Two SNPs rs1801133 and rs1801394 in folate pathway are associated with the risk of nonsyndromic cleft lip with or without cleft palate

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Abstract

Background Prenatal intake of folic acid is important for prevention of nonsyndromic cleft lip with or without cleft palate (NSCL/P). Associated genes in folate pathway are major enzymes of folic acid metabolism that is crucial for preventing birth defects. The present study aims to investigate the association between four single nucleotide polymorphisms (SNPs) in folate pathway genes and the risk of NSCL/P. Methods Comprehensive bioinformatics analysis was used to predict the functional pathogenicity of genetic variation. The PubMed, Embase database and Google Scholar were intensively searched by two researchers to ascertain all relevant studies. Stata 11.0 software was used to analyze the results. Subgroup analysis was carried out to assess the influence of genetic background. Sensitivity analysis, regression analysis and publication analysis were also conducted to enhance the strength of our results. Results It is estimated that the probability of two missense mutation rs1801133 in MTHFR and rs1801394 in MTRR are more likely to be damaging by bioinformatics analysis. A total of 34 publications were included in the present study analysis. Our results showed a significant association between rs1801133 and risk of NSCL/P in two genetic models: TT allele vs CC allele (OR=1.333 95%CI=1.062-1.674, P =0.013), and recessive model (OR=1.325 95%CI=1.075-1.634, P =0.008). A significant association between rs1801394 and the risk of NSCL/P in Asian (GG allele vs AA allele, OR=0.520 95%CI=0.321-0.841, P =0.008) was also observed. Meta-regression, sensitivity analysis, and publication bias analysis confirmed that the results of the present study were statistically significant. Conclusions The present study highlights two SNPs rs1801133 in MTHFR and rs1801394 in MTRR, associated with the increasing risk of NSCL/P. Further, larger studies should be performed to confirm these findings.
**Background**

Nonsyndromic cleft lip with or without cleft palate (NSCL/P) is one of the most common birth defects, characterized by craniofacial abnormality due to incomplete separation between the nasal and oral cavities [1]. NSCL/P can influence the quality of life by affecting communication problems and contributing to dysphagia. Cleft lip and palate occur in approximately one in 500-700 live births worldwide. This rate varies substantially across different ethnic groups and geographical areas (http://www.who.int/oral_health/publications/factsheet/en/). Cleft lip is a hereditary disease with polygenic inheritance, however, the underlying genetic cause and fundamental molecular mechanism of the disease remains still elusive.

Although folic acid and multivitamin supplementation in prescribed period of pregnancy has been indicated as an effective method to prevent the risk of oral facial cleft. The significance of genetic variants in folate pathway and folate metabolism involved in disease pathogenesis is not clear [2, 3]. Recently, many efforts have been made to find the genetic polymorphisms in folate pathway genes such as *MTHFR*, *MTRR*, *TCN2*, and *BHMT* and their susceptibility to cleft lip [4-10]. MTHFR plays an important role in primary circulation of folate and catalyzing the reaction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. The substrate and metabolites are important for DNA biosynthesis, cell division and process during development. Currently, there is no targeted therapy for NSCL/P patients carried with *MTHFR* mutations, while there are some reports on other genetic diseases. In 2017, Martinez Saguer et al. reported successful management of hereditary angioedema during pregnancy in a patient carried with heterozygous *MTHFR* mutation [11]. Lahiri et al. reported successful conservative treatment of myocardial infarction in a teenager carried with *MTHFR* mutation [12]. Recently, Al-Eitan et al. also showed that *MTHFR* polymorphism was associated with
treatment response in Jordanian population with epilepsy [13]. MTRR and TCN2 are essential in maintaining the levels of activated vitamin B12, and BHMT is vital for catalyzing betaine to dimethyl glycine (DMG), which are involved in remethylating homocysteine (Hcy) to methionine (Met) (Figure 1). The four genetic missense variations C677T in MTHFR (rs1801133), A66G in MTRR (rs1801394), C776G in TCN2 (rs1801198), and G716A in BHMT (rs3733890) have influence on protein function (Table 1), and have been reported to be associated with cleft lip. However, there are different conclusions regarding the influence of these SNPs in different populations [4-10, 14-36].

Here, therefore, we conduct a systematic review and performed an updated meta-analysis of all related studies published before April 2019, to provide more precise statistical results. Our study could provide basic data for exploring effective therapeutic strategies for NSCL/P.

Methods

2.1 Literature search

The present meta-analysis was conducted according to the recommendations of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis 2009 (PRISMA2009).

All published studies before April 2019 were searched using the PubMed database, Embase database, and Google Scholar with the following terms: “NSCL/P”, “cleft lip”, “SNP”, “polymorphism”, “genetic”, “variant”, “MTHFR”, “MTHFR C677T”, “rs1801133”, “MTRR”, “MTRR A66G”, “rs1801394”, “TCN2”, “TCN2 C776G”, “rs1801198”, “BHMT”, and “rs3733890”. Relevant references of related articles were also included.

2.2 Inclusion criteria

All studies were intensively reviewed by two researchers. Studies were included in the meta-analysis if they met the following criteria: (1) original observational study of human participants; (2) an association study between rs1801133 and/or rs1801194 and/or
rs1801398 and/or rs3733890 and NSCL/P; (3) case-control study or cohort study; (4) allele data were available; (5) largest sample size or sufficient useful data were included in duplicate publications from the same population; and (6) studies were published in English.

2.3 Quality score assessment

Study quality was assessed to guarantee the strength of results and conclusions. The Newcastle-Ottawa scale (NOS) score was calculated to assess the quality of studies. A maximum of nine scores, including selection, comparability and exposure items, could be awarded [37]. Any variances in comparison were decided by a third researcher.

2.4 Data extraction

The data were extracted independently by two researchers from all included studies using an integrated and standardized form. The following information was extracted: (1) first author name; (2) publication year; (3) population ethnicity; and (4) genotype distribution.

2.5 Computational and Statistical analysis

Polyphen2, SIFT, CADD, phyloP, and LRT were used for bioinformatics prediction. The Hardy-Weinberg equilibrium (HWE) test was calculated by the chi-square test. The distribution of allelic frequencies in controls were considered to deviate from HWE when \( P<0.05 \). STATA (11.0; Stata Corporation, College Station, TX, USA) software was used to calculate the results of meta-analysis. Heterogeneity across individual studies was assessed by Cochran’s \( Q \) test and \( I^2 \) statistic (\( P<0.10 \) and \( I^2 >50\% \) indicated evidence of heterogeneity). The fixed-effects model (Mantel-Haenszel method) was used to estimate the pooled OR when there was no evidence of the heterogeneity; otherwise, the random-effects model (DerSimonian and Laird method) was used. The odd ratios (ORs), with corresponding 95% confidence intervals (95% CIs), were calculated to assess the association between four genetic SNPs of folate pathway and NSCL/P risk. Four genetic
models were performed in the present meta-analysis. Using rs1801133 C>T as an example: (1) allele model, T allele vs. C allele; (2) dominant model, (CT+TT vs. CC); (3) recessive model, (TT vs. CT+CC); and (4) genotype model, (CT vs. CC; CT vs. TT). The same genetic models were performed for “rs1801394”, “rs1801198”, and “rs3733890”. A P value of $P<0.05$ was established as the significant threshold for each genetic model. Two subgroups, including Caucasian and Asian, based on ethnicity were analyzed to reduce the heterogeneity and influences from the genetic background. Meta-regression and one-way sensitivity analysis were also included in the meta-analysis. Egger’s regression test was used to evaluate the publication bias with quantitative analysis [38]. The trim and fill method was also used when publication bias exists.

Results

3.1 Study characteristics

Based on the search strategy, 926 publications were identified in the initial search. After evaluating the titles and abstracts, 801 publications were excluded, and 125 full-text publications were further reviewed. By applying the inclusion criteria, 34 publications were used for the final meta-analysis (Figure. 2). A total of 30 publications with 5517 cases and 7770 controls were included in the rs1801133 group; ten publications with 1767 cases and 2029 controls were included in the rs1801394 group; six publications with 1815 cases and 898 controls were included in the rs1801198 and five studies with 1253 cases and 1562 controls were included in the rs3733890 group. A total of seven studies in the control group (not excluded) were found to deviate from HWE. A total NOS assessment score of three or lower, four to six, and seven or greater were defined as low, medium and high quality, respectively. The main characteristics of the included publications are shown in Table 2.

3.2 Associations between the four SNPs of folate pathway gene and NSCL/P in
The overall population

The meta-analysis results showed that there was a significant association between rs1801133 and NSCL/P risk in two genetic models: TT allele vs CC allele (OR=1.333, 95%CI=1.062-1.674, \(P=0.013\)) and recessive model (OR=1.325, 95%CI=1.075-1.634, \(P=0.008\)) (Table 3, Figures 3-4). There was no statistically significant association between rs1801394 of the MTRR, rs1801198 of the TCN2, rs3733890 of the BHMT and NSCL/P risk in the overall population (Tables 4-6).

3.3 Subgroup analysis by ethnicity stratification

To assess the role of genetic background in the meta-analysis, we carried out a subgroup analysis, where the overall population was divided into two subgroups, (i) Asian and (ii) Caucasian. The subgroup analysis showed that there was significant association between rs1801394 and NSCL/P risk in Asian (GG allele vs AA allele, OR=0.520, 95%CI=0.321-0.841, \(P=0.008\)), but no associations in Caucasian (Table 4).

3.4 Meta-regression and influence analysis

The following covariates were considered for meta-regression: publication year, sample size and HWE in controls. The results showed that there was no influence for the four SNPs (\(P>0.05\)). To identify the degree to which a single study affected the overall OR estimates, one-way sensitivity analysis was performed by repeating the meta-analysis, sequentially excluding one study at a time. The results showed that no study was found to exert an excessive influence on the pooled effect.

3.5 Publication bias

There was publication bias for rs1801133 in the Asian population in genotype model CT vs CC (Table 3). Trim and fill results showed that the adjusted risk estimate unchanged, which confirmed that the results of present study are statistically robust.

Discussion
NSCL/P is a multifactorial disease caused by genetic and environmental factors. In previous years, various genomic susceptibility regions have been identified in association studies, linkage studies, family sequencing studies, and animal experiments suggesting that gene mutations influence the development of maxillofacial area. However, the underlying biological mechanisms remain unclear.

Folic acid is an important factor that influences the metabolism and the synthesis of nucleotides and amino acids. Previous studies have suggested that folic acid plays an important role in decreasing the risk of NSCL/P [2, 3]. Folic acid metabolism is a complex process and many genes are involved in the pathway, such as \textit{MTHFR}, \textit{MTRR}, \textit{TCN2}, and \textit{BHMT}. However, there are no consistent results regarding the association between the genetic variations of these genes and NSCL/P in different populations. To clarify these inconsistent results, we carried out the meta-analysis in this study.

The present meta-analysis results demonstrated a significant association between rs1801133 and NSCL/P risk in two genetic models: TT allele vs CC allele (OR=1.333 95%CI=1.062-1.674, \( P=0.013 \)) and recessive model (OR=1.325 95%CI=1.075-1.634, \( P=0.008 \)). There were a significant association found between rs1801394 and NSCL/P risk in Asian (GG allele vs AA allele, OR=0.520 95%CI=0.321-0.841, \( P=0.008 \)).

\textit{TCN2}, encode transcobalamin2, transports vitamin B12 to cells, have been reported to be associated with multiple diseases, such as cancer, Alzheimer and other congenital abnormalities [39-41]. In 2006, Martinelli \textit{et al.} found that the C776G in \textit{TCN2} was associated with risk of cleft lip, but subsequent studies didn’t get the significant results [10]. Similarly, the present study didn’t find the significant association between the C776G and NSCL/P.

\textit{BHMT}, a zinc dependent cytosolic enzyme, is important for homocysteine metabolism and methionine synthesis. In 2010, Mostowska \textit{et al.} first found that rs3733890 of the \textit{BHMT}
was associated with NSCL/P, and other studies also indicated its association with coronary
artery disease and neural tube defects [23]. In the present study, we found no evidence
showing rs3733890 playing any significant role [23]. We inferred several factors may
contribute to the result. First, we found a relative high value of heterogeneity among
studies, which cause a different distribution of genotype. Second, the number of included
studies and sample size are relatively small. So the subgroup analysis was not conducted
based on ethnicity.

MTRR plays a vital role in functional regeneration of methionine synthase, and it may be
associated with increasing the congenital heart disease risk [18]. But the meta-analysis
conducted by Zhang et al. in 2013 and Lei et al. in 2018 showed no association between
rs1801394 and the risk of NSCL/P [42, 43]. In the present study, we found a significant
association between rs1801394 and the NSCL/P risk in Asian, but no association in
Caucasian. The data from 1000 genomes and ExAC database was shown in Supplementary
table 1.

MTHFR is an important enzyme in homocysteine metabolism and C677T rs1801133 is one
of the most important functional polymorphisms. Prediction by bioinformatics tools
showed that the change of genetic variant will influence the protein function and
predispose to cause the disease (Table 1). However, the association between rs1801133
and the risk of NSCL/P is inconsistent. The allelic frequencies vary in different ethnic
groups and the minor allele frequency (MAF) of MTHFR rs1801133 in Asian are lower than
that in European and American, so it is very valuable to summarize and analyze by
systematic statistical methods. In the present study, we included 30 studies including
5517 cases and 7770 controls and found T allele can increase the risk of NSCL/P.
The strength of this meta-analysis is that it expands to a large number of related studies,
and the most updated publications were included. To guarantee the quality of the included
studies, a strict procedure for search strategy, literature inclusion, data extraction, and quality assessment was performed, which could avoid potential influences and increase the strength of the results. Meta-regression and sensitivity analysis were also performed to strengthen the conclusions. We confirmed the previous investigation by summarizing a larger number of closely related studies.

There are some limitations in the present meta-analysis. Firstly, studies published only in English were included in the meta-analysis, and studies published in other languages were excluded. Secondly, environmental factors also contribute to NSCL/P, and in the present study, non-genetic factors and other potential interactions, including environment-gene interactions, were limited. Additionally, some potential covariates (e.g., age, sex, folate etc) were not included in the analysis due to insufficient information.

Conclusion

In this study, we successfully identified two SNPs rs1801133 in MTHFR and rs1801394 in MTRR, associated with the increasing risk of NSCL/P. Further well-designed studies are required to established these findings.

Abbreviations

HWE: Hardy-Weinberg equilibrium

NOS: Newcastle-Ottawa scale

NSCL/P: Nonsyndromic cleft lip with or without cleft palate

OR: Odd ratio

SNP: Single nucleotide polymorphism

Declarations

Availability of data and materials

All the data in the present research is contained in this manuscript
**Ethics approval and consent to participate**

Not applicable.

**Consent to publish**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ Contributions**

All authors have contributed to the paper. Q.L, L.X, W.S, and S.F conceived and designed the study; Q.L and L.X, X.J, K.S, T.Z extracted and analyzed the data. Q.L, L.X, X.J, K.S, T.Z, W.S, and S.F drafted the manuscript. All authors revised and approved the final draft.
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References

1. Zucchero TM, Cooper ME, Maher BS, Daack-Hirsch S, Nepomuceno B, Ribeiro L, Caprau D, Christensen K, Suzuki Y, Machida J et al: Interferon regulatory factor 6 (IRF6) gene variants and the risk of isolated cleft lip or palate. N Engl J Med 2004, 351(8):769-780.

2. Tolarova M: Periconceptional supplementation with vitamins and folic acid to prevent recurrence of cleft lip. Lancet 1982, 2(8291):217.

3. Wehby GL, Murray JC: Folic acid and orofacial clefts: a review of the evidence. Oral Dis 2010, 16(1):11-19.

4. Wang W, Jiao XH, Wang XP, Sun XY, Dong C: MTR, MTRR, and MTHFR Gene Polymorphisms and Susceptibility to Nonsyndromic Cleft Lip With or Without Cleft Palate. Genet Test Mol Biomarkers 2016, 20(6):297-303.

5. Aslar D, Ozdiler E, Altug AT, Tastan H: Determination of Methylenetetrahydrofolate Reductase (MTHFR) gene polymorphism in Turkish patients with nonsyndromic cleft lip and palate. Int J Pediatr Otorhinolaryngol 2013, 77(7):1143-1146.

6. Bezerra JF, Oliveira GH, Soares CD, Cardoso ML, Ururahy MA, Neto FP, Lima-Neto LG, Luchessi AD, Silbiger VN, Fajardo CM et al: Genetic and non-genetic factors that increase the risk of non-syndromic cleft lip and/or palate development. Oral Dis 2015, 21(3):393-399.

7. Waltrick-Zambuzzi M, Tannure PN, Vieira TC, Antunes LS, Romano FL, Zambuzzi WF, Granjeiro JM, Kuchler EC: Genetic Variants in Folate and Cobalamin Metabolism-Related Genes in Nonsyndromic Cleft Lip and/or Palate. Braz Dent J 2015, 26(6):561-
8. Karas Kuzelicki N, Smid A, Kek T, Eberlinc A, Gersak K, Mlinaric-Rascan I: Common polymorphism in the glycine N-methyltransferase gene as a novel risk factor for cleft lip with or without cleft palate. Int J Oral Maxillofac Surg 2018, 47(11):1381-1388.

9. Hu Y, Chen E, Mu Y, Li J, Chen R: BHMT gene polymorphisms as risk factors for cleft lip and cleft palate in a Chinese population. Biomed Environ Sci 2011, 24(2):89-93.

10. Martinelli M, Scapoli L, Palmieri A, Pezzetti F, Baciliero U, Padula E, Carinci P, Morselli PG, Carinci F: Study of four genes belonging to the folate pathway: transcobalamin 2 is involved in the onset of non-syndromic cleft lip with or without cleft palate. Hum Mutat 2006, 27(3):294.

11. Martinez Saguer I, Escuriola Ettingshausen C: Successful management of hereditary angioedema during pregnancy in a patient with heterozygous MTHFR mutation. Ann Allergy Asthma Immunol 2017, 118(6):734-735.

12. Lahiri S, Cuglievan B, Gutierrez JL, Pefkarou A: Successful conservative treatment of myocardial infarction in a teenager with MTHFR mutation. Int J Cardiol Heart Vasc 2017, 15:24-25.

13. Al-Eitan LN, Al-Dalalah IM, Mustafa MM, Alghamdi MA, Elshammari AK, Khreisat WH, Aljamal HA. Effects of MTHFR and ABC2 gene polymorphisms on antiepileptic drug responsiveness in Jordanian epileptic patients. Pharmgenomics Pers Med 2019, 12: 87-95.

14. Brandalize AP, Bandinelli E, Borba JB, Felix TM, Roisenberg I, Schuler-Faccini L: Polymorphisms in genes MTHFR, MTR and MTRR are not risk factors for cleft lip/palate in South Brazil. Braz J Med Biol Res 2007, 40(6):787-791.

15. Jin LL, Chen EJ, Hou W, Liu XH, Hu Y: The Association between Folate Pathway Genes and Cleft Lip With or Without Cleft Palate in a Chinese Population. Biomed Environ Sci
Aşlar D, Taştan H: Prevalence of MTHFR, MTR and MTRR gene polymorphisms in Turkish patients with nonsyndromic cleft lip and palate, vol. 16; 2014.

Wyszynski DF, Diehl SR: Infant C677T mutation in MTHFR, maternal periconceptional vitamin use, and risk of nonsyndromic cleft lip. Am J Med Genet 2000, 92(1):79-80.

Verkleij-Hagoort A, Bliek J, Sayed-Tabatabaei F, Ursem N, Steegers E, Steegers-Theunissen R: Hyperhomocysteinemia and MTHFR polymorphisms in association with orofacial clefts and congenital heart defects: a meta-analysis. Am J Med Genet A 2007, 143A(9):952-960.

Sozen MA, Tolarova MM, Spritz RA: The common MTHFR C677T and A1298C variants are not associated with the risk of non-syndromic cleft lip/palate in northern Venezuela. J Genet Genomics 2009, 36(5):283-288.

Shotelersuk V, Ittiwut C, Siriwan P, Angspatt A: Maternal 677CT/1298AC genotype of the MTHFR gene as a risk factor for cleft lip. J Med Genet 2003, 40(5):e64.

Rafik A, Rachad L, Kone AS, Nadifi S: MTHFR C677T polymorphism and risk of nonsyndromic cleft lip with or without cleft palate in the Moroccan population. Appl Clin Genet 2019, 12:51-54.

Murthy J, Gurramkonda VB, Karthik N, Lakkakula BV: MTHFR C677T and A1298C polymorphisms and risk of nonsyndromic orofacial clefts in a south Indian population. Int J Pediatr Otorhinolaryngol 2014, 78(2):339-342.

Mostowska A, Hozyasz KK, Wojcicki P, Dzieglewska M, Jagodzinski PP: Associations of folate and choline metabolism gene polymorphisms with orofacial clefts. J Med Genet 2010, 47(12):809-815.

Mills JL, Molloy AM, Parle-McDermott A, Troendle JF, Brody LC, Conley MR, Cox C, Pangilinan F, Orr DJ, Earley M et al: Folate-related gene polymorphisms as risk
factors for cleft lip and cleft palate. Birth Defects Res A Clin Mol Teratol 2008, 82(9):636-643.

25. Marini NJ, Yang W, Asrani K, Witte JS, Rine J, Lammer EJ, Shaw GM: Sequence variation in folate pathway genes and risks of human cleft lip with or without cleft palate. Am J Med Genet A 2016, 170(11):2777-2787.

26. Little J, Gilmour M, Mossey PA, Fitzpatrick D, Cardy A, Clayton-Smith J, Hill A, Duthie SJ, Fryer AE, Molloy AM et al: Folate and clefts of the lip and palate--a U.K.-based case-control study: Part II: Biochemical and genetic analysis. Cleft Palate Craniofac J 2008, 45(4):428-438.

27. Kumari P, Ali A, Sukla KK, Singh SK, Raman R: Lower incidence of nonsyndromic cleft lip with or without cleft palate in females: is homocysteine a factor? J Biosci 2013, 38(1):21-26.

28. Jiang C, Yin N, Zhao Z, Wu D, Wang Y, Li H, Song T: Lack of Association Between MTHFR, MTR, MTRR, and TCN2 Genes and Nonsyndromic CL+/-P in a Chinese Population: Case-Control Study and Meta-Analysis. Cleft Palate Craniofac J 2015, 52(5):579-587.

29. Han Y, Pan Y, Du Y, Tong N, Wang M, Zhang Z, Wan L, Wang L: Methylenetetrahydrofolate reductase C677T and A1298C polymorphisms and nonsyndromic orofacial clefts susceptibility in a southern Chinese population. DNA Cell Biol 2011, 30(12):1063-1068.

30. Gaspar DA, Pavanello RC, Zatt M, Passos-Bueno MR, Andre M, Steman S, Wyszynski DF, Matioli SR: Role of the C677T polymorphism at the MTHFR gene on risk to nonsyndromic cleft lip with/without cleft palate: results from a case-control study in Brazil. Am J Med Genet 1999, 87(2):197-199.

31. Estandia-Ortega B, Velazquez-Aragon JA, Alcantara-Ortigoza MA, Reyna-Fabian ME,
Villagomez-Martinez S, Gonzalez-Del Angel A: 5,10-Methylenetetrahydrofolate reductase single nucleotide polymorphisms and gene-environment interaction analysis in non-syndromic cleft lip/palate. Eur J Oral Sci 2014, 122(2):109-113.

32. Ebadifar A, KhorramKhorshid HR, Kamali K, Salehi Zeinabadi M, Khoshbakht T, Ameli N: Maternal Supplementary Folate Intake, Methylenetetrahydrofolate Reductase (MTHFR) C677T and A1298C Polymorphisms and the Risk of Orofacial Cleft in Iranian Children. Avicenna J Med Biotechnol 2015, 7(2):80-84.

33. Chevrier C, Perret C, Bahuau M, Zhu H, Nelva A, Herman C, Francannet C, Robert-Gnansia E, Finnell RH, Cordier S: Fetal and maternal MTHFR C677T genotype, maternal folate intake and the risk of nonsyndromic oral clefts. Am J Med Genet A 2007, 143A(3):248-257.

34. Ali A, Singh SK, Raman R: MTHFR 677TT alone and IRF6 820GG together with MTHFR 677CT, but not MTHFR A1298C, are risks for nonsyndromic cleft lip with or without cleft palate in an Indian population. Genet Test Mol Biomarkers 2009, 13(3):355-360.

35. Abdollahi-Fakhim S, Asghari Estiar M, Varghaei P, Alizadeh Sharafi M, Sakhinia M, Sakhinia E: Common Mutations of the Methylenetetrahydrofolate Reductase (MTHFR) Gene in Non-Syndromic Cleft Lips and Palates Children in North-West of Iran. Iran J Otorhinolaryngol 2015, 27(78):7-14.

36. Murthy J: Genetic variant in MTRR A66G, but not MTR A2756G, is associated with risk of non-syndromic cleft lip and palate in Indian population. J Oral Maxillofac Surg Med Pathol 2015.

37. Stang A: Critical evaluation of the Newcastle-Ottawa scale for the assessment of the quality of nonrandomized studies in meta-analyses. Eur J Epidemiol 2010, 25(9):603-605.

38. Egger M, Davey Smith G, Schneider M, Minder C: Bias in meta-analysis detected by a
simple, graphical test. BMJ 1997, 315(7109):629-634.

39. Martinelli M, Scapoli L, Mattei G, Ugolini G, Montroni I, Zattoni D, Rosati G, Solmi R: A candidate gene study of one-carbon metabolism pathway genes and colorectal cancer risk. Br J Nutr 2013, 109(6):984-989.

40. Oussalah A, Levy J, Filhine-Tresarrieu P, Namour F, Gueant JL: Association of TCN2 rs1801198 c.776G>C polymorphism with markers of one-carbon metabolism and related diseases: a systematic review and meta-analysis of genetic association studies. Am J Clin Nutr 2017, 106(4):1142-1156.

41. Pawlik P, Mostowska A, Lianeri M, Sajdak S, Kedzia H, Jagodzinski PP: Folate and choline metabolism gene variants in relation to ovarian cancer risk in the Polish population. Mol Biol Rep 2012, 39(5):5553-5560.

42. Zhang T, Lou J, Zhong R, Wu J, Zou L, Sun Y, Lu X, Liu L, Miao X, Xiong G: Genetic variants in the folate pathway and the risk of neural tube defects: a meta-analysis of the published literature. PLoS One 2013, 8(4):e59570.

43. Lei W, Xia Y, Wu Y, Fu G, Ren A: Associations Between MTR A2756G, MTRR A66G, and TCN2 C776G Polymorphisms and Risk of Nonsyndromic Cleft Lip With or Without Cleft Palate: A Meta-Analysis. Genet Test Mol Biomarkers 2018, 22(8):465-473.

Tables

Table 1 Information of four SNPs in the present study

| SNP    | Gene | Codon | Polyphen2 score | Polyphen2 prediction | SIFT score | SIFT prediction |
|--------|------|-------|-----------------|----------------------|------------|----------------|
| rs1801133 | MTHFR | C677T | 0.998           | probability damaging | 0.027      | damage         | 25. |
| rs1801394 | MTRR  | A66G  | 1               | probability damaging | 0.064      | tolerable      | 23. |
| rs1801198 | TCN2  | C776G | 0.315           | benign               | 0.09       | tolerable      | 18. |
| rs3733890 | BHMT  | G716A | 0.064           | benign               | 0.218      | tolerable      | 21. |
Table 2 Characteristics of included studies about associations between four SNPs of folate pathway gene and NSCL/P

| Study                              | Year | Ethnicity | Genotype in case | P-value  |
|------------------------------------|------|-----------|------------------|----------|
| **MTHFR rs1801133**                |      |           |                  |          |
| Shaw et al.                         | 1998 | Caucasian | Total            |          |
| Tolarova et al.                    | 1998 | Caucasian | 310              | 143 127 40 | 0.334 |
| Gaspar et al.                      | 1999 | Caucasian | 111              | 43  49 19 | 0.392 |
| Wyszynski et al.                   | 2000 | Caucasian | 77               | 30  39  8 | 0.357 |
| Martinelli et al.                  | 2001 | Caucasian | 259              | 114 109 36 | 0.349 |
| Grunert et al.                     | 2002 | Caucasian | 64               | 22  30 12 | 0.422 |
| Shotelersuk et al.                 | 2003 | Asian     | 66               | 34  26 6 | 0.288 |
| van Rooij et al.                   | 2003 | Caucasian | 66               | 34  26 6 | 0.288 |
| Gaspar et al.                      | 2004 | Caucasian | 66               | 34  26 6 | 0.288 |
| Pezzetti et al.                    | 2004 | Caucasian | 66               | 34  26 6 | 0.288 |
| Brandalize et al.                  | 2007 | Caucasian | 66               | 34  26 6 | 0.288 |
| Chevrier et al.                    | 2008 | Caucasian | 66               | 34  26 6 | 0.288 |
| Little et al.                      | 2008 | Caucasian | 66               | 34  26 6 | 0.288 |
| Mills et al.                       | 2008 | Caucasian | 66               | 34  26 6 | 0.288 |
| Ali et al.                         | 2009 | Asian     | 66               | 34  26 6 | 0.288 |
| Sozen et al.                       | 2009 | Caucasian | 66               | 34  26 6 | 0.288 |
| Mostowska et al.                   | 2010 | Caucasian | 66               | 34  26 6 | 0.288 |
| Ebadifar et al.                    | 2010 | Asian     | 66               | 34  26 6 | 0.288 |
| Han et al.                         | 2011 | Asian     | 66               | 34  26 6 | 0.288 |
| Aslar et al.                       | 2013 | Caucasian | 66               | 34  26 6 | 0.288 |
| Kumari et al.                      | 2013 | Asian     | 66               | 34  26 6 | 0.288 |
| Murthy et al.                      | 2014 | Asian     | 66               | 34  26 6 | 0.288 |
| Estandia-Ortega et al.             | 2014 | Caucasian | 66               | 34  26 6 | 0.288 |
| Jiang et al.                       | 2015 | Asian     | 66               | 34  26 6 | 0.288 |
| Bezerra et al.                     | 2015 | Caucasian | 66               | 34  26 6 | 0.288 |
| Abdollahi-Fakhim et al.            | 2015 | Asian     | 66               | 34  26 6 | 0.288 |
| Wang et al.                        | 2016 | Asian     | 66               | 34  26 6 | 0.288 |
| Marini et al.                      | 2016 | Caucasian | 66               | 34  26 6 | 0.288 |
| Karas Kuzelicki et al.             | 2018 | Caucasian | 66               | 34  26 6 | 0.288 |
| Rafik et al.                       | 2019 | Africa    | 66               | 34  26 6 | 0.288 |
| **MTRR rs1801394**                 |      |           |                  |          |
| Brandalize et al.                  | 2007 | Caucasian | Total            |          |
| Mostowska et al.                   | 2010 | Caucasian | 144              | 36  69 9 | 0.382 |
| Aslar et al.                       | 2014 | Caucasian | 164              | 31  81 52 | 0.564 |
| Wu et al.                          | 2014 | Caucasian | 140              | 31  81 52 | 0.564 |
| Wang et al.                        | 2016 | African   | 140              | 31  81 52 | 0.564 |
| Marini et al.                      | 2016 | Caucasian | 140              | 31  81 52 | 0.564 |
| Karas Kuzelicki et al.             | 2018 | Caucasian | 140              | 31  81 52 | 0.564 |
| **TCN 2 rs1801198**                |      |           |                  |          |
| Martinelli et al.                  | 2006 | Caucasian | Total            |          |
| Mills et al.                       | 2008 | Caucasian | 218              | 85 110 23 | 0.358 |
| Mostowska et al.                   | 2010 | Caucasian | 218              | 85 110 23 | 0.358 |
| Jin et al.                         | 2015 | Asian     | 218              | 85 110 23 | 0.358 |
| Wang et al.                        | 2016 | African   | 218              | 85 110 23 | 0.358 |
| Marini et al.                      | 2016 | Caucasian | 218              | 85 110 23 | 0.358 |
| **BHMT rs3733890**                 |      |           |                  |          |
| Mostowska et al.                   | 2010 | Caucasian | Total            |          |
| Hu et al.                          | 2011 | Asian     | 140              | 31  81 52 | 0.564 |
| Jin et al.                         | 2015 | Asian     | 140              | 31  81 52 | 0.564 |
| Marini et al.                      | 2016 | Caucasian | 140              | 31  81 52 | 0.564 |
| Karas Kuzelicki et al.             | 2018 | Caucasian | 140              | 31  81 52 | 0.564 |

Note: AF: allele frequency of minor allele HWE Hardy-Weinberg equilibrium.
### Table 3 Associations between rs1801133 of MTHFR and NSCL/P

| Genetic model       | T allele vs C allele | TT vs CC         | CT vs CC         | Dominant model | Recessive model |
|---------------------|---------------------|-----------------|-----------------|---------------|----------------|
|                     | Overall             | Caucasian       | Asian           | Overall       | Caucasian     | Asian       |
|                     | $i^2$ (%)           | $P$ for heterogeneity | OR (95% CI)     | $P$ value     | $P$ value     | $P$ value |
| T allele vs C allele| Overall             | 73.1            | 0               | 1.111 (0.992-1.244) | 0.069         |             |
|                     | Caucasian           | 58.5            | 0.001           | 1.085 (0.976-1.206) | 0.131         |             |
|                     | Asian               | 19.1            | 0               | 1.244 (0.961-1.611) | 0.098         |             |
| TT vs CC            | Overall             | 62.6            | 0               | 1.333 (1.062-1.674) | 0.013         |             |
|                     | Caucasian           | 56.2            | 0.001           | 1.230 (0.976-1.551) | 0.080         |             |
|                     | Asian               | 70.5            | 0.001           | 1.701 (0.949-3.049) | 0.075         |             |
| CT vs CC            | Overall             | 57.6            | 0               | 1.026 (0.901-1.169) | 0.696         |             |
|                     | Caucasian           | 40.3            | 0.033           | 1.027 (0.904-1.166) | 0.686         |             |
|                     | Asian               | 62.4            | 0.006           | 1.081 (0.821-1.422) | 0.580         |             |
| Dominant model      | Overall             | 66.2            | 0               | 1.075 (0.936-1.234) | 0.305         |             |
|                     | Caucasian           | 52.8            | 0.003           | 1.067 (0.932-1.223) | 0.347         |             |
|                     | Asian               | 67.9            | 0.002           | 1.172 (0.883-1.557) | 0.272         |             |
| Recessive model     | Overall             | 62.5            | 0               | 1.325 (1.075-1.634) | 0.008         |             |
|                     | Caucasian           | 40.9            | 0.030           | 1.190 (0.989-1.433) | 0.066         |             |
|                     | Asian               | 79.1            | 0               | 1.737 (0.940-3.210) | 0.078         |             |

Note: Italic indicates statistically significance

### Table 4 Associations between rs1801394 of MTRR and NSCL/P
## Table 5 Associations between rs1801198 of TCN2 and NSCL/P

| Genetic model     | $i^2$ (%) | $P$ for heterogeneity | OR (95% CI)         | $P$ value |
|-------------------|-----------|------------------------|---------------------|-----------|
| C allele vs G allele |          |                        |                     |           |
| Overall           | 36.0      | 0.120                  | 0.986 (0.895-1.085) | 0.766     |
| Caucasian         | 0         | 0.938                  | 1.037 (0.928-1.158) | 0.520     |
| Asian             | 77.7      | 0.011                  | 0.843 (0.694-1.023) | 0.084     |
| GG vs AA          |           |                        |                     |           |
| Overall           | 30.7      | 0.172                  | 0.977 (0.781-1.223) | 0.841     |
| Caucasian         | 0         | 0.816                  | 1.176 (0.909-1.520) | 0.217     |
| Asian             | 0         | 0.820                  | 0.520 (0.321-0.841) | 0.008     |
| AG vs AA          |           |                        |                     |           |
| Overall           | 78.3      | 0                      | 0.879 (0.630-1.227) | 0.449     |
| Caucasian         | 31.9      | 0.184                  | 1.018 (0.813-1.275) | 0.877     |
| Asian             | 93.2      | 0                      | 0.644 (0.211-1.968) | 0.441     |
| Dominant model    |           |                        |                     |           |
| Overall           | 69.4      | 0.001                  | 0.921 (0.704-1.205) | 0.550     |
| Caucasian         | 0         | 0.514                  | 1.046 (0.886-1.236) | 0.595     |
| Asian             | 90.3      | 0                      | 0.746 (0.351-1.768) | 0.506     |
| Recessive model   |           |                        |                     |           |
| Overall           | 37.8      | 0.117                  | 1.032 (0.853-1.248) | 0.748     |
| Caucasian         | 45.1      | 0.090                  | 1.062 (0.858-1.315) | 0.581     |
| Asian             | 39.6      | 0.198                  | 0.922 (0.605-1.406) | 0.707     |

*Note: Italic indicates statistically significance*
| Genetic model         | $i^2$ (%) | $P$ for heterogeneity | OR (95% CI)       | $P$ value |
|----------------------|-----------|------------------------|-------------------|-----------|
| A allele vs G allele | 61.0      | 0.036                  | 0.994 (0.820-1.204) | 0.9       |
| AA vs GG             | 73.6      | 0.004                  | 0.993 (0.558-1.764) | 0.9       |
| GA vs GG             | 23.7      | 0.264                  | 0.966 (0.801-1.164) | 0.7       |
| Dominant model       | 33.2      | 0.200                  | 0.983 (0.812-1.190) | 0.8       |
| Recessive model      | 75.1      | 0.003                  | 1.002 (0.569-1.767) | 0.9       |

**Figures**

![Folate pathway](image)

**Figure 1**

Folate pathway.
926 publications searched in PubMed, Embase databases and Google scholar

801 were excluded based on reading titles and abstracts

125 full text reports were reviewed for inclusion

70 were excluded due to not fulfilling inclusion criteria (such as review articles, meta-analysis, family population, duplicate

55 publications fulfilled criteria

21 publications were filtered due to not provide sufficient information

34 publications were included in final meta-analysis

Figure 2
Study flow diagram.
| Study ID                      | OR (95% CI) | Weight |
|------------------------------|-------------|--------|
| Shaw et al. (1998)           | 0.89 (0.55, 1.43) | 4.89   |
| Tolarova et al. (1998)       | 2.54 (1.01, 6.41)  | 3.07   |
| Gaspar et al. (1999)         | 2.61 (0.78, 8.73)  | 2.26   |
| Wyszynski et al. (2000)      | 0.93 (0.56, 1.54)  | 4.75   |
| Martineau et al. (2001)      | 1.48 (0.60, 3.62)  | 3.16   |
| Grunert et al. (2002)        | 0.64 (0.24, 1.68)  | 2.91   |
| van Rooyen et al. (2003)     | 1.94 (0.52, 7.24)  | 2.02   |
| Gaspar et al. (2004)         | 0.80 (0.51, 1.27)  | 4.98   |
| Pezzetti et al. (2004)       | 1.89 (0.99, 3.64)  | 4.11   |
| Brandalize et al. (2007)     | 1.25 (0.56, 2.77)  | 3.52   |
| Chevrier et al. (2007)       | 0.52 (0.27, 0.99)  | 4.12   |
| Little et al. (2008)         | 0.83 (0.37, 1.87)  | 3.48   |
| Mills et al. (2008)          | 1.09 (0.77, 1.54)  | 5.45   |
| Sozen et al. (2009)          | 2.10 (0.83, 5.32)  | 3.05   |
| Mostowska et al. (2010)      | 1.02 (0.48, 2.17)  | 3.71   |
| Aslar et al. (2013)          | 11.35 (3.07, 41.87)| 2.04   |
| Estandia-Ortega et al. (2014)| 2.53 (1.47, 4.37)  | 4.59   |
| Bezerra et al. (2015)        | 0.69 (0.32, 1.50)  | 3.59   |
| Marini et al. (2016)         | 1.12 (0.71, 1.74)  | 5.02   |
| Karas Kuzelicki et al. (2018)| 1.36 (0.61, 3.04)  | 3.52   |
| Shotelersuk et al. (2003)    | 0.37 (0.02, 7.71)  | 0.51   |
| Ali et al. (2009)            | 4.30 (0.94, 19.66) | 1.65   |
| Ebadiifar et al. (2010)      | 4.12 (2.00, 8.49)  | 3.82   |
| Han et al. (2011)            | 1.94 (1.05, 3.59)  | 4.28   |
| Kumari et al. (2013)         | 3.34 (1.20, 9.29)  | 2.76   |
| Murthy et al. (2014)         | 0.15 (0.01, 2.88)  | 0.53   |
| Jiang et al. (2015)          | 0.71 (0.41, 1.23)  | 4.59   |
| Abdollahi-Fakhimi et al. (2015)| 0.81 (0.38, 1.72) | 3.69   |
| Wang et al. (2016)           | 2.77 (1.19, 6.41)  | 3.36   |
| Rafik et al. (2019)          | 0.10 (0.01, 1.65)  | 0.58   |
| Overall (I^2 = 62.6%, p = 0.000) | 1.33 (1.06, 1.67) | 100.00 |

NOTE: Weights are from random effects analysis

**Figure 3**

Forest plot for pooled ORs for the associations between TT vs CC model of rs1801133 and NSCL/P risk. Each square is proportional to the study-specific weight.
Figure 4

Forest plot for pooled ORs for the associations between recessive model of rs1801133 and NSCL/P risk. Each square is proportional to the study-specific weight.