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Dr. Sapna Kumari
Department of Orthodontics,
Rajiv Gandhi University,
Bangalore, Karnataka, India

Dr. Prashanth CS
Professor and HOD, Dept. of
orthodontics, Rajiv Gandhi
University, Bangalore,
Karnataka, India.

Dr. Amarnath BC
Professor, Department of
Orthodontics, Rajiv Gandhi
University, Bangalore,
Karnataka, India

Dr. Dharma RM
Professor, Department of
Orthodontics, Rajiv Gandhi
University, Bangalore,
Karnataka, India

Dr. Akshai Shetty
Professor, Department of
Orthodontics, Rajiv Gandhi
University, Bangalore,
Karnataka, India

Dr. Manjunath Hegde
Professor, Department of
Orthodontics, Rajiv Gandhi
University, Bangalore,
Karnataka, India

Corresponding Author:
Dr. Sapna Kumari
Department of Orthodontics,
Rajiv Gandhi University,
Bangalore, Karnataka, India

Correlation of the SNP's of fgfr1 and fgf10 with non-syndromic cleft lip with or without cleft palate in Indian population by DNA sequencing method

Dr. Sapna Kumari, Dr. Prashanth CS, Dr. Amarnath BC, Dr. Dharma RM, Dr. Akshai Shetty and Dr. Manjunath Hegde

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Abstract

Nonsyndromic cleft lip & palate is a congenital deformity of multifactorial origin. Various etiologic candidate genes have been reported with conflicting results, according to the race and population studied. Numerous efforts have been made to understand the etiology of cleft/palate so as to predict its occurrence and thus enable its prevention. The FGF regulation pathway also plays a major role in craniofacial development. FGFR1, FGFR2, FGFR3, FGF10 and FGF18 are expressed during the various stages of secondary palate development. Thus their inactivation leads to cleft palate. This study was done to evaluate the association of FGFR1 and FGF10 gene variants with nonsyndromic cleft lip and palate. DNA samples of 25 subjects with nonsyndromic cleft lip and palate and 25 unrelated controls, collected from the department were used for the study. The extracted DNA samples were subjected to Polymerase chain reaction later subjected to DNA sequencing. The results were documented in the form of electropherogram. The results indicated that for FGFR1 gene (rs13317), genotype CC ($P=0.02$) was statistically significant and FGF 10 gene (rs1448037), genotype AA ($P < 0.001$) was highly statistically significant. Thus suggesting a strong association between FGFR1 and FGF10 with NSCLP.

Keywords: Nonsyndromic Cleft Lip and Palate, FGFR1 gene variant rs13317 and FGF10 gene variant rs1448037

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 (Murthy *et al.* 2014) [8]. It is a polygenic, multifactorial disorder with both genetic and environmental factors contributing to the aetiology of this condition (Mossey *et al.* 2009) [10].

Cleft on the lip is called cheiloschisis and Cleft on the palate is called Palatoschisis. Individuals with orofacial clefts suffer initially from difficulties in feeding and also experience speech, hearing, and dental problems. Clefts can be surgically repaired but patients often undergo multiple craniofacial and dental surgeries, as well as speech and hearing therapy. Thus, this craniofacial anomaly warrants complex multidisciplinary treatment. Despite these interventions, patients can experience lifelong psychosocial effects from this malformation.

Non syndromic or isolated cleft lip and palate occur in a wide geographical distribution, with an average Asian populations having a higher birth prevalence of clefting, whites are intermediate, and African populations have the lowest (Alexandre *et al.* 2008) [1]. In India, the highest rates are reported in the states of Andhra Pradesh, Karnataka and Tamil Nadu, with Kerala being an exception because of the strict avoidance of consanguineous marriage amongst the large Christian population (Mossey *et al.* 2009) [10].

The lip development starts between the 3rd and 7th weeks, and palate development between the 5th and 12th weeks of intra-uterine period. Prenatal ultrasound can sometimes determine if a cleft exists in an unborn child.

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.

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. 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.

Numerous candidate genes like TGF α , TGF β 1, TGF β 2, TGF β 3, RARA, MTHFR, IRF6, BCL3 and MSX1 have been identified in association with cleft lip and palate in various studies that have been conducted on populations of different ethnic backgrounds.

Mammalian fibroblast growth factors (FGF1–FGF10 and FGF16, FGF23) control a wide spectrum of biological functions during development and adult life and are strong candidates for an etiological role in NSCLP. The FGF regulation pathway also plays a major role in craniofacial development. FGFR1, FGFR2, FGFR3, FGF10 and FGF18 are expressed during the various stages of secondary palate development. Thus their inactivation leads to cleft palate (Bridget *et al.* 2007) [3].

It is therefore 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 relationship of FGFR1 and FGF10 gene variants (rs13317 and rs1448037) 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 at the molecular level for correction of such problems.

Advances in genetic testing, gene therapy, Pharmacogenomics,

Mechanogenomics and stem cell therapy are likely to produce the most dramatic changes in orthodontic treatment.

Materials and Methods

The present study aims at investigating the association of FGFR 1 (Fibroblast growth factor receptor) gene variants (rs13317) and FGF 10(rs1448037) in cleft lip and palate.

The polymorphism in FGFR 1 (rs13317) and FGF 10 (rs1448037) gene variants 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 an electropherogram unlike other sequencing techniques.

2 ml venous blood samples from 25 cases with non-syndromic cleft lip with / without palate and 25 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: Twenty five subjects with Non syndromic cleft lip/ palate (P1- P25)

Group B: Twenty five controls (C1- C25)

Inclusion criteria for Group-A subjects:

1. The presence of Non syndromic cleft lip/ palate on clinical examination.

Exclusion criteria for Group-A subjects: Cleft lip/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.

Sequential listing of Reagents used in the present study:

Extraction of Genomic DNA

1. MgCl₂: It is added as a buffer component to maintain the pH of the solution.
2. Triton X-100 (1%) (50 μ l): It is a detergent which aids in preparation of RBC cell for lysis.
3. Sodium Dodecyl Sulphate (SDS 10%) (40 μ l): It is a detergent which binds to cell membrane which causes cell lysis and releases cell components.
4. NaCl (100 μ l): It helps in precipitation of proteins.
5. Isopropanol (600 μ l): It is used for precipitation of DNA.
6. Ethanol (70%) (200 μ l): It is used to remove the excess salt.
7. TE buffer (30 μ l): It contains Tris. Cl and EDTA. Its used for elution of DNA.

Column Purification of DNA

1. Equilibration Buffer (400 μ L): It contains guanidine thiocyanate. This buffer solubilizes gel slice, maintains the pH \leq 7.5 and provides appropriate conditions for the DNA fragments to bind to the silica membrane.
2. Wash Buffer (500 μ L): It contains Tris.HCl, NaCl and Alcohol. This buffer removes primers and unwanted impurities such as salt, enzymes.
3. Elution Buffer: It contains Tris. Cl. This buffer is used for elution of DNA during purification.

Polymerase Chain Reaction (PCR) reagents:

4. Taq Polymerase (0.5U/1 μ l): To carry on the polymerization reaction. (PCR)
5. PCR reaction buffer: This buffer consists of:
 - a) Tris. HCl & KCl: To maintain the pH of the PCR reaction buffer.
 - b) MgCl₂: It contributes Mg⁺² which act as a cofactor for Taq polymerase to function and carry out the

polymerization reaction.

6. dNTP: It is used to supply the necessary nucleotides (A, T, C and G) for the reaction to occur.
7. Primers: Short preexisting polynucleotide chain to which new deoxyribo nucleotides can be added by DNA polymerase.
8. Distilled Water: To make up for the volume so that the reaction is carried out.
9. Agarose gel: It is a polymer which has minute pores and enables separation of DNA fragments based on the size.
10. Ethidium bromide: It is a fluorescent dye which binds to the DNA and illuminates with U.V transilluminator on gel documentation.

DNA sequencing reagents

11. Fluorescent tagged ddNTPs

Sequential listing of Armamentarium used in the present study

Extraction of Genomic DNA

1. EDTA coated tubes: It was used for collection of blood samples.
2. Eppendorf tubes: Eppendorf tubes of 1.5ml were used as containers to keep solutions to isolate DNA from blood.
3. Incubator: It was used for incubation at 37°C for 30 min.
4. Ultracentrifuge: It was used to form pellets in the process of isolation of DNA from blood.

Column Purification of DNA

1. Spin Column: It was a tube used for placing isolated DNA.
2. Collection tube: It was a tube in which the spin column was placed and impurities were collected upon centrifugation.
3. Silica filter membrane: It was used for filtration of impurities and adsorption of DNA fragments.

Polymerase Chain Reaction (PCR) test

1. Micropipettes: Graduated micropipettes were used to add reagents according to their specific quantities
2. PCR tubes: PCR tubes of 0.5ml were used to add reagents according to their specific quantities.
3. PCR Machine: It is an automated thermo cycler which performs the polymerase chain reaction test, which is the quick and easy method for generating unlimited copies of desired fragment of DNA (Xeroxing of DNA). It changes and maintains the specific temperature for that particular time as set programmed for each of the steps in one cycle of the reaction.
4. Automated ABI sequencer: It was used for DNA sequencing with capillary electrophoresis.

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.

Step 1: Collection and storage of blood samples

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

Step 2: Extraction of genomic DNA

In general, the isolation of DNA from cells and cellular components with the help of reagents can be divided into five stages:

1. Cell disruption- salt buffer.
2. Lysis of Cell - Sodium Dodecyl Sulphate.
3. Removal of Proteins and Contaminants-NaCl.
4. Precipitation of DNA- Isopropanol.
5. Elution of DNA on agarose gel - TE buffer

Genomic DNA was extracted from the blood of the subjects according to the five stages mentioned. Venous blood was taken with a graduated micropipette into 1.5ml Eppendorf tube. It was treated with 900 µl of low salt buffer and 50 µl of Triton-X and the solution was mixed well. It was incubated for 10 minutes to lyse the RBCs and Cells were centrifuged at 8000 rpm for 3 minutes and the supernatant was discarded. This step was repeated 2-3 times with decreasing amount of Triton-X till RBC lysis was complete and a white pellet of WBCs was obtained. To the cell pellet, 600 µl of high salt buffer and 40 µl of 10% SDS were added, thus dispersing the pellet. The solution was mixed thoroughly and incubated at 65 °C for 15 minutes. At the end of incubation, 100 µl of 5M NaCl was added and vortexed to precipitate the proteins. Cells were centrifuged at 8000 rpm for 5 minutes. Then the supernatant was transferred into a new Eppendorf tube containing 600 µl of Isopropanol. Thus, precipitating the DNA by inverting the Eppendorf tube slowly. It was then centrifuged at 8000 rpm for 10 minutes to pellet down the DNA. The supernatant was discarded, 200 µl of 70% ethanol was added and mixed slowly to remove any excess salts. Finally the preparations were centrifuged at 8000 rpm for 5 minutes to pellet down the DNA. The supernatant was discarded and extracted DNA was air-dried. After thorough drying, 30 µl of TE buffer was added to elute the DNA. This was loaded and checked on 1% Agarose Gel.

Step 3: Column purification of DNA

Isolated DNA was collected into the spin column which was inturn placed in a collection tube. This was followed by addition of 400µl of equilibration buffer (Guanidine thiocyanate). This buffer Solubilizes gel slice, maintains the pH ≤ 7.5 and provides appropriate conditions for the DNA fragments to bind to the silica membrane. The tube was centrifuged at high speed for 1 minute at 10000 rpm. The collected buffer was discarded. This step was repeated till DNA sample was complete. Later, 500µl of wash buffer was added to remove the primer and unwanted impurities such as salt and enzymes. The tube was centrifuged at high speed for 1 minute. The wash was collected in the tube and discarded. Again the column was centrifuged with empty collection tube to completely remove the wash buffer for 2 minutes. The column was placed in new collection tube and added 50 µl of prewarmed Elution buffer (Tris. Cl) at the center of the filter membrane for separation of the DNA fragments. The tube was incubated for 2 minutes and centrifuged at high speed for 1 minute. The column was removed and the lid of collection tube was closed. Thus purified Genomic DNA was ready to use for PCR.

Step 4: Polymerase Chain Reaction (PCR) Test

The Polymerase chain reaction (PCR) is an *in vitro* technique which allows the amplification of a specific deoxyribonucleic acid (DNA).

For rs 13317

FGFR1FP1:5'CAGCAGGTGGCAGAAAGTAA'3
 FGFR1RP1: 5'CAGGAGGCTGAGGCAGAATC'3

For rs 1448037

FGF10FP2: 5'GCTGAGTAGGAGTCAGGTG'3
 FGF10RP2: 5'GAAGCATAGTCCATGAAAGAG'3

Genomic DNA was amplified by 0.2 Units of Taq polymerase which carries out the polymerization reaction in a final volume of 20µl TRIS HCl buffer containing 25mmol/l KCl, 0.25 mmol/l dNTP, 2.5mmol/l MgCl₂ and 250mmol/l PCR primers. The balance volume is added up, by 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 three staged cycle 35 times.

Stage 1: Separation/Denaturation: The double stranded DNA is denatured by heat, by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA templates at 95 °C for 3 min, followed by actual denaturation at 95 °C for 1 min.

Stage 2: Priming/Annealing: The primers anneal to the single-stranded DNA template at 58 °C for 1 min.

Stage 3: Polymerization/Elongation: The DNA polymerase recognizes the primer and makes a complimentary copy of the template at 72 °C for 1 min.

Step 5: DNA Sequencing

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.

Results

In the present study, the relationship between FGFR1 (rs 13317) and FGF 10 (rs1440837) gene variants with cleft lip with or without cleft palate was evaluated in 50 subjects consisting of group A (P1-P25) as cases and group B (C1-C25) as controls using polymerase chain reaction (PCR) test followed by DNA sequencing.

Results for FGF 10 RS 1448037 variants

For FGF 10 (rs 1448037) three genotype can be possible

G/G	Normal Homozygous Allele
A/A	Mutant Homozygous Allele
A/G	Mutant Heterozygous Allele

Later, the results of these fifty patients were tabulated based on the presence or absence of GG, AG and AA genotype of FGF10 (rs 1448037) variant (Table 1). The number of cases and controls with difference in their genotype frequencies has been tabulated (Table 2).

In group A

- 1 out of 25 cases showed the presence of GG genotype.
- 6 out of 25 cases showed the presence of AG genotype.
- 18 out of 25 cases showed the presence of AA genotype.

In group B

- 22 out of 25 controls showed the presence of TT genotype.
- 2 out of 25 controls showed the presence of TC genotype.
- 1 out of 25 controls showed the presence of CC genotype.

After statistical analysis (Z- test) (Table 3)

- There were statistically significant difference in GG and AA genotype frequencies between cases and controls
- GG genotype was found to be highly statistically significant with the controls (GROUP B) (p=<0.001)
- AA genotype was found to be highly statistically significant with the cases (GROUP A) (p=<0.001)
- AG genotype was neither statistically significant with cases (GROUP A) nor controls (GROUP B) (p=0.12)

Results for FGFR 1 RS 13317 variants

For FGFR1 (rs 13317) three genotype can be possible:

T/T	Normal Homozygous Allele
C/C	Mutant Homozygous Allele
T/C	Mutant Heterozygous Allele

Later, the results of these fifty patients were tabulated based on the presence or absence of TT,TC and CC genotype of FGFR 1, rs 13317 variant (Table 4). The number of cases and controls with difference in their genotype frequencies has been tabulated (Table 5).

In group A

- 9 out of 25 cases showed the presence of TT genotype.
- 5 out of 25 cases showed the presence of CC genotype.
- 11 out of 25 cases showed the presence of TC genotype.

In group B

- 21 out of 25 controls showed the presence of TT genotype.
- 0 out of 25 controls showed the presence of CC genotype.
- 4 out of 25 controls showed the presence of TC genotype.

After statistical analysis (Z- test) (Table 6)

- There were statistically significant difference in TT,TC and CC genotype frequencies between cases and controls
- TT genotype was found to be highly statistically significant with the controls (GROUP B) (p=0.005)
- TC genotype was found to be statistically significant with the cases (GROUP A) (p=0.03)
- CC genotype was found to be statistically significant with the cases (GROUP A) (p=0.02)

Statistical Methods

Z test has been used to find the significance of association of FGFR1, FGF10 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:

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

$$SEDp = \sqrt{\hat{P}(1 - P^*)(1/n_1 + 1/n_2)}$$
 and

$$P = \frac{x_1 + x_2}{n_1 + n_2}$$

$p1, \text{proportion}1 = x1 / n1$

$p2, \text{proportion}2 = x2 / n2$

$x1$ =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

Strongly significant $p \leq 0.001$

Not significant $p \geq 0.05$

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

Statistical interpretation

Table 1: Tabulated results for fifty subjects showing variation in presence of genotypes of FGF10 (rs 1448037) gene variant among cases and controls

Group A	GG genotype	AG genotype	AA genotype	Group B	GG genotype	AG genotype	AA genotype
1.	Absent	Absent	Present	1.	Present	Absent	Absent
2.	Absent	Present	Absent	2.	Absent	Present	Absent
3.	Absent	Absent	Present	3.	Present	Absent	Absent
4.	Absent	Absent	Present	4.	Present	Absent	Absent
5.	Present	Absent	Absent	5.	Present	Absent	Absent
6.	Absent	Absent	Present	6.	Absent	Present	Absent
7.	Absent	Present	Absent	7.	Present	Absent	Absent
8.	Absent	Absent	Present	8.	Present	Absent	Absent
9.	Absent	Absent	Present	9.	Present	Absent	Absent
10.	Absent	Absent	Present	10.	Present	Absent	Absent
11.	Absent	Absent	Present	11.	Present	Absent	Absent
12.	Absent	Absent	Present	12.	Present	Absent	Absent
13.	Absent	Present	Absent	13.	Present	Absent	Absent
14.	Absent	Present	Absent	14.	Present	Absent	Absent
15.	Absent	Absent	Present	15.	Present	Absent	Absent
16.	Absent	Absent	Present	16.	Present	Absent	Absent
17.	Absent	Absent	Present	17.	Present	Absent	Absent
18.	Absent	Absent	Present	18.	Absent	Absent	Present
19.	Absent	Present	Absent	19.	Present	Absent	Absent
20.	Absent	Absent	Present	20.	Present	Absent	Absent
21.	Absent	Absent	Present	21.	Present	Absent	Absent
22.	Absent	Absent	Present	22.	Present	Absent	Absent
23.	Absent	Present	Absent	23.	Present	Absent	Absent
24.	Absent	Absent	Present	24.	Present	Absent	Absent
25.	Absent	Absent	Present	25.	Present	Absent	Absent

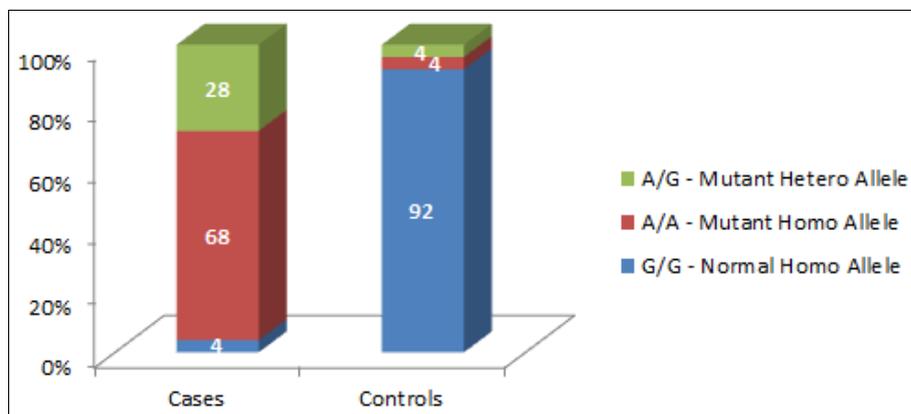
Table 2: The presence of GG, AG, AA genotype of FGF 10 (rs 1448037) gene variants among cases and controls

Genotype of FGF 10 (rs 1448037) Gene variant	Group A (Cases)	Group B (Controls)	Total
GG	1	22	23
AG	6	2	8
AA	18	1	19
Total	25	25	50

Table 3: The table denotes the statistical significance of the genotype when cases and controls are compared using z-test

Genotype of FGF 10 gene variant	Cases		Controls		Difference in proportions	Z	P value
	N	%	N	%			
G/G	1	4	22	88	0.84	-5.958	<0.001**
A/A	18	72	1	4	0.68	4.953	<0.001**
A/G	6	24	2	8	0.16	1.543	0.12

- The difference in the proportion of positive results between cases and controls for genotype GG was found to be statistically highly significant ($p < 0.001$)
- The difference in the proportion of positive results between cases and controls for genotype AA was found to be statistically highly significant ($p < 0.001$)
- The difference in the proportion of positive results between cases and controls for genotype AG was not statistically significant ($p = 0.12$)



Graph 1: Bar diagram showing the percentage distribution of different genotypes of FGF10 in case and control groups

Table 4: Tabulated results for fifty subjects showing variation in presence of genotypes of FGFr1 (rs13317) gene variant among cases and controls

Group A	Genotype TT	Genotype TC	Genotype CC	Group B	Genotype TT	Genotype TC	Genotype CC
1.	Present	Absent	Absent	1.	Present	Absent	Absent
2.	Absent	Present	Absent	2.	Present	Absent	Absent
3.	Absent	Present	Absent	3.	Present	Absent	Absent
4.	Absent	Present	Absent	4.	Absent	Present	Absent
5.	Present	Absent	Absent	5.	Present	Absent	Absent
6.	Absent	Absent	Present	6.	Present	Absent	Absent
7.	Present	Absent	Absent	7.	Present	Absent	Absent
8.	Present	Absent	Absent	8.	Present	Absent	Absent
9.	Absent	Present	Absent	9.	Present	Absent	Absent
10.	Absent	Absent	Present	10.	Present	Absent	Absent
11.	Present	Absent	Absent	11.	Present	Absent	Absent
12.	Absent	Present	Absent	12.	Absent	Present	Absent
13.	Absent	Present	Absent	13.	Present	Absent	Absent
14.	Absent	Present	Absent	14.	Present	Absent	Absent
15.	Absent	Present	Absent	15.	Absent	Present	Absent
16.	Present	Absent	Absent	16.	Present	Absent	Absent
17.	Absent	Absent	Present	17.	Present	Absent	Absent
18.	Present	Absent	Absent	18.	Present	Absent	Absent
19.	Absent	Present	Absent	19.	Present	Absent	Absent
20.	Absent	Absent	Present	20.	Present	Absent	Absent
21.	Absent	Present	Absent	21.	Absent	Present	Absent
22.	Present	Absent	Absent	22.	Present	Absent	Absent
23.	Absent	Present	Absent	23.	Present	Absent	Absent
24.	Absent	Absent	Present	24.	Present	Absent	Absent
25.	Present	Absent	Absent	25.	Present	Absent	Absent

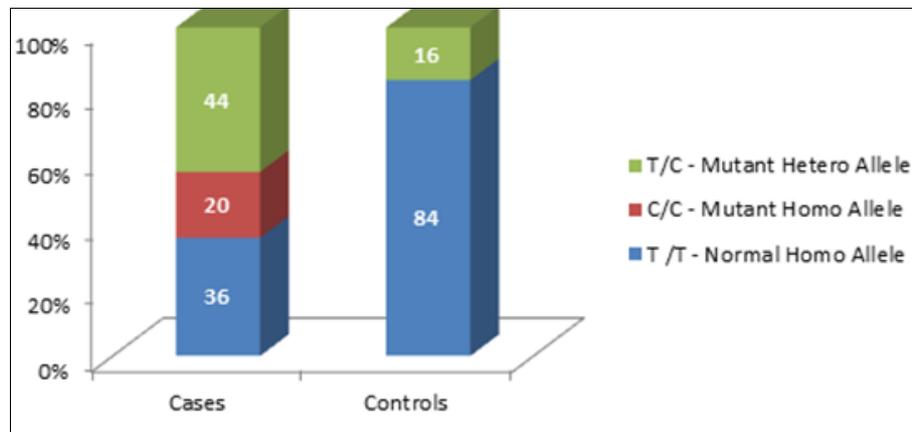
Table 5: The presence of TT, TC, CC genotype of FGFr 1(rs 13317) gene variants among cases and controls

Genotype of FGFr 1 (rs 13317) gene variant	Group A (cases)	Group B (controls)	Total
TT	9	21	30
TC	11	4	15
CC	5	0	5
Total	25	25	50

Table 6: The table denotes the statistical significance of the genotype when cases and controls are compared using Z-test

Genotype of FGFr 1 gene variant	Cases		Controls		Difference in proportions	Z	P value
	N	%	N	%			
T/T	9	36	21	84	0.48	-3.464	0.005
C/C	5	20	0	0	0.2	2.357	0.02
T/C	11	44	4	16	0.28	2.160	0.03

- The difference in the proportion of positive results between cases and controls for genotype TT was found to be statistically highly significant (p=0.005)
- The difference in the proportion of positive results between cases and controls for genotype CC was found to be statistically significant (p=0.02)
- The difference in the proportion of positive results between cases and controls for genotype TC was found to be statistically significant (p=0.03)



Graph 2: Bar diagram showing the percentage distribution of different genotypes of FGFR1 in case and control groups

Discussion

Development of the head and face comprises one of the most complex events during embryonic development, coordinated by a network of transcription factors and signaling molecules together with proteins conferring cell polarity and cell-cell interactions. Disturbance of this tightly controlled cascade can result in a facial cleft where the facial primordia ultimately fail to meet and fuse or form the appropriate structures. Collectively, craniofacial abnormalities are among the most common features of all birth defects (Stanier *et al.* 2004) [14].

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 seems 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 indispensable to highlight the gene involvement in CL±P patients according to literature survey (Haque *et al.* 2015) [6].

In 2008, the World Health Organisation (WHO) has recognized that non-communicable diseases, including birth defects cause significant infant mortality and childhood morbidity and have included cleft lip and palate in their Global Burden of Disease (GBD) initiative (Mossey *et al.* 2009) [10].

Due to their disturbing appearance in many cases, these deformities have attracted much attention in terms of treatment and research. The large impact of the defects on facial growth, function and social integration renders them a major public health problem worldwide (Rajion *et al.* 2007) [11]. Many researchers are currently studying the location and nature of gene mutations associated with CL/P. In recent years, advances in genetics and molecular biology have begun to disclose the basis of craniofacial development, and the number of genes associated with CL/P have been identified.

Collectively CL/P has a major clinical impact requiring surgical, dental, orthodontic, speech, hearing and psychological treatments or therapies throughout childhood. Patients undergo multiple rounds of surgical repair in the first year of life and may continue until 18 or 20 years of age (Stainer 2004) [14].

Polymorphism is a mechanism by which individuals may exhibit variations within the range of what is considered biologically normal. Gene polymorphisms are also associated to disease susceptibility. Most polymorphisms are single nucleotide exchanges that occur at a high frequency in the human genome and may affect the function of genes. Thus genes involved in craniofacial development are plausible candidates for oral clefts.

Fibroblast growth factors (FGF) signalling involves almost all structures of the craniofacial morphology from the development and outgrowth of the facial primordia during the entire embryonic development process and is now implicated in the genetic basis of both syndromic and non-syndromic CL/P. Fibroblast growth factors (FGFs) and their receptors (FGFRs) comprise a large, complex system of growth factor signalling. The human FGF family comprises of 22 members. The FGF signalling pathway is known to have an important role in craniofacial development, especially neural crest induction, skeletogenesis and epithelial-mesenchymal interactions. FGF signalling in the mouse is closely integrated with other pathways such as Bmp, Shh, Tgfb, Sox, Msx, Dlx, Egf and Wnt which are also known to be required for normal craniofacial development (Pauws *et al.* 2007) [5].

A study revealed the role of FGF signaling in mammalian palate formation. It explained that coordinated epithelial mesenchymal interactions are essential during the initial stages of palate development and require FGF-Shh signaling network (Rice *et al.* in 2004) [12].

Several studies support the role of FGF gene family in the etiology of cleft lip/palate. Knock out mouse models for FGF 10 and FGF2b develop cleft palate, establishing the necessity of epithelial FGF signaling in normal palatogenesis (Alison *et al.* 2010) [2]. A study done by Leibbrandt *et al.* in 2007 [9] concluded that FGF8 is essential for survival and normal development of the neural crest derived facial mesenchyme and suggest that other FGF receptors in addition to FGFR1 are involved in the reception of the FGF8 signal (Leibbrandt *et al.* 2007) [9].

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.

Our study showed a highly significant difference in presence of genotypes in cases and controls in both the FGFR1 gene variants rs13317 and FGF10 gene variants rs1448037. This is in accordance with a study done by Bridget *et al.* in Philippines population, which concluded the associations between NS CLP and SNPs in FGF3, FGF7, FGF10, FGF18, and FGFR1. Thus the data suggest that the FGF signalling pathway may contribute to as much as 3–5% of NS CLP and will be a consideration in the clinical management of CLP (Bridget *et al.* 2007) [3]. Our study also concurs with a study done by Hong Wang *et al.*, in which analysis of the entire data set for SNPs in ten FGF genes revealed a significant association with the risk of NSCL/P for the Asian population (Hong Wang *et al.* 2013) [7].

The results of our study are contrary to the study done by wan *et al.* in Chinese population, suggesting presence of FGFR1 gene variant is not associated with cleft lip and palate however the similar study shows association of FGF10 with an increased risk for cleft lip/palate (wan *et al.* 2009) [15]. The contradictory results are probably due to genetic heterogeneity, incomplete 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. But 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 micro array techniques, further identification of the candidate gene and genetic pathways involved in Non-syndromic clefting can be expected. Ultimately all of these advances will allow more 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 preventative and *in vivo* foetal therapy for these debilitating conditions.

Conclusion

The conclusions drawn from this study are:-

1. This study indicates that there is a strong association between the presence of FGFR1 gene variant rs13317 and FGF10 gene variant rs1448037 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 TC ($p=0.03$) & CC ($p=0.02$) genotype for FGFR1 gene variant rs13317 and AA ($p<0.001$) genotype for FGF10 gene variant rs1448037.
3. This study suggests that the incidence of Non syndromic cleft lip and palate is lesser in subjects having TT ($p=0.005$) genotype of FGFR1 gene variant rs13317 and GG ($p<0.001$) & AG ($p=0.12$) genotype of FGF10 gene variant rs1448037.
4. The findings of this study suggest that FGFR1 gene variant rs13317 and FGF10 gene variant rs1448037 can be considered as genetic markers for Nonsyndromic cleft lip and palate for our population.

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