

Original Article

MTHFD1 gene polymorphisms as risk factors involved in orofacial cleft: an independent case-control study and a meta-analysis

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Abstract: Background: Orofacial clefts (OFCs) were among the most familiar birth defects in the world, which had been reported to be influenced by the folic acid ingestion in pregnancy previously. Methylenetetrahydrofolate dehydrogenase1 (*MTHFD1*) gene was associated with the susceptibility of OFCs through a complex metabolism correlate with folic acid. The aim of our study was to evaluate the correlation of five single-nucleotide polymorphisms (SNPs) within *MTHFD1* related to the OFCs risk in a Chinese population. Methods: By the use of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), we genotyped 5 filtered SNPs (identified by Haploview 4.2 software with HapMap databases) on *MTHFD1* gene: 118913T>C, 31136A>G, 58893A>G, 1958G>A and 61869T>C of 216 subjects (108 OFCs cases and 108 healthy controls) from a Chinese population. The association between these SNPs and OFCs risk was investigated by student t-test, one-way analysis of variance (ANOVA) and chi-square test with GraphPad Prism 5.0 software. Furthermore, we also performed a meta-analysis of relevant studies to investigate the association between *MTHFD1* 1958G>A and the susceptibility of OFCs. Results: Through the genotyping, the AA genotype was found significantly correlated with the susceptibility of OFCs compared with other SNPs on *MTHFD1*, yielding an OR of 2.71 (95% CI = 1.12-6.58, $P = 0.025$) under the homozygous model and an OR of 2.37 (95% CI = 1.06-5.30, $P = 0.033$) under the recessive model. While other selected SNPs 118913T>C and 31136A>G were also associated with an increased OFC risk, the results were not statistically significant (all $P > 0.05$). However, the overall result of meta-analysis did not support the conclusion that the 1958G>A variant could be a genetic susceptible factor for OFCs (A allele vs. G allele: OR = 1.02, 95% CI = 0.85-1.23, AA vs. GG: OR = 1.06, 95% CI = 0.69-1.63, GA vs. GG: OR = 1.02, 95% CI = 0.81-1.27, AA vs. GG+GA: OR = 0.94, 95% CI = 0.61-1.46, AA+GA vs. GG: OR = 0.94, 95% CI = 0.74-1.19). Conclusions: The *MTHFD1* 1958G>A variant was significantly associated with the increased OFCs risk in Chinese population. However, this association was not supported by meta-analysis of all relevant studies. Further investigations about functional impact of this polymorphism were needed.

Keywords: Orofacial cleft, methylenetetrahydrofolate dehydrogenase (*MTHFD1*), polymorphism, folate metabolism

Introduction

Orofacial clefts (OFCs) are among the most familiar birth defects all over the world, which may leave a long distressing life for the patients [1]. OFCs could be classified in cleft lip with or without cleft palate (CLP), cleft palate only (CPO) and cleft lip only (CLO). Previous epidemiologic studies for OFCs suggested that the overall birth prevalence was around 0.1%-0.2%, but this value varies among different races and districts [2-5]. OFCs are chiefly suffered in Asian population, followed by Caucasian population while the risk in African

and American population is the lowest [6]. The risk of OFCs could be affected by both complex environmental and genetic factors [7]. Based on previous epidemiologic researches on this cohort, many environmental factors like folate supplementation and smoking were stated. Early in 1971, the study in twins by Hay et al. supported that the special environment in uterus is a robust risk factor within the craniofacial disorder [8]. Numerous researchers had conclusively supported that the ingestion of folic acid supplementation during pregnancy was probably an access to the precaution of several congenital anomalies [5], though these associ-

ations still remain controversial, with some others did not found the correlation [9]. On the other hand, it was hypothesized that some genes take part in the folate metabolism would be also correlated with the birth defects including OFCs. The observation of OFCs recurrence under inheritance act as substantial evidence, also suggesting the genetic factors might lead to OFCs. Further, piles of single-nucleotide polymorphisms (SNPs) were verified to result in birth defects occurrence or the manifestation of OFCs, and some gene-gene and gene-environment interactions were also observed to play a role in the aetiology.

Methylenetetrahydrofolate dehydrogenase1 (*MTHFD1*) gene is verified to be one of the genes involved in folate metabolism which locates on chromosome 14q23.3. *MTHFD1* is a nicotinamide adenine dinucleotide phosphate (NADP)-dependent trifunctional cytoplasmic enzyme (often referred to as “C1-THF synthase”) in three sequential reactions, and it plays an important role in the one-carbon (1C) derivatives of tetrahydrofolate, which could derive 10-formyltetrahydrofolate (10-FTH), 5, 10-methenyltetrahydrofolate cyclohydrolase (5, 10-methenylTHF cyclohydrolase), and 5,10-methylenetetrahydrofolate dehydrogenase (5,10-MTHFD) [10]. It had been reported that several SNPs on *MTHFD1* gene could influence the homocysteine and folic acid levels in serum [11, 12], and was further associated with several cancers, migraine, congenital anomalies like NTDs and congenital heart disease [13-15].

Though the *MTHFD1* gene had been world widely researched in the fields of OFCs, the findings were contradictory in the previous studies and the relative studies in Asian population still need more robust testimonies. Hence, in our study, we filtered 5 representative SNPs on *MTHFD1*: 18913T>C (rs8006686), 31136A>G (rs2357694), 58893A>G (rs8003567), 1958G>A (rs2236225) and 61869T>C (rs1256143) from the database of International HapMap Project (HapMap Data Rel 24/phasell Nov08, on NCBI B36 assembly, dbSNP b126) to clarify the association of the SNPs on *MTHFD1* with the OFCs risk in a Chinese population. Furthermore, considering the limitation of individual study with small sample size, we also performed a meta-analysis of the association between *MTHFD1* SNP 1958G>A and OFCs

susceptibility based on all the available public data, in order to reinforce the overall result.

Material & methods

Study population

The study we performed was consisted of 216 subjects in the protocol from April 2012 to May 2014, approved by the ethics review board of East Hospital (Shanghai, China). All the subjects recruited were filtered based on the characteristic of the neonate, the parents and their family history, regarding the level of OFCs and some possible influential factors which may lead to OFCs of the maternal. The subjects were comprised of 108 cases who were neonates OFCs sufferers from oral and maxillofacial surgery and other 108 normal controls randomly selected from the unrelated healthy neonates without family history of OFCs from inpatients of the gynecology and obstetrics department. We further classified the subjects in case group by the certainly diagnosis of their cleft sort in three subgroups: CLP, CPO and CLO (the characteristic details are showed in **Table 2**). For the entire specimens in our study, 5 ml peripheral blood samples were collected with EDTA vacutainer for next assays. Each specimen's data were available for following up. An informed consent granted by the institutional ethics committee of East Hospital must be signed by each volunteer (as for the neonates, the informed consent must be signed instead by their parents or guardians).

SNP selection

In this study, we select 16 SNPs on *MTHFD1* from the genome browser. The SNPs were from unrelated Chinese individuals in the public genotype database according to Chinese HapMap Consortium. The selection of the tag-SNPs was dependent on the pairwise option of the Haploview 4.2 software, the linkage disequilibrium (LD) pattern using HapMap genotype data and r^2 of 0.8 was selected as a threshold for the analyses [16, 17]. As a result, we filtered 5 SNPs as representatives on *MTHFD1*: 118913T>C, 31136A>G, 58893A>G, 1958G>A and 61869T>C. Through previous studies these SNPs had a high incidence and were supposed to result in amino acid change which would further vary the function of related enzymes [18]. The linkage disequilibrium plot

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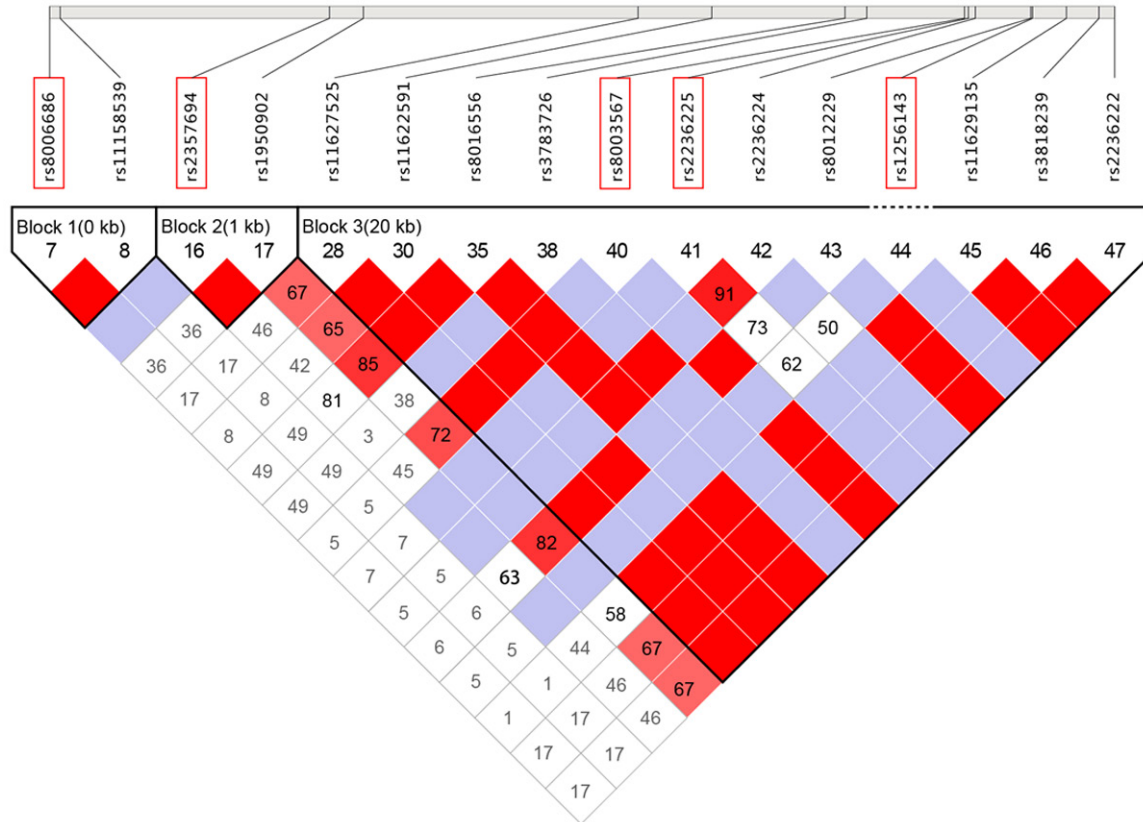


Figure 1. Linkage disequilibrium of the sixteen tag-SNPs.

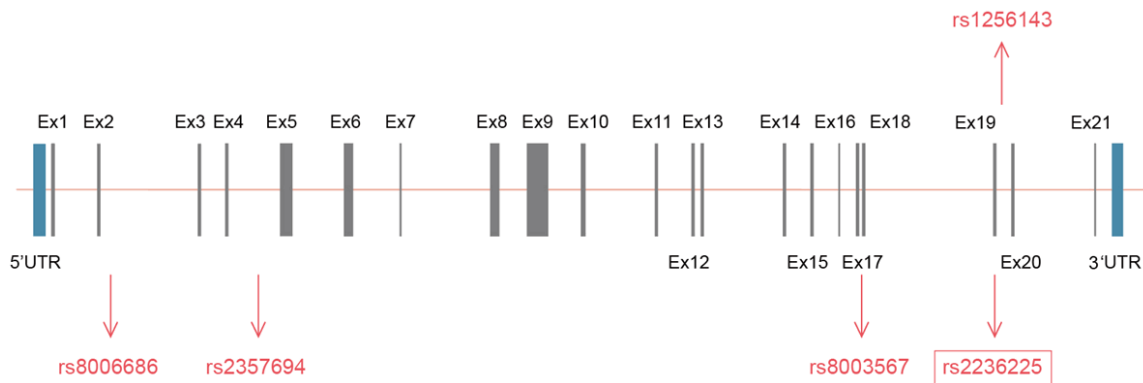


Figure 2. Genetic location of the selected five tag-SNPs.

of all the 16 SNPs on *MTHFD1* gene was presented by the Haploview 4.2 software and showed in **Figure 1**. The approximate locations of the selected SNPs on *MTHFD1* gene were as following: 118913T>C was on intron 2, the 31136A>G was on intron 4, the 58893A>G was on intron 17, the 1958G>A was on exon 19 and the 61869T>C was on intron 19 regions, as demonstrated in **Figure 2**.

Genotyping method

We isolated the whole genomic DNA from peripheral blood samples with the QIAamp DNA Blood Mini Kit (Qiagen Biosciences, Germany) as performed in Shaffer et al.'s assay [19]. For all polymorphisms, genotyping quality was verified by repeat analysis genotyping of at least 10% of the samples by the initial genotyping

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Table 1. Primers of *MTHFD1* gene polymorphisms for PCR amplification

SNP	Gen Pos	Alias name	Primers for PCR amplification	MAF
rs8006686	Intron 2	18913T>C	F: 5'-CCTGTATGGCTTAGTAGAA-3' R: 3'-AGGAAGGTCCATTAACAC-5'	0.237
rs2357694	Intron 4	31136A>G	F: 5'-TTGTTGTACTAGCCACTT-3' R: 3'-AGACGGGTGAGTTCGGAG-5'	0.176
rs8003567	Intron 17	58893A>G	F: 5'-TGTGACTGGGACGTTACTG-3' R: 3'-AAGGAAGGCTAAGGTTTA-5'	0.120
rs2236225	Exon 19	1958G>A	F: 5'-TCCTCCATCATTGCAGAC-3' R: 3'-TCCCAAACATCCAATCACAAA-5'	0.342
rs1256143	Intron 19	61869T>C	F: 5'-AGATGTACCTCACAAAAT-3' R: 3'-TTACTCATCTATCCCTTC-5'	0.110

SNP, single-nucleotide polymorphism; F, forward; and R, reverse.

Table 2. Comparison of patients and controls by selective characteristics

Clinical characteristics	Cases group	Controls group	P value
Total	108	108	
Age at conception (mean ± SD)	23.8±5.32	22.7±6.33	0.894
Maternal Smoking			
ever	67 (62.0%)	53 (49.1%)	0.055
never	41 (38.0%)	55 (50.9%)	
Alcohol drinking during pregnancy			
ever	77 (71.3%)	42 (38.9%)	< 0.001
never	31 (28.7%)	66 (61.1%)	
Folate supplementation			
no	76 (70.4%)	51 (47.2%)	< 0.001
yes	32 (29.6%)	57 (52.8%)	
Offspring sex			
male	60 (55.6%)	58 (53.7%)	0.785
female	48 (44.4%)	50 (46.3%)	
Type of cleft			
CLO	29 (26.9%)	-	
CLP	53 (49.1%)	-	
CPO	26 (24.0%)		

method or direct sequencing with > 99% agreement for the five polymorphisms [20]. The SNPs we assayed were selected through HapMap previously and had been studied for their mechanism. The genotyping of the 5 polymorphic sites on *MTHFD1*: 118913T>C, 31136A>G, 58893A>G, 1958G>A and 61869T>C were done by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. We used the Primer Premier 5 software designing the primers for the 5 selected SNPs as showed in **Table 1**. The cycle sequencing was performed according to the manufac-

turer's instructions. The primers and conditions of all assays are available upon request. And the gene positions, primers for PCR amplification and Minor Allele Frequency (MAF) were revealed in **Table 1**.

Statistical analysis

We managed the statistical analysis using the STATA 12.0 software. During the analysis, student t-test and chi-square (χ^2) test were applied to analyze the distribution of the several selected characteristics between OFCs sufferers and healthy controls. Differences of genotype frequencies were also assessed by the χ^2 test. We also implement a chi-square goodness-of-fit test by comparison of observed and expected genotype frequencies to verify the data quality in each subject class, exploring significant departure from Hardy-Weinberg equilibrium (HWE). The genotypes related OFCs risk was assessed by odds ratio (OR) and their corresponding respective confidence intervals 95% (CIs) value, for both combined and respective genotype.

A two-sided *P* value less than 0.05 was considered to be statistically significant for all analyses. Further, we conducted a meta-analysis of all relevant studies on the correlation between 1958G>A on *MTHFD1* and OFCs risk.

Results

Study characteristics

We recruited 216 specimens in this study as blood sample donators from East Hospital (Shanghai, China). The specimens were consisted of 108 OFCs sufferers (60 males and 48

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Table 3. Associations between five polymorphisms of *MTHFD1* gene and the risk of orofacial cleft

Genotype	Cases group (n = 258)	Controls group (n = 258)	OR (95% CI)	χ^2	P value
rs2357694 (31136A>G)					
AA	127	143	Ref.		
AG	81	67	1.36 (0.91-2.04)	2.263	0.133
GG	50	48	1.17 (0.74-1.86)	0.457	0.499
Recessive (AG+GG vs. AA)			1.28 (0.91-1.81)	1.989	0.159
Dominant (GG vs. AA+AG)			1.30 (0.89-1.91)	1.857	0.173
Allele (G allele vs. A allele)			1.17 (0.90-1.52)	1.413	0.235
rs2236225 (1958G>A)					
GG	122	146	Ref.		
GA	102	90	1.36 (0.94-1.97)	2.588	0.108
AA	34	22	1.85 (1.03-3.33)	4.282	0.039
Dominant (GA+AA vs. GG)			1.45 (1.03-2.06)	4.472	0.035
Recessive (AA vs. GG+GA)			1.63(0.92-2.87)	2.884	0.089
Allele (G allele vs. A allele)			1.40 (1.07-1.83)	6.043	0.014
rs8006686 (18913T>C)					
TT	158	186	Ref.		
TC	53	48	0.85 (0.55-1.31)	0.547	0.459
CC	47	24	0.98 (0.62-1.56)	0.005	0.942
Recessive (TC+CC vs. TT)			0.91 (0.64-1.29)	0.290	0.590
Dominant (CC vs. TT+TC)			1.03 (0.66-1.61)	0.013	0.909
Allele (C allele vs. T allele)			0.95 (0.73-1.25)	0.118	0.732

OR, odds ratio; CI, confidence interval.

females) and 108 healthy controls (58 males and 50 females) as two homogenous groups. Despite the difference among the gender, no significant difference was observed ($P = 0.785$). Furthermore, the cases group was classified into 29 CLO patients, 53 CLP patients and 26 CPO patients, as showed in **Table 2**. Besides, due to the possible factors that might have influence on the malformation during pregnancy, the detail characteristics of the neonates' mothers were also investigated. Through our previous filter, the difference of the age at conception of the mothers in the two groups was not significant ($P = 0.894$). For the concrete data showed the age at conception in the cases group was 23.8 (SD = 5.32) and in the control group was 22.7 (SD = 6.33). We also considered about the maternal smoking, alcohol drinking and the folate supplementation during pregnancy. The maternal smoking had a weak influence on the susceptibility of OFCs ($P = 0.055$) without significant difference, while both the alcohol drinking and folate supplementation during pregnancy showed a noteworthy significant difference between the two groups ($P < 0.001$), respectively. 71.3% moth-

ers in the cases group had ever drunk alcohol during pregnancy. Similarly, 70.4% mothers of the OFCs sufferers took no folate supplementation during the affected pregnancy, more than the mothers in the controls group. The data elucidated the mothers who drank alcohol and did not take appropriate folate supplementation during pregnancy were more likely to have a neonate suffered OFCs (The detailed data on the clinic characteristics are revealed in **Table 2**).

The associations between MTHFD1 and the risk of orofacial cleft

As indicated in **Table 3**, all the SNPs we assayed have positive effect with the OFCs susceptibility with their OR values over 1.0. Merely, only the association of *MTHFD1* 1958G>A on exon19 with the OFCs risk was statistically significant through our analysis, with a P value below 0.05. The *MTHFD1* 1958G>A genotype was more frequent in OFCs patients than in control groups. The significantly increased risk of OFCs was observed among the specimen with *MTHFD1* 1958G>A in allele model, homo-

Table 4. Characteristics of the included studies

First author	Year	Country	Racial descent	Sample size		Detection method	NTD cases			Healthy controls		
				Case	Control		GG	GA	AA	GG	GA	AA
Nan XR	2014	China	Asian	265	276	PCR-RFLP	113	127	25	100	141	35
Murthy J	2014	India	Asian	142	141	PCR-RFLP	21	83	38	42	68	31
de Aquino SN	2014	Brazil	Caucasian	181	478	TaqMan	64	91	26	181	211	86
Mostowska A	2010	Poland	Caucasian	174	176	PCR-RFLP	49	96	25	35	95	33
Bufalino A	2010	Brazil	Caucasian	106	184	PCR-RFLP	35	45	26	64	94	26
Palmieri A	2008	Italy	Caucasian	214	212	PCR-RFLP	56	118	40	39	110	63
Mills JL	2008	Ireland	Caucasian	962	1062	PCR-RFLP	161	327	152	290	570	202
Present study	2014	China	Asian	108	108	PCR-RFLP	31	56	21	40	58	10
Nan XR	2014	China	Asian	265	276	PCR-RFLP	113	127	25	100	141	35
Murthy J	2014	India	Asian	142	141	PCR-RFLP	21	83	38	42	68	31

zygote model and recessive model. Under the allele model, compared with the G allele genotype, the A allele genotype had a higher incidence in the OFCs sufferers (A allele vs. G allele: OR = 1.47, 95% CI = 1.00-2.16, $P = 0.050$). Similarly, a significant positive association was also noticed in recessive model (AA vs. GG+GA: OR = 2.37, 95% CI = 1.06-5.30, $P = 0.033$). And in homozygote model, a higher level of the significant was observed (AA vs. GG: OR = 2.71, 95% CI = 1.12-6.58, $P = 0.025$). Besides, no significant difference was noted in neither heterozygote model ($P = 0.469$) nor Dominant model ($P = 0.192$).

Meta-analysis for the association between MTHFD1 1958G>A and OFCs risk

We performed a meta-analysis to assess the association between *MTHFD1* 1958G>A and the susceptibility of OFCs. We recruited data from 8 eligible interrelated studies (including the present study) which were all selected through pre-specified search strategy [21-27]. In total, the studies provided the data of 4789 specimens. Among the 1040 specimens from Asian, 515 were OFCs cases and 525 were healthy controls. Besides, the other 3749 Caucasian specimens were consisted of 1637 OFCs cases and 2112 healthy controls. The detail characteristics of 8 included articles were revealed in **Table 4**. Due to the P -value of chi-squared test is below 0.05 and the I -square is over 50% in all the models we assayed, which means we observed significant heterogeneity among the studies we recruited. We performed the random-effects model in overall analysis. The data in our analysis were divided into two groups (Asian and Caucasian) by the ethnicity.

Through all the genetic models analysis, we found no significant association between the *MTHFD1* 1958G>A variant and the susceptibility of OFCs (A allele vs. G allele: OR = 1.02, 95% CI = 0.85-1.23, AA vs. GG: OR = 1.06, 95% CI = 0.69-1.63, GA vs. GG: OR = 1.02, 95% CI = 0.81-1.27, AA vs. GG+GA: OR = 0.94, 95% CI = 0.61-1.46, AA+GA vs. GG: OR = 0.94, 95% CI = 0.74-1.19). Though statistically significant of the AA genotype variant correlated with OFCs risk was perceived in several articles, the overall result still hold insignificant.

Discussion

In this study, we looked into the association between the polymorphism on *MTHFD1* gene and the risk of OFCs. We focused on 5 folic acid-related SNPs (118913T>C, 31136A>G, 58893A>G, 1958G>A and 61869T>C) on *MTHFD1* gene through our filter in HapMap database. Based on our analysis, only the SNP 1958G>A revealed a convincing association with the incidence of OFCs. Demonstrating that *MTHFD1* 1958G>A might be considered as a risk factor to OFCs, especially the AA genotype variant perform a high risk to the OFCs. However, the overall result of meta-analysis did not support the conclusion that the 1958G>A variant could be a genetic susceptible factor for OFCs.

It had been reported that the environmental factors such as the maternal smoking, alcohol drinking during pregnancy and vitamin supplementation all have an influence on OFCs susceptibility [21]. In our study, we analyzed the influence of smoking, alcohol drinking and folate supplementation during pregnancy on

the susceptibility of OFCs. Our results revealed that these environmental factors all associated with an increased susceptibility of OFCs. But this conclusion remains controversial. For instance, in James et al.'s study on a Dublin population, they failed to find the association between *MTHFD1* 1958G>A and other environment factors [28]. Besides the environment factors, genetic factors hold a big part of the pathogeny of OFCs. The *MTHFD1* gene was a folate related gene which might result in many kind of disease and had been widely researched in several different populations in previous studies. In our study, the minor allele frequency of *MTHFD1* 1958G>A was 45.37% in cases group and 36.11% in healthy group, close to the level of 34.19% from the public database in NCBI. The frequency of A allele in OFCs sufferers of us was equivalent with the study of Murthy et al. in an Indian population (46.1%) [29], but not as much as in the research of Rai et al., which was also conducted in India (55%) [30]. Demonstrating this SNP variant might be a risk factor for OFCs in Asian population. But this association was failed to be observed in some other countries like Polish [24] and Italy [22]. Nonetheless, since the participation of *MTHFD1* in folate metabolism, SNPs on *MTHFD1* were associated with many diseases which partly relied on the level of folic acid, such as neural tube defects (NTDs) and congenital heart defects [31].

The *MTHFD1* 1958G>A variant was verified to be a representative variant on *MTHFD1* gene which might change the glutamine (Gln) in 10-formyltetrahydrofolate synthetase region of the *MTHFD1* enzyme into arginine (Arg) [32]. In a previous investigation by Christensen et al., it was supported that this substitution could diminish enzyme stability, thermostability and inhibition of *de novo* purine biosynthesis [33]. Anyway, this shift of the amino acid might result in variation of folate levels and homocysteine status by disturbing the pathway of folate-mediated homocysteine [31, 34], and might further reduce the incidence of OFCs neonate. This interaction still requires functional study.

Our results from meta-analysis suggested that *MTHFD1* 1958G>A could not significantly associated with the OFCs risk in both Asian and Caucasian population, though several researches have reported strong association. Nevertheless, the correlation between this SNP

and the OFCs risk was stronger in Asian than in Caucasian, with the OR values in subtotal were all above 1 in Asian compared with the OR values in Caucasian were all below 1. Referable to the complex mechanism the reason was still controversial. It could be supposed that the significance of the risk might be locked in a more specific population, with a combination of non-genetic factors, environmental and social interacted genetic factors and polymorphism distributions.

In our study, the Chinese population we recruited was genetically homogeneous through our early filter. This reduced the probability of stratification and enhances the statistical power of our study's result. But on the other hand, several limitations should be acknowledged. The sample size of our study was relatively small for more convincing comparisons. The result of this study was not able to further extrapolate due to the bias which was resulted from races and regions related stratification. Regarding to the limitations above, more studies of this polymorphism in different areas and new research on the association between OFCs and other SNPs on *MTHFD1* are in need.

In conclusion, our study revealed a significant association between *MTHFD1* 1958G>A and the OFCs susceptibility in a Chinese population. However, the overall result of meta-analysis did not support the conclusion that the 1958G>A variant could be a genetic susceptible factor for OFCs. Nonetheless, further studies about the exact etiology mechanism of *MTHFD1* gene variant 1958G>A in the craniofacial development are recommended.

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Disclosure of conflict of interest

None.

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