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Detection of Single Nucleotide Polymorphism rs2013162 of IRF6 Gene in Patient with Cleft Lip and Palate

Husnain Shehzad[1*] and Osheen Sajjad[2]

Abstract:

Background: Cleft lip and palate are congenital disorders which induce affected individuals medically, socially and psychologically. The objective of this study was to investigate the association of Single Nucleotide Polymorphism(SNP); rs2013162 of *IRF6* Gene in Patient with Cleft Lip and Palate.

Materials and Methods: Fifty patients with non-syndromic CL/P were included in present study alongwith fifty individuals with no psychiatric history as controls. In all of the these individuals, search for Single nucleotide polymorphism was carried out by designing sequence specific primers. The sequence was amplified by using Real time PCR and products were investigated by visualizing high resolution melting curve upon HRM-PCR.

Results: The logistic regression and Hardy-Weinberg equilibrium were applied to investigate the association of *IRF6* SNP rs2013162 with disease. Results revealed no association of this polymorphism with non-syndromic CL/P.

Conclusion: We found no association of *IRF6* SNP rs2013162 in patients with non-syndromic CL/P. Further study is required with larger sample size to validate the findings of the present study in Pakistani population and along with this SNP other polymorphisms of the same gene should be analyzed to find out the association with the non-syndromic CL/P.

Keywords: Interferon regulatory factor-6 (IRF6) Gene, Single nucleotide polymorphism, Non-syndromic Cleft Lip and Palate

1. Gulab Devi Institute of Allied Health Sciences Lahore, Pakistan
2. Institute of Biomedical and Allied Health Sciences, University of Health Sciences Lahore, Pakistan

*Correspondence: ranahusnain401@gmail.com

Introduction:

Cleft lip and palate are considered most common birth defects that possess significant medical, psychological, social, and financial implications on the affected individuals and families**(1)**. Cleft lip and palate is a gap, which occurs when the lip or roof of the mouth does not completely fuse during the first trimester of foetal development

Cleft lip and cleft palate are congenital disorders also known as orofacial cleft, is a group of conditions that includes cleft lip (CL), cleft palate (CP), and both together (2, 3). Cleft lip and cleft palate are openings or splits in the upper lip, the roof of the mouth (4) or both. Cleft lip and cleft palate result when facial structures that are developing in an unborn baby don't close completely(5). Cleft lips come in many shapes and sizes like unilateral, bilateral, complete and incomplete cleft palate. Clefts of the palate can include just the back part of the roof of the mouth (**soft palate**) called a soft palate cleft. They

may also include the hard bony part of the roof of the mouth (**hard palate**) (6).

Anatomically, the philtrum and its pillars are a part of the upper lip. The surface of the lip is comprised of four zones: hairy skin, vermilion border, vermilion and oral mucosa. The normal shape of the lips varies with age, and is influenced by ethnicity [7]. The maxillary prominence and medial nasal prominence form the upper lip, it is form between 6th and 11th week of the pregnancy(8). Hard Palate is formed by Bones: Maxilla(Palatine Processes) Soft Palate formed by bones Fibromuscular shelf attached like a shelf to posterior portion of hard palate.(9) Primary Palate Forms during 4th to 7th week of Gestation Secondary Palate Forms in 6th to 9th weeks of gestation(10).

The earliest documented history of cleft lip is based on a combination of religion, superstition, invention and charlatanism. While Greeks ignored their existence, Spartans and Romans would kill these children as they were considered to Harbour evil spirits. When saner senses prevailed Fabricius ab Aquapendente (1537–1619) was the first to suggest the embryological basis of these clefts. The knowledge of cleft lip and the surgical correction received a big boost during the period between the Renaissance and the 19th century with the publication of Pierre Franco's Petit Traité and Traité des Hernies in which he described the condition as "lievré fendu de nativité" (cleft lip present from birth). The first documented Cleft lip surgery is from China in 390 BC in an 18-year-old would be soldier, Wey Young-Chi. Albucasis of Arabia and his fellow surgeons used the cautery instead of the scalpel and Yperman in 1854 recommended scarifying the margins with a scalpel before suturing them with a triangular needle dipped in wax.

The repair was reinforced by passing a long needle through the two sides of the lip and fixing the shaft of the needle with a figure-of-eight thread over the lip. Germanicus Mirault can be credited to be the originator of the triangular flap which was later modified by C.W. Tennison in 1952 and Peter Randall in 1959. In the late 50s, Ralph Millard gave us his legendary 'cut as you go' technique. The protruding premaxilla of a bilateral cleft lip too has seen many changes throughout the ages – from being discarded totally to being pushed back by wedge resection of vomer to finally being left to the orthodontists (11).

Abnormal anatomy CL\P Vermillion (wet/dry border) Cupid's bow Along the upper vermillion cutaneous border (white roll), two midline elevations form the bow Philtrum Philtral columns and dimple Tubercle (12). The complexity of this cranio-facial developmental pathway and the numerous developmental points at which clefting could be induced is reflected in the heterogeneity of the phenotypic expression of the condition. Cleft lip and/or palate: the maxillary, medial nasal, and lateral nasal prominences converge through a complicated process of epithelial bridging, programmed cell death, and sub epithelial-mesenchymal penetration(13). Cleft lip and/or palate is likely to be secondary to a defect of epithelial fusion or mesenchymal growth, processes involving many possible genetic loci or intracellular signalling pathways (14). This results in interrupted fusion of the maxillary and median nasal prominences.

Oral clefts have a multifactorial origin and are affected by genetic and environmental factors. Gender, geographic location, nationality, nutritional and peri-conceptional consumption of folic acid have an effect on the incidence rate of oral clefts (15, 16). It has also been reported that tobacco use,

antiepileptic drugs and possibly alcohol consumption (17), low birth weight (18), and mustard gas (19) increase the incidence rate of oral clefts in new-borns. Several studies have also reported that racial/ethnic factors(20, 21) and consanguinity (22).**There are several other risk factors which causes CLP such as :**family history, sex, race, exposure to certain substances during pregnancy, maternal cigarette smoking and alcohol consumption during pregnancy, having diabetes during pregnancy, being obese during pregnancy, X-ray during pregnancy and several environmental factors(23). Current research continues to investigate the extent to which folic acid can reduce the incidence of cleft(24)

Cleft lips and/or palates vary in severity. They can range from a small notch in the upper lip, to a gaping defect of the lip and palate, and sometimes the nose. With a cleft palate, the hole in the roof of the mouth means there's a connection between the mouth and the nose (25). Cleft lip/cleft palate can present a number of obstacles for a child, including possible: feeding issues, failure to gain weight, flow of milk through nasal passages during feeding, poor growth, dental and orthodontic problems, speech and language difficulties, hearing impairment, social and self-esteem challenges (26).

Orofacial clefting (OFC) is a common birth defect with worldwide incidence around 1 in 700 live births (27). Asian and Amerindian populations have the highest birth prevalence, often as high as 1/500, with European derived populations intermediate at about 1/1000, and African-derived populations the lowest at 1/2000 (28). The study was a 7-year retrospective study from March 1998 to March 2005. Twenty-five live births with cleft lip and/or palate (CL ± P) were born between 20 March 1998 and 20

March 2005 from the total of 11,651 live births in a maternity hospital in Tehran (18). Several descriptive epidemiologic studies have been carried out in many countries worldwide; however, no such study has ever been performed in Pakistan. Population-based data on the incidence of cleft lip and palate were obtained from birth registry information in northern Pakistan. A total of 117 cases from 61,156 live births reported were identified. The incidence for cleft lip and/or cleft palate was 1.91 per 1000 births (one per 523 births(29)

Fogh-Andersen in Denmark first proposed a role for genetic factors in NS CL/P ((30). Consistent with a multifactorial model of inheritance, the aetiology of NS CL/P may be explained by the interaction of as few as 2 or as many as 14 different genes (31). Several approaches have been employed to identify genes involved in craniofacial development and explain how specific genetic variants result in NS CL/P. These genetic approaches include genome-wide linkage and association analysis as well as study of candidate genes studies selected on the basis of genes causing syndromic forms of OFC, gene expression, or animal models with clefts.(32). Convincing genetic and/or biologic evidence exists for the contribution of many genes: IRF6, 8q24, FGFR2, FOXE1, BMP4, TGFβ3, MSX1, MAFB, PAX7, ABCA4, and VAX1, to NS CL/P(33)

Interferon Regulatory Factor 6 (IRF6):
(IRF6) located on 1q32.2 chromosome and is the poorly understood member of a family of nine transcription factors with highly conserved DNA binding domains and less conserved protein binding domains. This gene has 4 transcripts (splice variants), 83 orthologues, 8 paralogues, is a member of 1-Ensembl protein family and is associated with 11 phenotypes. Most IRFs have a role in

innate immunity, particularly after viral infection, but the role of IRF6 remains largely unclear (34). IRF6 acts as a tumor suppressor in mammary cells and helps induce keratinocyte differentiation (35). Mutations in IRF6 cause Van der Woude (VWS) and popliteal pterygium syndromes; these mutations typically interfere with DNA-binding or protein-protein interactions of this transcription factor and lead to haplo-insufficiency of this gene in the affected individual(34).

IRF6 association with NS CL/P has been consistently replicated in other populations (36-40) demonstrated by a genome-wide linkage scan (41), and a meta-analysis of 13 linkage studies (42), and has been confirmed in all GWA studies in NS CL/P (27, 43). In addition to craniofacial defects, animal models of IRF6 function exhibit skin and limb abnormalities (44). Irf6 is expressed in the epidermis of the skin and mice deficient for Irf6 exhibit abnormal proliferation and differentiation of cutaneous keratinocytes (45). This gene is polymorphic and many of the SNPs have been associated by various researchers and their colleagues with cleft lip and palate including rs1319435, rs2235371, rs2235375, rs2235543, and rs2013162. Polymorphism rs2013162 was analysed to test the association of this SNP with the risk of developing NSOC, using both case-parent trios and cases and controls.

The purpose of the present study is to recruit affected families with CL\P from the Pakistani population. From this population, molecular studies on large scale have been done and many candidate genes have been identified in variable phenotypes. In the present study, inherited CL\P related disorders (where innervations contributed in the disease pathway) are the focus for genetic studies. In the Pakistani health setup, it always remains difficult to diagnose genetic diseases due to the lack of required diagnostic tools and genetic testing modules.

Material and Methods:
Study Design: This was a case-control study.
Settings: The study was conducted in the Department of Human Genetics and Molecular Biology, University of Health Sciences, Lahore. Samples were collected from CLAP Hospital Faisal Town Lahore
Sample Size: Blood samples from 50 CLP patients and 50 sex matched healthy controls were taken.
Sampling Technique: Non-probability purposive sampling technique was used to collect the data.
Inclusion Criteria: Case group: Both male and female **p**atients with non-syndromic cleft lip and palate; **control group:** Male and female general population without cleft lip and palate.
Exclusion criteria: CLP with syndromic features will be excluded.
Sample collection and storage: After an informed written consent, all the relevant demographic and clinical history was collected and recorded in a structured questionnaire. Blood samples (3-5 ml) were collected from the schizophrenic patients and normal subjects, in 0.5 M EDTA (ethylene diamine tetra acetate) containing vials. The blood was then stored at -20°C until processed for DNA isolation.
DNA Extraction by Salting Out Method:
1.   Autoclaved eppendorf tubes were taken and were labeled properly.
2.   500 μl of blood samples were added in the respective tubes.
3.   700 μl of Tris-EDTA buffer was added in all the tubes for the lysis of RBCs.
4.   The lids of the tubes were closed and were mixed by vortexing.

5.   The tubes were left at room temperature for 10 to 15 minutes.

6.   They were then centrifuged at 13500 rpm for 10 minutes.

7.   The supernatant was discarded, and the pellet was broken properly by tapping with fingers.

8.   Step numbers 3 to 7 were repeated 4 times but the amount of Tris-EDTA added was reduced to 500 µl after the first wash.

9.   After the last wash with Tris-EDTA, the pellet was broken again as before and 25 µl of 10% SDS solution was added

10.   Then 375 µl of sodium acetate and 10 µl of proteinase K were added respectively.

11.   The solution in the eppendorf tubes was mixed properly and they were incubated at 37°C for 24 hours.

12.   After 24 hours incubation, the tubes were vortexed and 250 to 300 µl of ice cold chloroform and iso-amylalcohol (24:1) solution was added in each tube. Solution in tubes was mixed by inverting them several times.

13.   The tubes were centrifuged at 13500 rpm for 10 min.

14.   After centrifugation, three layers were formed in each tube. The central layer contained debris and the upper layer contained DNA.

15.   The upper layer was transferred into new labeled tubes.

16.   Equal volume (500 µl) of cold absolute ethanol was added in each tube containing the upper layer. It precipitated the DNA and the DNA threads were visible after inverting the tubes gently 3 to 4 times.

17.   The tubes were left at room temperature for 15 to 20 minutes.

18.   The tubes were centrifuged at 13500 rpm for 10 minutes.

19.   The supernatant was discarded by inverting the tubes over layers of tissue papers and 500 µl of cold 70% ethanol was added in the pellet for washing DNA.

20.   The tubes were centrifuged at 13500 rpm for 10 minutes.

21.   The supernatant was again discarded by inverting the tubes slowly over the layers of tissue papers.

22.   The pellet was left to be air dried for 30 minutes to one hour.

23.   After the pellet got dried, 50 µl of low TE was added and DNA was allowed to get dissolved in it.

24.   The tubes were incubated at 65°C for half an hour to deactivate DNase.

25.   The DNA was finally stored at -20°C (46).

DNA Quality Analysis by Gel electrophoresis:

1.   Gel electrophoresis was carried out for the detection of DNA isolated from the blood.

2.   2% agarose gel was prepared by adding 4g of agarose in 200ml of 1X TBE buffer and the solution was heated in microwave oven until it became clear.

3.   When the temperature of the solution decreased to $< 60^{O}$C, 10 µl of ethidium bromide (10 µg/ml) was mixed in 200 ml of the agarose solution.

4.   The gel-casting tray was washed and cleaned, and combs were inserted in it.

5.   The gel solution was immediately poured into the gel-casting tray before it solidified and was then allowed to solidify in the tray at room temperature.

6.   After solidification, the gel-casting tray containing the gel was shifted into

electrophoresis tank having 1X TBE buffer in it sufficient to cover the gel surface up to

volts for half an hour to 45 min until the dye reached two third of the gel.

No	Gene	db SNP	Allele	Position	NCBI No.	Position on Sequence
Table 1: Gene with its corresponding SNP selected in this study						
1	*IRF6*	rs2013162	(C/T)	Intron 1	**NC_000001. 11**	60953T>C

2mm.

7. The combs were removed smoothly after 15 min to allow the gel to be equilibrated with buffer.

11. The separated DNA bands on the gel were visualized under UV light using gel documentation system and the concentration of the DNA bands was estimated by

No	SNP	Primer	Sequence	GC content (%)	Product Size
Table 2 : Sequence of primers for DRD2 gene used in this study					
1	Rs2013162	*IRF6* -F	5'- CAGGGACTGATCCTGCTTTC-3'	60	160 bp
2		*IRF6* -R	5'- TGATGTTCAGGAAGGGGAAG-3'	55	

8. On a parafilm, 2 µl of DNA was mixed in 2µl of 6X loading dye and 8µl of 1X TBE buffer.

9. This mixture was then loaded in the wells within the gel alongside a DNA ladder/marker containing DNA fragments of

comparing its intensity with the nearby DNA fragment of the DNA ladder/marker.

DNA Quantity Analysis:

The purified DNA samples were quantified by Nanodrop analysis (spectrophotometric analysis). 10µL of genomic DNA sample

Figure 1: The thermal cycling conditions for PCR of rs2013162
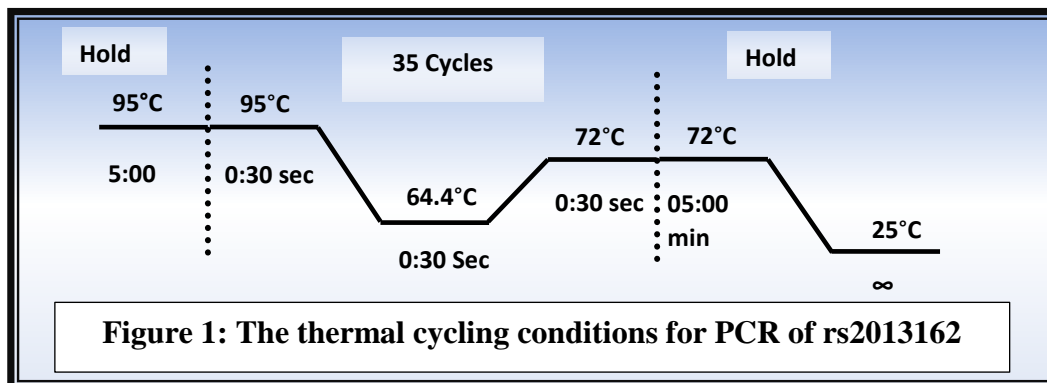
known concentration.

10. The power supply was turned on and the gel was run from cathode to anode at 150

diluted to 1 ml (dilution factor of 100) with autoclaved distilled water was subjected to spectrophotometric analysis. The quantity of DNA was found by taking absorbance at 260

nm and putting the value in the following formula.

DNA quantity (µg/ml) = 50 x optical density (OD) at 260nm x dilution factor.

The quantity of DNA was found to be in acceptable range if value is in between 50-70 ng/µl.

SNP Selection:

One SNP was selected in *IRF6* gene according to the dbSNP http://www.ncbi.nlm.nih.gov/snp/ which is rs2013162.

Primer Designing:

Sequences of selected human genes under study were retrieved from NCBI (National Centre for Biotechnology Information). Sequence was then BLAST (basic local alignment tool) in the bioinformatics tool (www.genome.ucsc.edu). Using this tool, the SNP 2005313 sequence regions was copied and saved. The sequence at least 50 base pair up and down stream of desired SNP was then subjected to primer designing in primer3 software (www.primer3.com). The designed primers were then BLAST in the bioinformatics tool. The sequences of the primers are given in Table 2.

Primer reconstitution and dilution:

100ρmoles oligoes or primers: The nmoles of lyophilized oligoes or primers were multiplied with 10 and the answer was the measure of low TE buffer in µl that was then added in lyophilized primers to make 100ρmoles solution and it was stored at -20ºC. For example, a lyophilized primer containing 40.2 nmoles was dissolved in 420 µl of low TE buffer so the concentration of the resultant solution was 0.1 nmoles or 100 ρmoles.

10ρmoles oligoes or primers: 100 µl from 100ρmoles oligoes or primers was mixed with 900 µl of distilled water and was stored at -20ºC.

PCR optimization:

Primers were optimized for optimum reaction conditions of temperature by using gradient PCR. These optimum conditions were then used in the experiment. The gradient temperatures and optimized annealing temperatures for the primers were:

Table 3: Optimization conditions for SNP of the present study		
SNP	Gradient temperatures	Annealing temperatures
IRF6	57 ºC-64 ºC	64ºC

Polymerase Chain Reaction (PCR):

PCR DreamTaq 2X Master Mix (Thermo Scientific) was used for amplification of DNA. 1 µl of DNA was amplified by the conventional PCR with the optimized conditions, using the synthesized forward and reverse gene specific primers. The reaction mixture was as given in Table 4. The optimized cycling conditions for IRF^ rs2013162 are shown in Figure 1.

Table 4: Components and concentrations of PCR reaction	
Components	**Concentrations**
DNA	1µl
Reverse Primer (10 pm)	0.5µl
Forward Primer (10 pm)	0.5µl
DreamTaq Master Mix (2X)	8 µl
Nuclease free H$_2$O	up to 15.0 µl

Restriction Fragment Length Polymorphism:
Procedure or steps:
Step I: Restriction digest

- Extraction of desired fragments of DNA using restriction endonuclease (RE). The enzyme RE has specific restriction site on the DNA, so it cut DNA into fragments.
- Different fragments of different sizes was generated along with the specific desired fragments.

Step II: Gel electrophoresis

- The digested fragments were run in agarose gel electrophoresis to separate the fragments based on size. Different sized fragments form different bands.

Step III: Denaturation

- The gel was placed in sodium hydroxide (NaOH) solution for denaturation so that single stranded DNA are formed.

Step IV: Blotting

- The single stranded DNA were transferred into charge membrane i.e., nitrocellulose paper by the process called capillary blotting or electro-blotting.

Step V: Baking and blocking:

- The nitrocellulose paper transferred with DNA was fixed by autoclaving. Then the membrane was blocked by using bovine serum albumin or casein to prevent binding of labelled probe non-specifically to the charged membrane.

Step VI: Hybridization and visualization:

- The labelled RFLP probe was hybridized with DNA on the nitrocellulose paper. The RFLP probes were complimentary as well as labelled with radioactive isotopes so they form colour band under visualization by autoradiography.

Result:

Subjects: The NSCLP patients were recruited from CLAP Hospital Faisal Town Lahore. After obtaining written informed consent from each patient, 50 NSCLP patients and 50 healthy controls were enrolled in the study. There were 66.0% male patients and 37.0% were females. The age of the patients ranged from 3 months to above 18 years. Most patients 33.0% were in the age group of 3 months to 5 years and in this group the percentage of male patients (30.0%) was greater than that of females (20.0%). Figure 2 illustrates the three age groups of the study population.

Consanguinity was observed to be associated with increased risk for as 31.0% of the patients were the product of consanguineous marriages. CL/P was seen occurring commonly in Rajput (9.0%), Arain (9.0%), and Jutt (7.0%), families. However, majority of the study population belongs to different castes like Mughal, Khokhar and Gujjar etc. 25.5% of patients were from different castes
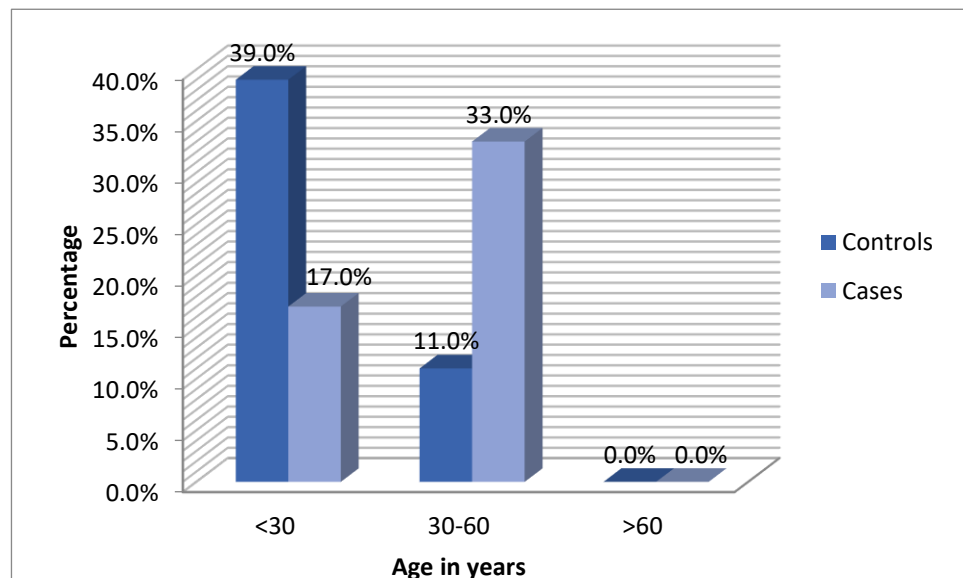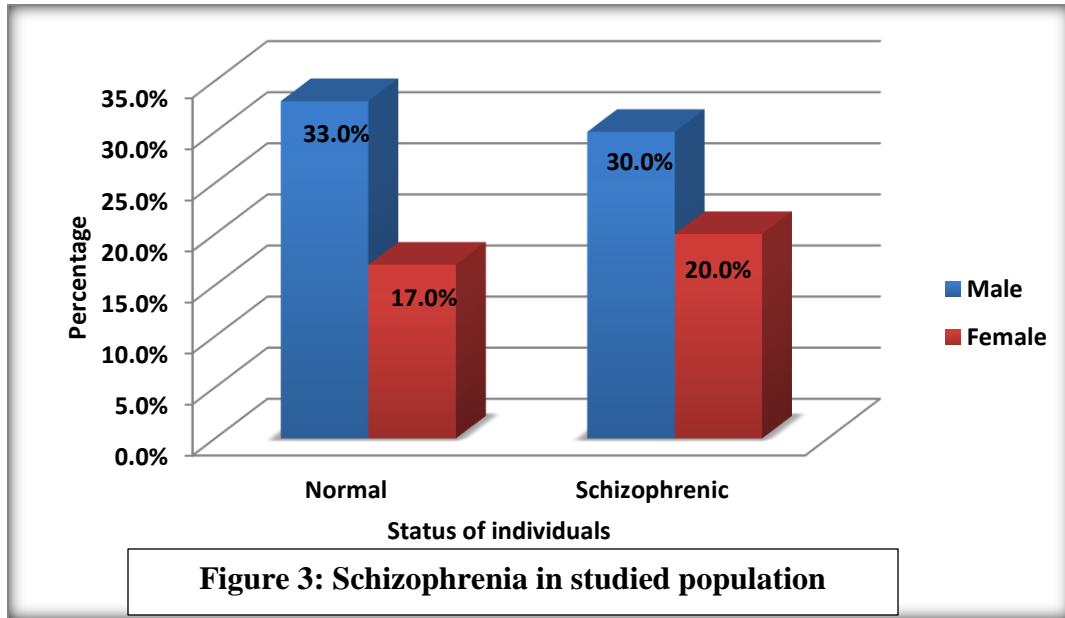

Figure 2: Age Groups in studied cases and controls

**Figure 3: Schizophrenia in studied population**

which reveals that CL/P is not caste specific disease.

In this study, 13 patients out of 50 have Cleft lip, 16 patients have cleft palate and 21 have both CL, CP.

In this study 11 patients having SNP were presented and 12 patients have cleft lip but have no SNP detected. (Chi square= 1.819, p-value= 0.173)

Table 5: Association of CL with SNP:				
		rs2013162		
		Present	Absent	Total
CL	Present	11	1	12
	Absent	27	10	37
Total		38	11	50

Figure 4: Cases distrinution among different castes
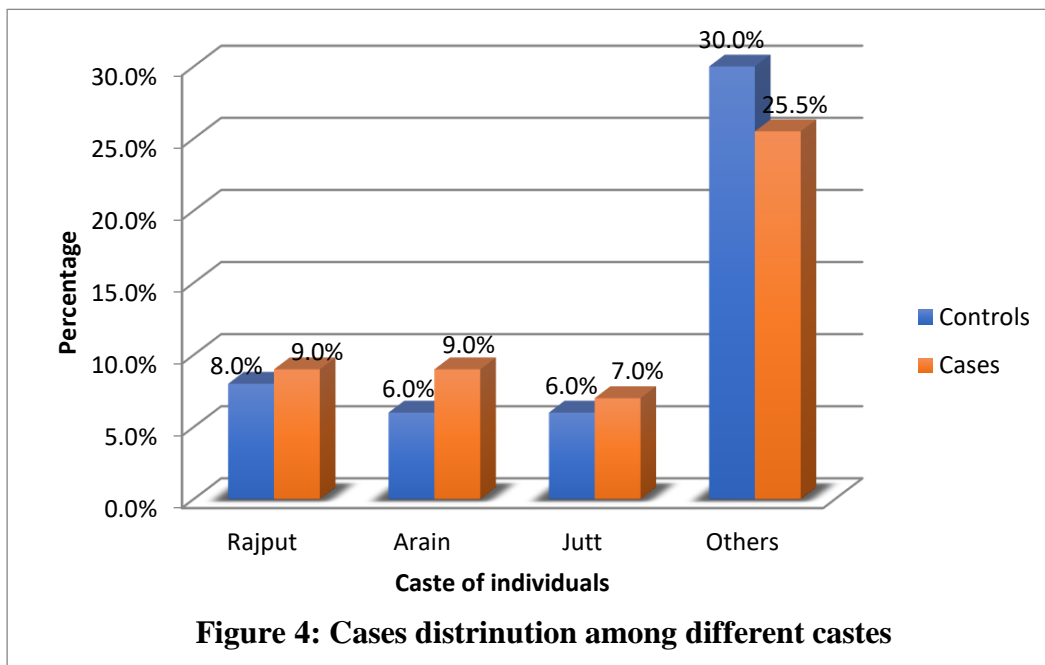
In this study 12 patients having CP showed SNP while others have CP but no SNP is

Table 6: Association of CP with SNP

		rs2013162		
		Present	Absent	Total
CP	Present	12	3	15
	Absent	27	8	35
Total		39	11	50

detected. (Chi square= 3.876, p value=0.57)

Discussion:

Orofacial clefting (OFC) is a common birth defect with worldwide incidence around 1 in 700 live births (4). Asian and Amerindian populations have the highest birth prevalence, often as high as 1/500, with European derived populations intermediate at about 1/1000, and African-derived populations the lowest at 1/2000 (47) cleft lip and palate is a gap, which occurs when the lip or roof of the mouth does not completely fuse during the first trimester of foetal development.

The effect of rs642961 on IRF6 expression has been investigated in foreskin keratinocytes and lip tissue from cleft patients (48) however, this study did not identify IRF6 expression differences based on SNP genotype for rs2235371 or rs642961. Current study was small with only 100 samples and could have resulted in decreased power to detect correlation. In addition, the two IRF6 SNPs have low minor allele frequencies in Caucasian populations and higher minor allele frequencies in Asian populations. This study may have failed to identify association because our mostly Caucasian population is homozygous for the common alleles, with 45 heterozygotes and only 3 individuals carrying the minor

homozygous genotype for rs642961 and 10 heterozygotes and no individuals with the homozygous for minor the minor alleles of rs2235371. Examining foreskin tissue of an Asian population or a population of cleft patients may increase the odds of detecting the effect of these two IRF6 SNPs on IRF6 gene expression.

Developmental genetic studies have shown that the mechanisms of disease onset are different between NSCL $\pm$ P and NSCP. Moreover, the incidences of these diseases also vary with gender: NSCL $\pm$ P is more common in men, whereas NSCP is more common in women. In a previous study, it was found that two SNPs in the transforming growth factor alpha (TGFα) gene are associated with NSCL $\pm$ P, but not NSCP, in a northern Chinese population(49) In recent years, various investigations have shown the IRF6 gene is involved in NSCL/P(50) but studies on the association between the IRF6 gene and risk to NSCP are very limited. In this study, the association between the SNPs rs2013162 and rs2235371 in IRF6 and NSCP in a northeast Chinese population was investigated using case-control and case-parent analysis.

A study by Jugessur et al. (2008) found a significant association between rs2013162 and NSCP (P = 0.025). In the present study, this association was confirmed via case-control analysis (P < 0.05). Furthermore, FBAT analysis showed over-transmission of the A allele at rs2013162 (P = 4.472, Z = 0.001). These results suggested that rs2013162 is a "risk allele" for NSCP in the northeast Chinese population (51). Its previously reported that rs2235371 and rs2013162 in the IRF6 gene are significantly associated with NSCL/P in a northeast Chinese population. Here, we found that those two SNPs are strongly associated with

NSCP in the same population. Jugessur et al. (2008) observed that rs2013162, but not rs2235371, is associated with NSCP in a Norwegian population. These contradicting results may be attributed to genetic heterogeneity of different populations.

Conclusion:

This study found strong association of IRF6 SNP rs201362 in patients with NSCLP. Further study is required with larger sample size to validate. It is more common in male than females

Outcome and Utilization:

Increased understanding of the molecular basis of CL/CP will arise from a greater understanding of the genes involved in disease development, pointing in the direction of molecular pathways that may lead to CL/CP. This information may help to understand the biology of the disease and to develop an appropriate

Conflict of interest: Authors do not have any conflict of interest.

Human/Animal Rights: No human or animal rights are violated during this study.

Informed Consent: An informed consent was obtained from both case and control groups.

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