

DNA Sequencing for AXIN 2 (SNP 7591 and 7224837) Gene Polymorphisms in Non-Syndromic Cleft Lip and/or Cleft Palate in the Local Population

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Abstract: Introduction: The aim of this study was to evaluate the association of AXIN2 gene variants rs 7591 and rs 7224837 with non-syndromic cleft lip and palate. Materials and Methods: Blood samples of 30 subjects with NSCLP and 30 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 DNA sequencing. Results: This study suggests that the likelihood of Non-syndromic cleft lip and palate is higher in subjects having TT ($p<0.001$) & AT ($p=0.03$) genotype for AXIN2 gene variant rs7591 and AG ($p=0.01$) genotype for AXIN2 gene variant rs7224837. Conclusion: The result suggests that AXIN2 gene variant rs7591 and AXIN2 gene variant rs7224837 can be considered as genetic markers for Non syndromic cleft lip and palate in local population.

Keywords: Non-syndromic Cleft Lip and Palate, AXIN2, AXIN2 gene variant rs7591 and AXIN2 gene variant rs7224837

1. Introduction

Isolated, non-syndromic cleft lip with or without cleft palate represents one of the most common human birth defects with significant medical, psychological, social and economic ramifications.[41]

Mammalian palatogenesis is a complex process involving highly regulated interactions between epithelial and mesenchymal cells of the palate to permit correct positioning of the palatal shelves and subsequent fusion of the palatal shelves which require matrix metalloproteinases [26]

Recent success in genome-wide linkage and association studies has identified novel loci significantly associated with Cleft lip and palate [10]. Researchers are currently striving to identify the etiologic variants at these novel loci to understand the developmental disturbances leading to Cleft lip palate, and this knowledge should eventually result in improved prevention, treatment and prognosis for individuals with this condition.

As orthodontists are intimately and ardently involved in the successful therapeutic management of the patients affected with cleft lip with or without cleft palate and its associated tooth anomalies, it becomes essential that they keep current knowledge on the etiology of these conditions. It is therefore necessary to study about these genetic variations to elaborate our understanding of the genetic control in various craniofacial determinants. The Axin2 mutation displays enhanced expansion of osteoprogenitors, accelerated ossification, stimulated expression of osteogenic markers and

increased mineralization.

In the present study, the focus of interest is to study the relationship of Axin2 gene variants (rs7591 and rs7224837) with Non Syndromic Cleft Lip/Palate in our population. This will help us in understanding the etiology of Non syndromic Cleft Lip / Palate so as to predict its occurrence and also to target the gene at the molecular level for correction of such problems.

2. Materials and Methods

2ml venous blood samples from 30 cases with non syndromic cleft lip with / without palate and 30 unrelated controls who visited Department of Orthodontics and Dentofacial Orthopedics, D.A.P.M.R.V.Dental College, were taken after the written informed consent. These were divided into two groups: **Group A:** Thirty subjects with Non syndromic cleft lip/ palate (P1- P30) **Group B:** Thirty controls (C1- C30)

Inclusion criteria for Group-A subjects: The presence of Non syndromic cleft lip/ palate on clinical examination.

Exclusion criteria for Group-A subjects: Cleft lip/palate 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), vitamin (before and after

conception) during pregnancy; Tobacco use, Smoking during Pregnancy; Ethanol intake during pregnancy.

The polymorphism in Axin 2 gene variants rs 7591 and rs 7224837 were detected using the Polymerase Chain Reaction (PCR) test followed by DNA Sequencing.

Automated DNA sequencing procedure was selected for the sequencing of DNA where each nucleotide was labelled with fluorescent dyes. Thus when the DNA fragments were placed on the electrophoresis gel and passed through a laser beam, the DNA sequence was detected more precisely and accurately on a electropherogram unlike other sequencing techniques.

3. Methodology

The methodology consisted of five steps:

- Step 1: Collection and storage of blood samples,
- Step 2: Extraction of Genomic DNA,
- Step 3: Column purification of Genomic DNA,
- Step 4: Polymerase Chain Reaction Test (PCR),
- Step 5: DNA sequencing (fig 1)



Figure 1: ABI sanger DNA sequencer

4. Statistical Methods

Z test has been used to find the significance of association of Axin 2(rs 7591 and rs 7224837) gene polymorphism with non-syndromic cleft lip and palate.

Z – test: It can be applied for qualitative as well as quantitative data. Here it was applied to test the difference between two proportions (cases and controls).

Z-test for proportions formula:

$$z = \frac{\hat{P}_1 - \hat{P}_2}{\text{SEDp}}$$

$$\text{SEDp} = \sqrt{\hat{P}(1 - \hat{P})(1/n_1 + 1/n_2)} \quad \text{and} \quad P = \frac{x_1 + x_2}{n_1 + n_2}$$

p1, proportion1= x1/ n1

p2, proportion2= x2/ n2

x1 =number of cases with the 3 genotypes of each gene.

x2 =number of controls with the 3 genotypes of each gene.

n1 =total number of cases

n2 =total number of controls

5. Statistical Interpretation

Strongly significant $p \leq 0.001^{***}$

Significant $p \leq 0.05^{**}$

Not significant $p > 0.05^*$

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.

6. Results

In the present study, the relationship between AXIN2 (rs 7591) and AXIN2 (rs 7224837) gene variants with cleft lip with or without cleft palate was evaluated in 60 subjects consisting of group A (P1-P30) as cases and group B(C1-C30) as controls using polymerase chain reaction(PCR) test followed by DNA sequencing.

Results for AXIN2 rs 7591 variants: For AXIN2 (rs 7591) three genotype can be possible:

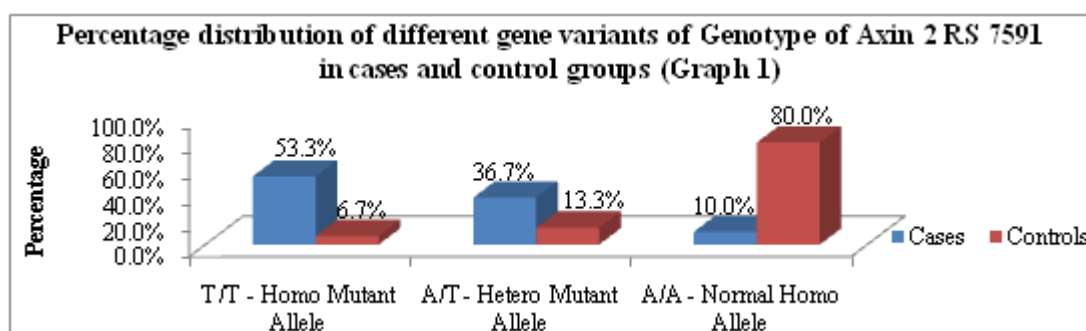
T/T	HOMOZYGOUS MUTANT ALLELE
A/T	HETEROZYGOUS MUTANT ALLELE
A/A	NORMAL HOMOZYGOUS ALLELE

In group A

- 16 out of 30 cases showed the presence of TT genotype.
- 11 out of 30 cases showed the presence of AT genotype.
- 3 out of 30 cases showed the presence of AA genotype.

In group B

- 2 out of 30 controls showed the presence of TT genotype.
- 4 out of 30 controls showed the presence of AT genotype.
- 24 out of 30 controls showed the presence of AA genotype.(graph 1)



- AA genotype was found to be highly statistically significant with the controls (GROUP B)(p=0.001)
- TT genotype was found to be highly significant with the cases (GROUP A) (p=0.001)
- AT genotype was found to be statistically significant with the cases (GROUP A) (p=0.03) (as shown in table 1)

Table 1: The table denotes the statistical significance of the genotype when cases and controls are compared using z-test

Genotype of Axin2 rs 7591 Gene Variant	Cases		Control		Differ in Prop	Z	P-Value
	n	%	n	%			
T/T	16	53.3	2	6.7	0.47	3.888	<0.001**
A/T	11	36.7	4	13.3	0.24	2.147	0.03*
A/T	3	10	24	80	-0.70	-5.450	<0.001***

Results for AXIN 2 RS 7224837 variants: For AXIN 2 (rs 7224837) three genotype can be possible

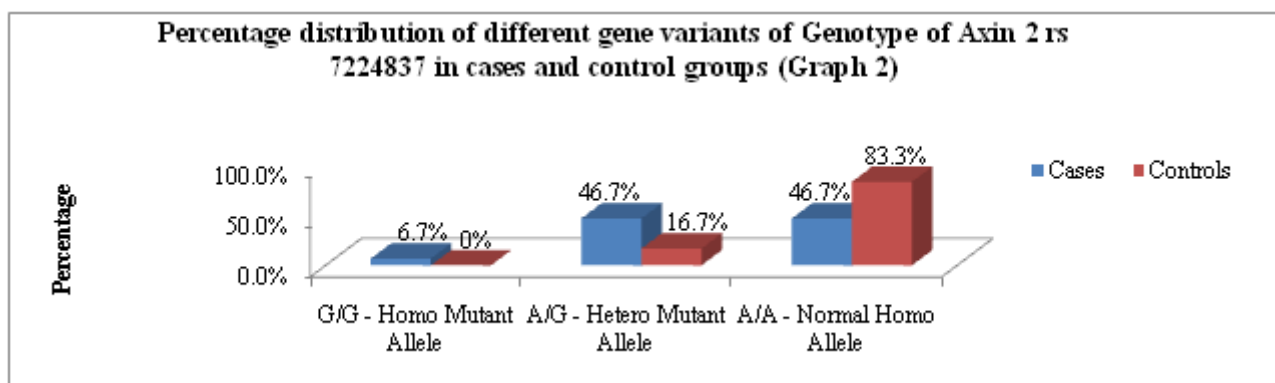
G/G	HOMOZYGOUS MUTANT ALLELE
A/G	HETEROZYGOUS MUTANT ALLELE
A/A	NORMAL HOMOZYGOUS ALLELE

In group A

- 2 out of 30 cases showed the presence of GG genotype.
- 14 out of 30 cases showed the presence of AG genotype.
- 14 out of 30 cases showed the presence of AA genotype.

In group B

- 0 out of 30 controls showed the presence of GG genotype.
- 5 out of 30 controls showed the presence of AG genotype.
- 25 out of 30 controls showed the presence of AA genotype. (Graph 2)



- There were statistically significant difference in AG genotype frequency between cases and controls (p=0.14)
- GG genotype was found to be statistically insignificant with the cases. (GROUP A)(p=0.14)
- AA genotype was found to be highly statistically significant with the controls (GROUP B)(p<0.007) (as shown in table 2)

Table 2: The table denotes the statistical significance of the genotype when cases and controls are compared using z-test

Genotype of Axin2 rs 7224837 gene variant	Cases		Controls		Difference Proportion	Z	P-value
	n	%	n	%			
G/G	2	6.7	0	0	0.07	1.475	0.14*
A/G	14	46.7	5	16.7	0.30	2.491	0.01**
A/A	14	46.7	25	83.3	-0.33	2.708	0.007***

7. Discussion

Craniofacial morphogenesis is regulated by complex interactions between the surface and neural ectoderms, endoderm paraxial mesoderm and cranial neural crest (Francis 1998). This morphogenetic process is highly dependent on the patterning information of emigrant cranial neural crest cells (Couly et al, 1996). Upon closure of the neural fold, these cells migrate ventro laterally to populate the head and neck regions and give rise to a wide variety of tissues. Majority of craniofacial malformations are caused by defects in these cells. Therefore understanding the mechanisms that control craniofacial development, particularly the cranial neural crest cells and its contribution

to various facial tissues and structures, might provide new insights into the molecular basis of these defects in humans. [17]

Studies of orofacial clefting have shown that CL/P has complex inheritance patterns as evidenced by a positive family history for clefting in 33% of the patients, no clearly recognizable mode of inheritance, and reduced penetrance. The relative risk for siblings, defined as the prevalence in siblings of an affected individual divided by the population prevalence, is 40; there is a 2–5% increased risk for offspring of affected individuals and a greater concordance in monozygotic than dizygotic twins, all providing evidence that genetic factors play an etiologic role. Yet, segregation analyses have not conclusively defined the mode of inheritance. Studies have estimated that 3–14 genes interacting multiplicatively may be involved, indicating that CL/P is a heterogeneous disorder. [19]

Determining the sequence of bases in DNA has become a major challenge of contemporary biology. DNA sequencing of human and other genome has been the center of interest in the biomedical field over the past several decades and is now leading toward the era of personalized medicine.

During this time, DNA sequencing methods have evolved from the labor intensive slab gel electrophoresis, through automated multicapillary electrophoresis systems using fluorophore labelling with multispectral imaging. DNA sequencing allows the use of four dideoxynucleotide chain terminator, tagged with dyes of different fluorescent emission wavelengths in a single sequencing reaction which

is depicted by a graph called as Electropherogram and Chromatogram. This graph contains peaks of four different colours which are universally coded for each nucleotide (Thymine-red, Adenine- green, Guanine-black, Cytosine-blue). Any change in normal nucleotide sequencing will be shown as different colour peak and if it is homozygous it will be shown as a single peak while if it is heterozygous it will be shown as double peak.

According to the interpretation of the electropherogram and statistical analysis, in our population, AXIN2 gene variant rs7591, showed statistically significant differences in genotype frequencies between cases and controls, with TT ($p < 0.001$) and AT ($p = 0.03$) genotypes found more in cases, with AA genotype ($p < 0.001$) found more in controls. (Table no.1 and Graph no.1).

According to the interpretation of the electropherogram and statistical analysis, in our population, AXIN2 gene variant rs7224837 showed statistically significant differences in genotype frequencies between cases and controls, with AG($p = 0.01$) genotypes found more in cases, with AA($p = 0.007$) genotype found more in controls. (Table no. 2 and Graph no. 2).

Our study showed a highly significant difference in presence of genotypes in cases and controls in both the AXIN2 gene variants rs 7591(TT and AT) but AXIN2 gene variants rs7224837 showed a significant difference only for AG genotype. Thus the data suggest that the WNT signalling pathway may contribute to as much as 3–5% of NS CLP and will be a consideration in the clinical management of CL/P [24].

The results of our study are contrary to the study done by Bjork, M.E.Cooper et al (2012) in multiple population, suggesting presence of AXIN2 gene variant 7224837 is strongly associated with cleft lip and palate .The contradictory results are probably due to genetic heterogeneity, incomplete penetrance, limited sample sizes and different study designs.

Gene manipulation can be employed to control the expression of any gene in several orthodontically relevant issues. In turn we may witness the introduction of both preventative and in vivo foetal therapy for these debilitating conditions.

The findings of this study indicate that Axin2 gene variants rs7591 and rs7224837 gene polymorphisms may be one of the genetic markers for cleft lip and palate in our population. Further studies, targeting a large sample size are required for a better insight to assess the complex genetics of Non syndromic cleft lip and palate.

8. Conclusion

1) This study indicates that there is a highly significant association between the presence of AXIN2 gene variant rs7591 and a significant association between AXIN2 gene variant rs7224837 with the incidence of Non syndromic cleft lip and palate.

- 2) This study suggests that the likelihood of Non syndromic cleft lip and palate is higher in subjects having TT ($p < 0.001$) & AT ($p = 0.03$) genotype for AXIN2 gene variant rs7591 and AG ($p = 0.01$) genotype for AXIN2 gene variant rs7224837.
- 3) This study suggests that the incidence of Non syndromic cleft lip and palate is lesser in subjects having AA ($p < 0.001$) genotype of AXIN2 gene variant rs7591 and GG($p = 0.14$) & AA($p = 0.007$) genotype of AXIN2 gene variant rs7224837.
- 4) The findings of this study suggest that AXIN2 gene variant rs7591 and rs 7224837 can be considered as genetic markers for Non syndromic cleft lip and palate for our population.

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