Original Article

Association of Wnt9B rs1530364 and Wnt5A rs566926 Gene Polymorphisms with Nonsyndromic Cleft lip and Palate in South Indian Population using Deoxyribonucleic Acid Sequencing

Abstract

Context: Nonsyndromic cleft lip with or without cleft palate (CL/CP) is a common congenital facial malformation without any other structural or developmental abnormalities. **Aims and Objectives:** To test the association of Wnt9B rs1530364 and Wnt5A rs566926 gene variants with the nonsyndromic CL/CP patients in South Indian population. **Methods:** Deoxyribonucleic acid (DNA) samples of 25 subjects with nonsyndromic cleft lip and palate (NSCLP) and 25 unrelated controls collected from the department were used for the study. Group A: DNA samples of 25 subjects NSCLP (P1–P25). Group B: DNA samples of 25 unrelated controls (C1–C25). The extracted DNA samples were subjected to polymerase chain reaction, and later, these amplified products were subjected to DNA sequencing. Results were documented in the form of electropherograms. **Results:** The results indicated that there is a strong association between the presence of Wnt9B rs1530364 gene with the incidence of NSCLP. This study also suggests that the likelihood of NSCLP is higher in subjects having CC (P = 0.02) genotype for Wnt9B gene variant rs1530364. **Conclusion:** We can conclude that Wnt9B gene variant rs1530364 can be considered as genetic marker for NSCLP for our population.

Keywords: *Nonsyndromic cleft lip and palate, Wnt5A gene variant rs566926, Wnt9B gene variant rs1530364*

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Introduction

Cleft lip with or without cleft palate (CL/CP), one of the most common craniofacial anomalies in humans, may occur as part of a syndrome or may be isolated, wherein the affected persons do not present any associated structural anomalies. More than 300 syndromes, including some that are chromosomal or Mendelian, may present a cleft of the lip and/or the palate as a feature, and these make up about 30% of all cleft cases. The remaining 70% are attributed to isolated, nonsyndromic oral clefts without any associated structural anomaly.[1]

CL/CP and cleft palate-only (CPO) are genetically distinct phenotypes in terms of their inheritance patterns. CPO is less common, with a prevalence of approximately 1/1500–2000 births in Caucasians, whereas cleft lip and palate is more common, with 1–2/1000 births.[2] The prevalence of CL/CP varies considerably with Asians and American-Indians having the highest rate and Africans the lowest. Nonsyndromic CL/CP is a common birth defect with a prevalence of 1/700 live births.[3] It is a polygenic, multifactorial disorder with both genetic and environmental factors contributing to the etiology of this condition.

Cleft lip or palate is caused by genetic variations in more than one gene because several processes are involved in lip and palate formation, including cell proliferation, differentiation, adhesion, and apoptosis. Different results have been obtained for the different populations investigated. The clinical manifestations of these defects are diverse, ranging from isolated clefts of the lip to complete bilateral clefts of the lip, alveolus, and palate.

Recent success in genome-wide linkage and association studies has identified novel loci significantly associated with cleft lip and palate.[4]

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The wingless-type Mouse Mammary Tumor Virus (MMTV) integration site family (Wnt) signaling pathway plays an important role in craniofacial development. Wnt signaling genes are conserved among species and are essential to the development of several processes, including face morphogenesis. Loss of function of Wnt genes is associated with defects in the facial region, incomplete penetrance of cleft lip, and defects in kidney morphogenesis in homozygous mice mutants.[5] Wnt expression is observed in the upper lip and primary and secondary palates, and Wnts are involved in regional specification of the vertebrate face.[6]

The objectives of this study was

1. To evaluate the relationship of Wnt9B rs1530364 and Wnt5A rs566926 genes with nonsyndromic cleft lip and palate (NSCLP).
2. To identify for our population, whether Wnt9B rs1530364 and Wnt5A rs566926 genes can act as genetic markers for NSCLP.

Methods

The polymorphism in Wnt9B (rs1530364) and Wnt5A (rs566926) gene variants was detected using the polymerase chain reaction (PCR) test followed by deoxyribonucleic acid (DNA) sequencing.

Saliva samples from 25 cases with nonsyndromic CL/CP and 25 unrelated controls were collected from the Department of Orthodontics and Dentofacial Orthopedics, D.A.P.M.R.V. Dental College and Swasthya Foundation, Mysore, after taking the written informed consent.

These were divided into two groups:

- Group A: Twenty-five subjects with NSCLP (P1–P25)
- Group B: Twenty-five controls (C1–C25).

Inclusion criteria for Group A subjects

The presence of NSCLP on clinical examination.

Exclusion criteria for Group A subjects

CL/palate associated with any:

1. 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
2. Family history of orofacial clefts and related conditions, including any additional major associated anomalies (e.g., cardiac defects and eye and brain anomalies)
3. History of maternal illnesses
4. Medication (e.g., anticonvulsants and retinoic acid derivatives), vitamin (before and after conception) during pregnancy
5. Tobacco use, smoking during pregnancy
6. Ethanol intake during pregnancy.

Methodology

The method was divided into four steps:

1. Step 1: Collection and storage of saliva samples
2. Step 2: Isolation of genomic DNA
3. Step 3: PCR test
4. Step 4: DNA sequencing.

Step 1: Collection and storage of saliva samples

The steps are outlined in Flowchart 1.

Step 2: Isolation of genomic deoxyribonucleic acid

The protocol is outlined in Flowchart 2.

Step 3: Polymerase chain reaction test

The PCR is an *in vitro* technique which allows the amplification of a specific DNA.

Primer sequence

For Wnt5A (rs 566926)

- Wnt5AFP: 5'GAGT TCCCTTCGCC TCTTCC'3
- Wnt5ARP: 5'GTTCTCTCAACCAAGGAGTCC'3.

For Wnt9B (rs 1530364)

- WntB9FP: 5'GGTCAGTCTGAGGCAAGTC'3
- WntB9RP: 5'ATGTTCCCAGATTCCCAGAG'3.

Step 4: Deoxyribonucleic acid sequencing [Flowchart 3]

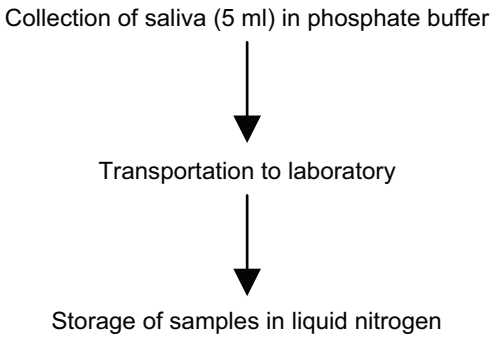
DNA sequencing was performed using Frederick Sanger's dideoxy sequencing method in an automated ABI sequencer machine based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during *in vitro* DNA replication.

Statistical methods

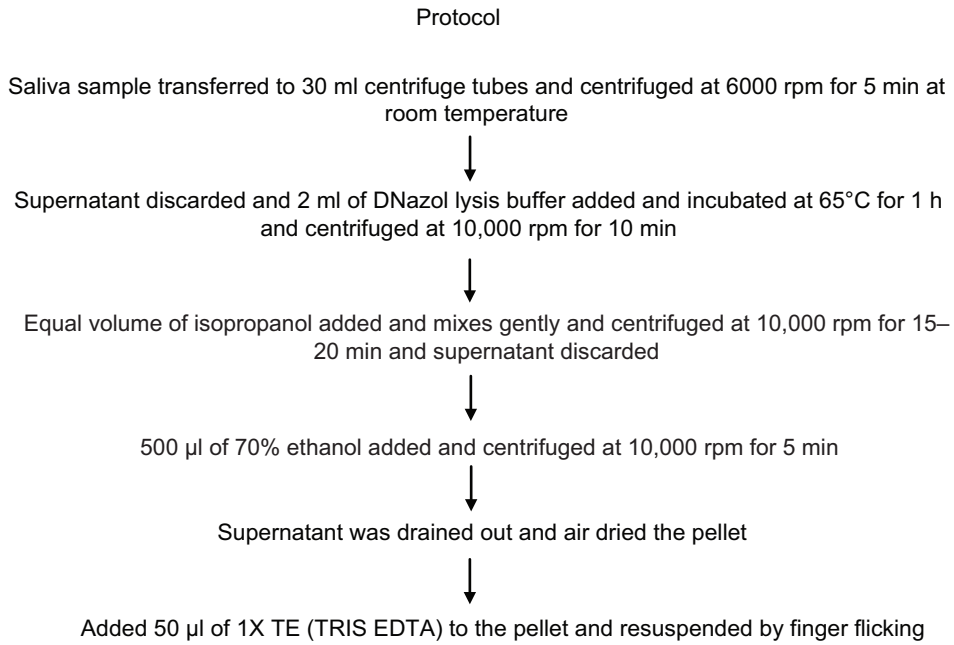
Z-test has been used to find the significance of association of *Wnt9B* and *Wnt5A* gene polymorphism with NSCLP.

Z-test 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:



Flowchart 1: Collection and storage of saliva sample



Flowchart 2: Isolation of genomic deoxyribonucleic acid

$$Z = \frac{\hat{P}1 - \hat{P}2}{SEDp}$$

$$SEDp = \sqrt{\hat{P}\left(1 - \hat{P}\right)\left(1/n1 + 1/n2\right)} \text{ and } P = \frac{X1 + X2}{n1 + n2}$$

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.

Statistical interpretation

- Highly statistically significant, $P < 0.001$
- Statistically insignificant, $P \geq 0.05$.

Statistical software

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

Results

In the present study, the relationship between Wnt9B (rs1530364) and Wnt5A (rs566926) gene variants with CL/CP was evaluated in 50 subjects consisting of Group A (P1–P25) as cases and Group B (C1–C25) as controls using PCR test followed by DNA sequencing.

Results for Wnt9B (rs1530364) variants [Flowchart 4]

For Wnt9B (rs1530364), three genotypes can be possible:

T/T	Normal homozygous allele
C/C	Mutant homozygous allele
T/C	Mutant heterozygous allele

Results for Wnt5A (rs566926) variants [Flowchart 5]

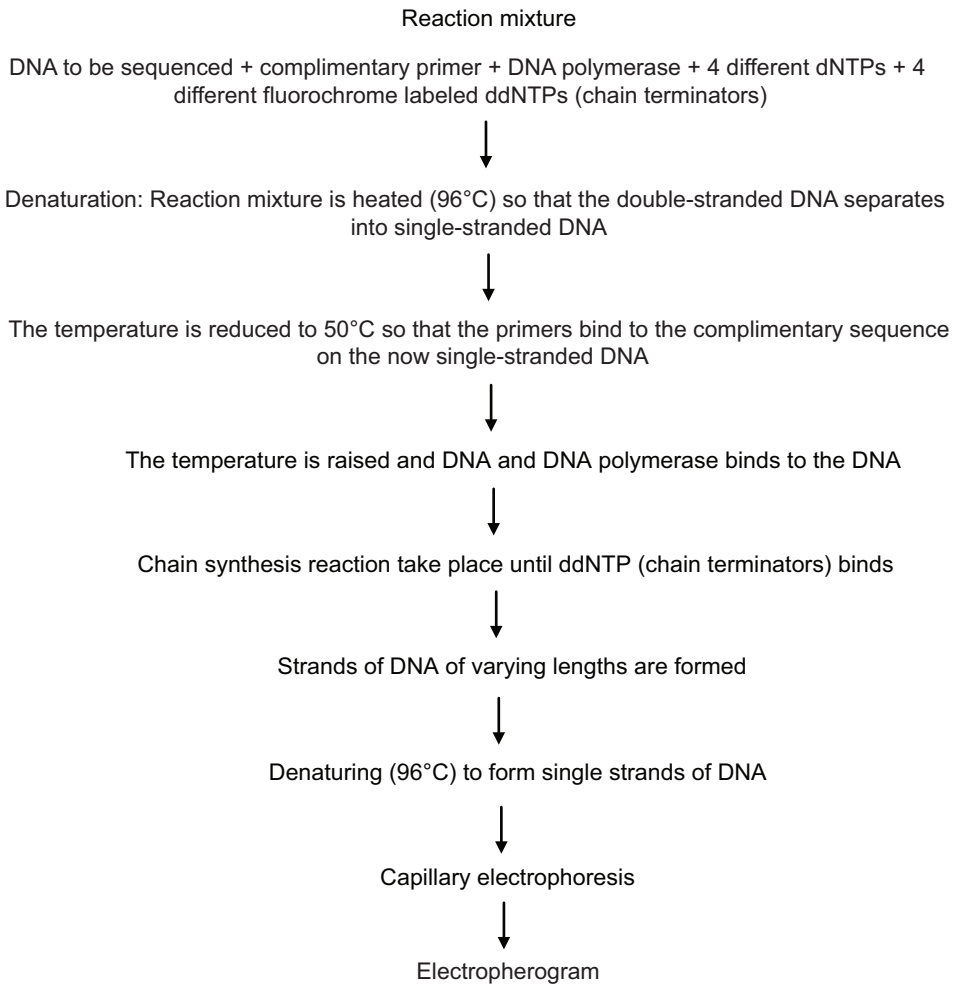
For Wnt 5A (rs566926), three genotypes can be possible.

AA	Normal homozygous allele
CC	Mutant homozygous allele
A/C	Mutant heterozygous allele

Discussion

The etiologies of CL ± P are multifaceted and occupy both major and minor genetic influences with erratic connections from environmental factors. Its complexity is exemplified by the large number of candidate genes and loci that seem to be involved. Although many studies have been done to find the genetic pattern of this malformation, there is still no precise answer. It is indispensible to highlight the gene involvement in CL ± P patients according to literature survey.[7]

Wnt signaling molecules constitute a family of conserved secreted glycoproteins that play fundamental roles in developmental and biological processes. The Wnt genes are involved in regulating midface development and upper lip fusion and are therefore candidates for an etiologic role in nonsyndromic CL/CP. Evidence supporting Wnt genes as possible clefting loci comes from studies with the inbred



Flowchart 3: DNA sequencing

A/WySn mouse strain, in which Wnt3 and Wnt9B genes are located on chromosome 11 within the clf1 locus and contribute to a clefting phenotype. This region is syntenic to the human chromosome 17q21, which is known to be associated with NSCLP in humans. Polymorphic variants in Wnt3, Wnt3A, Wnt5A, Wnt9B, and Wnt11 genes have been associated with NSCLP in different populations, including a case–control study in a Brazilian population. In humans, variations in Wnt genes have been described in patients with syndromic CL/P and NSCLP.[5,6,8]

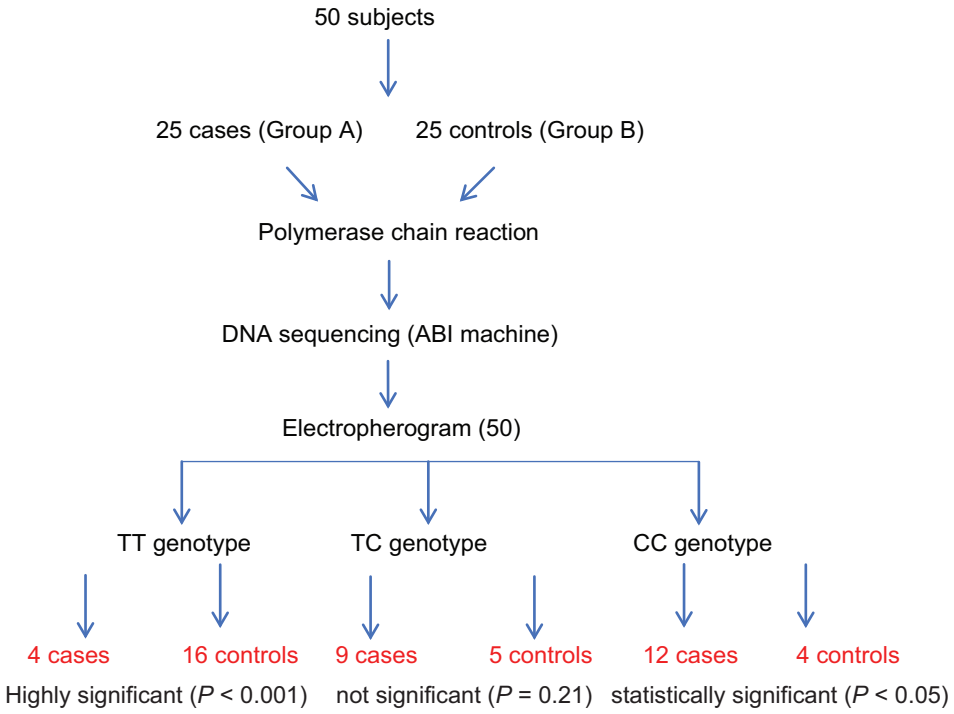
In this study, the presence of Wnt9B gene variants rs1530364 and Wnt5A gene variants rs566926 was assessed in a sample of 50 subjects comprising 25 cases (P1–P25) with NSCLP and 25 unrelated controls (C1–C25).

According to the interpretation of the electropherogram and statistical analysis, in our population, Wnt9B gene variant rs1530364 showed highly statistically significant differences in genotype frequencies between cases and controls, with CC ($P < 0.02$) genotypes found more in cases and TT genotype ($P < 0.001$) found more in controls [Table 1 and Graph 1].

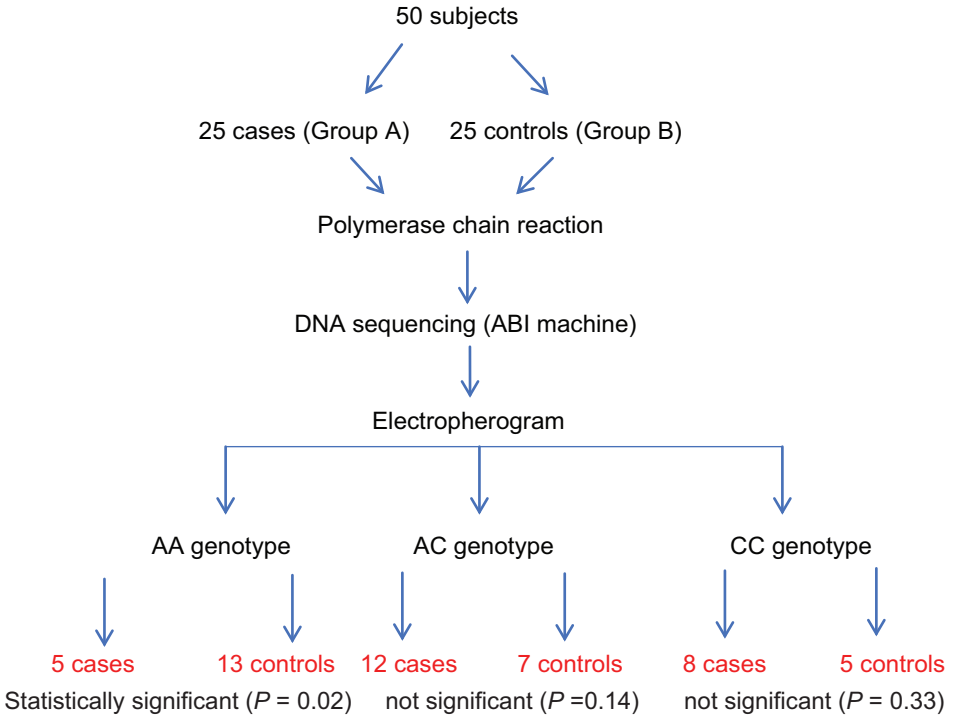
According to the interpretation of the electropherogram and statistical analysis, in our population, Wnt5A gene variant rs566926 showed statistically significant differences in genotype frequencies between cases and controls with AA ($P = 0.02$) genotype found more in controls and statistically insignificant differences in genotype frequencies between cases and controls with CC ($P = 0.33$) genotype [Table 2 and Graph 2].

Our study showed a highly significant difference in the presence of genotypes in cases and controls in Wnt9B gene variants rs1530364. This is in accordance with a study done by Fontour *et al*. in Brazilian Families, which concluded a positive association between NSCLP and single-nucleotide polymorphism rs1530364 in the Wnt9B gene.[5]

Our study showed statistically insignificant differences in genotype frequencies between cases and controls in Wnt5A gene variants rs566926. This is in contrary to the study done by Chiquet *et al*. in 2008 in European Americans and Hispanics, in which Wnt5A was found to be significantly associated with NSCLP.[6] The contradictory results are probably due to genetic heterogenecity, incomplete



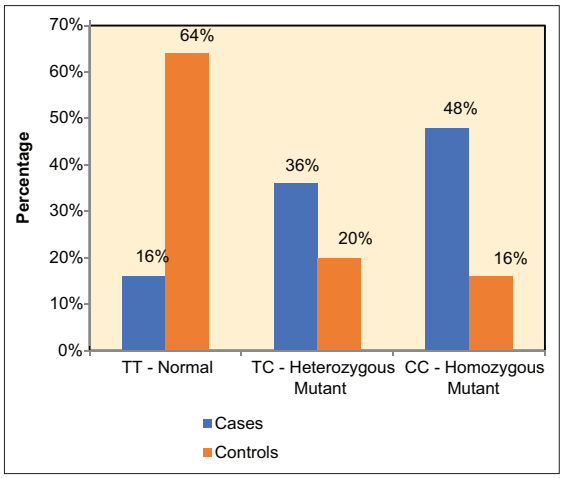
Flowchart 4: Results of Wnt9B (rs1530364)



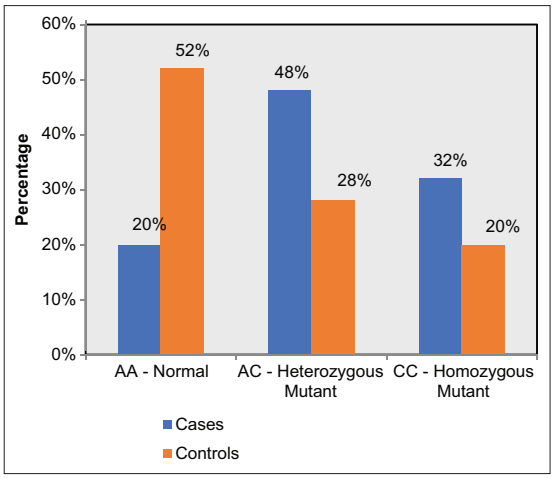
Flowchart 5: Results of Wnt5A (rs566926)

penetrance, limited sample sizes, and different study designs.

The completion of the genome sequence has contributed to recent successes in identifying novel CL/P genes. However, it is likely that advances in our understanding of both genetic and environmental etiology of CL/P will continue. With the recent DNA sequencing and microarray techniques, further identification of the candidate gene and genetic pathways involved in nonsyndromic clefting can be expected. Ultimately, all of these advances will allow more



Graph 1: Percentage distribution of different gene variants of genotype of Wnt9B rs1530364 in cases and control groups



Graph 2: Percentage distribution of different gene variants of genotype of Wnt5A rs566926 in cases and control groups

Table 1: The statistical significance of the genotype when cases and controls are compared using *Z*-test

Genotype of Wnt9B rs1530364 gene variant	Cases, *n* (%)	Controls, *n* (%)	Difference in proportions	*Z*	*P*
TT - normal	4 (16)	16 (64)	−0.48	−3.464	0.005**
TC - heterozygous mutant	9 (36)	5 (20)	0.16	1.260	0.21
CC - homozygous mutant	12 (48)	4 (16)	0.32	2.425	0.02*

Table 2: The statistical significance of the genotype when cases and controls are compared using *Z*-test

Genotype of Wnt5A rs566926 gene variant	Cases, *n* (%)	Controls, *n* (%)	Difference in proportions	*Z*	*P*
AA - normal	5 (20)	13 (52)	−0.32	−2.357	0.02*
AC - heterozygous mutant	12 (48)	7 (28)	0.20	1.457	0.14
CC - homozygous mutant	8 (32)	5 (20)	0.12	0.967	0.33

accurate methods of genetic screening, the identification of high-risk individuals or family groups, and improved prenatal diagnosis.

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. In turn, we may witness the introduction of both preventive and *in vivo* fetal therapy for these debilitating conditions.

The findings of this study indicate that Wnt9B gene variants rs1530364 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 and complex genetics of NSCLP.

Conclusion

The conclusions drawn from this study are:

1. This study indicates that there is a highly significant association between the presence of Wnt9B gene variant rs1530364 with the incidence of NSCLP
2. This study suggests that the likelihood of NSCLP is higher in subjects having CC (P = 0.02) genotype for Wnt9B gene variant rs1530364
3. This study suggests that the incidence of NSCLP is lesser in subjects having TT (P < 0.001) and TC (P = 0.21) genotype of Wnt9B gene variant rs1530364 and AA (P = 0.02), AC (P = 0.14), and CC (P = 0.33) genotype of Wnt5A gene variant rs566926
4. The findings of this study suggest that Wnt9B gene variant rs1530364 can be considered as a genetic marker for NSCLP for our population.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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