MTHFR* rs1801133 TT genotype and the *MTHFR* $G_{rs4845882}A_{rs4846048}T_{rs1801133}C_{rs9651118}A_{rs3753584}$ haplotype increased the risk of GCA.

The MTHFR gene produces methylenetetrahydrofolate reductase, which is a rate-limiting enzyme in folate metabolism and DNA methylation. It is an active 77 kDa protein that catalysis the conversion of 5, 10-methylenetetrahydrofolate into 5-methyltetrahydrofolate [17]. The *MTHFR* gene is highly polymorphic in the general population. There is evidence that *MTHFR* gene mutations lead to increased thymidylate synthase (TS) activity in cancer cells, as a consequence of increased level of 5, 10-methylenetetrahydrofolate. The latter supplies methyl for the methylation of dUMP to dTMP [18]. TS is a critical and rate-limiting enzyme for maintaining an appropriate supply of DNA to ensure

Table 2. Logistic regression analyses of associations between *MTHFR* polymorphisms and the risk of GCA

Genotype	Cases (n = 330)		Controls (n = 608)		Crude OR (95% CI)	*P*	Adjusted OR[a] (95% CI)	*P*
	n	%	n	%				
MTHFR rs1801133 C>T								
CC	102	31.48	170	28.72	1.00		1.00	
CT	148	45.68	318	53.72	0.78 (0.57-1.06)	0.112	0.76 (0.55-1.04)	0.086
TT	74	22.84	104	17.57	1.19 (0.81-1.75)	0.387	1.23 (0.83-1.82)	0.300
CT+TT	222	68.52	422	71.28	0.88 (0.65-1.18)	0.381	0.87 (0.65-1.17)	0.362
CC+CT	250	77.16	488	82.43	1.00		1.00	
TT	74	22.84	104	17.57	1.39 (0.99-1.94)	0.054	1.46 (1.04-2.05)	**0.029**
T allele	296	45.68	526	44.43				
MTHFR rs3753584 A>G								
AA	275	84.88	518	87.50	1.00		1.00	
AG	48	14.81	72	12.16	1.26 (0.85-1.86)	0.257	1.22 (0.82-1.82)	0.319
GG	1	0.31	2	0.34	0.94 (0.09-10.43)	0.961	1.03 (0.09-11.41)	0.984
AG+GG	49	15.12	74	12.50	1.25 (0.85-1.84)	0.266	1.22 (0.82-1.81)	0.324
AA+AG	323	99.69	590	99.66	1.00		1.00	
GG	1	0.31	2	0.34	0.91 (0.08-10.12)	0.942	1.00 (0.09-11.12)	1.000
G allele	50	7.72	79	6.67				
MTHFR rs4845882 G>A								
GG	216	66.26	416	69.10	1.00		1.00	
GA	99	30.37	161	26.74	1.18 (0.88-1.60)	0.268	1.18 (0.87-1.60)	0.287
AA	11	3.37	25	4.15	0.85 (0.41-1.76)	0.656	0.81 (0.39-1.68)	0.562
GA+AA	110	33.74	186	30.90	1.14 (0.85-1.52)	0.375	1.13 (0.84-1.51)	0.421
GG+GA	315	96.63	577	95.85	1.00		1.00	
AA	11	3.37	25	4.15	0.81 (0.39-1.66)	0.558	0.77 (0.37-1.59)	0.475
A allele	121	18.56	211	17.52				
MTHFR rs4846048 A>G								
AA	253	79.56	490	81.26	1.00		1.00	
AG	63	19.81	103	17.08	1.19 (0.84-1.68)	0.340	1.22 (0.86-1.74)	0.262
GG	2	0.63	10	1.66	0.39 (0.08-1.78)	0.223	0.33 (0.07-1.54)	0.158
AG+GG	65	20.44	113	18.74	1.11 (0.79-1.57)	0.534	1.14 (0.80-1.60)	0.473
AA+AG	316	99.37	593	98.34	1.00		1.00	
GG	2	0.63	10	1.66	0.38 (0.08-1.72)	0.208	0.32 (0.07-1.48)	0.145
G allele	67	10.53	123	10.20				
MTHFR rs9651118 T>C								
TT	129	40.31	241	41.13	1.00		1.00	
TC	148	46.25	276	47.10	1.00 (0.75-1.34)	0.990	0.98 (0.73-1.31)	0.874
CC	43	13.44	69	11.77	1.16 (0.75-1.80)	0.495	1.18 (0.76-1.83)	0.469
TC+CC	191	59.69	345	58.87	1.03 (0.78-1.37)	0.812	1.02 (0.77-1.34)	0.912
TT+TC	277	86.56	517	88.23	1.00		1.00	
CC	43	13.44	69	11.77	1.16 (0.77-1.75)	0.468	1.19 (0.79-1.80)	0.403
C allele	234	36.56	414	35.32				

[a]Adjusted for age, sex, smoking and drinking status; Bold values are statistically significant ($P < 0.05$).

accurate DNA synthesis and repair [19]. It follows that SNPs in the *MTHFR* gene may contribute to the genetic susceptibility to cancer [20].

Several earlier studies have also suggested that *MTHFR* rs1801133 TT genotype may increase the risk of GCA in the Chinese Han

Table 3. MTHFR haplotype frequencies (%) in cases and controls and risk of GCA

Haplotypes	Cases (n = 660)		Controls (n = 1216)		Crude OR (95% CI)	P
	n	%	n	%		
$G_{rs4845882}A_{rs4846048}T_{rs1801133}T_{rs9651118}A_{rs3753584}$	273	41.36	528	43.42	1.00	
$G_{rs4845882}A_{rs4846048}C_{rs1801133}C_{rs9651118}A_{rs3753584}$	210	31.82	407	33.47	1.00 (0.80-1.25)	0.985
$A_{rs4845882}G_{rs4846048}C_{rs1801133}T_{rs9651118}A_{rs3753584}$	64	9.70	118	9.70	1.05 (0.75-1.47)	0.780
$A_{rs4845882}A_{rs4846048}C_{rs1801133}T_{rs9651118}G_{rs3753584}$	42	6.36	73	6.00	1.11 (0.74-1.67)	0.607
$G_{rs4845882}A_{rs4846048}C_{rs1801133}T_{rs9651118}A_{rs3753584}$	27	4.09	48	3.95	1.09 (0.66-1.78)	0.738
$G_{rs4845882}A_{rs4846048}T_{rs1801133}C_{rs9651118}A_{rs3753584}$	22	3.33	8	0.66	5.32 (2.34-12.10)	< 0.0001
$A_{rs4845882}A_{rs4846048}C_{rs1801133}C_{rs9651118}A_{rs3753584}$	7	1.06	7	0.58	1.93 (0.67-5.57)	0.222
$G_{rs4845882}A_{rs4846048}T_{rs1801133}T_{rs9651118}G_{rs3753584}$	5	0.76	0	0.00	—	0.980
$A_{rs4845882}A_{rs4846048}C_{rs1801133}T_{rs9651118}A_{rs3753584}$	3	0.45	9	0.74	0.65 (0.17-2.40)	0.513
$A_{rs4845882}G_{rs4846048}C_{rs1801133}T_{rs9651118}G_{rs3753584}$	2	0.30	0	0.00	—	0.981
$G_{rs4845882}A_{rs4846048}C_{rs1801133}T_{rs9651118}G_{rs3753584}$	0	0.00	4	0.33	—	0.983
$A_{rs4845882}A_{rs4846048}T_{rs1801133}T_{rs9651118}A_{rs3753584}$	2	0.30	3	0.25	1.29 (0.21-7.76)	0.781
$A_{rs4845882}G_{rs4846048}C_{rs1801133}C_{rs9651118}A_{rs3753584}$	0	0.00	3	0.25	—	0.978
$G_{rs4845882}G_{rs4846048}T_{rs1801133}T_{rs9651118}A_{rs3753584}$	0	0.00	3	0.25	—	0.978
others	3	0.45	5	0.41	1.16 (0.28-4.89)	0.839

With the order of *MTHFR* rs4845882 G>A, rs4846048 A>G, rs1801133 C>T, rs9651118 T>C and rs3753584 A>G in gene position.

population [21-23]. It is reported that a C→T mutation at nucleotide 677 loci (in exon 4 at the folate-binding site) led to valine substitution for alanine (677 C>T, rs1801133 C>T) and that this is functionally relevant, causing a reduction in the activity of methylenetetrahydrofolate reductase [24]. Studies have shown that individuals who are heterozygous for *MTHFR* rs1801133 polymorphism have 70% of normal enzyme activity, but those who are homozygous have only 30% of the normal enzyme activity [25]. With regard to relationship between *MTHFR* and folate, some studies have suggested that compound heterozygosity for the 677T allele is associated with decreased plasma folate levels [26]. A different study found that the functional polymorphism rs1801133 C>T is associated with low plasma folate content and significantly decrease MTHFR activity [27]. MTHFR plays a role in the formation of dimers, with flavin adenine dinucleotide (FAD) being a cofactor. However, mutant *MTHFR* (677T) dissociates into monomers leading to decreased enzymatic activity. Docking studies have established that mutant *MTHFR* (677T) has less affinity with FAD than the wild type enzyme (677C) [11]. When combined with our results, findings suggest that a C-to-T mutation in *MTHFR* results in lower enzyme activity and lower folate concentrations and that these may be associated with an increased risk of GCA.

The rs9651118 T>C SNP is situated in the intron region of the *MTHFR* gene. Several recent studies have shown that it has moderate protective effect against carcinoma of the lung and breast [28, 29], but not against esophageal squamous cell carcinoma (ESCC) [13]. We did not find any association between it and GCA risk. Together, these findings indicate that *MTHFR* rs9651118 T>C has different effects depending on the type of cancers. *MTHFR* rs4845882 G>A is located on the intron region of the *MTHFR* gene, with almost complete linkage disequilibrium with rs1801131 A>C. A previous study showed that there was no significant association between the combined AC/CC variant genotypes and the risk of GCA [30]. Our results are consistent with this. *MTHFR* rs3753584 A>G is also situated in the intron region of the *MTHFR* gene. A previous study found that there was an increased risk of lung cancer in carriers of the variant allele of this SNP when compared with subjects who were homozygote for the wild type. The risk was more marked in those over 60 years [31]. No similar association was found with ESCC [13].

Our study failed to find an association between this SNP and GCA. *MTHFR* rs4846048 A>G is situated 463 base pairs (bp) up stream of a polyadenylation signal [32]. It has been associated with the decreased risk of ESCC [13], but no association has been found between it and the risk of breast cancer [33]. We found no evidence of an association between it and the risk of GCA. Further studies are required in order to better determine the biological significance of these SNPs in the pathogenesis of GCA.

We studies five potentially functional *MTHFR* SNPs in order to ascertain the association between *MTHFR* haplotypes and the susceptibility to GCA. Haplotype analysis suggested a significant association with susceptibility to GCA. In a previous reported study of the association between the haplotype of three SNPs (*MTHFR* rs1801133 C>T, rs1801131 A>C and rs2274976 G>A) and GCA risk, it was found that individuals with six mutant alleles had a significantly increased risk when compared to those with 0-2 mutant alleles [30]. However, further studies with larger sample sizes are needed to conform these findings.

Our study used a fine-mapping approach to obtain the *MTHFR* SNPs we used. It is the first reported study to investigate rs9651118 T>C, rs4846048 A>G, rs4845882 G>A and rs3753584 A>G *MTHFR* SNPs. Moreover, in comparison to previously reported study, its sample size was large.

However, the study did have several limitations. Given that the cases and controls were recruited from hospitals, the study population may not have been representative of the general Chinese Han population. Folate status may influence the association between *MTHFR* SNPs and GCA susceptibility. We did not have data on the folate intake of those we studied. Finally, an even larger sample size than we were able to recruit might be expected to lead to more definitive findings. Further studies are needed to better understand the role of interactions between genes and the environment in the causation of GCA.

In conclusion, our results suggest that the functional *MTHFR* rs1801133 C>T polymorphism and the *MTHFR* $G_{rs4845882}A_{rs4846048}T_{rs1801133}$-$C_{rs9651118}A_{rs3753584}$ haplotype may contribute to susceptibility to GCA in Chinese Han individuals.

Acknowledgements

This study was supported in part by the National Natural Science Foundation of China (81472332, 81370001, 81300037 and 81341006), Jiangsu Province Natural Science Foundation (BK2010333 and BK2011481), Fujian Province Natural Science Foundation (2013J01126 and 2013J05116), Fujian Medical University professor fund (JS12008) and Fujian Province science and technology programmed fund (2012Y0030). We appreciate all subjects who participated in this study. We wish to thank Dr. Yiqun Chen (Biowing Applied Biotechnology Company, Shanghai, China) for technical support.

Disclosure of conflict of interest

None.

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Table S1. Primary information for *MTHFR* rs1801133 C>T, rs3753584 A>G, rs4845882 G>A, rs4846048 A>G and rs9651118 T>C polymorphisms

Genotyped SNPs	*MTHFR* rs9651118 T>C	*MTHFR* rs4846048 A>G	*MTHFR* rs1801133 C>T	*MTHFR* rs4845882 G>A	*MTHFR* rs3753584 A>G
Chromosome	1	1	1	1	1
Function	intron	intron	missense	intron	nearGene-5
ChrPos (Genome Build 36.3)	11784801	11768839	11778965	11765754	11787173
Regulome DB Score[a]	5	3a	4	1f	4
TFBS[b]	Y	—	—	—	Y
Splicing (ESE or ESS)	—	—	—	—	Y
miRNA (miRanda)	—	Y	—	—	—
nsSNP	—	—	Y	—	—
MAF[c] for Chinese in database	0.382	0.105	0.439	0.198	0.093
MAF in our controls (n = 608)	0.353	0.102	0.444	0.175	0.067
P value for HWE[d] test in our controls	0.456	0.097	0.033	0.066	0.764
Genotyping method[e]	LDR	LDR	LDR	LDR	LDR
% Genotyping value	96.59%	98.19%	97.65%	98.93%	97.65%

[a]http://www.regulomedb.org/; [b]TFBS: Transcription Factor Binding Site (http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm); [c]MAF: minor allele frequency; [d]HWE: Hardy-Weinberg equilibrium; [e]LDR: ligation detection reaction.