Association of Transforming Growth Factor Alpha and Methylenetetrahydrofolate reductase gene variants with nonsyndromic cleft lip and palate in the Indian population

Asavari L. Desai, M. R. Dinesh[1], B. C. Amarnath[1], R. M. Dharma[1], K. R. Akshai[1], C. S. Prashanth[1]

Abstract

Objectives: The aim was to evaluate the relationship of the K-primer variant of the transforming growth factor-alpha (TGF-α) gene and C677T variant of the methylenetetrahydrofolate reductase (MTHFR) gene with nonsyndromic cleft lip and palate (CL/P) in the Indian population. **Setting and Sample Population:** The study group consisted of DNA samples of 25 subjects with nonsyndromic CL with or without cleft palate and 25 unrelated controls, already existing in the Department of Orthodontics, D.A.P.M.R.V. Dental College, Bengaluru, Karnataka, India. **Materials and Methods:** The DNA samples were divided into two categories: Group A which included the 25 subjects with nonsyndromic CL/P; and Group B, which consisted of the 25 unrelated controls. 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 enzyme HinfI, 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:** In Group A, the TGF-α gene variant was present in 16 subjects ($P = 0.001$) and MTHFR gene variant was present in 8 subjects ($P = 0.185$). A combination of both gene variants were present in seven subjects, which was an interesting finding. In Group B, four subjects tested positive for the TGF-α and MTHFR gene variants. **Conclusions:** The TGF-α gene variant and a combination of TGF-α + MTHFR gene variants significantly contribute to the development of nonsyndromic CL/P and can be considered as genetic markers for Indian population. The MTHFR gene variant, though a minor risk factor, cannot be considered as a genetic marker.

Keywords: Methylenetetrahydrofolate reductase, nonsyndromic cleft lip and palate, transforming growth factor-alpha

Introduction

Orofacial clefts, particularly nonsyndromic cleft lip (CL) with or without palate are the most common craniofacial deformities affecting 1 in every 700-1000 births worldwide, with significant medical, psychological, economic, and social ramifications.[1-3]

The etiology of orofacial clefts is multifactorial and involves a complex interplay of genetic and environmental factors. The key to the determination of the etiology of malocclusion, and its treatability lies in the ability to differentiate the effect of genes and environment on the craniofacial skeleton in a particular individual.[4]

Various efforts have been made to understand the etiology of CL and palate (CL/P) so as to predict its occurrence and to prevent it from occurring in the future. Recently, using a combination of epidemiology, careful phenotyping, genome-wide association studies and analysis of animal models, several distinct genetic and environmental risk factors have been identified and confirmed for nonsyndromic CL/P. These findings have advanced our understanding of developmental biology and created new opportunities for clinical research.[5] Several candidate genes like the transforming growth factor-alpha (TGF-α), methylenetetrahydrofolate reductase (MTHFR), VAX, BMP4, MSX1, TGF-β3, RARA, IRF6, BCL3 etc., have been identified in studies conducted on populations of diverse ethnic backgrounds, with conflicting results.

Transforming growth factor-alpha is located on human chromosome 2p13. During craniofacial development, it is expressed in the medial edge of epithelium of fusing palatal shelves and promotes synthesis of extracellular matrix and mesenchymal cell migration into the palate.[6]

Methylenetetrahydrofolate reductase gene is located on human chromosome 1p36 and it plays a key role in folate metabolism.

Departments of Orthodontics, Manipal College of Dental Sciences, Mangalore, [1]D.A.P.M.R.V. Dental College, Bengaluru, Karnataka, India

Correspondence: Dr. Asavari L. Desai,
Department of Orthodontics, Manipal College of Dental Sciences, Light House Hill Road, Mangalore - 575 001, Karnataka, India.
E-mail: desaiasavari@gmail.com

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Deficiency of nutritional folic acid during embryonic development has been proposed as a candidate environmental factor in the etiology of CL/P. It has also been hypothesized that genetic variants in the enzymes controlling folate metabolism might play a role in the susceptibility of oral clefts.[7-10]

This study was undertaken to evaluate the association of TGF-α K-primer variant and MTHFR C677T variants with nonsyndromic CL/P in the Indian population.

Materials and Methods

Patients and DNA samples

The study group consisted of DNA samples of 25 subjects with nonsyndromic CL/P and 25 unrelated controls, already existing in the Department of Orthodontics, D.A.P.M.R.V. Dental College, Bengaluru, Karnataka, India. The CL/P phenotype spectrum included unilateral or bilateral CL with or without cleft palate.

Inclusion criteria

The presence of nonsyndromic CL with or without cleft palate on clinical examination.

Exclusion criteria

Cleft lip and palate associated with any:

- History of developmental disabilities, including learning disabilities and attention deficits, hearing impairment or abnormalities, which may be the first 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
- Medications (e.g. anticonvulsants and retinoic acid derivatives) during pregnancy
- Tobacco use, smoking during pregnancy
- Ethanol intake during pregnancy.

They were categorized into two groups:

Group A: DNA samples of 25 subjects with nonsyndromic CL/P

Group B: DNA samples of 25 unrelated controls.

Materials used in the present study

The following reagents were used to carry out the polymerase chain reaction (PCR) test and digestion with restriction enzyme Hinf1:

1. Taq polymerase (0.5 U/L μl): The enzyme which selectively amplifies the target DNA
2. PCR reaction buffer (2.5 μl): This buffer consists of:
   a. Tris. HCl and KCl: To maintain the pH of the PCR reaction buffer
   b. $MgCl_2$: It contributes Mg^{+2} which acts as a cofactor for Taq polymerase to function and carry out the polymerization reaction.
3. Deoxynucleoside triphosphates (2.5 μl): It is used to supply the necessary nucleotides (A, T, C, G) for the reaction to occur
4. Distilled water: To make up for the volume so that the reaction is carried out
5. Agarose gel: It is a polymer which has minute pores and enables separation of DNA fragments based on the size
6. Ethidium bromide: It is a fluorescent dye which binds to the DNA and illuminates with U.V. transilluminator on gel documentation
7. Restriction enzyme Hinf1 (1 μl): It is an enzyme that cuts double-stranded or single stranded DNA at specific recognition nucleotide sequences known as restriction sites. It was added to the PCR amplified DNA for its digestion.

Genotype analysis

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

Gene variant primer sequence PCR conditions product size (bp).

K-primer GAGACGGACTCCTGTTCACCTAGG 94°C, 1 min 345.

C677T CAAAGGCCACCCCGAAGC 94°C, 1 min 246.

For restriction fragment length polymorphism analysis of the variants, the Hinfl restriction enzyme was used.

After digestion with the restriction enzyme, the DNA fragments were analyzed by electrophoresis using 2.5% agarose gels. A U.V. transilluminator was used to see the specific bands of base pairs of the digested PCR products.

Statistical analysis

The Chi-square test was carried out and $P < 0.05$ were considered to indicate statistical significance. The statistical software namely SPSS 11.0 (SPSS Inc.) and Systat 8.0 (Systat Software Inc.) were used for the analysis of the data and Microsoft word and Excel have been used to generate tables.

Results

Transforming growth factor-alpha K-primer variant

The size of the amplified PCR product was 345 bp [Figure 1]. After digestion with the Hinfl enzyme, the amplified product was completely digested with one restriction site and two specific bands of 67 bp and 278 bp [Figure 2] in 16 subjects from Group A ($P = 0.001$) and four subjects from Group B, indicating the presence of variant.

Methylenetetrahydrofolate reductase C677T variant

An amplified product of 246 bp was obtained after PCR [Figure 3]. Digestion of the amplified product with HinfI enzyme resulted in one restriction site and two specific bands of 175 bp and 71 bp [Figure 4] in only eight subjects from Group A ($P = 0.185$) and four subjects from Group B. This indicated the presence of variant.

Interestingly, it was observed that few subjects showed the presence of more than one gene variant. A combination of TGF-α + MTHFR was found in seven subjects ($P = 0.004$).

Discussion

Identification of the genes involved in the development of the human craniofacial region can serve as a first step towards developing a better understanding of the diagnosis, prevention and treatment of developmental anomalies of this region.

The identification of genetic risk factors of nonsyndromic CL/P has been the subject of intensive research and numerous candidate genes such as TGF-β3, RARA, IRF6, BCL3, TGF-α, and MTHFR have been identified in studies conducted on populations of diverse ethnic backgrounds. In a study done by Singh *et al*.[11] in the Indian population, the TGF-β3 gene was found to be associated with nonsyndromic CL/P. However, the role of TGF-α and MTHFR genes in causing nonsyndromic CL/P in the Indian population has not been previously studied.

Therefore in this study, the presence of the TGF-α and MTHFR gene variants was assessed in a sample of 50 subjects.

In Group A, 16 out of 25 subjects, tested positive for the presence of TGF-α variant which was statistically strongly significant [Table 1]. This indicates that the TGF-α gene variant contributes to the occurrence of nonsyndromic CL/P in our population. This is in accordance with studies done by Ardinger *et al*. in a Caucasian population,[12] Holder *et al*. in a British population,[13] Jara *et al*. in a South American

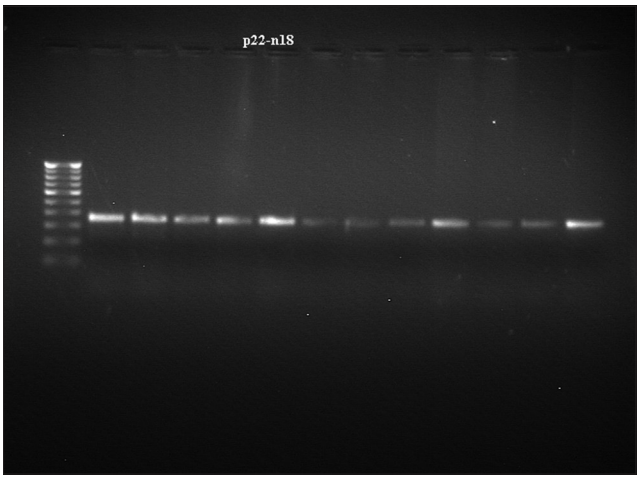

Figure 1: The amplified polymerase chain reaction product of the K-primer variant (345 bp)

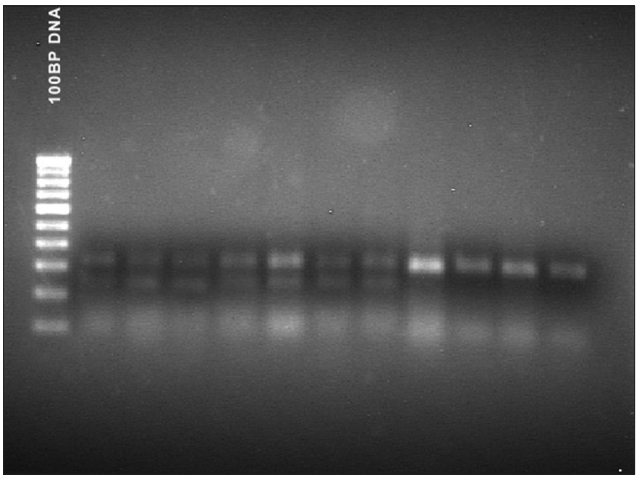

Figure 2: Polymerase chain reaction products for the K-primer variant after digestion with HinfI enzyme

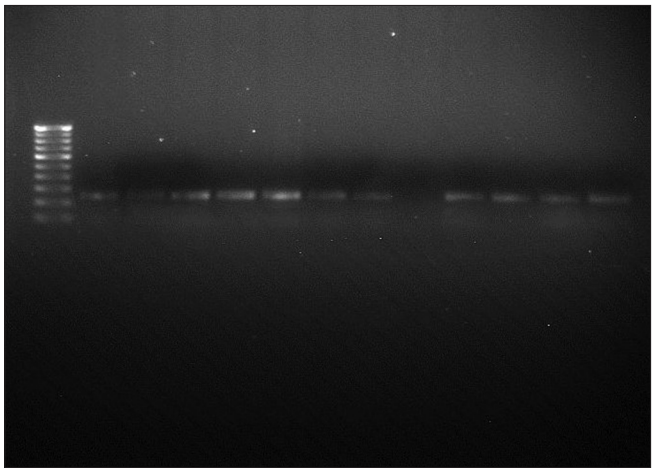

Figure 3: The amplified polymerase chain reaction product of the C677T variant (246 bp)

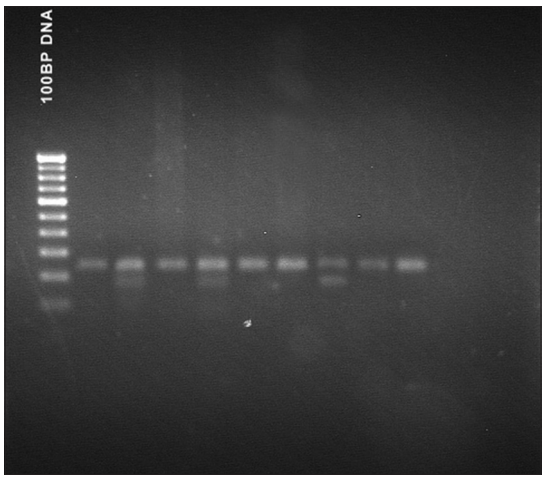

Figure 4: Polymerase chain reaction products for the C677T variant after digestion with HinfI enzyme

Table 1: The presence of TGF-α, MTHFR and a combination of TGF-α+MTHFR gene variants, in groups A and B

Gene variant	Group A (cases)	Group B (controls)	*P* value
TGF-α	16	4	0.001
MTHFR	8	4	0.185
TGF-α+MTHFR	7	0	0.004

MTHFR: Methylenetetrahydrofolate reductase; TGF-α: Transforming growth factor-alpha

population,[14] Tanabe *et al*. in a Japanese population,[15] Lu *et al*. in the Chinese population[16] and Letra *et al*. in the Brazilian population.[17] However, this is contrary to a study done by Lidral *et al*. in the Philipines[18] and Hecht *et al*. in Rochester,[19] which may be due to genetic differences in that population.

The MTHFR gene variant did not show any significant association with nonsyndromic CL/P cases, since only 8 out of 25 subjects, tested positive for the presence of variant [Table 1]. The results of this study indicate that MTHFR could be a minor risk factor but does not significantly contribute to orofacial clefting in the Indian population. This is in accordance with studies done by Brandalize *et al*. in a Brazilian population,[20] van Rooij *et al*. in The Netherlands,[21] Reutter *et al*. in a Central European population,[22] and Grunert *et al*. in a German population.[23] This is contrary to the results of the studies done by Jagomägi *et al*. in Estonia,[24] Jugessur *et al*. in Norway,[25] Shotelersuk *et al*. in Thailand[26] and Pezzetti *et al*. in Italy[27] which can be attributed to genetic differences in these populations.

It was observed that seven subjects in Group A showed the presence of both the gene variants, that is, TGF-α + MTHFR and none of the subjects in Group B. The correlation of TGF-α + MTHFR gene variants with nonsyndromic CL/P was found to be significant ($P = 0.004$) [Table 1]. This was an interesting observation as it throws light on the polygenetic nature of CL/P.

In Group B (controls), eight subjects showed the presence of the gene variants – four tested positive for TGF-α and four for the MTHFR gene variant [Table 1]. Whether this attributes to the pleotrophic nature of the TGF-α and MTHFR gene variants is a matter of further study.

Of 25 subjects with nonsyndromic CL/P, eight subjects did not show the presence of either of the gene variants. This suggests the possibility of the involvement of other candidate genes such as MSX1, TGF-β, IRF6, BCL3, RARA, etc., which have been established as factors contributing to the development of orofacial clefts in diverse populations.

The findings of this study suggest that the TGF-α gene variant and a combination of TGF-α + MTHFR gene variants significantly contribute to the development of nonsyndromic CL/P and can be considered as genetic markers for the Indian population. The MTHFR gene variant, though a minor risk factor, cannot be considered as a genetic marker.

Although genetic screens for various diseases currently exist, future progress in identifying the functions of genes in facial development and the mutations that affect these characteristics and functions could change orthodontic practice. In the near future, with rapid advances in the science of gene manipulation, the correction or alteration of genetic defects at the molecular level, remains a distinct possibility. Gene manipulation can be employed to control the expression of any gene in several orthodontically relevant issues. This will be critical to the development of clinically relevant tools.[28]

Further studies targeting a larger sample size and a number of genes are required for a better insight and understanding of the complex genetics of nonsyndromic CL/P. It is clear that there is no single major genetic risk factor for the development of orofacial clefts; and the development of the orofacial clefts in an individual depends on the interaction of several genes with environmental factors. Identifying specific genetic polymorphisms that influence orofacial cleft phenotypes will shed light on the molecular pathways involved in these complex disorders and provide a better understanding of the pathophysiology of orofacial clefts.

A major challenge is not only a complete cataloguing of all the genes but also identification of their polygenetic nature to have a thorough understanding of craniofacial development.

Conclusions

The results of this study indicated that there is a strong association between the presence of TGF-α gene variant and a combination of both TGF-α + MTHFR gene variants with the incidence of nonsyndromic CL/P in the Indian population and can be considered as genetic markers for the same.

The MTHFR gene variant however, did not show any significant correlation with nonsyndromic CL/P and cannot be considered a genetic marker for the Indian population.

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