

Association between Genetic Polymorphism in Interferon Regulatory Factor 6 (IRF6) & Non-Syndromic Cleft Lip & Palate Cases in Central Indian Population

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ABSTRACT

BACKGROUND

Orofacial clefts can transpire either, as part of complex malformation syndromes, or as an isolated entity, also called non-syndromic cleft. Cleft lip with or without cleft palate (CL/P), collectively termed oral clefts, are the second most commonly observed birth defects among newborns after congenital heart defects. We wanted to investigate the association between genetic polymorphism in Interferon Regulatory Factor 6 (IRF6) & non-syndromic cleft lip and palate (CL/P) cases in Central Indian population.

METHODS

In this cross-sectional observational study, the sample comprised of Group 1: 66 individuals and 7 affected families with non-syndromic CL/P; and Group 2: 30 normal individuals and 10 normal families. 5 ml blood sample was collected from each individual following proper surgical protocol using disposable syringes, in blood tubes containing EDTA, with proper labelling and coding for further identification. DNA extraction was done by phenol chloroform extraction protocol and amplification was done using Polymerase Chain Reaction (PCR). Genotyping for the IRF6 polymorphism was completed by restriction digestion of PCR products, also called Restriction Fragment Length Polymorphism (RFLP).

RESULTS

The correlative comparison between GG and GA polymorphism in IRF6 gene between affected cases and normal patients shows highly significant value. The comparison between GG & GA polymorphism shows that GG polymorphism is significantly higher in affected cases compared to Group 2. While GA polymorphism is significantly low or decreased in non-syndromic CL/P patients when compared to control group.

CONCLUSIONS

GG polymorphism is more frequently associated with non-syndromic CL/P as compared to GA polymorphism.

KEY WORDS

Genetics, Polymorphism, Cleft Lip and Palate, IRF6

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BACKGROUND

Orofacial clefts characterize a ubiquitous stratification of congenital malformations, and are some of the major public health concerns. Globally, the prevalence of clefts is predicted to be near about, 1 in every 1000 newborns, a figure that is higher in certain ethnic groups.^[1] Orofacial clefts can transpire either, as part of complex malformation syndromes, or as an isolated entity, also called non-syndromic cleft. According to the National Center on Birth Defects and Developmental Disabilities (NCBDDD), birth defects affect about 1 in every 33 babies born in the United States each year.^[2] They are the leading cause of infant deaths, accounting for more than 20% of all infant deaths. Cleft lip with or without cleft palate (CL/P), collectively termed oral clefts, are the second most commonly observed birth defects among newborns after congenital heart defects.^[3] These patients show many alterations in other functions of the craniofacial complex such as speech, and airway.^[4]

Rare syndromes with oral clefts have distinct genetic causes, whereas the more common non-syndromic form of cleft has a multifactorial aetiology with both genetic and environmental components, challenging the identification of underlying aetiologies.^[4,5] Studying genetic factors involved in CL/P is vital to better understand its underlying aetiologies and to improve the diagnosis, treatment, prognosis and eventually prevention, of this devastating birth defect. Fogh-Andersen gave initial reorganization of genetical contribution of orofacial clefting, and employed a plethora of approaches for identification of genes and loci involved in orofacial clefting.^[4]

IRF6 shows pronounced expression in the ectoderm which covers the fetal facial primordia and in the medial edge epithelia of the secondary palatal shelves.^[6] Human and murine IRF6 show high degree of evolutionary conservation. Mice exhibiting deficiency, for both alleles of IRF6 present with abnormally thick skin, and grave limb and craniofacial defects, which also include clefting of the secondary palate. However, mice heterozygous for the null allele show no apparent phenotype except occasional intraoral adhesions in only 4% of embryos.^[7] The thick skin in mutants has been shown to result from an excessive proliferation of the spinous layer and a failure of keratinocyte terminal differentiation, suggesting that IRF6 plays an important role in keratinocyte proliferation and differentiation. Lack of the outermost cornified layer of the skin exposes the underlying hyperproliferated and fusion-competent spinous layer, which is thought to promote abnormal adhesions between adjoining tissues, particularly in the oral cavity. The intra-oral adhesions in the knockout mice were observed between the epithelia covering the maxillary and mandibular prominences in the molar region.

The intra-oral adhesions in these mice were detected between the epithelia lining the floor of the oral cavity, and the ventral surface of the anterior portion of tongue. The clefting phenotypes observed in these mice seems to be caused by a failure of the palatal shelf elevation.^[8] These studies further suggest that the oral adhesions or compression of the oral cavity due to thickened skin prevent elevation of palatal shelves, resulting in the cleft of the secondary palate. Based on a study,^[9] genetic variation in IRF6 has been estimated to be responsible for 12% of the

component of non-syndromic CL/P, and tripled the recurrence risk in families with an affected child.

On the basis of previous studies, it is obvious that genetic variation in IRF6 gene is a substantial risk factor contributing to the pathogenesis of non-syndromic CL/P.^[10] Since mutation screening in the protein coding and splice site sequences of IRF6 did not reveal potentially etiological variants, we hypothesized that a common deleterious variant is in strong linkage disequilibrium with the valine allele and could reside in a regulatory element of IRF6. However, the regulatory elements that control IRF6 expression have not yet been well-defined.^[11]

The purpose of this study was to recognize potential regulatory elements that control IRF6 expression using comparative genomic sequence analysis coupled with in-vivo and in-vitro functional assays, and to search for potentially etiological variant (s) in such regions, in individuals affected with non-syndromic CL/P. Also, the association between polymorphism in IRF6 gene as an aetiology in orofacial clefting was established.

METHODS

A sample of total 96 subjects and 7 families were selected after screening, from the patients being treated at the Department of Orthodontics at Sharad Pawar Dental College & Hospital, and AVBRH, Datta Meghe Institute of Medical sciences. This study was approved by Institutional Ethical Committee of Datta Meghe Institute of Medical Sciences (Deemed University). In the present cross-sectional observational study, 96 individuals and 7 affected families constituted Group 1, with non-syndromic CL/P and 30 normal individuals and 10 normal families (father, mother & child) served as Group 2. Informed consent was obtained from all the individuals participating in the study. Detailed case history of the subjects was recorded before taking the samples.

Inclusion Criteria

Individuals with unilateral or bilateral cleft lip and palate were selected, with or without positive family history.

Exclusion Criteria

Individuals with craniofacial syndromes were excluded from the study. For example: Sticklers syndrome, Ectodermal dysplasia, etc.

Procedure

5ml blood was withdrawn from each subject following proper surgical protocol and the blood was collected in EDTA vacuum tubes (where EDTA was the anti-coagulant). The blood was immediately processed for isolation of genomic DNA. DNA extraction was done by phenol chloroform extraction protocol, and amplification was done by employing polymerase chain reaction (PCR), consisting primers IRF6E7F: 59- AGTGGCCTTCCTGAATGCTG-39 and IRF6E7R: 59- CTTGACCTCCTCCAGACTAA-39, precipitating a PCR product of 647 bases pairs (bp). Genotyping for the IRF6 820 G→A polymorphism was established by restriction digestion of PCR products with DpnII (New England Biolabs, Beverly, Massachusetts, USA), as per the recommendations of the

manufacturing company. DpnII causes digestion of the G variant into five fragments (322, 177, 80, 35, and 33 bp). The A variant aids in addition of another restriction site, which causes the digestion of 322 bp into two smaller fragments of 235 and 87 bp.

Statistical Analysis

The data obtained was subjected to statistical analysis. The correlation between the frequency of polymorphism in IRF6 gene and non-syndromic CL/P was assessed by using the Chi-square test. $p < 0.05$ was considered as the level of statistical significance, which was calculated with the help of GraphPad Prism Software (version 4.0).

RESULTS

Table no. 1 represents genotypic allelic frequency distribution and comparison of IRF6 gene in the affected cases as compared with normal patients. This data shows that GG allelic frequency (0.53) is significantly high while compared with Group 2 (0.33). This represents that high GG allelic frequency is directly associated with disease condition ($\chi^2 = 3.21, p = 0.073$). Genotypic allele G and A and their distribution in the affected family when compared with Group 2 families are represented in table no 2. Our data shows that allele G frequency (0.78) is significantly high when compared with allele A frequency (0.22). Overall study of affected families and normal families shows higher G allele frequency, which shows that it contributes an important role in disease condition and may act as a genomic alternative marker for disease condition.

Genotype	Group 1 (n=66)	Group 2 (n=30)
GG	0.53(35)	0.33(10)
GA	0.35(23)	0.60(18)
AA	0.06(8)	0.066(2)
Chi ²	3.21	
p value	0.073	

Table 1. Genotypic Allelic Frequency Distribution and Comparison of the IRF 6 Gene in Group 1 Patients as Compared with Group 2

Alleles	Patients	Father	Mother	Control
G	0.78 (6)	0.715 (5)	0.575 (4)	0.63 (18)
A	0.22 (1)	0.285 (2)	0.425 (3)	0.37 (12)
P value	0.204	0.459	0.606	

Table 2. Distribution of G/A Allele Frequency in the IRF 6 Gene in Group 1 and Group 2

DISCUSSION

A genetic condition that closely bears a resemblance to isolated CL/P is Van der Woude’s syndrome, which is an autosomal dominant cleft disorder, wherein presence of pits on the lower lip are the sole additional notable feature. A direct overlapping is observed between the phenotype of Van der Woude’s syndrome with that of isolated CL/P, as the clefts are typical and are accompanied by lower lip pits. Therefore, 15 % of such syndromic cases may not be clinically distinguishable from isolated CL/P. According to recent reports, genetic mutations for Interferon Regulatory Factor 6 (IRF6) are responsible for Van der Woude’s

syndrome.^[12] Thus, contribution of IRF6 to CL/P has been recognized. It was initially identified as a domain for investigations, as mutations were detected in this gene in Van der Woude syndrome patients. As per the findings of recent studies,^[8] DNA sequence variants correlated with IRF6 are key contributors to CL/P in several human populaces.

When correlative comparison between GG and GA polymorphism in IRF 6 gene was done for affected and normal cases in our study sample, GG polymorphism was observed in 60.34% and GA polymorphism was seen in 39.65% of affected cases. These results showed, that the prevalence of GG polymorphism is significantly high in affected cases as compared to GA polymorphism. Amongst the control group, GA polymorphism showed higher value of 64.28% as compared to GG polymorphism i.e. 35.71%, suggestive of prevalence of GA polymorphism in Group 2 cases. When correlative comparison between GA and GG polymorphism was done in both groups, the value was highly significant statistically (p value = 0.032). These findings tell us that the GG polymorphism may play an important role in causing non-syndromic CL/P.

Our study shows that attributable risk of GG genotype is significantly high (53.0%) as compared to Group 2 (33.3%) with estimated attributable risk of 29.57%. From this data it can be predicted that there is 29.57% chance of transmission of GG genotype in this study samples. Our findings are similar to a research conducted^[13] where in a significantly greater frequency of the 820 GG genotype was detected in CL/P patients, as opposed to normal controls with an odds ratio of 1.67 (95% CI, 1.13 to 2.47). Thus, results show a significantly greater frequency of 820GG genotype in affected CL/P cases. No correlation was observed between the GA and AA genotypes in the study conducted by C. Srichomthong et al.^[13] The present study also showed no association between GA & AA polymorphism.

The estimated attributable risk for the G allele and the GG genotype in CL/P is 17.21% and 29.57% respectively of the IRF6 820 G allele in the Indian population. These values are in agreement with the attributable risk of 11.6% in the Philipino population. Although the IRF 6 820 A allele is very rarely seen or nearly absent amongst Europeans, according to a recent investigation involving the Italian population^[9], a strong evidence of linkage disequilibrium was observed between polymorphisms at the IRF6 locus. These results confirm the contribution of this gene in the aetiology of non-syndromic CL/P in various populaces.

The IRF6 820 G → A is the site for substitution of an isoleucine, for an evolutionarily preserved valine residue at codon 274 in a protein binding domain. Although the specific functions of IRF6 still remain unknown, previous data^[14] is suggestive of its role in the transforming growth factor beta signalling pathway. The A allele, i.e., the protective allele is explicit to humans playing a major role in human evolution. Because of changes in the commonest of the G allele in human populations, mutations in IRF6 gene are the most commonly known cause of this birth defect (CL/P). GG polymorphism was highest in affected group (53.03%), whereas GA polymorphism was more in normal individuals. Thus, GG polymorphism is more associated with non-syndromic CL/P. Estimated attributable risk (A.R.) for GG genotype is 29.57. Hence it can be said that there is 29.57% chance of transmission of GG genotype. Also, significantly greater

frequency of the GG genotype was detected in CL/P cases with an odd's ratio of 2.26 at 95% CI (0.84 – 6.34).

Newer horizons for improved understanding the human CL/P are opening up, with the increasing number of researches being planned in this field. While syndromic gene mutations may be associated with not more than 10% of the total cases, these findings will enlighten towards a better knowledge of the gene pathways and interactions in non-syndromic CL/P cases. For many such patients, the profits of accurate diagnosis, precise risk assessment and genetic counselling can be attained. On integration with tissue specific expression, profiling and targeted developmental investigations, the potential for therapies and preventive treatments may become an achievable reality.

CONCLUSIONS

IRF6 G→A is associated with non-syndromic CL/P. DNA sequence variants associated with IRF6 have the genetic contribution to CL/P. Recognition of genes can improve genetic counselling of families and provides a basis for identification of other genes or environmental factors linked to CL/P and provides pathways for treatment or prevention.

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