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Research Article

ASSOCIATION OF TRANSFORMING GROWTH FACTOR BETA 3 GENE (RS3917168, RS3917177) VARIANTS WITH NONSYNDROMIC CLEFT LIP/PALATE IN SOUTH INDIAN POPULATION

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A B S T R A C T

Introduction: Non-syndromic Cleft lip & Palate(NSCLP) is a congenital deformity of multi factorial origin. The aim of this study was to evaluate the association of TGFβ3 gene variants rs3917168 and rs3917177 with NSCLP.
Methods: DNA samples of 25 subjects with NSCLP and 25 unrelated controls were used for the study. The extracted DNA samples were subjected to Polymerase chain reaction in which amplification of the selected gene segments was done; later these amplified products were subjected to Restriction Fragment Length Polymorphism using XapI(Xanthomonas ampelina) and PaeI (Pseudomonas aeruginosa) restriction enzyme.
Results: This study suggests that the likelihood of NSCLP is higher in subjects having AT genotype of TGFβ3 gene variant rs3917168 and CC and GG genotype of rs3917177 and lesser in subjects having AA and TT genotype of TGFβ3 gene variant rs3917168 and CG genotype of rs3917177.
Conclusion: The result suggests that TGFβ3 gene variants rs3917168 and rs3917177 can be used as genetic markers for NSCLP in local population.

INTRODUCTION

"Genetic information, or genetic factors in general, are both the Queen of the cells and the grandmother of all living organisms"- M. Matjusaitis, Lithuania.

The development of the head and face is one of the most complex and tightly controlled events during embryonic development. Disturbances during the period critical for the formation of face (7-10 weeks for humans) may lead to orofacial clefts.[2]

Cleft lip and palate (CL/P) is one of the most common congenital malformations. Immediately after birth, individuals with CL/P have facial deformities, feeding problems and frequent middle ear infections, the treatment of which requires interventions from multiple disciplines.[2]

CL/P have a wide geographic distribution, with average birth prevalence in the world ranging from 1/300 to 1/2500 births. Approximately two thirds of the cases are not accompanied by other anomalies and are called Non-syndromic (NS).[4]

The etiology seems complex, but genetics plays a major role. Various candidate genes MSX1, TGFβ1, TGFβ2, TGFβ3, RARA, MTHFR, IRF6, BCL3 and TGF-alpha have been associated with NSCL/P in different populations, but TGFβ3 are found to be strongly related in various populations on a consistent basis.

TGFβ molecules are proposed to act as cellular switches that regulate processes such as immune function, proliferation, and epithelial- mesenchymal transition. TGFβ1 is involved in haematopoiesis and endothelial differentiation. TGFβ2 affects development of cardiac, lungs, craniofacial, limb, eye, ear, and urogenital systems and TGFβ3 influences palatogenesis and pulmonary development.

TGFβ3 is located on human chromosome 14q 24. TGFβ3 expression coordinates palatogenesis. TGFβ3 gene determines extracellular matrix protein accumulation by stimulating extracellular matrix protein neosynthesis thereby inhibiting many enzymes which are responsible for extracellular matrix protein degradation. Thus, the reduced activity of TGFβ3 gene due to genetic variation may increase the risk for oral clefts. Knockout mice studies with TGFβ3 deletion have shown development of cleft palate.[9]

The mutations and polymorphisms of TGFβ3 have been shown to be diverse. It is necessary to study about these genetic variations to elaborate our understanding of the genetic control in various craniofacial determinants. In the present study, the focus of interest is to study the relation of TGFβ3 gene variants to NSCL/P. This helps in understanding the etiology of NSCL/P so as to predict its occurrence and also to target at

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the molecular level for correction of such problems in the future.

METHODOLOGY

The sample consisted of gene data of 25 subjects who were patients at D.A.P.M.R.V. Dental College & hospital and 25 unrelated controls. They were grouped into two groups

Group A: Twenty five subjects with NSCL/P (cases)
Group B: Twenty five control subjects.

Venous blood samples (2 ml) were obtained from both Subjects and controls with informed consent.

Inclusion Criteria

The presence of NSCL/P on Clinical examination.

Exclusion criteria

CL/P associated with any

- History of developmental disabilities, including learning disabilities and attention deficits, hearing impairment, and speech deficits or abnormalities 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,
- Medication (e.g., anticonvulsants and retinoic acid derivatives), vitamins (before and after conception) during pregnancy.
- Tobacco use, Smoking during Pregnancy
- Ethanol intake during pregnancy8

METHODOLOGY

Method: The method was divided into four steps:
Step 1: Collection and storage of blood samples,
Step 2: Isolation of Genomic DNA,
Step 3: Polymerase Chain Reaction Test,
Step 4: Digestion with Restriction Enzymes PaeI (Pseudomonas aeruginosa) and XapI(Xanthomonas ampelina).

Step 1: Collection and Storage of Blood Samples

Whole blood (2 ml) was collected in a tube containing EDTA from each subject and stored for later examination in liquid nitrogen (-70 ºC).

Step 2: Isolation of Genomic DNA

Genomic DNA was extracted from the blood of the subjects by the following method. Whole blood (100µl) was taken with a graduated micropipette into 1.5ml micro centrifuge tube. It was treated with 400µl of 10mmol/l TRIS.HCl basic buffer (pH 7.5) solution containing 5mmol/l MgCl2, 0.32mmol/l sucrose and 1% Triton X-100. Later, proteinase K (10mg/ml) and 10% Sodium Dodecyl Sulphate (SDS) were added to the mixture and then incubated at 37ºC for 30 min.

Then the mixture was subjected to phenol treatment which dissolves all the proteins and then to chloroform treatment to remove the phenol. In this way, the genomic DNA was extracted by phenol and chloroform. Ethanol (100% alcohol) was added and ripped in the ultracentrifuge at 12,000 rpm for five minutes at room temperature. The genomic DNA was precipitated by ethanol as a clear layer in the micro centrifuge tube.

Step 3: Polymerase Chain Reaction (PCR) Test

Once the isolated genomic DNA precipitate is obtained, 2.0µl was transferred to 0.5ml PCR tube with a graduated micropipette. It was used as a template for the polymerase chain reaction (PCR) test.The TGFβ3(gene variant-rs3917177,rs3917168) are determined and the partial sequence of the TGFβ3 gene were amplified by PCR. The primers used in this study were procured from New England Laboratories,USA having the following base pair sequence.

rs3917177	Primers (5' 3')
Forward-CCCCAGCTCTGGTTATAGCA	
Reverse -CTGCCACACCTGGCTAATTT	

rs3917168	Primers (5' 3')
Forward-TGGCAAAACCCCATCTCTAC	
Reverse-TTTGATCTCCCAGGCAATTC	

Genomic DNA was amplified by 0.5U or 1µl of Taq polymerase which carries out the polymerization reaction in a final volume of 20µl TRIS HCl buffer containing 25mmol/l KCl, 0.25mmol/l dNTP, 2.5mmol/l MgCl2 and 250mmol/l PCR primers. The balance volume is added up by the distilled water till it reaches 20µl volume in the PCR tube.

This reaction mixture in the PCR tubes was placed in the PCR machine which was set programmed to repeat the following cycle (stages 1,2,3) 35 times.

Stage 1: Separation: The double stranded DNA is denatured by heat into single strands. (1 min at 94 ºC)

Stage 2: Priming: The primers anneal to the end of the strand. (2 min at 52ºC).

Stage3: Polymerization: The DNA polymerase recognizes the primer and makes a complimentary copy of the template. (2 min at 72 ºC)

The PCR products 246 and 234 base pairs [bp] thus obtained were run on 1.5% agarose gel containing ethidium bromide to assess the initial amplified PCR products.

Step 4: Digestion with Restriction Enzymes Paei And Xapi

The amplified PCR products (15µl) were transferred to new PCR tubes. To this 3µl of buffer solution (pH – 7.8) containing TRIS HCl, NaCl, MgCl2, 1µl of restriction enzyme PaeI and XapI (New England Biolabs, USA) were added and the remaining volume was balanced with distilled water (11µl). Here, the amplified PCR products were digested with the specific restriction enzymes PaeI and XapI at 37°C. The method adopted for PCR was Restriction Fragment Length Polymorphism (RFLP).

Later, 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. Easy Win-32 software by Microsoft Inc. was used to visualize the bands in picture format by attaching the gel documentation to the computer.

The ethical clearance for the study was obtained from Ethical Committee and Institutional Review Board (IRB).

Statistical Methods

Chi-Square test has been used to find the significance of association of TGFβ3 with NSCL/P.

Statistical software

The Statistical software namely SPSS 11.0 and Systat 8.0 were used for the analysis of the data and Microsoft word and Excel have been used to generate graphs, tables etc.

RESULTS

Results of the Polymerase Chain Reaction Test: -rs3917168

The initial PCR product of the TGFβ3 gene was obtained for the fifty subjects of both the group A and group B. Three genotypes were determined namely AA, AT, TT in Fig 1, 2, 3.

In group A: Eight out of 25 showed the presence of AA genotype.

11 out of 25 showed the presence of AT genotype.
Two out of 25 showed the presence of TT genotype.

In group B: Seven out of 25 showed the presence of AA genotype.

Zero out of 25 showed the presence of AT genotype.
11 out of 25 showed the presence of TT genotype.

There were statistically significant differences in genotype frequencies between cases and controls, with AA and AT genotypes found more in cases. The association between TT genotype and the groups was found to be statistically significant (p-value < 0.01), with genotype seen more in controls. Also, the association between AT genotype and the groups was found to be significant statistically (p-value < 0.001), with AT genotype seen more in cases. However, the association between AA genotype and groups was not found to be statistically significant. (P-value>0.05).

Results of the Polymerase Chain Reaction Test: -rs3917177

The initial PCR product of the TGFβ3 gene was obtained for the fifty subjects of both the group A and group B. Three genotypes were determined namely CC, CG, GG in Fig 6,7,.

In group A: 16 out of 25 showed the presence of CC genotype.
Nine out of 25 showed the presence of GG genotype.
One out of 25 showed the presence of CG genotype.
In group B: Seven out of 25 showed the presence of CC genotype.
Zero out of 25 showed the presence of GG genotype.
Zero out of 25 showed the presence of CG genotype.

There were statistically strong significant differences in genotype frequencies between cases and controls, with CC genotype found more in cases. The association between CC genotype and the groups was found to be statistically significant (p-value < 0.05), with CC genotype seen more in cases. Also, the association between GG genotype and the groups was found to be significant statistically (p-value < 0.05), with GG genotype seen more in cases. However, the association between CG genotype and groups was not found to be statistically significant. (P-value>0.05).

DISCUSSION

Craniofacial development is a highly complex phenomenon with a large array of genes implicated, combined with multigenic inheritance and the influence of non-genetic factors. Thus identifying the key genes implicated in human cleft lip and palate represents a major challenge.[5]

The process of palatal fusion is also controlled by interactive signalling from the mesenchyme to the epithelium which is mediated by growth factors and extracellular matrix (ECM) proteins. One of the key components in this process is TGFβ 3 expression which coordinates palatogenesis (Britto *et al*). [16] This gene (TGFβ3) determines extracellular matrix protein accumulation by stimulating extracellular matrix protein neosynthesis thereby inhibiting many enzymes which are responsible for extracellular matrix protein Degradation (Blavier et al.).[11] This reduced activity of TGFβ3 due to genetic variation may increase the risk for oral clefts.

In this study, the presence of TGFβ3 gene variants (rs3917168, rs3917177) was assessed in a sample of 50 subjects consisting of Group A (25 subjects) with NSCL/P and Group B (25 unrelated controls) Our study showed a significant difference in presence of genotypes in cases and controls.

In our study TGFβ3 gene variant rs3917168 showed the presence of three genotypes AA, AT and TT. AA genotype was statistically not significant(p-value=0.758) as it was found in eight cases and seven controls. AT genotype was statistically highly significant (p-value<0.001) as it was found in 11 cases and zero controls. This significant difference in presence of AT genotypes in cases and controls suggests that subjects with AT genotype are more likely to have CL/P in our population.TT genotype was statistically significant (p-value=0.004) among controls, as it was found in two cases and 11 controls. In our study,11 out of 25 controls 71 had TT genotype, suggesting that the subjects with TT genotype are less likely to have CL/P. But as it is seen in two cases also, the results could not be confirmed and a larger sample size is required to further confirm the findings.

In our study TGFβ3 gene variant rs3917177 showed the presence of three genotypes CC, GG and CG. CC genotype was statistically significant (p-value=0.011) as it was found in 16 cases and seven controls. The presence of CC genotype in both the groups could be due to pleiotropic nature of genes (one gene can affect many characters).Whether this attributes to the nature of TGFβ3 gene variants is a matter of further study. This significant difference in presence of CC genotypes in cases and controls suggests that subjects with CC genotype are likely to have CL/P, in our population.GG genotype was statistically significant(p-value=0.002) as it was found in nine cases and zero controls. This significant difference in presence of GG genotypes in cases and controls suggests that subjects with GG genotype are more likely to have CL/P in our population.CG genotype was statistically not significant (p-value=0.312) as it was found in one case and zero controls.

In this study 20% of the cases (Samples 3,4,10,13,24) having NSCL/P did not show the presence of any of these gene variants. This may suggest the possibility of involvement of other genes as NSCL/P is of polygenetic nature and studies suggest that other genes like RARA, BCL3, TGFα, MTHFR and IRF6 are involved in the etiology of NSCL/P.

Our study thus suggests that TGFβ3 gene variant contributes to the occurrence of NSCL/P in south Indian population.

This study was done to evaluate the association of TGFβ3 gene variants rs3917168 and rs3917177 with NSCL/P. The results thus proved that there was a statistically significant association between the incidence of NSCL/P and TGFβ3 gene variants.The finding of this study conclude that AT genotype can be implicated as a genetic marker for the TGFβ3 gene variant rs3917168 and CC and GG genotype can be implicated as a genetic marker for the TGFβ3 gene variant rs3917177 for our population. 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 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 possibility. Gene manipulation can be employed to control the expression of any gene in several orthodontically relevant issues.

CONCLUSION

The conclusions drawn from this study are:

1. This study indicates that there is a strong association between the presence of TGFβ3 gene variants rs3917168 and rs3917177 with the incidence of NSCL/P higher in subjects having AT genotype of TGFβ3 gene variant rs3917168 and CC and GG genotype of rs3917177 and lesser in subjects having AA and TT genotype of TGFβ3 gene variant rs3917168 and CG genotype of rs3917177.
2. The findings of this study suggest that AT genotype for variant rs3917168 and CC, GG genotype for variant rs3917177 can be considered as genetic markers for NSCL/P in south indian population.

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