MTR, *MTRR*, and *MTHFR* Gene Polymorphisms and Susceptibility to Nonsyndromic Cleft Lip With or Without Cleft Palate

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Objective: To examine the associations of *methionine synthase* (*MTR*), *methionine synthase reductase* (*MTRR*), and *methylenetetrahydrofolate reductase* (*MTHFR*) gene polymorphisms with the susceptibility to nonsyndromic cleft lip with or without cleft palate (NSCL/P). **Methods:** Between May 2012 and August 2014, 147 NSCL/P patients (case group) and 129 healthy volunteers (control group) were recruited for the study. The *MTR* A2756G, *MTRR* A66G, *MTHFR* C677T and *MTHFR* A1298C polymorphisms were assessed by polymerase chain reaction–restriction fragment length polymorphism. Haplotype analyses were performed with SHEsis software. Logistic regression analysis was used to evaluate the possible risk factors for NSCL/P. Generalized multifactor dimensionality reduction (GMDR) was applied to detect gene–gene interactions. **Results:** *MTR* A2756G, *MTRR* A66G, and *MTHFR* C677T gene polymorphisms were associated with the risk of NSCL/P (all $p < 0.05$). Logistic regression analysis revealed that *MTR* A2756G, *MTR* RA66G, and *MTHFR* C667T might increase the risk of NSCL/P (odds ratio [OR] = 0.270, 95% confidence interval [95% CI] = 0.106–0.689; OR = 0.159, 95% CI = 0.069–0.368; OR = 0.343, 95% CI = 0.139–0.844). The CA haplotype in the *MTHFR* gene may serve as a protective factor for NSCL/P (OR = 0.658, 95% CI = 0.470–0.923), and the TA haplotype might be a risk factor (OR = 2.001, 95% CI = 1.301–3.077). GMDR revealed that the optimal models were two- and four-dimensional models with prediction accuracies of 75.73% ($p = 0.001$) and 77.21% ($p = 0.001$) and the best cross-validation consistencies of 10/10 and 10/10, respectively. **Conclusion:** *MTR* A2756G, *MTRR* A66G, and *MTHFR* C677T polymorphisms may be related to NSCL/P, and interactions were detected between the *MTR* A2756G, *MTRR* A66G, and *MTHFR* C677T and A1298C polymorphisms.

Introduction

OROFACIAL CLEFTS (OFCs) ARE a group of conditions that include cleft lip, cleft palate, and combined cleft lip and palate. As a common congenital anomaly, OFCs have average birth prevalences of ~7.75 per 10,000 persons in the United States and ~7.94 per 10,000 internationally, but the prevalence varies with geography, ethnicity, and socioeconomic status (Mossey *et al.*, 2011; Tanaka *et al.*, 2012; Shaye *et al.*, 2015). OFCs occur in ~1–2 per 1000 births in the developed world and 3.27 per 1000 births in China, and males account for 63.5% of all cases (Li *et al.*, 2008; Kling *et al.*, 2014; Watkins *et al.*, 2014). Nonsyndromic cleft lip with or without cleft palate (NSCL/P) without any associated anomalies accounts for ~70–95% of all OFC cases worldwide and elicits heavy health and economic burdens (Carinci *et al.*, 2007; Borges *et al.*, 2015).

Variations in the prevalence of NSCL/P result from ethnic and environmental differences, and multifactorial etiologies involving both genetic and environmental factors have been

documented with accumulating evidence (Erickson, 2010; Ludwig *et al.*, 2012, 2014; Jia *et al.*, 2015). Moreover, genetic polymorphisms that encode folate metabolism enzymes, such as *methionine synthase* (*MTR*), *methionine synthase reductase* (*MTRR*), and *methylenetetrahydrofolate reductase* (*MTHFR*), may potentially influence the molecular pathophysiology of NSCL/P (Guo *et al.*, 2009; Blanton *et al.*, 2011; Pan *et al.*, 2012).

Folate metabolism is an intricate process that depends on a series of enzymatic reactions involving numerous genes and pathways that produce active tetrahydrofolate (THF) derivatives (Biselli *et al.*, 2012; Nazki *et al.*, 2014). Aberrantly interacting folate metabolism pathways can result in frank folate deficiencies and affect DNA methylation and synthesis with the downstream effects of disrupting important biological processes, such as craniofacial development (Bhaskar *et al.*, 2011; Blanton *et al.*, 2011). The involvement of the *MTR*, *MTRR*, and *MTHFR* enzymes in folate metabolism and methyl group metabolism makes these enzymes crucial for the maintenance of proper DNA methylation and nucleic acid synthesis (Lopez-Cortes *et al.*, 2013; Weiner *et al.*, 2014).

MTR is encoded by the *MTR* gene and is responsible for the regeneration of methionine from homocysteine, and *MTR* gene mutations may contribute to various diseases, including cardiovascular diseases, cancers, birth defects, and congenital anomalies (Weiner *et al.*, 2012; Coppede *et al.*, 2013; Hosseini, 2013; Yang *et al.*, 2013, 2014). In addition, *MTR* A2756G (rs1805087) has been demonstrated to contribute to breast and prostate cancer, but no previous study has observed associations of *MTR* A2756G (rs1805087) with NSCL/P in Chinese populations (Guo *et al.*, 2009; de Cassia Carvalho Barbosa *et al.*, 2012; Lopez-Cortes *et al.*, 2013). *MTRR* regulates the homocysteine metabolic pathway, and the *MTRR* gene A66G and C524T polymorphisms have been suggested to be related to congenital heart defects (Scazzone *et al.*, 2009; Zeng *et al.*, 2011).

A meta-analysis indicated that the *MTRR* A66G polymorphism, but not the *MTR* A2756G polymorphism, may increase the maternal risk for neural tube defects among Caucasians (Ouyang *et al.*, 2013). In addition, a recent study reported that the *MTRR* A66G polymorphism, but not the *MTR* A2756G polymorphism, may contribute to NSCL/P in Indian populations (Murthy *et al.*, 2015). *MTHFR* influences folate and homocysteine metabolisms, and *MTHFR* gene mutations may increase the occurrence of methylene THF reductase deficiency (Goyette *et al.*, 1994; Trimmer, 2013). Evidence has demonstrated that the *MTHFR* C677T and A1298C polymorphisms decrease the enzymatic activity and these polymorphisms are the most commonly studied variants in the folate pathway with respect to NSCL/P (Blanton *et al.*, 2011; Zhao *et al.*, 2014; Ebadifar *et al.*, 2015).

However, less information is currently available regarding the importance of the interactions between the *MTR* A2756G, *MTRR* A66G, and *MTHFR* C677T and A1298C polymorphisms and their haplotypes in the modulation of NSCL/P. Therefore, the present study aimed to assess the functional *MTR* A2756G, *MTRR* A66G, and *MTHFR* C677T and A1298C polymorphisms and NSCL/P susceptibility among a Chinese population.

Materials and Methods

Study subjects

Between May 2012 and August 2014, a total of 147 NSCL/P patients (Han nationality, 86 males and 61 females; mean age: 10.23 ± 2.78 years) were randomly recruited from the Department of Oral Maxillofacial Surgery of the First Affiliated Hospital, Harbin Medical University. Congenital NSCL/P patients were diagnosed based on the International Classification of Diseases (ICD-10) developed by the World Health Organization (Tanno *et al.*, 2014).

The exclusion criteria were as follows: (1) cheilopalatognathus associated with other congenital disorders or congenital malformations, such as neural tube defects or congenital heart diseases with concomitant cheilopalatognathus, kabuki make-up syndrome, Van der Woude syndrome, Meckel syndrome, or velocardiofacial syndrome and (2) NSCL/P patients with associated hypertension, coronary heart disease, or other important organ diseases. In addition, 129 volunteers confirmed to be healthy by physical examinations with the corresponding period were enrolled in the control group (Han nationality, 76 males and 53 females; mean age, 10.35 ± 2.86 years). The characteristics of the case and control groups were comparable, including basal state of illness and age (all P s > 0.05).

Importantly, this study was approved by the Ethics Committee of the First Affiliated Hospital, Harbin Medical University, and we obtained informed consent from the parents or legal guardians of the study subjects. The study protocols followed the ethical principles for medical research involving human subjects of the Declaration of Helsinki (Glas *et al.*, 2013).

Single-nucleotide polymorphism screen and selection

Using the NCBI-Database of Single-Nucleotide Polymorphisms (dbSNPs; www.ncbi.nlm.nih.gov/SNP/) and the HapMap database (http://SNP.cshl.org/cgi-perl/gbrowse/hapmap27_B36/), we searched and downloaded data packets related to the *MTHFR*, *MTRR*, and *MTR* SNPs in the human gene pool. The *MTHFR*, *MTRR* and *MTR* SNPs were screened with Haploview 4.2 software. The parameters were set as follows: Chinese Han population, minor allele frequency (MAF) = 0.05, and $r^2 > 0.8$. The linkage disequilibrium (D') 95% confidence interval (95% CI) was calculated to classify the adjacent SNPs with D' 95% CI of 0.70–0.98 into the same haplotype block. After selection, *MTR* rs1805087 (A2756G), *MTRR* rs1801394 (A66G), *MTHFR* rs1801133 (C677T) and rs1801131 (A1298C) were found to be eligible for further assessment (Table 1).

Polymerase chain reaction–restriction fragment length polymorphism

Blood samples from veins in the elbow (5 mL) were collected from all subjects after overnight fasting, placed in tubes with EDTA anticoagulant, and stored in a -70°C refrigerator until further use. The DNA of each subject was extracted using the phenol/chloroform extraction method. The *MTR* rs1805087 (A2756G), *MTRR* rs1801394 (A66G), *MTHFR* rs1801133 (C677T) and rs1801131 (A1298C) genotypes were sequenced and analyzed by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP). The

TABLE 1. SNP VARIATION INFORMATION OF *MTR*, *MTRR*, AND *MTHFR*

Gene	dbSNP	Function	Alleles	Allele frequency (CHB)
<i>MTR</i>	rs1805087 (A2756G)	Missense	A/G	A:0.892, G:0.108
<i>MTRR</i>	rs1801394 (A66G)	Missense	A/G	A:0.750, G:0.250
<i>MTHFR</i>	rs1801133 (C677T)	Missense	C/T	C:0.561, T:0.439
	rs1801131 (A1298C)	Missense	A/C	A:0.756, C:0.244

CHB, Chinese Han population; dbSNP, database of single-nucleotide polymorphisms; *MTHFR*, methylenetetrahydrofolate reductase; *MTR*, methionine synthase; *MTRR*, methionine synthase reductase; SNP, single-nucleotide polymorphism.

TABLE 2. PRIMERS DESIGNED FOR *MTR*, *MTRR*, AND *MTHFR* GENE POLYMORPHISM LOCI

Genes	Primers	Product length (bp)
<i>MTR</i> A2756G	Forward: 5'-CATGGAAGAATATGAAGATATTAGAC-3' Reverse: 5'-GAACTAGAAGACAGAAATTCTCTA-3'	189
<i>MTRR</i> A66G	Forward: 5'-GCAAAGGCCATCGCAGAAGACAT-3' Reverse: 5'-AAACGGTAAAATCCACTGTAACGG-3'	151
<i>MTHFR</i> C677T	Forward: 5'-TGAAGGAGAAGGTGTCTGCGGGA-3' Reverse: 5'-AGGACGGTGCGGTGAGAGTG-3'	198
<i>MTHFR</i> A1298C	Forward: 5'-AAGGAGGAGCTGCTGCTGAAGATG-3' Reverse: 5'-CTTTGCCATGTCCACAGCATG-3'	237

PCR primer sequences (Biological Engineering Co., Ltd.) are summarized in Table 2. We then hired the Beijing Genomics Institute to verify the sequencing of a randomly selected 10% of the PCR products.

The PCR-amplified products were digested overnight at 37°C with specific restriction enzymes (*Hae*III for *MTR* A2756G, *Nde*I for *MTRR* A66G, *Hinf*I for *MTHFR* C677T, and *Mbo*II for *MTHFR* A1298C) in a total volume of 20 μ L containing 17 μ L PCR-amplified products, ddH₂O, 2 μ L 10 $\times$ reaction buffer, and 1 μ L restriction enzyme (10 U/ μ L). Six percent polyacrylamide gel electrophoresis was used to separate the enzyme-digested products. The genotypes were determined according to the enzyme map.

Statistical analyses

The continuous variables are presented as the means $\pm$ standard deviations. The categorical variables are presented as frequencies or percentages. We conducted *t*-tests to com-

pare the continuous variables and chi-square or Fisher's exact tests as appropriate to compare the categorical variables. The deviations from the Hardy-Weinberg equilibrium were estimated. Odds ratios (ORs) and their 95% CI were calculated for the associations of the *MTR*, *MTRR*, and *MTHFR* polymorphisms with NSCL/P, which were obtained from logistic regression analysis. Haplotype analysis was performed with the SHeSis software.

Generalized multifactor dimensionality reduction (GMDR) was performed using a JAVA-based free software (GMDR 0.9) to detect the gene-gene interactions (Lou *et al.*, 2007). After sorting according to NSCL/P and health control status, four SNPs of the *MTR*, *MTRR*, and *MTHFR* genes were introduced into the GMDR model as variables and subjected to sign and permutation tests to calculate the cross-validation consistencies and balance test accuracies of the different factor combinations in the various dimensions.

The cross-validation consistency was used to measure the degree of consistency and to determine whether the selected

TABLE 3. THE GENOTYPE AND ALLELE FREQUENCY DISTRIBUTIONS OF *MTR* A2756G, *MTRR* A66G, *MTHFR* C677T, AND *MTHFR* A1298C BETWEEN THE CASE GROUP AND THE CONTROL GROUP

SNPs		Case (n = 147), n (%)	Control (n = 129), n (%)	χ^2	p	OR (95% CI)
<i>MTR</i> A2756G	AA	99 (67.3)	70 (54.3)	Ref		
	AG	48 (32.7)	58 (44.9)	4.629	0.031	1.709 (1.047–2.789)
	GG	0 (0.0)	1 (0.8)	1.403	0.236	4.234 (0.169–105.5)
	AG+GG	48 (32.7)	59 (45.7)	4.954	0.026	0.575 (0.353–0.938)
	A	246 (83.7)	198 (76.7)	Ref		
<i>MTRR</i> A66G	G	48 (16.3)	60 (23.3)	4.193	0.041	1.553 (1.017–2.371)
	AA	71 (48.3)	29 (22.5)	Ref		
	AG	26 (17.7)	59 (45.7)	30.091	<0.001	5.556 (2.953–10.45)
	GG	50 (34.0)	41 (31.8)	5.289	0.021	2.008 (1.104–3.650)
	AG+GG	76 (51.7)	100 (77.5)	19.821	<0.001	0.310 (0.184–0.525)
<i>MTHFR</i> C677T	A	168 (57.1)	117 (45.3)	Ref		
	G	126 (42.9)	141 (54.7)	7.654	0.006	1.607 (1.147–2.250)
	CC	28 (19.1)	19 (14.7)	Ref		
	CT	66 (44.9)	97 (75.2)	5.373	0.020	2.166 (1.118–4.196)
	TT	53 (36.0)	13 (10.1)	5.811	0.016	0.362 (0.156–0.838)
<i>MTHFR</i> A1298C	CT+TT	119 (80.9)	110 (85.3)	0.907	0.341	0.734 (0.388–1.389)
	C	122 (41.5)	135 (52.3)	Ref		
	T	172 (58.5)	123 (47.7)	6.476	0.011	0.646 (0.461–0.905)
	AA	66 (44.9)	57 (44.2)	Ref		
	AC	67 (45.6)	57 (44.2)	0.003	0.953	0.985 (0.597–1.625)
<i>MTHFR</i> A1298C	CC	14 (9.5)	15 (11.6)	0.273	0.602	1.241 (0.552–2.789)
	AC+CC	81 (55.1)	72 (55.8)	0.014	0.906	0.972 (0.604–1.564)
	A	199 (67.7)	171 (66.3)	Ref		
	C	95 (32.3)	87 (33.7)	0.123	0.726	1.066 (0.747–1.521)

95% CI, 95% confidence interval; OR, odds ratio; Ref, reference.

TABLE 4. MULTIPLE LOGISTIC REGRESSION ANALYSIS FOR NSCL/P

Variables	β	SE	Wald	Significance	Exp(β)	95% CI
MTR A2756G	-1.310	0.479	7.488	0.006	0.270	0.106–0.689
MTRR A66G	-1.840	0.428	18.464	<0.001	0.159	0.069–0.368
MTHFR C667T	1.071	0.460	5.419	0.020	0.343	0.139–0.844

β , partial regression coefficient; Exp(β), exponent function (partial regression coefficient); NSCL/P, nonsyndromic cleft lip with or without cleft palate; SE, standard error.

interaction was the optimal model, and the balance test accuracy scores, which ranged from 0.50 (indicating an unsatisfactory model) to 1.00 (indicating the optimal model), were used to accurately predict the degree of the interaction. The predictive accuracy of the permutation test was used to measure the significance of each model ($p < 0.05$). SPSS 19.0 software (SPSS, Inc.) was utilized for the statistical analyses. A two-tailed p value below 0.05 indicated statistical significance.

Results

Genotype and allele frequency distributions

The genotype distributions of the studied polymorphisms were consistent with the Hardy–Weinberg predictions in both groups (all P s > 0.05). The MTR A2756G polymorphism was related to the development of NSCL/P (AA vs. AG: OR = 1.709, 95% CI = 1.047–2.789, $p = 0.031$; A vs. G: OR = 1.553, 95% CI = 1.017–2.371, $p = 0.041$). The MTRR A66G polymorphism increased the risk of NSCL/P (AA vs. AG: OR = 5.556, 95% CI = 2.953–10.45, $p < 0.001$; AA vs. GG: OR = 2.008, 95% CI = 1.104–3.650, $p = 0.021$; A vs. G: OR = 1.607, 95% CI = 1.147–2.250, $p = 0.006$), and an obvious association between the MTHFR C677T polymorphism and NSCL/P risk was observed (CC vs. CT: OR = 2.166, 95% CI = 1.118–4.196, $p = 0.020$; CC vs. TT: OR = 0.362, 95% CI = 0.156–0.838, $p = 0.016$; C vs. T: OR = 0.646, 95% CI = 0.461–0.905, $p = 0.011$).

In addition, no significant differences were observed in terms of the genotype or allele frequency distributions of MTHFR A1298C polymorphism between the case group and control group (all P s > 0.05) (Table 3).

Multiple logistic regression analysis

In the multiple logistic regression analysis, the MTR A2756G, MTR RA66G, and MTHFR C667T polymorphisms were used as independent variables, and NSCL/P was used as the dependent variable. The results revealed that the MTR A2756G (OR = 0.270, 95% CI = 0.106–0.689, $p = 0.006$), MTR RA66G (OR = 0.159, 95% CI = 0.069–0.368, $p < 0.001$), and MTHFR C667T (OR = 0.343, 95% CI = 0.139–0.844,

$p = 0.020$) polymorphisms might increase the risk of NSCL/P (Table 4).

MTHFR C677T and A1298C haplotype analysis

Haplotype analysis was performed with SHEsis software, and the haplotypes with frequencies below 3% were ignored regarding the MTHFR at different loci. The haplotype analysis demonstrated that the CA haplotype frequency was significantly lower in the case group than in the control group ($\chi^2 = 5.907$, $p = 0.015$), whereas the TA haplotype frequency was significantly greater in the case group ($\chi^2 = 10.201$, $p = 0.001$; Table 5).

Interactions of the MTR, MTRR, and MTHFR genotypes and the NSCL/P risk

The four SNPs of MTR, MTRR, and MTHFR were incorporated as variables into the GMDR model according to the NSCL/P or health control status. The result revealed that the two-, three-, and four-dimensional model combinations were statistically significant. The optimal models were the two- and four-dimensional models, which had prediction accuracies of 75.73% ($p = 0.001$) and 77.21% ($p = 0.001$) and the best cross-validation consistencies of 10/10 and 10/10, respectively. These results indicated that the MTR, MTRR, and MTHFR SNPs exhibited interaction effects on NSCL/P (Table 6).

Discussion

Extensive studies have explored the relationships of gene polymorphisms related to the folic acid/homocysteine metabolic pathway with NSCL/P, and the results have revealed that the relationships of genetic mutations with NSCL/P susceptibility vary between different populations (Birnbau et al., 2009; Bohmer et al., 2013; Wang et al., 2013; Borges et al., 2015). Regarding the findings of the present study, the MTR A2756G, MTRR A66G, and MTHFR C677T gene polymorphisms, but not the MTHFR A1298C (glutamate-alanine) polymorphism in exon 7, may increase the risk of NSCL/P among the Chinese population, and these findings were further confirmed by logistic regression analysis.

TABLE 5. HAPLOTYPE ANALYSES OF MTHFR C677T AND MTHFR A1298C POLYMORPHISMS

Haplotype	Frequency		χ^2	p	OR (95% CI)
	Case	Control			
CA	122.00 (0.415)	132.52 (0.514)	5.907	0.015	0.658 (0.470–0.923)
TA	77.00 (0.262)	38.48 (0.149)	10.201	0.001	2.001 (1.301–3.077)
TC	95.00 (0.323)	84.52 (0.328)	0.036	0.849	0.966 (0.676–1.381)

TABLE 6. INTERACTIONS OF *MTR*, *MTRR*, AND *MTHFR* AND NSCL/P RISK IN GENERALIZED MULTIFACTOR DIMENSIONALITY REDUCTION

Dimensional model	Combinations	Cross-validation consistency	Prediction accuracy (%)	p
1	<i>MTHFR</i> C677T	9/10	63.66	0.055
2	<i>MTRR</i> A66G/ <i>MTHFR</i> C677T	10/10	75.73	0.001
3	<i>MTHFR</i> A1298C/ <i>MTRR</i> A66G/ <i>MTHFR</i> C677T	5/10	73.74	0.001
4	<i>MTHFR</i> A1298C/ <i>MTRR</i> A2756G/ <i>MTRR</i> A66G/ <i>MTHFR</i> C677T	10/10	77.21	0.001

These three SNPs can alter homocysteine levels and reduce enzymatic activity, which may lead to DNA damage associated with folate deficiency that may further contribute to the development of NSCL/P (Lopez-Cortes *et al.*, 2013; Wang *et al.*, 2013). More specifically, *MTR*A2756G (rs1805087) might result in an Asp919Gly amino acid substitution that has been demonstrated to contribute to alterations in the homocysteine and folate levels (Diwakar *et al.*, 2008; Weiner *et al.*, 2012). The *MTRR* enzyme catalyzes methylcobalamin regeneration and plays roles in maintaining the activation of the *MTR*. The *MTRR* polymorphism at nucleotide 66 (A-to-G) may result in variant enzyme activity with lowered affinity for *MTR* (Han *et al.*, 2012).

MTHFR C677T causes the substitution of alanine to valine and reduces enzyme activity, and the *MTHFR* 677T allele might increase the risk of NSCL/P in Asian populations, including Chinese populations (Nan *et al.*, 2014; Zhao *et al.*, 2014). Furthermore, *MTHFR* 677T homozygotes are involved in NSCL/P, and 677CT heterozygosity is a minor risk factor, whereas *MTHFR* A1298C poses no risk for NSCL/P with any combination of alleles among Indian population (Ali *et al.*, 2009). Partially consistent with our study results, the heterozygous genotypes of *MTHFR* 677AT, *MTR* 2756AG, and *MTRR* 66AG may be associated with increased risks of NSCL/P among Western Ukrainian inhabitants, especially regarding the risk of mothers delivering NSCL/P infants (Chorna *et al.*, 2011).

However, conflicting results regarding the *MTR* A2756G polymorphism and the risk of NSCL/P have been reported among Chinese and Indian populations; thus, future studies among Chinese populations are needed (Guo *et al.*, 2009).

Three SNPs (i.e., *MTR* rs6428977, rs12060264, and *MTRR* rs7730643), but not *MTHFR*, exhibit nominally significant associations with NSCL/P based on a transmission disequilibrium test of 806 NSCL/P trios among a Chinese population (Bohmer *et al.*, 2013). In addition, a significant association of the CA haplotype (i.e., the C allele of rs1801133 and the A allele of rs2274976) with NSCL/P has been observed and may be driven by the strong individual effect of the rs2274976 polymorphism. Moreover, the combination of *MTHFR* rs2274976, *MTHFD1* rs2236225, and *SLC19A1* rs1051266 best predicted the maternal risk for NSCL/P among a Brazilian population (Bufalino *et al.*, 2010).

The allelic combination of *MTHFR* rs1801131 and rs1801133 has been found to be associated with haplotype A-T overtransmission in the affected NSCL/P offspring among a Brazilian population (Mostowska *et al.*, 2012; de Aguiar *et al.*, 2015). The aforementioned information indicates the important role of gene–gene interactions and haplotype analyses in predicting NSCL/P risk among Chinese popula-

tions. Another significant finding suggested that the CA haplotype may serve as a protective factor against NSCL/P, while the TA haplotype acted as a risk factor, and the *MTR*, *MTRR*, and *MTHFR* SNPs exhibited interactive effects regarding NSCL/P, particularly in the two- and four-dimensional models.

A recent study indicated that the interaction between the *MTR* and *BHMT* genes plays a vital role in NSCL/P pathogenesis among Chinese populations (Jin *et al.*, 2015). Interestingly, significant differences were observed in the allele frequencies and haplotype analyses of rs4077829 and rs10802565 in *MTR* between the NSCL/P and control groups, but these differences were not significant after correction with 10,000 permutations. Polymorphisms related to alterations in folate metabolism were not found to be involved in NSCL/P among a Chinese population (Jiang *et al.*, 2014).

There are some limitations to the present study. First, the limited sample size of this study may have biased the associations of the *MTR* A2756G, *MTRR* A66G, and *MTHFR* C677T gene polymorphisms with the NSCL/P risk. Second, this study did not consider the possibility of linkage disequilibrium between SNP–SNP interactions. Third, gene–gene or gene–environment interactions may have influenced the results. These limitations could be overcome with more specific studies with larger samples and with more advanced molecular genetics technology.

In summary, these preliminary findings revealed that the *MTR* A2756G, *MTRR* A66G, and *MTHFR* C677T gene polymorphisms, but not the *MTHFR* A1298C polymorphism, were associated with increased risks for NSCL/P in a Chinese population. Future studies will uncover additional mechanistic insights and potential gene–environment interactions of the *MTR* A2756G, *MTRR* A66G, and *MTHFR* C677T gene polymorphisms with NSCL/P.

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