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RESEARCH ARTICLE

CO-RELATION BETWEEN A1298C AND C677T MTHFR GENE POLYMORPHISM WITH THE SUSCEPTIBILITY OF NON SYNDROMIC CLEFT LIP AND PALATE

Dr. Ashim Roy, Dr. Rahul Maheshwari, Dr. Soumyarup Sen, Dr. Alisha Dey and *Dr. Rajashekar

#3Srinivasa Complex, Hosahalli road, Hunasemarana Halli, Bengaluru -562157, India

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ABSTRACT

Aims and objectives: To determine which among the two MTHFR gene polymorphisms viz , C677T and A1298C has greater contribution to non syndromic cleft palate and lip (nsCL/P).
Materials and methods: The study group consisted of the DNA samples of 20 patients with nsCL/P and 20 unrelated controls. DNA sample was subjected to Polymerase Chain Reaction and products are digested with restriction enzymes. The digested products were subjected to electrophoresis to ascertain the presence of MTHFR gene variants (A1298C AND C677T) in the samples.
Results: The cleft cases were evaluated for having polymorphism in the MTHFR C677T gene for 3 genetic variants namely C/C (homozygous wild type), T/T (homozygous mutant) and C/T (heterozygous mutant).The odds ratio for T/T variant of C677T was 3.3 and for the C/T variant was 2.3, indicating that presence of both T/T variant and C/T variant of C677T was associated with presence of nsCL/P. For the polymorphism in the MTHFR A1298C gene namely A/A (homozygous wild type), C/C (homozygous mutant) and A/C (heterozygous mutant).The odds ratio was 1.5 for the C/C variant and 2.1 for the A/C variant indicating that both the mutated variants of A1298C gene were associated with nsCL/P.
Conclusion: Both the polymorphisms of the MTHFR gene namely MTHFR C677T and MTHFR A1298C may be implicated in the etiopathogenesis of Non Syndromic Cleft Lip/Palate in our population, with the mutations in the MTHFR C677T gene showing a stronger association with nsCL/P.

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INTRODUCTION

Cleft Lip and Palate (CL/P) is one of the most common congenital defects affecting the or ofacial region, caused by improper embryonic fusion of the palatal shelves with each other or, the palatal shelves with the median nasal process. CL/P can be classified as Syndromic (sCLP) and Non Syndromic (nsCLP). Both forms of CL/P are characterized by a strong genetic components (Ngantung and Yusuf, 2014). In recent years, great efforts have been made to identify the genes involved in the causation of nsCL/P to provide a better insight into the pathogenic mechanism that leads to this malformation. Recent research has found an association between the methyl netetrahydrofolate reductasegene (MTHFR) with nsCL/P. This gene is thought to play an important role in folate metabolism. Two common single nucleotide polymorphisms (SNPs) namely, C677T (C>T, alanine to valine substitution) and A1298C (A>C, glutamate to alanine) in the MTHFR gene have been linked to neural tube defects, congenital heart disease and orofacial clefts. Substitution of a single nucleotide causes change in the amino acid sequence, leading to synthesis of thermo-labile MTHFR enzyme with reduced activity, elevated plasma homocysteine levels and low folate synthesis (Ebadifar et al., 2015). Though a co –relation between MTHFR C677T and MTHFR A1298C gene polymorphism and nsCL/P has been established in other populations, there has been no literature to substantiate their association in our local population. Hence the objective of this study was to assess the co-relation between MTHFR C677T and MTHFR A1298C gene polymorphisms and nsCL/P patients in the local population.

Corresponding author: **Dr. Rajashekar**
#3Srinivasa complex, Hosahalli road, Hunasemarana Halli, Bengaluru -562157, India.

MATERIALS AND METHODS

The sample consisted of 20 patients with non-syndromic cleft lip and palate and 20 unrelated controls selected from the out-patient Department of Orthodontics and Dentofacial Orthopaedics, Dayananda Sagar College of Dental Sciences, Bangalore, INDIA. Both the test group and the control group were selected on the basis of a thorough case history and clinical examination.

The source genetic material for testing the MTHFR gene variants (A1298C AND C677T**)** in both groups was derived from a 2ml venous blood sample for all 40 patients after taking informed consent for participation in the study. The inclusion and exclusion criteria for choosing the

Patients for both groups was as follows

Inclusion Criteria

This included only those patients who had the presence of non-syndromic cleft lip/ palate which was established after ruling out the genetic predisposition through clinical examination, detailed case history and pedigree analysis.

Exclusion criteria

- Cleft lip/palate associated with any history of developmental disabilities, including learning disabilities and attention deficits, hearing impairment, and speech deficits or abnormalities which may be an indication of an underlying syndromic genetic disorder.
- Family history of orofacial clefts and related conditions, including any additional major associated anomalies (e.g., cardiac defects and eye and brain anomalies).
- History of maternal illnesses.
- History of use of medication during gestation (e.g., anticonvulsants and retinoic acid derivatives), excessive vitamin intake during pregnancy.
- Tobacco use, smoking during pregnancy.
- Ethanol intake during pregnancy.

Materials used in the Present Study

Taq Polymerase (0.5U/1µl): To carry on the polymerization reaction. (PCR).

TRIS. HCl buffer: It is a basic buffer solution which maintains pH at 7.5.

Proteinase K: To add proteolytic enzymes to digest all the proteins.

Sodium Dodecyl Sulphate (SDS 10%): It is a detergent which binds to cell membrane which causes cell lysis and releases cell components.

dNTP (2.5µl): It is used to supply the necessary nucleotides (A, T, C, G) for the reaction to occur.

Primers: Short preexisting polynucleotide chain to which new deoxyribonucleotides can be added by DNA polymerase. (Primer - 1.5µl)

Distilled Water: To make up for the volume so that the reaction is carried out.

Agarose gel: It is a polymer which has minute pores and enables separation of DNA fragments based on the size.

Ethidium bromide: It is a fluorescent dye which binds to the DNA and illuminates with U.V.transilluminator on gel documentation.

Restriction endonuclease Hinf1: (1µl) It is the specific restriction enzyme which was added to the PCR amplified DNA for its digestion or restriction for variant C677T**.**

Restriction endonuclease MboII: (1µl) It is the specific restriction enzyme which was added to the PCR amplified DNA for its digestion or restriction for variant A1298C**.**

Genotype analysis: Genotyping of all subjects was based on PCR amplification of DNA fragments .the primer sequence for each of gene variants and conditions of the PCR are mentioned below

A1298C /C677T Primer mix5'-TCTTCAT CCC TCGC CTT GA AC-3' 128 bp 5'-AGGACGGTGCGGTGAGAGTG-3' 5'-CTTCTACCTGAAGAGCAAGTC3' 264 bp5'-CATG TC C ACA GCATGGAG-3'.

After the polymerase chain reaction (PCR) test was done for amplification of the region of interest from the DNA samples. Restriction digestion was then performed on the amplified product using the restriction enzymes, separately for each of the variants. The digested PCR products were separated into channels on a 1.5% agarose gel containing ethidium bromide in an electrophoretic chamber. A U.V. transilluminator was used to see the specific bands of base pairs of the digested PCR products.

RESULTS

Methylenetetrahydrofolate reductase gene (MTHFR) has two common single nucleotide polymorphisms (SNPs) viz C677T (C>T, alanine to valine substitution) and A1298C (A>C, glutamate to alanine substitution). (Pramodkumar and Shobha Tandon, 2001) Substitution of a single nucleotide causes change in the amino acid sequence, which leads to synthesis of thermo labile MTHFR enzyme which results in reduced activity, elevated plasma homocysteine levels and low folate synthesis#. According to the presence or absence of mutant T allele in the C677T region, cases are genotyped as T/T if homozygous for mutant allele, C/T if heterozygous for C677T allele and C/C if homozygous for "wild type" allele. Similarly, cases are genotyped for the A1298C polymorphism as C/C homozygous (mutant genotype), A/C heterozygous and A/A "wild type" homozygous. Ngantung (Ngantung and Yusuf, 2014) For the C677T gene the odds ratio at 95% CI in C/C Variant for controls was 4.4 indicating that the CC variant was associated with the absence of nsCL/P. Similarly odds ratio for T /T Variant was 3.3 and that for the C/T variant in the cleft cases was 2.3 indicating that the chances of finding nsCL/P with the T/T variant was higher as compared to the C/T variant of C677T gene. (Table-I)

Table 1. Odds ratio (OR) for C677T polymorphisms for MTHFR gene (a. OR for Cases, b. OR for Controls)

Genotype of C677T	OR	95% CI		P-value
		Lower	Upper	
C/C – Variant	4.4[b]	1.8	10.9	<0.001*
T /T – Variant	3.3[a]	1.2	6.6	<0.001*
C/T – Variant	2.3[a]	0.7	7.8	0.24

Note- * - statistically significant

For the A1298C gene the odds ratio at 95% CI in A/A variant for controls was 2.2 indicating that the A/A variant was associated with the absence of nsCL/P. Similarly odds ratio for C/C- variant was 1.5 and that for the A/C variant in the

cleft cases was 2.1 indicating that the chances of finding ns CL/P with the A/C variant was higher than the C/C variant of A1298C gene. (Table-II)

Table II. Odds ratio (OR) for A1298C polymorphisms for MTHFR gene (a. OR for Cases, b. OR for Controls)

Genotype of A1298C	OR	95% CI		P-value
		Lower	Upper	
C/C – Variant	1.5[a]	0.5	2.8	0.10
A/C – Variant	2.1[a]	0.9	5.2	0.02*
A/A – Variant	2.2[b]	1.2	4.2	0.02*

Note- * - statistically significant

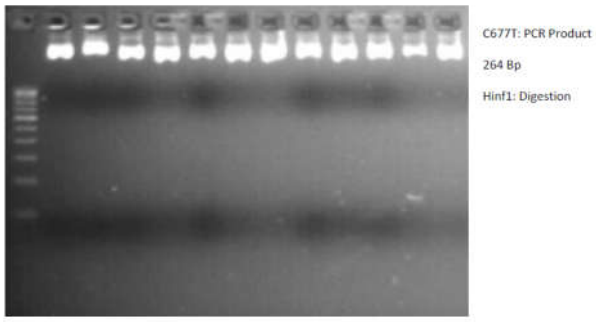

Fig. 1. Showing the amplified polymerase chain reaction product of C677T variant (264bp)

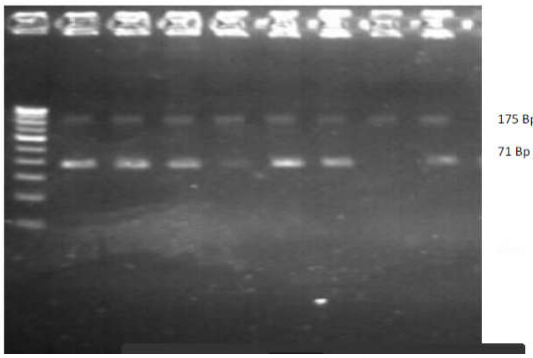

Fig. 2. Polymerase chain reaction of C677T variant after digestion with Hinfl enzyme

Restriction mapping for MTHFR C677T variant is shown in Fig 1 and 2. Amplification of polymerase chain reaction product of C677T showed 264 base pairs (bp). Digestion with restriction Hinfl enzyme gave two restriction site and two specific bands of 175 bp and 75 bp in 10 subjects with nsCL/P indicating that the presence of this variant contributed to the incidence of nsCL/P in our studied population. Restriction mapping for MTHFR A1298C variant is shown in fig 3 and 4.

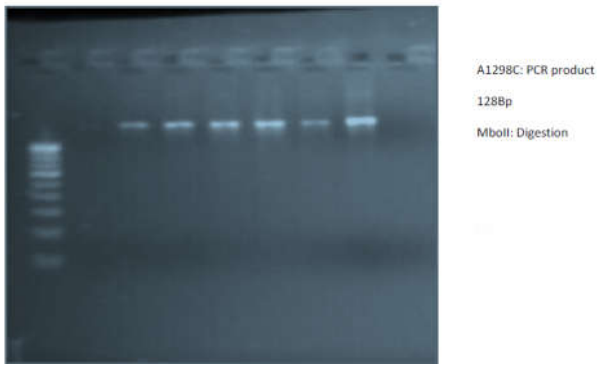

Fig. 3. showing the amplified polymerase chain reaction product of A1298C variant (128bp)

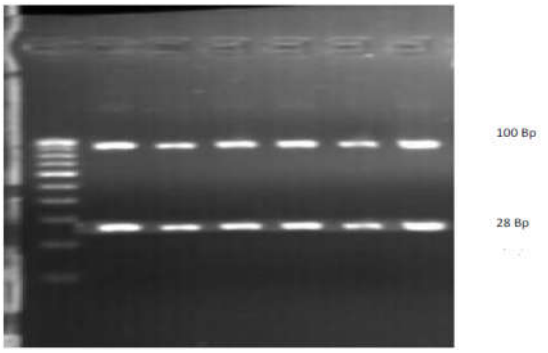

Figure 4. Polymerase chain reaction of A1298C variant after digestion with Mboll enzyme

Amplification of polymerase chain reaction product of C677T showed 128 base pairs (bp). Digestion with restriction MboII enzyme gave two restriction site and two specific bands of 100 bp and 28 bp in only 2 subjects with nsCL/P.

DISCUSSION

There was a significant difference in T/T variant of C677T genotype between case and controls (p value = 0.003). The odds ratio for this variant was 3.3 indicating that presence of T/T variant was strongly associated with presence of non-syndromic cleft lip/palate with 50 % of the cleft cases showing this genetic variant. However, for the C/T variant of C677T (heterozygous mutant allele) the odds ratio was 2.3 indicating that this variant was also associated with the presence ns CL/P but the chances of its association was less than the homozygous mutant allele (T/T). There was a significant difference between controls and cases, for the presence of C/C variant, (homozygous wild type) of C677T with majority of the controls showing this genetic variant, indicating that this variant was the normal allele with the Odds ratio for controls being 4.4. Only one control sample did not show any of the three genetic variant of C677T, which could be attributed to a null mutation. The results of the present study was in agreement with the study done in the Indonesian (Northern Sulawesi) population in which the prevalence of this gene polymorphism of C677T of MTHFR gene was higher in ns CL/P cases.[3] Our findings are also in agreement with the study done by Jhu et al who also found higher frequency of the T/T genetic variant of C677T gene in subjects with nsCL/P in Northern Chinese population (Zhu et al., 2006). However, polymorphism of C677T was not associated with nsCL/ in the Central European population, indicating that its presence may not be indicative of nsCL/P in all populations (Desai et al., 2014).

The present study also showed that for the A1298C polymorphisms of MTHFR there was a significant difference between A/A genetic variant of A1298C between cases and controls (p value = 0.01) indicating that the presence of A/A variant (odds ratio 2.2) was associated with the absence of nsCL/P. For the C/C variant of A1298C (homozygous mutant allele)the odds ratio was 1.5 and that for the A/C variant of A1298C(heterozygous mutant allele) the odds ratio was 2.1 .In our study 10% the cleft cases showed C/C variant of A1298C and 50% of the cleft cases showed A/C variant of A1298C . This indicated that the presence of both A/C variant and C/C variant of A1298C gene showed a strong association with ns CL/P. The results of the present study are in accordance with the study done by Fakhim SA et al in the

population of North Western Iran, which also showed a significant association between the A1298C polymorphism of the MTHFR gene (A/C genetic variant) and non-syndromic cleft palate.[6]The results of the present study were in agreement with a meta-analysis study done by Pan et al, who concluded that genotypes of *MTHFR* C677T and A1298C were related to the development of nsCL/P in Asiatic populations. (Pan et al., 2015) As against this Sozen et al have found no association of risk for nsCL/P in the genetic variants of MTHFRgene (C677T and A1298C) in the North Venezuelan population. (Sözen et al., 2009) The presence of the variant was confirmed by recognizing the specific band on the DNA sequence using restriction endonucleases. Restriction endonucleases are enzymes that recognize a specific DNA base sequence and cleave both strands of a double-stranded DNA molecule at or near the recognition site. For the MTHFR C677T variant the restriction enzyme used for amplifying the DNA products was HinfI enzyme. The amplified product was completely digested with two restriction sites and two specific bands of 71 bp and 175 bp in 10 subjects with nsCL/P .This indicated that the presence of C677T variant of the MTHFR gene contributes to the incidence of nsCL/P in our studied population. (Desai et al., 2014) For the MTHFR A1298C variant the restriction enzyme used for amplifying the DNA products was MboII enzyme. The amplified product was completely digested with two restriction sites and two specific bands of 100 bp and 28 bp in only 2 subjects with nsCL/P. (Desai et al., 2014). This finding could be attributed because of low population size analysed in our study. Thus it was concluded in our study that, both the genetic polymorphisms of MTHFR gene C677T and A1298C are associated with nsCL/P in our population. The Odds ratio for the genetic variants of C677T was higher than the Odds ratio for the genetic variants of A1298C polymorphisms of MTHFR gene, indicating that the genetic variants of C677T are most strongly associated with nsCL/P than the mutations of A1298C.

Conclusion

Both the polymorphisms of the MTHFR gene namely MTHFR C677T and MTHFR A1298C may be implicated in the etiopathogenesis of non syndromic cleft lip/palate in our population. However the findings of our study were limited due to small sample size .Further studies targeting a generously proportioned sample size would provide a better insight into the use of this mutant alleles as genetic markers for screening and prevention of orofacial clefts in our population.

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