

Single-nucleotide polymorphisms of methylenetetrahydrofolate reductase gene in a South Indian cohort with nonsyndromic cleft lip with or without palate

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Abstract

Objective: Clefts of the lip, with or without cleft palate and cleft palate only, collectively called as orofacial clefts (OFCs) are one of the most common congenital malformations with varying degrees of penetrance and phenotype expressions. The aim of this study was to investigate the association between methylenetetrahydrofolate reductase (MTHFR) cytosine-to-thymine (c. 677 C>T), adenine-to-cytosine (c.1298 A>C) single-nucleotide polymorphisms (SNPs) and South Indian patients with the nonsyndromic cleft lip with or without palate (NSCL ± P).

Methods: A cohort consisting of 25 cases of NSCL ± P and 18 controls from a South Indian cohort were included in this case-control study. Genetic analysis of c.677C>T and c.1298A C polymorphisms in the MTHFR gene was carried out using Sanger sequencing and analyzed from chromatogram profiles. Data interpretation was done using statistical software MedCalc Statistical Software version 16.2 and the Statistical Package for the Social Sciences (SPSS version 22.0).

Results: DNA sequence analysis of the MTHFR gene revealed c. 677C>T and c. 1298A>C polymorphisms in 16% and 76% of NSCL ± P cases, respectively. Heterozygous variant in MTHFR c. 1298A>C polymorphism was found to be a significant risk factor ($P = 0.0164$) for NSCL ± P in South Indian ethnic population. c.677C>T polymorphism, in particular, was apparently dormant overall in the study population. These results offer certain novelty in terms of the distinctive pattern in SNPs of genotypes observed in the study.

Conclusion: NSCL ± P is one of the most common and challenging congenital malformations with complex etiological basis. Common risk factors such as MTHFR SNPs, namely c.677C>T and c.1298A>C, are subjected to variations in terms of ethnic group, geographic region and micro/macro-environmental factors. Overall, our study has explored part of South Indian ethnic population and revealed a different and unique distribution of mutations in this sample population.

Keywords: c.1298A>C, c.677C>T, homocysteine-folate metabolism, methylenetetrahydrofolate reductase, nonsyndromic cleft lip with without palate, single-nucleotide polymorphisms

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INTRODUCTION

Clefts of the lip, with or without cleft palate and cleft palate only, collectively called as orofacial clefts (OFCs) are one of the most common congenital malformations with varying degrees of penetrance and phenotype expressions.^[1] The formation of craniofacial structures during embryonic development entails a complex series of molecular developmental pathways that program cell migration, growth, differentiation and apoptosis processes to occur in close coordination. Any disturbance in growth in these critical stages might result in an incomplete anatomical development of craniofacial structures and further causing OFCs.^[2] Approximately two-thirds of the cases are not accompanied by other anomalies and hence are called as nonsyndromic cleft lip with or without palate (NSCL ± P).^[3] Prevalence rates are subjected to ethnic group, geographic region and sex. North American Indians and Asians have the highest prevalence rates (1/500), while Caucasians have intermediate rates (1/1000) and Africans having the lowest prevalent rates (1/2500).^[1]

Methylenetetrahydrofolate dehydrogenase 1 and 5,10-methylenetetrahydrofolate reductase (MTHFR) genes encode enzymes involved in reactions of reduction and oxidation in folate/homocysteine (Hcy) metabolism. Methionine synthase (MTR) and MTR reductase are the methylating and reducing enzymes involved in the conversion of Hcy and circulatory form of folate (5-methyltetrahydrofolate) into intermediates for the continuation of the cycle and biosynthesis of purines and pyrimidines [Figure 1].^[4] Hence, the MTHFR gene expression plays a crucial role in the folate/Hcy metabolism

and influences various phenotypic expressions driven by these biochemical pathways. Although there is a clear interaction between genetic and environmental factors in the etiology of this complex defect, multiple correlation exists with factors such as maternal folate intake, genetic variations that influence Hcy and folate metabolism and other environmental risk factors such as maternal exposure to tobacco smoke, alcohol, poor nutrition, viral infection, medicinal drugs and teratogens.^[5-7] Confounding effects of nutrition, smoking, stress or drug abuse are also found to be associated with the prevalence of cleft cases. Maternal smoking during pregnancy has been linked consistently with increased risk of both cleft lip with or without palate and isolated cleft palate, with a population attributable risk as high as 20%.^[8]

In the studies carried out so far, maternal alcohol consumption and risk of cleft lip in the offspring have yielded contradictory findings with some studies ascertaining positive associations^[9,10] while others not.^[11,12] Findings of observational studies have linked multivitamin supplements in early pregnancy to decreased risk of OFCs, albeit assessments of dietary intake or biochemical measures of nutritional status are challenging and generally are not available in many impoverished populations with the highest risk of OFCs.^[2]

Here, our focus was on two single-nucleotide polymorphisms (SNPs) of MTHFR gene, i.e., NG013351.1: c.677C>T and NG013351.1:c.1298A>C gene, that are extensively studied in cleft cases. This polymorphism was reported to be located on the short arm of chromosome 1 (1p36.3, MIM 607093, GenBank ID 4524). Altogether,

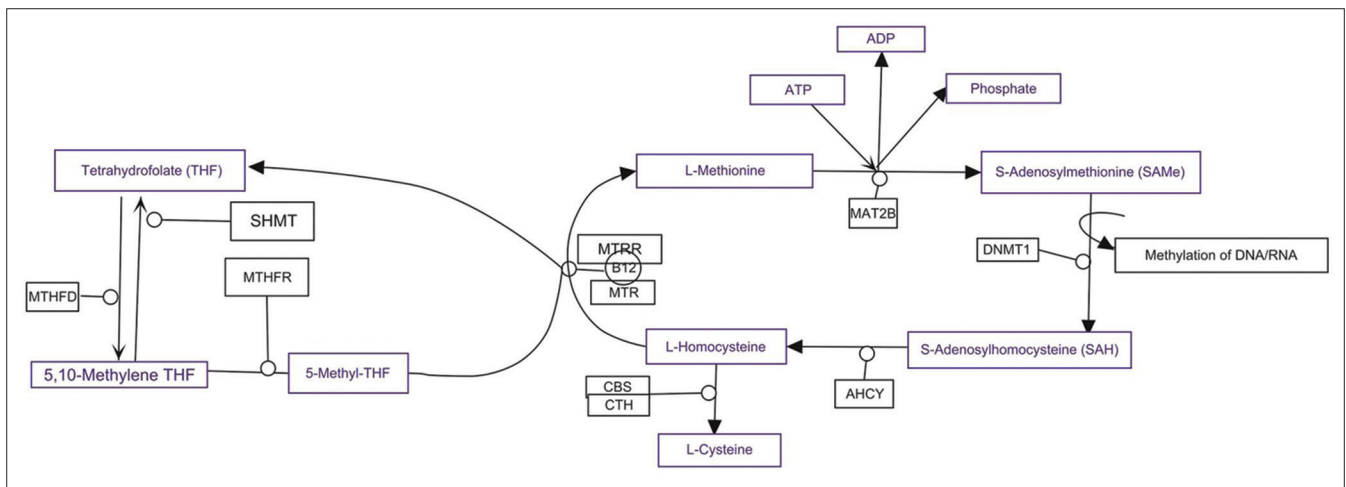


Figure 1: Homocysteine-folate biochemical pathway (27). SHMT: Serine hydroxymethyltransferase, MTHFD: Methylenetetrahydrofolate dehydrogenase, MTHFR: Methylenetetrahydrofolate reductase, MTRR: Methionine synthase reductase, MTR: Methionine synthase, CBS: Cystathionine β synthase, CTH: Cystathionine γ lyase, AHCY: Adenosylhomocysteinase, MAT2B: Methionine adenosyltransferase, DNMT1: DNA methyltransferase

MTHFR presents 10 polymorphisms, of which c. 677C>T and c. 1298A>C are the most studied, causing the greatest effect on enzymatic function and consequently leading to a high level of plasma Hcy. Exons 4 and 7 of MTHFR gene harbor c.677C>T and c. 1298A>C, respectively, and both of them are nonsynonymous with former resulting in alanine-to-valine substitution and latter providing for valine-to-glutamic acid change.^[13] A case–control study was carried out on a South Indian cohort using Sanger sequencing molecular technique. Significance and association of genetic variants in the study population were explored using chromatogram analysis, haplotype test and odds ratio (OR) statistical methods to compare controls and NSCLP cases.

METHODS

Patients

The study was approved by the Institutional Ethics Committee, Yenepoya University (Ethical Clearance Reference Number: YUEC127/26/06/13), following which patient consent was obtained from 25 cases and 18 controls from South Indian states of Karnataka and Kerala. Age- and sex-matched participants were enrolled from the pediatric and general ward of Yenepoya Medical College Hospital. Participants in the control group were the ones not affected with any kind of orofacial deformities, and there were ten males and eight females with an average age of 11.72 ± 8.45 years. All study participants were evaluated for individual organ system before the surgical procedure as per the protocol. There were no associated syndromes or defects in control group of patients as verified by both case record details, physical and other systemic diagnostic examinations. Systemic details such as respiratory, cardiac, ENT and skin were evaluated from the medical records department of the institution before the patients were posted for surgery of the clefts. Condition/diseases where Hcy levels were elevated such as congenital vascular diseases, neurodegenerative and psychiatric conditions were excluded in both study group and control group. These detailed clinical investigations were also assessed to rule out any deformities and medical illness for the administration of general anesthesia for the surgical intervention of the patients. The study group comprised participants having NSCL ± P with varying degrees of penetrance and phenotypic expressions. Out of a total of 25 cases (12 males and 13 females), there were 12 isolated cleft palate, 8 cleft lip and 5 cleft lip with palate cases with an average age of 11.84 ± 5.2 years.

Genetic analysis

For genetic analysis, 3–5 ml of peripheral blood sample was collected in a Vacutainer™ EDTA tube (Becton Dickinson, Franklin Lakes, NJ, USA). Genomic DNA was extracted

from whole blood using QIAamp DNA mini kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. For polymorphism analysis, the exons 4 and 8 of the MTHFR gene were polymerase chain reaction (PCR) amplified using the two sets of primer sequences: Forward primer 5'-CTGATCCTCAACTGCACCGT-3' and Reverse primer 5' CAGTCAGCCTCTGACAAGCA-3'. The final PCR products were sequenced using Sanger sequencing (ABI 3730 DNA analyzer, Thermo Fisher Scientific). The sequences were aligned to a genomic reference sequence, NG_013351.1, in the NCBI GenBank database to detect mutations, namely c.677C>T/rs1801133 and c.1298A>C/rs1801131. FASTA sequences were extracted from the chromatogram files and analyzed for homozygous (TT, CC), heterozygous (CT, AC) and absent (CC, AA) data for c.677C>T and c.1298A>C polymorphisms, respectively.

Statistical analysis

Genotype and allele frequencies were calculated by genotype count^[14] followed with test sample prediction by the assumption of Hardy–Weinberg (HW) equilibrium,^[15] and both the control and cases were found to be in HW equilibrium. Statistical significance was accepted at $P < 0.05$. The ORs and 95% confidence interval were calculated and were assessed between c.677C>T, c.1298A>C polymorphisms and NSCL ± P. OR tabulation was generated using MedCalc Statistical Software version 16.2. Haplotype analysis was carried out for four haplotypes CA, TA, CC and TC in c.677C>T and c. 1298A>C polymorphisms and the frequency values were compared and tested for significance ($P < 0.05$) between controls and cases using Chi-square test (Statistical Package for the Social Sciences (SPSS) version 22.0 for Windows; SPSS, Chicago, IL, USA).

RESULTS

Upon analyzing sequencing chromatogram profiles of samples [Figure 2], heterozygous, homozygous and absent data for both the polymorphisms were compiled. Results revealed that all three genotypes were occurring at the same rate in both control and NSCL ± P cases for c.677C>T polymorphism [Table 1]. Moreover, heterozygous and homozygous mutations for c.677C>T were accounting for mere 16% of individuals in both control and cleft cases, respectively, implying their low expression in the study population [Table 1]. With respect to c.1298A>C polymorphism, 76% of cases had mutation as compared to 42% in controls and 60% of cases had heterozygous change as compared to 24% of controls. Fifty-eight percent of controls did not exhibit c.1298A>C polymorphism as compared to 24% of cases [Table 1].

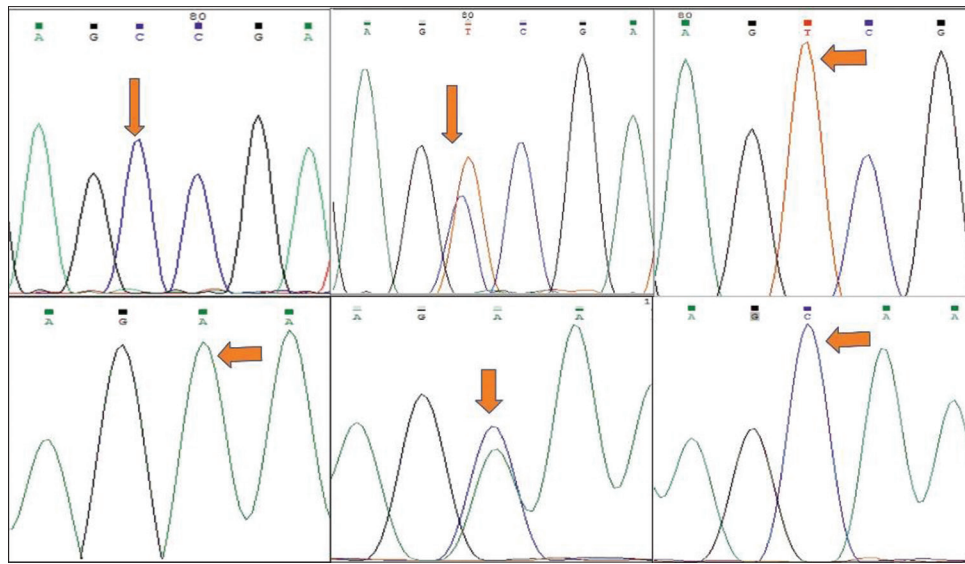


Figure 2: Representative sequencing chromatogram profiles of controls/cases. Single-nucleotide polymorphisms marked by arrows; (a) Methylene tetrahydrofolate reductase c.677C>T absent, (b) Methylene tetrahydrofolate reductase c.677C>T heterozygous mutation, (c) MTHFR c.677C>T homozygous mutation, (d) Methylene tetrahydrofolate reductase c.1298A>C absent, (e) Methylene tetrahydrofolate reductase c.1298A>C heterozygous mutation, (f) Methylene tetrahydrofolate reductase c.1298A>C homozygous mutation

Table 1: Distribution of Genotype and Allele frequencies of MTHFR c. 677C >T and c. 1298A >C polymorphisms among NSCL±P cases and controls

Variable	Control (n=18) (%)	NSCL±P (n=25) (%)	OR (%) CI	P
Genotype				
c. 677C>T				
CC	15 (0.83)	21 (0.84)	1	-
CT	2 (0.11)	4 (0.16)	1.43 (0.2-8.8)	0.7
TT	1 (0.05)	0	0.24 (0.009-6.3)	0.39
Allele C	32 (0.88)	46 (0.92)	1	-
Allele T	4 (0.11)	4 (0.08)	0.95 (0.2-4.5)	0.95
c. 1298A>C				
AA	10 (0.55)	6 (0.24)	1	-
AC	4 (0.22)	15 (0.6)	6.25 (1.3-27.9)	0.016*
CC	4 (0.22)	4 (0.16)	1.66 (0.2-9.2)	0.55
Allele A	24 (0.66)	27 (0.54)	1	-
Allele C	12 (0.33)	23 (0.46)	0.48 (0.19-1.23)	0.12

CI, Confidence interval; OR, Odds ratio

OR statistical test showed that there was a statistically significant difference between genotype frequency of heterozygous expression of MTHFR c.1298A>C polymorphism in NSCLP cases compared with controls ($P = 0.016$) [Table 1]. The c.1298A>C mutation was hence observed to be a significant risk factor for NSCL ± P condition in our study population. Allele frequencies of both C, T of c.677C>T and A, C of c. 1298A>C did not show any association with NSCL ± P cases. Haplotype analysis was carried out to identify the occurrence of any two nucleotide variations simultaneously in c.677C>T and c.1298A>C. Among four haplotypes derived from two polymorphisms, CA haplotype showed a significant difference ($P = 0.0293$) in their frequencies between controls and NSCL ± P cases [Table 2].

DISCUSSION

The results on the relationship between SNP patterns and nonsyndromic cleft lip and palate have been often inconsistent and are attributed to multiple interacting genes, with their own variable tendency to be expressed.^[16,17] Although physiologic/pharmacologic and genetic studies in animal models and human populations have identified several candidate genes and pathways that regulate various factors concerning transcription, growth, cell signaling and detoxification metabolisms, genetic variations resulted by SNPs and their association with NSCL ± P is still controversial.^[18,19] A number of meta-analysis studies and extensive research work have come out with a conclusion that c.677C>T is a major SNP that perturbs the efficiency of MTHFR enzyme so that inverse relation between production of circulating folate and balancing serum Hcy levels is curtailed.^[20,21] Moreover, it is supported by the fact that the mutation lies in exon 4 of the catalytic N-terminal domain of MTHFR, while c.1298A>C polymorphism is located in exon 7 of the regulatory C-terminal domain.^[22] Both c.677C>T and c.1298A>C reduce respective enzyme activity, with former getting thermolabile and more predominant with its effect.^[23] Nonetheless, there is no clear-cut conclusion on the significance of these genetic alterations affecting the metabolism of folate/Hcy, which in turn might have a causative role to play in the embryogenesis and molecular development to finally affect craniofacial anomalies like NSCL ± P. In a study on Brazilian population, c.677C>T genotype was revealed to be significantly modulating the folate-Hcy relationship

Table 2: Haplotype analysis of c. 677C>T and c. 1298A>C polymorphisms in Controls and NSCL±P cases

Haplotype	Frequency in control group (%)	Frequency in NSCL±P group ^a (%)	P
CA	7 (0.38)	3 (0.12)	0.0293*
TA	3 (0.16)	3 (0.12)	0.9816
CC	8 (0.44)	17 (0.68)	0.1747
TC	0	2 (0.08)	0.2321
Total	18	25	

^aHaplotype order is MTHFR 677C: T e 1298 A: C

in the studied sample with a mild folate deficiency.^[24] A case–control triad study in The Netherlands revealed that a detrimental effect of low periconceptional folate intake on the risk of giving birth to a cleft lip/palate child was more pronounced in mothers with the MTHFR c. 677C>T or MTHFR c.1298A>C polymorphisms.^[25] A multiethnicity-based study was carried out with 169 NSCLP probands from 3 different geographical regions, namely Bangladesh, Iran and Tibet. While there was exclusive evidence for Italian population regarding association for polymorphisms in MTHFR, TCN2 and CBS genes, no evidence of association with NSCL/P was observed for the genes with the remaining two populations.^[26,27] In a study on North Indian population, MTHFR A1298C did not show any risk in any combination of alleles such as interferon regulatory factor CIRF6C>T and MTHFR c. 677C>T. IRF6C>T too formed a minor risk. However, combined genotypes IRF6C>T and MTHFR c. 677C>T together and c. 677C>T alone formed a greater risk for NSCL ± P. Furthermore, there was a significant association of elevated Hcy levels with c. 677C>T variant in cleft cases.^[28,29]

From the heterogeneous rate of incidence of this developmental anomaly, it could be well understood that the genetic, epidemiological and environmental factors responsible for NSCL ± P could be often varied and contradicted. Our study results deviated from general observation that c. 677C>T being the most commonly occurring SNP in NSCLP cases. There was no difference observed in c. 677C>T polymorphism between controls and cases. Eighty-four percent of controls and cases did not show C to T change indicating seemingly dormant nature of c. 677C>T polymorphism in the sample population. However, c. 1298A>C polymorphism was observed in 76% of cases and heterozygote genotype frequency (60%) was significantly linked with NSCL ± P cases. Furthermore, haplotype analysis revealed a significant difference between controls and cases for CA haplotype which represents wild-type genes in both the polymorphisms. A meta-analysis study comprising 4 population groups from China, 2 from North India and 1 each from Turkey and Thailand studied an

association between MTHFR c.677C>T and c.1298A>C polymorphisms and NSCL ± P risk in Asians and reported that c.677C>T was significantly associated with NSCL ± P risk while c.1298A>C polymorphism was not related to children's or mother's NSCL ± P susceptibility under any of the genetic models, even in the subgroup analysis by geographical location.^[30] The apparent indifference with c.1298A>C and NSCL ± P risk was evident since there have been no studies yet on South Indian cohort in particular to SNPs under study. However, an investigation on South Indian population on the role of polymorphic variants at 1p22 (near ABCA4 gene) revealed a contributory role in the pathophysiology and risk of NSCL/P, while the variations in 1p22 (near MAFB gene) did not underlie the pathophysiology of NSCL±P.^[31] A preliminary study has shown a significant elevation of serum Hcy levels in NSCL±P cases in a South Indian cohort as compared to controls.^[32] Along with their role in predisposing neural tube defects, MTHFR mutations are found to be associated with increased risk of recurrent pregnancy loss, colon cancer, diabetes and genetic syndromes. Studies on the correlation of MTHFR mutations, blood Hcy concentration and cardiovascular disease have mixed results. Some studies show a correlation of the MTHFR mutations with blood Hcy levels and cardiovascular disease risk, while others show no correlation. MTHFR genotyping is often used to identify the risk factors for venous thrombosis and atherosclerosis. However, based on the mixed results of research studies and several factors that influence Hcy levels, the American College of Medical Genetics and Genomics' 2013 practice guideline is that MTHFR genotyping has minimal clinical utility.^[34]

CONCLUSION

Differential expression of genotypes under study in NSCLP cases can be considered as a distinctive evidence with regard to high prevalence of c.1298A>C polymorphism in comparison to c.677C>T polymorphism in South Indian cohort. The present findings may be helpful in correlating MTHFR gene expression and its association with the Hcy-folate metabolic pathway. It will be useful for genetic diagnosis, carrier detection and genetic counseling to families with a similar disease condition. Furthermore, studies with larger and diverse sample size in South India would offer a better perspective in understanding the etiological basis of NSCL±P.

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Conflicts of interest

There are no conflicts of interest.

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